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Special Issue I

RESERVE BANK OF INDIA – AN OVERVIEW

Dr.S.DENESH SINGH

Christian College, Kattakada,Tvm

Introduction:

A bank is a financial institution that provides banking and other financial services to their customers. A bank generally provides fundamental banking services such as accepting deposits and providing loans. In addition banking sector offers several facilities and opportunities to their customers. All the banks safeguards the money and valuables and provide loans, credit, and payment services, such as checking accounts, money orders and cashier's cheques. The banks also offer investment and insurance products. As a variety of models for co-operation and integration among finance industries have emerged, some of the traditional distinctions between banks, insurance companies, and securities firms have diminished. In spite of these changes, banks continue to maintain and perform their primary role—accepting deposits and lending funds from these deposits. The Banks play a vital role in the financial system in India which results in the economic development of the country.

There are also non-banking financial institutions which provide certain banking services without meeting the legal definition of a bank.

A very simple meaning for bank, it is a place where people deposit their money with full hearted trust. Literally speaking at present we have to think the fact, whether the public deposits are safe in bank? Why the banks are always changing their rules? Is it really needed for a bank to exist, every customer has to keep a minimum balance in their account? For what reasons bank loans are easy for business and rich people?

The banking system in India should not only be hassle free but it should be able to meet the new challenges posed by the technology and any other external and internal factors. . For the past three decades, India's banking system has several outstanding achievements to its credit. But in the present scenario the banks are punishing the public for their and government blunders. It is to be changed. Will it? If Reserve Bank of India thinks everything can be changed.

Objective of the study

1.To gain knowledge about Reserve Bank of India
2.To study about the various functions of Reserve Bank of India
3.To highlight the role of Reserve Bank of India in the economic growth of the country

Methodology of the study

The data and information has been collected from secondary sources like magazines, business newspaper, journals, periodicals, reports, text books and website.

Reserve Bank of India:

Establishment

The Reserve Bank of India was established on April 1, 1935 in accordance with the provisions of the Reserve Bank of India Act, 1934. The Central Office of the Reserve Bank was initially established

in Calcutta but was permanently moved to Mumbai in 1937. The Central Office is where the Governor sits and where policies are formulated. Though originally privately owned, since nationalisation in 1949, the Reserve Bank is fully owned by the Government of India.

Preamble

The Preamble of the Reserve Bank of India describes the basic functions of the Reserve Bank as:"to regulate the issue of Bank notes and keeping of reserves with a view to securing monetary stability in India and generally to operate the currency and credit system of the country to its advantage; to have a modern monetary policy framework to meet the challenge of an increasingly complex economy, to maintain price stability while keeping in mind the objective of growth."

Central Board

The Reserve Bank's affairs are governed by a central board of directors. The board is appointed by the Government of India in keeping with the Reserve Bank of India Act.

Functions: General superintendence and direction of the Bank's affairs

Indian currency

The Indian rupee is the official currency of the Republic of India. The rupee is subdivided into 100 paise (singular paisa), though as of 2018, coins of denomination 25 paise and less are no longer legal tender. The issuance of the currency is controlled by the Reserve Bank of India. The Reserve Bank manages currency in India and derives its role in currency management on the basis of the Reserve Bank of India Act,1934. The rupee is named after the silver coin, rupiya, first issued by Sultan Sher Shah Suri in the 16th century and later continued by the Mughal Empire. In 2010, a new rupee symbol "₹"was officially adopted. The first series of coins with the new rupee symbol started in circulation on 8 July 2011. On 8 November 2016 the Government of India announced the demonetisation of 500 and 1000 banknotes with effect from midnight of the same day, making these notes invalid. A newly redesigned series of 500 banknote, in addition to a new denomination of 2000 banknote is in circulation since 10 November 2016. The new redesigned series is also expected to be enlarged with banknotes in the denominations of 1000, 100 and 50 in the coming months. On 25 August 2017, a new denomination of 200 banknote was added to Indian currency to fill the gap of notes due to high demand for this note after demonetisation. In July 2018, the Reserve Bank of India released the 100 banknote

Bank notes

The Reserve Bank has the sole authority to issue banknotes in India. Reserve Bank, like other central banks the world over, changes the design of banknotes from time to time. The Reserve Bank has introduced banknotes in the Mahatma Gandhi Series since 1996 and has so far issued notes in the denominations of Rs.5, Rs.10, Rs.20, Rs.50, Rs.100, Rs.500 and Rs.1000 in this series.

Functions of RBI

a) Traditional functions
b) Development functions
c) Supervisory functions

(a) TRADITIONAL FUNCTION OF RBI

1.Issue of Currency Notes

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Special Issue on:

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on

Insights into the Interdisciplinary Perspectives of Chemical and Bio Sciences

From 26th to 28th February 2018

Guest Editor

Dr. K. Murugan

Principal

Government Arts College,

Thycaud, Thiruvananthapuram, Kerala 695014

Government Arts College
Thycaud, Thiruvananthapuram, Kerala 695014
Phone : 0471 2323040
Email: artscollegeofficetvpm@gmail.com
Web: www.gactvm.org

Dear Sir/ Madam

Research in emerging areas and interdisciplinary/ multidisciplinary approaches are inevitable for making major breakthroughs in scientific world..I am delighted to write the foreword for the compendium of papers of the **National seminar on Insights into the Interdisciplinary Perspectives of Chemical and Bio Sciences** by **Govt. Arts College, Trivandrum, Kerala**. The papers in connection with the seminar are an enthusiastic celebration of diversity with respect to molecules, of natural origin, intricate structure and biological relevance. It is also a unique tribute to the many young researchers who are involved in chemical synthesis. On the top of that, there are innumerable data that interweave chemistry and biology in a very appealing way. Being the focus of chemical and biological research moving from structure to function, dynamic interactions between large biopolymers and small organic molecules that cause and control processes in living organisms need to be investigated with a new vision. Chemical Biology is sure to become even more important. Science is objective and it is human intelligence and endeavor that discover and create interesting new substances. Researchers and scientists have discovered or designed more than thirty million compounds/molecules which possess biological potentialities. Blending chemistry, biology and medicine through this seminar will revolutionize the concept of life. I am confident that in the future the young minds will elucidate the chemical mechanisms of cell functions, thought and even memory. It is my hope and expectation that the papers related with the theme of the National seminar will provide an experience and referenced resource for all Science professionals caring.

Thanking you

Yours truly

Dr. K Murugan

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Address for communication:

Prof. Dr. A.K. Sharma,
BPAS Publications
115-RPS-DDA Flat, Mansarover Park, Shahdara, Delhi-110032, India
(Phone-011-22138726, 9212200909)
Email: editorbulletin8@gmail.com, bpaspublications@gmail.com
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Synthesis of 1,2,3–benzotriazol–1–yl–[n–(n'–arylthiocarbamoyl)] amidines

Akhila V.R. [1], Sophia K. Philip[1] and K.N. Rajasekharan[1,*]

Author Affiliations
[1]Department of Chemistry, University of Kerala, Kariavattom campus, Thiruvananthapuram 695581, Kerala, India.

Corresponding Author
***K.N. Rajasekharan,** Department of Chemistry, University of Kerala, Kariavattom campus, Thiruvananthapuram 695581, Kerala, India.

E-mail: kn.rajasekharan@gmail.com

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Abstract

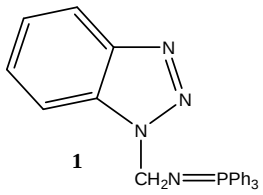
Synthesis of hitherto unreported 1,2,3–benzotriazol–1–yl–[N–(N'–arylthiocarbamoyl)] amidines from benzo–1,2,3–triazol–1–ylamidinium p–toluene sulfonate is reported.

Keywords: Benzo–1,2,3–triazole, Thiocarbamoylamidine, Microwave irradiation

1. INTRODUCTION

The chemistry of 1,2,3–benzotriazole was extensively studied and developed by Katrizky during the last two decades and consequently, 1,2,3–benzotriazole has emerged as a major synthetic auxillary in organic synthesis. The 1, 2, 3–benzotriazole moiety is seen to serve as a better leaving group and 1, 2, 3–benzotriazole based reagents act as acyl anion and isothiocyanate equivalents [1–3]. Benzotriazole derivatives have also been used in the synthesis of bioactive triazinones [4], guanidine's, thioureas [5] and other heterocyclic systems [6]. Apart from its use as an auxilliary in synthesis, recent studies highlight the application of benzotriazole derivatives in medicinal chemistry due to their anticancer, antifungal, antibacterial, antitubercular, antioxidant and antiviral activities [7]. Synthetic strategies have recently been reported for the preparation of primary, secondary and tertiary amines in high yields using a variety of 1,2,3–benzotriazole based reagents such as 1–(triphenylphosphoroylideneaminomethyl) benzotriazole **(1)** [8]. The use of 1–acyl–1,2,3–benzotriazoles as convenient trifluoroacylation agents [9] and as acylating agents for furan and thiophene rings [10] further exemplify the use of benzotriazole based reagents in synthesis. The use of 1–amidino–1,2,3–benzotriazoles as guanylating agents for the conversion of amines to guanidines have been explored [5,11]. We have reported the synthesis of 1–(N–arylamidino)–1,2,3–benzotriazole derivatives and their use in guanidine synthesis [12]. We have also reported that the N–thiocarbamoylamidine derivatives of 3,5–dimethylpyrazole can be used as an efficient thiocarbamoyl transfer reagent [13]. Thus, such pyrazolyl thiocarbamoy lamidines on reaction with amines produce thiocarbamoylguanidines, which in turn are useful precursors for heterocycle synthesis. We have also shown that pyrazolylthiocarbamoylamidines are also good precursors for thiazole and triazole ring synthesis [14]. In this context, we now report the synthesis of thiocarbamoylamidine derivatives of

1,2,3–benzotriazole, starting from benzo–1,2,3–triazol–1–ylamidinium p–toluenesulfonate (**2**). The required amidinium salt (**2**) has now been prepared easily under microwave activation.



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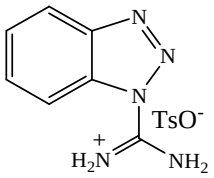


2

2. EXPERIMENTAL

2.1. Materials and Methods

All solvents and reagents used were of analytical grade, purchased from Merck– India. Melting points of all compounds were determined by open capillary method and are uncorrected. The FTIR spectra were recorded on Agilent Technologies FTIR spectrophotometer. The ^{1}H and ^{13}C nmr spectra were recorded in DMSO on Bruker DPX 400 MHZ NMR spectrometer by using TMS as the internal standard. The Thin Layer Chromatography (TLC) was performed on aluminium foil– backed, silica gel 60 F_{254} TLC plates from Merck. The spots were visualized under UV light or in iodine vapour. Samsung domestic microwave oven model M183 DN was used for microwave experiments.

2.2. Preparation of benzo–1,2,3–triazol–1–ylamidinium p–toluene sulfonate (2) under microwave irradiation

To a mixture 1,2,3–benzotriazole (119 mg, 1 mmol) and p–toluene sulphonic acid (190 mg, 1 mmol), cyanamide (50 mg, 1.2 mmol) followed by distilled water (0.04 mL) was added. The whole reaction mixture was mixed well to obtain a homogeneous, pale brown coloured paste which was microwave irradiated at 180W for 30 s with a resting interval of 10 s after each 10 s irradiation to avoid overheating. The mixture was triturated well with a glass rod at each interval to maintain homogeneity. Cooling afforded benzo–1,2,3–triazol–1–yl– amidinium p–toluene sulfonate (**2**) as a white solid. The crude compound was crystallized from 2–propanol. Crude yield 0.318 g, 95%; crystallized yield 64%; mp 178–180^{0}C (Lit. mp[12] 181–183°C). Analysis: Found: C, 50.57; H, 4.60; N 20.92: Calc. For $C_{14}H_{15}N_5SO_3$ (333.37): C, 50.44, H, 4.54; N, 21.01%; IR (KBr): 3662, 3297, 3234, 3074, 1712, 1546, 1224, 1161, 1124, 1035, 1010, 822, 785, 765, 744, 681 cm^{-1}; ^{1}H NMR: (300 MHz, DMSO–d_6): δ 2.07–2.28 (s, 3H), 7.11–7.13 (d, 2H, ArH), 7.47–7.49 (d, 2H, ArH), 7.63–7.68 (t, 1H, ArH) 7.82–7.87 (t, 1H, ArH), 7.99–8.02 (d, 1H, ArH), 8.30–8.32 (d, 1H, ArH), 9.99 (br s, NH).

2. 3. Preparation of benzo–1,2,3–triazol–1–ylamidine (3)

Benzo–1,2,3–triazol–1–yl–amidinium p–toluenesulfonate (**2**) (3.33 g, 10 mmol) was dissolved in hot distilled water (40 mL). Potassium carbonate (1.38g, 10 mmol) was added with stirring and after gas evolution, the solution was cooled in ice–salt mixture whereupon the free base crystallised. On recrystallization from chloroform, benzo–1,2,3–triazol–1–ylamidine (**3**) was obtained as white prisms. Crude yield 1.1 g, 68%; Crystallised yield 58%; mp: 129–131°C.
Analysis: Found: C, 51.97; H, 4.47; N, 43.28: Calc. for $C_7H_7N_5$ (161.17): C, 52.16; H, 4.38; N, 43.46%; IR (KBr): 3387, 3275, 3120, 2360, 2208, 2164, 1672, 1492, 1472, 1054, 1011, 779, 766, 744, 721 cm^{-1}; ^{1}H NMR: (300 MHz, $CDCl_3$): δ 5.22–6.46 (br, NH), 7.44–7.49 (t, 1H, ArH), 7.59–7.64 (t, 1H, ArH), 8.09–8.12 (d, 1H, ArH), 8.44–8.47 (d, 1H, ArH); ^{13}C NMR: (75 MHz, $CDCl_3$): δ 115.0, 119.9, 125.2, 129.4, 131.4, 146.7, 150.4; ESI MS: m/z: 162 $[M+1]^{+}$.

2.4. General procedure for the synthesis of benzo–1,2,3–triazol–1–yl–[N–(N'–aryl) thiocarbamoyl] amidines (4a–e)

Method A: Directly from benzo–1,2,3–triazol–1–yl–amidinium p–toluenesulfonate (2)

To DMF (2 mL), containing dry powdered KOH (28 mg, 0.5 mmol), benzo–1,2,3–triazol–1–ylamidinium p–toluenesulfonate **(2)** (138 mg, 0.5 mmol) and a pinch of tetrabutylammonium bromide

(TBAB) were added. The mixture was stirred magnetically in an ice bath for 20 min followed by the addition of aryl isothiocyanate (0.5 mmol) in DMF (1 mL). Then the ice bath was replaced by water bath of 60°C and stirred for another 2 hr. Then, the reaction mixture was added to ice–water (30 mL) with stirring and stored for coagulation. The solid product so formed was filtered and washed with petroleum ether (5mL x 4) and dried. The purity of compounds was checked by TLC using ethyl acetate: pet. ether (1:3) as eluent. The crude compounds were crystallized from ethanol.

Method B: From benzo–1,2,3–triazol–1–ylamidine free base (3)

To a solution of benzo–1,2,3–triazol–1–ylamidine (**3**), obtained as described above, (81 mg, 0.5 mmol) in DMF (2 mL) aryl isothiocyanate was added (0.5 mmol) and the solution was stirred for 6–7 h. The progress of the reaction was monitored by TLC using ethyl acetate– petroleum ether (1:3) as eluent. When the reaction was over, the reaction mixture was added to ice water (50 mL) with stirring and stored for coagulation. The solid product formed was filtered, washed with petroleum ether (4 x 5 mL) and dried.

2.4.1. Benzo–1,2,3–triazol–1–yl–[N–(N'–phenyl) thiocarbamoyl] amidine (4a)

m. p. 160–61°C; IR: 3354, 3157, 3000, 1651, 1591, 1524, 1453, 1371, 1118, 1047, 793, 738, 689 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.05–7.48 (m, 5H), 7.48–8.20 (b m, 3H), 8.45–8.90 (b m, 1H), 11.00–11.50 (b, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 189.61, 154.27, 146.79, 138.12, 130.54, 129.94, 129.64, 128.93, 127.17, 126.99, 126.66, 124.60, 119.99, 119.20; ESI MS: m/z: 162 $[M+1]^+$.

2.4.2. Benzo–1,2,3–triazol–1–yl–[N–(N'–4–chlorophenyl) thiocarbamoyl] amidine (4b)

m. p. 162–64°C; IR: 3347, 3153, 2989, 1647, 1528, 1483, 1438, 1375, 1244, 1170, 1043, 857, 823, 745, 674 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.15–7.40 (m, 4H), 7.45–8.02 (m, 2H), 8.05–8.30 (b m, 1H), 8.40–8.75 (b m, 1H), 11.00–11.50 (b, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.44, 136.65, 135.79, 130.48, 129.93, 129.69, 129.57, 126.84, 125.86, 120.59, 120.17; FAB MS: m/z: 331.5 $[M+H]^+$

2.4.3. Benzo–1,2,3–triazol–1–yl–[N–(N'–4–methylphenyl) thiocarbamoyl]amidine (4c)

m. p. 158–60°C; IR : 3475, 3356, 3024, 2995, 1649, 1587, 1483, 1436, 1373, 1103, 1070, 1047, 860, 796, 746, 719, 698 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 2.30–2.50 (m, 3H), 7.05–7.40 (m, 4H), 7.45–7.95 (m, 2H), 8.00–8.18 (b m, 1H) 8.45–8.90 (b m, 1H), 11.10–11.40 (b, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 189.55, 154.09, 146.78, 137.02, 136.71, 135.67, 129.93, 129.78, 129.54, 129.39, 126.12, 125.81,120.45, 119.95, 21.13; ESI MS: m/z: 333 $[M+Na]^+$, 311 $[M+H]^+$.

2.4.4. Benzo–1,2,3–triazol–1–yl–[N–(N'–4–methoxyphenyl) thiocarbamoyl] amidine (4d)

m. p. 160–62°C; IR: 3429, 3287, 3004, 1640, 1509, 1390, 1300, 1166, 1103, 1013, 834, 752, 719 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 3.80–3.95 (m, 3H), 6.80–7.05 (m, 2H), 7.10–7.40 (m, 2H), 7.45–7.95 (m, 2H), 8.00–8.20 (m, 1H), 8.35–8.85 (m, 1H), 11.15–11.45 (b, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 189.84, 158.73, 158.24, 154.11, 147.02, 131.20, 129.92, 129.47, 127.82, 127.72, 121.39, 120.47, 115.62, 115.49, 55.56; FAB MS (m/z): 327.5 $[M+H]^+$.

2.4.5. Benzo–1,2,3–triazol–1–yl–[N–(N'–4–ethoxyphenyl) thiocarbamoyl] amidine (4e)

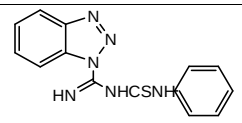
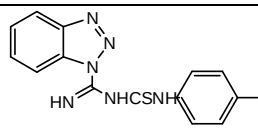
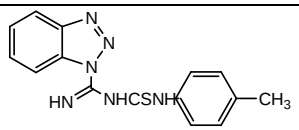
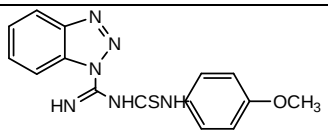
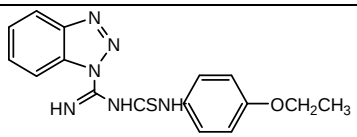
m. p. 161–63°C; IR: 3377, 3209, 3026, 2983, 1651, 1597, 1512, 1442, 1384, 1305, 1244, 1109, 1045, 802, 758, 711 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 1.35–1.55 (m, 3H), 3.95–4.15 (m, 2H), 6.85–7.05 (m, 2H), 7.15–7.45 (m, 2H), 7.60–7.95 (m, 2H), 8.00–8.20 (b m, 1H), 8.35–9.00 (b m, 1H), 11.10–11.45 (b, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 189.76, 158.10, 154.10, 146.78, 131.02, 130.58, 127.71, 126.56, 125.63, 120.47, 119.95, 114.92, 114.69, 63.88, 14.84; FAB MS: m/z: 341 $[M+H]^+$.

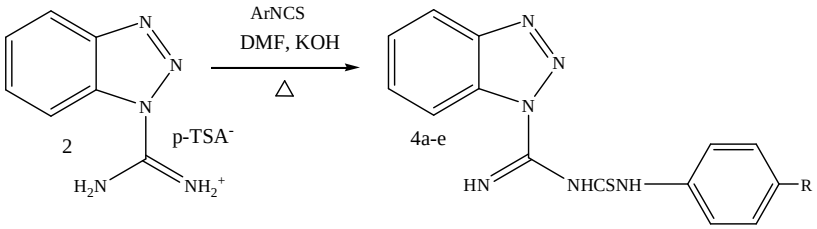
3. RESULTS AND DISCUSSION

The reaction of benzo–1,2,3–triazol–1–ylamidinium p–toluenesulfonate with heterocumulenes, such as isothiocyanates, have not been reported from elsewhere. We now wish to report the conversion of benzo–1,2,3–triazol–1–ylamidinium p–toluenesulfonate (**2**) to benzo–1,2,3–triazol–1–

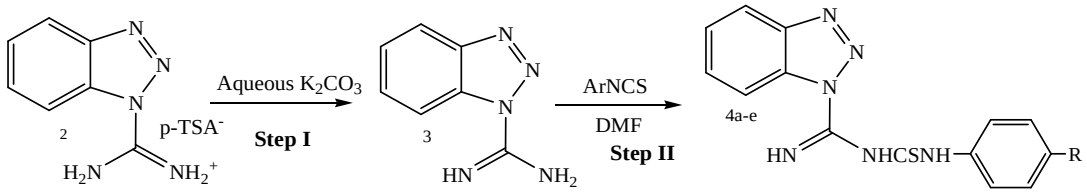
yl–[N–(N'–arylthiocarbamoyl)] amidines (**4**). Thus, it was now found that benzo–1,2,3–triazol–1–ylamidinium p–toluenesulfonate (**2**) in a polar aprotic solvent such as N,N'–dimethylformamide (DMF) in presence of dry powdered KOH and a trace of a phase transfer catalyst such as tetra–n–butylammonium bromide, would react with aryl isothiocyanates to afford benzo–1,2,3–triazol–1–yl–[N–(N'–aryl)thiocarbamoyl] amidines (**4a–e**) (Scheme 1, Method A). In order to establish that the reaction proceeded through the formation of the free base, the above reaction was also conducted in two steps, by first converting the benzo–1,2,3–triazol–1–ylamidinium p–toluenesulfonate (**2**) to its free base (**3**) and then reacting it with aryl isothiocyanates (Scheme 2, Method B). However, the two–step approach required the mechanical stirring of the reaction mixture for 7h for the completion of reaction as shown in Scheme 2, while in the reaction as shown in Scheme 1 was completed within 2.5 h. It is also notable that the yield of compounds **4a–e** by the one–step method **(**Scheme 1) was better than the overall yield by the two–step method (Scheme 2). For example, the yield of benzo–1,2,3–triazol–1–yl–[N–(N'–phenyl)thiocarbamoyl] amidine (**4a**) by Method A in Scheme 1 was 58%. Though the second step of Scheme 2 yielded the compound (**4a)** in 81%, the step 1 yielded benzo–1,2,3–triazol–1–ylamidine free base (**3**) in 63% only, thus resulting in an overall yield of compound (**4a**) by Method 2 of 51% (Table 1). The structure of the synthesized compounds was established based on IR, ^{1}H, ^{13}C NMR and mass spectral evidences. The ^{1}H NMR spectra seemed to indicate that these benzo–1,2,3–triazol–1–yl–[N–(N'–aryl)thiocarbamoyl] amidines show tautomerism in that the spectral peaks were generally broad, appearing as multiplets. Chromatographic analysis and the fact that identical products were formed by Methods A and B further established the homogenity of the presently obtained benzotriazolylthiocarbamoylamidines. Careful crystallization and a preliminary single crystal x–ray diffraction studies showed the product to be homogenous and the presence of only one tautomer in solid state (data not shown). These studies along with variable temperature, solvent dependent and 2D nmr analysis are in progress.

Table 1: Comparison of yields of compounds (**4a–e**) from scheme 1 and scheme 2.

Sl. No.	Compound	Scheme1 Yield (%)	Scheme 2		
			Step I Yield (%)	Step II Yield (%)	Overall Yield (%)
1.	 **4a**	58	63%	81	51
2.	 **4b**	64		91	57
3.	 **4c**	49		62	39
4.	 **4d**	47		65	41
5.	 **4e**	60		72	45



Scheme 1: Method A: Direct conversion of benzo–1,2,3–triazol–1–ylamidinium p–toluenesulfonate (**2**) to benzo–1,2,3–triazol–1–yl–[N–(N–aryl)thiocarbamoyl]amidines (**4a–e**).
(4a: R=H, 4b: R=Cl, 4c: R=CH_3, 4d: R=OCH_3, 4e: R=OCH_2CH_3 for both Scheme 1 and II)



Scheme 2: Method B: Two step conversion of benzo–1,2,3–triazol–1–ylamidinium p–toluenesulfonate (**2**) to benzo–1,2,3–triazol–1–yl–[N–(N–aryl)thiocarbamoyl]amidines (**4a–e**)

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Structural, Bonding and Spectral Analysis of an anti-malarial drug 5-(4-chloro phenyl)-6-ethyl-2,4-pyrimidinediamine

Y. Sheeba Sherlin[1], T.Vijayakumar[2], J.Binoy[3], S.D.D. Roy[4] and V.S. Jayakumar[5,*]

Author Affiliations
[1]Dept. of Physics, Scott Christian College (Autonomous), Nagercoil-629003, Tamil Nadu.
[2] Dept. of Physics & Nanotechnology, SRM University, Kattankulathur-603203, Tamil Nadu, India.
[3] Dept. of Physics, Govt. College for Women, Thiruvananthapuram-695014. Kerala, India
[4] Dept. of Physics, Nesamony Memorial Christian College, Marthandam-629165, Tamil Nadu, India.
[5]Mar Baselios Institute of Technology, Anchal-691306, Kerala, India.

Corresponding Author
*__V.S. Jayakumar__, Mar Baselios Institute of Technology, Anchal-691306, Kerala, India.

E-mail: vsjkumar@gmail.com

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Abstract

Antimalarial drug 5–(4–chlorophenyl)–6–ethyl–2,4–pyrimidinediamine (PYR) was investigated using FT IR and FT Raman spectra along with its molecular geometry, natural bond orbital and its molecular electrostatic potential computed using B3LYP/6–311++G (d,p) basis set. Dihedral angle between pyrimidine and phenyl rings has been extensively studied which plays an important role in the proper docking of the drug molecule in the active site of the enzyme. The strong splitting of the stretching vibrations of amino group in PYR explains the existence of intermolecular N–H···N and N–H···Cl hydrogen bonding network. MPA scheme shows that Cl_{17} atom attached to the phenyl ring is the most reactive site during electrophilic attack.

Keywords: FT-Raman, Anti-malarial, Drug activity, DFT, Pyrimidine ring

1. INTRODUCTION

Pyrimethamine (PYR) drug acts by inhibiting the dihydrofolate reductase activity of the Plasmodium falciparum enzyme dihydrofolate reductase-thymidylate synthase (PfDHFR-TS), which is involved in the reproduction of the parasite. PYR interferes with the biosynthesis of parasite by inhibiting the enzyme DHFR of plasmodia thereby blocking the biosynthesis of purine and pyrimidine which are essential for DNA synthesis and cell multiplication leading to failure of nuclear division at the time of schizont formation in the erythrocytes and liver [1]. The PYR drug shows enhanced activity in combination with Sulfadoxine. When used in combination, it produces a synergistic effect on the parasite and can be effective even in the presence of resistance to individual component [2]. The present work deals with the vibrational spectral investigations of PYR using FT-IR and NIR FT-Raman spectra along with DFT computations to analyze the structural and bonding features responsible for drug activity, nature of hydrogen bonding and charge transfer interactions.

2. EXPERIMENTAL AND COMPUTATIONAL

A Nikon Eclipse 50i microscope using an Nd: YAG laser at 1064 nm of 300 mw output as the excitation source and a liquid nitrogen-cooled Ge-diode detector with the powder sample in a capillary tube to measure the NIR-FT Raman spectrum of PYR. FT-IR spectrum of the sample was recorded using FT-IR 8400s Shimadzu spectrometer in the region 4000–400 cm^{-1} with the standard KBr pellet technique and spectral resolution was 4 cm^{-1}.

Density Functional Theoretical (DFT) computations were performed by B3LYP/ 6-311++G(d,p) basis set using GAUSSIAN'09 program package [3]. Natural bond orbital (NBO) analysis of PYR was performed using NBO 3.1 program at same level.

3. RESULTS AND DISCUSSION

3.1. Structural analysis

Optimized structural parameters of PYR (Figure 1) obtained by DFT and compared with XRD data are listed in Table 1. Slight deviations computed geometry from experimental values is probably due to the intermolecular interactions in the crystalline state. C–C bond lengths in the pyrimidine ring of C_3–C_4 (1.420 Å) is slightly increased compared with C_4–C_5 (1.397 Å) due to the presence of nitrogen. In the benzene ring, the simulated angles of C7–C8–C9 (121.39°), C8–C9–C10 (119.13°), C8–C7–C12 (117.95°), C9–C10–C11 (121.0°), C10–C11–C12 (119.11°) and C7–C12–C11 (121.42°). This deviation of the C–C–C bond angles from the normal value of 120° in the benzene ring shows the asymmetry in the benzene ring due to substitution of chlorine and pyrimidine ring.

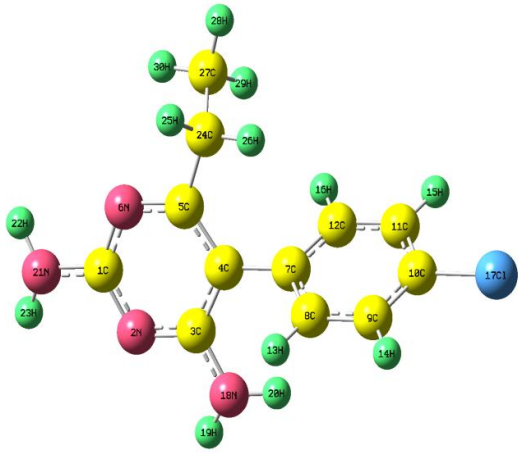

Fig. 1: Optimized molecular structure of PYR

Dihedral angles C_3–C_4–C_7–C_8 is 82.4° (78.04°) and C_5–C_4–C_7–C_{12} is 74.4° (78.92°) between 2,4–diaminopyrimidine and *p*–chlorophenyl rings confirmed pyrimethamine molecules can be described with two dihedral angles involving the pyrimidine and phenyl rings. Thus, the phenyl ring avoids coplanarity with the pyrimidine ring and attains a position approximately perpendicular to it. The same observation has been made in the modelling studies on dihydrofolate reductase-pyrimethamine complexes [4, 5] and it reveals that the relative orientation of the two rings of PYR play a key role in drug binding.

Similarly, another torsion angle C_4–C_5–C_{24}–C_{27} connecting ethyl group with pyrimidine ring is calculated to be 110.2° which represents the deviation of the ethyl group from the benzene plane while the experimental torsion angle is measured to be 97.8°. The formation of dihydrofolate reductase–pyrimethamine complexes indicate that this dihedral angle plays an important role in the proper docking of the drug molecule in the active site of the enzyme and that the change in the torsion

angle representing the orientation of the ethyl group does not affect the overall binding energy of the enzyme-drug complex [6].

Table 1: Geometrical parameters of PYR by DFT along with XRD data

Bond Length	Exp (Å)	Cal (Å)	Bond Angle	Exp. (°)	Cal. (°)	Torsion Angle	Exp. (°)	Cal. (°)
$C_1 - N_2$	1.336	1.339	$C_1–N_2–C_3$	116.47	116.36	$C_1– N_2 – C_3 – C_4$	–0.74	–0.71
$N_2 – C_3$	1.353	1.334	$N_2–C_3–C_4$	122.03	122.45	$N_2– C_3 – C_4 – C_5$	0.88	0.82
$C_3 – C_4$	1.376	1.420	$C_3–C_4–C_5$	116.17	115.56	$C_3– N_2 – C_1 – N_6$	–0.36	–0.32
$C_4 – C_5$	1.414	1.397	$N_2–C_1–N_6$	126.64	126.58	$N_2– C_3– C_4 – C_7$	179.9	117.69
$N_6 – C_1$	1.336	1.337	$C_3–C_4–C_7$	120.22	120.12	$C_3– C_4– C_7– C_8$	–81.2	–78.05
$C_5 – N_6$	1.338	1.343	$C_4–C_7–C_8$	121.08	121.23	$C_4– C_7 – C_8 – C_9$	179.6	179.60
$C_1 –N_{21}$	1.350	1.368	$C_7–C_8–C_9$	120.95	121.29	$C_7– C_8– C_9 – C_{10}$	0.17	–0.18
$C_4 – C_7$	1.492	1.492	$C_8–C_9–C_{10}$	118.80	119.13	$C_8–C_9– C_{10}– C_{11}$	–1.42	–0.14
$C_7 – C_8$	1.381	1.402	$C_9–C_{10}–C_{11}$	121.65	121.00	$C_9–C_{10}–C_{11}– C_{12}$	1.02	0.02
$C_8 – C_9$	1.389	1.392	$C_{10}–C_{11}–C_{12}$	118.91	119.11	$C_1–N_2–C_3–N_{18}$	179.6	178.24
$C_9– C_{10}$	1.366	1.391	$N_2–C_3–N_{18}$	117.12	116.16	$N_2–C_3–N_{18}–H_{19}$	178.8	164.77
$C_{10}–C_{11}$	1.359	1.391	$C_3 –N_{18}–H_{19}$	116.15	116.56	$N_2–C_3–N_{18}–H_{20}$	–13.05	11.93
$C_{11} – C_{12}$	1.385	1.393	$C_3–N_{18}–H_{20}$	122.05	119.68	$C_3–N_2–C_1–N_{21}$	179.5	177.48
$C_{12} – C_7$	1.374	1.402	$N_2–C_1 –N_{21}$	116.89	116.53	$N_2–C_1–N_{21} –H_{22}$	–172.5	–166.00
$N_{21} – H_{22}$	0.852	1.006	$C_1–N_{21} H_{22}$	117.80	117.08	$N_2–C_1–N_2 –H_{23}$	–10.01	–15.50
$N_{21} – H_{23}$	0.808	1.006	$C_1–N_{21}–H_{23}$	116.09	117.49	$C_3–C_4–C_5–C_{24}$	177.8	–179.80
$C_3 – N_{18}$	1.340	1.367	$C_5–C_{24}–H_{25}$	109.41	107.21	$C_4–C_5–C_{24} –H_{26}$	23.67	–13.16
$N_{18} – H_{19}$	0.891	1.007	$C_5–C_{24}–H_{26}$	109.41	110.35	$C_4– C_5– C_{24}–C_{27}$	–97.24	110.22
$N_{18} – C_{20}$	0.877	1.006	$C_5– C_{24}–C_{27}$	111.08	112.53	$C_5–C_{24}–C_{27}–H_{28}$	61.41	176.85
$C_{27}– H_{28}$	0.970	1.093	$C_{24}–C_{27}–H_{29}$	109.47	110.53	$C_5–C_{24}–C_{27}–H_{30}$	178.5	56.84
$C_{10}– Cl_{17}$	1.748	1.759	$C_9 –C_{10}–Cl_{17}$	119.35	119.49	$C_8–C_9–C_{10}–Cl_{17}$	178.3	179.98

3.2. Natural Bond Orbital Analysis

NBO analysis of PYR was performed and presented in Table 2 for both monomer and dimer. Lone pair interaction of $LP(1)N_{21}$ and $LP(1)N_{18}$ with anti-bonding $\pi^*(C_1–N_6)$ and $\pi^*(N_2–C_3)$ has a considerable energy difference (~55 kJ mol^{-1}), which is an evidence for charge transfer from nitrogen atom to the $\pi^*(C_1–N_6)$ and $\pi^*(N_2–C_3)$ and induces partial π character. Intermolecular charge–transfer interactions are formed by the orbital overlap between the bonding (π) and anti-bonding (π^*) orbitals. This investigation obviously clarifies the formation of two intermolecular H–bonded interactions between $LP(1)N_2$, $LP(1)N_{31}$ and $\pi^* N_{40}–H_{42}$, $\pi^* N_{18}–H_{20}$ anti-bonding orbitals respectively, illustrating the existence of strong N–H···N intermolecular hydrogen bonding in PYR supported by the XRD data.

Table 2: 2nd order perturbation theory analysis of Fock matrix in NBO basis for PYR

Donor NBO (i)	Acceptor NBO (j)	$E^{(2)}$ kcal/ mol	E(j) –E(i) a.u.	F(i,j) a.u.
$\pi C_1 – N_6$ (Monomer)	$\pi^* C_4 – C_5$	33.78	0.34	0.096
$\pi N_2 – C_3$ (Monomer)	$\pi^* C_1 – N_6$	39.14	0.31	0.104
$\pi C_4 – C_5$ (Monomer)	$\pi^* N_2 – C_3$	34.18	0.26	0.088
LP_1N_{18} (Monomer)	$\pi^* N_2 – C_3$	56.68	0.28	0.119
LP_1N_{21} (Monomer)	$\pi^* C_1 – N_6$	50.27	0.28	0.113
LP_1N_2 (Dimer)	$\pi^* N_{40} – H_{42}$	8.14	1.06	0.085
LP_1N_{31} (Dimer)	$\pi^* N_{18} – H_{20}$	7.77	1.13	0.085
LP_1N_{37} (Dimer)	$\pi^* C_{32} – N_{33}$	62.15	0.26	0.121
LP_1N_{40} (Dimer)	$\pi^* N_{31} – C_{36}$	59.58	0.26	0.119

3.3. Vibrational spectra analysis

The vibrational analysis of Pyrimethamine is performed on the basis of the characteristic vibrations of amine, Pyrimidine ring, Phenyl ring, Methyl, Methylene groups and skeletal bonds. Raman and Infrared spectra are shown in Figure 2 and 3 as well as calculated vibrational wave numbers assignments are given in Table 3.

3.3.1. Amino group vibrations

Asymmetric and symmetric amino group vibrations are usually expected in the region 3380–3350 cm^{-1} and 3310–3280 cm^{-1}, respectively [7]. Intense IR bands at 3481 and 3468 cm^{-1} correspond to the asymmetric stretching of the amino groups attached to the pyrimidine ring and symmetric stretching vibrations of the amino groups manifest as a strong IR band at 3311 cm^{-1} and at 3311 and 3324 cm^{-1} as split bands in Raman. Lowering of N–H bands provides a strong diagnostic point for detection of interaction between the drug and other molecules due to the possibility of intermolecular N–H···N and N–H···Cl hydrogen bonding hydrogen bonding.

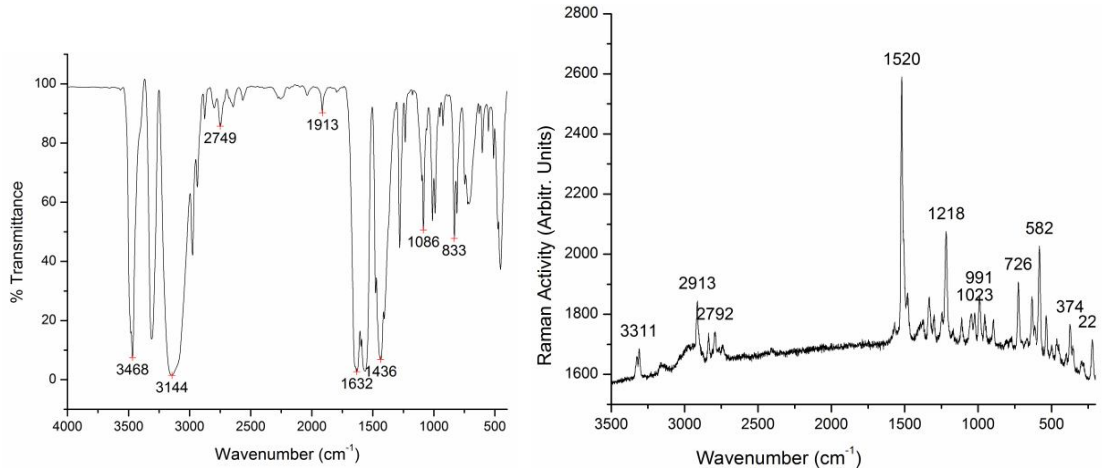

Fig. 2: FT IR and Raman spectra of PYR

3.3.2. Pyrimidine/Phenyl ring vibrations

Numbering of the vibrations of pyrimidine can generally be made by analogy with those for benzene and its derivatives proposed by Wilson. Most of the substituted pyrimidines exhibit four bands in the range 1600–1375 cm^{-1} due to aromatic ring stretches [8]. In pyrimidines, pairs of absorption bands of 8a, 8b, 19a, and 19b have been assigned as the CC and CN coupled vibrations [9]. In PYR, the vibration 8a appears as a very strong band in IR at 1560 cm^{-1} and a weak band in Raman spectrum at 1569 cm^{-1}. The most intense Raman band in PYR at 1520 cm^{-1} and at 1436 cm^{-1} in IR are correlated to very intensive C=C/C-N stretching vibrations (8b and 19b modes) of the pyrimidine ring. Phenyl ring mode *8a* manifests as very strong band in IR at 1596 cm^{-1} and the *8b* mode appears as medium Raman band at 1535 cm^{-1} being calculated at 1584 and 1552 cm^{-1}. With strong donor and acceptor substituents, the *19a* mode in *p*-disubstituted benzene can be expected in the region 1460–1530 cm^{-1} with larger intensity and *19b* appears as medium band between 1370 and 1470 cm^{-1}. The intense IR band observed at 1506 cm^{-1} corresponds to the *19a* mode while the corresponding computed wave numbers are calculated to be 1481 cm^{-1}. *19b* mode appears in Raman at 1374 cm^{-1} (calculated at 1374 cm^{-1}). Carbon-hydrogen stretching vibrations give rise to bands in the region 3100–3000 cm^{-1} in all the aromatic compounds [10]. C–H stretching vibrations in the benzene derivatives arises from two nondegenerate modes a_{1g} (3072 cm^{-1}), b_{1u} (3060 cm^{-1}) and two degenerate modes e_{2g} (3047 cm^{-1}), e_{1u} (3099 cm^{-1}), i.e. vibrations *2, 13, 7* and *20*, respectively. C–H stretching vibration *20b* is observed in IR at 3087 cm^{-1} as strong shoulder bands and the most intense IR band at 3144 cm^{-1} corresponds to ring C–H stretching mode *2*.

Table 3: Vibrational assignments for PYR

$\nu_{calculated}$	ν_{IR}	ν_{Raman}	**Assignments**
3576 3558	3481(vvssh) 3468 (vvs)		NH_2 asymmetric stretch
3459 3445	3311 (vvs)	3324 (w) 3311 (w)	NH_2 symmetric stretch
3100	3144 (vvs)	3158 (w)	2 Benzene ring vibration
3099	3087 (vssh)		20b Benzene ring vibration
3021	2975 (s)		CH_3 asymmetric stretch
3001	2937 (m)		CH_2 asymmetric stretch
2944		2913 (m)	CH_3 symmetric stretch
2933	2877 (w)		CH_2 symmetric stretch
	2800 (w) 2749 (w)	2796 (w) 2785 (w)	N-H· · ·Cl stretch / N-H· · ·N stretch
1605	1651 (vvs) 1631 (vvs)		NH_2 Scissoring
1584	1596 (vs)		8a, Benzene ring vibration
1564	1566 (vvs) 1558 (vvs)	1569 (w)	8a, PYM ring vibration
1552		1535 (vw)	8b, Benzene ring vibration
1537		1520 (vvs)	8b, PYM ring vibration
1481		1506 (ssh)	19a, Benzene ring vibration
1480		1481 (msh)	CH_3 asymmetric bending
1453	1477 (vs)		CH_2 Scissoring
1417	1436 (vvs) 1404 (vvs)		19b, PYM ring vibration/ CH_2 twisting
1379		1374 (vw)	19b, benzene ring vibration
1365		1332 (m)	CH_3 symmetric bending
1317		1298 (w)	CH_2 wagging
1282	1280 (s)		3, benzene ring vibration
1268		1246 (w)	14, benzene ring vibration
1257	1233 (m)		CH_2 Twisting
1229		1218 (s)	CH_2 Twisting
1165	1170 (vvw)	1169 (w)	9a, benzene ring vibration
1139		1113 (w)	NH_2 rocking
1092	1085 (s)		18b, benzene ring vibration
1068		1047 (w)	18a, benzene ring vibration
1046		1022 (m)	CH_3 asymmetric deformation/ NH_2 rocking
1008	1011 (s)		CH_2 Rocking

3.3.3. Methyl/Methylene vibrations

CH_3 asymmetric stretching appears at 2975 cm^{-1} in IR as strong bands and corresponding mode is computed at 3021 cm^{-1}. Symmetric CH_3 stretching mode band is observed at 2913 cm^{-1} as medium Raman band, which is calculated at 2944 cm^{-1} and the asymmetrical and symmetrical bending vibrations of methyl group occur near 1450 and 1375 cm^{-1} respectively [11]. Asymmetrical bending vibrations appear in IR at 1481 cm^{-1} as medium band while the medium Raman band at 1332 cm^{-1} is due to the symmetrical umbrella mode, which are predicted at 1480 and 1365 cm^{-1} by DFT calculations.

Methylene asymmetric and symmetric stretching bands are usually observed near 2953 and 2868 cm^{-1} respectively. Asymmetric stretching modes appear as medium IR band at 2937 cm^{-1} (calculated at 3001 cm^{-1}) while the symmetric stretching vibration is observed at 2877 cm^{-1} (calculated at 2933 cm^{-1}) in IR. Scissoring mode of the CH_2 group gives rise to a characteristic band near 1465 cm^{-1} in IR and Raman spectra which is correlated to the strong band observed at 1477 cm^{-1} in IR.

3.4. Molecular Electrostatic Potential (MESP) Analysis

Molecular electrostatic potential surface of PYR (MESP) was determined by B3LYP/6-311++G(d,p) level in order to understand the relative polarity of the molecule. The variation in electrostatic potential produced by a molecule is largely responsible for the binding of a drug to its receptor binding sites, as the binding site in general is expected to have opposite areas of electrostatic potential. The different values of the electrostatic potential at the surface are represented by different colors: red represents regions of most negative electrostatic potential, blue represents regions of most positive electrostatic potential, and green represents regions of zero potential. It is evident that the region around the hydrogen atoms of the carbon and nitrogen atoms are electron deficient (light blue color), therefore binding site for electrophiles (Figure 3). The region around the nitrogen of the pyrimidine ring represents the most electron rich region and it is the binding site for nucleophiles.

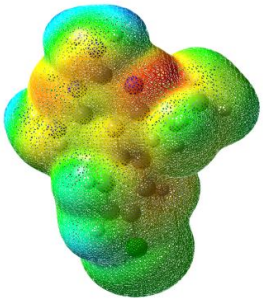

Fig. 3: Electrostatic potential at the surface

4. CONCLUSIONS

Vibrational spectra and DFT quantum chemical computations at B3LYP/6-311++G(d,p) level were performed on PYR to investigate the structural and bonding features responsible for drug activity, nature of hydrogen bonding and charge transfer interactions. Dihedral angle between the pyrimidine ring and phenyl ring indicating the coplanarity of the phenyl ring with the pyrimidine ring. NBO analysis explains the formation of two intermolecular H-bonded interactions illustrating the existence of strong N-H···N intermolecular hydrogen bonding in PYR supported by XRD data. C=C stretching vibrations are expected to be sensitive to possible drug-target interactions. From MESP map the binding sites of electrophiles and nucleophiles have been identified. MPA scheme shows that Cl_{17} atom attached to the phenyl ring, C_9 atom of the phenyl ring and few carbon atoms of the pyrimidine ring are the most reactive sites during electrophilic attack.

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Solid state synthesis of 5–anilino–3–(3,5–dimethylpyrazol–1–yl)–1,2,4–thiadiazoles

K.G. Krishnapriya, K.N. Rajasekharan*

Author Affiliations
Department of Chemistry, University of Kerala, Kariavattom campus, Thiruvananthapuram 695581, Kerala, India.

Corresponding Author
***K.N. Rajasekharan,** Department of Chemistry, University of Kerala, Kariavattom campus, Thiruvananthapuram 695581, Kerala, India.

E-mail: krishnapriyakg06@gmail.com

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Abstract

We wish to report the synthesis of 5–anilino–3–(3,5–dimethylpyrazol–1–yl)–1,2,4–thiadiazoles by a solid state, mechanochemical oxidative cyclization of 3,5–dimethyl–1–[N–(N'–arylthiocarbamoyl) amidino] pyrazoles, which in turn have also been accessed through a solvent free, green technique.

Keywords: 1,2,4–thiadiazole, mechanochemistry, hetarylthiocarbamoylamidine, oxidative cyclization, NMR analysis

1. INTRODUCTION

The synthesis of 1,2,4–thiadiazoles, a versatile class of S–N heterocycles, has claimed recent attention owing to their remarkable biological activities since a wide spectrum of therapeutic applications have been reported for substituted 1,2,4–thiadiazoles [1]. The clinically prescribed antibiotic cefozopram is a 1,2,4–thiadiazole derivative. Several reports exist on the pharmacophore properties of 1,2,4–thiadiazoles. For example, 3,5–bis(indolyl)–1,2,4–thiadiazole derivative showed potent cytotoxicity against human cancer cell lines [2] and a substituted 1,2,4–thiadiazole has been identified for the treatment of multiple sclerosis and rheumatoid arthritis [3]. The bioactive properties of 1,2,4–thiadiazole have also received recent attention in G protein coupled receptor modulation [4] and glycogen synthase kinase inhibition [5]. Literature also exists on the antimicrobial, anti–inflammatory, anticancer, anticonvulsant, antidepressant, antioxidant, radio protective, and anti–leishmanial activities of 1,2,4–thiadiazoles [6-8]

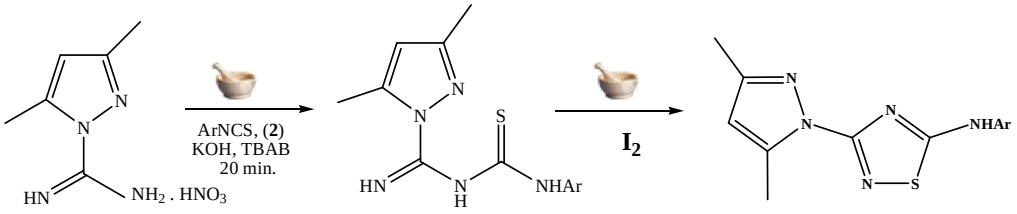
Mechanochemical reactions are effected by either hand grinding or by mechanical milling in the absence of solvents [9]. The latter is carried out using using steel balls in a mixer, shaker or planetary mill. Mechanochemical synthesis has been suggested to be advantageous over the conventional solution phase thermal synthesis due to its energy efficiency, product selectivity, operational

simplicity, health and environmental safety. This field of synthesis has attracted much recent attention as it also aims to establish safe alternatives to synthesis using solvents. The application of mechanochemical method to a wide variety of organic reactions have recently been reviewed [10]. These reactions include Michael additions, Knoevanagel and Barbier reactions, pinacol rearrangement, Grignard reactions, Wittig and Horner–Wadsworth–Emmons reactions as typical examples. A number of metal–catalyzed transformations, including Suzuki, Heck–Mizoroki, Sonogashira and Glaser couplings, have been done mechanochemically, [11]. Mechanochemistry sometimes becomes the preferred method for reactions that are low–yielding and tedious to conduct in solution. For example, N–heteroacenes are mechanochemically synthesised by cyclocondensation between 1,2–dicarbonyl compounds and 1,2–diaminoarenes, avoiding the formation of side products seen in the corresponding solution phase synthesis [12].

Oxidation of thiourea derivatives to form substituted ureas, formamidinium disulfide and 2–aminothiazoles and 1,2,4–thiadiazoles has been recently reviewed [13]. Oxidative cyclisation of suitable thiocarbamoylamidines, thiocarbamoylguanidines and thioureas resulted in 1,2,4–thiadiazoles [1,15]. A recent, green version of this approach the involved iodine–catalyzed oxidative dimerization of thioamides in water using molecular oxygen as an oxidant [15]. We have earlier reported that 1–[N–(N'–arylthiocarbamoyl)amidino]–3,5–dimethylpyrazoles are useful starting materials for 5–acyl–2,4–diaminothiazole and 3,5–diamino–1,2,4–triazole synthesis [16]. We now wish to report the synthesis of 1–[N–(N'–arylthiocarbamoyl)amidino]–3,5–dimethylpyrazoles and their oxidation leading to 5–anilino–1,2,4–thiadiazoles under solid state conditions mechanochemically.

2. RESULTS AND DISCUSSION

We had reported the synthesis of 1–[N–(N'–arylthiocarbamoyl)amidino]–3,5–dimethylpyrazoles earlier by the reaction of 3,5–dimethylpyrazol–1–ylamidine (**1),** either as a salt, or as its free base, with isothiocyantes **(2)** under thermal condition in presence of solvents [16]. Now we report the application of mechanochemistry for the preparation of hitherto unreported 1–[N–(N'–arylthiocarbamoyl)amidino]–3,5–dimethylpyrazoles (**3a–e**). The reaction methodology involves a simple hand grinding of reactants; 1–amidino–3,5–dimethylpyrazole nitrate and aryl isothiocyanates in an agate mortar with a pestle for 20 min in presence of solid KOH and tetra–n–butylammonium bromide TBAB as a solid phase transfer catalyst (Scheme 1).



1 **3a-e** **4a-e**

3a :Ar= 3-CH_3OC_6-H_5
3b :Ar= 3-CH_3C_6-H_5
3c : Ar= 3-ClC_6-H_5
3d : Ar= 4-FC_6H_5
3e : Ar= 4-BrC_6H_5

4a :Ar= 3-CH_3OC_6-H_5
4b :Ar= 3-CH_3C_6-H_5
4c : Ar= 3-ClC_6-H_5
4d : Ar= 4-FC_6H_5
4e : Ar= 4-BrC_6H_5

Scheme 1:

The compounds prepared by this mechanochemical approach are found to be identical with those obtained by corresponding, but lengthy, solution phase thermal reaction by TLC analysis, melting point and FTIR spectral data. The newly obtained 1–[N–(N'–arylthiocarbamoyl)amidino]–3,5–dimethylpyrazoles were characterised by FTIR, ^{1}H NMR, ^{13}C NMR and HRMS. The IR spectrum of the product 3**a** obtained by the reaction of 3,5–dimethylpyrazol–1–ylamidine (**1**) with 3–methoxyphenyl isothiocyanate showed N–H stretching vibrations at 3385 and 3242 cm^{-1}. Bands due

to C–H stretching of the aromatic ring appeared at 3055 $cm^{-1.}$ Bands due to C=C bending of aromatic ring were observed at 1633 cm^{-1}. The NMR spectrum of **3a**, recorded in $CDCl_3$ first seemed to indicate the formation of a mixture of products since discreet peaks with well defined integral hydrogen counts were not present in the spectrum. Instead, peaks were generally broad and showed non– integral hydrogen counts. For example, the peak due to the H4 of the pyrazole ring appeared as a cluster of two broad peaks centered at δ5.87 and δ 6.01 respectively. It was then observed that normalisation of peak areas of other peak clusters, based on the combined integrated area under the above cluster of two broad peaks representing H4, indicated that the cluster of three peaks in the region δ1.90–2.80, with peak–centres at δ 1.99, 2.22 and 2.70, accounted for the six hydrogens of the two methyl groups. Similarly, the broad peak centered at δ 3.78 accounted for three hydrogens of the methoxy group and the peak clusters in the region δ 6.70–7.70 accounted for four aryl hydrogens. The three NH hydrogens appeared as a broad peak at δ 7.89, a cluster of two broad peaks in the region δ 8.30–8.80 and another cluster of two peaks in the region δ 10.40–11.30 three NH hydrogens respectively. The ^{13}CNMR spectrum showed peaks at δ 55.40 corresponding to the methoxy carbon, and benzene ring carbons and the pyrazole ring carbons in the region δ 109–160. The reason for the above peak heterogenity and broadening in the NMR spectra in $CDCl_3$ is attributable to the presence of tautomers. The other possibilities of the formation of a mixture of products, or due to product decomposition or dimerisation, were ruled out by the following observations. The repetition of the reaction under entirely different conditions developed earlier by us [16] afforded the same product as evidenced by FTIR, mp and mixed mp studies and in comparable yield, thus excluding the formation of a product mixture. The HRMS data showed no evidence for any other side products. Based on the above data, the structure of the compound obtained from 3,5–dimethylpyrazol–1–ylamidine (**1)** with 3–methoxyphenyl isothiocyanate was assigned as 1–[N–(N'–3–methoxyphenylthiocarbamoyl) amidino]–3,5–dimethylpyrazole **(3a)**. Additional confirmation of the product homogenity of **3a** arose from its subsequent oxidative cyclisation under very mild conditions leading to a single product, as described below. Similar reaction of 3,5–dimethylpyrazol–1–ylamidine nitrate (**1**) with 3–methylphenyl, 3–chlorophenyl, 4–bromophenyl and 4–fluorophenyl isothiocyanates (**2b–e)** afforded 1–[N–(N'–arylthiocarbamoyl)amidino]– 3,5–dimethylpyrazoles **(3b–e)** in fairly good yield.

In the light of our earlier success in the synthesis of heterocycles including 2,4–diaminothiazoles and 1,2,4–triazoles starting from 1–[N–(N'–arylthiocarbamoyl)amidino]–3,5–dimethylpyrazoles [16] [Jenardanan *et al.,* 1997] and recently reported mechanochemical heterocycle synthesis [Sherin *et al*., 2015], we have now attempted a solid state synthesis of 5–anilino–3–pyrazol–1–yl–1,2,4–thiadiazoles by the oxidative cyclisation of 1–[N–(N'–arylthiocarbamoyl)amidino]–3,5–dimethylpyrazoles. Such pyrazole substituted 1,2,4–thiadiazoles had been synthesised by Godfrey and Kurzer by the reaction of acetylacetone on –anilino–3–hydrazino–1,2,4–thiadiazole [17]. Our solid state synthesis of thiadiazoles involved the grinding of 1–[N–(N'–arylthiocarbamoyl)amidino]–3,5–dimethylpyrazoles (**3a–e**) with iodine for 5–10 mins. The resulting compounds are found to be identical with that obtained from corresponding solution phase reaction. Thus, our work now provides a rapid, solventless alternative to access the title compounds. The IR spectrum of the product **4a** formed by the oxidative cyclisation of 1–[N–(N'–3–methoxyphenylthiocarbamoyl)amidino]–3,5–dimethylpyrazole showed N–H stretching vibrations at 3267 and 3216 cm^{-1}. Bands due to C–H stretching of the aromatic ring appeared at 3066 $cm^{-1.}$ Bands due to C=C stretching of aromatic ring were observed at 1628 cm^{-1}. In the ^{1}H NMR spectrum, two methyl groups in the pyrazole ring were appeared as singlet each at δ 2.19 and 2.56. A singlet at δ 3.78 is assignable to the methoxyphenyl group and another one at δ 6.12 is attributed to the H4 of the pyrazole ring. The four aryl hydrogens appeared as two doublets at δ 6.70 and 7.05, a singlet at δ 7.23 and a triplet at δ 7.33. A singlet at δ 11.19 is ascribed to the NH hydrogen. In the ^{13} C NMR spectrum peaks at 13.82 and 14.10 corresponds to methyl carbons, methoxy carbon appeared at 55.61. The pyrazole, benzene and thiadiazole carbons were seen between 104–180 ppm. The spectral evidences confirm the structure of the compound obtained as 5–(3–methoxyanilino)–3–(3,5–dimethylpyrazol–1–yl)–1,2,4–thiadiazoles (**4a**).

Table 1: Yield and melting point of 1–[N–(N'–arylthiocarbamoyl)amidino]–3,5–dimethylpyrazoles (**3a–e**)

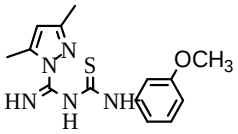
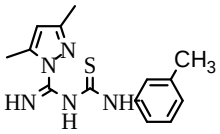
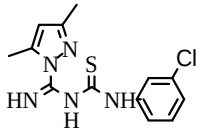
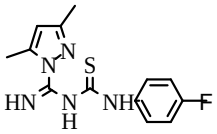
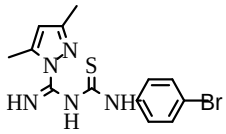
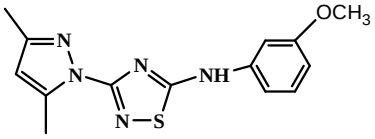
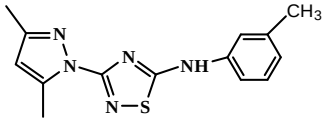
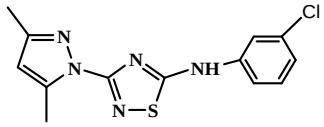
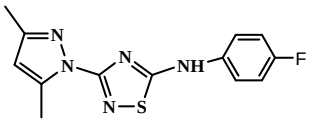
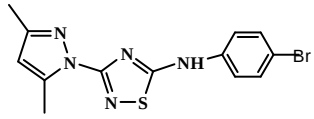
Sl. No.	Compound	Yield (%)	Melting point (°C)
1	 **3a**	65%	115–117
2	 **3b**	63%	86–88
3	 **3c**	75%	136–138
4	 **3d**	60%	121–122
5	 **3e**	62%	123–125

Table 2: Yield and melting point of 5–anilino–3–(3,5–dimethylpyrazol–1–yl)–1,2,4–thiadiazoles (**4a–e**)

Sl. No.	Compound	Yield (%)	Melting point (°C)
1	 **4a**	68%	225–27
2	 **4b**	64%	240–242
3	 **4c**	63%	275–276

4	 **4d**	68%	253–254
5	 **4e**	62%	222–223

3. EXPERIMENTAL

All melting points are uncorrected and were determined by open capillary method. The thin layer chromatography analyses were performed using silica gel–G. coated on glass plates or commercial plastic plates. The spots were visualized in iodine vapour or under UV light. The IR spectra were recorded on Shimadzu FTIR spectrophotometer. The NMR spectra were recorded in $CDCl_3$ and DMSO on Bruker DPX 400 MHZ NMR spectrometer at room temperature. The HRMS–ESI analysis was performed on an Agilent 6520 QTOF–MS/MS system. All chemicals used were from Sigma Aldrich and E. Merck, India.

3.1. *Synthesis of 1–[N–(N'–arylthiocarbamoyl)amidino]–3,5–dimethylpyrazoles (3a–e)*

Method A:

1–Amidino–3,5–dimethyl–pyrazole nitrate (100 mg, 0.5 mmol), powdered potassium hydroxide (28 mg, 0.5 mmol) and tetra–n–butylammonium bromide TBAB (5 mg) were ground in a mortar and pestle for 3 min. To this mixture, aryl isothiocyante (0.5 mmol) was added and the grinding continued at room temperature for 15–20 minutes. An initially observed oil like appearance of the ground mixture turned to a solid mass. The solid then was worked up by grinding with petroleum ether, followed by water, to obtain 1–[N–(N'–arylthiocarbamoyl)amidino]–3,5–dimethylpyrazoles. The products were then collected by decantation and filtration. Purification involved crystallization from ethanol–water (1:1).

Method B:

The above reactions between 1–amidino–3,5–dimethyl–pyrazole nitrate (**1**) and aryl isothiocyanates (**2a–**e) were repeated under conditions developed earlier in our laboratory. Thus, to a suspension of **1** (100 mg, 0.5 mmol mmol) in N,N–dimethylformamaide (DMF, 2 mL), containing powdered KOH (28 mg, 0.5 mmol), aryl isothiocyanates (0.5 mmol) in DMF (1 mL)was added dropwise with stirring at 0 ^{o}C during 20 mins. The temperature of the reaction mixture was then raised to 65–70 ^{o}C and the stirring was continued for another 2 h. The reaction mixture was then poured into ice water to obtain solid product.

The compounds prepared by this method were:

1–[N–(N'–3–Methoxyphenylthiocarbamoyl)amidino]–3,5–dimethylpyrazole (3a): Yield: Method A: 65%, m.p**.** 115–117 °C.; Method B: 71%; m. p. 118 °C; IR: 3385, 3242, 3055, 1633, 1539, 1390, 1317, 1114, 968, 825, 684 cm^{-1} ; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ : 1.90–2.80, bm, 6H; 3.70–3.90, b, 3H; 5.80–6.10, b, 1H; 6.40–7.60, m, 4H; 7.70–8.00, b, 1H; 8.30–8.80, b 1H; 10,45–11.25, b,1H ; ^{13}C NMR (100MHz, $CDCl_3$, 25 °C): δ = 14.13, 14.98, 16.16, 55.40, 111.43, 111.89, 117.85, 129.44, 139.41, 143.96, 151.69, 155.68, 188.64.

HRMS (ESI): $[M+H]^+$ 304. 1154 (calcd), 304.1226 (found)

1–[N–(N'–3–methylphenylthiocarbamoyl)amidino]–3,5–dimethylpyrazole (3b): Yield: Method A: 63%, m.p**.** 86–88 °C. Method B: 70 %; m. p. 87 °C ; IR: 3194, 3034, 1641, 1481, 1392, 1313, 1124, 975, 840, 775, 684 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ : 1.85–2.80, bm, 9H; 5.75–6.10, b, 1H; 6.90–7.55, m, 4H; 7.80–8.10, b, 1H; 8.35–8.55, b 1H, 10.40–11.15, b,1H.
^{13}C NMR (100MHz, $CDCl_3$, 25 °C): δ = 14.13, 14.90, 14.23, 21.26, 111.33, 115.63, 119.47, 122.62, 12515, 125.44, 126.19, 128.56, 138.63143.95, 151.63, 155.61, 188.62.
HRMS (ESI): $[M+H]^+$ 288. 1205 (calcd), 288.1275 (found).

1–[N–(N'–3–Chlorophenylthiocarbamoyl)amidino]–3,5–dimethylpyrazole (3c): Yield: Method A: 75%, m.p**.** . 136–138 °C. Method B: 79 %; m. p. 137 °C ; IR : 3401, 3174, 3058, 1613, 1523, 1479, 1363, 1013, 911 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ : 1.90–2.85, bm, 6H; 5.80–6.15, b, 1H; 7.05–7.55, m, 4H; 7.85–8.20, b, 1H, 8.25–8.60, b 1H, 10.60 –11.20, b,1H.; ^{13}C NMR (100MHz, $CDCl_3$, 25 °C): δ = 13.66, 13.71, 15.10, 111.63, 116.59, 118.61, 122.47. 122.96, 124.22125.71, 129.73, 130.65, 134.22, 139.39, 143.82, 151.94, 156.16, 188.65; HRMS (ESI): $[M+H]^+$ 308. 0658 (calcd), 308.0723 (found).

1–[N–(N'–4–Fluorophenylthiocarbamoyl)amidino]–3,5–dimethylpyrazole (3d): Yield: Method A: 60%, m.p**.** 121–122 °C. Method B: 65 %; m. p. 122 °C ; IR : 3453, 3200, 3032, 1635, 1501, 1326, 1214, 964, 823 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ : 1.85–2.75, bm, 6H; 5.75–6.15, b, 1H; 6.95–7.65, m, 4H; 7.80–8.05, b, 1H, 8.15–8.90, b 1H, 10.65 –11.25, b,1H ; ^{13}C NMR (100MHz, $CDCl_3$, 25 °C): δ = 13.70, 14.88, 16.19, 111.48, 115.50, 115.72, 126.88, 127.76, 127.84, 134.52, 143.85, 151.81, 155, 88, 162.23, 189.18.
HRMS (ESI): $[M+H]^+$ 292. 0954 (calcd), 292.1031 (found).

1–[N–(N'–4–Bromophenylthiocarbamoyl)amidino]–3,5–dimethylpyrazole (3e): Yield: Method A: 62%, m.p**.** 123–125 °C. Method B: 68 %; m. p. 122 °C; IR : 3408, 3360, 3061, 1620, 1579, 1428, 1285, 1114, 968 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ : 1.80–2.85, bm, 6H; 5.80–6.10, b, 1H; 7.05–7.65, m, 4H; 7.80–8.15, b, 1H, 8.15–8.90, b 1H, 10.45 –11.30, b,1H ; ^{13}C NMR (100MHz, $CDCl_3$, 25 °C): δ = 13.71, 15.10, 16.06, 111.65, 119.34, 125.96, 126.95, 132.00, 137.34, 143.79, 144.79, 151.49, 155,94, 188.67.
HRMS (ESI): $[M+H]^+$ 352. 0153 (calcd), 352.0206 (found).

3.2. *Synthesis of 5–anilino–3–(3,5–dimethylpyrazol–1–yl)–1,2,4–thiadiazoles*

Method A: solid stae reaction

1–[N–(N'–Arylthiocarbamoyl)amidino]–3,5–dimethylpyrazoles (**3a–e**) (0.5 mmol) and iodine (0.5 mmole) were ground for 5–10 mins in an agate mortar. Work up by grinding with few drops dilute aq. ammonia solution, followed by water, afforded solid 5–anilino–3–(3,5–dimethylpyrazol–1–yl)–1,2,4–thiadiazoles (**4a–e**). The products could be collected by filtration. Purification involved crystallization from ethanol.

Method B:Solution phase reaction

To a solution of **3a–e** (0.5 mmol) in methanol (5mL), iodine solution in methanol (0.5mmol) was added and kept closed at room temperature with occasional shaking for 1 h. Work up with dilute aq. ammonia solution, followed by water, afforded solid 5–anilino–3–(3,5–dimethylpyrazol–1–yl)–1,2,4–thiadiazoles (**4a–e**). The compounds prepared by the above two methods were identical as shown by TLC, FTIR, m.p. and mixed m. p. anaylsis.

5–(3–Methoxyanilino)–3–(3,5–dimethylpyrazol–1–yl)–1,2,4–thiadiazole (**4a**): Yield: Method A: 68%, m.p**.** 225–27 °C. Method B: 64%; m. p. 226 °C ; IR: 3267, 3216, 3066, 1628, 1531, 1463, 1378, 1218, 842, 771 cm^{-1} ; ^{1}H NMR (400 MHz, DMSO, 25 °C): δ = 2.19 (s, 3H, CH_3), 2.56 (s, 3H, CH_3), 3.78 (s, 3H, OCH_3), 6.12 (s, 1H, pyrazole H), 6.70 (d, 1H, ArH, J= 9.2 Hz), 7.05 (d, 1H, ArH, J=7.8 Hz), 7.23 (s, 1H, ArH), 7.33 (t, 3H, ArH, J= 8.2 Hz), 11.19 (s, 1H, NH) ; ^{13}C NMR (100MHz, DMSO,

25 °C): δ = 13.82, 14.10, 55.61, 104.37, 109.05, 109.12, 110.68, 130.80, 140.94, 141.91, 149.90, 158.94, 160.55, 179.12

5–(3–Methylanilino)–3–(3,5–dimethylpyrazol–1–yl)–1,2,4–thiadiazole (**4b**): yield: Method A: 64%, m.p. 240–42 °C. Method B: 65%; m. p. 240–42 °C ; IR: 3252, 3204, 3081, 1620, 1531, 1382, 1166, 931 cm^{-1} ; ^{1}H NMR (400 MHz, DMSO, 25 °C): δ = 2.19 (s, 3H, CH_3), 2.33 (s, 3H, CH_3), 2.57 (s, 3H, CH_3), 6.12 (s, 1H, pyrazole H), 6.94 (d, 1H, ArH, J= 7.2 Hz), 7.28–7.36 (m, 3H, ArH), 11.14 (s, 1H, NH) ; ^{13}C NMR (100MHz, DMSO, 25 °C): δ = 13.82, 14.09, 21.65, 109.05, 115.52, 118.94, 124.57, 129.78, 139.29, 139.78, 141.93, 149.89, 158.89, 179.22

5–(3–Chloroanilino)–3–(3,5–dimethylpyrazol–1–yl)–1,2,4–thiadiazole (**4c**): Yield: Method A: 63%, m.p. 275–76 °C. Method B: 65%; m. p 275°C ; IR: 3241, 3118, 3058, 1613, 1531, 1423, 1382, 1121, 976, 801 cm^{-1} ; ^{1}H NMR (400 MHz, DMSO, 25 °C): δ = 2.20 (s, 3H, CH_3), 2.58 (s, 3H, CH_3), 6.14 (s, 1H, pyrazole H), 7.17(s, 1H, ArH), 7.44 (s, 2H, ArH),7.87 (1H, ArH), 11.35 (s, 1H, NH) ; ^{13}C NMR (100MHz, DMSO, 25 °C): δ = 13.83, 14.09, 109.20, 116.80, 117.87, 123.27, 131.52, 134.16, 141.07, 141.97, 150.07, 178.87

5–(4–Fluoroanilino)–3–(3,5–dimethylpyrazol–1–yl)–1,2,4–thiadiazole (**4d**): yield: Method A: 68%, m.p. 253–254 °C. Method B: 72%; m. p. 253 °C ; IR: 3271, 3218, 3036, 1628, 1589, 1501, 1441, 1393, 1218, 1106, 827 cm^{-1} ; ^{1}H NMR (400 MHz, DMSO, 25 °C): δ = 2.19 (s, 3H, CH_3), 2.57 (s, 3H, CH_3), 6.12 (s, 1H, pyrazole H), 6.94 (d, 1H, ArH, J= 7.2 Hz), 7.28–7.36 (m, 3H, ArH), 11.14 (s, 1H, NH) ; ^{13}C NMR (100MHz, DMSO, 25 °C): δ = 13.82, 14.04, 109.04, 116.41, 116.64, 120.29, 136.30, 141.94, 149.86, 157.34, 158.90, 159.73, 179.25

5–(4–Bromoanilino)–3–(3,5–dimethylpyrazol–1–yl)–1,2,4–thiadiazole (**4e**): Yield: Method A: 90%, m.p. 222–223 °C. Method B: 96%; m. p 222 °C ; IR: 3241, 3181, 3048, 1609, 1557, 1479, 1397, 1300, 1110, 81 cm^{-1} ; ^{1}H NMR (400 MHz, DMSO, 25 °C): δ = 2.19 (s, 3H, CH_3), 2.54 (s, 3H, CH_3), 6.12 (s, 1H, pyrazole H), 7.54–7.61(m, 4H, ArH), 11.26 (s, 1H, NH)
^{13}C NMR (100MHz, DMSO, 25 °C): δ = 13.83, 14.06, 109.09, 115.20, 120.28, 131.92, 132.61, 139.10, 141.99, 149.94, 158.86, 178.87

4. CONCLUSION

In conclusion, we have now shown that 3,5–dimethyl–1–[(*N*–arylthiocarbamoyl)amidino] pyrazoles are useful starting materials for accessing 5–anilino–1,2,4–thiadiazole derivatives, thus complementing our earlier report on their use to prepare 2,4–diaminothiazoles and 3,5–diamino–1,2,4–triazoles. We have also demonstrated that the above oxidative heterocyclization of 3,5–dimethyl–1–[(*N*–arylthiocarbamoyl) amidino] pyrazoles can effectively be achieved by mechanochemically in a green chemical approach.

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Investigations of vibrational spectra and bioactivity of ethyl N–[1–(piperidin–1–ylmethyl)benzimidazol–2–yl]carbamate

G.P. Sheeja Mol[1], D. Aruldhas[1,*]

Author Affiliations
[1]Department of Physics & Research Centre, Nesamony Memorial Christian College, Marthandam-629165, TamilNadu, India

Corresponding Author
***D. Aruldhas,** Department of Physics & Research Centre, Nesamony Memorial Christian College, Marthandam-629165, TamilNadu, India

E-mail: aruldhas2k4@gmail.com

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Abstract

In this work, FT–IR and FT–Raman spectra of Ethyl N–[1–(piperidin–1–ylmethyl) benzimidazol–2–yl]carbamate have been recorded in the regions 4000–400 cm^{-1} and 3500–50 cm^{-1} respectively. The molecular structure, geometry optimization, intensities and vibrational wave numbers were obtained by DFT levels of theory B3LYP with 6–311G(d,p) standard basis set. The complete vibrational distributions were performed on the basis of the potential energy distribution (PED) of the vibrational energy distribution analysis (VEDA 4) program. The harmonic frequencies were calculated and scaled values were compared with experimental FT–IR and FT–Raman data. The oscillator strength, wavelength and energy were also calculated by TD–DFT method. Molecular electrostatic potential (MESP), total electron density distribution and frontier molecular orbitals (FMOs) are constructed at B3LYP/6311G(d,p) level to understand the electronic properties. The charge density distribution and site of chemical reactivity of the molecules has been obtained by mapping electron density isosurface with electrostatic potential surfaces (ESPs. Ethyl N–[1–(piperidin–1–ylmethyl) benzimidazol–2–yl]carbamate was screened for its antifungal activity.

Keywords: Ethyl N–[1–(piperidin–1–ylmethyl) benzimidazol–2–yl]carbamate, HOMO, LUMO, MESP, Antifungal

1. INTRODUCTION

EthylN–[1–(piperidin–1ylmethyl) benzimidazol–2–yl]carbamate is a systemic agricultural fungicide used to control of certain fungal diseases of stone fruit. Title compound belongs to the family of Benzimidazoles. These are organic compounds containing a benzene ring fused to an imidazole ring. It is a systemic benzimidazole fungicide that is selectively toxic to microorganisms and invertebrates, interfering with cell functions, such as meiosis and intracellular transportation. The selective toxicity of benomyl as a fungicide is possibly due to its heightened effect on fungal rather

than mammalian microtubules [1]. Calmon etal. [2] explained the Kinetics and mechanisms of conversion of methyl 1–(butylcarbamoyl)–2–benzimidazole carbamate (benomyl) to methyl 2–benzimidazolecarbamate (MBC). Decreased permeability as a mechanism of resistance to methyl benzimadazol–2–yl carbamate (MBC) in Sporobolomyces roseus was explained by Nachmias et al. [3]. Van ketel [4] reported the Sensitivity to the pesticide benomyl. Fiscor etal. [5] supported the mutagenicity testing of benomyl, methyl–2–benzimidazole carbamate, streptozotocin and N–methyl–N'–nitro–N–nitrosoguanidine in Salmonella typhimurium in vitro and in rodent host–mediated assays. The main objective of the present study is on the structural analysis of present compound using theoretical and experimental techniques. To the best of our knowledge no detailed spectroscopic studies of these compounds were performed.

2. EXPERIMENTAL

Compound was purchased from Sigma Aldrich (St.Louis, MO, USA) and used without further purification. The room temperature fourier transform infrared spectra of the title compound was measured in the region 400–4000cm^{-1} , at a resolution of +1 cm^{-1}, using Perkin Elmer Spectrometer equipped with mercury vapour lamp and globar as source. The UV-Visible spectra of compound were examined in the range 190–900 nm using CARY100 BIO UV-Visible Spectrometer in acetone solvent. The antifungal activity of compound was analysed by the well diffusion method against the fungi Aspergillus niger and Candida albicans.

2.1. Computational

The combination of spectroscopic methods with DFT calculations are the powerful tools for understanding the fundamental spectral properties and the electronic structure of the compounds. Gaussian 09 software program package was used for theoretical calculation [6]. The quantum chemical calculations were performed by applying density functional theory [7–8] method with the three parameter hybrid functional (B3) [9–10] for the exchange part and the Lee–Yang–Par (LYP) correlation function [11]. The wave number values computed contain known systematic errors and therefore, scaling factor 0.9682 [12] has been used. The potential energy distribution (PED) was calculated with the help of VEDA 4 program package [13]. The Raman activities (S_i) calculated by Gaussian 09 program has been converted to relative Raman intensities (I_i) using the following relationship derived from the basic theory of Raman scattering [14].

$$I_i = \frac{f(\nu_0 - \nu_i)^4 S_i}{\nu_i\left[1 - \exp\left(-\frac{hc\nu_i}{k_b T}\right)\right]}$$

where ν_0 is the exciting wavenumber, ν_i the vibrational wavenumber of the normal mode, h, c and k_b are the universal constants and f is the suitably chosen common normalization factor for all the peak intensities.UV-Visible spectra, electronic tranistions, vertical excitation energies and oscillator strengths were computed with the time-dependent DFT method. Gaussview 5.0.8 visualization program [15] has been utilized to the shape of highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO).

3. RESULTS AND DISCUSSIONS

3.1. Structural analysis

The optimized molecular structure of compound was performed by using Gaussian 09 program. The results of calculated geometrical parameters (bondlengths, bond angles and dihedral angles) were compared with the experimental values [16–17]. In benzimidazole region, the bondlength C_{12}–N_{11}(1.327Å) shortened while comparing to other C–N bondlengths like C_{12}–N_{13}(1.338 Å) and C_{12}–N_{14} (1.343 Å) due to the influence of N_{14}–H_{15}…O_{24} strong intramolecular hydrogen bonding. The optimized structure of compound as shown in Figure 1.

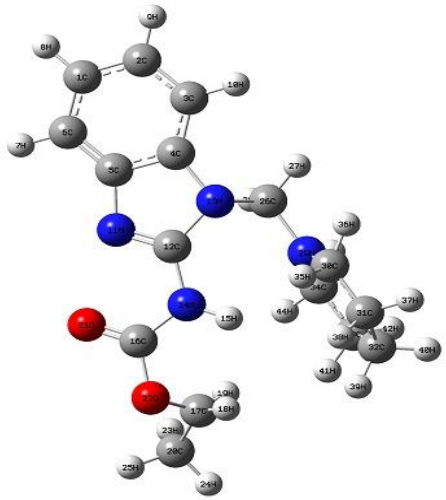

Fig. 1: Optimized molecular structure

The bondlengths N_{11}–C_{12}–N_{13}(109.3°), N_{11}–C_{12}–N_{14}(123.8°) decreases while comparing to N_{13}–C_{12}–N_{14}(127.0°) .In title compound, the dihedral angles N_{11}–C_{12}–N_{14}–H_{15}(–178.8°), N_{11}–C_{12}–N_{14}–C_{16} (1.1°), N_{13}–C_{12}–N_{14}–H_{15}(0.5°) and N_{13}–C_{12}–N_{14}–C_{16}(–179.6°) exhibit the nonplanarity nature of the benzimidazole region.

3.2. Vibrational analysis

The compound consists of 44 atoms, it has 126 normal vibrational modes. The calculations were made for a free molecule in vacuum, while experiments were made for a solid sample. Complete assignments of the fundamentals were propsed based on the calculated PED values. The FT–IR spectrum of compound shown in Figure 2.

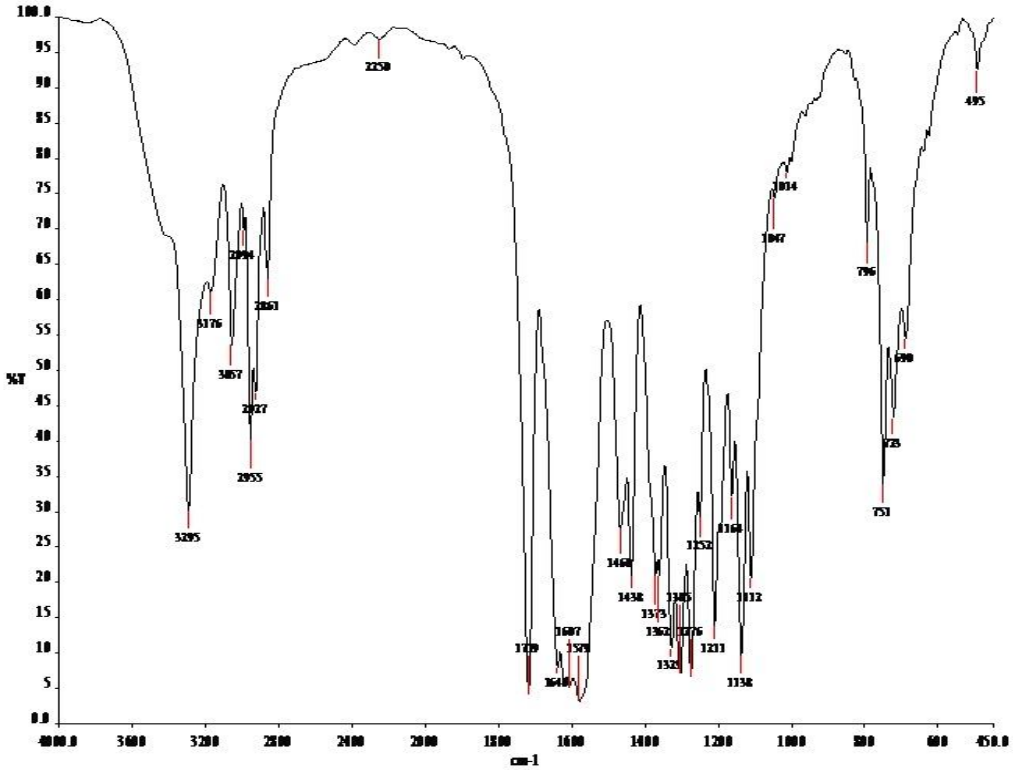

Fig 2: FT–IR Spectrum

3.2.1. N–H vibrations

Normally, in all the heterocyclic compounds, the N–H stretching vibration occurs in the region 3500–3000 cm^{-1} [18]. The band appear at 3295 cm^{-1}in FTIR spectrum is assigned to the N–H stretching mode of the compound.

3.2.2. C=N and C–N vibrations

In general, imidazoles have three or four bands in the regions1660–1450 cm^{-1} due to C=N and C=C stretching vibrations. The intensities of these bands depend on the nature and position of the substituents. Benzimidazoles absorbs at 1560–1520 cm^{-1} due to C=N stretching vibration.. Thus, the band observed at 1487 cm^{-1} is assigned to C=N stretching vibration. The red shift of the C=N stretching frequency of title compound is due to the presents of carbonyl group at C_{12} carbon. The fundamental modes observed at 1398, 1249, 1215 cm^{-1} and 1259cm^{-1} in the IR spectrum, are assigned to C–N stretching modes. The CNC in–plane bending mode is calculated at 957 cm^{-1}. The CNC out of plane bending mode is assigned at 628 cm^{-1} in the IR spectrum. The NCN out of plane bending mode is found at 598 cm^{-1} in IR spectrum. The CCN out of plane bending mode is attributed to 329 cm^{-1} in Raman spectrum.

3.2.3. C–H vibrations

The strong to medium intensity bands occur in the region 3100–3000 cm^{-1} is common for aromatic structure. In the present study, the aromatic C–H stretching vibrations are observed at 3047 cm^{-1} in IR spectrum. The bands due to C–H in–plane bending vibrations occur in the region1290–950 $cm^{-1.}$ The bands observed at 1154, 1124, 1008 cm^{-1} in IR spectrum are assigned to C–H in–plane bending vibrations of the molecule. The C–H out of plane bending modes is usually medium intensity and is observed in the region 950–600 $cm^{-1.}$ The out of plane C–H bending vibrations are observed at 931 and 851 cm^{-1} in IR spectrum.

3.2.4. C–C vibrations

The ring C–C stretching vibrations occur in the region 1650–1430 cm^{-1} [19]. In the present study, the C–C stretching vibrations of compound are observed at 1629 and 1596 cm^{-1} in the infrared spectrum. The C–C stretching vibrations are assigned to the modes 1353 and 1314 cm^{-1}in the IR .The ring CCC in–plane bending vibrations are generally weak often being masked by other stronger absorptions due to the substituent groups. The in–plane bending vibrations of compound are theoretically determined at 891, 808, 582 $cm^{-1}$and the calculated out of plane vibrations are 431, 392, 249, 139 and 101 $cm^{-1.}$These assignments are in good agreement with literature.

3.3 UV–visible spectral analysis

Electronic transitions have been investigated by UV–Visible spectroscopy. Absorption maximum (λ_{max}) was calculated by TD–DFT method. The experimental UV–Visible absorption spectrum of the sample is shown in figure 3. The UV–Visible spectrum was measured in acetone and it is found that the absorption bands are observed at 210nm. A very strong band at 210nm is a characteristic peak of aromatic system due to $\pi \rightarrow \pi^*$ transition.

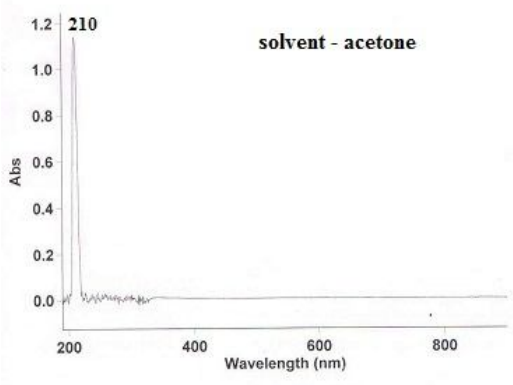

Fig. 3: UV–Visible spectrum

3.4 HOMO–LUMO ANALYSIS

Molecular orbitals, both the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) and their properties such as energy are very useful for physicists and chemists are the main orbital taking part in chemical reaction while the energy of the HOMO is directly related to the ionization potential, LUMO energy is directly related to the electron affinity [20–21]. This is also used by the frontier electron density for predicting the most reactive position in π–electron system and also explains several types of reaction in conjugated system [22]. The conjugated molecules are characterized by a small highest occupied molecular orbital–lowest unoccupied molecular orbital separation, which is the result of a significant degree of intramolecular charge transfer from the end–capping electron–donor groups to the efficient electron–acceptor group through π–conjugated path[23]. Surfaces for the frontier orbitals were drawn to understand the bonding scheme of compound1 and it's related compounds. The energy difference between HOMO and LUMO orbital which is called as energygap is a critical parameter in determining molecular electrical transport properties because it is a measure of electron conductivity.

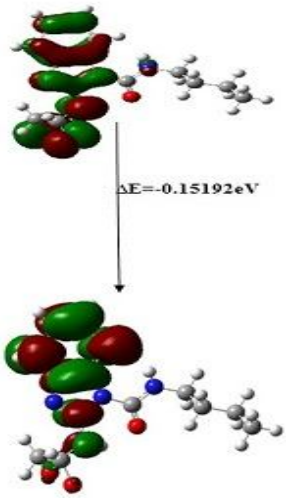

Fig. 4: HOMO–LUMO ENERGY GAP

3.5. MESP

The electrostatic potential is also well suited for analyzing processes based on the "recognition" of one molecule by another, as in drug–receptor, an enzyme–substrate interactions, because it is through their potentials that the two species first "see" each other [24–25]. To predict reactive sites of electrophilic and nucleophilic attacks for the investigated molecule, MESP at the B3LYP/6–311G (d,p) optimized geometry was calculated. The negative (red and yellow) regions of MESP were related to electrophilic reactivity and the positive (blue) regions to nucleophilic reactivity (Fig.5). The predominance of green region in the MESP surfaces corresponds to a potential halfway between the two extremes red and blue colour. As can be seen from the MESP map of the title compound, negative regions are mainly localized over the carbonyl groups The maximum positive regions are localized over the benzimidazole ring. As can be seen from the MESP map, regions having the negative potentials are over the electronegative atoms.

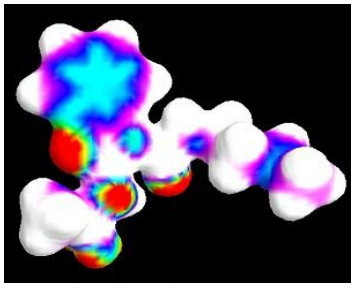

Fig. 5: Molecular Electrostatic potential

3.6. Antifungal activity

The antifungal activity of title compound lwere evaluated by the well–diffusion method against the fungi viz, Aspergillus niger and candida albicans cultured on potato dextrose agar as medium. The stock resolution was prepared by dissolving the compounds in DMSO and the solutions were serially diluted to find MIC (Minimal Inhibitory Concentration) values wells of approximately 10 mm was bored using a well cutter and samples of different concentration was added. The zone of inhibition was measured after overnight incubation and compared with that of standard antibiotics. The well was filled with the test solution using a micropipette and the plate was incubated 72H for fungi at 35 °C. During this period the test solution diffused and the growth of the inoculated microorganisms was affected. The inhibition zone was developed, at which the concentration was noted [26].The antifungal and solvent test for fungal strains were increased and mentioned in Table 1. It is noticed that itself has no activity on the figure.

Table 1: In vitro Antifungal activity

Sl. No.	Fungal pathogen	Zone of inhibition(mm)			
		250(µg)	500(µg)	1000(µg)	Clotrimazole(µg)
1	Aspergillus niger	15	25	25	30
2	Candida albicans	nil	nil	15	27

The experimental values show considerable activity observed in candida albicans against aspergillus niger, Clotrimazole as the standard drug used for this study. The inhibitory activity of the synthesized compound on the organism shows high activity in high concentration of the title compound increases; the inhibition of fungal strains also increases. Figure 6(a) and 6(b) indicates the antifungal activity of title compound at different concentrations for two antifungal pathogens (aspergillus niger and candida albicans). The presence of benzimidazole region in title compound is responsible for the antifungal action.

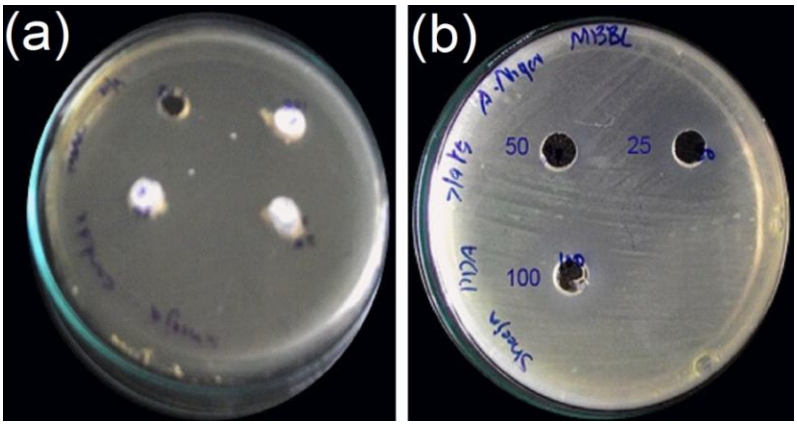

Fig. 6: Antifungal activity with (a) aspergillus niger and (b) candida albicans

5. CONCLUSIONS

In the present work, the optimized geometric parameters (bondlengths, bond angles and dihedral angles) were theoretically determined and compared with the experimental results. The increase in wavenumber from the expected value leads to the redshift and exhibits the possibility of intramolecular hydrogen bonding. The lowering of HOMO-LUMO bandgap supports fungicidal activity of title compound. The experimental UV-Visible spectrum shows an absorption maxima at 210 nm($n\rightarrow\pi^*$) in the acetone solvent. The maximum positive regions are localized over the benzimidazole region. Thus from above investigations, it can be concluded that title compound is a good antifungal agent to treat diseases and further work can be responsible for biological activity.

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Optical and dielectric investigations of nano crystalline scheelite $A_{0.5}B_{0.5}MoO_4$ (A=B=Ba, Sr and Ca)

S. Vidya[1]*, J.K. Thomas[2]

Author Affiliations
[1]Department of Physics, Sree Narayana College, Kollam, India - 691001
[2]Electronic Materials Research Laboratory, Department of Physics, Mar Ivanios College, Thiruvananthapuram, Kerala, India-695 015

Corresponding Author
***S. Vidya,** Department of Physics, Sree Narayana College, Kollam, India – 691001

E-mail: vidyaasnair@gmail.com

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Abstract

The synthesis of nanocrystalline $A_{0.5}B_{0.5}MoO_4$ by an auto-igniting combustion technique is reported. The structural characterization done by X–ray diffraction, Fourier transform Raman spectroscopy and infrared spectroscopy reveals that the as-prepared powder itself is phase pure with tetragonal structure. The particle size determined from Transmission electron microscopy are in the range 23–25 nm. The optical band gap determined show that the compounds are wide bandgap semiconductors. The photoluminescence spectrum of $A_{0.5}B_{0.5}MoO_4$ shows green emission, associated with the perfect order and crystallinity of the sample. All the samples are sintered at a temperature less than 875°C. The dielectric constant and loss factor of the samples are measured at radiofrequency range and its variation with temperature is also studied. The effect of change in composition of A^{2+} site of scheelite AMO_4 compounds comparative are also presented. The experimental results show that nano $A_{0.5}B_{0.5}MoO_4$ is an excellent luminescent material and also a promising 'Low temperature Co–fired Ceramic. Also it is inferred that we can fine tune both optical and dielectric properties to desired values according to our requirements by adjusting the cationic stoichiometric ratio in $[AO_8]$ octahedron.

Keywords: nanocrystalline, scheelite, AMO_4 compounds, FT Raman

1. INTRODUCTION

The quest for new and novel materials with excellent properties is increasing day by day due to the rapid technological development and processing techniques all over the world. Materials in the form of crystals, films, micron to nano sized powders and composites have caught much attention of the researchers. Among them, nanomaterials stand prominent due to their exceptional properties which are completely different from their other forms.

Scheelite compounds characterized by tetragonal structure with general formula AMO_4 are extensively studied due to their remarkable optical and electrical properties and wide range of applications including solid-state lasers, optic fibre, stimulated Raman scatters, low-temperature radiation detectors, scintillators and microwave applications [1–5]. The unique properties of these materials can be tailored by trying different stoichiometric ratios or combinations of these compounds such as $A_xB_{1-x}MO_4$, $AMo_xW_{1-x}O_4$ etc. Only a few reports have appeared in literatures on such combinations of scheelite group materials and their vast potential has not yet been exploited. The structural properties and photoluminescence behaviour in the $Ca_xSr_{1-x}WO_4$ system at room temperature was investigated by Porto et al [6] and that of $Ba_{0.5}Sr_{0.5}MoO_4$ by Wei et al [7]. Zhuravlev et al [8] has studied the stability of solid solutions in scheelite type molybdates and tungstates. $Sr_{1-x}Ca_xWO_4$ solid–solution films were prepared by Cho and Yoshimura [9]. Red–luminescence phosphors $Ca_{0.5}Sr_{0.5}MoO_4:Eu^{3+}$ for white LED have been reported by Shi et al [10]. The gas sensing property of $Ba_{0.5}Sr_{0.5}MoO_4$ thick film [11] and the enhancements of magneto resistance in $La_{0.7}Ca_{0.15}Sr_{0.15}Mn_{1-x}Mo_xO_3$ compound have also been investigated [12]. The different synthesis techniques used in these studies are soft chemical method [6], molten salt method, citrate method [8], sol–gel method [10] and solid state reaction [12, 13]. The preparation of $Ba_{1-x}Sr_xWO_4$ and $Ba_{1-x}Ca_xWO_4$ films by mechanically assisted solution reaction by Rangappa et al [14] at room temperature has also been reported.

Among the reported works much attention is devoted to the tuning of luminescence properties of the scheelite compounds while studies on its dielectric properties, sintering behaviour, band gap studies remain restricted. In view of this fact in mind we had made an attempt to study the structural, optical, sintering and dielectric behaviour of $Ba_{0.5}Sr_{0.5}MoO_4$, $Sr_{0.5}Ca_{0.5}MoO_4$ and $Ba_{0.5}Ca_{0.5}MoO_4$ of nanopowder synthesized through a modified combustion technique, for the first time. Brief comparisons between the parent compounds $AMoO_4$ and the compositions were also presented in the chapter and its suitability for various applications have also been discussed.

2. EXPERIMENTAL

Similar procedure was adopted for the preparation of the compositions $A_{0.5}B_{0.5}MO_4$ (A=B=Ba, Sr, and Ca) through modified combustion technique. The only distinction is in the stoichiometric amount of initial reagents to be taken.

As a typical instance, the preparation of $Ba_{0.5}Sr_{0.5}MoO_4$ nanopowder through the modified combustion process was detailed. For the preparation of $Ba_{0.5}Sr_{0.5}MoO_4$, aqueous solutions containing Ba, Sr and Mo ions were prepared by dissolving stoichiometric amounts of $Ba(NO_3)_2$, $Sr(NO_3)_2$ and ammonium molybdate in double distilled water. Citric acid was then added to the solution as complexing agent. The amount of citric acid to be added was calculated based on the total valence of the oxidizing and the reducing agents for maximum release of energy during combustion. Oxidant to fuel ratio of the system was adjusted by adding concentrated nitric acid and ammonium hydroxide solution and the ratio was kept at unity. The precursor solution of pH ~7.0 was stirred well for uniform mixing and a clear solution with no precipitate or sedimentation was obtained. The solution was then heated using a hot plate at ~250 °C in a ventilated fume hood. The solution boils on heating and undergoes dehydration accompanied by foam. On persistent heating the foam gets autoignited giving a voluminous fluffy powder of nano $Ba_{0.5}Sr_{0.5}MoO_4$. Thus obtained nano powder itself is used for further characterisation. A similar pattern was followed in case of $Sr_{0.5}Ca_{0.5}MoO_4$ and $Ba_{0.5}Ca_{0.5}MoO_4$.

Structure of the as-prepared powder was examined by powder X-ray diffraction (XRD) technique using a Bruker D–8 X-ray Diffractometer with Nickel filtered Cu Kα radiation. Particulate properties of the combustion product were examined using transmission electron microscopy (TEM, Model-Hitachi H-600 Japan) operating at 200 kV. The Infrared (IR) spectra of the samples were recorded in the range 400–4000 cm^{-1} on a Thermo-Nicolet Avatar 370 Fourier Transform Infrared (FTIR) Spectrometer using KBr pellet method. The Fourier transform–Raman spectra of the samples were carried out at room temperature in the wave number range 50–1200 cm-1 using Bruker RFS/100S

Spectrometer. The photoluminescence (PL) spectra of the samples were measured using Flurolog@-3 Spectroflurometer. For low frequency dielectric studies the pellets were made in the form of a disc capacitor with the specimen as the dielectric medium. Both the flat surfaces of the sintered pellet were polished and then electroded by applying silver paste. The capacitance of the sample was measured using an LCR meter (Hioki–3532–50 LCR HiTester) in the frequency range 100 Hz–5 MHz at different temperature from 30–250 °C.

3. RESULTS AND DISCUSSIONS

3.1. STRUCTURAL ANALYSIS

3.1.1. XRD Analysis

The XRD pattern of as-prepared $Ba_{0.5}Sr_{0.5}MoO_4$, $Sr_{0.5}Ca_{0.5}MoO_4$ and $Ba_{0.5}Ca_{0.5}MoO_4$ nanopowder is shown in the figure 1. All the peaks are indexed for a perfect tetragonal structure based on the spectral data in the JCPDS file 30-0157 for $Ba_{0.5}Sr_{0.5}MoO_4$ and file 30-1287 for $Sr_{0.5}Ca_{0.5}MoO_4$. The XRD pattern of $Ba_{0.5}Ca_{0.5}MoO_4$ is found to be iso-structural with the other two compounds of similar composition. Thus $Ba_{0.5}Ca_{0.5}MoO_4$ also possesses tetragonal structure and the XRD pattern is indexed on the basis of JCPDS files reported for $Ba_{0.5}Sr_{0.5}MoO_4$ and $Sr_{0.5}Ca_{0.5}MoO_4$.

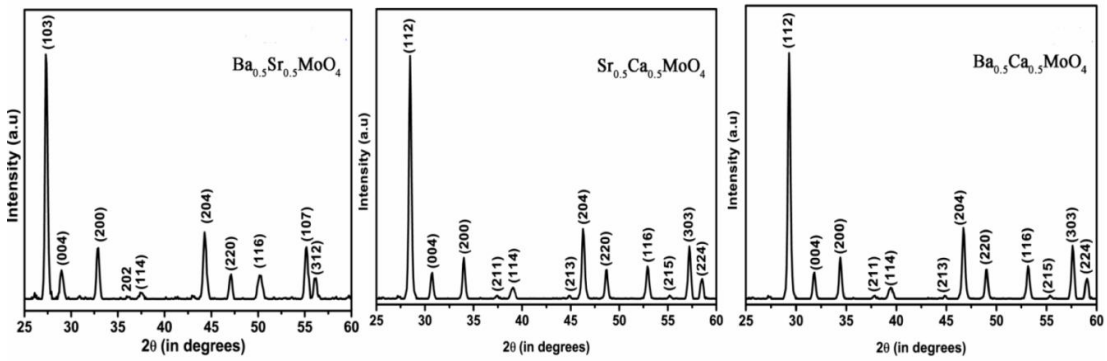

Fig. 1: XRD pattern of as-prepared nano $A_{0.5}B_{0.5}MoO_4$ (A=B=Ba, Sr, Ca)

Table 1: Calculated lattice parameters, unit cell volume and crystallite size estimated by the Scherer formulae

Compounds	Crystal Structure	Experimental lattice parameters (Å)	Unit cell Volume (Å^3)	Crystallite size (nm)
$Ba_{0.5}Sr_{0.5}MoO_4$	Tetragonal	a=b=5.490, c=12.495	376.006	23
$Sr_{0.5}Ca_{0.5}MoO_4$	Tetragonal	a=b=5.310, c=11.759	331.557	22
$Ba_{0.5}Ca_{0.5}MoO_4$	Tetragonal	a=b=5.482, c=12.488	375.293	24

No additional peak representing impurities or secondary phase or un-reacted multi could be observed in the XRD patterns. This suggests that all the compounds are single phase with scheelite structure. Thus the XRD patterns confirm that the modification of cation ratio in the A^{2+} site of AMO_4, did not lead to structural transformation. This may be due to the fact that all the substituted elements (Ba, Sr, Ca) have comparable ionic radii as they belong to same group of the periodic table. So they adhere well into the lattice structure without structure distortion. Phase formation was also complete during the combustion process itself, without any calcination step. Thus the modified combustion method for the synthesis of nanopowder offers an economic and time saving technique.

3.1.2. Vibrational Spectroscopic Studies

In order to study the vibrational spectra and conduct a detailed structural investigation, Raman and FTIR spectra of $A_{0.5}B_{0.5}MoO_4$ is recorded which are given in figure 2, and 3, respectively.

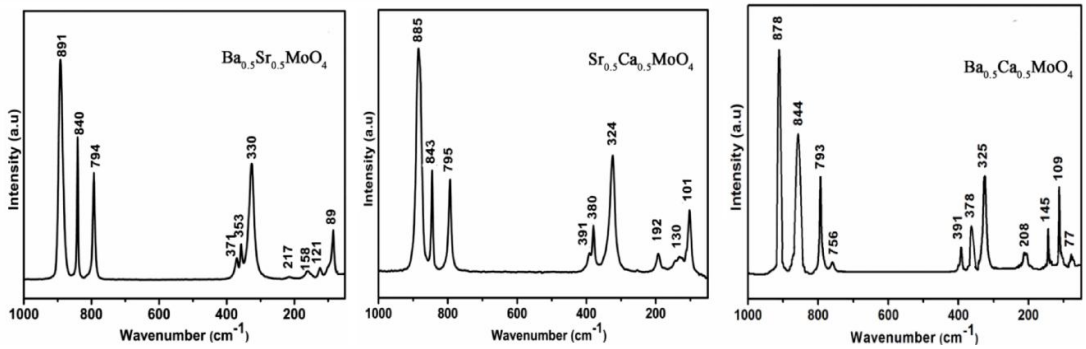

Fig. 2: Raman spectra of as-prepared nano $A_{0.5}B_{0.5}MoO_4$ (A=B=Ba, Sr, Ca)

On comparison with the vibrational spectra reported for the compounds $BaMoO_4$, $SrMoO_4$, $CaMoO_4$, $BaWO_4$, $SrWO_4$ and $CaWO_4$, the sample $A_{0.5}B_{0.5}MO_4$ also seem to exhibit a similar spectral pattern with respective shifts in the wave number positions [15–19].

The internal modes include the vibrational and rotational modes of the tetrahedron and are observed in the region 900−180 cm^{-1} whereas the external group consists of lattice phonon modes corresponding to the motion of Ba^{2+} /Sr^{2+} cations and the rigid molecular units and are attributed to peaks in the region 160−78 cm^{-1}.

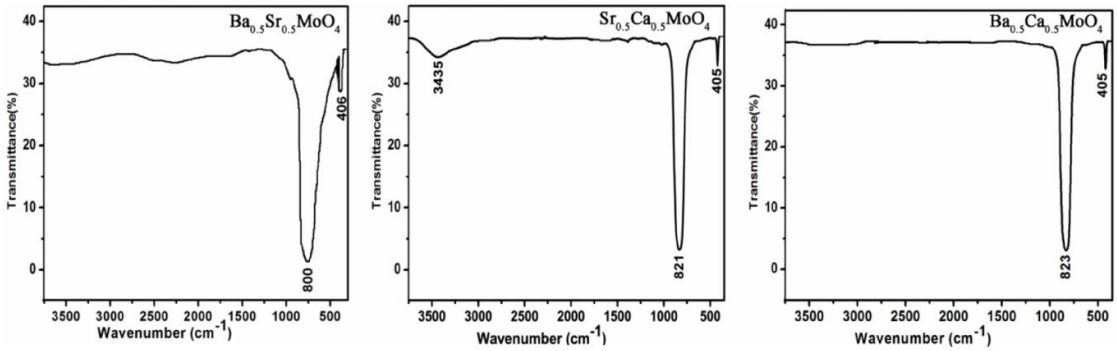

Fig. 3: FTIR spectra of as-prepared nano $A_{0.5}B_{0.5}MoO_4$ (A=B=Ba, Sr, Ca)

The most intense band of Raman spectra observed above 850 cm^{-1} in all the spectra are attributed to the A_g mode of vibrations. The B_g modes are assigned to peaks in the wave number range 845–830 cm^{-1}.The next highest bands of Raman spectra responds to internal mode of vibration, Eg..The modes involving the free rotation in the limiting case of the uncoupled MoO_4 ions in $Ba_{0.5}Sr_{0.5}MoO_4$, $Sr_{0.5}Ca_{0.5}MoO_4$, $Ba_{0.5}Ca_{0.5}MoO_4$ are observed above 200 cm^{-1} which has the E_g symmetry. This vibration mode shows highest wave number shift on comparing with AMO4 compounds where this mode is assigned to peaks around 180 cm^{-1}. The external modes $2B_g$ and $2E_g$ are assigned to the lowest frequency bands.

The IR spectrum shows a very strong absorption a band in which higher wavenumber is due to Metal–Oxygen (M–O) stretching vibration mode and the latter due to the weak Metal– Oxygen (M–O) bending vibration. These correspond to the IR active ν_3A_u and ν_4A_u modes of vibrations.

The splitting up of non-degenerate peaks, superposition of certain peaks into a broad peaks, small shift in the vibration mode frequencies, inactivity of certain active peaks in the Raman spectra of the compounds points to lowering of symmetry of the crystal structure. Thus all these compositions possess certain degree of shot range structural disorder. However there is no evidence for secondary phase or any other impurities. A trace of MoO_3 unit which was noticed in the Raman spectra of

$SrMoO_4$ was absent in this case. So the distortion is only short range. The vibrational analysis confirms the XRD result that $A_{0.5}B_{0.5}MO_4$ own tetragonal structure.

3.2. TEM analysis

The TEM studies on the morphology of the as-prepared nanopowder obtained by the combustion route are shown in the figure 4. The micrograph showed that the particles are of submicron size in the range of 20–25 nm. The nano particles of all the compounds possess nearly spherical morphology and the absence of agglomeration of the particles indicates the good nanocrystalline nature of the sample. The majority particle size obtained from the TEM are 23nm, 22nm and 25nm for A0.5B0.5MO4 (A=B=Ba, Sr, Ca) respectively.

SAED patterns are composed of a number of bright spots arranged in concentric rings. The ring nature of the electron diffraction pattern is indicative of the polycrystalline nature of the crystallites, but the spotty nature is due to the fact that the fine crystallites having related orientations are agglomerated together resulting in a limited set of orientations.

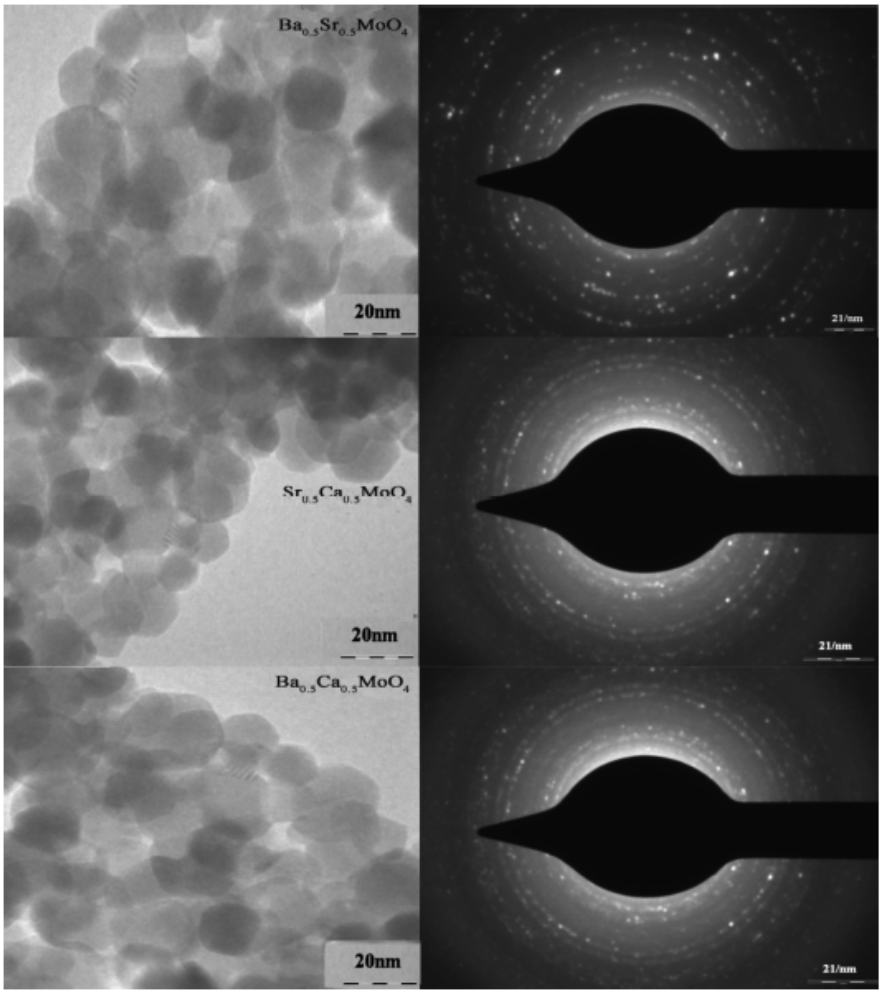

Fig. 4: TEM image of as-prepared nano $A_{0.5}B_{0.5}MoO_4$ (A=B=Ba, Sr, Ca)

3.3. Optical properties

3.3.1. Photoluminescent Studies

The PL emission spectrum of $A_{0.5}B_{0.5}MoO_4$ nano powder is shown in the figure 6. The sample shows intense green emission. The reason for the PL emission of scheelite structure is explained in literatures in many ways viz. due to structural disorder, morphology, charge–transfer transitions into

the $[MoO_4]^{2-}$ complex, the existence of MoO_3 and distorted MoO_4 complex clusters, particle sizes, crystalline degree, morphology, and surface defects [24–26].

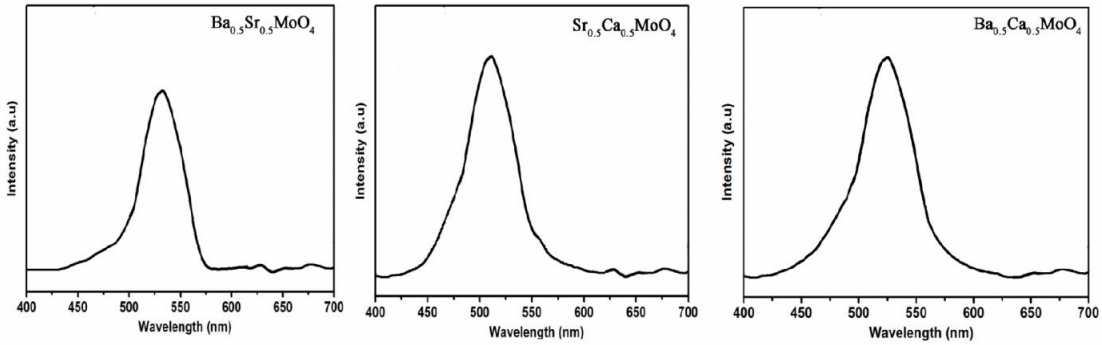

Fig. 5: PL spectra of $A_{0.5}B_{0.5}MoO_4$ (A=B=Ba, Sr, Ca)

For our sample, as there is no long range structural disorder, the main reason for the green emission in the PL emission spectrum can be the charge–transfer transitions within the $[MoO_4]^{2-}$ complex, in the ordered system. Wei et al reported a blue emission for $Ba_{0.5}Sr_{0.5}MoO_4$ and ascribed it to the $^1T_2 \rightarrow {}^1T_1$ electronic transitions into the $[MoO_4]$ tetrahedron groups [7]. A striking green luminescence was observed by varying the ratio of Ca and Sr in $Sr_{1-x}Ca_xMoO_4:Tb^{3+}$ by Wang et al [27]. The PL emission of $BaMoO_4$ $SrMoO_4$ and $CaMoO_4$ prepared through the same combustion method also showed an intense green emission along with a weak red emission which is because of the presence of MoO_3–MoO_4 clusters and on annealing, the red emission peak vanished [20,21]. Thus the observed green emission is associated with the perfect order and crystallinity of the sample.

Accordingly we can also make out that luminescence behaviour primly depends on $[MoO_4]^{2-}$ in molybdates scheelite class of compounds. The change of stochiometry in A^{2+} of AMO_4 could persuade a small wavelength shift. Hence when the composition is altered systematically as A_1 and $B_{(1-x)}$, we can obtain different compositions which yield blue emission as well as green emission. Thus we can fine tune the PL emissions which have much advantage in laser applications.

3.4. SINTERING

The sintering behaviour of the nanocrystals of $A_{0.5}B_{0.5}MO_4$ powder synthesized through the present combustion route was studied.

Table 2: Sintering temperature, soaking time and relative density of sintered AMO_4 and $A_{0.5}B_{0.5}MO_4$ (A=B=Ba, Sr, Ca and M=Mo, W)

Compounds	Sintering temperature (°C)	Soaking time (h)	Relative Density achieved (%)
$BaMoO_4$	750	3	95
$SrMoO_4$	850	2	96
$CaMoO_4$	775	3	96
$Ba_{0.5}Sr_{0.5}MoO_4$	820	3	96
$Sr_{0.5}Ca_{0.5}MoO_4$	875	3	96
$Ba_{0.5}Ca_{0.5}MoO_4$	800	3	96

A highly sintered specimen was obtained on sintering the compacted nanopowder for all the samples in the temperature range 815-875 °C. The change in composition has influenced the sintering temperatures of the compounds. The sintering temperatures with respective soaking time, density achieved by the compounds along with the same details of AMO_4 compounds prepared by the same method are also depicted in the Table 2.

The change in composition had resulted in an increase of sintering temperature. The maximum density that can be achieved is 96% whereas it is 98% in the case of $SrMoO_4$.The soaking time required for all the compounds are 3 hours. It is to be noted that as all the samples are sintered at a temperatures below 900 °C. Hence these samples are ideal candidates for LTCC applications.

3.5. Dielectric Properties

The dielectric properties of a material determine its functionality. The dielectric behaviour of the sintered samples of $A_{0.5}B_{0.5}MO_4$ nanocrystals are studied in the frequency range 100 Hz to 5MHz. The variation of dielectric constant (ε_r) and the loss factor (tanδ) with frequency are shown in the figure 8.

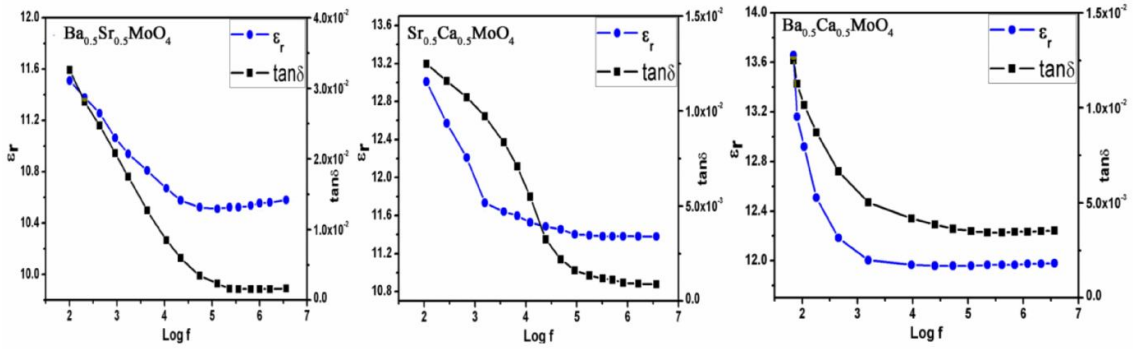

Fig. 8: Variation of ε_r and $\tan_\delta$ with frequency of sintered $A_{0.5}B_{0.5}MoO_4$ (A=B=Ba, Sr, Ca) pellets

The dielectric constant and the loss factor of the samples also show significant change. It can be clearly noted that loss factor decreases as frequency increases while the dielectric constant remains almost unaltered in the high frequency above 1 kHz. On comparing with AMO_4 compounds the dielectric constant of the composition seems to be higher. All the compositions show low loss factor values. The values of dielectric constant and very low loss factor indicate the suitability of the sample as a candidate for electronic and dielectric applications.

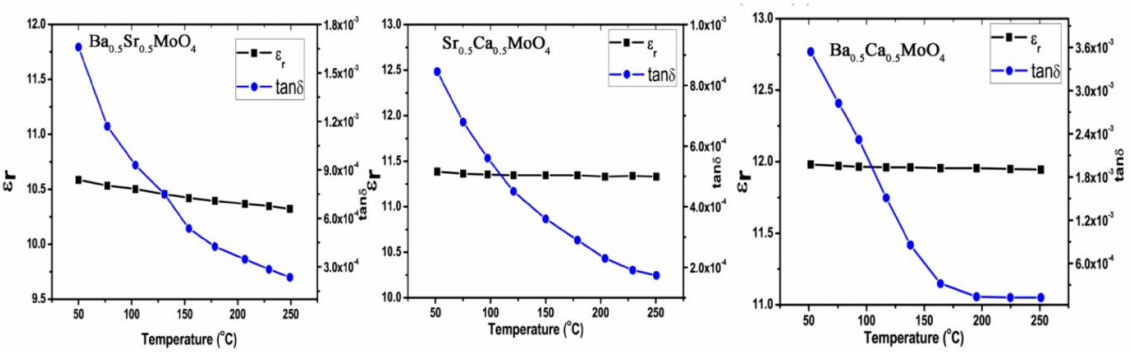

Fig. 9: Variation of ε_r and tanδ with temperature of sintered $A_{0.5}B_{0.5}MoO_4$ (A=B=Ba, Sr, Ca) pellets

The variation of ε_r and tanδ with temperature, in the range 30–250 °C is shown in Figure 9. It is evident from the graph that the variation of dielectric constant with temperature is very minimal in the measured temperature range. The loss factor value lowers with the increase of temperature and is of order 10^{-3} at temperature above 100 °C. The temperature coefficient of dielectric constant (TCK) is determined using the equation given below between temperature 250°C and 30°C at 5MHz.

$$T_{CK} = \left(\frac{\left[\frac{K_{250} - K_{30}}{220} \right]}{K_{30}} \right) \times 10^6 \, \text{ppm}/\circ\text{C}$$

where K_{30} and K_{250} are the dielectric constants at 30 °C and 250 °C respectively, and 220 is the temperature difference.

The obtained T_{CK} values are negative. This indicates that $A_{0.5}B_{0.5}MO_4$ nano powder have relatively low temperature coefficient of dielectric constant when compared to $AMoO_4$. It is clear from the graph that the temperature dependence of dielectric constant is very minimal in the measured range. The loss factor decreases with increase of temperature and is of order 10^{-4} at temperature above 150 °C. Thus nano $A_{0.5}B_{0.5}MO_4$ compositional compounds are suitable for temperature dependent dielectric applications.

The lower dielectric constant and the low loss factor value make nano structured $A_{0.5}B_{0.5}MO_4$ prepared by the present method a promising candidate for LTCC applications, substrate application and as electronic packing materials. That the dielectric constant remains almost constant on heat treatment ensures the suitability of $A_{0.5}Mo_{0.5}MO_4$ and $A_{0.5}W_{0.5}MO_4$ as LTCC material in multi layer circuits.

The main advantage noticed in varying the A^{2+} cation composition in AMO_4 compounds are changes in the dielectric properties. Thus we can infer that the dielectric properties can be fine tuned by adjusting the cation composition, as needed. Thus dielectric constant of these compositional materials can be varied from low to moderate dielectric constant materials with low dielectric loss according to our requirement.

It is also to be noted that even though the sintering temperature of these solid solutions is higher than scheelite compounds, it is less than 950 °C which makes these materials ideal for LTCC applications. As dielectric constant of the solid solution can be lowered or raised, and are low loss materials with negative TCK value, we can finely mould these materials according to our needs. Bivalent metal molybdates and tungstates with scheelite structure allows us to conclude that the chosen solid solutions are excellent luminescent wide band gap materials with moderate dielectric constant and are suitable for numerous optical and electrical properties as discussed.

4. CONCLUSIONS

Nanocrystalline semiconducting $A_{0.5}B_{0.5}MO_4$ (A=B=Ba, Sr, Ca and M=Mo) were synthesized through a modified combustion process. The X-ray diffraction studies have shown that the nanopowder were single phase and possess tetragonal structure. The splitting up of non-degenerate peaks, superposition of certain peaks into a broad peaks, small shift in the vibration mode frequencies, inactivity of certain active peaks in the Raman spectra of the compounds points to lowering of symmetry of the crystal structure. Thus all these compositions possess certain degree of short range structural disorder. FT-IR and Raman spectral analysis confirms the XRD results that the as prepared powder itself is phase pure. TEM analysis confirms the nanocrystalline nature of the sample having particle size in the range of 20–25nm and all the compositions possess nearly spherical morphology. The band gaps of the compositions are found to be higher than $AMoO_4$. The nanocyrstalline $A_{0.5}B_{0.5}MO_4$ are found to be a excellent photo luminescent material with strong green and shows a blue shift in emission wavelength with respect to change of substitutions elements. Thus we can fine tune the emission wavelength from blue to green region according to our needs. These nanocrystals could be sintered at a relatively low temperature in the range 800-875 °C for 3h to a high density. Sintering temperature seems to be increased with respect to AMO4 compound prepared through the same method. The temperature coefficients of dielectric constant for all the samples were negative which makes them suitable for temperature sensitive applications. Thus by varying cationic ration in AMO_4 scheelite system, we can fine tune its properties to the desired values in a limited range. The

low sintering temperature, dielectric constant and minimum loss makes nano $A_{0.5}B_{0.5}MO_4$ excellent composition for low temperature co-fired ceramics, substrate material, and electronic packing materials.

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Photocatalytic degradation of an acidic dye and a basic dye using heterostructured photocatalyst

Baiju V[1,*], Dedhila Devadathan[1], Raveendran R[1]

Author Affiliations
[1]Nanoscience Research Laboratory, Department of Physics, Sree Narayana College, Kollam, 691001, Kerala.

Corresponding Author
*__Baiju V,__ Nanoscience Research Laboratory, Department of Physics, Sree Narayana College, Kollam, 691001, Kerala.

E-mail: baijuvkollam@gmail.com

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Abstract

Most of the natural resources of drinking water are found to be contaminated with various toxic materials. One of the most relevant pollutants is organic dyes. Photocatalysis is a promising green technology for the removal of dyes. In the present work heterostructured NiO/ZnO nanocomposite was prepared using co–precipitation method, analyzed using XRD, SEM and UV/Vis spectroscopy. For comparison purpose NiO was also prepared. Comparison of XRD's of metal oxides with JCPDS confirmed the formation of heterojunctions. UV/Vis absorption studies confirmed the shift of the optical absorption towards the visible region. The photocatalytic degradations of dyes namely Malachite Green, a basic and Congo Red, an acidic dye were carried out using the prepared composite. Factors affecting photocatalytic degradation like effect of contact time, amount of photocatalyst and dye concentration were also investigated. Results suggested an increase in degradation efficiency in the case of heterojunctions nanocomposite as compared to individual metal oxide.

Keywords: Photocatalysis, Heterojunction, Nanocomposite, Photocatalyst, Co–precipitation

1. INTRODUCTION

Nature has its own mechanism for water recycling to provide us with sufficient quantity of fresh water with consumable purity level. Modern human activities have however disrupted the balance between the usage and natural purification process, leading to a shortage of potable water. Most of the natural resources of drinking water are found to be contaminated with various toxic materials and pathogenic microorganisms. One of the most relevant pollutants is organic dyes. The dyes have diverse applications in various industries of paper, leather, cosmetics, drugs, electronics, plastics and printing and approximately 80% of the synthetic dyes are consumed by the textile industry. Dyes are non biodegradable compounds and human exposure to wastewater which contains dyes is highly dangerous and these carcinogenic compounds show high resistance against biological, physical and

chemical reactions. Many researchers consider advanced oxidation process as the most effective, economically feasible and applicable technology for the removal of organic dye pollutants. Nanotechnology is widely applied for purification and treatment of waste water. The novel properties of nanomaterials such as large surface area, potential for self assembly, high specificity, high reactivity and catalytic potential make them excellent candidates for this application. An expanding trend for the nanomaterials is the fabrication of composite structures with materials capable of enhancing the properties. Many efforts have been made for the synthesis of different coupled semiconductors [1–4]. The synthesized coupled semiconductors significantly improve the photocatalytic efficiency by decreasing the recombination rate of the photogenerated electron–hole pairs and present potential application in water splitting, organic decomposition and photovoltaic device [5]. In the present study heterojuction NiO/ZnO nanocomposite is prepared and applied as photocatalyst.

2. MATERIALS AND METHODS

AR grade chemicals obtained from Merck were used for the preparation of NiO/ZnO heterojunction. NiO (NF) and NiO/ZnO (NZF) were prepared by the co–precipitation method in presence of capping agent. NF and NZF annealed at 5000c for three hours, was used for analysis. XRD studies were carried out using XPERT–PRO model powder diffractometer (PAN analytical, Netherlands) employing Cu–Kα radiation (λ = 1.54060Ao) operating at 40kV, 30mA. SEM of the samples was obtained using JEOL/EO Model JSM–6390LV scanning electron microscope. The absorbance spectra, reflectance spectra and photocatalysis studies of the sample was studied using JASCO V 650, UV/Vis spectrophotomer.

Metal oxides prepared in the present study were obtained in the form of hydroxides. The nickel hydroxide was obtained as dark green powder where as in the case of NiO/ZnO was obtained as light pista green powders. But both the annealed samples were black in colour.

3. RESULTS AND DISCUSSION

X–ray diffractograms of NF and NZF is shown in Figure 1. The well defined X–ray diffraction peaks in the case of NF and NZF indicate that NiO and NiO/ZnO formed are crystalline in nature. Also, the diffraction peaks are notably broadened indicating the smaller crystallite size. In order to confirm the phase purity of the samples prepared, the interplanar spacing (dhkl values), 2θ values and relative intensity values corresponding to the observed diffraction peaks were compared with the standard values of NiO in case of NF and in case of NZF the observed diffraction peaks were compared with the standard values of NiO and ZnO as reported by JCPDS–International Centre for Diffraction Data.

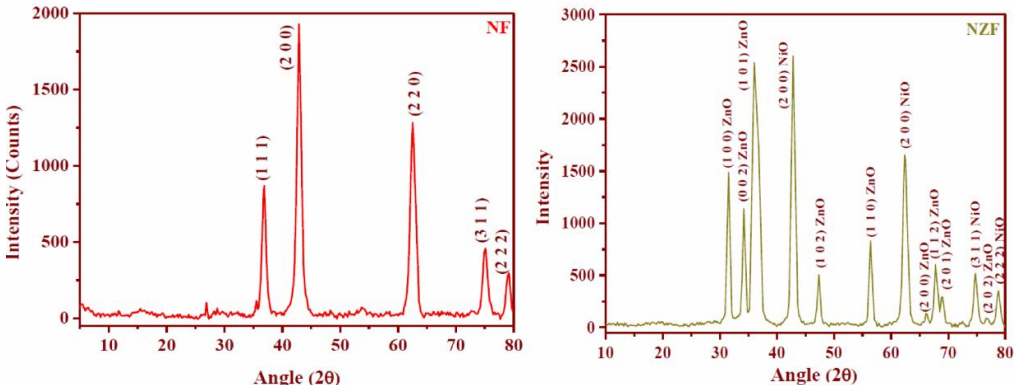

Fig. 2: XRD Spectrum of NF and NZF

XRD pattern for ZnO prepared using the very same procedure in our lab was used for JCPDS comparison in case of ZnO [6]. The obtained pattern for ZnO was found to match well with JCPDS–ICDD pattern number #79–0205. From the comparison of diffraction peaks NF with JCPDS–ICDD pattern number #78–0423 it is clear that NiO formed was a cubic system with FCC lattice. The data obtained for NiO/ZnO matched with JCPDS–ICDD pattern number #78–0423 of NiO and JCPDS–ICDD pattern number #79–0205 of ZnO separately. From JCPDS, NiO shows cubic system with FCC lattice and ZnO shows hexagonal system with primitive lattice. The variation observed in the d values of the crystal planes in case of NZF when compared to NiO and ZnO, confirms the formation of composites. This is also supported by the disappearance of the peak corresponding to (1 0 3) of ZnO in the XRD of the nanocomposite. The average crystallite size was calculated from the line broadening of the XRD pattern, making use of Scherrer formula. The crystallite size calculated using FWHM (Full width at half maximum) values of five major peaks in the XRD spectrum of NF using Scherrer equation are shown in the Table 1. In case of NZF the average crystallite size of NZF calculated from the line broadening of the XRD pattern, using FWHM values of seven major peaks in the XRD spectrum making use of Scherrer formula are shown in Table 2.

Table 1: Crystallite size calculation of NF using Scherrer equation

2θ	θ	Cosθ	FWHM	D (nm)
36.845	18.42	0.9488	0.719	11.65
42.873	21.44	0.9309	0.774	11.03
62.518	31.26	0.8549	0.858	10.84
75.075	37.54	0.7932	0.914	10.96

Average Crystallite size = 11.12± 0.3 nm

Table 2: Crystallite size calculation of NS using Scherrer equation

2θ	θ	Cosθ	FWHM	D (nm)
31.54	15.77	0.9624	0.383	21.56
34.20	17.10	0.9558	0.397	20.95
36.04	18.02	0.9509	0.353	23.67
42.85	21.42	0.9309	0.577	14.79
47.34	23.67	0.9159	0.459	18.91
56.41	28.20	0.8814	0.460	19.60
62.44	31.22	0.8553	0.801	11.60

Average Crystallite size = 18.73± 3 nm

The SEM images of NF and NZF are shown in Figure 2. NF showed agglomeration of spherical particles or cauliflower like morholopgy and NZF showed agglomeration of very small spherical particles of almost simlar uniform dimensions.

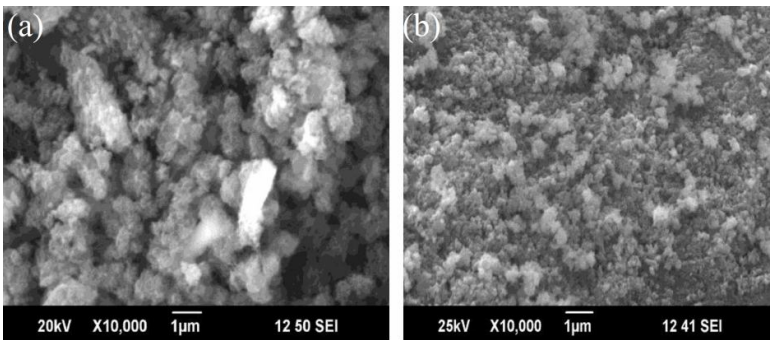

Fig. 2: SEM images of (a) NF and (b) NZF

The UV–Vis absorption spectrum of the NF taken in the wavelength range 210 to 850 nm with 1 nm resolution is shown in the Figure 3. Presence of an absorption band is observed in the range 225 nm – 270 nm with λ_{max} situated at 249nm in case of NF.

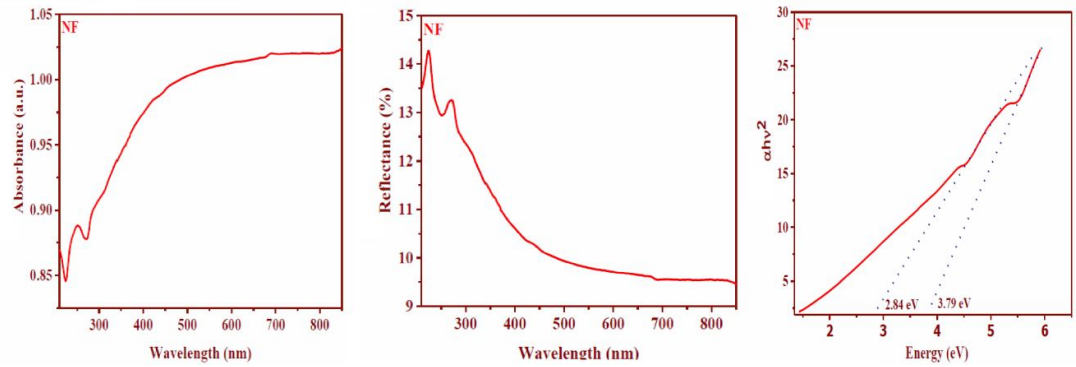

Fig. 3: UV/VisAbsorbance, Reflectance spectra and Tauc Plot of NF

An additional absorption band is observed in the wavelength range 270 nm – 305 nm. It is reported in literature that optical energy band gap of NiO lies in the range (3.6–4.0 eV) [Adler and Feinleib, 1970] and is attributed to charge–transfer transition between the valence bands of O (p) states to conduction bands of Ni d (e_{2g}) states [7]. The absorption edge is found to be 4.98 eV for NF. Figure 3 also shows the reflectance spectrum obtained for NF. The percentage reflectance corresponding to λ_{max} situated at 249 nm was found to be 12.94 %.

The materials also showed sub band gaps. This might be due to d–d transitions. The optical band gap of NF determined from the absorption spectra using Tauc's relation were 2.84 eV and 3.79 eV. Tauc's plot drawn for NF is also shown in Figure 3.

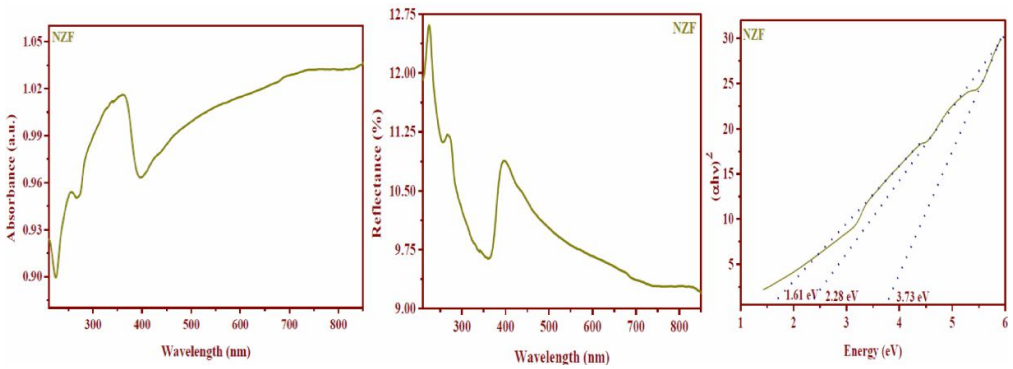

Fig. 4: UV/Vis Absorbance, Reflectance spectra and Tauc Plot of NZF

The UV–Vis absorption and reflectance spectra of NZF taken in the wavelength range 210 to 870 nm with 1 nm resolution is shown in the Figure 4. Presence of a weak absorption band was observed in the range 225 nm – 270 nm with λ_{max} situated at 256nm and also another strong absorption band in the range 260 nm – 400 nm with λ_{max} situated at 360 nm was also obtained. The shift of peak from 252 nm of NF to 256 nm in NZF can be attributed to the interaction between NiO and ZnO. The second band can be attributed to the presence of ZnO which could be assigned to the intrinsic band–gap absorption of ZnO due to the electron transitions from the valence band to the conduction band ($O_{2p} \rightarrow Zn_{3d}$) [8]. The absorption edge was found to be 3.44 eV for NZF. Figure 4 also shows the

reflectance spectrum obtained for NZF. The percentage reflectance corresponding to λmax values for NZF were found to be 9.6%. The optical band gap determined from the absorption spectra using Tauc's relation for NZF is shown in Figure 4.

Surface area and surface defects play an important role in photocatalytic efficiency and many other practical properties. In the present work the nanocomposite showed good visible light absorption in addition to UV absorption. Hence proper tuning could make these nanocomposites excellent candidates for many practical applications such as anti–bacterial, deodorizing, air purifying, anti fogging and as other surface purification agents.

The prepared sample was used as photocatalyst for the degradation of Malachite Green, and Congo Red. Organic dyes of 1000 ppm concentration were prepared and the desired concentrations were taken and used as dye samples. The photocatalytic activity of samples for the degradation of the dyes; Congo red (CR) and Malachite green (MG) and were studied using the respective photocatalyst in the presence of UV illumination in a photoreactor. The experiments were performed by suspending the desired amount of photocatalyst into 300 ml of dye solution of desired concentration which was varied from 25 ppm to 75 ppm. The experiment was carried out isothermally at 300 K. The concentration of residual dye in the solution after irradiation for 2 hrs was determined by monitoring the absorbance intensity of solution samples at their maximum absorbance wavelength by using UV–Vis spectrophotometer (JASCO V 650 UV–Vis spectrophotometer). In the present work various factors affecting photocatalytic degradation like effect of contact time, amount of photocatalyst and dye concentration were also investigated. To study the effect of contact time on the photodegradation of organic dyes, the following procedure was adopted. To the respective dye solution of concentration, 25 ppm in 300 ml solution kept at 300 K, 0.1 g of the photocatalyst was added and was kept under UV light. After desired time intervals [30, 60, 90 and 120 min], 10 mL of the solution was taken out, centrifuged and UV–Vis absorption spectra was recorded. In the above experiment, the amount of photocatalyst was varied from 0.025g to 0.125g to study its effect on photodegradation. Dye solutions of three different concentrations were selected (25 ppm, 50 ppm and 75 ppm) to study the effect of initial dye concentration on photodegradation efficiency.

The photocatalytic degradation efficiency was calculated as follows:

$$\text{Photocatalytic degradation efficiency (\%)} = \frac{(C_0 - C_t)}{C_0} \times 100 \quad \cdots\cdots(1)$$

C_0: initial concentration of dye solution [mgL–1],
C_t: final concentration of dye solution [mgL–1],

In the present work NiO/ZnO composites prepared are coupled heterogeneous system. When NiO/ZnO coupled metal oxide is irradiated by visible light, the electrons in the valence band (VB) of ZnO and NiO are excited to their conduction bands (CB) or to the defect levels present in the band gap. The excited electrons from CB of NiO are transferred to the low lying CB of ZnO. The holes are transferred from the VB of ZnO to the VB of NiO. This in turn leads to the efficient separation of photogenerated electron–hole pairs. As a result, the photocatalytic activity of the NiO/ZnO coupled metal oxide is significantly enhanced. The efficient visible light photocatalytic degradation shown by the coupled metal oxides could be due to the stoichiometry deficiency induced on coupling and the formation of defect energy levels. Figure 5 shows the suggested mechanism in the case of photocatalytic degradation process using NiO/ZnO. In contrast to single phase photocatalysts, heterojunction semiconductors or integrated multi–semiconductor systems possess significant advantages in promoting the separation of electron–hole pairs and keeping reduction and oxidation reactions of photocatalysis at two different reaction sites. Formation of heterojunction greatly diminishes the electron–hole pairs recombination and increases the life time of charge carriers, thus promoting the photocatalytic efficiency. A schematic diagram of the mechanism of photocatalysis is given below.

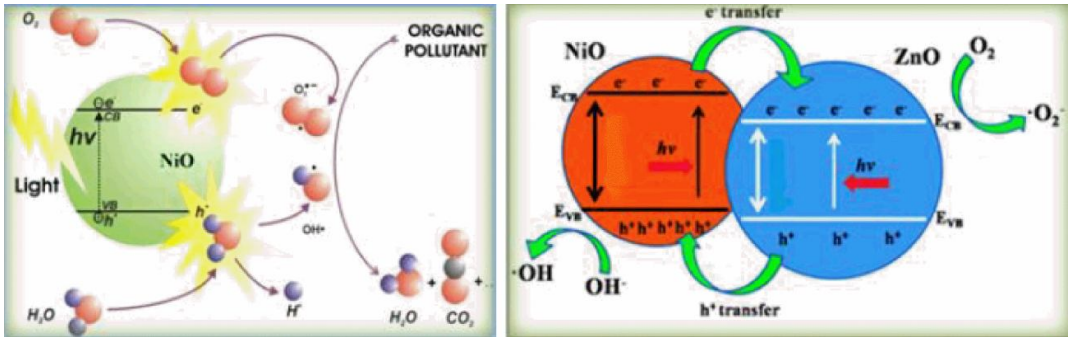

Fig. 5: Mechanism of Photocatalytic degradation process using NiO and NiO/ZnO hetrojunction nanocomposite

The percentage of photodegradation increased with increase in contact time of the dye and the catalyst. The amount of catalyst loading is one of the main parameters for the degradation efficiency. In order to avoid the use of excess catalyst it is necessary to find out the optimum loading for efficient removal of dye. The results showed that when catalyst dosage was increased from 0.025 to 0.075 g, the percentage de–colorization increased. However, on further increase in dosage of the catalyst beyond 0.1 g, there was a slight decrease in the degradation percentage. In the absence of photocatalyst it was found that CR dye is difficult to be oxidized by only UV light. The increase in degradation rate with increase in the catalyst loading is due to increase in total active surface area i.e. availability of more active sites on catalyst surface [9]. However, it increases significantly upon addition of photocatalyst due to the generation of higher amount of hydroxyl radical through the interaction of UV light with photocatalyst. But above 0.1 g, the percentage degradation significantly decreased due to decrease of formation of hydroxyl radicals. It should be pointed out that, the catalyst loading affects both the number of active sites on photocatalysts and the penetration of UV light through the suspension [10]. With increasing catalyst loading the number of active sites increases, but the penetration of UV light decreases due to shielding effect [11]. It should also be noted that the optimum value of catalyst loading is strongly dependent on the type and initial concentration of the dye and the operating conditions of the photoreactor [12]. The optimum concentration of the catalyst for efficient UV photodecolorization and degradation was found to be 0.1 g/300ml.

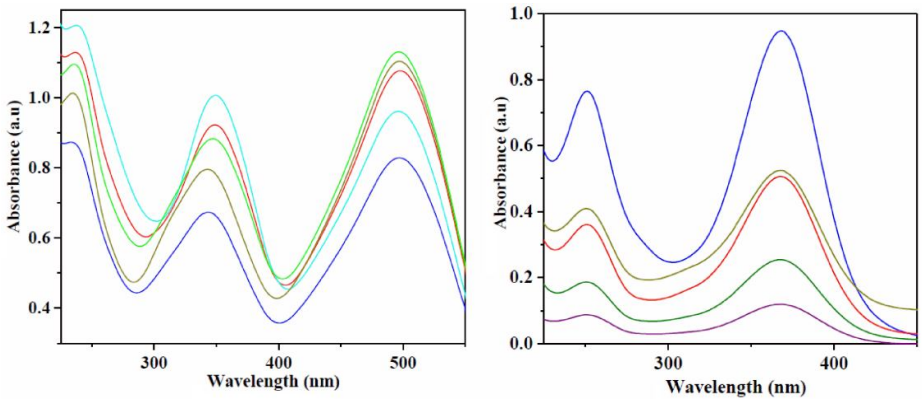

Fig. 6: Photocatalytic degradation of CR and MG

The rate of photocatalytic degradation decreased with the increasing initial dye concentration. As the initial concentration of a dye increases, the colour of dye solution becomes deeper which results in less penetration of light to the surface of the catalyst, decreasing the number of excited dye molecules. With increase in initial concentration of dye more and more organic substances are adsorbed on the surface of the photocatalyst. Therefore, the generation of hydroxyl radicals is reduced, since there are only fewer active sites in the system [13]. Similar results have been reported by other researchers for

the photocatalytic oxidation of pollutants [9]. The activity of the nanocomposite was also compared with the counterpart.

4. CONCLUSIONS

It was observed from the UV/Vis spectral analysis that the nanocomposites showed absorption in visible range in addition to UV light. Hence these materials could be used as photocatalyst in the degradation of organic dyes under solar ray irradiation. Results suggested an increase in degradation efficiency in the case of heterojunction nanocomposite when compared to metal oxide. It is suggested that these materials could be efficiently used as anti–bacterial, deodorizing, air purifying, anti fogging and as other surface purification agents.

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Antibacterial studies of polyindole based metal oxide nanocomposites

Dedhila Devadathan[1]*, Baiju V[1], Raveendran R[1]

Author Affiliations
[1]Nanoscience Research Laboratory, Department of Physics, Sree Narayana College, Kollam 691001

Corresponding Author
***Dedhila Devadathan,** Nanoscience Research Laboratory, Department of Physics, Sree Narayana College, Kollam 691001

E-mail: dedhila@yahoo.com

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Abstract

In the present work, polyindole based metal oxide nanocomposites were synthesised through chemical oxidation method. For comparative study, the counterparts, polyindole was synthesised through chemical oxidation method and the metal oxides were synthesised through chemical co-precipitation method. Structural studies were done using XRD. Phase purity and crystallinity of these samples were confirmed from XRD. XRD confirmed the formation of nanocomposites. Crystallite sizes were calculated using Scherrer equation for metal oxides. The present study investigates the antimicrobial activity of the synthesized samples as antibacterial agents. The gram positive bacteria: S. epidermidis and C. perfringens, gram negative bacteria: E. coli and V. cholera were the microbes used in the present study. The study confirms that the polyindole based metal oxide nanocomposites samples prepared in the present study showed antibacterial agent activity. The results suggest that proper tuning can make them good antimicrobial agents.

Keywords: Nanoparticles, Antibacterial agents, polyindole,

1. INTRODUCTION

The advances in the field of nanosciences and nanotechnology led to the development of inorganic and organic nanocomposites that are nowadays widely used as antimicrobials. It is a matter of fact that the binding of the particles to the bacteria depends on the surface area available for interaction. Since the reactions takes place at the surface of a chemical or material; the greater the surface for the same volume, the greater will be the reactivity. The smaller size of nanoparticles helps them to easily reach the nuclear content of bacteria which will give more bactericidal effect than the larger particles.

Conducting polymers because of its ability to be doped in reduced or oxidized form (redox properties) has facilitated its usefulness in certain biological applications [1]. Also they can be used in the form of powders, aqueous dispersions and as coatings on variety of substrates. In addition to

polymer materials which possess an intrinsic antibacterial activity, these properties can be induced into polymer. This can be achieved through adsorption or coating of an antibacterial agent onto the polymer surface, by direct incorporation of an antibacterial agent into the polymer structure during its synthesis or by immobilization of an antibacterial agent onto the polymer through ionic or covalent bonding [2, 3]. Recent studies indicate that among the polymers inherently showing antibacterial properties includes conducting polymers such as polyaniline. Polyaniline contains quarternary ammonium salts (QAS) and halamines. The antibacterial activity of QAS and halamines are due to the presence of charged nitrogen as well as due to chloride ions. A perfect recipe for making an antibacterial agent is the presence of QAS and halamines [4].

The polymeric matrices are considered as good host materials for metal and metal oxide nanoparticles. They provide additional qualities, such as processability, solubility and thermal stability to the systems formed [5]. Several efforts have been made for fabrication of polymeric material with potential antimicrobial activity [6]. Moreover, the nanocomposites prepared using inorganic metal oxide nanoparticles and organic polymers can find better utilization due to the enhanced antimicrobial activity. All these open the possibility of formulation of a new generation of bactericidal materials. Thus it was thought of interest to study the antimicrobial properties of polyindole having similar structure like PANI and Polypyrrole.

In the present work nickel oxide (NiO), nickel oxide-magnesium oxide (NiO-MgO) and nickel oxide-zinc oxide (NiO-ZnO) was prepared in presence of capping agent, citric acid using co-precipitation method. Polyindole and polyindole based metal oxide nanocomposites were prepared using chemical oxidation method. The aim of the work was to study the variations in the properties of the counterparts when compared to the nanocomposites. The samples synthesizedwas characterized using X-ray Diffraction Spectroscopy (XRD). The present study investigates the antimicrobial activity of the synthesized samples as antibacterial agents. The results of all the samples were also compared.

2. EXPERIMENTAL

AR grade chemicals obtained from Merck were used for the preparation of all the samples. NiO annealed at 500 °C (NF), NiO annealed at 700 °C (NS), NiO–MgO annealed at 500 °C (NMF), NiO–MgO annealed at 700 °C (NMS), NiO–ZnO annealed at 500 °C (NZF) and NiO–ZnO annealed at 700 °C (NZS) were the metal oxide parts prepared in the present study using co-precipitation method. Polyindole (PI) was the conducting polymer selected for the work. Polyindole based NiO (PIN), polyindole based NiO–MgO (PINM) and polyindole based NiO–ZnO (PINZ) were the polyindole based based metal oxides prepared in the present study. PI, PIN, PINM and PINZ were prepared using chemical oxidation method. XRD study was carried out using XPERT–PRO model powder diffractometer (PAN analytical, Netherlands) employing Cu–K_α radiation (λ = 1.54060A^o) operating at 40kV, 30mA.

3. RESULTS AND DISCUSSIONS

3.1 XRD Analysis

The XRD pattern of PI was found to match well with the XRD patterns in literature [7]. The XRD of polyindole shows the presence of numerous sharp crystalline peaks in the diffraction pattern having 2θ values between 15° and 30°. This can be related to the scattering from bare polymeric chains at the inter-planar spacing. Figure 1 shows the XRD diffraction pattern of PI.

An XRD peak is known to be the measure of crystallinity and the appearance of peaks in the XRD of the polymer confirms the crystalline nature rather than amorphous nature. Higher the degree of regularity in arrangement or ordering of the polymer chain, higher is the crystallinity [8]. The presence of sharp crystalline peaks in the XRD graph indicates good electrostatic (dipole–dipole) interactions among the adjoining molecular chains in the PI matrix and also their highly ordered state [9]. The XRD results justify the PI nanostructures as crystalline in nature.

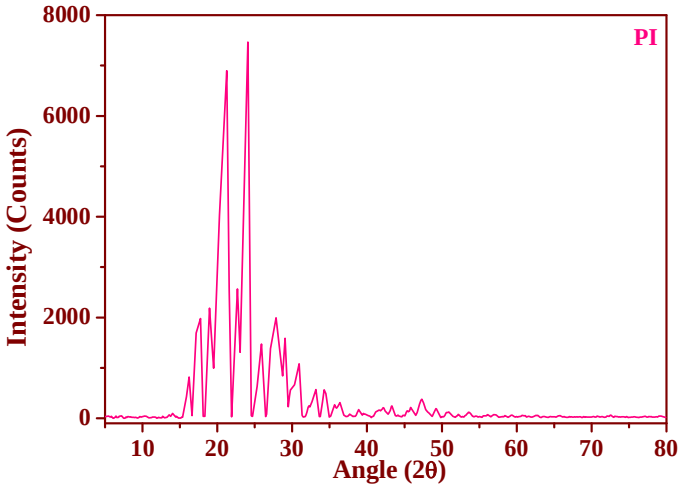

Fig. 1: XRD Spectrum of PI

X-ray diffractograms of NF and NS are shown in Figures 2 and 3 respectively. The well defined X-ray diffraction peaks in the case of NF and NS indicate that NiO formed are crystalline in nature. Also, the diffraction peaks are notably broadened indicating the smaller crystallite size.

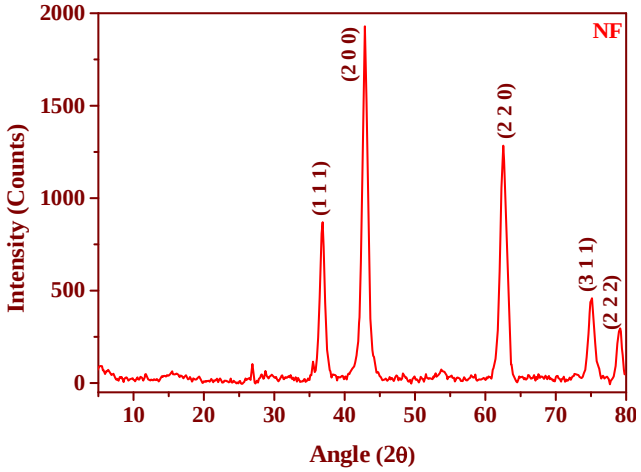

Fig. 2: XRD Spectrum of NF

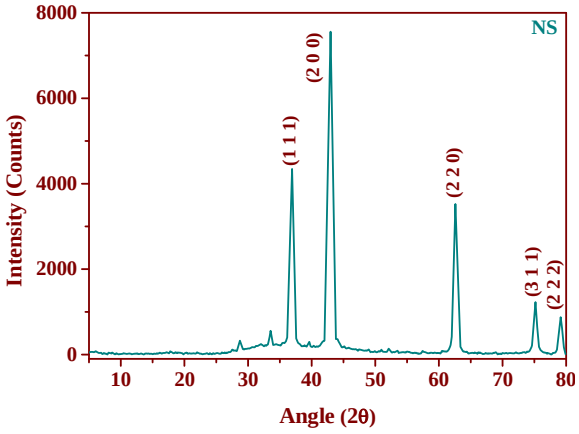

Fig. 3: XRD Spectrum of NS

In order to confirm the phase purity of the samples prepared, the interplanar spacing (d_{hkl} values), 2θ values and relative intensity values corresponding to the observed diffraction peaks were compared with the standard values of NiO reported by JCPDS-International Centre for Diffraction Data. From the comparison of diffraction peaks NF and NS with JCPDS-ICDD pattern number #78-0423 it is clear that NiO formed was a cubic system with FCC lattice.

The average crystallite size were calculated from the line broadening of the XRD pattern, making use of Scherrer formula [10,11] The crystallite size calculated using FWHM (Full width at half maximum) values of five major peaks in the XRD spectrum of NF and NS using Scherrer equation and was found to be 11 nm and 19 nm respectively. It is found that the crystallite size and crystallinity increase with increase in annealing temperature. This is due to agglomeration of particles at higher temperatures.

From the XRD analysis of PIN it is clear that the NiO particles are well distributed in the polymer matrix. The well defined peaks of planes (1 1 1), (2 0 0), (2 2 0) and (3 1 1) and (2 2 2) of NiO were found to be incorporated into the XRD diffraction pattern of polyindole. Also the crystalline peaks in the region between 15° and 30° observed in PIN showed variations when compared to PI which also supports the formation of nanocomposite. Figure 4 shows the XRD pattern of PIN.

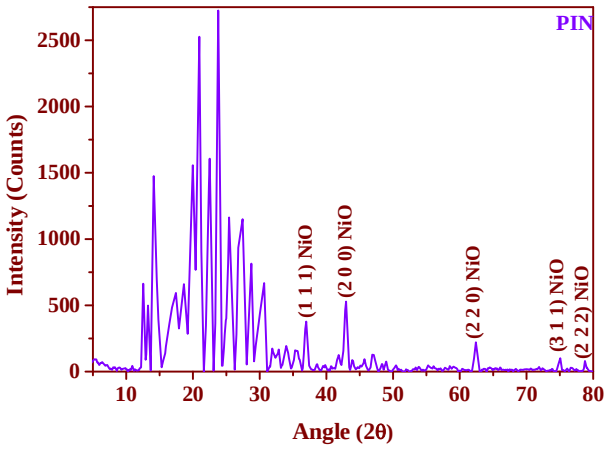

Fig. 4: XRD Spectrum of PIN

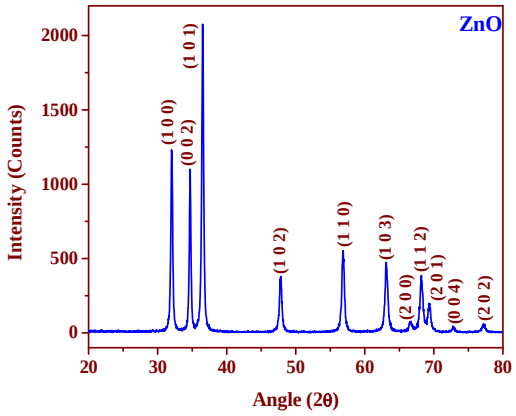

Fig. 5: XRD Spectrum of ZnO

Similar to NF and NS, NZF and NZS also showed well defined X-ray diffraction peaks which indicated that NZF and NZS too had crystalline nature. Again, the broader diffraction peaks indicated the smaller crystallite size. The interplanar spacing (d_{hkl} values), 2θ values and relative intensity values of NZF and NZS corresponding to the observed diffraction peaks were compared with the standard values of NiO and ZnO as reported by JCPDS-International Centre for Diffraction Data. The obtained pattern for ZnO (ZF) was found to match well with JCPDS-ICDD pattern number #79-0205. The data obtained for NiO/ZnO matched with JCPDS-ICDD pattern number #78-0423 of NiO and JCPDS-ICDD pattern number #79-0205 of ZnO separately. From JCPDS, NiO shows cubic system with FCC lattice and ZnO shows hexagonal system with primitive lattice. Figures 6 and 7 show XRD patterns obtained for NZF and NZS respectively.

The variation observed in the d values of the crystal planes in case of NZF and NZS when compared to NiO and ZnO, confirmes the formation of composites. This is also supported by the disappearance of the peak corresponding to (1 0 3) of ZnO in the XRD of the nanocomposite.

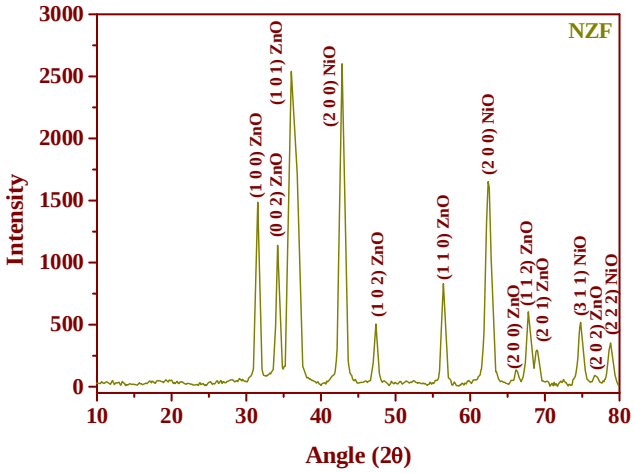

Fig. 6: XRD Spectrum of NZF

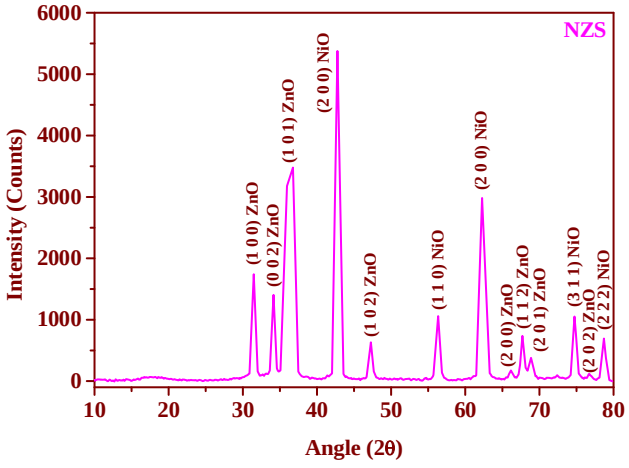

Fig. 7: XRD Spectrum of NZS

The average crystallite size of NZF and NZS calculated from the line broadening of the XRD pattern, using FWHM values of seven major peaks in the XRD spectrum making use of Scherrer formula and was found to be 18 nm and 22 nm respectively. Due to agglomeration of particles at higher temperatures the crystallite size and crystallinity was found to increase with increase in annealing temperature.

XRD of PINZ confirms the formation of nanocomposite. The well defined peaks of planes (1 0 0), (0 0 2) and (1 0 1) of ZnO and (2 0 0), (2 2 0), (3, 1 1) and (2 2 2) of NiO were found to be incorporated into the XRD spectrum of polyindole. It is clear that the metal oxide particles are well distributed in the polymer matrix. Here also the crystalline peaks in the region between 15° and 30° observed in PINZ showed variations when compared to the XRD of PI which also supports the formation of nanocomposite. Figure 8 shows the XRD pattern of PINZ.

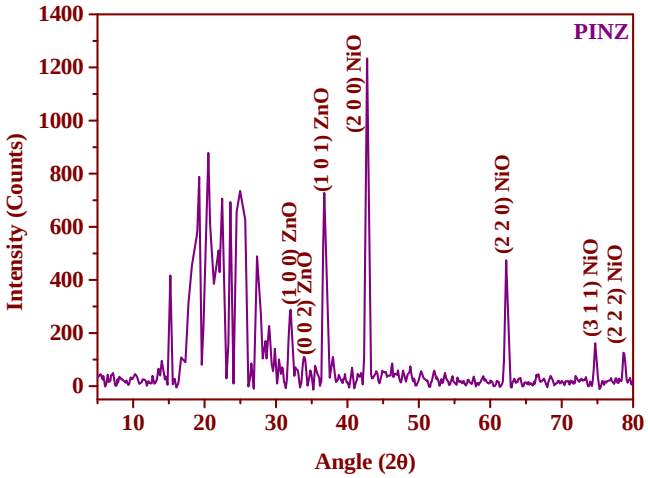

Fig. 8: XRD Spectrum of PINZ

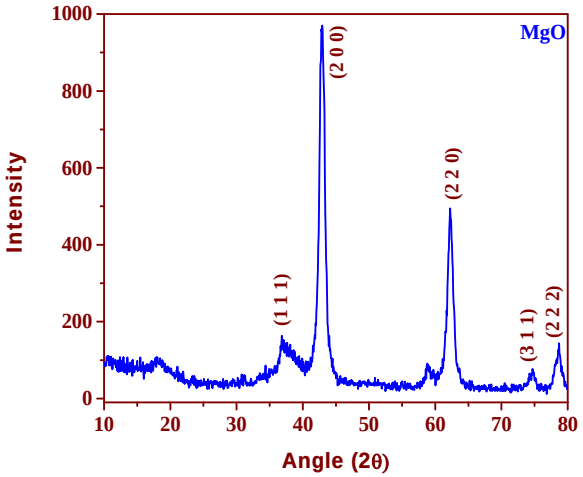

Fig. 9: XRD Spectrum of MgO

NMF and NMS also showed well defined X-ray diffraction peaks which indicated the crystalline nature of NMF and NMS. Here also the broader diffraction peaks obtained indicated the smaller crystallite size. The interplanar spacing (d_{hkl} values), 2θ values and relative intensity values of NMF and NMS corresponding to the observed diffraction peaks were compared with the standard values of NiO and MgO separately. Figure 9 shows XRD diffraction pattern obtained for MgO. The obtained pattern for MgO was found to match well with JCPDS-ICDD pattern number #89-7746. The obtained data for NMF and NMS were matched with JCPDS-ICDD pattern number #78-0423 of NiO and JCPDS-ICDD pattern number #89-7746 of MgO separately. From JCPDS comparison it is clear that MgO like NiO has a cubic system with FCC lattice. Hence it could be concluded that NMF and NMS also formed a cubic system with FCC lattice. Figures 10 and 11 show XRD pattern obtained for NMF and NMS respectively.

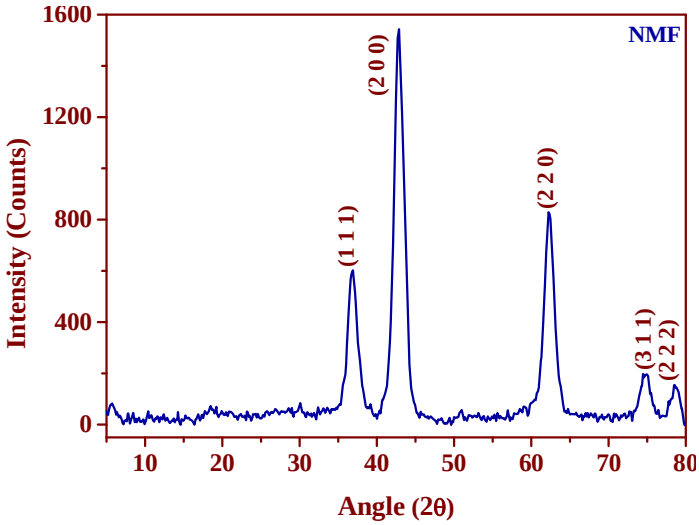

Fig. 10: XRD Spectrum of NMF

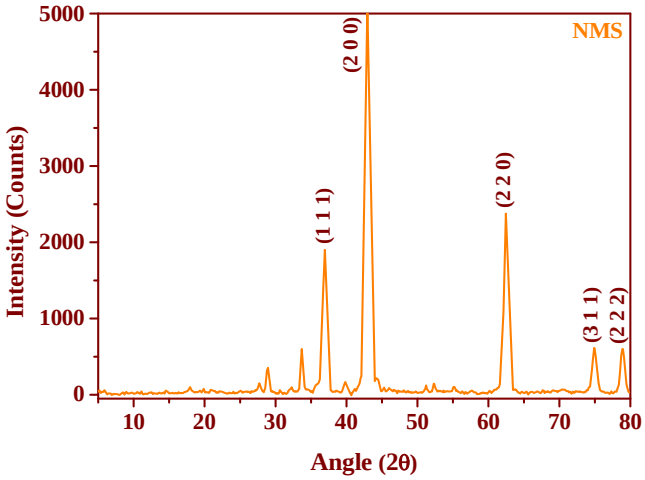

Fig. 11: XRD spectrum of NMS

The average crystallite size of NMF and NMS calculated from the line broadening of the XRD pattern, using FWHM values of three major peaks in the XRD spectrum making use of Scherrer formula and was found to be 6 nm and 16 nm respectively. Due to agglomeration of particles at higher temperatures the crystallite size and crystallinity was found to increase with increase in annealing temperature.

Both NiO and MgO are cubic systems with FCC lattice and the ionic radii of Ni^{2+} and $Mg^{2+}$are also similar. Their lattice parameters are also very close very close. For these reasons, the two oxides can easily form nanocomposite in which the ratio between the components can vary without disturbing the homogeneity. As NMF is formed, the individual XRD peaks of NiO and MgO merges together resulting in the same crystal planes.

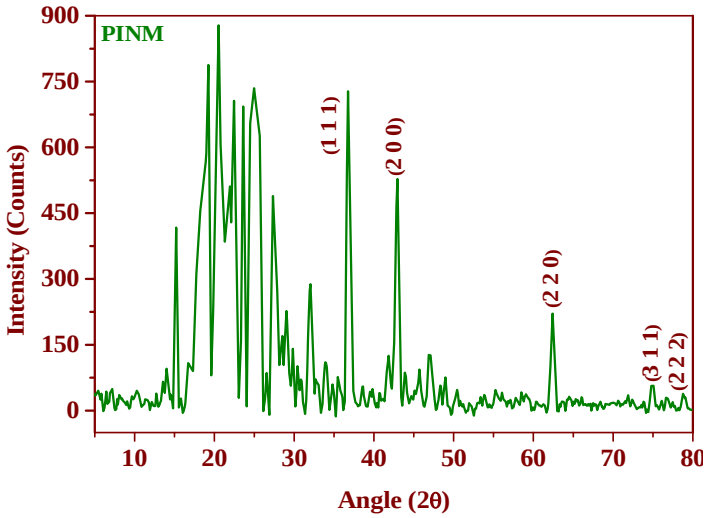

Fig. 12: XRD Spectrum of PINM

XRD of PINM confirms the formation of nanocomposite. The well defined peaks corresponding to the planes (1 1 1), (2 0 0), (2 2 0), (3, 1 1) and (2 2 2) of NiO/MgO were found to be incorporated into the XRD spectrum of polyindole. The variation in the peaks observedin PINM and PI in the region between 15° and 30° supports the formation of nanocomposites. It is clear that the metal oxide particles are well distributed in the polymer matrix. Figure 12 shows the XRD pattern of PINM.

3.2 Antibacterial Studies

The polyindole based metal oxide nanocomposites: PIN, PINZ and PINM showed significant antibacterial effects on the gram positive bacteria: S. epidermidis and C. perfringens, gram negative bacteria: E. coli and V. cholera.

In the case of metal oxides the activity was found to be annealing temperature dependent. The activity of NF was found to higher than NS (Table 1). Similarly the activity of the nanocomposite metal oxides NZF and NMF annealed at 500 °C was found to higher than those of NZS and NMS, the metal oxide nanocomposites annealed at 700 °C (Tables 2 and 3). This decrease in activity of samples annealed at higher temperature can be attributed to decrease in surface to volume ratio due to increase in particle size. The antimicrobial activity of the nanoparticles is generally known to be a function of the surface area which is in contact with the microorganisms. Reactions take place at the surface of a chemical or material. Hence, the smaller size and the higher surface to volume ratio *i.e.*, larger surface area of the NF when compared to NS has enhanced its interaction with the microbes. Table 1 gives the values of zone of inhibition area of NF and NS. Similarly, and NZF and NMF nanocomposites when compared to NZS and NMS have enhanced activity (Table 2 and 3).

Among NF, NZF and NMF the activity was found to be highest for the metal oxide composite, NZF. Here the activity can be attributed to the combined activities of NiO and ZnO. Antimicrobial effects of ZnO nanoparticles can be attributed to several mechanisms:

1. Induction of oxidative stress due to ROS (reactive oxygen species) generation,
2. Membrane disorganization due to accumulation of ZnO nanoparticles in the bacterial membrane and also their cellular internalization,
3. Release of Zn ions that may be responsible for antimicrobial activity by binding to the membrane of microorganisms.

These factors when combined with the activity of Ni^{2+}could have resulted in its higher antimicrobial activity when compared to NF. The zone of inhibition area of NZF is shown in Table 2.

In the case of NMF also the activity was found to be higher than NF. Here the inhibition of microbial growth can be attributed to the combined effect of NiO and MgO. The mechanism of the antibacterial activity of the MgO nanoparticles are mainly attributed to the presence of defects or oxygen vacancies at the surface of the nanoparticles which lead to the lipid peroxidation and reactive oxygen species generation. The activity was higher for NZF. This might be due to the specific activity of NZF to the selected microbes when compared to NMF. Furthermore, other factors such as nanoparticle diffusion rates may also affect bacterial strain differently. The zone of inhibition area of NMF and NMS are given in Table 3.

These materials also being good photocatalysts, their antibacterial mechanism can also be attributed to the destruction of the outer membrane of bacteria by the generated superoxide anion radicals ($\bullet O^{2-}$) as the reactive species. The reactive species such as •OH and $\bullet O^{2-}$ are generated at the catalyst's surface, hence the high surface area is very beneficial for degradation of bacteria.

Polyindole prepared in the present study, was found to have significant antimicrobial activity. Like polyailine, polyindole also contains quarternary ammonium salts (QAS) and halamines. Hence the antibacterial activity of PI can be attributed to the activity of QAS and halamines which are present in the polymer chain due to the presence of charged nitrogen as well as due to chloride ions. Nitrogen is present in the PI structure itself and the presence of chloride ions in the polymer chain as dopant. The presence of these two combined with the polycationic nature of polymer and the presence of polyfunctional groups is supposed to be a perfect recipe for making an antibacterial agent.

Table 1: Comparison of Antibacterial and Antifungal activity of NF, NS, PI and PIN

Samples	E. coli ZOI (mm)	C. perfringens ZOI (mm)	V. cholerae ZOI (mm)	S. epidermidis ZOI (mm)
NF	10	12	9	13
NS	9	10	7	11
PI	8	9	7	10
PIN	15	18	14	19

Composite of PI with metal oxides; PIN, PINZ and PINM have higher activities when compared to their counter parts. All the factors of PI combined with the activity of metal oxides was found to enhance the activity of the respective nanocomposite. The activity was found to be highest for PINZ than for PINM. Among the polyindole nanocomposites, PIN had lowest activity. The value of zone of inhibition area (ZOI) for PIN is given in Table 1. The corresponding values for PINZ and PINM are given in Table 2 and 3 respectively.

Table 2: Comparison of Antibacterial and Antifungal activity of NZF, NZS, PI and PINZ

Samples	E. coli ZOI (mm)	C. perfringens ZOI (mm)	V. cholerae ZOI (mm)	S. epidermidis ZOI (mm)
NZF	17	19	15	20
NZS	11	13	9	15
PI	8	9	7	10
PINZ	20	23	18	25

Table 3: Comparison of Antibacterial and Antifungal activity of NMF, NMS, PI and PINM

Samples	E. coli ZOI (mm)	C. perfringens ZOI (mm)	V.cholerae ZOI (mm)	S. epidermidis ZOI (mm)
NMF	16	17	13	18
NMS	10	12	8	14
PI	8	9	7	10
PINM	18	20	16	22

Among the microbes the activity of these samples was highest for S. epidermis and then for C. perfringens. Both these are gram positive bacteria. For all the samples, the activity was highest for gram positive than for gram negative bacteria. Among gram negative bacteria the activity was higher for E. coli than for V. cholerae. The variation in the sensitivity or resistance to both gram positive and gram negative bacteria populations could be due to the differences in the cell structure, physiology, metabolism or degree of contact of organisms with nanoparticles. Gram negative bacteria have a special cell membrane structure which possesses an important ability to resist antimicrobial agents; it has a relatively impermeable lipid based bacterial outer membrane. Greater sensitivity among gram positive bacteria can also be attributed to the greater abundance of amines and carboxyl groups on their cell surface than gram negative bacteria and the greater affinity of the antimicrobials used in the present work towards these groups.

4. CONCLUSIONS

The antimicrobial activity of all the synthesized nanocomposites were studied and compared. The results varied considerably. Antimicrobial activity was found to decrease withincrease in particle size in case of metal oxides. The presence quarternary ammonium salts (QAS) and halamines, the polyfunctional group and polycationic nature of polymer chain when combined with the activity of metal oxides enhanced antimicrobial activity of polyindole based metal oxide nanocomposites. The results indicate that both nanometal oxides and nanocomposites are effective against gram positive bacterial strains and gram negative bacterial strains. Activity was found to be highest for gram positive bacteria. Antimicrobial activity was found to be highest for polyindole based NiO/ZnO nanocomposite. Proper tuning of the nanocomposite is expected to improve its antimicrobial activity.

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Original Article

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2D and 3D QSAR model generation of CDK4 inhibitors

Divya V[1], Pushpa V.L.[1]*, Sarithamol S, Manoj K.B[1]

Author Affiliations
[1]Department of Chemistry, Sree Narayana College, Kollam, Kerala, India.

Corresponding Author
***Pushpa V.L,** Department of Chemistry, Sree Narayana College, Kollam, Kerala, India.

E-mail: drpushpavl2017@gmail.com

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Abstract

2D and 3D QSAR studies were applied on a set of CDK4 inhibitors which were assayed by same method to model and understand inhibitory activities. Among the various 2D QSAR models generated, ten models created by Kernel-Based Partial Least Square (kpls) regression method had high scores with an average value 0.8561. Top ranked model kpls_ linear_15 has a score value of 0.8804, R^2 0.8947 and Q^2 0.8909. This model considers linear fragments and ring closures. Bioactive conformation of the ligand molecules were identified by docking analysis of highly active molecule **2 (**pIC50 =8.796) in the Extra Precision mode with CDK4 homology model and the binding affinity was obtained as XP GScore of –8.072 which is the total contribution of coulomic interactions, binding interactions and van der Waals interations. All optimized structures were aligned and an atom based 3D QSAR model was established (R^2=0.9651, R^2_{cv}=0.8191 and Q^2=0.7368). This model was assigned large contribution of hydrophobic/ nonpolar features, electron withdrawing features and hydrogen bond donor features to the biological activity. The influences of these structural features were validated by analyzing the docking results. These outcomes will direct advance structural alteration and prediction of new CDK4 inhibitors.

Keywords: QSAR, Homology model, CDK4, Docking, Drug design

1. INTRODUCTION

Cell cycles are regulated by different types of Cyclins and Cyclin Dependent Kinases. Of these, the *holoenzyme* [1] formed by the complexation of G1/S-Specific Cyclin D1 and Cyclin Dependent Kinase 4 and 6 (CDK4/6) are of particular interest because the mutation in its genome level [2–5] or over expression of either the proteins (Cyclin D1 and CDK4) or the holoenzyme is very significant in the proliferation of different types of cancers.

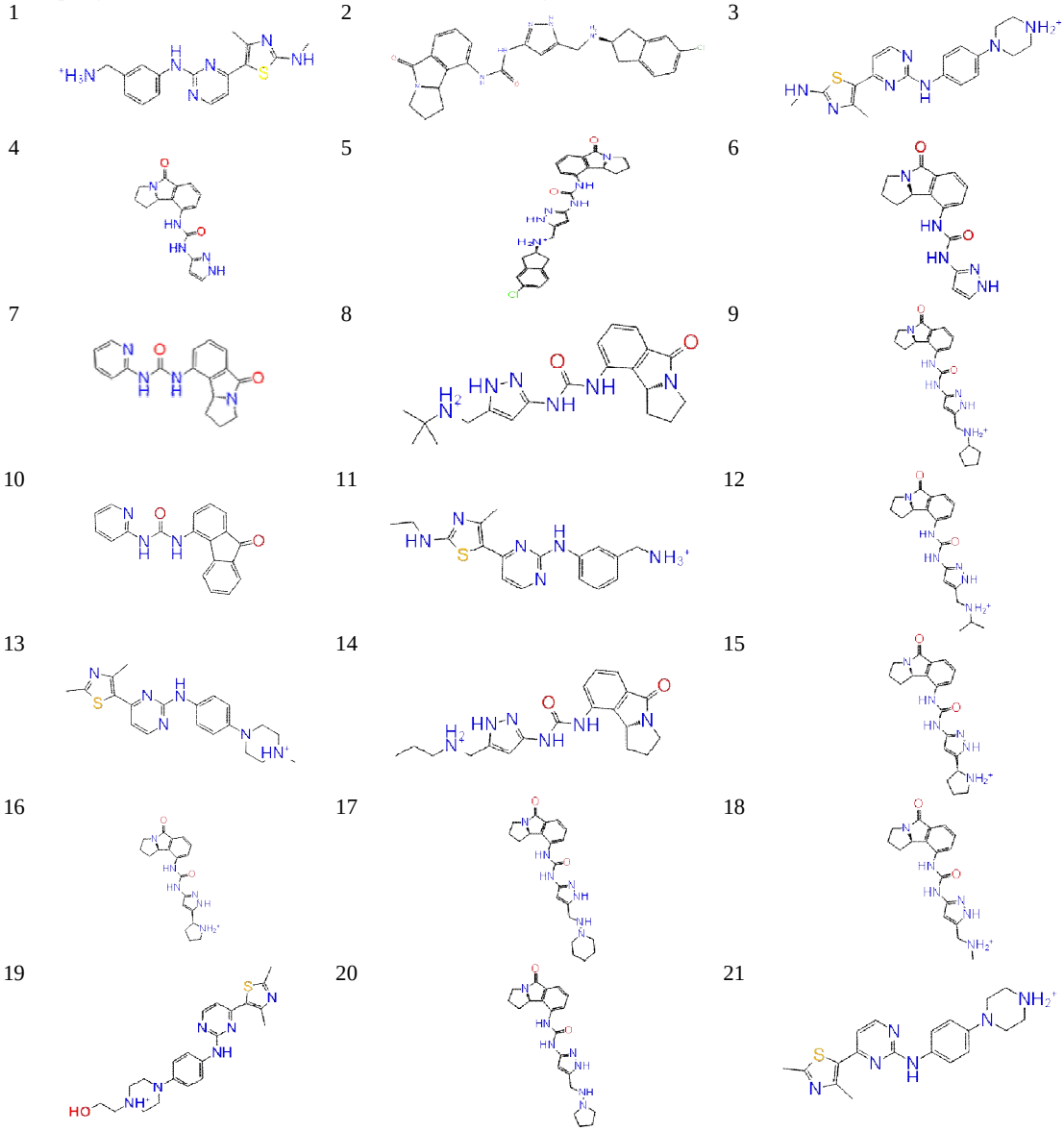
ATP competitive CDK4 inhibitors have shown to cause apoptosis at the G1/S level and thus promote cancer cell death. In the present study, we performed 2D and 3D QSAR studies on 43 ATP competitive CDK4 inhibitors founded on their structures and biological activities to get understanding into the vital structural aspects that influence selective inhibition. 2D QSAR models were generated following a best-practices methodology like generation of descriptors, validate, generate a large

number of QSAR models and apply models for prediction [6]. Atom based 3D QSAR study was employed for finding out the significance of electrostatic features, steric factors, non-bonding interactions etc of the same data set. For finding out the bioactive conformation of the ligand molecule, several structure-based designing strategies including homology modeling of CDK4 and docking analysis of highest biologically active molecule (**2**) were carried out. Assuming the docked form of the molecule **2** as the active conformation, all the prepared molecules were aligned on it and went for Atom based 3D QSAR modeling.

2. EXPERIMENTAL

2.1. Data Set

3D structures of 43molecules having inhibitory activity towards CDK4 in terms of IC50 [7–10] were drawn and prepared by using the ligprep module of Schrodinger software suits. The molecules were randomly divided into training sets (33 molecules 75%) and test sets (11 molecules, 25%) for developing the robust 2D and 3D QSAR models (Figure 1).



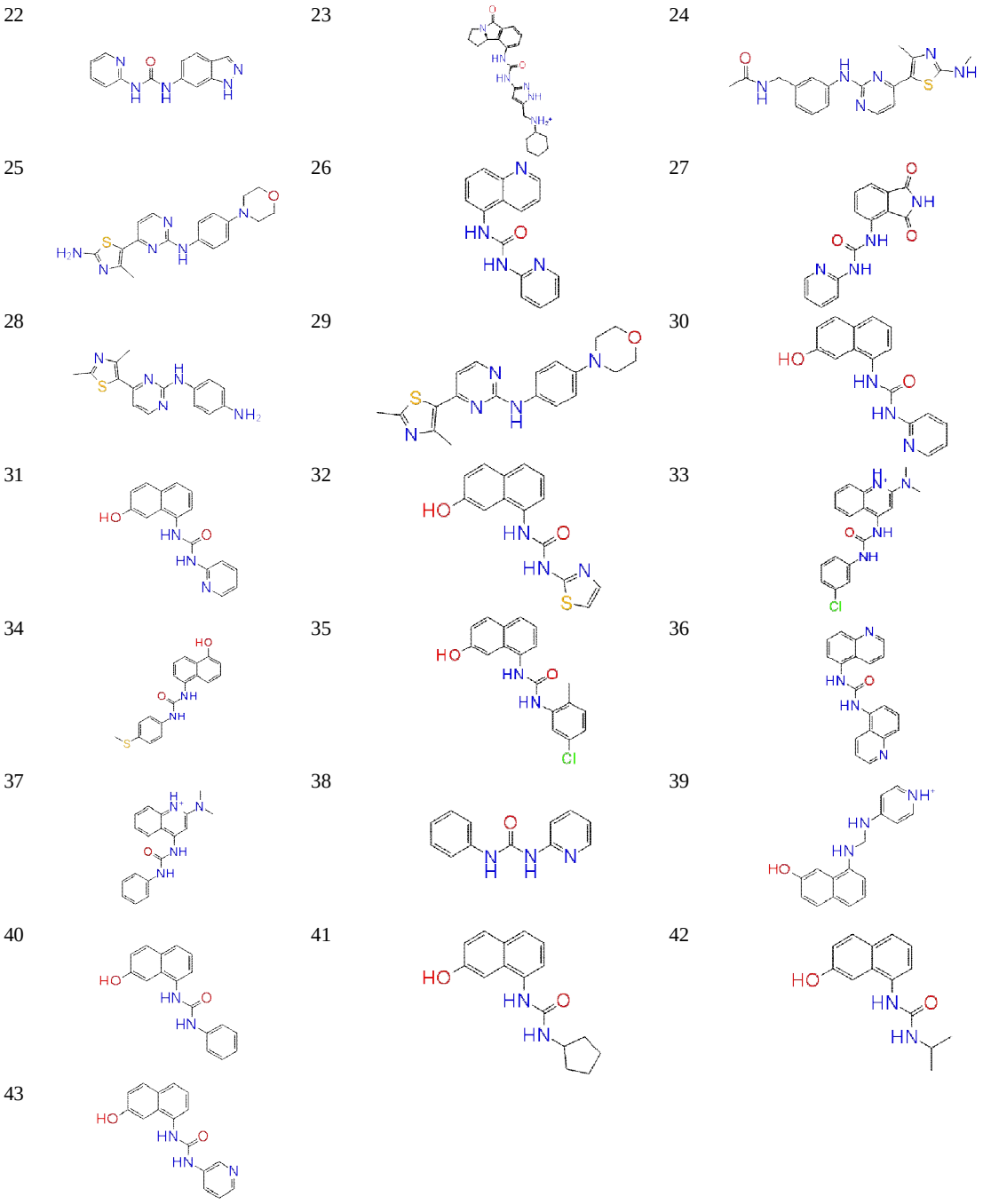


Fig. 1: Structure of molecules with CDK4 inhibitory activity

2.2. 2D QSAR study

AutoQSAR is a fully automated QSAR model generator from Schrodinger Software suit. By considering all the 1D, 2D and 3D structural data as input and computed descriptors and fingerprints and best suitable QSAR modelsare also generated using CANVAS module of Schrodinger Software suit. It adopts different approaches like Kernal- based partial least squares, Naïve Bayes and ensemble-based recursive partitioning for model generations. Different QSAR models are ranked basedon their performance. Diverse machine learning approaches were executed for training individualistically using these descriptors and fingerprints. Those descriptors were eliminated which

were having the same value for all compounds since they do not provide any significant data. Various descriptors with substantial collinearities were also eliminated since they do not provide any useful contributions to the development of QSAR. The maximum allowed correlations between any pair of independent variables were adjusted to 0.80.Reduction was achieved by performing hierarchical, agglomerative clustering on the absolute Pearson correlation matrix of the descriptors, which is a proper similarity matrix. 50 different random splits of learning set into 75% training set and 25% test set and went for QSAR model generations. A total of 400 models are generated.A quality score is assigned to each model based on its performance on both its training set and test sets and are sorted by decreasing score and the top ten retained.

2.3. Modeling and 3D QSAR studies

Conformations of ligands in the bioactive form, its orientation in the lattice and alignments are the main contributing factors for the 3DQSAR analysis [11]. Since no inhibitor bound CDK4 protein crystal structure was reported, a homology model of CDK4 was constructed using the crystal structure of CDK2 (1GIH; 2.8Å resolution) complexed with CDK4 inhibitor, **10** [12]. Knowledge based model building method was adopted by which insertions were constructed and gaps were closed with segments from known structures and returned to a single model of the structure. The inhibitor molecule bound with the template protein structure was also selected while building the model.

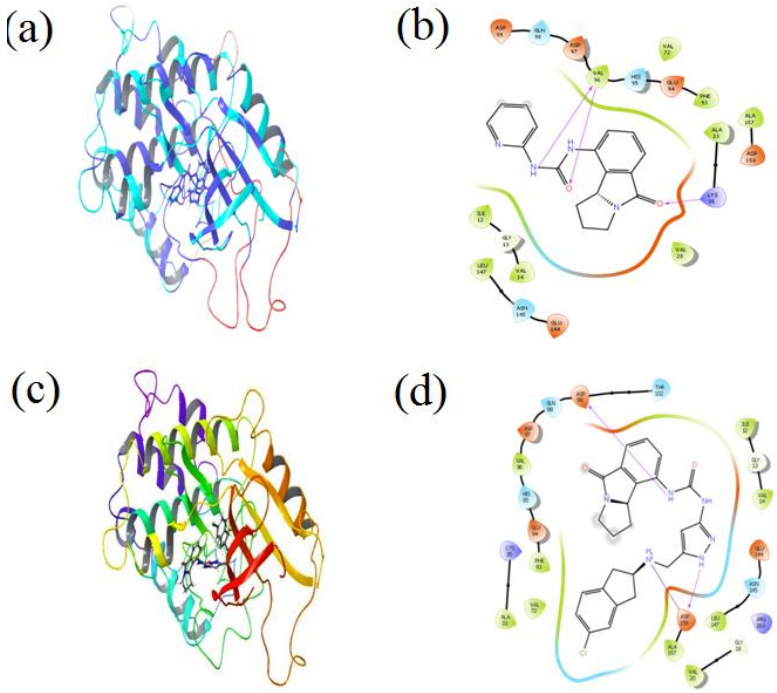


Fig. 2: (a) Homology model of CDK4 with natural ligand, 10 (b) Ligand interaction diagram of modeled CDK4 and natural ligand,10 (c) Homology model of CDK4 with compound 2 and (d) ligand interaction diagram of modelled CDK4 and compound 2.

Molecular alignment is crucial for the construction of reliable 3D QSAR models. The sensitivity of alignment based 3D-QSAR was determined by the superimposition of bioactive conformations of molecules on each other [13]. In this study, docking based alignment was adopted since this method is comparatively accurate without much subjective influence. Docked conformation of **2** was selected as a template for alignment since it had a high biological activity (Figure 2). Docking was performed in extra precision mode. Receptor Grid was generated around the centroid of workspace ligand by selecting the molecule. The scaling factor corresponding to van der Waals radii of non-polar receptor atoms was adjusted to 1.0 and the partial atomic charge cut off was specified below 0.25. All other

prepared molecules were aligned on the bioactive conformation of the template molecule, **2.** Figure 3 represents the alignment of molecules on the bioactive conformation of **2**.

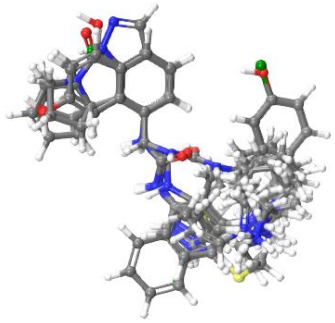


Fig. 3: The alignment of molecules on the bioactive conformation of **2**.

Both internal and external cross validation techniques were used for developing model since the consistency of the model and prediction of biological activity of those molecules which were not included in the training set were dependent on its statistical implication. Same molecules were considered for training and test set as that of 2D QSAR model. An atom based QSAR model was developed based on these molecules.

The 3D grid covering the space occupied by the ligands was specified as 1Å .maximum number of partial least square factors was chosen as 3. With an aim to get better predictions of test set molecules, we have to avoid unproductive variables. This had to be achieved by eliminating variables with l**t level**l less than 2. Since the number of training molecules was 33, leave one out (LOO) cross validation statistics was used for internal validation the QSAR model.

3. RESULTS AND DISCUSSIONS

3.1. 2D QSAR study

Based on 2D structural information and biological activity of 43 structurally prepared molecules, 497 physiochemical and topological descriptors along with a variety of Canvas fingerprints were generated and based on these independent variables, models were built. A large number of models were built and validated using different random training and test sets. The quality of the model was evaluated by analyzing the score. Top ranked ten models were listed in Table 1. From the analysis, it was found that Kernel-Based Partial Least Square (kpls) regression method using both descriptors and fingerprints generates high scored model.

The robustness of the model can be assessed by its score value. As the accuracy level approaches to 1, corresponds to perfect predictions. The score value can be calculated as

$$Score_M = accuracy_{test}\ (1.0 - |accuracy_{train} - accuracy_{test}|)$$

In the present study, all the top ranked ten models have score values greater than 0.82 and has an average value of 0.8561. Scatter plot of top scored model is presented in Figure 4.

The correlation coefficients $R^2$of the models between the observed and predicted activity of the training set have an average greater than 0.85 and that of the test sets also have an average Q^2 value 0.8675.The average RMSE and standard deviations of all these models were 0.4897 and 0.5222 respectively. And all these statistical results were largely established for high performance. Top ranked model, kpls_linear_15shows the highest score (0.8804), R^2(0.8947) and Q^2 (0.8909)and does considerably better than all other fingerprint types. This finding advocates that this model is a robust fingerprint method that has utmost chance of perfect execution with any settings.

Table 1: Statistical parameters of 2D QSAR models

Model Code	Score	S.D	R^2	RMSE	Q^2	MW(Null Hypothesis)
kpls_linear_15	0.8804	0.4693	0.8947	0.4693	0.8909	0.5264
kpls_molprint_2D_50	0.8801	0.5216	0.8762	0.3431	0.9342	0.4234
kpls_dentritic_17	0.8691	0.5231	0.8767	0.4985	0.8546	0.515
kpls_dentritic_49	0.866	0.5522	0.8632	0.4673	0.8701	0.5475
kpls_molprint_2D_15	0.8609	0.5455	0.8577	0.4728	0.8892	0.5264
kpls_linear_49	0.8532	0.5136	0.8816	0.5105	0.8439	0.5475
kpls_dentritic_20	0.852	0.4882	0.8885	0.5056	0.869	0.1848
kpls_molprint_2D_49	0.8394	0.5614	0.8586	0.5432	0.8238	0.5475
kpls_dentritic_15	0.839	0.5082	0.8765	0.5295	0.861	0.5264
kpls_molprint_2D_24	0.8206	0.5384	0.8671	0.5554	0.8371	0.2262

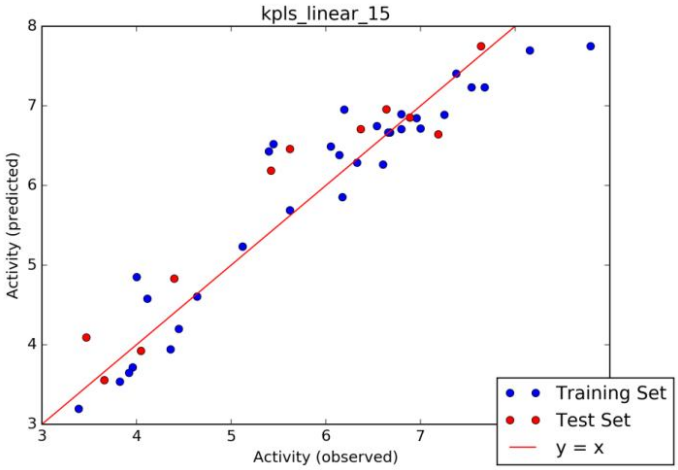


Fig. 4: kpls linear 2D QSAR model

3.2. Modelling and 3D QSAR studies

Homology model of CDK4 was constructed in the ligand bound form considering CDK2 as a template using the Prime module of Schrodinger software suitIle12, Val 20, Ala 33, Val 77, Phe 93, Glu 94, His 45, Val 96, Gln 98, Asp 99, Thr 102, Glu 143, Leu 147, Ala 157 and Asp158 are the binding region residues [14].

Both Intrinsic forces and extrinsic forces considerably stimulate the bioactive conformation of the molecule [15–16]. Consistency of 3D QSAR model was governed by the determination of bioactive conformations. In order to find out the bioactive conformation of the ligands, docking procedure was adopted. The molecule, **2** with highest biological activity in terms of pIC50 (8.796) was selected for flexible docking with CDK4 in its active site in which the natural ligand **10** was bound. Figure 2(a) and (b) represents the bound conformation and the ligand interaction diagram of natural ligand **10** (pIC50=7.00) and the modelled CDK4. The bound molecule formed two hydrogen bonds with Val 96 of CDK4 and another hydrogen bond with Lys 35. The natural ligand is a diaryl urea derivative, **10** with specific inhibition towards CDK4. Figure 2(c) and (d) characterizes the docked conformation of ligand, **2** (pIC50=8.7959) which had a high biological activity in terms of pIC50 with modelled CDK4. Compound **2** forms two hydrogen bonds with CDK4 at residues Asp99 and Asp158; both were in the hinge region. A salt bridge was also formed between Asp158 and the ligand molecule. These two hydrogen bonding connections together with salt bridge contributes

positively towards its high XPGScore of –8.072, docking score of –7.826 with glide energy of –58.781. Glide XP GScore is the total contribution of Coulombic interactions, binding interactions and van der Waals interactions [17]. Desolvation energy as well as strain energy caused by either ligand molecule or the protein obstructs binding. These penalties were also considered for calculating the XP GScore. It is symbolized as follows:

$$\text{XP GScore} = E_{coul}+E_{vdW}+E_{bind}+E_{penalty}$$
$$E_{bind} = E_{hyd\_enclosure}+E_{hb\_nn\_motif}+E_{hb\_cc\_motif}+E_{PI}+E_{hb\_pair}+E_{phobic\_pair}$$
$$E_{penalty} = E_{desolv}+E_{ligand\_strain}.$$

Observed biological activity and predicted biological activities predicted by both 2D QSAR and 3D QSAR methodology are summarized in Table 2 and the scatter plot is shown in Figure 5.

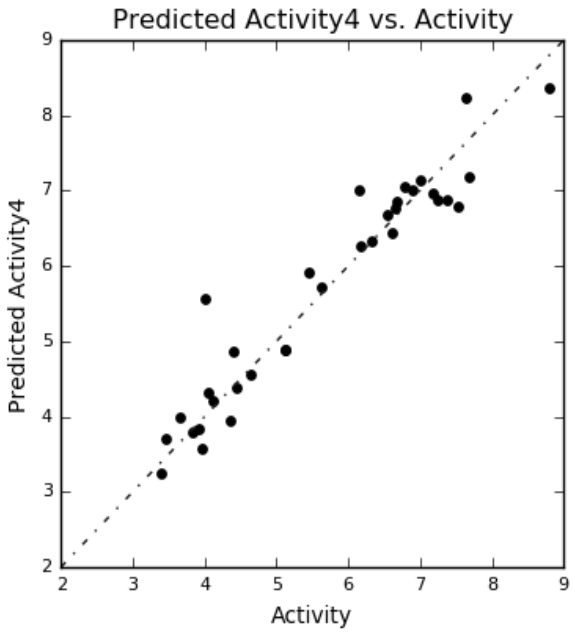


Fig. 5: Scatter plot of Atom Based 3D QSAR model

Predictiveness of the model can be assessed by foretelling the activity of an internal test set and external test set of molecules. The robustness of the model was depend on the R^2_{cv} and R^2_{cv}=($PRESS_0$-PRESS)/($PRESS_0$) where $PRESS_0$ is the mean of the observed biological activity while PRESS is the sum of the squares of the differences between the predicted and the observed activity values [18]. Q^2 is the Predictive correlation coefficient is a measure of predictive ability of the derived QSAR model and is calculated by Q^2 [19]. Q^2= (SD – PRESS) / SD where SD is the sum of squared deviations between the biological activities of the test set molecules and the mean activity of the training set molecules, while PRESS is the sum of squared deviations between the observed and the predicted activities of the test set molecules. A greater F value (Fischer Statistic value) infers that a more noteworthy correlation has been reached. This value can be considered as a degree of the statistical implication of the regression model [20]. It is counted as a norm to define whether a more complex model is significantly better than the other.

Table 2: Experimental and predicted inhibitory activities of CDK4 inhibitors

Title	Set	Y(Obs)	2D QSAR		3D QSAR	
			Y(Pred)	Error	Y(Pred)	Error
1	test	6.6383	6.9581	0.3198	6.6423	0.004
2	train	8.7959	7.7489	–1.047	8.357	–0.4389
3	train	8.1549	7.6977	–0.4572	7.2029	–0.9520
4	train	7.6778	7.2336	–0.4342	7.1773	–0.5005
5	test	7.6383	7.7489	0.1106	8.2422	0.6039
6	train	7.5376	7.2336	–0.304	6.7842	–0.7534
7	train	7.3768	7.4071	0.0303	6.8681	–0.5087
8	train	7.2518	6.8874	–0.3643	6.8692	–0.3826
9	test	7.1871	6.6438	–0.5433	6.9593	–0.2278
10	train	7	6.716	–0.284	7.1362	0.1362
11	train	6.9586	6.8466	–0.112	6.7248	–0.2338
12	test	6.8861	6.8536	–0.0324	7.0125	0.1264
13	train	6.7959	6.8946	0.0987	6.534	–0.1884
14	train	6.7959	6.7073	–0.0886	7.0568	0.2609
15	train	6.6778	6.6646	–0.0132	6.8627	0.1849
16	train	6.6576	6.6646	0.007	6.7753	0.1177
17	train	6.6021	6.2655	–0.3366	6.4314	–0.1607
18	train	6.5376	6.7453	0.2077	6.6921	0.1545
19	test	6.3665	6.7073	0.3407	6.4845	0.1180
20	train	6.3279	6.2884	–0.0395	6.3393	0.0114
21	train	6.1938	6.9522	0.7583	6.2408	0.0469
22	train	6.1739	5.8557	–0.3182	6.2738	0.0999
23	train	6.1427	6.381	0.2383	7.0082	0.8655
24	train	6.0506	6.4888	0.4382	6.1108	0.0602
25	train	5.6198	5.6885	0.0687	5.2709	–0.3489
26	test	5.6198	6.4599	0.8401	5.7231	0.1033
27	train	5.4337	6.5224	1.0787	5.9143	0.4706
28	test	5.4202	6.1872	0.767	5.7141	0.2939
29	train	5.3979	6.4291	1.0312	5.7598	0.3618
30	train	5.1192	5.2328	0.1136	4.8807	–0.2385
31	train	5.1192	5.2328	0.1136	4.8807	–0.2385
32	train	4.6383	4.6038	–0.0343	4.5532	–0.0851
33	train	4.4337	4.197	–0.2467	4.3804	–0.0633
34	test	4.3979	4.8321	0.4341	4.8762	0.4783
35	train	4.3565	3.9429	–0.4137	3.9519	–0.4046
36	train	4.1135	4.5766	0.4631	4.2018	0.0883
37	test	4.0458	3.9236	–0.1222	4.3203	0.2745
38	train	4	4.8517	0.8517	5.5669	1.5669
39	train	3.9586	3.7166	–0.242	3.5797	–0.3789
40	train	3.9208	3.647	–0.2738	3.8295	–0.0913
41	train	3.8239	3.5352	–0.2887	3.8021	–0.0218
42	test	3.6576	3.5555	–0.1021	3.9973	0.3397
43	train	3.4685	4.0912	0.6227	3.7151	0.2466

Table 3: Results of Atom based 3D QSAR

# Factors	4
Standard Deviation	0.3052
R^2	0.9651
R^2_{CV}	0.8191
R^2 Scramble	0.6922
Stability	0.91
F	145.2
P	5.55E–15
RMSE	0.75
Q^2	0.7368
Pearson R	0.8696
Hydrogen bond donor	0.090997
Hydrophobic/non-polar	0.577229
Positive ionic	0.02014
Electron withdrawing	0.31158

From the analysis it is found that R^2 for regression is 0.9651and cross validated R^2 computed from predictions obtained by a leave one out (LOO) procedure is 0.8191. High values of both R^2 and R^2_{cv} are indicators of the high predictive ability of the model. Stability nearer to one (0.91) indicates the model predictions should be stable. Greater value of variance ratio (F=145.2) specifies high statistical significance. Greater degree of the confidence of the model was indicated by smaller value of P (5.55E–15).Q^2 is the Predictive correlation coefficient is a measure of predictive ability of the derived QSAR model and in the present study; it is found that Q^2 is 0.7368. This result was pointing to fact that the proposed model has best predictive ability.

The field contributions of factors of Hydrophobic or nonpolar influence were 57.7% and 31.1% respectively signifying that the hydrophobic or nonpolar fields have greater impact on the model.

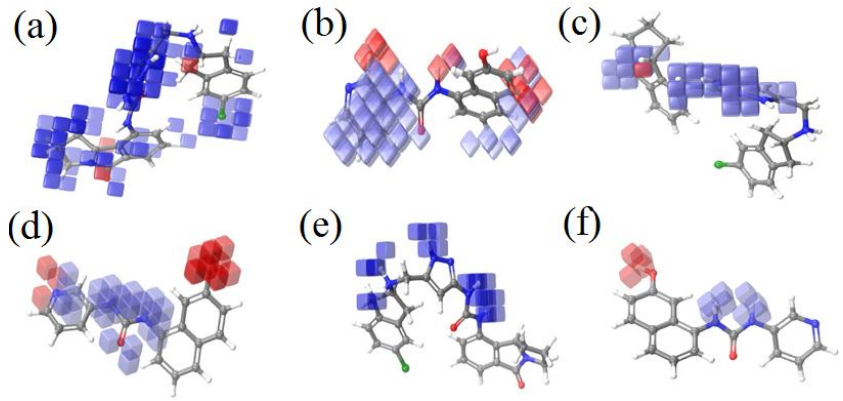


Fig. 6: Contour maps of atom based QSAR model in combination with compound 2 and 43 (a) and (b) hydrophobic/ nonpolar feature, (c) and (d) Electron withdrawing effect and (e) and (f) hydrogen bond donor feature

In atom based QSAR, a molecule is treated as a set of overlapping van der Waals spheres [21]. The consequence of spatial arrangement of structural features on CDK4 can be visualized in the Contour plot analysis. Figure 6 (a) and (c) represents the contour maps of atom based QSAR model of compound **2** of highest activity (pIC50=8.7959) and Figure 6(b) and (d) represent that of compound **43**(pIC50=3.4685). Here, blue cubes represent positive coefficient towards the inhibition and the red cubes represent negative coefficient towards inhibition of CDK4. From Figure 6(a) which represents the hydrophobic or non-polar feature, the blue cubes around benzopyrrolizin–5–one and the pyrazole

moiety contribute positively towards its hydrophobic nature whereas the carbonyl group in the 5th position of benzopyrrolizine contributes negatively. Influence of chlorine is remarkable towards inhibitory activity as it is covered with blue contours. Blue contours which favor better activity were absent in the case of **43** and this is supported by its low pIC50. Introduction of carbons, halogens and C–H in the regions favouring hydrophophic/ nonpolar regions may enhance the inhibitory activity of the molecule. Other significant component that impacts on the activity is the electron withdrawing character which includes different types of hybridization, hydrogen bond acceptor features, л-electron donation features etc and is shown in Figure.6 (c) and (d). Presence of diamide linkage, pyrazole moiety and benzopyrrolizin–5–one contribute positively towards the biological activity of the compound **2**whereas the presence of hydroxyl group in the naphthyl ring of **43** has a negative electron withdrawing effect and thus it has a lower bioactivity. Insertion of non-ionic nitrogens and oxygens may intensify pIC50 value. Hydrogens attached to polar atoms which are categorized as hydrogen bond donor attributes also contribute significantly towards the inhibitory activity (Figure 6(e) and (f)). High activity of **2** is also supported by the presence of hydrogen bonding groups as it is specified by the blue contour areas. Compound 43 lacks hydrogen bonding feature at certain specific areas. Nitrogen in the pyrrazole ring forms a hydrogen bond with Asp158 as per the docking analysis. Also, hydroxyl group (–OH) at the 7th position of the naphthyl ring reduces the inhibitory effect.

4. CONCLUSIONS

A set of molecules with CDK4 specific inhibition was examined to relate pIC50 values against CDK4 to the molecular structure. High predictive ability of 2D and 3D QSAR models was substantiated by leave one out (LOO) and internal and external validation procedures. The performance of KPLS approach was resulted in 10 best predictive models with an average score value of 0.8561. 3D QSAR results proved that electron withdrawing, hydrogen bonding as well as hydrophobic non-polar interactions affects considerably the biological activity. The scrutiny of the 3D contour maps permitted us to spot areas of identified inhibitors that require a physiochemical features to increase activity. Docking study of compound **2** support these results.

The biologically active conformation of the inhibitor molecules was provided by the docking study of the homology model of CDK4. This helped in the substantiation of QSAR models with respect to the protein environment and give exquisite perceptions for the structure activity explanations. With the aid of this information, we can design new active molecules with enhanced pharmacological properties.

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Sensitized luminescence from novel pyrazolone based β-diketonate complexes of Eu^{3+}

D.B. Ambili Raj[1]*, S.V. Manoj[1], and B.S. Vinod[1]

Author Affiliations
[1]P.G. & Research Department of Chemistry, SreeNarayana College, Kollam 691001

Corresponding Author
***D.B. Ambili Raj,** P.G. & Research Department of Chemistry, SreeNarayana College, Kollam 691001

E-mail: ambilrajdb@gmail.com

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Abstract

New pyrazolone based fluorinated ligand**,** 3–methyl 4–(2,2,3,3,3–penta fluoro–1–hydroxypropylidene)–1–phenyl–1H–pyrazol–5(4H)–one has been synthesized and well characterized. Europium–containing complexes were synthesized by adduct formation between 3–methyl 4–(2, 2, 3, 3, 3-penta fluoro–1–hydroxypropylidene)–1–phenyl–1H–pyrazol–5(4H)–one and europium (III) complex and a bidentate neutral donor 1,10–phenanthroline (phen). The emission spectra of the complexes display characteristic sharp peaks in the 575–700 nm region associated with the $^5D_0 \rightarrow {}^7F_J$ (J = 0–4) transitions of the Eu^{3+} ion.

Keywords: Lanthanide complex, PL properties, pyrazolone, fluorinated ligand, Eu^{3+}

1. INTRODUCTION

Lanthanide ions possess fascinating optical properties and are largely involved in various technological applications which are largely governed by their interaction with light. Light emitting diodes (LED's), television and computer displays, optical fibres, optical amplifiers, lasers, as well as responsive luminescent stains for biomedical analysis, medical diagnosis, and cell imaging rely heavily on lanthanide ions [1]. The two most useful lanthanides, Eu^{3+} and Tb^{3+}, have unusual spectroscopic characteristics, including longer lifetime (milliseconds), very sharp emission bands, and large Stokes shifts [2]. Unfortunately, as a consequence of the parity (Laporte) forbidden nature of the 4*f* transitions, the direct absorption of Ln(III) cations is only very weak, and they hence have very low molar absorption coefficients (typically less than 10 $M^{-1}cm^{-1}$) and hence a lower emission which limits their practical usage. In order to circumvent these lower values of extinction coefficients, the luminescent metal ion can be chelated to a chromophore–containing group which functions as an 'antenna,' by absorbing incident light and transferring this excitation to the metal ion, which can then deactivate by undergoing its typical luminescent emission. This process of 'antenna effect' will only be effective if the triplet level of the ligand is situated slightly above the accepting energy levels of the

lanthanide ion. This is because the accepting energy levels of the visible luminescent lanthanides are all situated at relatively high energy position of UV region in the spectra. Among these antenna molecules, β–diketone ligand is one kind of important antennae for the Eu^{3+} and Tb^{3+} ions [3]. β–diketonates, as they are negatively charged bidentate ligands can form 3:1 complexes with metals, which are overall neutral. Neutral ligands like phenanthroline can be coordinated in addition, because the coordination number of the ions is eight. Among the widely known ligands β–diketone appears to meet the requirements for lanthanide luminescent purpose. The advantage of the use of β–diketone ligands is that they form stable complexes with Ln^{3+}cation.The π–π^*transition of β–diketones is intense and occurs over a significant range of wavelengths that is appropriate for sensitization of Ln^{3+}cation luminescence. The replacement of C–H bonds in a β-diketone by C–F bonds is significant in the design of highly luminescent lanthanide complexes concerned the efficient emission. C–F oscillators lower the vibrational energy of the ligands and decrease the energy loss caused by ligand vibration and thereby enhances the emission intensity of the lanthanide ion. Further due to the heavy-atom effect, which facilitates inter system crossing, the lanthanide–centered luminescent properties are enhanced [4].

These are of special interest in the fabrication of OLED devices because the ternary complexes with phenanthroline or bathophenanthroline have a much lower sublimation point that allows easy sublimation of these materials on OLED devices. The lowered sublimation points are caused by distortion of the symmetry in the complexes and vacuum deposition of the complexes is relatively easily achieved.

2. EXPERIMENTAL

2.1. Materials and Methods

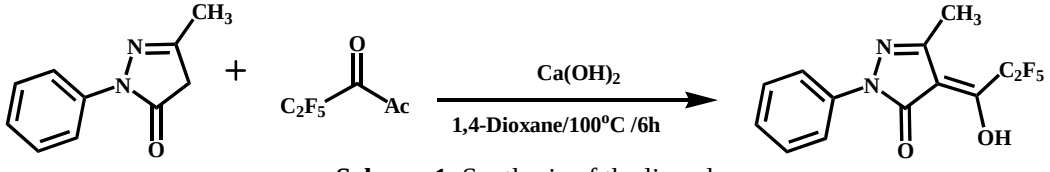
Commercially available chemicals: Europium (III) nitrate fluoropropionate (TCL), 1,10–phenanthroline are used without further purification. Infrared spectra of all samples were performed on KBr pellets in the 4000–400 cm^{-1} region with a resolution of 4 cm^{-1}, by accumulating 64 scans using a Perkin–Elmer Spectrum One FT–IR Spectrometer. A Bruker 300 MHz NMR spectrometer was used to record the ^1H NMR spectrum of the ligands. The excitation and emission spectra of the samples spectra were recorded on a Spex–Fluorolog FL3 22 spectrofluorimeter equipped with a double grating 0.22m Spex 1680 monochromator and a 450W Xe lamp as the excitation source operating in the front face mode.

2.2. Synthesis of the Ligand

Synthesis of 3 – methyl 4-(2,2,3,3,3–penta fluoro–1–hydroxypropylidene)–1–phenyl -1H–pyrazol–5(4H)–one (HMPP)

0.25g (1.435 mmol)1–Phenyl–3-methyl–5–pyrazolone and 0.111g (1.498mmol) calcium hydroxide was taken in a RB flask. It is dissolved in 1,4dioxane (10ml). Heated in a water bath at 60°C for about half an hour. It is cooled and 0.275g (1.432mmol) of ethyl pentafluoropropionate ($C_5H_5F_5O_2$) was added and stirred for 6 hours. The resulting solution was quenched with water, added 2M HCI, and the solution was extracted twice with chloroform. The organic layer was dried over sodium sulphate (Na_2SO_4), and the solvent was evaporated. The reaction mixture is then purified by chromatography on a silica gel column with mixture of chloroform (20%) and hexane (80%) as the eluent to get the yellow solid as the product. 3–methyl 4–(2,2,3,3,3–pentafluoro–1–hydroxypropylidene)–1–phenyl–1H–pyrazol–5(4H)–one (HL).

^1H NMR ($CDCI_3$) :δ = 7.26–7.86(m, 5H, phenyl); 1.73(s, 3H, CH_3 of the pyrazole ring) (ppm) IR (KBr)ν_{max}: 2929, 1628(C=O), 1597, 1503, 1358, 1213, 749cm^{-1}



Scheme 1: Synthesis of the ligand

2.3. Synthesis of the binary complex

The Eu(III) complex with the ligand **HL** was synthesized according to the following method

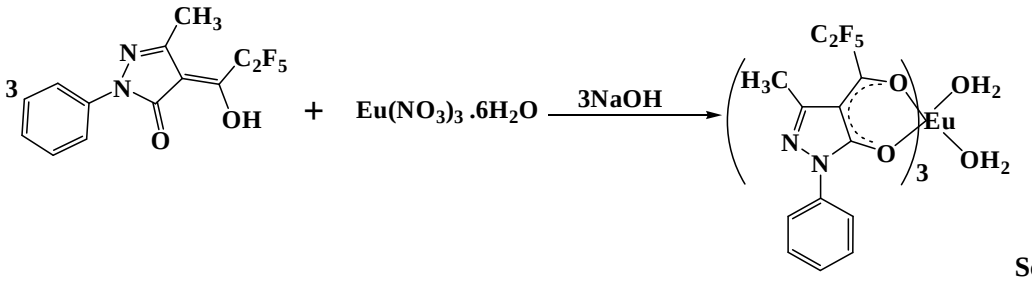
Eu $(L)_3.2H_2O$: An ethanolic solution of $Eu(NO_3)_3.6H_2O$ (0.5 mmol) was added to a solution of HL (1.5 mmol) in ethanol in presence of NaOH (1.5 mmol). Precipitation took place immediately, and the reaction mixture was stirred for 10 hrs at room temperature (Scheme **2**). The product was filtered, washed with ethanol, then with water. The complex was then purified by recrystallization from acetone–water mixture, dried and stored in a desiccator.

IR (KBr) ν_{max}: 3391, 1612, 1577,1484, 1400, 1232, 1154, 757 cm^{-1}.

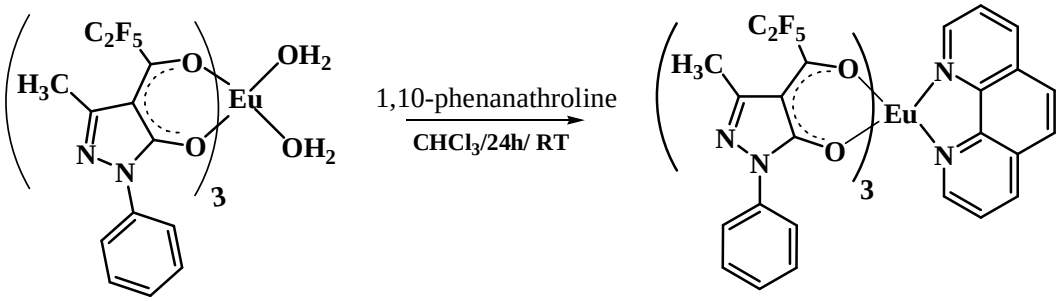
2.4. Synthesis of ternary complex

Synthesis route of the complex is shown in Scheme **3**. The complex was prepared by stirring equimolar solutions of $Eu(L)_3.2H_2O$ and 1,10–phenanathroline in $CHCl_3$ for 24 hrs at room temperature. The product was obtained after solvent evaporation and is purified by recrystallization from chloroform-hexane mixture.

$Eu(L)_3$phen: IR (KBr) ν_{max}: 3065,1641, 1613, 1485, 1439,1316, 1235, 1152, 1053, 757 cm^{-1}.



Scheme 2. Synthesis of binary complex $Eu(L)_3.2H_2O$



Scheme 3: Synthesis of ternary complex $Eu(L)_3$(phen)

3. RESULTS AND DISCUSSIONS

3.1. Synthesis and characterization of the ligand

The ligand HL was prepared as described as in Scheme **1** and was characterized by ^1H NMR. In the ^1H NMR spectrum of the ligand, no peak corresponding to the enolic–OH has been observed. However, the absence of a peak at δ 3.4 ppm, corresponding to the methylene proton at the fourth position of the pyrazolone ring, confirms the existence of the ligands in the enolic form.

3.2. Structure and characterization of Europium(III) complex

The synthesis procedure for europium complexeswere shown in Schemes **2** and **3**. The IR spectra of the binary complex show a broad absorption in the region 3000–3500 cm^{-1}, indicating the presence of solvent molecules in the complex. The existence of solvent molecules in lanthanide complexes with β–diketones is well documented [5]. On the other hand, the absence of the broad band in the region 3000–3500 cm^{-1} for the ternary complex, suggests that water molecule has been displaced by the bidentate ligands. The carbonyl stretching frequency of the ligands has been shifted to lower wave

numbers in all these complexes indicating the involvement of carbonyl oxygen in the complex formation with Eu^{3+} ion. Red shifts are observed in the C=N of 1,10-phenanthroline in the ternary complexes show the involvement of nitrogen in complex formation with Eu^{3+} ion in these complexes.

3.3. UV–Vis Spectra

UV–Vis absorption spectra of the β-diketone, 1,10–phenanthroline, and their corresponding Eu(III) complexes are measured in CH_3CN (c=2×10^{-5}) are shown in Figure 1. The maximum absorption bands at 288 nm for the ligands **HL** is attributed to singlet–singlet π–π* enol absorption of β–diketonates. Furthermore, compared with the spectra of the ligands **HL**, the absorption band of the corresponding europium complexes are all red shifted by 2 nm, which is a consequence of the enlargement of the conjugate structure of ligands after coordinating to the Eu^{3+} ion. The spectral shapes of the absorption spectra of Eu(III) complexes in CH_3CN are similar to those of the corresponding ligands, indicating that the coordination of the europium ion does not significantly influence the energy of the singlet state of the β–diketone ligands.

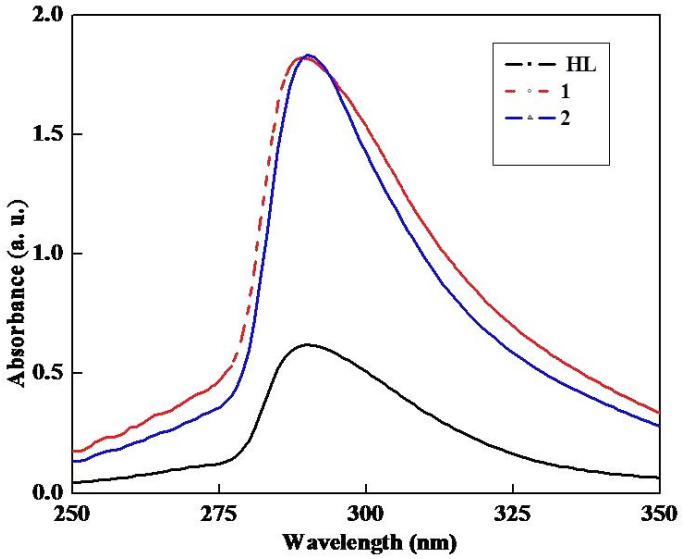


Fig. 1: UV–Visible spectra of **HL** and complexes **1–2** in acetonitrile (c = 2 × 10^{-5} M).

The molar absorption coefficients (ε) of the ligands **HL** is calculated as 2.81×10^4 (288 nm) $Lmol^{-1}cm^{-1}$, revealing that the ligand has a strong ability of absorbing light. Similarly, the determined molar absorption coefficients (ε) of the complexes Eu(**L**)$_3$(H2O)$_2$ and Eu(**L**)$_3$(phen) were 7.6×10^4 (290 nm) L $mol^{-1}cm^{-1}$, which is about three times of those of the corresponding ligand, indicating the presence of three ligands in each complex molecule and is in good agreement.

3.4. PL Properties of Europium (III) Complexes 1–2

The solid state excitation and emission spectra of the europium complexes **1–2**, recorded at 298 were shown in Figure 2. The excitation spectra were obtained with emission monitored at $^5D_0 \rightarrow {}^7F_2$ hypersensitive transition around 612 nm. The room temperature excitation spectra of the europium complexes exhibit a broad band between 250 and 450 nm, which can be assigned to ^{1}π–π* electron transition of the ligands [6–7].

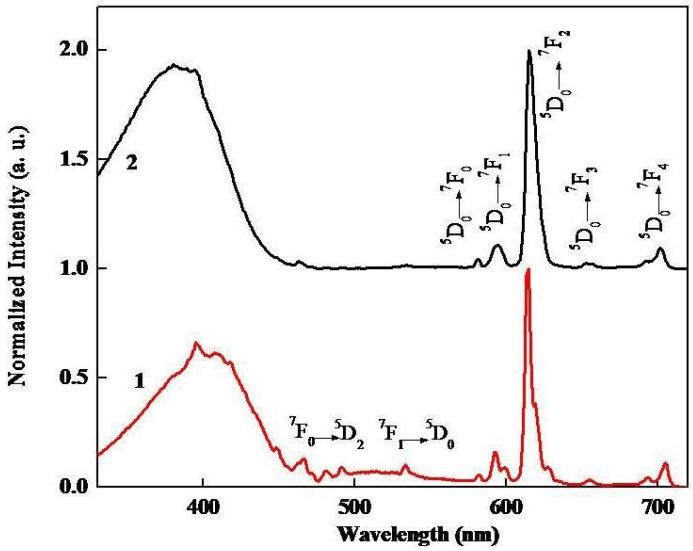


Fig. 2. Room-temperature excitation and emission spectra of complexes **1**–**2** (λ_{ex}= 380 nm), emission monitered around 613 nm.

The excitation spectra also show a series of narrow bands assigned to the 4f-4f transitions from the $^7F_0$ ground level to the excited states. However, these bands are less intense than those bands corresponding to the $S_0 \rightarrow S_1$ transition of the ligands. This result gives evidence of europium luminescence sensitization through intramolecular energy transfer from the β-diketones. Further, it also proves that luminescence sensitization via excitation of the ligand is much more efficient than the direct excitation of the Eu^{3+} ion absorption level [8]. The normalized photoluminescence spectra of the complexes **1–2** in solid state under the excitation wavelengths that maximize the Eu^{3+} emission intensity are shown in Figure 2. The emission spectra of the complexes display characteristic sharp peaks in the 575-700 nm region associated with the $^5D_0 \rightarrow {}^7F_J$ (J = 0-4) transitions of the Eu^{3+} ion. The five expected peaks of the $^5D_0 \rightarrow {}^7F_{0\text{-}4}$ transitions are well resolved, and the hypersensitive $^5D_0 \rightarrow {}^7F_2$transition is very intense, pointing towards a highly polarizable chemical environment around the Eu^{3+} ion, and is responsible for the red emission colour of the complexes. Further, the emission spectra of complexes show only one peak for the $^5D_0 \rightarrow {}^7F_0$, indicating the presence of a single chemical environment around the Eu^{3+} ion and also show that the metal ion occupies a low–symmetry site [9]. It is also important to mention that the emission spectra of the europium complexes do not exhibit ligand centered transitions, indicating that there is an efficient intramolecular energy transfer from the ligands to the Eu^{3+} ion.

3.5. Energy transfer between ligands and Europium(III)

Generally, the sensitization pathway in luminescent europium complexes consist of the excitation of the ligands from the ground state to their excited singlet states, and subsequently through the intersystem crossing of the ligands to their triplet states, following the energy transfer from the triplet state of the ligand to the central ion [10]. In this process, the 4f electrons of the Eu^{3+}ion are excited to the $^5D_0$ manifold ion from the ground state, finally the Eu^{3+}ion emits when the 4f electrons undergo a transition from the excited state of $^5D_0$to the ground state [11].

4. CONCLUSIONS

New pyrazolone based fluorinated ligand, 3–methyl 4–(2,2,3,3,3–penta fluoro–1–hydroxypropylidene)–1–phenyl–1H–pyrazol–5(4H)–onehas been synthesized and well characterized. Europium-containing complexes were synthesized by adduct formation between 3-methyl 4–(2, 2, 3, 3, 3–penta fluoro–1–hydroxypropylidene)–1–phenyl–1H–pyrazol–5(4H)–one and europium (III) complex and a bidentate neutral donor 1,10–phenanthroline (phen). The emission spectra of the

complexes display characteristic sharp peaks in the 575–700 nm region associated with the $^5D_0 \rightarrow ^7F_J$ (J = 0–4) transitions of the Eu^{3+} ion. The sensitization mechanism for luminescent europium complexes involves a triplet pathway, in which the transfer of energy absorbed by the ligand to the Eu^{3+} ion takes place from the ligand–centered triplet excited state. The results demonstrate that the substitution of solvent molecules by bidentatephen molecule in $Eu(L)_3(H_2O)_2$greatly enhances the metal–centered luminescence.

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Waste water treatment by removal of heavy metals using EDTA-functionalized chitosan-graphene oxide nanocomposites

Asha Radhakrishnan[1]*, Arya Sugathan[2], Deepa Vijayan[2], B. Beena[3]

Author Affiliations
[1,2]Department of Chemistry, D.B. Pampa College, Parumala , Pathanamthitta, India
[3]Department of Chemistry, K.S.M.D.B. College Sasthamcotta, Kollam, India

Corresponding Author
***Asha Radhakrishnan,** Department of Chemistry, D.B. Pampa College, Parumala, Pathanamthitta, India.

E-mail: ashagopan2009@gmail.com

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Abstract

This study focuses on the preparation of Ethylenediaminetetraacetic acid (EDTA)-functionalized magnetic chitosan (CS) graphene oxide (GO) nanocomposites (EDTA-MCS/GO) by biogenic method and its removal efficiency for heavy metals namely, Pb(ll) and As(III) from aqueous solutions. The synthesized nanocomposite was characterized by XRD, SEM, and FTIR analysis. The influence of various operating parameters, such as pH, metal ion concentration, and contact time on the removal of the metal ions, was investigated. The equilibrium data was evaluated by Langmuir, Freundlich and sips isotherms, while the heavy metal adsorption reaction kinetics was analyzed by Lagergren pseudo-first-order and pseudo-second-order kinetic models. The adsorption–desorption studies conducted over 6 cycles illustrate the viability and repeated use of the adsorbent for the removal of Pb(ll)and As(lII) from aqueous solutions.

Keywords: Adsorption, Heavy metals, Nanocomposites, Kinetics, Isotherms

1. INTRODUCTION

The discharge of wastewater containing heavy metals into the environment has increased continuously as a result of various human activities. Heavy metal contamination in such effluents presents a serious threat to the environment and human health because of their toxicity, non-biodegradability, carcinogenicity, and bioaccumulation in living organisms. Heavy metals such as Lead (Pb) and Arsenic (As) poses several risks to human health and long-term exposure in drinking water may cause cancer, muscular weakness, loss of appetite, and nausea. These issues require effective treatment of wastewater to decrease pollutants to acceptable levels. In this regard, many techniques have been developed for the removal of heavy metals from wastewater, such as chemical

precipitation, ion exchange, reverse osmosis, and adsorption. Among these, adsorption is the preferred technology because of its simplicity, effectiveness, and low cost [1].

In recent years, carbonaceous nanomaterials such as graphene oxide (GO), a two- or three dimensional nanosheet, have attracted the attention of researchers because of their unique properties and large composition diversity. The presence of a wide range of consecutive oxygen functional groups on the GO surface and large surface area provides abundant attachment sites for the functionalization of other compounds, such as chitosan (CS) and ethylene diamine tetra acetic acid (EDTA). This can ultimately increase the number of surface functional groups, which might enhance heavy metal adsorption. CS with primary amino groups is easily functionalized with different organic ligands, such as GO and EDTA, to improve its adsorption capacity. EDTA chelates with divalent metals, in which one carboxyl group is freely available and one water molecule is coordinated to the metal center [2].

In the present study EDTA–functionalized magnetic chitosan graphene oxide (EDTA-MCS/GO) nanocomposites is synthesized using biogenic method. Adsorption behavior of synthesized EDTA-MCS/GO nanocomposites for divalent Pb^{2+} and trivalent As^{3+} ions in aqueous solution is studied.

2. MATERIALS AND METHODS

2.1. Synthesis of EDTA-functionalized magnetic chitosan graphene oxide (EDTA-MCS/GO)

GO was synthesized from sugar cane bagasses oxidation. About 0.5 g of grounded sugar cane bagasse powder mixed with 0.1 g of ferrocene was taken in a china dish and directly kept in a muffle furnace at 300°C for 10 min under atmospheric condition. The black solid product was subjected for further analysis. The synthesis of EDTA-MCS/GO was a two-step process. In the first step, $FeCl_3$ (2 g) was dispersed in HCl. CS (2.0%) solution was obtained by dissolving low weight CS in HCl. GO solution, CS solution, and distilled water were added together and vigorously stirred for 1 h at room temperature. Then, NH_3 solution was added to induce the precipitation of a blackish material. The obtained product (MCS/GO) was washed several times with DI water and absolute ethanol, and finally dried in an oven. In the second step, EDTA and dried ground MCS/GO dispersions were mixed and allowed to react for 24 h at room temperature. Finally, the synthesized EDTAMCS/ GO nanocomposite was oven dried at 600C then manually grinded into a fine powder for adsorption studies.

2.2. Adsorption Experiments: Batch type contact method

Adsorption experiments were carried out in a thermostatic water bath shaker at 150 rpm rate at a particular temperature for predetermined time intervals using 100ml clean and dried Stoppered bottles. The metal ion concentrations in liquid phase were determined by atomic absorption spectroscopic studies. The amount of metal ions adsorbed in (mg/g) at equilibrium (qe) was calculated from the mass balance of initial and final metal concentrations in the aqueous phase. The amount of metal ion adsorbed at time $q_e$was calculated from mass balance equation.

$$q_e = \frac{(C_o - C_e)V}{m} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where q_e is the adsorbed metal ions per unit mass of adsorbent(mg/g) and V is the sample volume in L, m is the weight of the adsorbents.

The removal efficiency of various nanoadsorbents was calculated by using the equation,

$$\% \text{ removal} = \frac{(C_o - C_e)}{C_o} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

where, C_o and C_e are the initial and the equilibrium concentrations of heavy metal ions.

The quantity of metal ions adsorbed at selected time intervals was determined and used for kinetic analysis. Through the batch adsorption experiments it is possible to collect the details of the

applicability of adsorbent materials. But for studying the practical application in a bulk, continuous column studies are to be conducted in waste water effluents.

The adsorption conditions for the adsorbents were optimized by varying experimental parameters such as contact time, pH, initial concentration of adsorbate, pH, and adsorbent dose by batch method.

2.3. Instrumentation

The XRD measurement was performed on an XPERT-PRO powder diffractometer with Cu–Kα radiation as the X-ray source in the 2θ range of 10-90^0.The surface morphology was measured by a scanning electron microscope (SEM) of model JED-2300 system. FTIR spectra were recorded using Perkin-Elmer FTIR Spectrophotometer in the wave number range of 400 cm^{-1} to 4000cm^{-1} by KBr disc method. Adsorption studies were carried out using GBC-AAS spectrometer having lamp current 5milli ampere and a wave length 270nm.

3. RESULTS AND DISCUSSION

3.1. XRD Analysis

The XRD pattern of the GO is shown in Figure 1a. The peak at 2θ = 11.6° indicates that the agricultural sugarcane bagasse is fully oxidized into graphene oxide with the interlayer distance of 0.79 nm. This XRD pattern can be attributed to well graphitized two-dimensional structures made of GO sheets. The synthesized nanocomposite shows (Figure 1b), several sharp and strong diffraction peaks in, ranging from 2θ = 30–70 ^{o}, attributed to magnetic nature of nanocomposite. The XRD peak at 2θ=30^o indicated the amorphous behaviour of CS. A peak around 2θ=18^o might be the evidence of the presence of EDTA. These XRD peaks confirmed the formation of EDTA-MCS/GO nanocomposite [2].

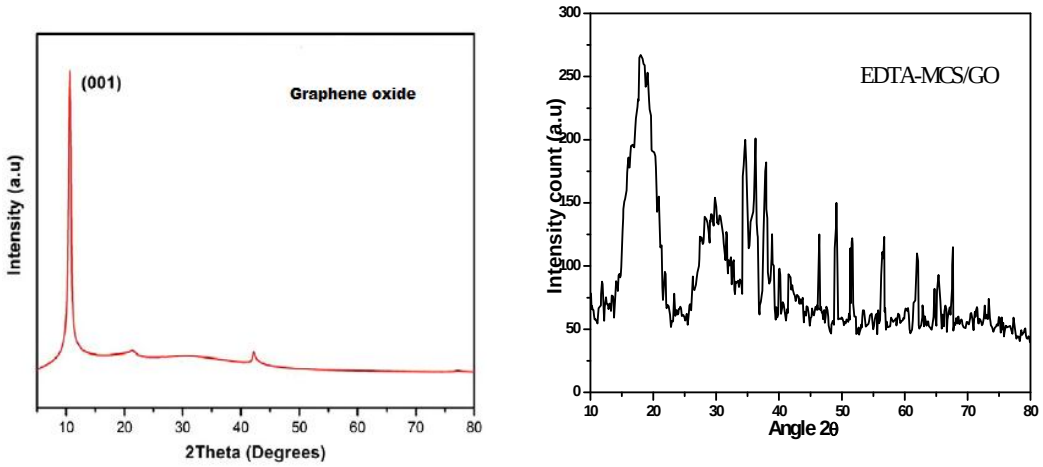

Fig.1: XRD spectrum of (a) Graphene Oxide (b) EDTA-MCS/GO nanocomposite

3.2. SEM Analysis

Surface morphologies and structures of the EDTA–MCS/GO nanocomposites were obtained from SEM analysis (Figure 2). The SEM image of GO (Figure 2a) revealed a sheet-like structure of sp^2–hybridized carbon atoms with a smooth surface and crumpled edges. The SEM image of EDTA-CS (Figure 2b) revealed the spherical nanoparticles with smooth particle surface. The SEM image in Figure 2c shows small rounded magnetic chitosan (MCS) particles distributed roughly onto the GO surface. CS was well dispersed with GO and showed a close attachment, which indicated bonding between GO and CS. By adding EDTA, the size of magnetic particles increased. Therefore, in the surface of the GO sheets, comparatively large, rounded magnetic EDTA–CS particles were observed. This phenomenon increased the surface availability of the nanocomposites for adsorption and

prevented GO agglomeration. EDTA functional groups and amino groups present in CS could form chemical bonds with the GO sheet surface.

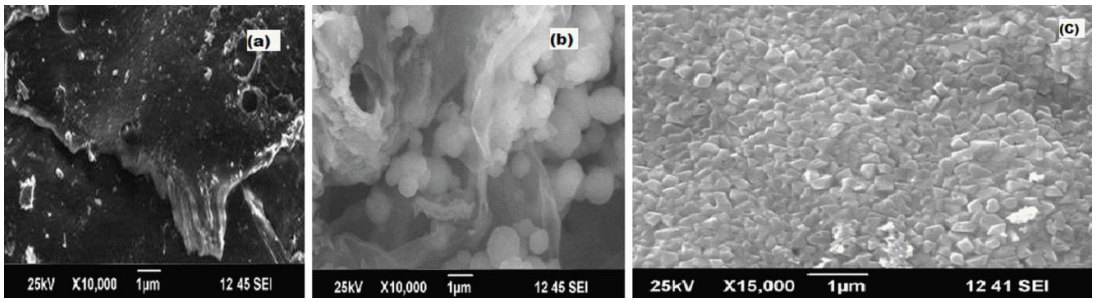

Fig. 2: SEM image of (a)GO,(b) EDTA–CS (c) EDTA–MCS/GO nanocomposites

3.3. FTIR Analysis

The FT-IR result shows (Figure 3) that EDTA–MCS/GO contains numerous oxygen and nitrogen functional groups. A broad band observed at 3386 cm^{-1} was assigned to O–H or N–H stretching vibrations from adsorbed H_2O or $–NH_2$ groups of CS on the surface of EDTA-MCS/GO.The band at 563 cm^{-1} was recognized as FeO bond stretching in EDTAMCS/ GO. The stretching vibration and bending vibration of the N–H bond appeared in a band at 1080cm^{-1}, which was assigned to $–NH_2$ groups in EDTA or CS on the surface of EDTAMCS/GO. Another vibrational band at 1375cm^{-1} was assigned to C–OH. A strong band at 1640 cm^{-1} was attributed to CO bond vibrations in carboxyl functional groups. Bands at 2920 and 2845 cm^{-1} resulted from C–H bonds in alkane groups. These O and N functional groups could act as available adsorption sites and play important roles in the removal of metal ions from aqueous solutions.

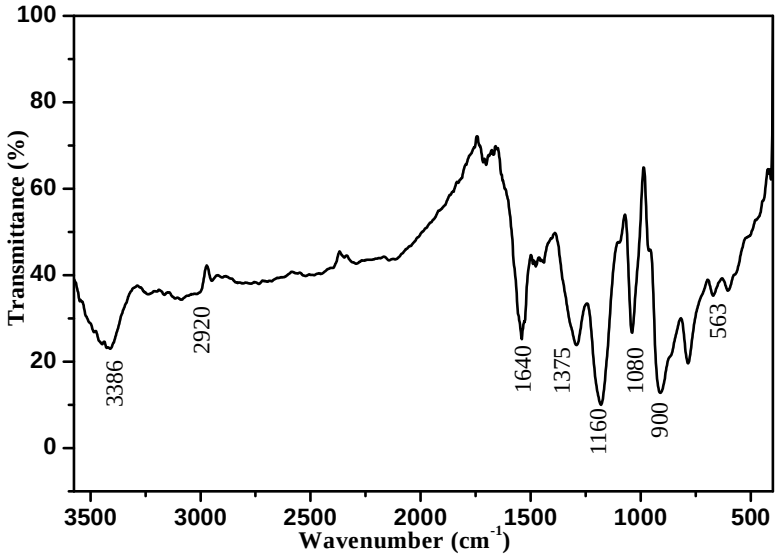

Fig. 3: FTIR spectrum of EDTAMCS/GO

3.4. Adsorption Studies

Present study focuses on the feasibility of synthesized EDTAMCS/GO as high efficient adsorbents for the removal of Lead ions (Pb(ll)) and Arsenic ions (As(lII)) from aqueous solutions. The effect of various operational conditions such as pH, adsorbent dosage and contact time was systematically studied. Adsorption kinetics and isotherm models were also analyzed to reveal the adsorption mechanisms.

The effect of pH on the adsorption capacity of metal ions such as Pb(ll) and As(lII) was evaluated in a pH range of 2.0 to 9.0 by agitating 100 ppm metal ion solution with 0.1g of EDTA-CS, GO and EDTA-MCS/GO separately. From Figure 4 it is observed that the percentage of adsorption of metal ions increased with increase in the solution pH for all adsorbents. The low adsorption in the acidic region can be attributed to the competition between hydrogen and metal ions for the same sites. The increase in pH makes the EDTA-MCS/GO surface more negative, thus enhancing electrostatic interactions between metal ions and the adsorbent, resulting in higher retention of metal species [3].

In the case of lead it exists as Pb^{2+}, $PbOH^{+}$ and $Pb(OH)^{3-}$ depending on pH. At lower pH value, low metal ion uptake was observed. At low pH values, the adsorbent is positively charged due to the presence of H^{+} ions on its surface and hence offers repulsive force to approaching Pb^{2+} ions. However, more Pb^{2+} uptake is observed as the pH increases which are due to the fact that at high pH values, lead still has a net positive charge but exists as $PbOH^{+}$ while most active sites on the adsorbent are deprotonated. This leads to net attractive force that is responsible for high Pb removal from solution. But at elevated pH values i.e, in strong basic region, the uptake of metal ions decreased, may be due to the hydroxide formation. In the present study the removal efficiency in the case of all the three samples was found to increase with pH and attained maximum at pH 6 and then it was found to decrease. At all pH values EDTAMCS/GO showed maximum removal efficiency.

The pH of the solution was found to have a great effect on the adsorption of As(lII) ions also. The adsorption increased sharply with pH and attained almost a constant value from pH 8 onwards and then showed a slight decrease in adsorption level [Figure 4]. The hydrolysis and precipitation of metal ions affect adsorption by changing the concentration and form of soluble metal species those are available for adsorption. Depending upon the pH of the solution, various species of arsenic can be formed during the hydrolysis. The hydrolysis extent of As(III) ions is unimportant up to approximately pH 8. [4]. In this case also at all pH values EDTAMCS/GO showed maximum removal efficiency. After each adsorption process, the final pH of the solution was recorded. It was found that after adsorption pH of the solution was lower compared to initial pH. This decrease in pH is an indication for ion exchange mechanism for removal of metal ions and the adsorption of metal ions onto EDTAMCS/GO surface by the release of H^{+} ions [5].

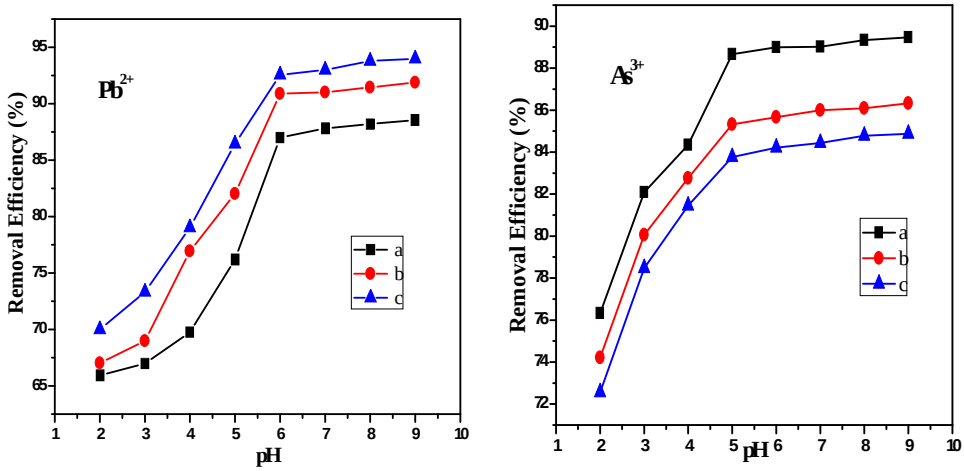

Fig. 4: Removal efficiency *vs* pH for Pb(II) and As(III)

The dependence of adsorbent dosage on metal ion adsorption was studied by varying the amount of adsorbents from 0.025 to 0.2 mgL^{-1}. From Figure 5, it can be observed that the removal efficiency of the adsorbents are enhanced by raising the adsorbent dosage and reached a saturation level at higher dosage. This is expected due to the fact that at higher concentrations of adsorbents greater is the availability of exchangeable sites for the ions. But increase in dosage beyond a certain level results

in no further increase in adsorption, may be due the fact that the amount of ions bound to the adsorbent and the amount of free ions in the solution remains constant [6].

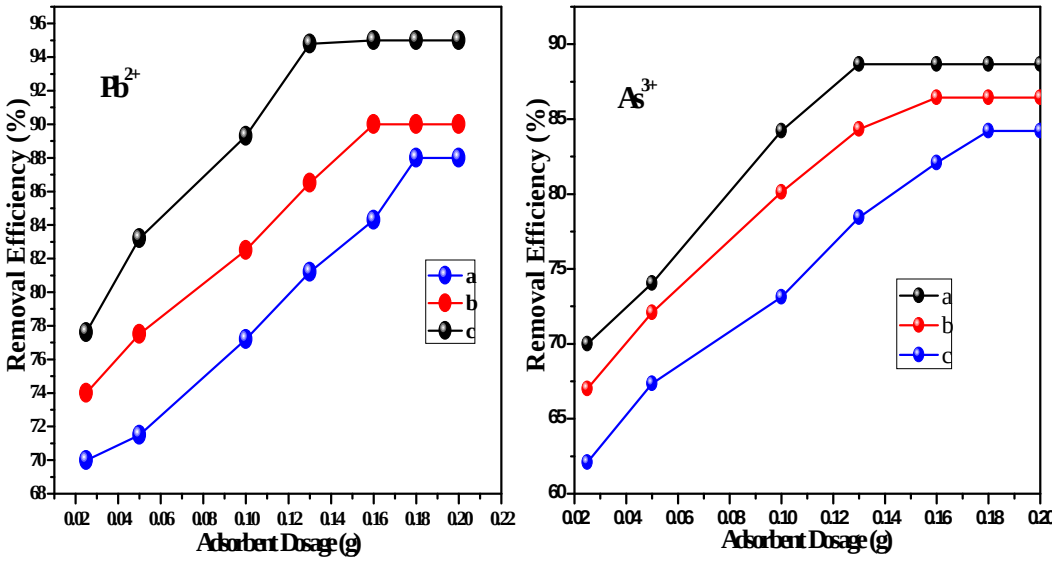

Fig. 5: Removal Efficiency *vs* adsorbent dosage for Pb(II) and As(III)

The dependence of adsorption capacity of EDTAMCS/GO on the initial concentrations of Pb(II) and As(III) ranging between 50 to 200 mg/L using 0.1 gm of the adsorbent is represented in Figure 6. The adsorption capacities increased steadily with increase in metal ion concentrations. The increase in the concentration gradient acts as a driving force for the adsorption process. When the concentration of metal ions increases, the active sites of EDTA-MCS/GO are surrounded by more metal ions, and the number of metal ions competing for available binding sites increases leading to an increased uptake of metal ions from the solution. Therefore the value of q_e increased with increase of initial metal ion concentration (C_0). It is also evident that adsorption capacity of EDTA-MCS/GO is higher as compared to EDTA–CS and GO; this is because of the presence of more active sites in EDTA-MCS/GO.

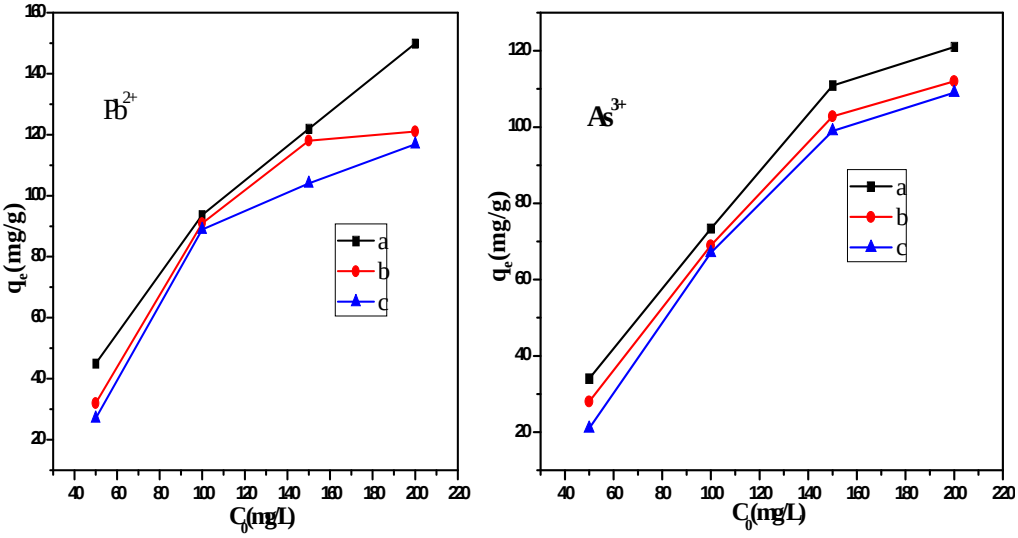

Fig. 6: Adsorption efficiency vs initial metal ion concentrations for Pb(II) and As(III)

The kinetic data were analyzed using the pseudo-first-order and pseudo-second- order kinetic models for EDTA-MCS/GO [Figure 7].

The kinetic data were analyzed using the following pseudo-first-order and pseudo-second- order kinetic models:

$$q_t = q_e(1-e^{-k_1 t}) \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots \quad (3)$$

$$q_t = \frac{k_2 q_e^2 t}{1+k_2 q_e t} \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots \quad (4)$$

where q_e and q_t are the amounts of solute adsorbed per unit mass of the adsorbent at equilibrium and time t (min), respectively, k_1 is the pseudo-first-order rate constant (min^{-1}) and k_2 is the pseudo-second-order rate constant of adsorption (g/mg/min). The pseudo-first-order and pseudo-second-order kinetic parameters were estimated from the experimental data using non-linear curve fitting procedure and the results are given in Table 3.3. The pseudo-first-order equation showed a poor correlation with experimental data, whereas the pseudo-second-order equation indicated a good correlation as indicated by a lower value of χ^2 and higher value of R^2 (nearing unity). In addition, q_e, which is the adsorption capacity, agreed very well with both the experimental and calculated values. The kinetic data indicates that the adsorption process is controlled by pseudo second–order equation. Also, this suggests the assumption behind the pseudo-second-order model that the metal ion uptake process is due to chemisorptions involving valence forces through the sharing or exchange of electrons between adsorbent and adsorbate [7].

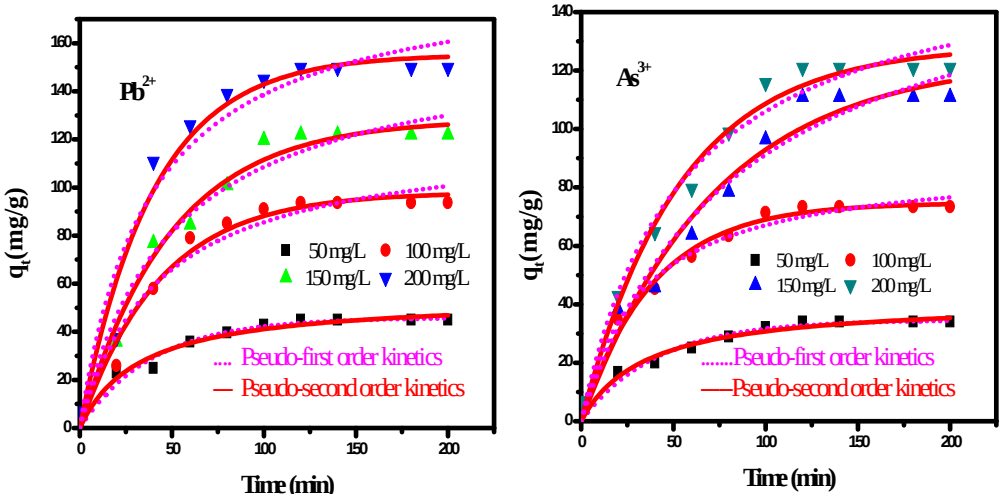

Figure 7: Kinetic plots for Pb(II) and As(III)

Table 1: Kinetic parameters for the adsorption of Pb(II) and As(III)

Metal ion	q_e (mg/g)	Pseudo-first-order				Pseudo-second-order			
	Exp	q_e(mg/g)	k_1/min	R^2	χ^2	q_e(mg/g)	k_2 min^{-1}	R^2	χ^2
Pb(II)	44.98	54.6	0.02	0.96	3.5	45.82	0.5×10^{-4}	0.96	0.61
	93.65	122	0.02	0.97	6	97.99	0.1×10^{-4}	0.95	0.23
	121.91	162.11	0.02	0.98	8	128.32	0.1×10^{-4}	0.96	0.57
	149.87	190.61	0.01	0.95	9	155.32	0.1×10^{-4}	0.99	0.17
As(III)	34	41.44	0.02	0.96	3.5	34.59	0.6×10^{-4}	0.96	3.21
	73.33	89.66	0.02	0.98	8	74.69	0.3×10^{-4}	0.98	3.62
	110.91	163.65	0.01	0.96	4.7	124.22	0.4×10^{-4}	0.95	5.71
	121	168.81	0.01	0.98	2.9	128.47	0.1×10^{-4}	0.97	4.62

The adsorption of metal ion on EDTA-MCS/GO was examined by well–known models to interpret equilibrium isotherm data. The experimental data of equilibrium adsorption were analyzed using the non-linear forms of Langmuir, Freundlich and Sips, isotherm equations:

$$q_t = \frac{q_m b C_e}{1 + b C_e} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (5)$$

$$q_e = K_F C_e^{1/n} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (6)$$

$$q_e = \frac{Q_s K_s C_e^{1/n_s}}{1 + K_s C_e^{1/n_s}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (7)$$

where qe and Ce are the equilibrium concentrations when an adsorbate gets adsorbed onto the adsorbent and q_m denotes the maximum adsorption capacity and b is the Langmuir constant. k_F (function of energy of adsorption and temperature) and 1/n (also called as the heterogeneity coefficient) are the Freundlich constants. If the 1/n value is less than 1, then it can be assumed that the adsorbent material is heterogeneous in nature. Q_s, is the Sips maximum adsorption capacity, k_s is the Sips equilibrium constant and $1/n_s$ is the Sips model exponent.

The experimental and model fits of Langmuir, Freundlich and Sips isotherms of Pb(II) and As(III) adsorption onto EDTA-MCS/GO are shown in Figure 8. The isotherm constants were calculated using non linear regression analysis and the results are listed in Table 2. Here also the values of R^2 and χ^2 showed that the adsorption of metal ions onto EDTA-MCS/GO was best fitted with Sips isotherm model over the concentration ranges studied. Sips isotherm model is a combination of Langmuir and Freundlich adsorption isotherm models. At higher concentration Sips favors Langmuir model while at lower concentration it favors Freundlich model. The R^2 values for all the three isotherms models were beyond 0.99 which indicate the suitability of models in explaining adsorption mechanism. Lower value of χ^2 and higher value of R^2 show a very good mathematical agreement with Sips isotherm model. Sips isotherm gives the idea of multilayer adsorption at lower concentration and monolayer at higher concentration of the metal ion.

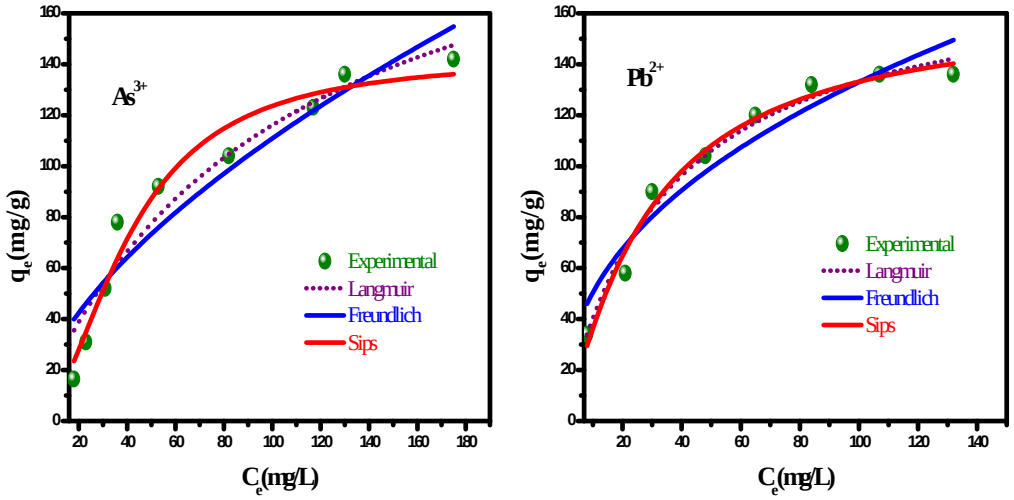

Fig. 8: Experimental and model fits of Langmuir, Freundlich and Sips isotherms of Pb(II) and As(III) adsorption onto EDTA-MCS/GO

Table 2: Langmuir, Freundlich and Sips isotherm constants for the adsorption of Pb(II) and As(III) onto EDTA-MCS/GO

		Pb(II)	As(III)
Langmuir	q_m mg/g	166.67	130.62
	b L/mg	0.19	0.01
	R^2	0.99	0.93
	χ^2	4.5	14
Freundlich	k_F mg $^{1-1/n}$L $^{1/n}$/g	44.47	7.12
	1/n	0.27	0.59
	R^2	0.98	0.88
	χ^2	2.0	2.2
Sips	Q_s mg/g	169.23	149.21
	K_s L/mg	0.10	0.005
	$1/n_s$	0.73	2.03
	R^2	0.99	0.95
	χ^2	1.1	8.3

In the present work the maximum adsorption capacity obtained using EDTA-MCS/GO for Pb^{2+} adsorption was 169.23 mg g^{-1} and for As(III) it was 149.21 mg g^{-1} under optimum conditions. This result suggests that the EDTA-MCS/GO prepared in the present study can be considered as a promising material for the removal of heavy metal ions from industrial waste water.

The desorption capacity of the prepared EDTA-MCS/GO was checked with HCl having different concentrations, ranging from 0.001 to 0.1 M . It was observed that the adsorbed Pb(II) and As(III) could be desorbed from the spent adsorbent using 0.1 M HCl and hence it could be used for the regeneration of EDTA-MCS/GO The adsorption–desorption process was carried out for six cycles with 0.1M HCl [Figure 9] and it was found that there was only a slight decrease in the adsorption capacity with respect to the regeneration cycles. However desorption capacity decreases to some extent because of the possible penetration of metal ions into inner cavities (pore diffusion or intraparticle diffusion). Therefore it could be included in the list of very effective adsorbent for Pb(II)and As(III) in real process such as industrial waste water treatment .

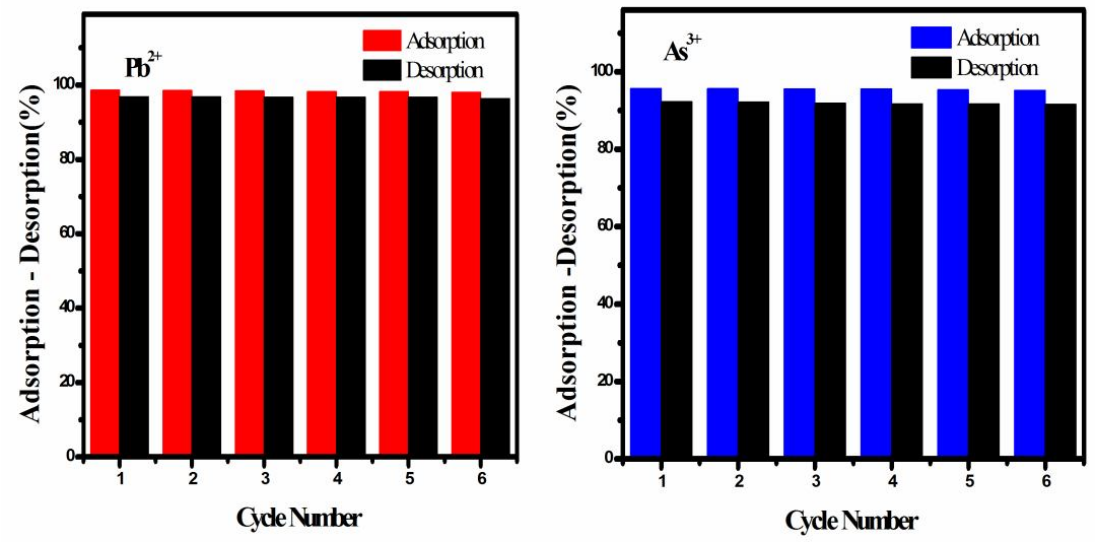

Fig. 9: Adsorption–desorption cycles for Pb(II) and As(III)

The practical efficiency and effectiveness of the adsorbents prepared were tested with industrial waste water. The collected waste water sample had Pb(II) and As(III) concentrations higher than the acceptable concentration for drinking water. Batch experiments conducted with industrial waste water showed that higher amount of adsorbent was needed compared to original metal ion solution. This discrepancy was due to the competitive adsorption of matrix ions with binding sites/screening effect by other metal ions.

4. CONCLUSION

This work demonstrated the efficient removal of divalent (Pb^{2+}) and trivalent (As^{3+}) metal ions using EDTA-MCS/GO nanocomposite. The synthesized nanomaterial was characterized by XRD, SEM, and FT-IR. The pseudo-second-order kinetic model gave better correlation with the adsorptions of all the metal ions than the pseudo-first order model. Sips isotherms were the best fits isotherm model for all metal ion adsorption. The synthesized nanocomposite exhibited a good regeneration capacity. Efficient removal of metal ions in real wastewater was also tested. Therefore, EDTAMCS/ GO nanocomposite is a promising adsorbent metal ion removal from wastewater.

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Effect of a pyramidine ring formation at the Linker part on the non linear optical property of a D–π–A type chalcone: An In silico study

Lakshmi C S Nair[1], Balachandran S[1*], Hubert Joe I[2]

Author Affiliations
[1]Department of Chemistry, Mahatma Gandhi College, University of Kerala, Thiruvananthapuram 695004, Kerala, India
[2] Centre for Molecular and Biophysics Research, Department of Physics, Mar Ivanios College, University of Kerala, Thiruvananthapuram, 695015, Kerala, India

Corresponding Author
***Balachandran S,** Department of Chemistry, Mahatma Gandhi College, University of Kerala, Thiruvananthapuram 695004, Kerala, India

E-mail: sbcnair@gmail.com

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Abstract

Theoretical calculations of energies, geometries polarizability, hyperpolarizabilities of (2*E*)–3–(4–aminophenyl)–1–(4–nitrophenyl)prop–2–en–1–one(ANC) and 6–(4–aminophenyl)–4–(4–nitrophenyl)–1,6–dihydropyrimidin–2–ol(ANCU) were studied based on density functional theory(DFT) using hybrid functional B3LYP with basis set 6–311G(d,p) and UV–Visible spectral analysis using time–dependent (TD–DFT) with same basis set. Calculated HOMO and LUMO energies represent the stability of the molecule. Stabilization energies of strong interactions are obtained from NBO analysis. The hyperpolarizability values of ANC and ANCU are thousand times than that of standard NLO material urea. The MESP analysis show variation in activity site thus the whole chemistry of the molecules changed with the condensation of chalcone with urea.

Keywords: Chalcone, Pyramidine, Hyperpolarizability, Intramolecular charge transfer

1. INTRODUCTION

Chalcones are the important class of organic compounds which possess non linear optical properties which varies with the substituent's present in the chalcone skeleton [1.2]. In chalcones two phenyl rings are connected through enone system, commonly benzoyl ring is designed as ring A and phenylene ring as ring B (Figure 1). Carbonyl group attached to the ring A can have a polar nature C^{+}–O^{-} which will be enhanced by electron donation from the skeleton and thus act as acceptor end, especially when electron donating substituent are attached to ring B in ortho and para position. The presence of donor acceptor entities with a conjugated linker is a characteristic feature shown by compounds with intramolecular charge transfer (ICT) and show non linear optical properties (NLO).

Compounds with chalcone skeletons have different pharmacological activities such as anticancer, antileishmanial, antiinflammatory, anti TB, antimicrobial, antifungal, antioxidant, cytotoxic, antitumour activities [3–8]. In the present study (2*E*)–3–(4–aminophenyl)–1–(4–nitrophenyl)prop–2–en–1–one(ANC) is selected in such a way the donor property of ring B is enhanced by p–amino substitution and the acceptor property was enhanced by p–nitro substitution. The effect of linker chain is altered by the condensation urea at the enone moiety which resulted in a heterocyclic pyramidine ring formation (Ring C) in 6–(4–aminophenyl)–4–(4–nitrophenyl)–1,6–dihydropyrimidin–2–ol(ANCU). The ring can effectively change the NLO properties of chalcone derivative. A detailed investigation was done based on the geometry optimization, NBO analysis, HOMO–LUMO and their energies and band gap, MESP analysis on the difference in first order hyperpolarizability of ANC and ANCU.

2. EXPERIMENTAL

2.1. Computational details

The computation of ground state geometries, electronic structures, fundamental frequencies, polarizability, hyperpolarpolarizabilities, electronic absorption, natural charge and natural bond orbital analysis for the title compounds was done using DFT with Gaussian '09 package [9]. The Becke's three parameter exchange–corrected exchange potential and the Lee–Yang–Parr gradient corrected correlation potential(B3LYP) along with double zeta split valence basis set 6–311G(d,p) was used to perform geometry optimization [10]. The electron absorption spectra require excitation and oscillator strengths, these calculations were done using TD–DFT [11] with the same basis set. Graphical representation of MESP, highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) data in structure optimized files were made using the Gaussview visualization program [12].

3. RESULTS AND DISCUSSIONS

3.1. Geometry Optimization

The important optimized geometry parameters of ANC and ANCU obtained from Gaussian 09 and were shown in Table 1. The optimized geometries visualized from Gaussview program and the numbering scheme for ANC and ANCU are in given in Figure 1.

Table 1: Selected geometrical parameters of ANC and ANCU

Theoretical bonds	(Å)	
	ANC	**ANCU**
$C_6 – C_7$	1.512	1.486
$C_7 – C_8$	1.472	1.348
$C_8 – C_9$	1.351	1.508
$C_9 – C_{10}$	1.449	1.523
$C_{13} – N_1$	1.379	1.389
$C_3 – N_2$	1.481	1.474
$C_7 – O_1$	1.224	–
Theoretical dihedral angles (º)		
$C_5 – C_6 – C_7 – C_8$	–18.1	–15.3
$C_6 – C_7 – C_8 – C_9$	176.8	179.4
$C_7 – C_8 – C_9 – C_{10}$	179.0	–123.8
$C_8 – C_9 – C_{10} – C_{11}$	–1.3	–124.5
$C_5 – C_6 – C_7 – O_1$	162.7	–
$C_5 – C_6 – C_7 – N_3$	–	166.4

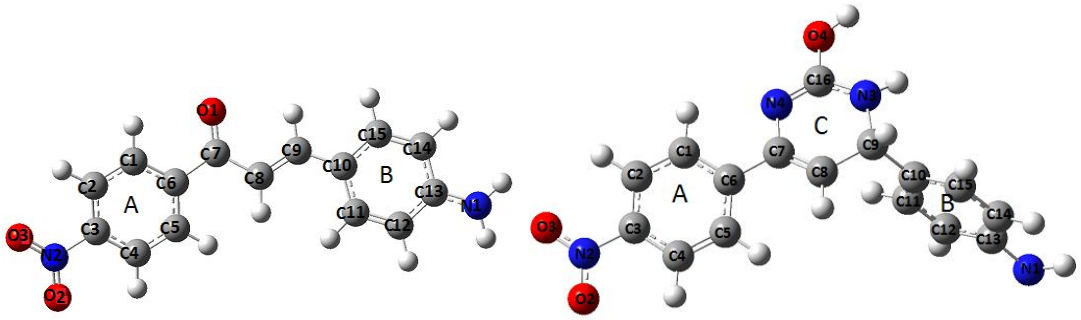

Fig. 1: Optimized geometries

The pyramidine ring formation at enone moiety not only alters the bond lengths in alkene skeleton but also in the substituent's as shown by C_3–N_2 and C_{13}–N_1. The bond length of C_6–C_7 is decreased and C_9–C_{10} was increased. The single and double bond characters C_6–C_7 and C_7–C_8 were reversed by ring formation. Increase of bond length of C_9–C_{10} from 1.449 to 1.523Å in illustrates the lesser conjugation of donor end ANCU however enhanced conjugation is observed in acceptor end (The C_6 – C_7 bond length shortened from 1.512Å to 1.486Å). The pyramidine ring formation alters the overall geometry of the compounds, the ring B flipped away however ring A become more planar to the newly formed ring C as indicated by the dihedral angle C_7–C_8–C_9– C_{10} and C_8–C_9–C_{10}–C_{11} which support the view that the conjugation in ring A increased and ring B reduced.

3.2. Non-linear Optical Properties

The inter related properties, dipole moment(μ), polarizability(α_0), average polarizability (Δα), first order polarizability(β_0) of the investigated molecules are calculated with B3LYP/6-311G(d,p) basis set, using finite-field approach. All these properties depend on the presence of applied electric field and the molecular structure of a system. The total dipole moment defines as

$$\mu = (\mu_x^2 + \mu_y^2 + \mu_z^2)^{1/2}$$

The mean polarizability and anisotropy of polarizability are defined as

$$\alpha_0 = \frac{1}{3}(\alpha_{xx} + \alpha_{yy} + \alpha_{zz})$$

$$\Delta\alpha = \frac{1}{\sqrt{2}}[(\alpha_{xx} - \alpha_{yy})^2 + (\alpha_{yy} - \alpha_{zz})^2 + (\alpha_{zz} - \alpha_{xx})^2 + 6\alpha_{xz}^2]^{1/2}$$

The components of the first hyperpolarizability can be calculated using following equation,

$$\beta_i = \beta_{iii} + 1/3\sum(\beta_{ijj} + \beta_{jij} + \beta_{jji}), (i \neq j)$$

Using the x,y,z components, the magnitude of the first hyperpolarizability tensor was calculated by

$$\beta_{tot} = (\beta_x^2 + \beta_y^2 + \beta_z^2)^{1/2}$$

The values of hyperpolarizability in a.u have been converted to esu by conversion β: 1 a.u. = 0.008639×10^{-30}esu

The dipole moment of the title compounds ANC, ANCU is 9.61D and 12.11D respectively and hyperpolarizability (β) components of ANC, ANCU calculated using DFT/B3LYP 6–311G(d,p) method are listed in Table 2. The static first order hyperpolarizability of ANC (86.95×10^{-30} esu) is reduced to 40.12×10^{-30} esu by the ring formation in ANCU. Both molecules have same donor and acceptor groups(amino and nitro respectively), The pyramidine ring formation in eneone skeleton at

the middle of the compound decrease the effective π electron conjugation from donor to the acceptor end which reduces the NLO activity of urea condensed product ANCU to half of the ANC. However both the molecules have higher NLO activity than the standard NLO material urea (0.13×10^{-30} esu) [13]. The NLO activity of ANC and ANCU were 67,000 and 31,000 times than that of urea.

Table 2: Hyperpolarizability components of ANC and ANCU

Components	ANC	ANCU
	10^{-30} esu	
β_{xxx}	84.11	14.7
β_{xxy}	13.84	1.75
β_{xyy}	2.01	0.15
β_{yyy}	0.27	0.58
β_{xxz}	–3.82	12.97
β_{xyz}	–0.42	1.57
β_{yyz}	0.02	1.05
β_{xzz}	–0.36	12.25
β_{yzz}	–0.15	4.95
β_{zzz}	0.31	14.59
β_{tot}	**86.95**	**40.12**

3.3. Frontier Molecular Orbitals and excitation spectra

Chemical reactivity of a molecule is highly dependent on the orbital properties and energy of frontier molecular orbitals [14], highest occupied molecular orbital(HOMO) and lowest unoccupied molecular orbital(LUMO). Lower HOMO–LUMO energy difference that is small band gap enhances effective overlapping of these orbitals which resulted in easy ICT. Ionization potential of a molecule depends on the ability of donating electron and is associated with HOMO and the characteristics of LUMO are associated with electron affinity [15].

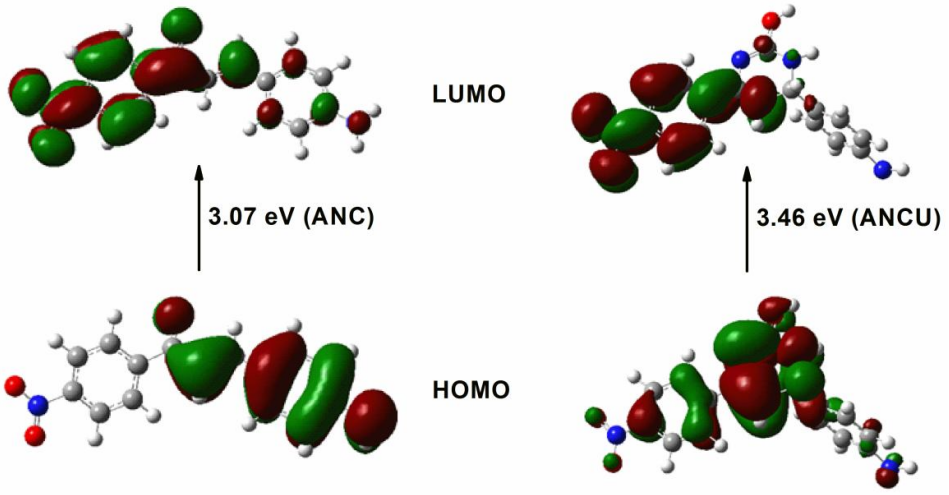

Fig. 2: Frontier molecular orbitals of ANC and ANCU

The Figure 2 represents the FMO's of ANC, ANCU. In ANC the energies of HOMO and LUMO are –5.95 and –2.89 eV respectively. HOMO localized over ring B (donor end), ethylenic bond and carbonyl group, LUMO delocalized over phenyl ring A (acceptor end). The band gap is 3.07eV. In ANCU HOMO energy is –5.81eV and mainly localized on the pyramidine ring at the middle region of the molecule this indicates the less conjugation of lone pair of electrons from amino group to the

pyramidine ring which is observed by the flipping of ring B. The lowest unoccupied molecular orbital lies at –2.35 eV delocalized on the electron withdrawing nitro substituted phenyl ring A, and the band gap is 3.46eV. This demonstrates intramolecular charge transfer in both cases but in different part of the molecule, the extent of ICT reduces in ANCU thus the NLO property. More easily charge excitation takes place in case of ANC due to the lower band gap it helps to develop enhance hyperpolarizability. The electronic translational behaviour of ANC and ANCU have been studied using time-dependent density functional theory with B3LYP/6-311G(d,p) basis set in gas phase. The calculated UV-Vis spectrum of ANC consists a maximum absorption peak at 446nm corresponds to the π→π*(homo to lumo) transition (98%), the λ_{max} value of ANCU at 403nm also attribute to π→π*(H to L) excitation (95%).

3.4. Molecular Electrostatic Potential

Molecular electrostatic potential (MESP) plot specifies the net electrostatic effect produced at a point by the total charge distribution over the molecule [16]. MESP surface helps to assess the nucleophilic and electrophilic binding sites in order to assign biological recognition and H–bonding interactions [17]. The different electrostatic potentials are represented by different colours. More electron dense region is represented by red colour and more positive region as blue colour. Green represents the neutral potential. Fig. 3 depicts the MESP map of ANC, ANCU. In ANC, the partial negative potential(orange colour) are mainly situated over nitro group and the highest negative potential on carbonyl oxygen atom, positive potential are localized over hydrogen atoms of amino group. In ANCU no change is observed for the electrophilic attacking centre, however the carbonyl centre has reduced nature due to more conjugation with the ring A as suggested by the geometry parameters. The shift in nucleophilic attack centre is observed, the highest at the ring C with a lowering nature in amino groups of ring B indicating a total variation in the chemistry of molecule by the condensation with urea.

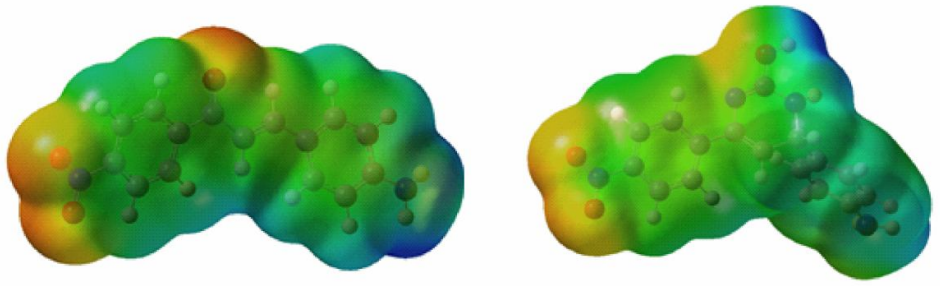

Fig. 3: MESP plots of tile compounds

3.5. Natural Charge Analysis

The charge on each atoms were determined on the basis of natural charge analysis calculated using NBO method at B3LYP/6–311G(d,p) basis set in vacuum. The atomic charges of selected atoms are tabulated in Table 3. The graphical representation of charge analysis of equivalent atoms (C_1–C_{15}) of ANC, ANCU are given in Figure 4. All hydrogen atoms in both cases are positively charged. More positively charged carbon atom among C_1–C_{15} are C_7 due to the presence of electronegative oxygen atom. Alternative higher and lower electronic charges represent the electron conjugation in both cases. In ANCU, two nitrogen and hydroxyl oxygen atom of the pyramidine ring possess negative charge and the carbon atom in between nitrogen atoms have positive charge (0.5029e).

Table 3: The natural atomic charges

Atoms	ANC	ANCU
C_3	0.1274	0.1259
C_6	–0.1283	–0.110
C_7	0.2565	0.1613
C_8	–0.1002	–0.0462
C_9	0.1064	0.2145
C_{10}	–0.0843	–0.1417
C_{13}	0.1316	0.1105
N_1	–0.0312	–0.0469
N_2	0.1720	0.1678

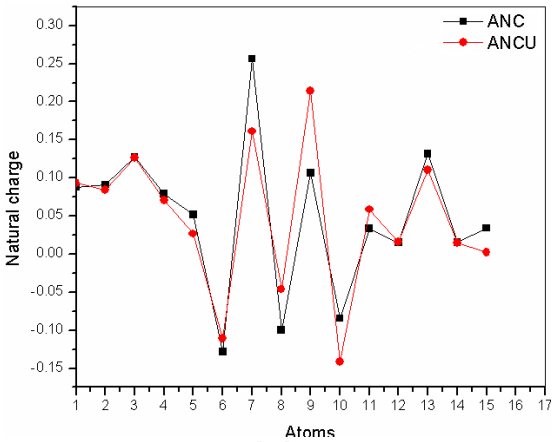

Fig. 4: Natural charge Diagram

3.6. Natural Bond Orbital Analysis

The Natural Bond Orbital(NBO) study using NBO program [18] executed in Gaussian '09 characterize all the intra–, inter molecular interactions and corresponding stabilization energies($E^{(2)}$). Stabilization energy depends on the strength of donor-acceptor interface. Tabulation of intensive intramolecular interactions of ANC, ANCU are in Table 4.

From the table 4 it is clear that π to π* interactions within the phenyl rings are of comparable stabilization energies. Maximum stabilization energy is reported in case of interaction within the nitro group. In the donor end, amino group interact with the phenyl ring B with stabilization energies 137.90, 123.05kJmol^{-1} described in case of ANC and ANCU respectively. It illustrate that in ANC the donor interaction is better, when it condensed with urea that donor interaction decreases and the intra molecular charge transfer (ICT) within the molecule reduced. The significant condition for a molecule to be NLO active is it must possess an ICT from donor end to acceptor end, as distance between donor to acceptor end increases the NLO activity also increases. In case of ANCU it weakens due to the presence of pyramidine ring, stabilization energy of 4.98 kJmol^{-1} shows that weak σ to σ* interaction of that ring with the acceptor end, the presence of ring C decreases the p–electron delocalization. As a result the first order hyperpolarizability value of ANC is 2 times that of ANCU.

Table 4: Second-order perturbation theory analysis of Fock matrix in NBO basis corresponding to the intramolecular interactions of ANC and ANCU computed at B3LYP/6–311G (d,p) level

ANC			ANCU		
Donor NBO(i)	Acceptor NBO(j)	$E^{(2)}$ ($kJmol^{-1}$)	Donor NBO(i)	Acceptor NBO(j)	$E^{(2)}$ ($kJmol^{-1}$)
$\pi C_5 - C_6$	$\pi^* C_3 - C_4$	96.36	$\pi C_1 - C_6$	$\pi^* C_2 - C_3$	103.30
$\pi C_1 - C_2$	$\pi^* C_7 - O_1$	62.43	$\pi C_1 - C_6$	$\pi^* C_7 - C_8$	56.15
$\pi C_1 - C_2$	$\pi^* C_3 - C_4$	92.93	$\pi C_2 - C_3$	$\pi^* N_2 - O_2$	105.65
$\pi C_1 - C_2$	$\pi^* C_5 - C_6$	87.95	$\pi C_7 - C_8$	$\pi^* C_1 - C_6$	47.74
$\pi C_3 - C_4$	$\pi^* N_2 - O_2$	110.21	$\sigma C_7 - N_3$	$\sigma^* C_6 - C_7$	4.98
$\pi C_8 - C_9$	$\pi^* C_7 - O_1$	97.99	n(2) O_4	π^* N_3–C_7	129.87
$\pi C_{10} - C_{15}$	$\pi^* C_8 - C_9$	86.36	n(2) N_4	π^* N_3–C_{17}	216.52
n(1) N_1	$\pi^* C_{13}$ –C_{14}	137.90	n(1) N_1	π^* C_{13}–C_{14}	123.05
n(2) O_1	σ^* C_6–C_7	81.84	n(3) O_3	π^* N_2–O_2	683.62
n(3) O_3	$\pi^* N_2$ –O_2	691.99			

$E^{(2)}$ means energy of hyperconjugative interactions (stabilization energy).

4. CONCLUSIONS

Using density functional theory with hybrid functional B3LYP, the geometries, electronic structures, dipole moment, hyperpolarizability, natural charge and natural bond orbital analysis, frontier molecular orbital analysis, corresponding energies, band gap and electrostatic potential map of two potential nonlinear optical materials, (2*E*)–3–(4–aminophenyl)–1–(4–nitrophenyl)prop–2–en–1–one(ANC) and 6–(4–aminophenyl)–4–(4–nitrophenyl)–1,6–dihydropyrimidin–2–ol(ANCU)were performed using Gaussian software. The optimized geometries shows that ANC is almost planar and ANCU is non planar in nature. The hyperpolarizability calculations reveals that both molecules are thousands of times more NLO active than standard urea, among them ANC is more active. This is due to the strong ICT in ANC. ANC and ANCU posses a band gap of 3.07eV and 3.46 eV respectively. FMO plots illustrate a charge transfer within the molecules for both cases. Both molecules are polar in nature. NBO analysis confirms a prominent intramolecular interaction on nitro group. Natural charge analysis shows all hydrogen atoms are positively charged and presence of two electronegative nitrogen atoms adjacent to carbon atom in ring C of ANCU have maximum positive charge(0.5029e).

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Conflict of Interest

The authors confirm that there are no known conflicts of interest associated with this publication. It is also certify that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

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Discovery and validation of potential impact of cannabinoid receptor agonists by insilico means

Sarithamol S[1], Divya V[2], V.L. Pushpa*[1], K.B. Manoj[3]

Author Affiliations
[1]P G and Research Department of Chemistry, Sree Narayana College, Kollam, Kerala.
[2]Department of Chemistry, MSM College, Kayamkulam, Alapuzha, Kerala, India.
[3]Sree Narayana College, Cherthala, Alapuzha, Kerala, India.

Corresponding Author
***V.L. Pushpa**, P G and Research Department of Chemistry, Sree Narayana College, Kollam, Kerala.

E-mail: drpushpavl2017@gmail.com

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Abstract

Endocannabinoid receptor agonists are highly recognized for its anti–inflammatory property. Their immunomodulatory effect can be utilized for the treatment of different types of immunomodulatory diseases. The present study comprised of 3 D QSAR analysis of a well aligned set cannabinoid receptor agonists. The model generated has revealed its statistical significance and ability for external test set prediction by producing better R^2 and Q^2 values. A five feature pharmacophore, AAADH generated from the cannabinoid receptor agonistic activity has produced valid information on the agonistic activity of the compounds. The systematic analysis of the QSAR model has furnished a clear output on how to improve the activity of the compounds through structural modifications. Since cannabinoid receptor agonists are having the property of reducing the expression of macrophages and T cells, especially interleukin 4, interleukin6, and interleukin 5, they are highly recommended for drug designing for those diseases managed by T–cell signaling pathways.

Keywords: Endocannabinoid receptors, 3D QSAR study, Pharmacophore, Regression analysis, Model validation

1. INTRODUCTION

Cannabinoids are a class of compounds showing activity towards cannabinoid receptors (CB). Cannabinoid receptors include CB 1 and CB2. CB1 is expressed in brain and CB2 is seen in the cells of immune system. Both the cannabinoid receptors play a vital role in the regulation of immune system. Several studies have been suggested for the therapeutic effect of cannabinoids and their endogenous ligands [1]. Arachidonic acid metabolites were found to have properties similar to the compounds seen in the plant cannabis sativa. These compounds are indicated as endocannabinoids. Cannabinoids are explored as potent anti–inflammatory agents and the actions include induction of apoptosis, inhibition of cell proliferation, suppression of cytokine production and induction of T–

regulatory cells. Cytokines are important cell signaling proteins works behind immunomodulatory diseases. Asthma is a chronic inflammatory disease of the airways characterized by eosinophilia, increased vascular permeability in the bronchial mucosa, mucus hyper secretion and airway hyper responsiveness [2]. Although there is a general consensus about the use of corticosteroids and bronchodilators as main therapeutic measures for the prevention and management of asthma, the identification and development of promising new substances with anti–asthmatic effects that can flank and co–operate with the above drugs is a fertile field for basic and clinical research [3] because of its primary medical interest. Arachoidonic acid metabolic pathway is a causative for asthma like autoimmune diseases [4]. Recently, claims have been made for the beneficial effects of cannabis and cannabinoids, the active components of Cannabis sativa. Many works have been reported for the antiasthmatic activity of cannabinoids. Δ 9–tetrahydrocannabinol (THC), the principal component of cannabis sativa has reported for reduction in the expression of interleukin 4 by creating inhibition in the IL4 signaling pathways. The THC is the first reported cannabinoid receptor ligand[1]. Asthma is one among those diseases, managed by IL4 signaling pathways. Δ 9–tetrahydrocannabinol (THC) is a well known cannabinoid receptor agonist. Many compounds including phytochemical compounds were reported to act as cannabinoid receptor agonists.

Quantitative structure activity relationship (QSAR) is a well accepted insilico technique utilized by leading pharmaceutical companies for effective, systematic and speedy method of drug designing process. The QSAR model is a statistically derived structure activity relationship which can make use of successful activity prediction of new set of compounds before going for its synthesis and can design compounds with better activity properties. The process can reduce the time of discovery of drugs and can do the whole process in a more economic way [5]. The present study include QSAR analysis of a set of cannabinoid receptor agonists to derive functional alert for the receptor agonistic property and the responsible functionality can be utilized for designing drugable compounds for asthma like immunomodulatory diseases.

2. EXPERIMENTAL

2.1. Insilico analysis – Data set

A bioassay of 82 cannabinoid receptor agonists was reported by Angela Berry et al, in their patent work US 7,928,123 B2, Apr. 19, 2011 [6]. The data set comprised of a diverse set of compounds having the scaffold of sulfonamides with activity (EC50) concentrations ranging from 0.04nM to 99nM. The present study has executed a three dimensional (3 D) QSAR analysis on the dataset for generating a successful QSAR model for the activity prediction of new series of compounds (Figure 1). The dataset was divided into training and test set in the ratio 75:25. The splitting were done in such a way that test set should have all the features of the training set, means, maximum structural and activity diversity were seen among training set compounds [7–8]. The EC50 values were converted into their negative logarithmic representations for statistical significance. The data set with activity values are included in supplementary information (Table SI. 1)

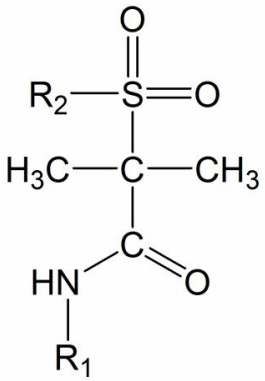


Fig. 1: General structure of bioassay compounds

2.2. Ligand preparation for QSAR study

All the 82 compounds were drawn in the 2D sketcher of maestro interface of the schrodinger software. Structure optimization and energy minimization were done using the ligprep module at a force field of OPLS_2005 and pH 7.4.

The structural alignment (Figure 2) is an essential feature for a meaningful QSAR modeling [8]. The present study has been aligned all the structures with the most active compound from the bioassay through flexible ligand based alignment using largest common Bemis–murcko scaffold alignment. A total of 68 compounds showing very good alignment with the active compound were obtained. Those 68 compounds were subjected to atom based 3D QSAR analysis after splitting them into training and test set in the ratio 75: 25.

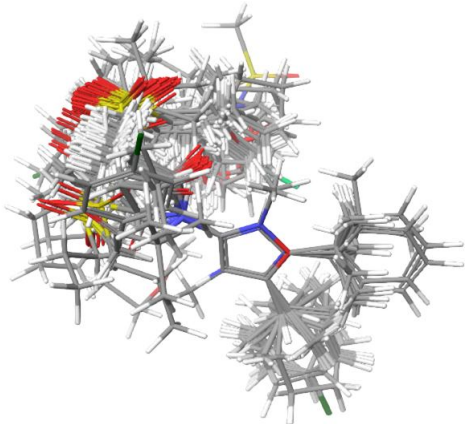


Fig. 2: Structural alignment with the active bioassay compound number 9 (EC50= 0.04nM)

2.3. Generation of pharmacophore

Pharmacophoric feature is a 3 dimensional feature of a set of compounds meant for biological activity [9]. Compounds were assigned as actives and inactives by assuming that compounds with activity (pEC50) below 7.25 as inactive and compounds with activity above 9.6 as actives. A total of 9 active and 5 inactive compounds were considered for pharmacophore generation. The activity threshold for dataset was generated on the basis of activity distribution (7.047–10.398). Pharmacophore feature generation was done by using the phase module of Schrodinger software. The module has offered some 6 pharmacophoric features including aromatic feature, hydrophobic, hydrogen bond acceptor, hydrogen bond donor, negatively charged and positively charged groups. A common pharmacophoric feature has derived from a set of 14 least and top active compounds using a tree–based partition algorithm which clusters similar pharmacophore based on their intersite distances and angles. The best pharmacophoric feature, the feature which is better aligned with the most active compounds, among a set of generated features was selected on the basis of survival active and survival inactive score. The survival score is the net result of volume and selectivity scores of aligned ligands, contributions from number of matches, relative energy of reference ligand and the activity of reference ligand. The pharmacophoric features were also mapped for least active compounds to discriminate between active and inactive compounds. A feature with very good active–inactive distinguishing ability should have high survival active score and less survival inactive scores. The scoring methodology include alignment of sites, vector and volume scores, selectivity, number of compounds matched, activity and relative conformational energy.

2.4. 3D QSAR analysis

A total of 68 better aligned sulfonyl amide compounds were chosen for atom based 3D QSAR modeling. 17 compounds were considered as test set while remaining 51training set compounds were utilized to develop a meaningful QSAR model using the 3D descriptor properties such as hydrogen

bond donors, hydrophobic effect, negative ionic effect, positive ionic effect and electron withdrawing effect. Partial least square analysis was executed to derive the model from the dataset [10]. The PLS factor should be 1/5 of the number of compounds in the training set. The PLS factor for the present study was 5. Leave one out cross validation technique was done for checking the validity and robustness of the model.

3. RESULTS AND DISCUSSIONS

3.1. Pharmacophore generation

All the 68 structurally aligned sulfonamide derivatives were subjected for pharmacophore generation using the phase module. After careful analysis of the scores and alignment of the active ligands to the generated hypothesis, the pharmacophore hypothesis AAADH was selected as the potential feature responsible for the biological activity. This five feature pharmacophore encompasses three hydrogen bond acceptors, one hydrogen bond donor and a hydrophobic group. Oxygen atom in the oxazole ring, carbonyl group of the amide linkage and S=O of the sulfonyl group contributed to the biological activity as hydrogen bond acceptors, the 3° carbon attached to the oxazole ring stands for hydrophobic effect and amino group of amide linkage could work behind hydrogen bond donor effect.

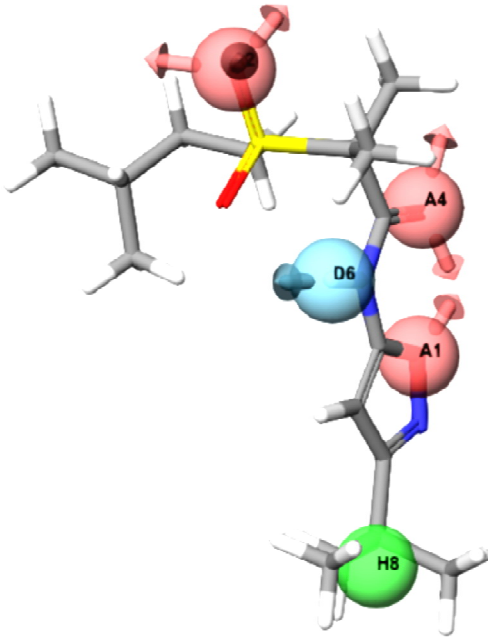


Fig. 3: (a) Pharmacophoric feature furnished by the cannabinoid receptor agonists, highest active compound aligned with the pharmacophore

The pharmacophoric feature should retain as such for an effective cannabinoid receptor agonistic activity. The highest active compound 3–tert–butyl–N–{3–methyl–3–[(3–methylbutane) sulfonyl] but–1–en–2–yl}–1, 2–oxazol–5–amine could align better with the feature with a finess score of 2.82 (Figure 3). Pharmacophoric feature with inter atomic distances and angles is included in supplementary information (Figure SI 1)

The feature AAADH has got highest survival score (Table 1), survival inactive score and lowest energy of conformation and all the top 9 active compounds could furnish high fitness score with the feature. These findings shed light on the potential effect of the five feature pharmacophore AAADH towards the cannabinoid receptor as very good agonists. This agonistic feature can be exploited for the treatment of immunomodulatory diseases like asthma, cancer etc.

Table 1: Pharmacophore scoring data

Pharmacophoric feature	Survival score	Survival inactive score	Matches	Activity	Energy
AAADH.1170	3.783	1. 811	9	9.699	0.000
AAADH.1509	3.742	1. 617	9	9.699	1.162
AAADR.90	3.780	1.329	9	10.000	1.767
AAADR.54	3.763	1.408	9	9.699	2.338
AAADR.371	3.771	1.473	9	10.000	1.767

3.2. 3D QSAR analysis

Atom based 3D QSAR model has been furnished with 68 cannabinoid receptor agonists by partial least square method keeping 51 compounds as training and 17 compounds as test. The PLS factor was chosen as 5. Five models were generated. The statistical data table is shown below (Table 2).

Table 2: Statistical analysis, F– variance, p– statistical significance, RMSE– deviation from reference ligand, SD– standard deviation

PLS factor	SD	R2	F	P	RMSE	Q2	Pearson–r
1	0.7354	0.2433	14.8	0.000367	0.75	0.2672	0.5333
2	0.571	0.5537	27.9	1.31E–08	0.56	0.5962	0.7866
3	0.491	0.6774	30.8	6.95E–11	0.55	0.6118	0.7869
4	0.3562	0.8341	54	3.19E–16	0.51	0.6623	0.8336
5	0.2683	0.9081	83	1.18E–20	0.39	0.8002	0.9042

Among the five models, model having PLS factor 5 has chosen as the stable better predictive model. The robustness and stability of the model has revealed through the regression coefficient, R^2= 0.9081, statistical significance of the model was expressed through high F value and low p value, here the values obtained were 83 for F and 1.18E–20 for p. external predictability of the model was assessed by cross correlation coefficient, Q^2 = 0.8002. The value of Q^2 >0.5 indicate external predictability of the model. Linearity of the model was checked by plotting experimental and predicted activity for training set and test set (Figure 4). Contour plots for the QSAR model could explain the structural requirement for a better cannabinoid receptor agonistic activity (Figure 5). Experimental and predicted activities are seen in supplementary information (Table SI. 2).

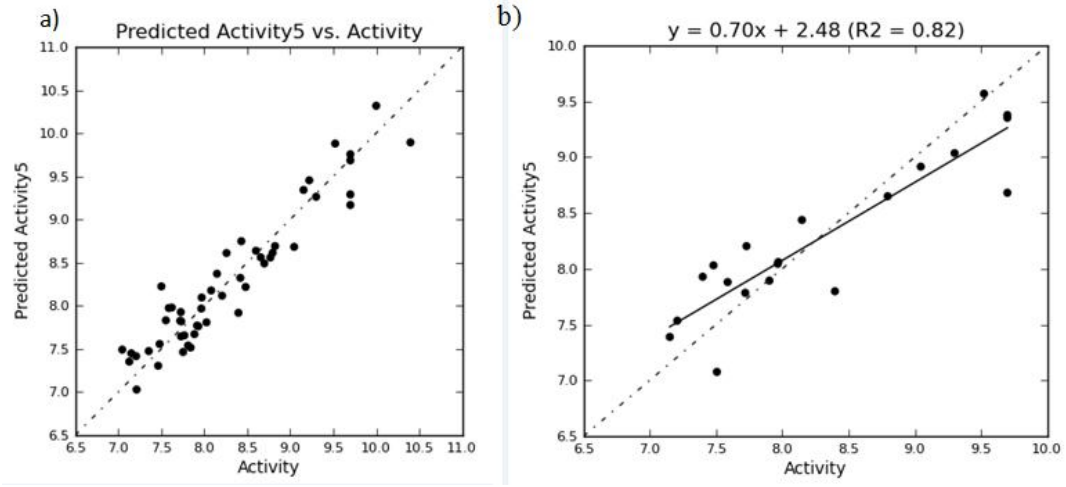


Fig. 4: (a) training set, (b) test set linear relationship

Red cubes in the contour map indicate negative contribution towards activity property and blue cubes indicate positive contribution towards receptor agonistic property. Hydrophobic effect, hydrogen bond donor effect, negative ionic effect and electron withdrawing effect were found to have contribution towards cannabinoid receptor agonist property. Hydrogen bond donor effect of oxazole ring and amide NH2 group had a positive contribution towards the agonistic property. The hydrophobic effect of isopropyl group has positive contribution towards the agonistic property. If a methyl group occupy the position of isopropyl group (compound 36), then the activity was found to be considerably decreases. The hydrophobic effect of tertiary methyl group near to the oxazole ring favors the receptor agonistic property. The negative ionic effect of oxygen atom of oxazole ring disfavors the activity. Electron withdrawing effect of the same group again degrades the agonistic property. This QSAR model of cannabinoid receptor agonists can make use of finding out of drug like compounds for immunomodulatory diseases like asthma.

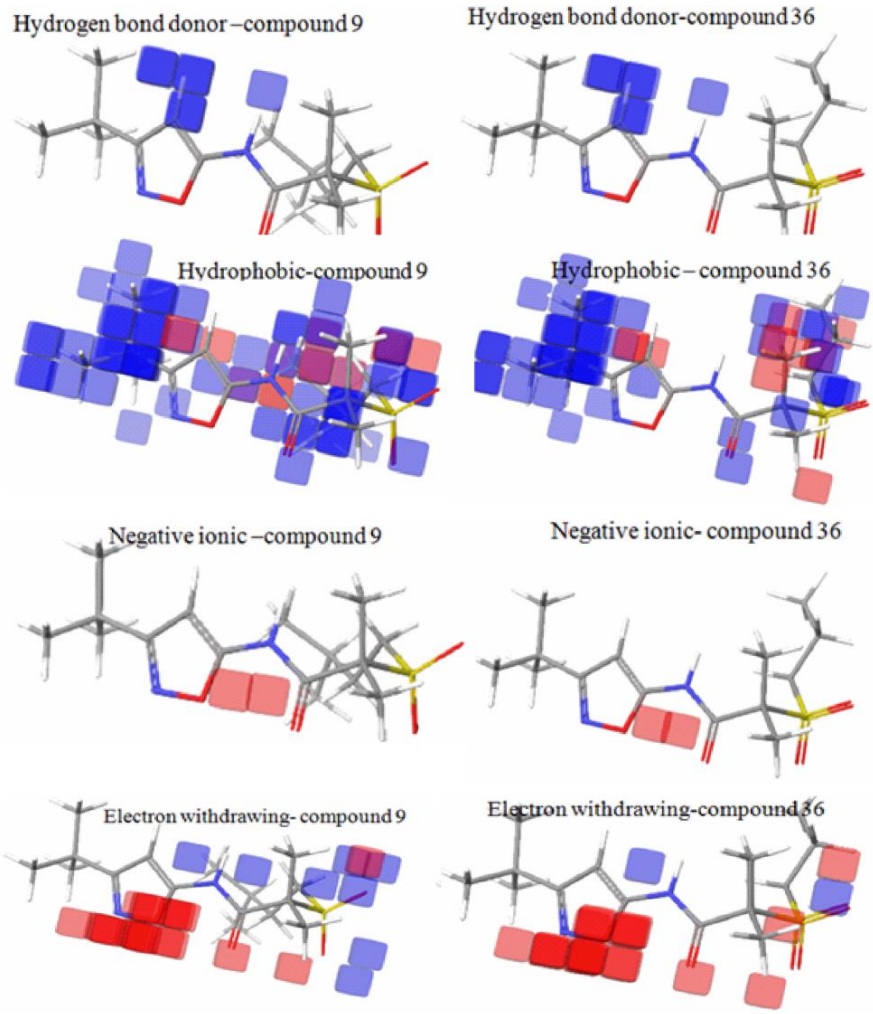


Fig. 5: QSAR contour plots, blue cubes– activate the system, red cubes– deactivate the system, compound 9– top active , compound 36– least active

Supplementary Information

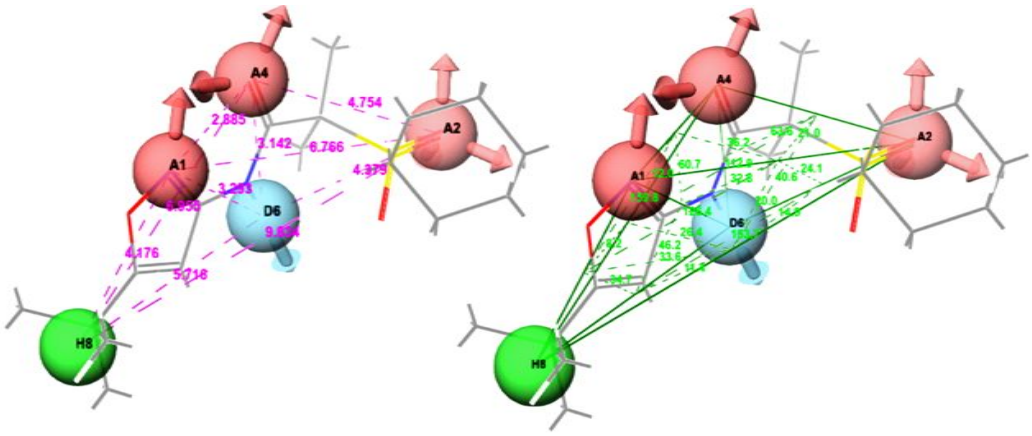
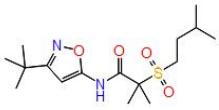
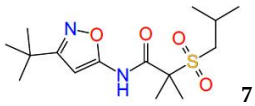
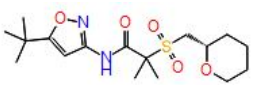
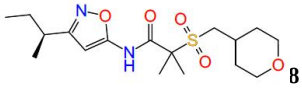
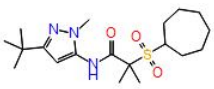
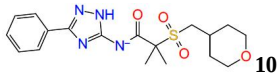
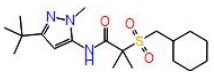
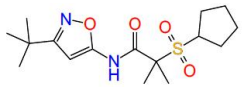
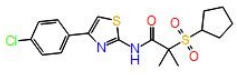
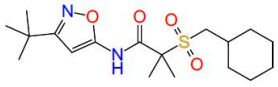
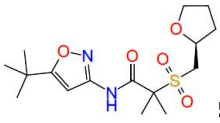
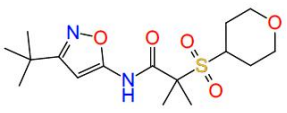
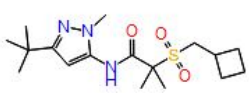
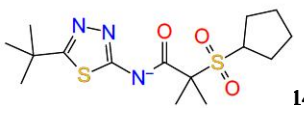
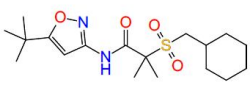
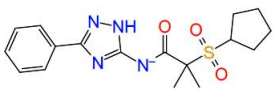
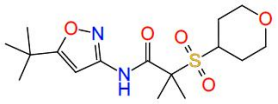
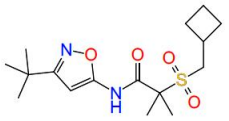
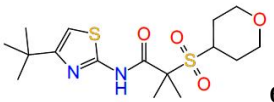
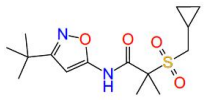
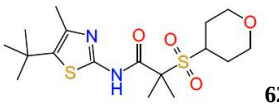
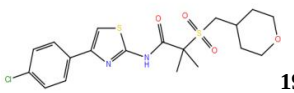
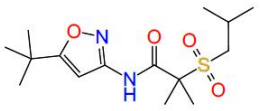
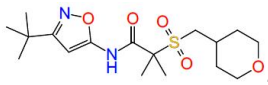
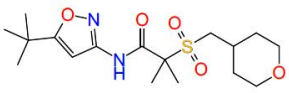
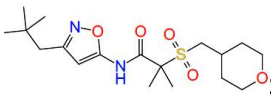
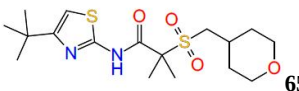
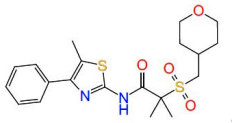
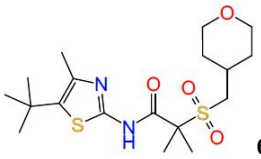
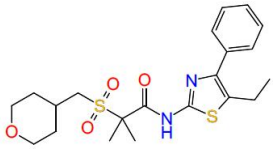
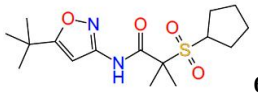
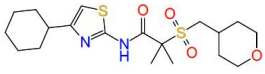
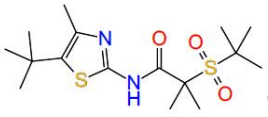
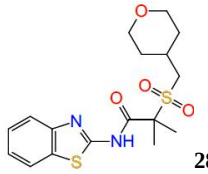
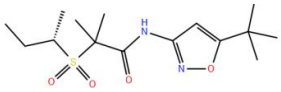
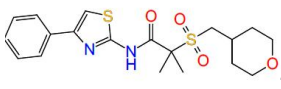
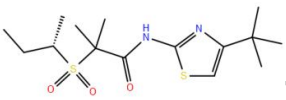
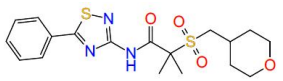
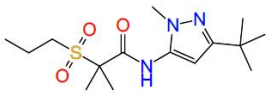
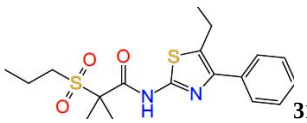
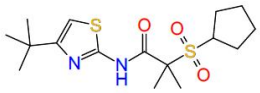
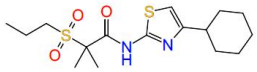
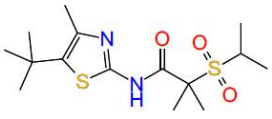
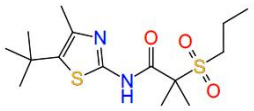
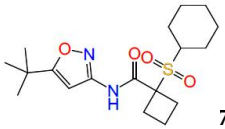
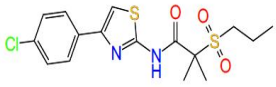
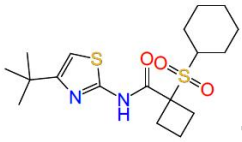
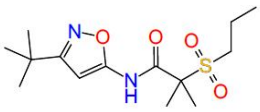
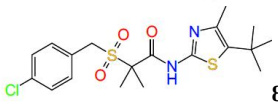
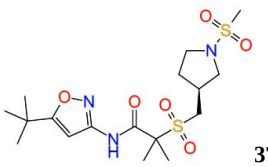
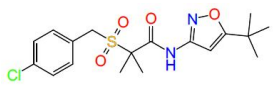
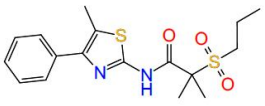
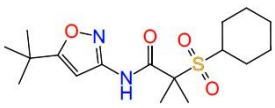
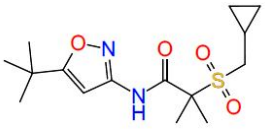
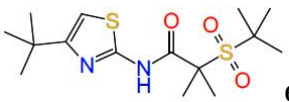
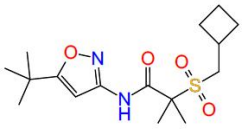
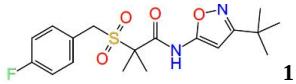
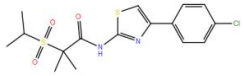


Fig. SI 1: Pharmacophore with (a) inter atomic distances and (b) angles

Table SI 1: Data set for QSAR analysis

Structure and Compound ID	EC50 (nM)	pEC50	Structure and Compound ID	EC50 (nM)	pEC50
 **9**	0.04	10.398	 **7**	1.6	8.796
 **51**	0.2	9.699	 **8**	6.2	8.208
 **52**	6.5	8.187	 **10**	1.7	8.77
 **53**	23.8	7.623	 **11**	0.9	9.046
 **55**	1.5	8.824	 **12**	0.1	10
 **56**	70.5	7.152	 **13**	0.1	10
 **57**	33	7.481	 **14**	18.6	7.73

 **58**	0.6	9.222	 **15**	3.8	8.42
 **59**	0.7	9.155	 **16**	3.7	8.432
 **60**	12.4	7.907	 **17**	12.5	7.903
 **62**	0.3	9.523	 **19**	2.2	8.658
 **63**	44.6	7.351	 **21**	4	8.398
 **64**	4.5	8.347	 **22**	74.3	7.129
 **65**	23	7.638	 **25**	2.5	8.602
 **66**	0.2	9.699	 **26**	2	8.699
 **68**	2.4	8.62	 **27**	8.3	8.081
 **70**	10.7	7.971	 **28**	27.9	7.554
 **71**	18.7	7.728	 **29**	7.1	8.149

 **72**	14.4	7.842	 **30**	17.6	7.754
 **73**	61.9	7.208	 **31**	10.8	7.967
 **75**	19	7.721	 **32**	31.1	7.507
 **77**	25.8	7.588	 **33**	7.2	8.143
 **78**	0.5	9.301	 **35**	13	7.886
 **79**	11.7	7.932	 **36**	89.7	7.047
 **80**	12.1	7.917	 **37**	44.1	7.356
 **81**	16.6	7.78	 **38**	52.2	7.282
 **82**	0.2	9.699	 **39**	34.3	7.465
 **69**	61.1	7.214	 **40**	3.4	8.469
 **1**	17	7.77	 **41**	44.9	7.348

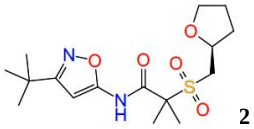
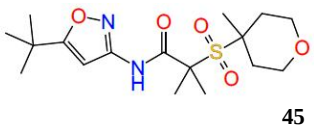
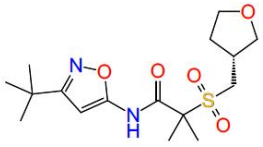
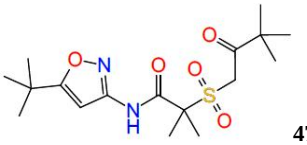
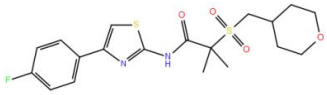
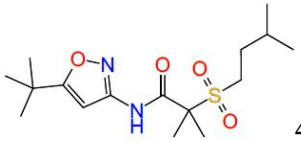
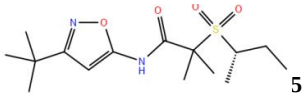
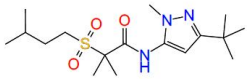
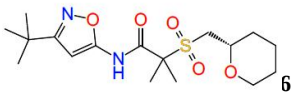
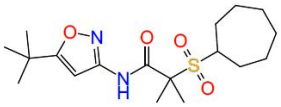
 **2**	31.5	7.502		 **45**	9.4	8.027
 **3**	18.5	7.733		 **47**	39.8	7.4
 **4**	5.5	8.26		 **48**	0.2	9.699
 **5**	3.3	8.481		 **49**	15.4	7.812
 **6**	0.2	9.699		 **50**	0.1	10

Table SI. 2: Data set experimental activity vs predicted activity

Comp. ID	QSAR set	pEC50	Predicted pEC50	Prediction error		Comp. ID	QSAR set	pEC50	Predicted pEC50	Prediction error
9	training	10.398	9.6801	-0.717842		7	training	8.796	8.77226	-0.02362
51	test	9.699	9.47192	-0.227052		8	training	8.208	8.35615	0.148539
52	test	8.187	8.77964	0.592551		10	training	8.77	8.67728	-0.0922718
53	training	7.623	7.89008	0.266656		11	training	9.046	9.07498	0.029225
55	training	8.824	8.61947	-0.204441		12	training	10	9.96743	-0.0325713
56	training	7.152	7.19752	0.0457043		13	test	10	9.85228	-0.147721
57	training	7.481	7.54609	0.0646071		14	training	7.73	7.69398	-0.036506
58	training	9.222	9.18004	-0.0418104		15	training	8.42	8.3951	-0.025117
59	training	9.155	9.34103	0.18613		16	training	8.432	8.53315	0.10135
60	test	7.907	8.12637	0.219788		17	test	7.903	8.02274	0.119648
62	training	9.523	9.60147	0.0785926		19	training	8.658	8.65333	-0.0042489
63	test	7.351	8.62973	1.27907		21	training	8.398	8.03332	-0.364619
64	test	8.347	8.94083	0.594042		22	training	7.129	7.10838	-0.0206345
65	test	7.638	8.12787	0.48959		25	training	8.602	8.63269	0.0306333
66	training	9.699	9.29249	-0.406476		26	training	8.699	8.73257	0.0336
68	test	8.62	8.57511	-0.044674		27	training	8.081	8.07824	-0.0026833
70	training	7.971	7.97145	0.00083213		28	training	7.554	8.11246	0.558066
71	training	7.728	7.73938	0.0112258		29	training	8.149	8.25114	0.102402
72	training	7.842	7.83926	-0.0023731		30	training	7.754	7.76457	0.0100847
73	training	7.208	7.19677	-0.0115426		31	training	7.967	7.87896	-0.0876144
75	training	7.721	7.64738	-0.0738618		32	test	7.507	7.63933	0.132094
77	training	7.588	7.86684	0.278461		33	test	8.143	8.10202	-0.0406441
78	training	9.301	9.20349	-0.0975403		35	training	7.886	7.67424	-0.211814
79	training	7.932	7.88681	-0.0450086		36	training	7.047	7.58875	0.541542
80	training	7.917	7.83818	-0.0790376		37	training	7.356	7.36949	0.0139261
81	training	7.78	7.80597	0.0260798		38	test	7.282	7.73844	0.456107
82	test	9.699	9.67298	-0.0259857		39	training	7.465	7.49711	0.0324086
69	training	7.214	7.26149	0.0475314		40	test	8.469	8.43126	-0.0372636
1	training	7.77	7.73025	-0.0392977		41	test	7.348	7.30492	-0.0428357
2	training	7.502	8.49557	0.993884		45	training	8.027	8.08945	0.0625777
3	training	7.733	7.95356	0.220728		47	test	7.4	7.85626	0.456146
4	training	8.26	8.24248	-0.017154		48	training	9.699	9.58391	-0.11506
5	training	8.481	8.54184	0.06035		49	training	7.812	7.59503	-0.217451
6	training	9.699	9.42945	-0.269517		50	test	10	9.18682	-0.81318

4. CONCLUSION

The pharmacophoric feature AAADH derived from the cannabinoid receptor agonists comprised of 5 potential features. The features include three hydrogen bond acceptors, one hydrogen bond donor and one hydrophobic feature. This feature gave a clear idea on the potential feature of the ligands responsible for the receptor agonistic property. The 3D qsar model derived from the aligned date set of 68 sulfonamide compounds revealed its statistical significance by the regression coefficient R^2= 0.9081, analysis of variance, F= 83, statistical significance, p = 1.18E–20 and cross correlation coefficient, Q^2= 0.8002. $Q^2 > 0.5$ stands for external test set predictability of the model. The model analysis has revealed the significance of electron withdrawing effect of alkyl substituents attached to the sulfonyl group, hydrophobic effect of oxazole ring substituents and electron withdrawing effect of sulfonyl group behind the receptor agonistic property. The observations suggested that the present QSAR model of cannabinoid receptor agonists can be utilized for rational drug designing for immuno modulatory diseases.

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Synthesis, structural characterization and uv analysis of praseodymium zirconate oxides

S. Srijith[1*], V. T. Kavitha[2], L.R. Asitha[1]

Author Affiliations
[1]Research and Post graduate Department of Physics, SN College, Kollam, Kerala, India.
[2]Research and Post graduate Department of Physics, Mahatma Gandhi College, Thiruvananthapuram, Kerala, India.

Corresponding Author
***S. Srijith,** Research and Post graduate Department of Physics, SN College, Kollam, Kerala, India.

E-mail: srijithkeanu@gmail.com

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Abstract

Nanoceramics can be utilized for various applications like electronic, ionic, thermal, optical and catalytic. In particular, the combination of a relatively large portion of interfacial or grain boundary atoms along with the interactions of photons, electrons, or dislocations can lead to unusual mechanical and electrical behavior. In this work, we synthesize $Pr_2Zr_2O_7$ oxides through solution combustion synthesis and study the band gap of the material. The effect of strain on the broadening of X ray peaks were also analysed using Hall Williamson analysis.

Keywords: Nanoceramics, Oxides, $Pr_2Zr_2O_7$, Crystallite size

1. INTRODUCTION

Nano–ceramics have received a considerable attention during the last two decades due to their capability to demonstrate improved or unique characteristics as compared to conventional bulk ceramic materials. Nano–ceramics can be utilized for various applications like electronic, ionic, thermal, optical and catalytic. In particular, the combination of a relatively large portion of interfacial or grain boundary atoms along with the interactions of photons, electrons, or dislocations can lead to unusual mechanical and electrical behaviour.

Among the ternary metallic oxides, compounds of the general formula, $A_2B_2O_7$ (A and B are metals), represent a family of phases isostructural to the mineral pyrochlore, (NaCa) $(NbTa)O_6F/(OH)$ [1]. Thespace group of the ideal pyrochlore structure is Fd3m and there are eight molecules per unit cell (Z=8). In $A_2B_2O_7$ pyrochlores, A is usually a trivalent rare earth ion, but can also be a mono, divalent cation and B may be 3d, 4d or 5d transition element having an appropriate oxidation state required for charge balance to give rise to the composition $A_2B_2O_7$ [2]. Pyrochlore unit cell contains eight A_2 B_2 $O(1)_6$ O(2) formula units, and for better and easy understanding one–eighth of the

pyrochlore unit cell is shown in figure 1. With the choice of B atom as origin, the cation sublattice is an alternate arrangement of A–site (16*d*) and B–site (16*c*) cations along the 110 direction in an fcc lattice. The anion sublattice is comprised of three different oxygen sites, two of which are occupied (8*b*) and (48*f*), and the third site (8*a*) is vacant. Hence, the anion vacancies are ordered. The A–site cations are eight coordinated and are located within scalenohedra (distorted cubes). The B–site cations are six–coordinated and are located within trigonal antiprisms. All anion sites are tetrahedrally coordinated: the 8*a* site is surrounded by four B cations, and the 8*b* site by four A cations. The 48*f* oxygen is surrounded by two A and two B cations that are slightly displaced from the center of the tetrahedral site towards the unoccupied 8*a* site. The magnitude of the displacement is represented by the positional parameter, *x*, of oxygen at the 48*f* site, which is 0.375 for the ideal fluoritestructure. The pyrochlore crystal structure also tolerates vacancies at the A and O sites to a certain extent with the result that cation and anion migration within the solid is feasible. Recently, it has been shown by our group that anion rich pyrochlores like $Ce_2Zr_2O_8$ are also possible [3]. The pyrochlore structure is closely related to the fluorite structure AX_2, except that there are two cation sites and one–eighth of the anions are absent as shown in the Figure 1 for the ordered pyrochlore, $A_2B_2O_7$, the phase stability of the superstructure is basically determined by the A and B site cation radius ratio.

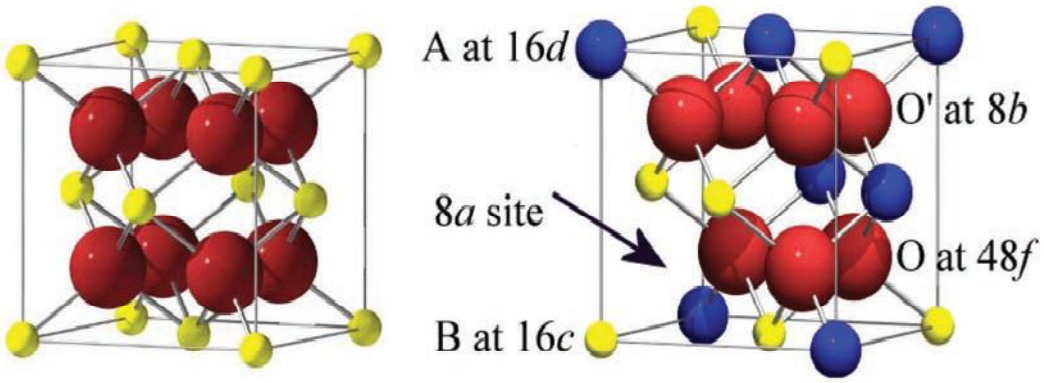

Fig. 1: (a): Unit cell of fluorite (Fmm), yellow atoms represent A^{4+} cations and the red O^{2-} and (b): Unit cell of pyrochlore (Fd3m), Blue spheres represent A^{3+} cations, yellow $B^{4+}$and red O^{2-}

It is worth noting here that compounds with similar cationic radii are more likely to form as disordered fluorites than ordered pyrochlores, for instance,$Er_2Zr_2O_7$ having r_A/r_B ~1.39 crystallizes as a disordered fluorite structure whereas $Er_2Ti_2O_7$ with r_A/r_B ~1.66 crystallizes as a ordered pyrochlore structure. Since more number of (3+, 4+) ions are possible, therefore, large number of pyrochlores having (3+, 4+) cations are reported in literature compared to pyrochlores having (2+, 5+) cations. Recently, (1+, 6+) pyrochlores like KOs_2O_6 are reported in the literature [4,5,6]. In general, pyrochlores exhibit a wide variety of interesting physical properties because a diverse range of constituent ions can be chosen to obtain the desired properties. The pyrochlore structure exhibits a greater degree of complexity than the fluorite structure. The cubic pyrochlore structure is similar to the cubic fluorite structure but with a large number of the oxygen atoms displaced (and one in eight missing). In this work, we synthesize $Pr_2Zr_2O_7$ oxides through solution combustion synthesis and study the band gap of the material.

2. EXPERIMENTAL

Among the available, solution chemistry routes, the solution combustion techniques are capable of producing the nanocrystalline powders of oxide ceramics at a lower calcination temperature in a surprisingly short time [7, 8]. Generally the powder obtained by this technique has compositional homogeneity, the highest degree of phase purity coupled with the improved powder characteristics like narrow particle size distribution, higher surface area and better sinterability;but those depend heavily on the adopted processing parameters. All the oxide materials were prepared by the combustion of aqueous solutions containing stoichiometric amounts of corresponding metal nitrates

and citric acid redox mixtures in a Pyrex dish. Stoichiometry of the redox mixture for the combustion is calculated based on the total oxidizing and reducing valencies of the oxidizer (O) and of the fuel (F), which serve as numerical coefficients so that the equivalence ratio, f_e (O/F), becomes unity and the heat released is at a maximum [9,10]. According to the principles used in propellant chemistry, the oxidizing and reducing valencies of various elements are considered as follows: C = 4, H = 1, O = –2, N = 0,M = 2, 3, 4, etc. Thus, the oxidizing valency of a divalent metal nitrate, M $(NO_3)_2$ becomes –10; $M(NO_3)_3$ = –15; $M(NO_3)_4$= –20; and the reducing valency of HMT ($C_6H_8O_7$) becomes +18. Therefore, the molar ratio of the divalent metal nitrate to fuel (citric acid is) is 15/36, i.e., 1:0.4166. The exothermicity of combustion reactions as well as combustion time can be increased by the presence of redox compounds, such as NH_4NO_3/NH_3ClO_4. This will facilitate the formation of the pure crystalline phase and doping.

A mixture of high purity Pr_2O_3 (2.0205gm), ZrO $(NO_3)_2$ $2H_2O$ (2.9794gm) and citric acid (5.2585gm) are taken in a beaker. After mixing the metal nitrate solutions in beaker, 30 ml nitric acid is added and stirred continuously for 30 minutes. The fuel used for the synthesis is citric acid. In order to attain a pH of 7, liquor ammonia is added drop by drop and the solution is heated at 100 ºC to remove as much of water content. After evaporation of the water content, the slurry was then introduced into a muffle furnace preheated to 400°C. The mixture frothed and ignited to combust with a flame, giving a voluminous and foamy $Pr_2Zr_2O_7$.The structure and phase purity of the powder were examined by XRD using Regaku, DMax C, Japan X– ray diffractometer with Nickel filtered Cu K_α radiation. The UV–visible spectra of samples were recorded by dispersing the sample in ethanol medium using an ultrasonic bath. The absorption spectra were recorded at room temperature using SHIMADZU–2550 UV–Visible Spectrophotometer in the range from 200–800 nm.

3. RESULTS AND DISCUSSIONS

3.1 XRD Analysis

The XRD pattern of the as prepared powder sample obtained by the present combustion synthesis is shown in Figure 2.

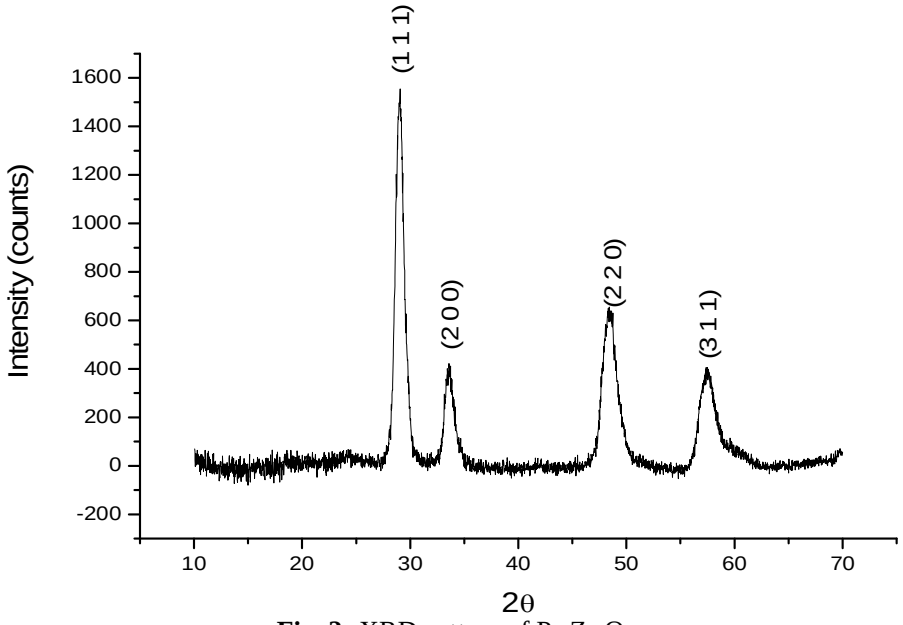

Fig. 2: XRD pattern of $Pr_2Zr_2O_7$

The powder XRD patterns of the sample confirm that the sample is crystalline in nature. Also, the diffraction peak is slightly broadened indicating small crystallite size. High intensity peaks with even–numbered miller indices, representing average fluorite–type unit cells. The superstructure peaks

at 2θ *at*≈14˚, 38˚, 46˚, etc. are very weak in intensity [11, 12]. Progressive structural transformation into defect pyrochlore structure can also lead to a decrease in intensity of the superstructure peaks. Hence it is very difficult to directly determine from the XRD pattern whether the average structure of the compounds is defect pyrochlore or defect fluorite. From the absence of the superstructure peaks, it can be concluded that the structure is defect fluorite [13,14,15]. All the reflections in the Xrd pattern shows that $Pr_2Zr_2O_7$ has cubic lattice, with lattice parameter a = 10.43 Å. Another important observation is that some of the Bragg peaks show asymmetric broadening in contrast to symmetric broadening expected due to strain.

3.2. Determination of Average Crystallite Size Using Scherrer equation

A perfect crystal would extend in all directions to infinity, so we can say that no crystal is perfect due to its finite size. This deviation from perfect crystalline leads to a broadening of the diffraction peaks. However, above a certain size (100–500 nm) this type of broadening is negligible. Crystallite size is a measure of the size of a coherently diffracting domain. Due to the presence of polycrystalline aggregates, crystallite size is not generally the same thing as particle size. In this section the crystallite sizes of sample is determined from X–ray diffraction line broadening using Scherrer equation [16]. Scherrer equation is the simplest method of determining the average crystallite size of a nanocrystalline sample from X–ray diffraction line broadening. Scherrer equation is,

$$t = K\lambda / (\beta_{hkl})_{measured} \times \mathrm{Cos}\theta_{hkl}$$

Here, t is the average crystallite size normal to the reflecting planes, K is the shape factor which lies between 0.95 and 1.15 depending upon the shape of the grains (K=1 for spherical crystallites), λ is the wavelength of X–rays used and $(\beta_{hkl})_{measured}$ is the measured FWHM of the diffraction line in radians and θ_{hkl} is the Bragg angle corresponding to the diffraction line arising from reflections from the planes designated by the Miller indices (h k l). In order to estimate the FWHM the following procedure was adopted. A Pseudo–Voigt 1 function which assumes the peak shape is symmetrical was used to fit the peak profiles. Pseudo–Voigt 1 is one of the most convenient functions used for the profile fitting of X–ray diffraction peaks and it is represented by the expression,

$$y = y_0 + A\left[\frac{2\omega}{\pi\left(x - x_c\right)^2 + \omega^2} + \frac{(1-\mu)\sqrt{4\ln 2}\left(e^{-\frac{4\ln 2(x-x_c)^2}{\omega}}\right)}{\sqrt{\pi}\omega}\right]$$

where, y is the dependent variable (Intensity in the present case), X is the dependent variable (2θ in the present case), ie, X_c is the peak centre, A is the measure of maximum intensity and W is the Full Width at Half Maximum and μ is the profile shape factor. The curve fitting was done using Microcal Origin Version 9. The best fit was chosen taking into account the minimum error as well as the realistic values for individual variables. From Table 1, it can be noted that the average crystallite size of the sample ~18 nm.

Table 1: Scherrer size of crystallites

2θ (°)	θ (°)	FWHM (°)	Crystallite size t, (nm)	
29.02	14.51	0.7957	11.6	9.4nm
33.64	16.82	0.8271	11.3	
48.34	24.17	1.2402	7.9	
57.41	28.71	1.4843	6.9	

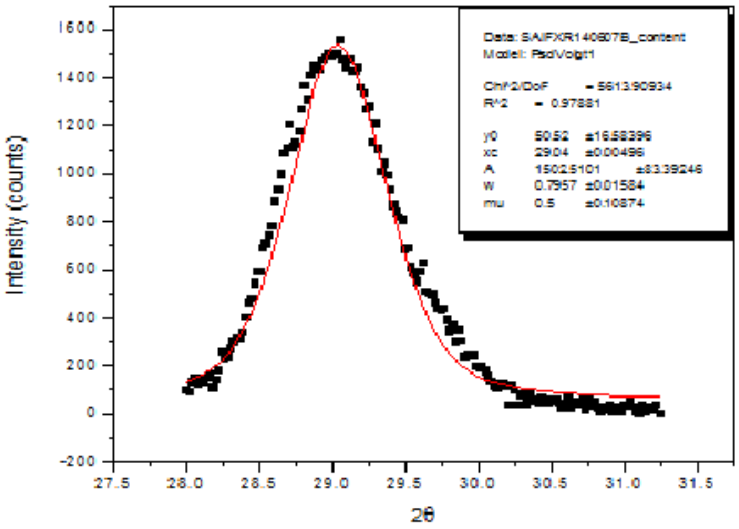

Fig. 3: Peak fitting using pseudo Voigh 1 function

3.3. Hall–Williamson Method to Separate Crystallite Size and Strain Broadening

Hall–Williamson analysis of the nanocrystalline samples is carried out assuming Uniform deformation model [17]. In crystals, we can observe two types of strain, Uniform strain and non–uniform strain. Uniform strain causes the unit cell to expand or contract in an isotropic way. This simply leads to a change in the unit cell parameters and shift of the peaks. There is no broadening associated with this type of strain. Non–uniform strain leads to systematic shifts of atoms from their ideal positions and to peak broadening. This type of strain arises due to point defects (vacancies, site–disorder), plastic deformation (cold worked metals, thin films) films) and poor crystalline. This model doesn't take into account the anisotropic nature of the crystal. In this model, microstrain **ε** is assumed to be uniform in all crystallographic directions and Hall– Williamson equation can be written as

$$\beta_{hkl}\cos\theta_{hkl} = (K\lambda/t) + (4\langle\varepsilon\rangle \sin\theta_{hkl}).$$

Plotting the measured values of $(\beta_{hkl})_{corrected}$ as a function of $(4\sin\theta_{hkl})$, the microstrain $\langle\varepsilon\rangle$ can be estimated from the slope of the line and the crystallite size from the intersection of the vertical axis. The Hall–Williamson plot assuming uniform deformation model for the sample is shown in Figure 4.

It can be noted that the microstrain and crystallite size contribution can be separated using Hall–Williamson analysis. Further, the microstrain values obtained for the sample is small in magnitude. Strain and particle size are calculated from the slope and y–intercept of the fitted line respectively. From the lattice parameters calculations it was observed that this strain might be due to the lattice shrinkage. The crystallite size obtained when considering the strain contribution using Hall Williamson analysis assuming uniform deformation model is found to be 70 nm. Presence of defects can also affect the anisotropy of the crystal which leads to non–uniform nature of the crystal. For confirmation some other techniques such as Warren–Averbach model and Rietveld analysis have to be employed.

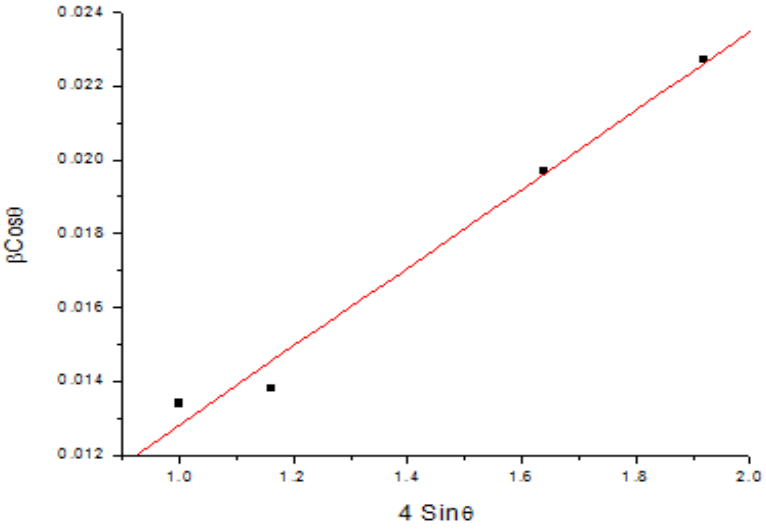

Fig. 4: Hall Williamson Plot of the sample

3.4. Determination of Band gap energy from UV–Visible Absorption Spectra

UV–Visible absorption spectroscopy is widely used for calculating the bang gap energy of semiconductor materials including nanostructured materials. Also UV–Visible spectrum of the samples gives information about excitonic or inter band transition of nanocrystalline materials. From the absorbance value, knowing the concentrations of the samples, absorption coefficient **α** was estimated. The optical band gap of a semiconductor can be estimated using the following equation for semiconductor,

$$\alpha h\nu = A\,(h\nu - E_g)^n$$

where, A is a constant, **α** is the absorption coefficient and E_g isthe band gap of the material. The value of the exponent determines the transition. If the transition is allowed direct n=1/2, for allowed indirect n=2, forbidden direct n=3/2 and forbidden indirect n=3. The exact value of band gap can be determined by plotting $(\alpha h\nu)^{1/n}$ versus hν graph and extrapolating the straight line portion of the graph to hν axis. The UV–Visible spectrum of the sample is shown in Figure 5.

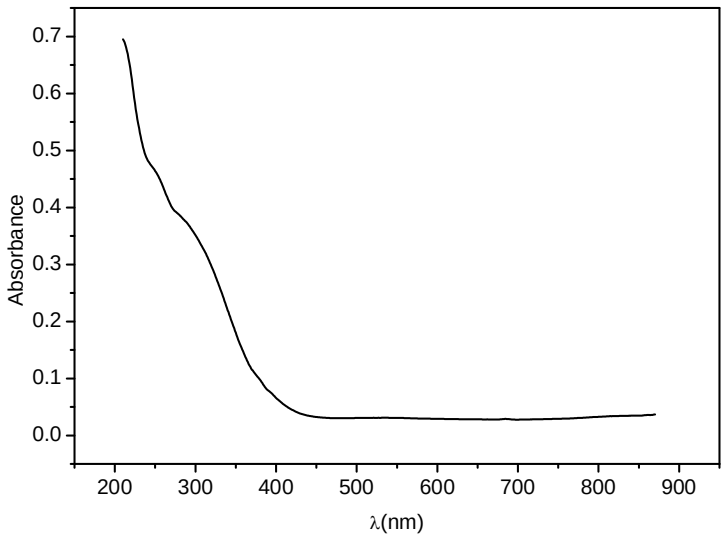

Fig. 5: UV–Visible spectrum of $Pr_2Zr_2O_7$

The optical transition of the sample is reported to be an allowed direct one. Hence in the following analysis the value of n is set as 1/2. Figure 6 shows the plot of $(\alpha h\nu)^2$ versus hν for sample. By extrapolating the straight line portions of the plots, the exact value of the band gap are obtained and was found to be 2.69 eV[18].The Pr compound shows moderate transmittance at UV wavelengths and the blue end ofthe visible range, gradually increasing to a maximum at the red end.The valance band and conduction band of $Pr_2Zr_2O_7$ consist of Pr 4f orbitals and Zr 4d orbitals. The energy gap between these two orbitals strongly depends on the crystallite size. The O 2p orbitals lie below the Pr 4f orbital and do not affect the bandgap of the materials. Hence, the quantum size effect and Pr 4f orbitals affect the bandgap, resulting in the variation of the bandgap energies for these nanoceramics.

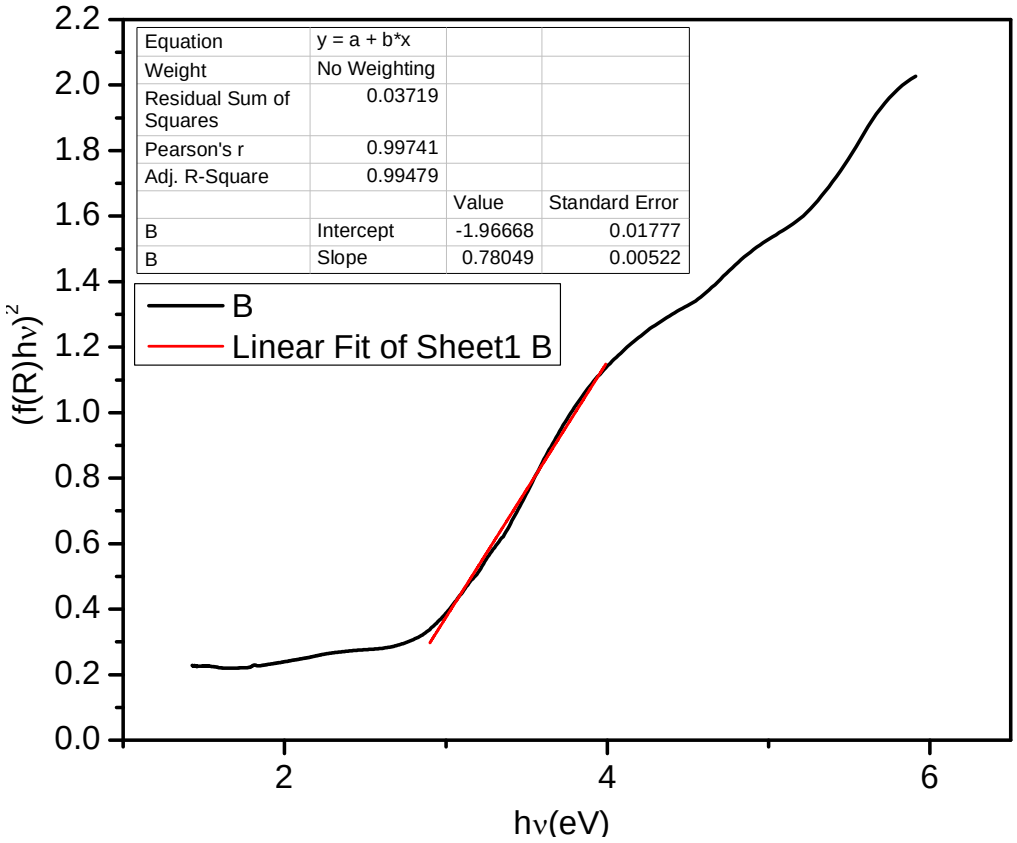

Fig. 6: (f(R)hν)1/n versus hν plot of $Pr_2Zr_2O_7$

4. CONCLUSIONS

A modified combustion process has been developed for the synthesis of nanoparticles of $Pr_2Zr_2O_7$. The xrd analysis concluded that the structure is defect fluorite, having cubic lattice. Average Crystallite size of the sample was calculated from Debey–Scherrer equation is to be 9 nm. Hall Williamson analysis was carried out to separate the strain contribution in X–ray line broadening. The size obtained from the analysis was found to be 70 nm, which is many times higher than as that obtained from Scherrer calculation. This may be due to the anisotropy of the crystal. Band gap energy of the sample was obtained from UV–Visible spectroscopic analysis of the sample. It was found to be 2.69 eV which is in agreement with the wide band gap nature of defect fluorite. This also supports the larger anisotropy of the structure.

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Synthesis and characterization of ZnO@zinc terephthalate metal organic framework

Harisankar. A[1], Asif. A[1]*

Author Affiliations
[1]Department of Chemistry, University College, Thiruvananthapuram

Corresponding Author
***Asif. A,** Department of Chemistry, University College, Thiruvananthapuram

E-mail: asifibnuazeez@gmail.com

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Abstract

Zinc oxide nano particles prepared using starch as capping agent were prepared and then incorporated into a zinc terephthalate framework. ZnO@Zinc terephthalate framework composites were then characterised by FTIR, XRD, SEM and thermogravimetric analysis. N_2 adsorption isotherms were obtained to calculate BET surface area and pore parameter of the framework.

Keywords: Zinc oxide, Nano particles, MOF, zinc terephthalate, BET

1. INTRODUCTION

Metal-organic frameworks (MFs) otherwise called inorganic coordination polymers are formed from metal or metal ion clusters which are linked by multitopic organic linkers [1–3]. Porous network structures have been used in the fields including storage of gas [4], sensing [5], catalysis [6] and for medicinal filed and as ailments in biomedicine [7, 8]. Functionality and acidic and basic moieties in the microporous structures gives them unique identification mark among other inorganic polymers. Supramolecular entities make them more flexible for host guest interactions [9, 10]. Properties of materials used in the field of ceramics, biopolymers can be enhanced by incorporating the nanomaterials into the porous metal organic framework [11].

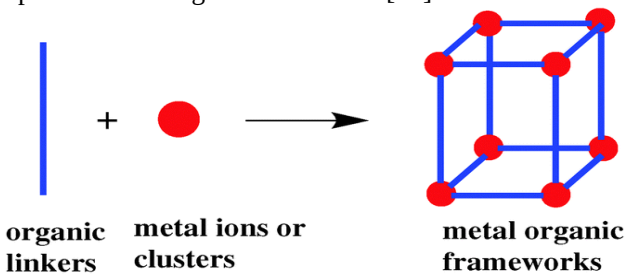


Fig 1: Formation of MOF's.

The present work focuses on the synthesis and characterisation of Zinc oxide nanoparticles @ Zinc terephthalate MOF.

2. EXPERIMENTAL

2.1. Preparation of Zinc terephthalate framework (H6J)

$Zn(NO)_3.6H_2O$ (0.012 mol) and terephthalic acid (0.012 mol) were dissolved in Dimethyl formamide with mild stirring, and 3 mL of trimethyl amine (TEA) was added to the solution drop wise and the system was and heated at 100 °C for 3 hours in an air oven. The resulting crystals were collected and dried.

2.2. Preparation of ZnO @ Zinc Terephthalate MOF (H7J)

Prepared Zinc terephthalate framework and Zinc acetate was dissolved in dilute HCl solution. Then solution of NaOH was added to the above solution until precipitation occurs, refluxed for 2 hours. The precipitates were then collected by filtration. The compound was dried at 60°C. These compounds are named as ZnO@ Zinc terephthalate framework (H7J).

ZnO@Zinc terephthalate framework (H7J) was then calcined at 450 °C for 3 hrs. This calcined product was named as H8J.

2.3. Preparation of zinc oxide nanoparticles using starch as capping agent (H9J)

0.01 M starch solution was prepared in 100 ml water. 100 mL of 0.1 M zinc acetate solution was added drop wise to the starch solution. Then 100 mL of 1 M NaOH solution was added to it till the pH was 12 and the mixture were stirred continuously for 3 hours. The resulting precipitate was filtered, washed, dried and calcined at 450 °C for 3 hours.

The nanoparticles and the MOF's were characterised by FTIR, XRD, SEM, BET and thermogravimetric analysis.

3. RESULTS AND DISCUSSIONS

3.1. FTIR Analysis

Figure 2 shows the FTIR spectrum of Zinc terephthalate framework (H6J), ZnO@Zinc terephthalate framework (H7J), Calcined ZnO@Zinc terephthalate framework (H8J) and ZnO nanoparticles (H9J).

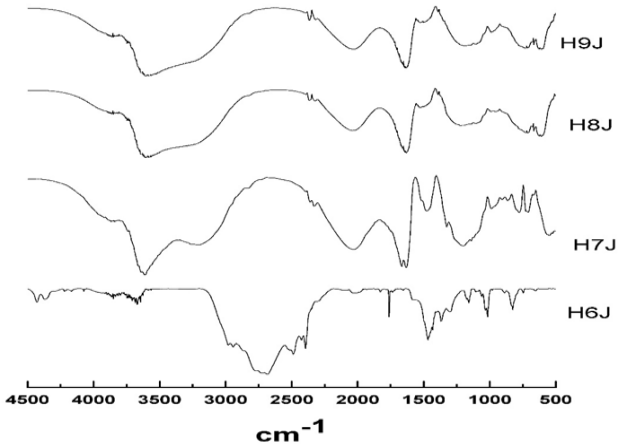


Fig 2: FTIR spectrum of ZnO@Zinc terephthalate framework composites.

In Zinc terephthalate framework, the peaks at 1400 and 1762 cm^{-1} corresponds to the symmetric and anti-symmetric vibration of –CO group in H_2BDC respectively. Peaks ranging from 1300–825 cm^{-1} correspond to in-plane C–H bending which identifies the possibility of formation of zinc terephthalate framework. The peaks in ZnO@ Zinc terephthalate framework with a slight deviation of

fingerprint values show the possibility of entrapping other moieties in the framework. Peaks at 1400–1500 cm^{-1} corresponds to C=O bonds. Peaks at 3600 cm^{-1} corresponds to OH stretching vibrations which indicates the formation of zinc oxide. In Zinc oxide nanoparticles using starch as capping agent shows peaks at 450–500 cm^{-1} corresponds to metal oxide bond (Zn–O) and the broad peak at 3500 cm^{-1} range corresponds to water molecules. The IR spectrum of calcined ZnO@MOF is exactly similar to that of zinc oxide prepared by starch as capping agent, which reveals the possibility of the degradation of ZnO@MOF structure to ZnO.

3.2. X-ray diffraction analysis

Figure 3 represents the XRD pattern of Zinc terephthalate frame works and its ZnO composites. The powder XRD pattern of Zinc terephthalate (H6J) frameworks shows characteristic peaks at 2θ values in the range of 5° to 30° such as at 6.5, 9, 13, 15, 23.7, 24.0, 26.3 and 30. Similar XRD patterns are reported for Zinc terephthalate framework prepared from different zinc precursor [12].

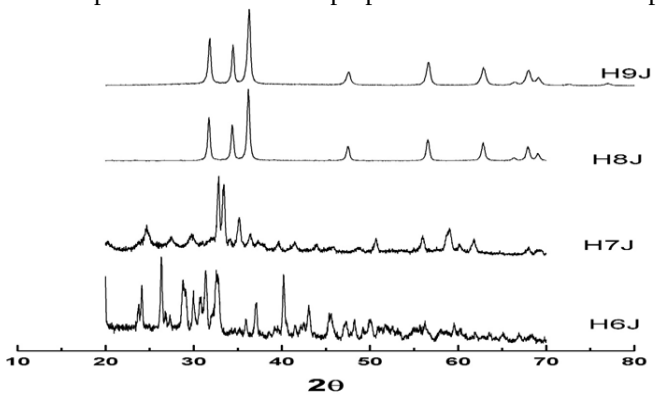
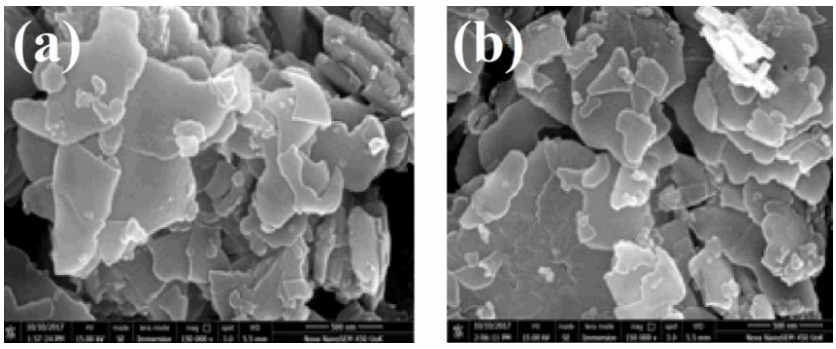


Fig. 3: XRD pattern of ZnO@Zinc terephthalate framework composites

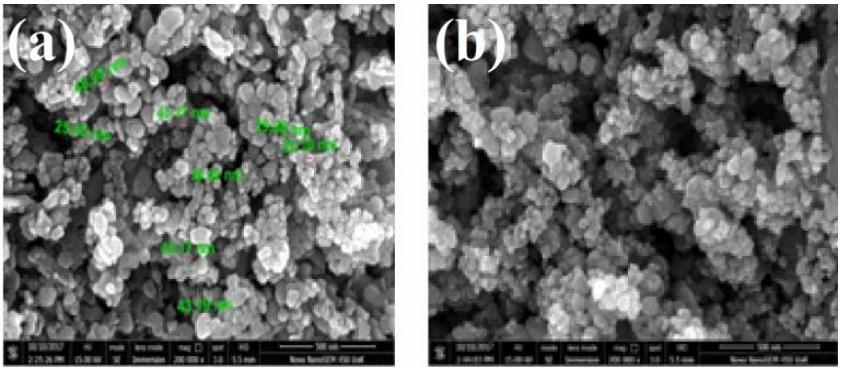
XRD pattern of ZnO@Zinc terephthalate framework reveals that the product is composed of both zinc terephthalate frameworks and ZnO nanoparticles as it is clear from the peaks observed at 31.7, 34.4 and 36.2 corresponds to pure zinc oxide, in addition to the above said peaks of the Zinc terephthalate framework structure. ZnO and calcined ZnO@MOF show same XRD pattern indicating the complete conversion of ZnO@MOF to ZnO nanoparticles and their average particle size calculated from Debye Scherer equation is 25nm.

3.3. SEM analysis

Figures 4 represent the scanning electron micrographs of zinc oxide @ zinc terephthalate frame work. It shows an irregular flaky crystalline morphology which is different from small crystals with well-defined cubic morphology for Zinc terephthalate framework reported by Biemmil et al. [12]. The destruction of the cubic morphology to this flaky morphology is attributed to the incorporation of ZnO nanoparticles.



Figs. 4: SEM images of zinc oxide @ zinc terephthalate frame work.



Figs. 5: SEM images of calcined zinc oxide @ zinc terephthalate frame work.

The SEM image of the calcined zinc oxide @ zinc terephthalate frame work is shown in Figure 5 Micrograph reveals that the flaky structure has been completely converted into granular morphology with particle size of 25-40 nm as calculated from XRD. This conversion of morphology clearly confirms the formation of Zinc oxide by the calcination of zinc oxide @ zinc terephthalate frame work, which is also clear from the XRD patterns

3.4. BET surface area analysis

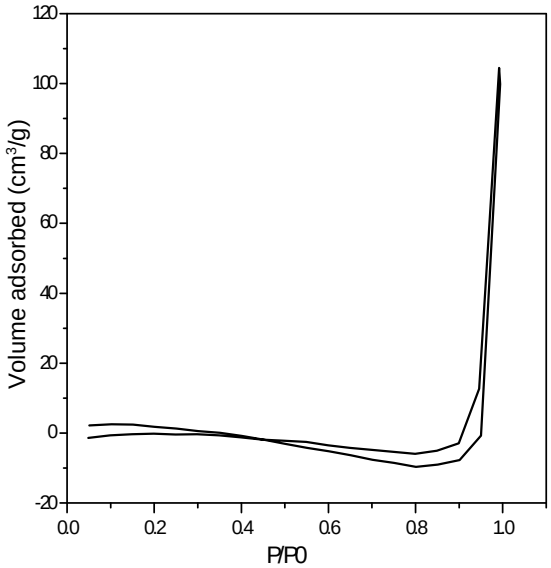


Fig. 6: Nitrogen adsorption and desorption isotherm of ZnO@zinc terephthalate frame work

The BET surface area and Pore parameter of zinc oxide@zinc terephthalate frame work were calculated according to the N_2 adsorption isotherms (Figure 6) at 77.35 K. The adsorption and desorption isotherms are almost same indicating that the configuration of MOF is retained during this process. Adsorption isotherm resembles type IV isotherm indicating the mesoporous nature of the framework. BET surface area of the compound is found to be 5.524 m^2/g. This value is insignificant when compared with BET surface area of zinc terephthalate framework, which is 839.6 m^2/g [13]. The decrease in BET surface area is attributed to the encapsulation of ZnO nanoparticles in Zinc terephthalate framework. Similarly pore volume is found to be 0.175 cc/g which is found to be smaller than the reported value of 0.34 cc/g [13].

3.5. Thermogravimetric analysis

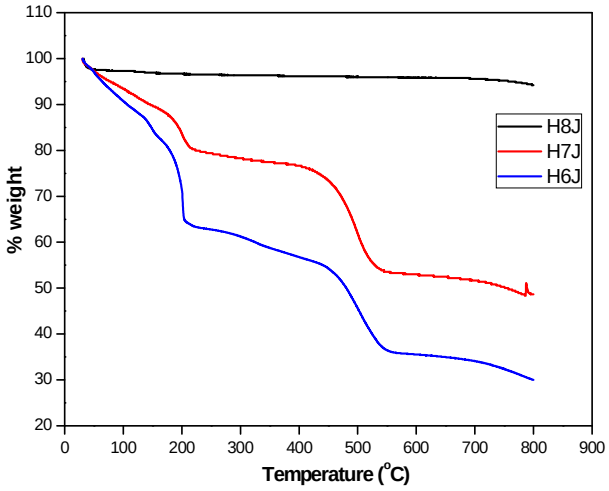


Fig. 7: TG curves of ZnO@Zinc terephthalate framework composites

Thermogravimetric analysis of ZnO@Zinc terephthalate framework composites are shown in Figure 7. Thermograms of both Zinc terephthalate framework and ZnO@Zinc terephthalate framework show two major weight losses. The first weight loss, between 40 to 206°C, is assigned to the removal of solvent molecules occluded within the pores of framework structure. The second weight loss from 440 to 550°C is attributed to the destruction of terephthalate molecules in the framework structure. The percentage of weight loss is found to be more for ZnO@Zinc terephthalate framework. Indicating incorporation of ZnO nanoparticles, which replaces the solvent molecules occluded in the framework. In the case of Zinc terephthalate framework, the first and second weight loss is 17% and 22% respectively, but it is 7.7% and 24% respectively for ZnO@Zinc terephthalate framework. The same kind of two stage decomposition thermogram for zinc terephthalate framework was reported by Loiseau et al [14]. Thermogram of calcined ZnO@Zinc terephthalate framework shows no significant weight loss in the whole temperature range of 40 to 800°C. This high thermal stability is attributed to the formation of ZnO nanoparticles by the calcination of ZnO@Zinc terephthalate framework.

4. CONCLUSIONS

FT-IR spectrum reveals the presence of characteristic functional group within the MOF structure and ZnO@MOF composites of terephthalate systems. The formation of ZnO nanoparticles is also evidenced from FTIR. The crystal structures of all these materials have been identified from XRD patterns. XRD pattern and FTIR spectrum of ZnO and calcined ZnO@Zinc terephthalate composites are found to be same indicating the degradation of framework to ZnO nanoparticles with average particle size of 25 nm. SEM images of ZnO@Zinc terephthalate show the flaky crystalline morphology which is different from the normal cubic structure of Zinc terephthalate framework. The destruction of cubic topology of framework structure is attributed to the incorporation of ZnO nanoparticles. SEM image of calcined ZnO@ Zinc terephthalate framework structure shows a granular morphology of size between 25-45 nm which clearly confirms the formation of zinc oxide. N_2 adsorption and desorption isotherm is a type-IV isotherm indicates mesoporous framework structure. Surface area and pore volume calculated from BET equation is less than that of reported value that confirms the encapsulation of ZnO in Zinc terephthalate framework. Thermal analysis shows a two stage decomposition thermogram for both Zinc terephthalate framework and ZnO@ Zinc terephthalate framework systems. But, the percentage of weight loss is found to be more for Zinc terephthalate framework structures compared to ZnO@ Zinc terephthalate framework composite systems. This indicates the encapsulation of ZnO in Zinc terephthalate framework

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Synthesis, spectral, thermal and antibacterial studies of oxovanadium(IV) complex of an azo dye derived from 4–aminoantipyrine

Athira L S[1], Balachandran S[1,*], Harikumaran Nair M L[2]

Author Affiliations
[1]Department of Chemistry, Mahatma Gandhi College, University of Kerala, Thiruvananthapuram-695004, Kerala, India.
[2]Department of Chemistry, University College, University of Kerala, Thiruvananthapuram-695034, Kerala, India.

Corresponding Author
*__Balachandran S,__ Department of Chemistry, Mahatma Gandhi College, University of Kerala, Thiruvananthapuram-695004, Kerala, India.

E-mail:sbcnair@gmail.com

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Abstract

Synthesis of oxovanadium (IV) complex with anazodye 4–[(*E*)–(4–hydroxy–2,5–dimethylphenyl) diazenyl]–1,5–dimethyl–2–phenyl–1,2–dihydro–3*H*–pyrazol–3–one (HL) derived from 4–aminoantipyrine and 2,5-dimethylphenol are reported. The complex has been characterized by elemental analysis, molar conductance, magnetic susceptibility data, FT–IR, UV–Vis, NMR and ESR spectra.The physico–chemical studies and spectral data indicate that HL acts as a bidentate chelating ligand. The thermal behaviour of the complex was investigated by thermogravimetric techniques. The complex is found to be neutral and a square pyramidal geometry has been tentatively proposed. The ligand HL and complex were screened for antibacterial activity.

Keywords: Azo dye, Oxovanadium(IV) complex, Thermal studies, Antibacterial

1. INTRODUCTION

In the last few years, there was growing interest in the synthesis and physico-chemical studies of first row transition metal complexes with a number of azo dye ligands [1]. Azo dyes are used for dyeing of polyester fabrics,cotton and wool [2], certain metal complexes of azo dyes were also found to be effective for non–linear optical materials [3,4], IR cut–off filters, water ink-jet links, recordable digital video disks, etc [5]. Metal complexes of azo dye ligand have played a major role in the development of coordination chemistry.The interest of chemists in the study of oxovanadium(IV) complexes is due to their powerful influence in inhibiting the function of a large range of enzymes and promoting the function in effect of diabetic animals and these types of complexes are reported to have antiviral, antitumor and antibacterial activities [6,7].They are known to be involved in a number of biological reactions such as inhibition of DNA, RNA and protein synthesis [8].In the present study

the synthesis and spectral characterization of oxovanadium(IV) complex of an azo dye, HL was reported along with its thermogravimetric analysis and antibacterial activity.

2. EXPERIMENTAL

The chemicals used for the synthesis of the compounds were obtained from Merck Chemical Company, Mumbai, India and used without any further purification.The solvents used were of AR grade.

2.1. Synthesis of ligand (HL)

Synthesis of ligand, HL ($C_{19}H_{21}N_4O_2$) was carried out by diazotization of 4-aminoantipyrine followed by coupling reaction with 2,5–dimethylphenol [9]. The product (HL) obtained was filtered under suction, washed with cold water, dried and recrystallized from methanol.The purity was ascertained by TLC. The structure of HL is shown in Figure 1.

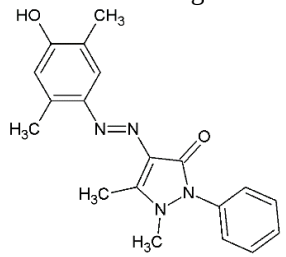

Fig. 1: Structure of dimethylphenol azoantipyrine, HL

2.2. Synthesis of oxovanadium(IV) complex

The oxovanadium complex was prepared by the method reported earlier [10]. A hot ethanolic solution (20 mL) of vanadyl sulphate hydrate (2 mmol) was added to a hot ethanolic solution (20 mL) of the ligand (2 mmol) and refluxed on a water bath for about 3–5 hours.The resulting solution was concentrated to half of its volume and allowed to cool. The solid complex formed was filtered and washed with ethanol and finally dried over calcium chloride in a desiccator.

2.3. Characterization

The melting points of the compounds were studied by using Analab melting point apparatus. FT-IR spectra of ligand and complex were recorded using KBr pellets on a Perkin Elmer Spectrum 65 FT-IR spectrophotometer in the region 400–4000 cm^{-1}. The UV-Vis absorption spectrum was measured in the range 200–800 nm using quartz cuvette on a Perkin Elmer Lambda 25 UV-Vis Spectrophotometer in methanol solution and its ^1H NMR spectrum was recorded on a (DMSO-d6, ppm) 500 MHz Bruker/ TOPSIN NMR instrument using TMS as a reference. Elemental analysis(C, H, N) were carried out by CHNS Rapid analysis instrument.The electron spin resonance (ESR) measurements of the complex at liquid nitrogen temperature were recorded on Electron Spin Resonance Spectrometer-JEOL equipment.Vanadium and sulphate were estimated in the complex by standard methods [11,12].The molar conductance of the complex in methanol solution (10^{-3} M) at room temperature was measured using a Systronics direct reading conductivity meter. Magnetic susceptibility of the complex was measured at room temperature (28±2 °C) on a Sherwood Scientific Magnetic Susceptibility Balance.The thermo gravimetric measurements were made on Shimadzu DTG–60 differential thermogravimetry equipment at a heating rate of 10 °C/ min in nitrogen atmosphere with the temperature range 0–1000 °C.The antibacterial studies of the ligand and complex were conducted at Biogenix Research Centre, Valiyavila, Thiruvananthapuram.

3. RESULTS AND DISCUSSIONS

The ligand, HL and complex are non-hygroscopic solids. The ligand melt in the range 239–240 °C and the complex remain intact up to 300 °C. The complex is soluble in organic solvents like chloroform, DMF and DMSO. The molar conductance of 10^{-3} M solution of the complex exhibited

non-electrolyte behaviour in methanol. The results of elemental analysis and magnetic susceptibility values of the complex are listed in Table 1.

Table 1: Analytical data of ligand and complex

Ligand/ complex	Color	% of C		% of H		% of N		% of S		% of V		μ_{eff} in BM
		Obs.	Cal.	Obs.	Cal.	Obs.	Cal.	Obs.	Cal.	Obs.	Cal.	
HL	Red	66.87	67.85	4.55	5.93	15.96	16.21	–	–	–	–	
[VO(HL)(SO_4)]	Dark brown	45.01	45.69	4.43	4.01	11.49	11.22	6.62	6.41	10.86	10.2	1.74

3.1. Spectral measurements

The characteristic IR spectral assignments of ligand and complex are presented in Table 2. A new band is formed at ~ 3434 cm^{-1} (instead of 3118 cm^{-1}) [13] which implies that the H-bond of ligand was broken and the OH group in the ligand does not participate in coordination with the VO(IV) ion. The stretching vibration of C=O in the pyrazolone ring [14],of the ligand is shifted to ~1596 cm^{-1} in the complex showing the participation of the C=O group in coordination. The stretching vibration N=N group exhibits a negative shift to 1444 cm^{-1} [15] in the complex which indicates the participation of N=N group of the ligand in the complex formation. A new band is formed at 974 cm^{-1} in the spectrum of the complex, which indicate a strong V=O stretching band [16,17]. Thus IR data suggest neutral bidentate ligand behaviour in the complex. Also the IR spectra of the complex shows a weak intense band which may be due to νV–N (490 cm^{-1}) [18] and νV–O respectively.The presence of an ionic sulphate group in the complex is confirmed by the appearance of three bands at 1130–1135, 955–960 and 602–608 cm^{-1} [17] respectively.

Table 2: Spectral measurements of VO(IV) complex of HL

Ligand/ complex	IR spectra ν(cm^{-1})	NMR (ppm)	UV-Vis spectra (in ethanol) ν(cm^{-1})	ESR
HL	3118 (OH), 1635(C=O), 1494 (N=N)	δ 9.85 (s, proton of OH group), δ2.65 (s, proton of >C–CH_3), δ3.37 (s, proton of N–CH_3), δ 7.25-7.42 (m, protons of phenyl rings)	39062 ($\pi\rightarrow\pi^*$) 26881 ($n\rightarrow\pi^*$)	-
[VO(HL)(SO_4)]	3434 (OH), 1596 (C=O), 1444 (N=N), 974 (V=O), 490 (V–N), 1130-1135(SO_4) 955-960 (SO_4)	δ 10.2 (s, proton of OH group)	38910 ($\pi\rightarrow\pi^*$) 31545 ($n\rightarrow\pi^*$) 14814 (d- d)	A_{ll} (200), A⊥lar (80), A_{av} (120), g_{ll}(2.543), $g_{\perp lar}$(2.19), g_{av}(2.3076)

The assignments of ^{1}H NMR spectrum of HL is based on earlier reports [19, 20] and listed in Table 2. On analyzing the spectrum of [VO(HL)SO_4], the presence of singlet due to phenolic proton indicates the non-participation of the –OH group during coordination, which is also confirmed by the IR spectra.

The electronic spectral bands of VO (IV) complexes of HL recorded in methanol are given in Table 2. The $\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$ transitions observed in the electronic spectra of the ligand appear in the spectra of the complex. One more band is observed in complex in the region 14814 cm^{-1} which may be due to d-d transition [21, 22].

3.2. ESR spectrum

The X-band ESR spectrum (Figure 2) of an oxovanadium(IV) complex was recorded in DMSO at room temperature and at nitrogen temperature (77K) and analyzed by the method of Mishra et al [23,24].The room temperature ESR spectra show eight lines,which are due to hyperfine splitting arising from the interaction of the unpaired electron with a ^{51}V nucleus having the nuclear spin which confirms the presence of a single oxovanadium(IV) cation as the metallic centre in the complex.The anisotropy is not observed due to rapid tumbling of molecules in solution at room temperature and only g-average values are obtained. However the anisotropy is clearly visible at liquid nitrogen temperature and eight bands are observed separately which are in good agreement for a square pyramidal vanadyl complexes [25–27].The values are measured from the spectra are A_{ll}, $A\perp_{lar}$,A_{av},g_{ll}, $g\perp_{lar}$, g_{av}(Table 2) which are in good agreement for a square-pyramidal structure.

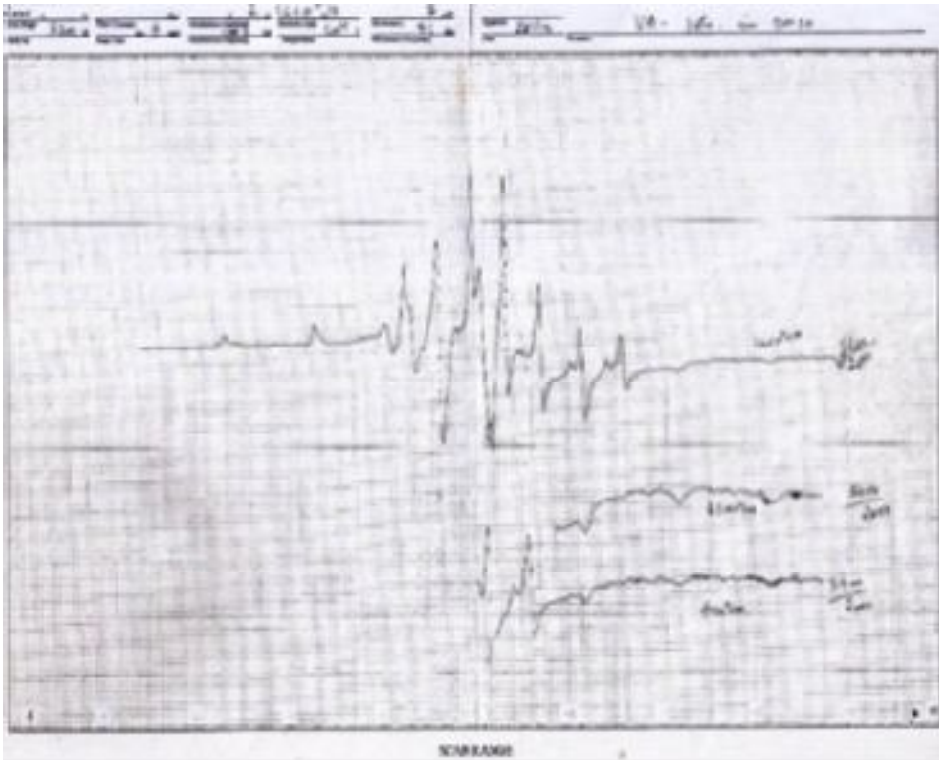

Fig. 2: ESR spectrum of $[VO(HL)SO_4]$

3.3. Magnetic susceptibility

The oxovanadium(IV) complex was found to be air stable and exhibit a magnetic moment at room temperature in the solid state. Oxovanadium(IV) has an electronic configuration of [Ar] $3d^1$ and hence has a single unpaired electron.The magnetic moment values of the vanadyl complexes ranges from 1.71–1.76 BM which corresponds to a single electron of the $3d^1$ system of square pyramidal [28] oxovanadium(IV) molecule.The magnetic moment value of synthesized $[VO(HL)SO_4]$ is 1.74 BM and is shown in Table 1.

3.4. Thermo gravimetric analysis

Thermal studies were carried at the heating rate 10 °C/ min in nitrogen gas is given in Figure 3. The complex shows a plateau up to 210°C which indicates that the complex is stable up to 210 °C and no coordinated water molecule is present in the complex. The TG curve shows two stage

decomposition, the first stage corresponds to a mass loss of 16.61% (210–290 °C) which can be assigned to the anionic part,the second stage corresponds to a mass loss of 80.76% (410–550 °C),to the complete decomposition of the complex to give VO_2. The16.61 and 80.76% percentage mass loss during the first and second stage decomposition is in good agreement with the theoretical value.

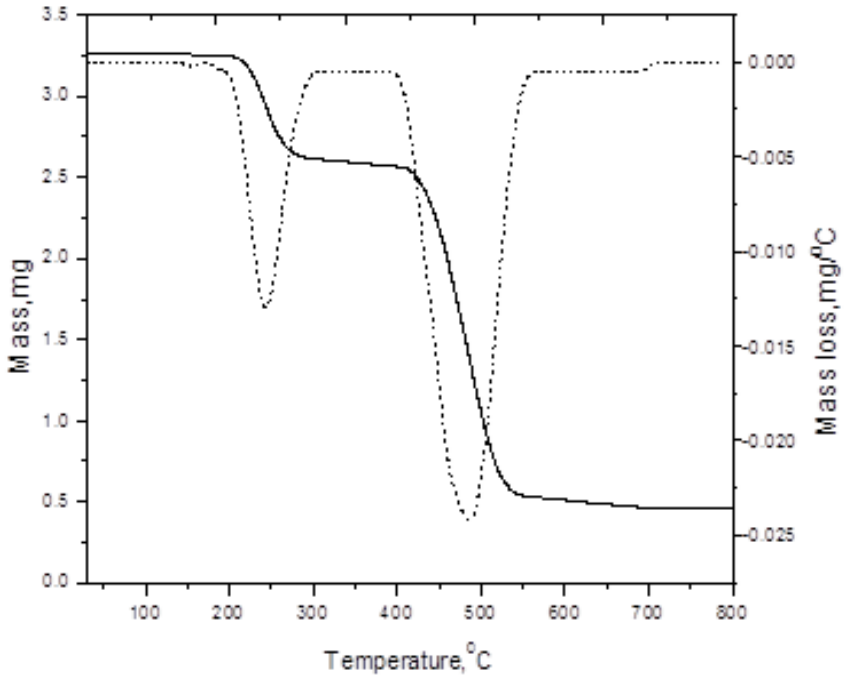

Fig. 3: TG and DTG curves of $[VO(HL)SO_4]$

The computational details and the kinetic parameters of the two stages of thermal decomposition of the complex $[VO(HL)SO_4]$ are given in the Table 3. The Coats-Redfern plots of the two stages of decomposition are given in Figures 4(a) and (b).

The energy of activation for the first stage decomposition is found to be 216.27 kJ/mol and that of second stage is 236.63 kJ/mol. The values of the correlation coefficient (r) are closer to unity indicating that the calculated kinetic parameters (Table 4) fit perfectly with the experiment.

Table 3: Computational details of the I and II stages of decomposition of $[VO(HL)SO_4]$

Temp (°C)		mg		TK		RT		W		A		gα		CR	
I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
210	410	3.24	2.56	483	683	2.07039	1.46413	0	0	0	0	0	0	-	-
220	420	3.20	2.53	493	693	2.02840	1.44300	0.04	0.03	0.0645	0.0148	0.0689	0.0149	-15.0751	-17.283
230	430	3.12	2.45	503	703	1.98807	1.42248	0.12	0.11	0.1935	0.0543	0.2400	0.0566	-13.8683	-15.981
240	440	2.99	2.34	513	713	1.94932	1.40252	0.25	0.22	0.4032	0.1086	0.6756	0.1183	-2.87259	-15.272
250	450	2.85	2.19	523	723	1.91205	1.38313	0.39	0.37	0.6290	0.1827	1.6956	0.2123	-11.9911	-14.716
260	460	2.74	2.00	533	733	1.87617	1.36426	0.5	0.56	0.8064	0.2765	4.1666	0.3513	-1.12993	-14.240
270	470	2.67	1.76	543	743	1.84162	1.3459	0.57	0.8	0.9193	0.3950	11.400	0.5714	-0.16061	-13.781
280	480	2.64	1.54	553	753	1.80832	1.32802	0.6	1.02	0.9677	0.5037	30.000	0.8389	-9.22952	-13.423
290	490	2.62	1.28	563	763	1.77620	1.31062	1.0	1.28	-	0.6321	-	1.2973	-	-13.014
-	500	-	1.05		773	-	1.29366	-	1.51	-	0.7456	-	1.9658	-	-12.624
-	510	-	0.84		783	-	1.27714	-	1.72	-	0.8494	-	3.1533	-	-12.177
-	520	-	0.69		793	-	1.26103	-	1.87	-	0.9234	-	5.2289	-	-11.697
-	530	-	0.6		803	-	1.24533	-	1.96	-	0.9679	-	9.1631	-	-11.161

$CR=\ln\frac{g(\alpha)}{T^2}$, n=2, r=0.99945

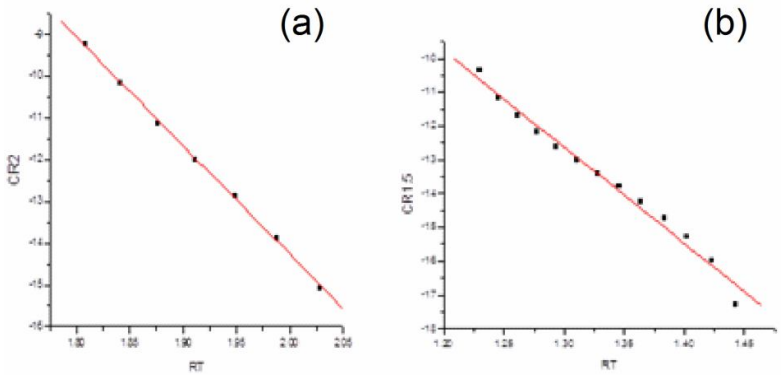

Fig. 4: (a) Coats–Redfern plot of stage I decomposition (b) Stage II decomposition

Table 4. Kinetic parameters for the thermal decomposition of $[VO(HL)SO_4]$

Stage	Peak (°C)	Activation Energy (E) (kJ/mol)	Pre-exponential Term (A) (S^{-1})	Entropy of activation(ΔS) ($JKmol^{-1}$)
I	243	216.27	1.0898×10^{14}	–214.842
II	486	236.63	1.8274×10^{14}	–243.967

On the basis of all the above spectral data and physico-chemical studies, a square pyramidal geometry (Figure 5) has been tentatively proposed for the complex.

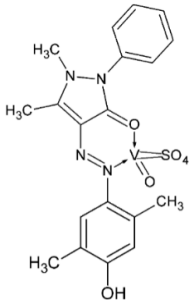

Fig. 5: Suggested structure of [VO(HL) SO4]

3.5. Antibacterial activity

The ligand HL and the complex $[VO(HL)(SO_4)]$ have been screened for their possible antibacterial activity by Agar–Well Diffusion method against Staphylococcus aureus and E coli bacteria at different concentrations (25, 50, and100 µl).The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well. Gentamycin was used as a positive control. From the result, it is clear that both the ligand and the complex show the antibacterial activity (Staphylococcus aureus and E–coli) at different concentrations.

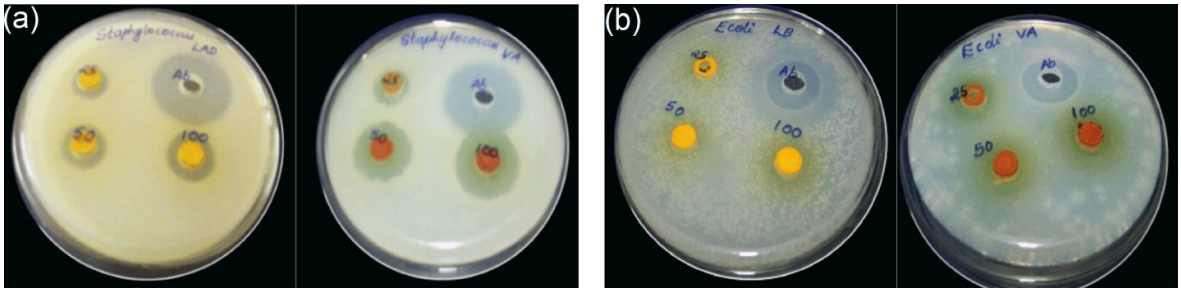

Fig. 6: Antibacterial activity of the ligand HL and its complex $[VO(HL)SO_4]$ towards (a) Staphylococcus (b) E-coli

4. CONCLUSIONS

The dimethylphenol azo antipyrine (HL) was synthesized by diazotization and coupling reaction. The synthesized oxovanadium complex was characterized by elemental analysis, molar conductance, magnetic susceptibility data, FT-IR, UV–Vis, NMR and ESR spectral studies. The molar conductance of 10^{-3} M solution of the complex exhibited non–electrolyte behaviour in methanol. The physico-chemical studies and spectral data indicate that the complex has square-pyramidal structure and the ligand HL coordinate to metal ion through the carbonyl oxygen and nitrogen of azo moiety. The thermal behaviour of the complex was investigated by thermogravimetric techniques and verifies the Coats-Redfern equation.The ligand HL and complex were screened for antibacterial activity towards staphylococcus aureus and E-coli.

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A comparative study on the antioxidant properties of curcuminoids and its rubrocurcumin analogues

Jeena John[1], Sudha Devi R[1] and Balachandran S[1]*

Author Affiliations
[1]Department of Chemistry, Mahatma Gandhi College, University of Kerala,Thiruvananthapuram.

Corresponding Author
***Balachandran S**, Department of Chemistry, Mahatma Gandhi College, University of Kerala, Thiruvananthapuram.

E-mail: sbcnair@gmail.com

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Abstract

The yellow colored pigment, curcumin present in turmeric is responsible for its various biological activities like antioxidant, anti-inflammatory and anticarcinogenic activity. Different functional group present in curcumin includes a β–diketone group, carbon–carbon double bond and two phenyl rings with hydroxy and methoxy substituents. A controversy exists in literature on the site and mechanism for the antioxidant activity (AOA), the keto–enol/phenolic moiety present in curcumin. In the present work AOA of curcuminoids and its boron complex with oxalic acid – rubrocurcumin analogues were conducted which differ in the substituent in the benzene ring. Since in rubrocurcumin analogue β-diketone group is blocked through bonding with boron its influence will be absent in its antioxidant activity.

Keywords: curcumin, antioxidant, rubrocurcumin, β–diketone

1. INTRODUCTION

Curcumin isolating from the rhizomes of the plant turmeric shows remarkable pharmacological activities and is widely used as a spice and food coloring agent [1–2]. The antioxidant activity of curcumin is mainly responsible for its biological activities [3–6]. However its instability in water mediated system and its low bioavailability reduces its application as an oral drug. Curcumin derivatives and analogues are better options for drug applications and many reviews are observed in literature. A closely related molecule of curcumin having enhanced AOA is the best choice. Since curcumin has different functional groups structure activity relationship study will help to find the actual antioxidant site for curcumin and the ways to enhance its activity [7–9]. Commercial curcumin contains three different curcuminoids; curcumin (C1), demethoxycurcumin (C2) and bisdemethoxycurcumin (C3) and their antioxidant nature decreases in the order C1>C2>C3 [10–11].

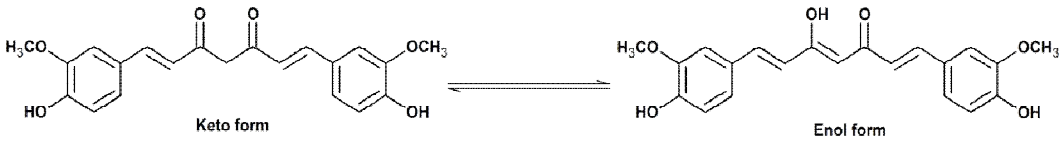

Fig. 1: Keto-enol tautomeric forms of curcumin

Curcumin exist in equilibrium with the keto enol tautomeric forms (Figure 1) and in solution it exists mainly in enol form. NMR spectroscopic studies in solvents of various polarities have shown that curcumin exists predominantly as enol tautomer [12–13]. The physical, chemical and biological activities of curcumin may be due to its unique chemical structure and the existence of keto-enol tautomeric forms. Curcumin in the enol form has better activity because of its ability to accept and donate hydrogen bonds and its ability to chelate positively charged metal ions [14].

The enol form of curcumin is excellent metal chelators and will form complexes with all metal ions and helps to prevent Alzheimer's disease caused by metal deposition in brain tissues [15–18]. The metal complexation deny the possibility of enolic group to act as the antioxidant centre and the present group demonstrated a slight decrease in the antioxidant property of transition metal complexes [19]. In the present work the antioxidant property rubrocurcumin analogues were revealed in which curcumin complexes with boric acid along with oxalic acid. Rubrocurcumin classically used for the estimation of boron in different matrices even at low concentration i.e. less than 1 ppm [20]. The less hydrolytic stability of these boron complexes is its main drawback in using them as spectrophotometric reagent for boron determination [20–21]. However the low hydrolytic stability of these complexes makes them a suitable candidate for the carrier system for the delivery of curcumin in human body.

All curcuminoids; curcumin (C1), demethoxycurcumin (C2) and bisdemethoxycurcumin (C3) form the corresponding rubrocurcumin analogues RC1, RC2 and RC3 when reacted with boric acid and oxalic acid (Figure 2) [22]. Another rubrocurcumin analogue (RC4) was prepared using a curcumin analogue which doesn't have any substituent in benzene ring (C4). AOA studies of these compounds will give clear indication regarding the structure activity relationship in the antioxidant mechanism of curcumin and the influence of methoxy group in its AOA.

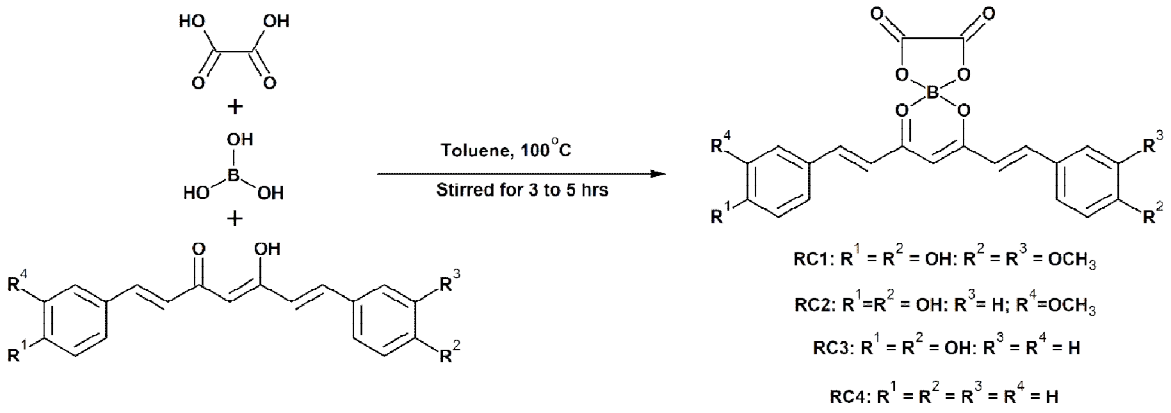

Fig. 2: Synthesis of rubrocurcumin analogues.

2. MATERIALS AND METHODS

2.1. Antioxidant activity assay

The AOA of curcuminoids and rubrocurcumin analogues were assayed as reported in literature [25]. To 0.5 mL of diluted ABTS solution having absorbance 0.8 to 0.9, 2.5 mL of sample in methanol at different concentrations ($1–9\times10^{-5}$ µg/mL) were added and the percentage of radical

scavenged was determined for each concentrations of sample relative to a blank containing no sample. The percentage inhibition was calculated using the equation

$$\% \text{ inhibition } = \left(1 - \frac{A_S}{A_C}\right) \times 100$$

Where A_s is the absorbance of the remaining ABTS radical in presence of sample and A_c is the absorbance of blank without sample. All the analyses were done in duplicate and the averaged results were used for analysis of the data. From the % inhibition vs. concentration graph, 50% fall in ABTS solution was determined as IC_{50} value in µM which determines the AOA of a compound.

3. RESULTS AND DISCUSSIONS

DPPH method was commonly employed for the study of free radical scavenging activity however not suitable for rubrocurcumin analogues since they absorb in the same region of DPPH radical. The AOA of four different curcuminoids along with all the synthesized rubrocurcumin analogues were assessed using ABTS method. The AOA values depends on the method used, the time and reaction conditions such as solvent polarity, pH, temperature and concentration of the reactive species [19, 26–29]. The radical scavenging activity of curcuminoids and its rubrocurcumin analogues are shown in Table 1 as IC_{50} values. A larger IC_{50} value indicates low AOA. The AOA of curcuminoids decreases in the order C1 > C2 > C3 and are consistent with reported antioxidant activity of curcuminoids in DPPH method [10]. C4 shows no AOA up to the concentration of 30×10^{-5} µg/mL supporting the antioxidant property of curcuminoids is due to the presence of hydroxyl group present in the phenyl rings [7–8]. The *o*-methoxy group can form an intramolecularhydrogen bond with the phenolic hydrogen making the H-atom abstraction from the *o*-methoxy phenols surprisingly easy which resulted in the enhance antioxidant activity of C2 and C1 over C3 [30]. However Somparn *et al.*, suggested that the antioxidantactivities of curcumin and its derivatives can arise both from the *o*-methoxyphenol and from central methylenic hydrogen in the central seven carbon chain and β-diketone moiety [10]. However the absence of antioxidant activity of C4 and the antioxidant activity shown by the transition metal complexes [19] of curcumin show that the enol moiety have no role in initiation [28] or the antioxidant activity of curcumin and its analogues.

Table 1: IC_{50} values in µM of curcumin and its rubrocurcumin analogues

Curcuminoids	IC_{50}(µM)	Rubrocurcumin analogues	IC_{50}(µM)
C1	11.65	RC1	11.82
C2	15.74	RC2	13.73
C3	21.50	RC3	18.02
C4	No activity	RC4	No activity

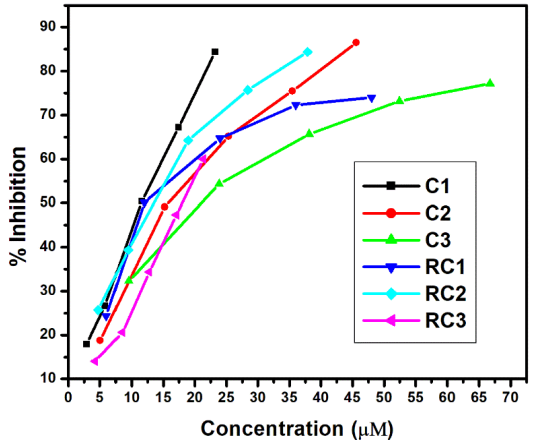

Fig. 3: ABTS scavenging activity of curcuminoids and its rubrocurcumin analogues.

The AOA order of rubrocurcumin analogues is RC1 > RC2 > RC3 indicating the influence of lactone ring of rubrocurcumin in antioxidant behaviour. Similar to C4, RC4 doesn't show any AOA up to the concentration of 30×10^{-5} µg/mL. The percentage inhibition vs. concentration graph used to calculate the IC_{50} values for the curcuminoids and its rubrocurcumin analogues are shown in Figure 3. The IC_{50} value of C1 and RC1 are comparable and is within the error percentage. However a comparatively large variation is observed for C2 and C3 with its rubrocurcumin analogues.

Antioxidant reaction mechanism for rubrocurcumin

Litwinienko *et al.,* suggested that the antioxidant activity of curcumin take place by SPLET mechanism, where the first stage is the ionization of Ar–OH into $Ar–O^-$ anion by the solvent. In the second stage electron transfer occurs from this anion to free radical [19], which is highly depended on the hydrogen bonding interaction of solvent with the phenolic moiety [28].The formation of phenoxide ion determines the AOA activity and is largely depend on the nature of diketone moiety. In polar medium the enol moiety exchange its hydrogen with the solvent and the formed anionic group displaces electrons towards the phenolic groups through the extended conjugated structure of curcumin making the conversion of phenolic OH to $Ar–O^-$ anion less susceptible. In rubrocurcumin analogues, the diketone moiety complexes with boron atom to form [BO_4^-] moiety which is less negative and will promote the formation of $Ar–O^-$ anion in faster rate than curcumin. The influence of charge delocalization is prominent in free Ar–OH rather than intramolecularly hydrogen bonded Ar–OH as in C1 and C2. In RC1 methoxy group is present in ortho position forms intramolecular hydrogen bond which retard the influence electron flow of from [BO_4^-] moiety thus its activity is similar to that of C1.

The results of AOA of rubrocurcumin analogueis a clear indication that the OH group is not independently acting as AOA group as claimed by Barclay [7]. The electron flow and the molecular dissociation via free radical rupture process may be totally different and require detailed investigation by studying in different solvent systems and rubrocurcumin analogues with changes in lactone rings.

4. CONCLUSIONS

Rubrocurcumin is an admirable molecule which can be used as the biological carriers for curcumin in human body. Not many studies were reported so far on its biological activities. In this work an attempt were made to study its AOA using ABTS method. A comparative study was also conducted between curcuminoids and their corresponding rubrocurcumin analogues to check the influence of boron complexation in its AOA. From the result it is clear that the rubrocurcumin analogues are good antioxidant agents similar to curcumin and RC2 and RC3 are more active than its corresponding curcuminoids. The increase in AOA of these compounds may due to the electronic interaction of [BO_4^-] moiety towards the antioxidant site.

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An integrated approach on evaluation of hydrochemical parameters of riverine systems in Trivandrum urban area along with phosphate removal studies

Karthika V. S[1,2], Harsha Mahadevan[1,3], V. Viswadas[1,3], Vinu V. Dev [1,3], K. Anoop Krishnan[1,*]

Author Affiliations
[1]Hydrological Processes (HyP) Group, National Centre for Earth Science Studies (NCESS), Akkulam, Trivandrum-695011, India
[2]Department of Chemistry, Government Arts College, Thycaud, Trivandrum-695001, India
[3]Research Scholar, University of Kerala, Trivandrum, India

Corresponding Author
***K. Anoop Krishnan.** Hydrological Processes (HyP) Group, National Centre for Earth Science Studies (NCESS), Akkulam, Trivandrum-695011, India

E-mail: sreeanoop@rediffmail.com

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Abstract

The present work focuses on the quality aspects of riverinesystems in Trivandrum urban area during premonsoon season of 2017along with phosphate removal studies. Altogether, 11 water samples collected from Karamanariver, Killiyar and Parvathy Puthanar canal, andsubsequently analysed for various physic-chemical parameters. *In situ* measurements made for finding out the parameters such as pH, electrical conductivity, total dissolved solids and dissolved oxygen, which was in the range of 6.6–6.9, 265.6–6317.0 µS/cm, 4.7–65.5 mg/L and BDL–4.9 mg/L, respectively. The biochemical oxygen demand and alkalinity were found to be in the range of BDL–3.8 mg/L and 19.5–53.7 mg/L, respectively. Other parameters such as chloride (1.4–28.1 mg/L), sulphate (177.6–585.0 mg/L), nitrite (BDL–1.95 mg/L), silicate (7.1–27.3 mg/L) and phosphate (BDL–5.5 mg/L) were also determined. The results showed that most of the hydrochemical parameters were found to be within the standard limit prescribed by BIS(2012) except hardness, conductivity, TDS and Phosphate. In general, the study was helpful in evaluating the level of pollution in Trivandrum urban area during pre monsoon season. In the application side, the phosphate removal studies from Parvathy Puthanar were carried out using a novel zirconium impregnated bentonite clay.

Keywords: Physic-chemical parameters, Trivandrum urban area, bentonite clay, phosphate adsorption

1. INTRODUCTION

Freshwater resources are deteriorating day-by-day and now become a global problem. Therefore, the continuous monitoring of these precious resources are highly warranted to know the extent of pollution levels. The physic-chemical and biological characteristics of water bodies showed the health of a particular environmental system. Any of these parameters beyond the limit of maximum permissible level may cause serious threat to flora and fauna. Most of the rural communities depends upon rivers, streams, water reservoirs, ponds, lakes, etc for their domestic as well as agricultural needs, whereas urban people depends on these water sources for domestic and industrial purposes. The domestic, agricultural and industrial wastes have been discharged back to these water sources, from which these water resources get polluted and ultimately lead to different types of diseases and toxic effects. The human activities includeurbanization and conversion of agricultural land to impervious building and road increase the rate of urban runoff, the highly contaminated water streams enriched with nutrients deteriorates the water resources instantaneously leading to eutrophication of water bodies. Higher pollutant concentration leads to the degradation of water to a very large extend. Therefore monitoring of physic-chemical and microbiological quality of water required for the safe and sustainable life on earth.

Nutrient pollution monitoring studies give an input about their dynamics in the riverine systems. These studies are highly helpful in developing viable treatment techniques to reduce the concentration of nutrients such as phosphate, below the permissible limits. Among the various techniques, adsorption is found to be an efficient and ecofriendly technique in removing phosphate from aqueous phase. In this context, we designed our work by integrating the nutrient monitoring studies in the riverine systems along with its removal studies for maintaining a sustainable ecosystem in the river basins.

2. MATERIALS AND METHODS

Thiruvananthapuram district is the capital city of Kerala, situated between north latitudes 8º 17' and 8º 54' and east longitudes 76º 41' and 77º 17'., Karamana river, Killiyar river and Parvathy Puthanar are the three major rivers flowing through the urban part of the district, which is being mainly polluted by municipal wastes and sewerages. To monitor the nutrient flux, the fieldwork was conducted in these rivers during the pre monsoon season of 2017 and the study area is as shown in Figure 1.

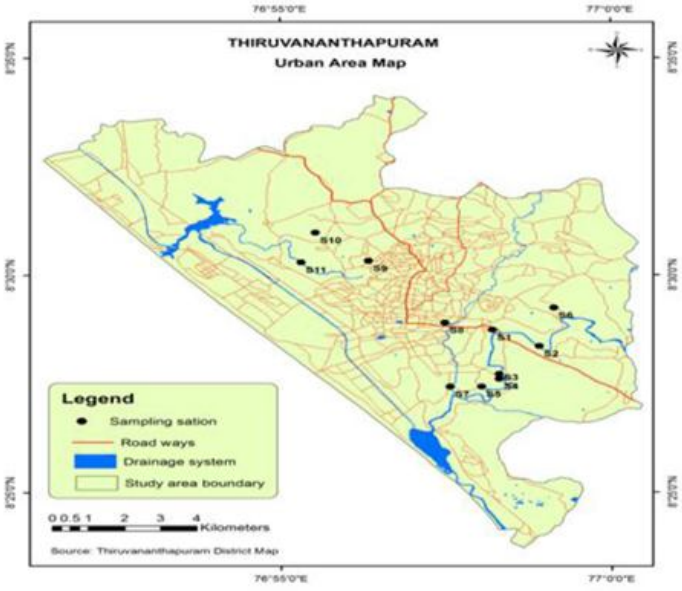

Fig. 1: Study Area

The laboratory analysis of samples was carried out using standard procedures [1]. The titrimetric methods were used for the determination of total alkalinity and acidity. The amount of DO and BOD was determined by EDTA titrimetric method. The colorimetric methods of analysis include the estimations of nitrite, sulphate, silicate and inorganic phosphate. All the chemicals used were of GR grade.The Mohr's argentometric titration method was used for chloride determination. At the same time calcium, magnesium and total hardness was determined by EDTA titrimetric method. The adsorption experiments were carried out using the riverine water collected from different riverine streams of Trivandrum urban area using the zirconium pillared bentonite clay.

3. RESULTS AND DISCUSSIONS

3.1. Hydrochemical parameters

The permissible limit for pH prescribed by WHO is 6.5–8.5. The pH below 4.8 and above 9.2 is deleterious for aquatic organisms especially for fish [2]. The selected samples shows a range of 6.6–6.9, implies slightly acidic nature of water.Conductivity defines the water quality and it indicates the level of dissolved solids in water. Collected water samples show conductivity values varies from 265.6 –6317µS/cm. Conductivity was found to be high at station 4 and low at station 7. The TDS values are ranged from 4.73– 645.50 mg/L. The variation of nitrite in all three riverine systems is presented in Figure 2.

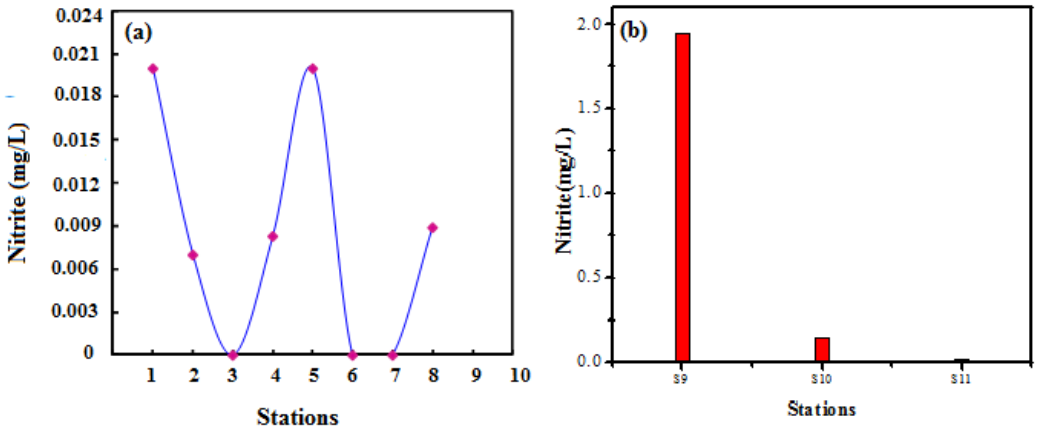

Fig. 2: Variation of Nitrite (mg/L) (a) Karamana (S2-S7), Killi River (S1, S6- S8) Bar diagram showing Variation of Nitrite (mg/L) (b) Parvathy Puthanar

The DO concentration more than 5 mg/ L favours good growth of flora and fauna. In the present study, DO was varied between BDL–4.48 mg/L.In the present study, BOD ranges between BDL–3.84 mg/L. Water sample collected from Karamana river Basin (station 3) having high value of BOD.

Alkalinity is usually imparted by the carbonate or bicarbonate ions of natural water. The collected samples have alkalinity in the range of 19.52– 53.68 mg/L. In natural freshwater, high concentration of chlorides may be treated as an indicator of sewer pollution. In the present study, chloride ranges between 1.4–28.1 mg/L. A maximum value of 28.1 mg/Lwas found at S11, while a minimum value of 1.4 mg/L was found in S1.Domestic sewage and industrial effluents, besides biological oxidation of reduced species may add Sulphate to natural water. In our study area, Sulphate concentration varies from 5.85–177.6 mg/L. Station 5 show higher value of Sulphate concentration.Nitrite concentration in the present study varies from BDL–1.95 mg/L. Water sample from Parvathy Puthanar exhibit maximum nitrate (1.95 mg/L).Phosphate determination may help to judge if the pollution is due to domestic sewage or not [3]. Under normal condition, the concentration of phosphate should not exceed 5mg/L. In the selected samples phosphate present in the range 0.014–5.47 mg/L, which indicates high degree of phosphate pollution.In our study area, silicate values range from 7.07–27.33 mg/L. Overload of silicate may influence the composition of other nutrients especially phosphate and nitrite. Enrichment of silicate reduced the nutrient concentration. This also

implies serious environmental issues and deterioration.Hardness of water is caused by polyvalent ions like Ca^{2+}, Mg^{2+}, iron etc, which is dissolved in water. Water containing calcium and magnesium are said to be hard.In the present study, most of the stations exhibit higher hardness.

3.2 Application of zirconium pillared bentonite clay for cleaning Parvathy Puthanar

Adsorption is not only an economical technique, but also simple in operation as the variables are rather flexible and the separation is almost complete with in a short time.In order to study phosphate adsorption,water samples collected from Parvathy Puthanar (man–made canal) is used. The concentrations of phosphate in Parvathy Puthanar at different stations are shown in Table 1.

Table 1: The phosphate concentration at different stations, Parvathy Puthanar

Stations (Parvathy Puthanar)	Amount of Phosphate (mg/L)
Station 1	5.47
Station 2	3.71
Station 3	2.23

Batch adsorption studies were performed at normal experimental conditions for the removal of phosphate from the samples collected from Parvathy Puthanar using the zirconium pillared bentonite clay. In our experiments, phosphate concentration of 15mg/L showed a maximum adsorption of 91.0 %, when the real samples are spiked with 10 mg/L of phosphate solution. The % of adsorption and spiking details are given in Table 2.

Table 2: The adsorption percentage of phosphate at various spiking events

Station	Phosphate Concentration (mg/L)	Adsorption (%)
Station 1	5.47 mg/L (phosphate present in real system) + 10 mg/L (Spiking)	91%
Station 2	3.71 mg/L (phosphate present in real system) + 12 mg/L (Spiking)	79%
Station 3	2.23 mg/L (phosphate present in real system) + 13 mg/L (Spiking)	87%

4. CONCLUSIONS

A detailed hydrochemical profiling of three major riverine systems in the urban area of Trivandrum district carried out. Among the parameters, conductivity, hardness, total dissolved solids and Phosphatewere found to be above the maximum permissible levels. Considering the nutrient profile, sulphate shows maximum concentration (5.85–177.66 mg/L) compared to other nutrients such as nitrite (BDL–1.95 mg/L), silicates (7.07–27.33 mg/L) and phosphate (BDL–5.47 mg/L). The water samples collected from Parvathy Puthanar exhibit maximum concentration of nitrates and phosphates shows the high degree of pollution among other rivers.The result of the study concluded that the present status of the river water system in Trivandrum Urban area is almost suitable for all aquatic lives, domestic and agricultural uses.Necessary initiatives, therefore, should be taken against river bank erosion, use of excessive fertilizers and pesticides to improve the overall quality of the water for sustainable management. Moreover, further research and periodic monitoring of river water quality is of importance for the improvement or maintenance of the river waters. Also, the newly developed adsorbent was found to be highly efficient for phosphate removal.

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Synthesis and characterization of calcium ferrite nanoparticles by solution combustion method

Vidya VG[1]*, Revathy L[1]

Author Affiliations
[1]Department of Chemistry, University College, Trivandrum-695034

Corresponding Author
***Vidya VG,** Department of Chemistry, University College, Trivandrum-695034

E-mail: vg_vidya@yahoo.co.in

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Abstract

The current paper has focussed on the synthesis and characterization of calcium ferrite nanoparticles by solution combustion method. Calcium ferrite nanoparticles were prepared by a solution based method using calcium chloride ($CaCl_2$), ferrous sulphate ($FeSO_4$), dl–Alanine and sodium hydroxide (NaOH) as a precipitant and the obtained precipitation was calcined under 500 °C for 4 hours. The resulting material was characterized by using X–ray diffractometry, Scanning electron microscopy, FTIR Spectroscopy and UV–Vis spectroscopy. The magnetic characterization was done by using a vibrating sample magnetometer. The electrical conductance, salinity and total dissolved solid measurements of the prepared nanoparticles were conducted. From PXRD results the calculated crystalline size is 12 nm. SEM micrograph reveals the formation of spherical structures. The FTIR spectrum confirms the Ca–O and Fe–O bonds in the sample. The VSM analysis reveals that the particle shows superparamagnetism.

Keywords: Calcium ferrite, Nanoparticles, Combustion, Super paramagnetism

1. INTRODUCTION

Ferrites are iron based oxides with technologically fascinating magnetic properties, making them a prominent category in magnetic materials.The behaviour of ferrite compounds depends on the method of preparation as well as the purity, crystallinity and magnetic properties of the input materials [1]. Ferrites are divided into three families; spinels, hexagonal ferrites and garnets. Spinel type oxides with general formula AB_2O_4 are the most widely used in the family of ferrites [2].The ferrite particles in nano–regime with significant change of physical properties provide more advantages over the bulk ferrites. In the nano–regime ferrites were found to have undergone changes in magnetic properties due to superparamagnetism and surface spin effects until the stable magnetism occurs below the blocking temperature [3,4].

The $CaFe_2O_4$ type structure includes edge and corner sharing BO_6 octahedral, constituting a very distinctive network similar to the one formed in pervoskite related compounds, this structural network suggests that interesting physical properties may exist in the $CaFe_2O_4$ compounds, where B–site atoms are transition metal magnetic elements, such as high–T_C superconductivity in cuprates, quantum magnetic characters in ruthenates, strongly correlated features in magnetates [5]. In comparison with the other ferrites such as $MnFe_2O_4$, $NiFe_2O_4$, $ZnFe_2O_4$, $CoFe_2O_4$ and $CuFe_2O_4$, calcium ferrite has a significant advantages; it is biocompatible and eco–friendly due to the presence of Ca^{2+} instead of heavy metals [6].Calcium ferrites have extensive applications in the optical memory devices [7], magnetoplumbite structure [8,9] and steel making industries [10,11]. Also it has considerable applications in information storage devices, magnetic bulk cores, magnetic fluids, microwave absorbers, catalysts and medical diagnostics [2,3,12].

Combustion synthesis has emerged as an important technique for the synthesis and processing of advanced ceramics, catalysts, composites, alloys, intermetallicand nanomaterials. In this method, the exothermicity of the redox chemical reaction is used to produce useful materials. Combustion synthesis processes are characterized by high temperatures, fast heating rates and short reaction times. These features make combustion synthesis an attractive method for the manufacture of technologically useful materials at lower cost compared to conventional ceramic processes [13,14].

2. MATERIALS AND METHODS

Calcium chloride ($CaCl_2$), Ferrous sulphate ($FeSO_4$), Sodium hydroxide (NaOH), dl–alanine and absolute ethanol (99.9%). Calcium chloride and ferrous sulphate were purchased from SD fine chemicals. All other chemicals were of analytical grade. All the aqueous solutions were prepared using distilled water.

The first solution 0.2 M $CaCl_2$, 0.4 M $FeSO_4$ and 0.2 M dl–alanine was dissolved in 250 mL of distilled water and the second solution was prepared by 3M NaOH pellets in 250 mL distilled water. The second solution was added drop by drop with continues stirring on magnetic stirrer. The obtained particle solution was taken in the condenser and boiled at 100^0C temperature for about 2 hours then the hot solution was filtered using Whatmann filter paper (G–41) and dried at 100 °C in a hot air oven for about 1 hour, then obtained powder was washed with ethanol 3–4 times to remove the impurities present in the $CaFe_2O_4$ nanoparticles.

Solution combustion derived product was characterised by PXRD. Powder X–ray diffraction patterns were collected on X'PERT– PRO– PHILIPS X–ray Diffractometer with CuKα radiation with diffraction angle range $2\theta = 20°– 80°$ operating at 40 kV and 30 mA. TheFT–IR studies have been performed on a Shimadzu DR–43S FTIR Spectrophotometer with KBr pellet technique in the frequency range 4000– 400 cm^{-1}.The UV–Vis spectra of the prepared calcium ferrite nanoparticles were recorded on Perkin Elmer λ25 UV–Vis Spectrophotometer. The Product was morphologically characterized by SEM analysis which was performed on a JEOL Model JSM–6390LV electron scanning microscope.The VSM analysis of the prepared $CaFe_2O_4$ nanoparticles were done on Lakeshore VSM 7410and the conductance were measured on Thermoscientific Orion 5 Star.

3. RESULTS AND DISCUSSIONS

The calcium ferrite nanoparticles are prepared by solution combustion method and its properties were characterized by Powder XRD, FT–IR spectroscopy, UV–Vis spectroscopy, Scanning electron microscopy and VSM analysis.

The crystalline size of the prepared calcium ferrite nanoparticles was obtained from PXRD results. Infrared spectra give the different vibrational modes present in the sample. The optical property can be obtained from UV–Vis absorption spectrum. The surface morphology of the prepared nanoparticles can be studied by SEM micrographs. VSM analysis gives the magnetic properties of the

calcium ferrite nanoparticles. Also the electrical conductance, salinity and TDS measurements of the prepared nanoparticles were conducted.

3.1. PXRD studies for phase formation and crystalline size

The formation of nanocrystalline phase of prepared sample was confirmed by PXRD measurements. The XRD pattern of the obtained calcium ferrite nanoparticle is shown in Figure 1. All peaks can be well indexed to the structure of calcium ferrite (matched with JCPDS PDF No 72– 1199) with highly crystalline nature. All the diffraction peaks can be indexed to (220), (320), (040), (131), (311), (331), (401), (520), (260), (600), (170), (022) and (042) reflections. The crystalline size is calculated from the full width at half maximum (FWHM) of the diffraction peaks using Debye Scherer's formula method [15] using the following equation.

$$d = \frac{k\lambda}{\beta\cos\theta}$$

Where'd' is the average crystalline dimension perpendicular to the reflecting phases; 'λ' is the X– ray wavelength, k is Scherer's constant (0.94). '$\boldsymbol{\beta}$' is the full width at half maximum (FWHM) intensity of a Bragg reflection excluding instrumental broadening and '$\boldsymbol{\theta}$' is the Bragg's angle. Here (040) reflection has maximum intensity. Therefore, full width at half maximum of (040) reflection is considered as $\boldsymbol{\beta}$. The calculated crystalline size of the sample is found to be 12 nm.

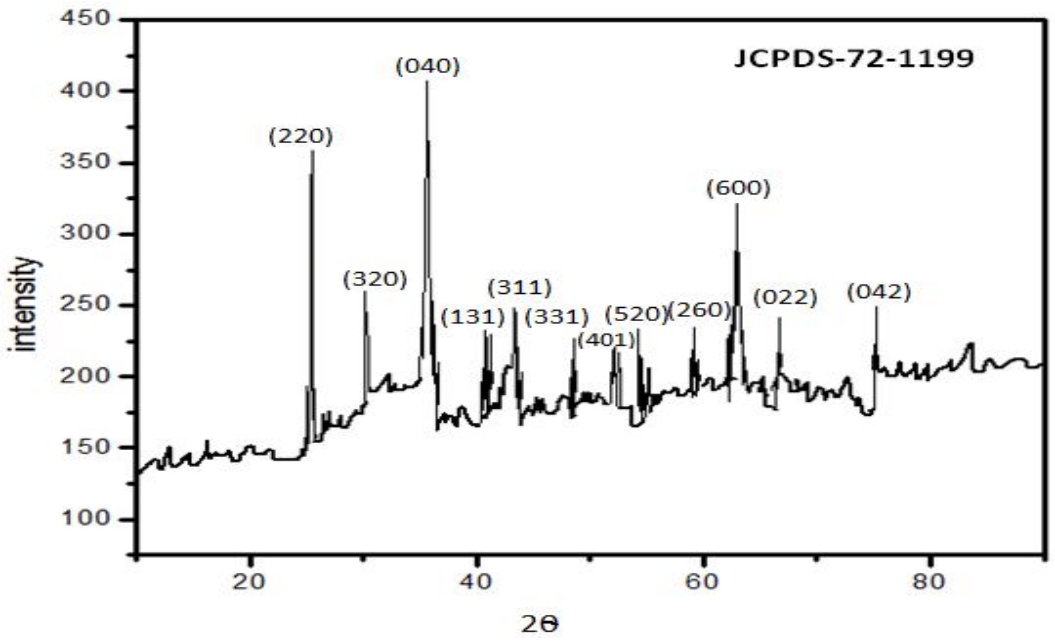

Fig. 1: XRD pattern of the obtained calcium ferrite nanoparticle

3.2. FT–IR spectroscopic studies

FTIR spectroscopy is an imperative vibrational technique to identify and construe the different bond formation in the material. The FTIR spectrum of calcium ferrite nanoparticles recorded in the range of 4000–400 cm^{-1}. The bands at 420 cm^{-1}, 557 cm^{-1} and 590 cm^{-1} correspond to the stretching vibrations of Ca–O & Fe–O bonds. The bands at 1138 cm^{-1}, 1163 cm^{-1} corresponds to the stretching vibrations of C–O [16,17].

3.3. UV–Vis spectroscopy studies

In order to determine the optical property, the UV–Vis spectrum was recorded. The sample shows a strong absorption peaks (λ_{max}) at 262 nm in the UV region and two absorption bands at 364 nm and 463 nm in the visible region. This can be attributed to photo excitation of electron from valence band to conduction band.

3.4. Morphological analysis

Figure 2 shows SEM micrographs of $CaFe_2O_4$ nanoparticles. The micrographs revealed that the particles are spherically agglomerated having uniform size and distribution. Due to this particle size cannot be measured and so it can be found out from Transmission Electron Microscopy TEM analysis.

The agglomeration of $CaFe_2O_4$ was caused by the high surface energy and magnetic dipole interaction between ferrite particles [18]. The particles are having large number of voids due to the release of gaseous products.

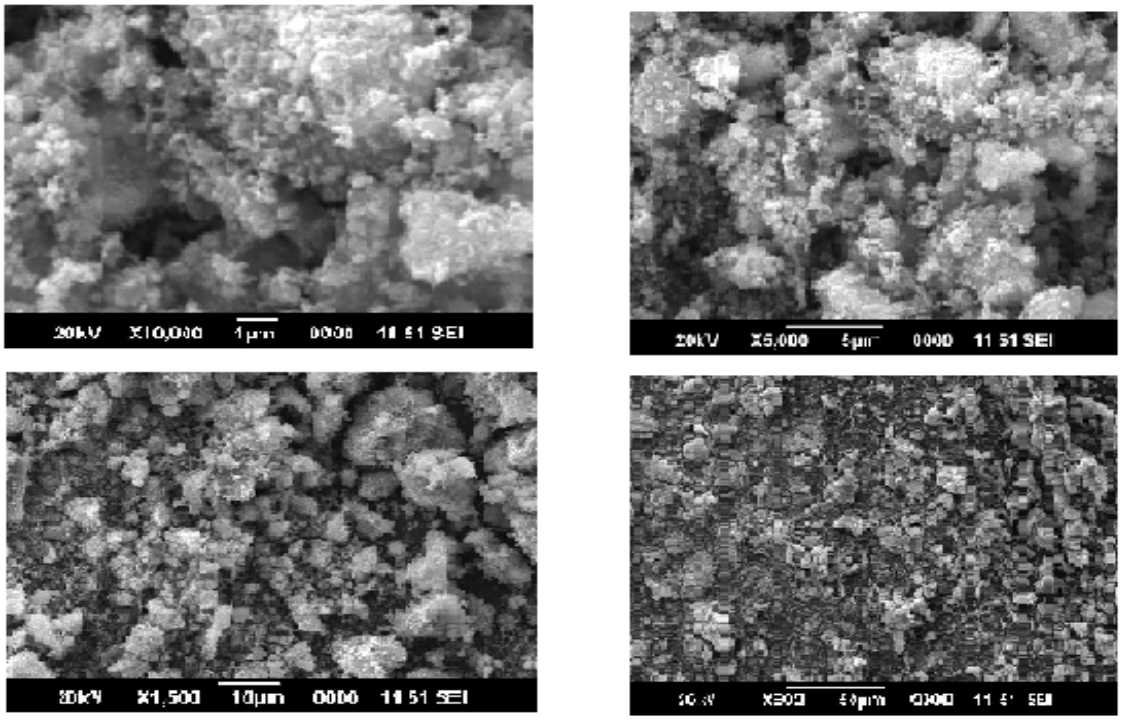

Fig.2: SEM micrographs at different magnifications

3.5 Magnetic characterization based on VSM

The hysteresis curve of the synthesized $CaFe_2O_4$ nanoparticles was measured using a VSM (Vibrating sample magnetometer) at room temperature (Fig.3). It is apparent from the figure that the magnetization of calcium ferrite nanoparticles results in high superparamagnetic behaviour at room temperature. The magnetic saturation (Ms) value of 1.236 emu/g is calculated per gram of the sample at room temperature. The coercivity (Hci) value is 8.73 G. Superparmagnetism is a form of magnetism, which appears in small ferromagnetic or ferromagnetic nanoparticles [19]. Superparamagnetism occurs in nanoparticles which are single domain, ie; composed of a single magnetic domain. This is possible when their diameter is below 3–50 nm, depending on the materials [20].

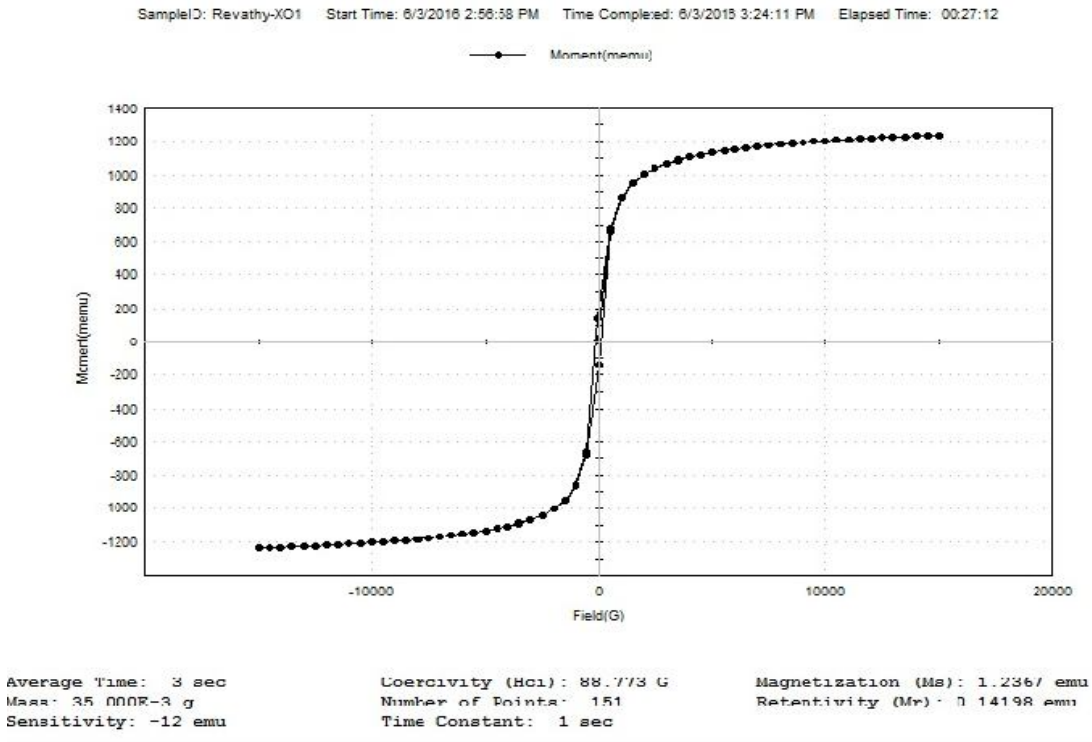

Fig. 3: VSM of $CaFe_2O_4$ nanoparticles

3.6. Measurement of Electrical conductance, salinity and TDS

Calcium ferrite clearly indicates that they are highly clustered, compact have flake like morphology and well–connected grains. Some of the particles are in spindle like shape with well–connected grain to each others. The conductivity of calcium ferrite nanoparticle is found to be 2.8 μScm^{-1}. The low electrical conductivity of calcium ferrite and high dielectric constant can be utilized in microwave materials [21].

Salinity is a measure of all the salts dissolved in water [22]. Salinity is usually measured in parts per thousand (ppt). The salinity of calcium ferrite nanoparticle is 0 ppt. Thus it is insoluble in water.

Total dissolved solids (TDS) is the measure of the combined content of all inorganic and organic substances contained in a liquid in molecular, ionized or micro–granular suspended form [23,24]. The two principal methods of measuring total dissolved solids are gravimetric analysis and conductivity. The TDS of prepared calcium ferrite nanoparticle is 1 mg/L; it is not a health hazard.

4. CONCLUSIONS

$CaFe_2O_4$ nanoparticles were successfully prepared by the simple solution combustion method using $FeSO_4$, $CaCl_2$ and dl–alanine. Powder XRD results shows that the synthesized product is well matched with JCPDS file no: 72–1199. The average crystalline size is 12 nm which is calculated from Debye–Scherer's formula. The FT–IR spectrum confirms the Ca–O and Fe–O bonds in the sample. The VSM analysis of the prepared nanoparticles revealed their superparamgnetic behaviour. The electrical conductance of the sample is found to be 2.8μScm^{-1}. SEM micrographs reveal that the particles are spherically agglomerated.

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Synthesis, characterization and biological studies of transition metal complexes of Mn(II), Cu(II) and Zn(II) with an azo dye (E)–4–((2,6–dihydroxyphenyl) diazenyl)benzoic acid

S. Mini[1]*, Aravind S.S. [1]

Author Affiliations
[1]University College, M G Road, Palayam, Thiruvananthapuram 695034 Kerala, India

Corresponding Author
***S. Mini,** University College, M G Road, Palayam, Thiruvananthapuram 695034 Kerala, India

E-mail: minissadasivan@yahoo.com

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Abstract

The present work includes the synthesis and characterization of complexes of (E)–4–((2,6–dihydroxyphenyl)diazenyl)benzoic acid with metal ions Mn(II), Cu(II) and Zn(II) and characterized by elemental analysis, spectroscopic data including FT–IR, ^{1}H–NMR and electronic spectra. It has been found that the azodye behaves as a neutral bidentate N, O donor which chelates with the metal ions Cu(II) and Mn(II) in 1:2 and with Zn(II) in 1:1 stoichiometry. Magnetic moment and electrolytic conductance data confirms this. The azodyes and complexes were screened for antimicrobial activity. The ligand and the complexes show better antimicrobial activity.

Keywords: 4–aminobenzoic acid, resorcinol, antimicrobial activity, NMR spectra

1. INTRODUCTION

Many of the metal complexes are deeply colored and are used as dye and pigment before they were recognized as co-ordination compounds [1]. The structure and constitution of metal complexes of azo dyes are extensively studied by co-ordination chemists. It was Morgan and his students, who made significant contribution on the importance of co-ordination chemistry in dyeing technology. Variation in color or shade, resulting from changes in metal ion present in the bath, as on the fibre during dyeing supports the formation of co-ordination compounds [2–3].

The azo dyes having a donor group in the ortho position to the azo group are generally chelating agents. Stability and instability refers to the position of equilibrium, i.e., to the equilibrium constant. This constant is a measure of enthalpy and entropy changes and hence a thermodynamic property. The thermodynamic stability of metal chelates is influenced by the basicity of the ligand, chelate ring size, number of chelate rings per ligand and nature of metal. The stability of metal complexes increases with increase in basic strength [4]. It is found that the chelate ligands form stable complexes than those formed by their mono–dentate analogues. The chelate stability arises largely from favorable entropy changes [5]. In this work we have prepared an azo dye from resorcinol and 4–amino benzoic acid; (RABA). The complexes of Zn(II), Mn(II) and Cu(II) were prepared using RABA.

2. EXPERIMENTAL

2.1. Materials and Methods

All the chemicals used are of analytical grade and purchased from Merck. The complexes were analyzed for metal and halide content by standard methods [8]. The electrical conductance of the complexes in methanol and DMF (10^{-3} m solution) were measured at room temperature using a Systronics direct reading conductivity meter. The Infrared spectra of the ligands and complexes were recorded in the range of 4000–400 cm^{-1} on a Perkin Elmer spectrum 65 IR spectrophotometer. Electronic spectra of the ligands and the complexes in methanol were measured in the range 200–900 nm on Perkin Elmer Lamda 25 UV–Visible spectrophotometer. The Elemental analyses (C, H, N) were carried out on a Vario EL–III CHN Elemental analyzer at the SAIF, Cochin University of Science and Technology. The magnetic moments were measured at room temperature on a Sherwood Scientific magnetic susceptibility meter. The proton NMR spectrum of the ligand and zinc complex was recorded in Brucker, Ascend^{TM} 400 NMR spectrometer at 400MHz.

2.2. Preparation of ligand

The ligand used for the present study is resorcinol azo benzoic acid [RABA]. 3.425g of 4–aminobenzoic acid was dissolved in 14ml 1:1 HCl. Then it is cooled. This solution was diazotized by using a cooled solution of 1.725g of $NaNO_2$ in 10ml of water. This solution was filtered and cooled. Prepared another solution by dissolving 2.75g of resorcinol in 22.5ml 2%NaOH. Into this the diazotized mixture was added with constant stirring. The red precipitate obtained was allowed to attain room temperature. It was suction filtered and dried over anhydrous $CaCl_2$ in a desiccator.

2.3. Preparation of complexes

The complexes are synthesized by a general method. Methanolic solution of the metal salt (0.01mol) and ligand (0.01mol) are mixed. The ligand solution is added gradually in small portions with good stirring to the metal salt solution when sudden color change was occurred indicating the complex formation. Then it was kept under reflux for 2–3 hours, on a water bath for completion of reaction. Afterwards, the solid complexes formed were filtered, washed with ethanol to remove excess ligands. It was then dried in vacuum desiccator.

3. RESULTS AND DISCUSSIONS

The complexes reported here are stable, colored and non-hygroscopic amorphous solids. They are partially soluble in acetone and methanol, and completely soluble in DMSO, but insoluble in water, chloroform and ethanol. The microanalytical data are shown in the Table 1 given below. The experimental values are in good agreement with the theoretical values. Based on the elemental analysis , the empirical formulae of Zn(II), Mn(II) and Cu(II) complexes can be formulated as $[Zn(RABA)(H_2O)_2]CH_3COO^-$, $[Mn(RABA)_2(H_2O)_2]$ and $[Cu(RABA)_2]$ respectively.

Table 1: Microanalytical data of the complexes

Compound	C %		H%		N %		O %		Metal %	
	Cal	Obs	Cal	Obs	Cal	Obs	Cal	Obs	Cal	Obs
RABA	60.47	60.54	3.88	3.81	10.85	10.91	24.81	23.5	–	–
$[Zn(RABA)(H_2O)_2]CH_3COO^-$	43.53	43.57	3.63	3.67	7.81	7.78	26.79	26.71	18.24	18.27
$[Mn(RABA)_2(H_2O)_2]$	51.58	51.52	3.64	3.61	9.26	9.30	26.45	26.47	9.08	10.01
$[Cu(RABA)_2]$	54.03	54.10	3.12	3.08	9.70	9.63	22.16	22.21	11.00	10.98

The magnetic moment value calculated for Mn(II) and Cu(II) complexes are 6.12 BM and 1.81 BM respectively. The magnetic moment value supports octahedral structure for Mn(II) complex and square planar structure for Cu(II) complex. The molar conductance of the complexes (10^{-3} molar concentration) were carried out in DMSO indicated that Mn and Cu complexes are non–electrolytes [6]. But Zn(II) complxes act as 1:1 electrolyte. The values are presented in the Table 2 below.

Table 2: Molar Conductance of the complexes in DMSO

Complex	Molar Conductance in DMSO ($ohm^{-1}cm^2mol^{-1}$)	Assignment
$[Zn(RABA)(H_2O)_2]CH_3COO^-$	65	1:1electrolyte
$[Mn(RABA)_2(H_2O)_2]$	27	Non-electrolyte
$[Cu(RABA)_2]$	32	Non-electrolyte

3.1. UV-Vis Spectra

Table 3 given below shows the electronic spectral details of the synthesised compexes. The ligand is characterized by two absorption bands in the UV region. A high intensity band at 393nm is attributed to $n\rightarrow\pi^*$ transition and low intensity band at 259 nm is attributed to $\pi\rightarrow\pi^*$ transition of azo group. The absorption bands in the complexes are shifted and new bands are appeared due to d→d transition. The band at 648 nm in Mn(II) complex suggests an octahedral geometry to the complex. The bands at 597 nm and 684 nm in Cu (II) complex suggests a square planar geometry to the complex.

Table 3: Absorptions of the ligand and complexes

Compound	λ_{max} (nm)	Assignment
RABA	393	$n\rightarrow\pi^*$
	259	$\pi\rightarrow\pi^*$
$[Mn(RABA)_2(H_2O)_2]$	394	$n\rightarrow\pi^*$
	259	$\pi\rightarrow\pi^*$
	648	d→d transition
$[Cu(RABA)_2]$	390	$n\rightarrow\pi^*$
	259	$\pi\rightarrow\pi^*$
	597	$^2B_{1g}\rightarrow{}^2E_g$
	684	$^2B_{1g}\rightarrow{}^2B_{2g}$

3.2. FT–IR Spectra

The IR spectral data (Table 4) of the ligand RABA and complexes with Zn(II), Mn(II) and Cu(II) are in agreement with an expected range.

Table 4: Infrared spectral data of the ligand and complexes

RABA ($\bar{\upsilon}$ cm^{-1})	$Zn(RABA)(H_2O)_2]CH_3COO^-$ ($\bar{\upsilon}$ cm^{-1})	$[Mn(RABA)_2(H_2O)_2]$ ($\bar{\upsilon}$ cm^{-1})	$[Cu(RABA)_2]$ ($\bar{\upsilon}$ cm^{-1})	Assignment ($\bar{\upsilon}$ cm^{-1})
1477	1417	1412	1420	$\bar{\upsilon}_{N=N}$
1242	1236	1241	1229	$\bar{\upsilon}_{C-O\ (chelated)}$
1602	1597	1596	1601	$\bar{\upsilon}_{C=O\ (free)}$
–	778	728	768	$\bar{\upsilon}_{M-N}$
–	690	667	653	$\bar{\upsilon}_{M-O}$

The band at $1477cm^{-1}$ in the ligand is attributed to azo group. This is shifted to $1417cm^{-1}$ in Zn complex, $1412cm^{-1}$ in manganese complex and 1420 cm^{-1} in copper complex suggesting a coordination of metal ion to nitrogen of azo group. The band at $1242cm^{-1}$ in the ligand is attributed to C–O stretching. This is shifted to $1236cm^{-1}$ in Zn complex, $1241cm^{-1}$ in manganese complex and $1229cm^{-1}$ in copper complex. The carbonyl absorption of ligand and complexes are given by the bands at $1602cm^{-1}$ (in ligand), $1597cm^{-1}$ (in Zn complex), $1596cm^{-1}$ (in Mn complex) and at $1601cm^{-1}$ (in Cu complex).

3.3. NMR Spectra

Proton NMR spectra (Figure 1) of the ligand RABA gives the following signals: Multiplet of aromatic proton of the benzoic acid part at 7.6–8.1 ppm. Multiplet of aromatic proton of the resorcinol part at 6.3–6.6 ppm. Singlet signal due to –OH of –COOH group at 12.39 ppm. Singlet signal due to –OH of resorcinol part at 10.89 ppm.

The proton NMR spectrum of the Zn(II) complex shows the following signals: Multiplet of aromatic proton of the benzoic acid part at 7.6–8.1 ppm. Aromatic proton of the resorcinol part shows a multiplet at 6.3–6.5 ppm. The singlet obtained due to –OH of –COOH group is seen at 12.57 ppm. Singlet signal of –OH (resorcinol part) is obtained at 10.79 ppm. From the spectra of the ligand and the complex we can confirm that one of the –OH group in the resorcinol is coordinated to the metal. The less intense peak at 10.78 ppm indicates the second –OH group of the resorcinol remain unchanged in its position. It is not coordinated to the metal.

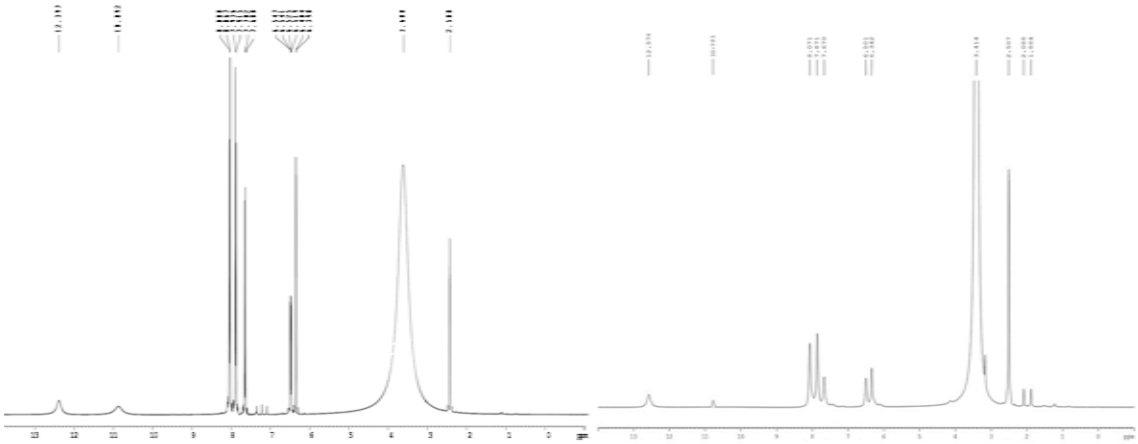

Fig. 1: NMR spectrum of RABA and $[Zn(RABA)(H_2O)_2]CH_3COO^-$

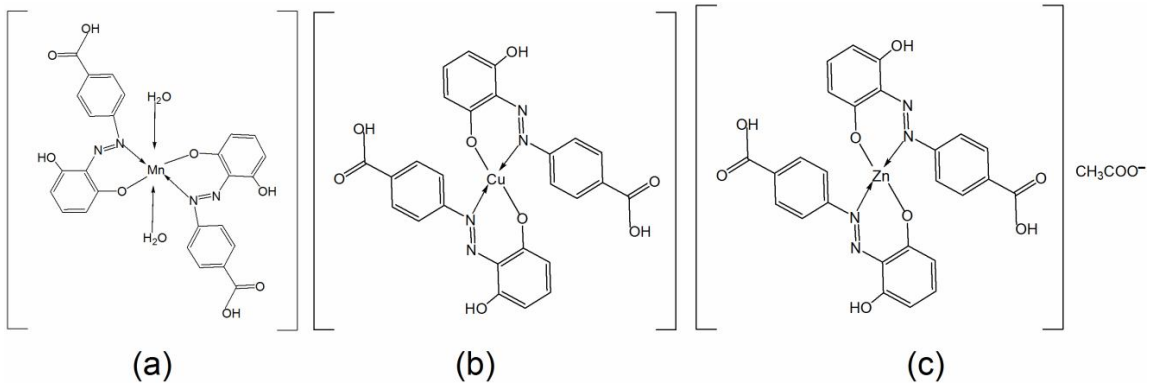

Fig. 2: Proposed structure for (a) $[Mn(RABA)_2(H_2O)_2]$, (b) $[Cu(RABA)_2]$ and (c) $[Zn(RABA)(H_2O)_2]CH_3COO^-$

On the basis of these observations and discussions it can be suggested that RABA is acting as a bidentate ligand. The Zn (II) and Cu(II) complexes have co-ordination number four and assumes tetrahedral and square planar geometry respectively and Mn(II) complex has co-ordination number six and assume octahedral geometry (Figure 2).

3.4. Antibacterial activity

The invitro biological screening effect of the investigated compounds were tested against the bacteria *Staphylococcus aureus* by using Agar-well diffusion method by taking DMSO as solvent for RABA and $[Mn(RABA)_2(H_2O)_2]$.

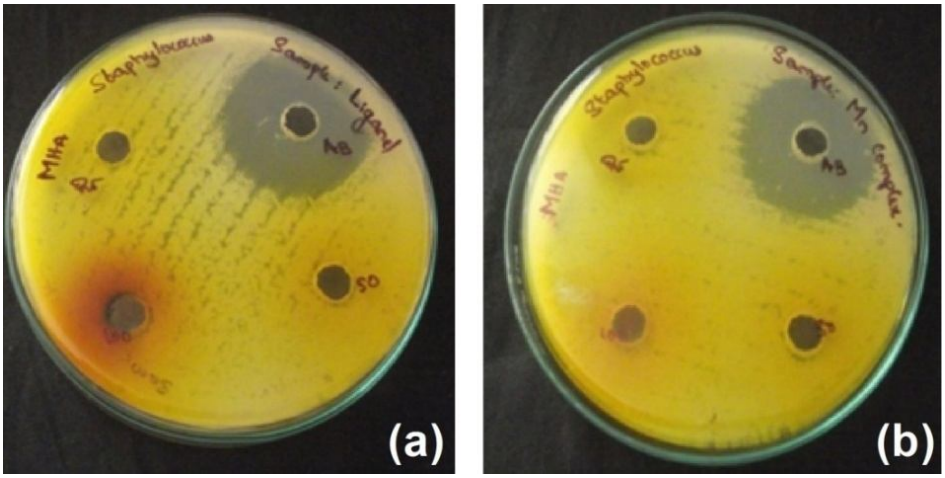

Fig. 3: Antibacterial activity of (a) RABA and (b) $[Mn(RABA)_2(H_2O)_2]$

The result indicates that the $[Mn(RABA)_2(H_2O)_2]$ shows greater antibacterial activity (Figure 3) than RABA against *Staphylococcus aureus*. Stock concentration was taken as 10mg/ml. The details are given in Table 5.

Table 5: Antibacterial activity of the RABA and $[Mn(RABA)_2(H_2O)_2]$

Sample	Concentration (µg/mL)	Zone of inhibition (cm)
Ligand RABA	Streptomycin (10µg)	3.5
	250	Nil
	500	Nil
	1000	1.0
Mn complex $[Mn(RABA)_2(H_2O)_2]$	Streptomycin (10µg)	3.5
	250	Nil
	500	Nil
	1000	1.3

3.4. Antifungal activity

The antifungal activity was determined by Agar well diffusion method with concentration of stock as 10mg/ml against the organism *Candida albicans* (Figure 4) and the results are shown in Table 6. The results indicate that the $[Mn(RABA)_2(H_2O)_2]$ shows greater antifungal activity than RABA against *Candida albicans.*

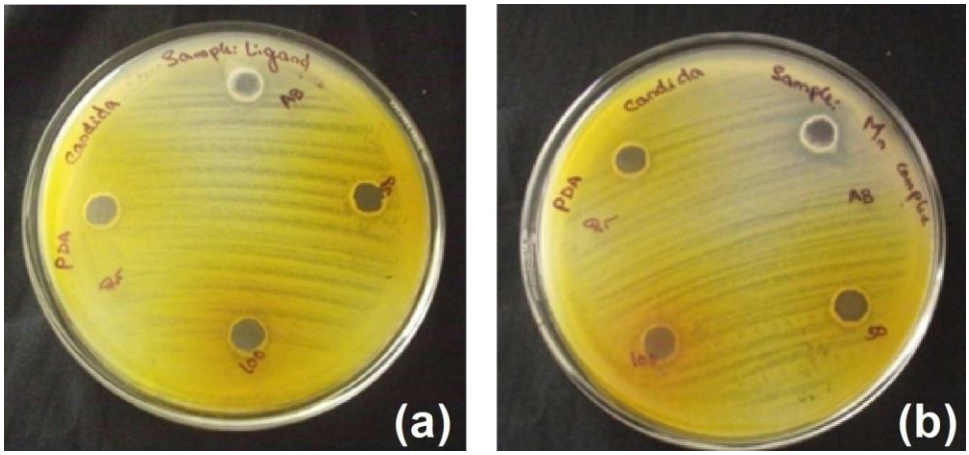

Fig. 4: Antibacterial activity of (a) RABA and (b) $[Mn(RABA)_2(H_2O)_2]$

Table 6: Antibacterial activity of the RABA and $[Mn(RABA)_2(H_2O)_2]$

Sample	Concentration (µg/mL)	Zone of inhibition (cm)
Ligand RABA	Clotrimazole	1.5
	250	Nil
	500	Nil
	1000	1.0
Mn complex $[Mn(RABA)_2(H_2O)_2]$	Clotrimazole	1.5
	250	Nil
	500	Nil
	1000	1.3

4. CONCLUSIONS

An azo dye is prepared from resorcinol and 4-amino benzoic acid (RABA). The complexes of Zn(II), Mn(II) and Cu(II) were prepared using RABA. Characterization of the ligand and complexes has been done on the basis of analytical and physico-chemical methods. From their spectral and magnetic data it is concluded that the manganese complex possess octahedral geometry having the formula $[Mn(RABA)_2(H_2O)_2]$ and copper complex possess square planar geometry having formula $[Cu(RABA)_2]$. Tetrahedral geometry is assigned for the zinc complex [Zn (RABA) $(H_2O)_2$] on the basis of conductance measurements, IR and NMR spectral studies. The ligand and metal complexes were screened for their biological activities against *Staphylococcus aureus* and *Candida albicans*. The ligand and the complexes showed better antimicrobial activity.

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One pot synthesis of 1–(3–methyl–4*H*–benzo [1,4]thiazin–2–yl) ethanone and its antimicrobial properties

Shajeelammal[1], Minitha R.[1,]*, Reena Ravindran[2]

Author Affiliations
[1]Department of Chemistry, University College, Thiruvananthapuram
[2]Department of Chemistry, Sree Narayana College, Chempazhanthy, Thiruvananthapuram

Corresponding Author
***Minitha R.,** Department of Chemistry, University College, Thiruvananthapuram

E-mail: minithabalan1@gmail.com

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Abstract

A novel 1,4–benzothiazine derivative, 1–(3–methyl–4*H*–benzo[1,4]thiazin–2–yl) ethanone (ATPAC) is synthesized from 2–aminothiophenol and acetylacetone and is characterized by elemental analysis, ^{1}H NMR, IR spectroscopy and electronic spectroscopy. Antimicrobial property of the title compound was determined by MIC using *E. coli*, *V. cholerae* and *C. albicans*.

Keywords: 1,4–benzothiazine, ATPAC, Antimicrobial,

1. INTRODUCTION

Heterocyclic thiazine derivatives are important because they are biological constituents of many biomolecules and drugs [1]. It has been noticed that structural modifications in the thiazine and its derivatives results in valuable medicinal properties that should be further explored through structure activity relationship (SAR) methods for the development of highly potent compounds against multi–drug resistant microorganisms and other diseases. Thus, the research to explore different avenues of chemical modifications of thiazine and its available derivatives to obtain novel active compounds should be continued [2, 3]. They also act as multidentate ligands for different metals due to the presence of nitrogen and sulfur atoms and are thus used extensively in coordination chemistry to obtain new frameworks with potential bioactivity [4]. Several heterocyclic compounds in the developmental phase have the potential to be part of new drugs and also play an important role in modern drug discovery [5]. A large number of thiazine ring containing drugs with versatile type of applications are being used clinically while their chemosensing properties are not explored.

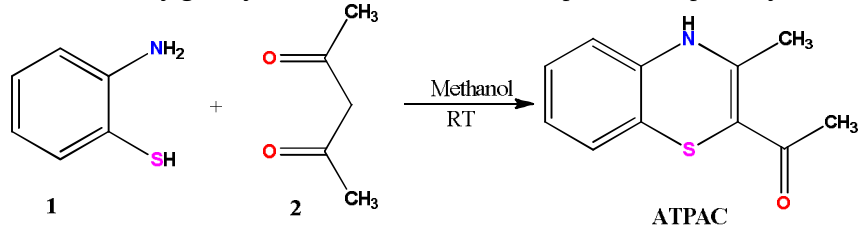
2. EXPERIMENTAL

Synthesis of ATPAC [1–(3–methyl-4H–benzo[1,4]thiazin–2–yl)ethanone]

2–aminothiophenol and acetylacetone were taken in 1:1 stoichiometric ratio and are separately dissolved in methanol. The two solutions mixed together, stirred for 10 minutes and kept aside for two days. Orange red crystals obtained were filtered, washed with ethanol and stored in desiccator.

3. RESULTS AND DISCUSSIONS

A novel 1,4–benzothiazine derivative, [1–(3–methyl–4H–benzo[1,4]thiazin–2–yl)ethanone] is synthesized from the reaction between 2–aminothiophenol and acetylacetone (Scheme 1). Formation of ATPAC in different reaction conditions were explored (Table 1). The reaction was studied: (a) without solvent at room temperature by intimate mixing in a mortar; (b) in ethanol (i) RT, (ii) with heating; (c) in ethanol–acetic acid (i) RT, (ii) with heating; (d) in methanol (i) RT, (ii) with heating (e) in methanol–acetic acid (i) RT, (ii) with heating. The maximum yield was compared and the optimum reaction condition was fixed according to the reaction temperature, time, solvents etc. Under all conditions 1,4–benzothiazine was formed exclusively and the best yield was obtained under RT methanol conditions. Fairly good yields were obtained, with separation of pure crystalline product.

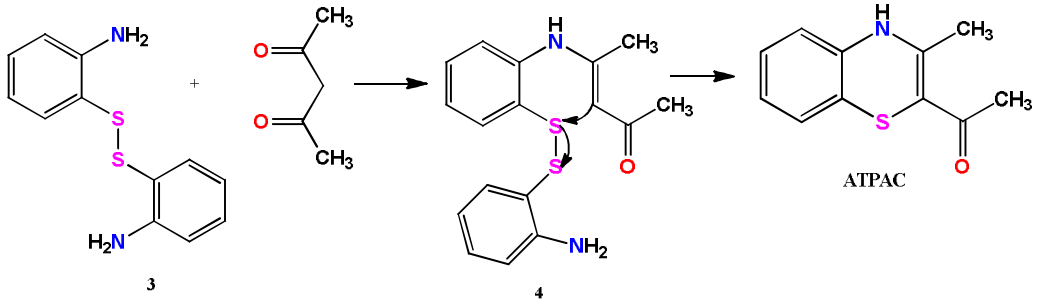


Scheme 1: Formation of ATPAC

Table 1: Reaction conditions and the corresponding yields of ATPAC

Solvents	Reaction conditions		Yield (%)
	Temperature	Time	
Without solvent	RT	2 days	95
Methanol	RT	2 days	**98**
	150 °C	5 hours	95
Ethanol	RT	2 days	70
	150 °C	5 hours	65
Ethanol + Acetic acid	RT	2 days	75
	150 °C	5 hours	80
Methanol + Acetic acid	RT	2 days	85
	150 °C	5 hours	85

The new ring system was obtained *via* oxidative cyclization by reacting a mixture of acetylacetone (**1**) and 2–mercapto aniline (**2**) in methanol at room temperature. Since the 2–mercapto aniline (**2**) readily oxidize to bis(*o*–aminophenyl)disulfide (**3**) under these reaction conditions, the reaction is considered to proceed via an intermediate (**4**), which is readily cyclized by the scission of the sulfur–sulfur bond upon the attack by the nucleophilic enaminone system. The possible mechanism is proposed in Scheme 2.



Scheme 2: Mechanism of oxidative cyclization

The result of elemental analysis and physical properties of ATPAC are given in Table 2. The percentages of C, H, N and S showed close agreement with the molecular formula $C_{11}H_{11}NSO$.

Table 2: Physical and analytical data of ATPAC

Compound	Colour	M. P. (°C)	Elemental percentage Calc. (found)				
			C	H	N	S	O
ATPAC	Orange Red	242	64.077 (64.390)	5.340 (4.890)	6.796 (6.950)	15.530 (15.290)	8.257 (8.480)

IR Spectrum of ATPAC

The benzothiazine, ATPAC exhibit a characteristic peak in the region 3443 cm^{-1}. This shift to a lower frequency suggests the possibility of a strong –NH…O intermolecular hydrogen bond. The IR band observed at 1798 cm^{-1} in ATPAC may be due to carbonyl group. IR spectra of the compound ATPAC is given in Figure 1.

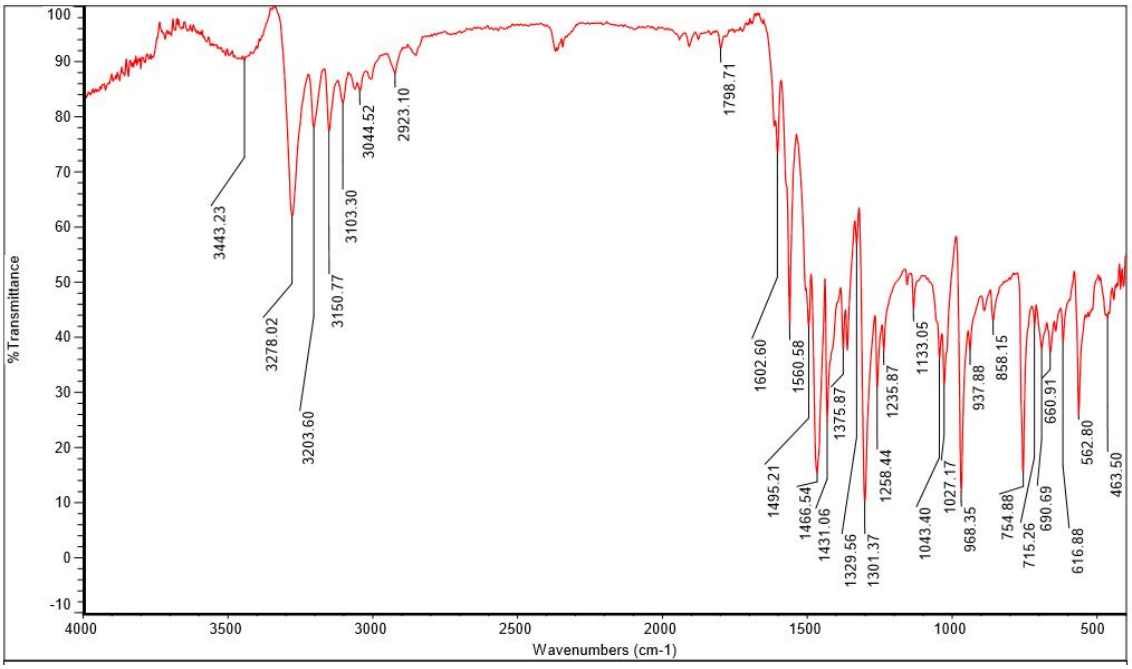


Fig. 1: IR Spectrum of ATPAC

^{1}H NMR Spectrum of ATPAC

In the ^{1}H NMR spectra of ATPAC, we have a signal at 11.100 ppm, which corresponds to the N–H proton frequency. Signals in the range 8.866–9.177 ppm with relative intensities in the rato 1:2:1 represent 4 aromatic protons in the benzene ring. We have another signal at around 4.452 ppm. It is given by methyl protons attached to C=O. Signal at 4.413 ppm also shows the presence of methyl protons, but attached to Nitrogen. The shift in the NMR frequency of two types of methyl protons is due to the presence of electronegative oxygen and nitrogen atoms in the vicinity of methyl protons. There is a sharp signal at around 5.6 ppm which is of DMSO. ^{1}H NMR results agree well with the assigned structure of ATPAC. ^{1}H NMR spectrum of ATPAC is shown in Figure 2.

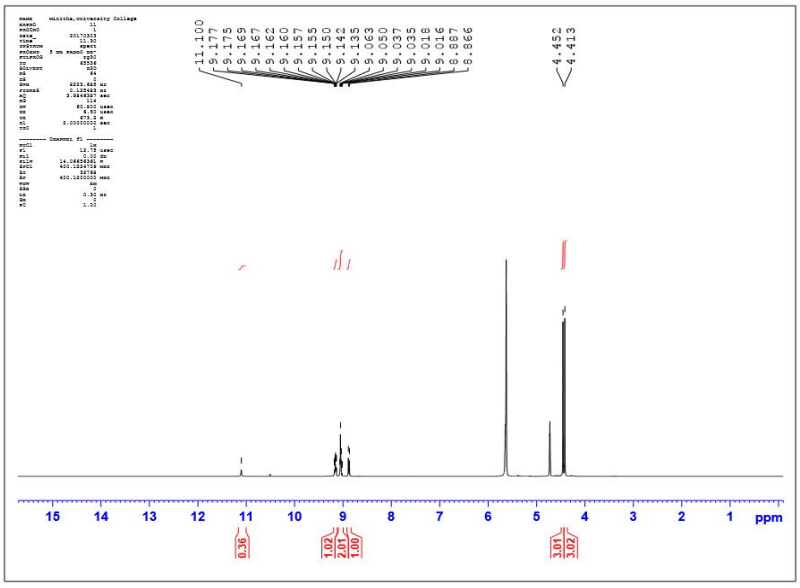


Fig. 2: ^{1}H NMR Spectrum of ATPAC

Electronic spectrum of ATPAC

Electronic spectrum of ATPAC was recorded in acetonitrile (Figure 3). The spectrum of ATPAC shows a relatively weak band at 298 nm, assigned to n→π* transition of the thiazine and carbonyl group. In addition, one strong band also appeared at 220 nm assignable to π→π* transitions, respectively.

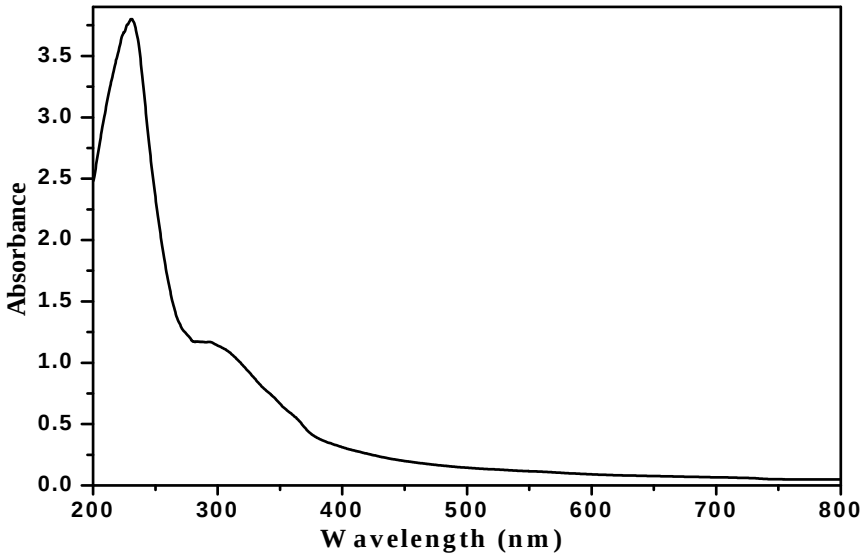


Fig. 3: Electronic spectrum of ATPAC

Antimicrobial properties

The synthesized compound ATPAC is screened for the antimicrobial activities against two bacteria and one fungus. The bacteria used for the study are *Escherichia coli* and *Vibrio cholerae*. The fungus used is *Candida albicans*. The test was performed using the disc diffusion agar technique. The minimum inhibitory concentration (MIC) is determined by different concentrations such as 100%, 75%, 50% and 25%. The activity was compared with known antibiotic Erythromycin. The results are tabulated in Table 3. Photographs showing antimicrobial activities are given below.

The antimicrobial activity results show that the synthesized ATPAC is active only against *V. cholera* and is inactive towards *E. Coli* and *C. Albicans*. The MIC value for *V. Cholerae* is 50%.

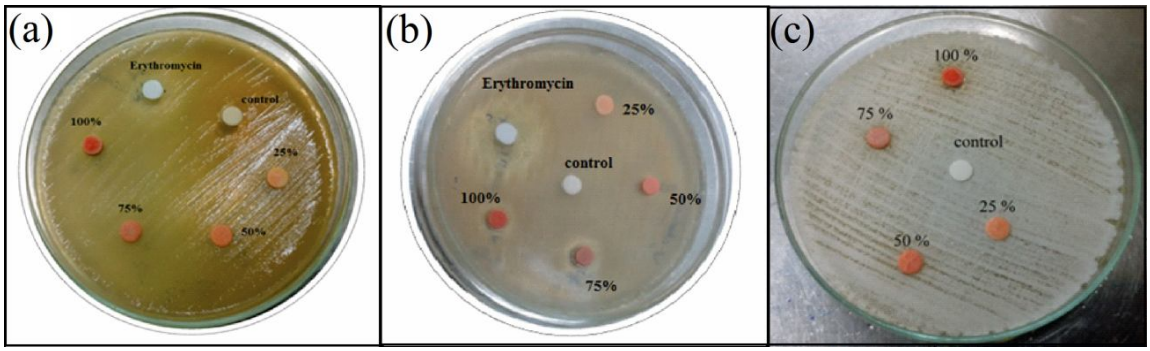


Fig. 4: Antimicrobial activity against (a) *E. Coli* (b) *V. Cholera* and (c) *C. Albicans*

Table 3: Antimicrobial activity of ATPAC

Organism	Zone of inhibition in mm					
	100%	**75%**	**50%**	**25%**	**Control**	**Erythromycin**
E. coli	NZ	NZ	NZ	NZ	NZ	12
V. cholerae	13	10	8	NZ	NZ	18
C. albicans	NZ	NZ	NZ	NZ	NZ	–

NZ: No Zone

4. CONCLUSIONS

An 1,4–benzothiazine derivative was synthesized from 2–aminothiophenol and acetylacetone by oxidative cyclization reaction. Different reaction conditions are chosen to optimize the best reaction condition which gives the maximum yield and it was observed that reaction carried out in methanol at room temperature produced an yield of 98%. The synthesized compound was characterized by means of elemental analysis, FT–IR, ^{1}H NMR and electronic spectroscopy. The compound is active against the bacteria *V. cholera* with MIC 50%. Thus, the acetylacetone based benzothiazine provided good antibacterial agent for *V. cholerae*.

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Development of hetero metal organic frameworks of transition metal ions

Rani Pavithran[1,]*, Lekshmi O[1]

Author Affiliations
Department of Chemistry, University College, Thiruvananthapuram

Corresponding Author
***Rani Pavithran,** Department of Chemistry, University College, Thiruvananthapuram

E-mail: ranipavithran@gmail.com

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Abstract

Heterometallic metal organic frameworks (MOFs) of transition metal ions have been hydrothermally synthesized with 1,4–benzenedicarboxylic acid as the organic linker in ethanol medium. The synthesized MOFs are found to be nanocrystalline and photoluminescent.

Keywords: MOF, hydrothermal synthesis, 1,4–benzenedicarboxylic acid, nano crystalline

1. INTRODUCTION

The discovery of MOFs in eighties created a revolution in chemistry. MOFs are hybrid crystalline materials with distinct structural properties and tuneable pore size, formed by the connectivity of metal ions and organic linkers [1]. MOFs find applications in gas storage, separation, photocatalysis, optoelectronics, sensors, bioimaging etc. Bimetallic MOFs find applications in gas storage and separation [2–4]. But the research in this area is still in its infancy. Hence we have synthesized bimetallic MOFs of transition metal ions such as zinc(II), copper(II) and manganese(II) and were characterised using FT–IR, SEM–EDS, PXRD and photoluminescent spectroscopy with a view to explore their applications in various fields.

2. EXPERIMENTAL

The heterometallic MOFs have been synthesized under solvothermal conditions by the reaction of 1,4–benzenedicarboxylic acid with corresponding metal salts. Heterobimetallic MOFs have been synthesised using various combination of transition metal ions such as Zn(II), Cu(II) and Mn(II) with 1,4–BDC in ethanol solvent. The reaction was carried out in an autoclave at 150^{o}C for 3 days under autogeneous pressure and cooled afterwards to get crystals. The crystals were filtered off, washed thoroughly with ethanol and dried at room temperature.

3. RESULTS AND DISCUSSIONS

3.1. IR spectral studies

In the IR spectrum of the ligand, there is a strong band at 1673cm^{-1}, due to COOH stretching of benzene ring. The presence of absorption around 3200–2800cm^{-1} is due to OH stretching of COOH bond. The band at 3200–2800cm^{-1}corresponding to OH is absent in various MOFs. The absorption band at 1673cm^{-1} in the ligand is shifted from 1673cm^{-1} to values between 1668cm^{1} to 1524cm^{-1} in all MOFs which indicates that the metal is bonded to carboxylic group.

3.2. Powder XRD Analysis

The PXRD data show the crystalline nature of MOFs. The particle sizes are calculated using Debye–Scherrer equation, D= Kλ/βcosθ. The calculated grain sizes in all the cases show that the frameworks are nanosized. The nature of the peaks indicates the crystalline nature of MOFs. In the PXRD patterns of heterobimetallic Zn/Cu –BDC MOF (Fig.1) and Cu/Mn–BDC MOF (Fig.2) the high intensity Bragg diffraction peaks are observed at 2θ = 14.77, 17.69, 24.17, 27.74, and 33.24° and at 2θ=18.35, 26.11, 28.06 and 36.48° respectively. The average grain sizes of these MOFs are calculated as 4.01 nm and 4.08nm respectively, from Debye – Scherrer formula and are found to be nano sized. The peaks indicate the crystalline nature of MOFs

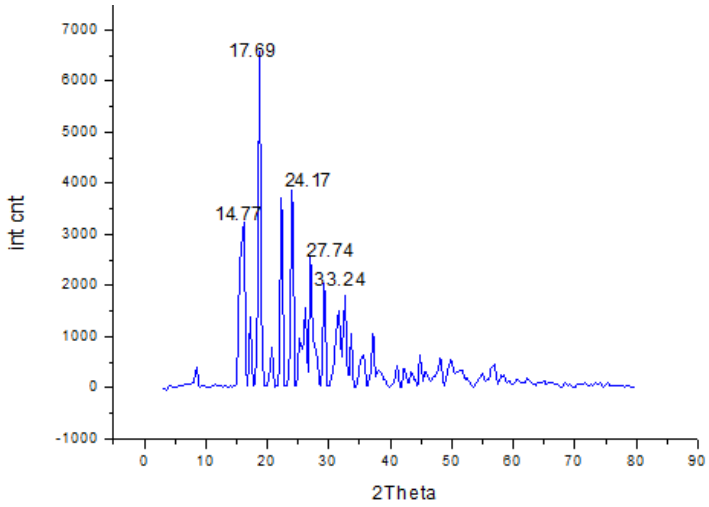

Fig.1: Powder XRD of heterobimetallic Zn/Cu-BDC MOF

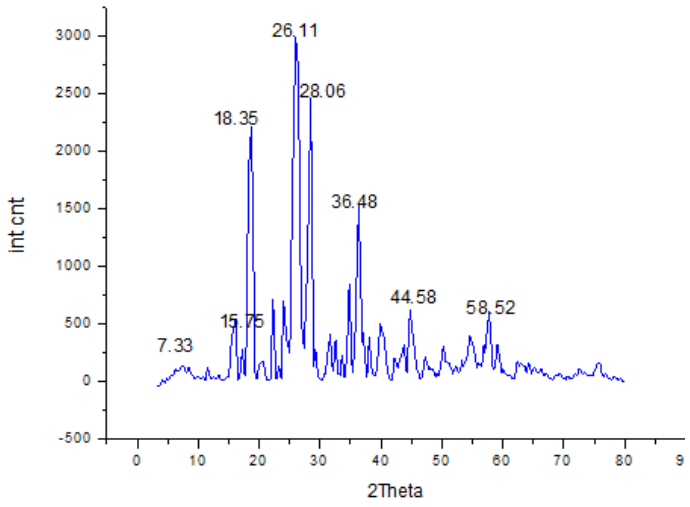

Fig. 2: Powder XRD of heterobimetallic Cu/Mn–BDC MOF

3.3. SEM–EDS Analysis

The morphology of MOF crystals were studied by SEM analysis. The SEM image of heterobimetallic Zn/Cu–BDC MOF (Fig.3) shows an agglomeration of particles. The SEM image of heterobimetallic Cu/Mn–BDC MOF (Fig.4) shows that the particles are crystalline in nature and has a structure similar to a cauliflower.

The elemental analyses of the newly synthesized MOFs of Zn/Cu & Cu/Mn with 1,4–Benzenedicarboxylic acid were carried out using EDS (Fig.5 & Fig.6). The results show that all the compounds contain the expected elements in their energy dispersive spectra. The EDS spectra confirm the successful synthesis of all these MOFs.

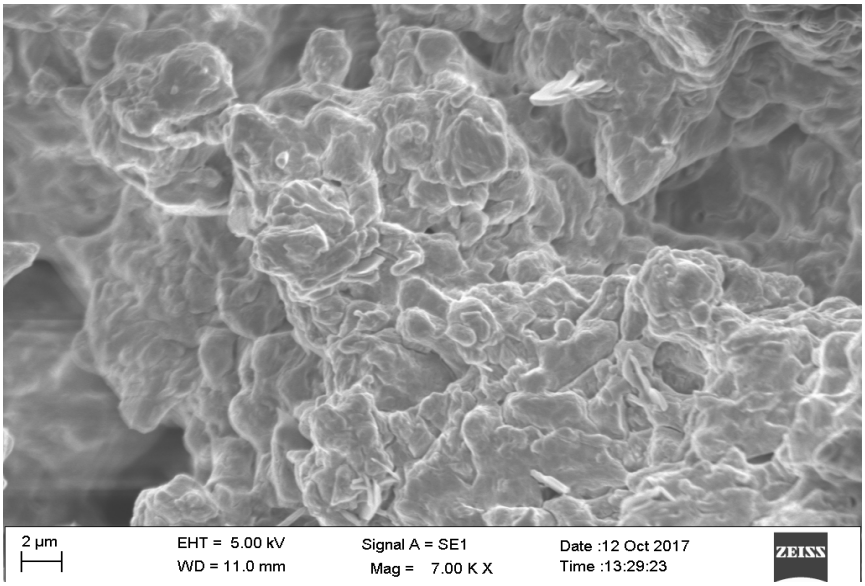

Fig. 3: SEM image of heterobimetallic Zn/Cu –BDC MOF

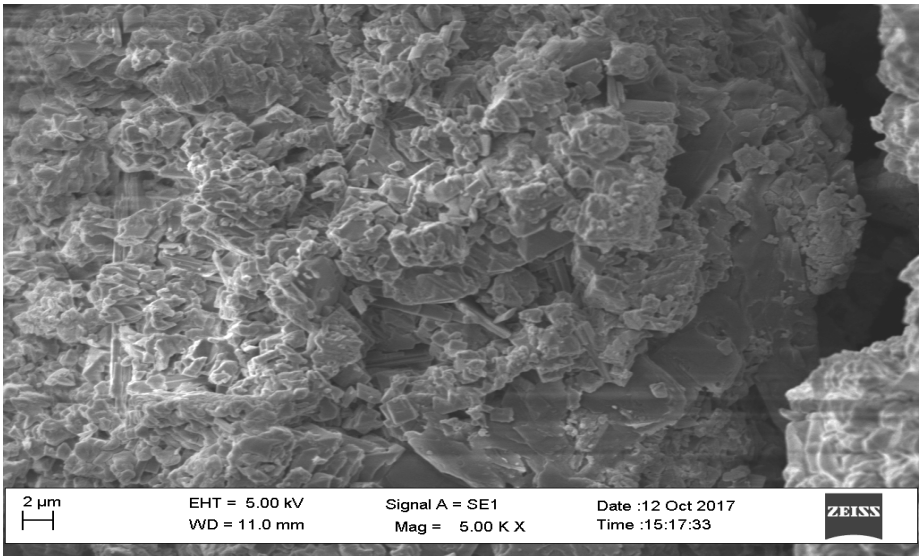

Fig. 4: SEM image of heterobimetallic Cu/Mn –BDC MOF

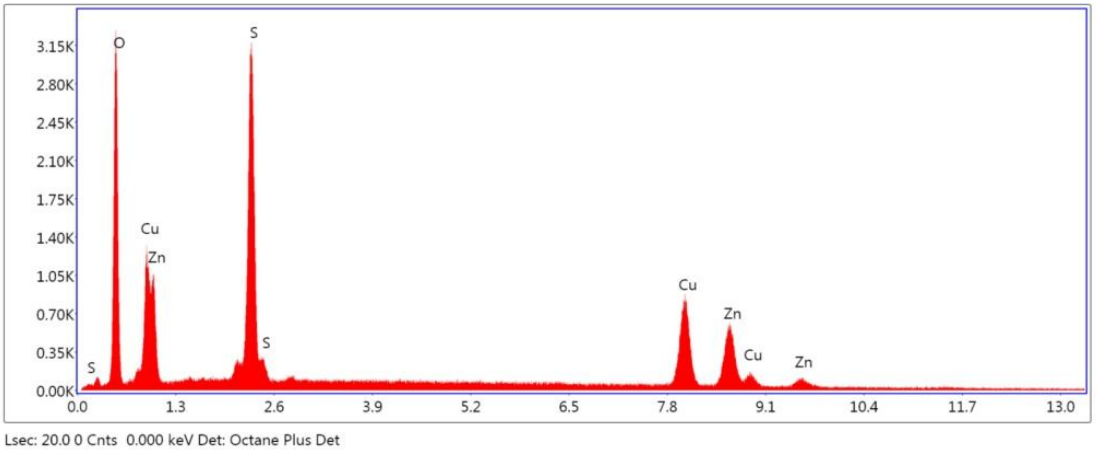

Fig. 5: EDS of heterobimetallic Zn/Cu –BDC MOF

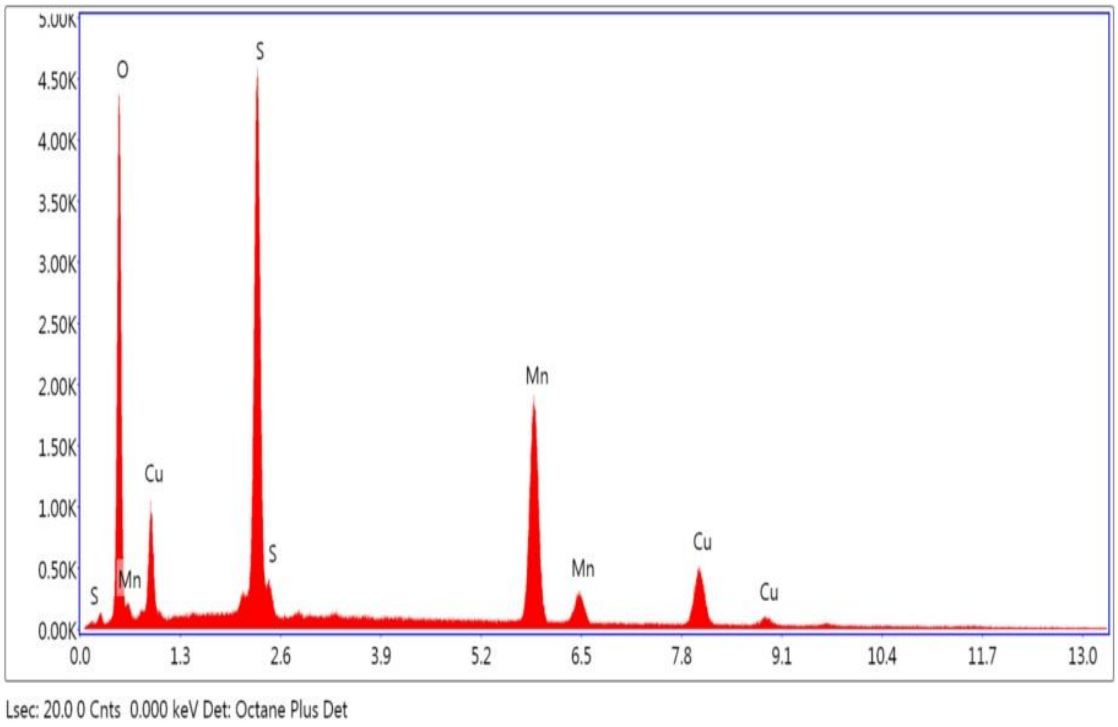

Fig. 6: EDS of heterobimetallic Cu/Mn –BDC MOF

3.4. Photoluminescent studies

The solution state photoluminescence properties of the ligand and the synthesized MOFs have been investigated in methanol at room temperature from 400 –750nm. The ligand shows emission maxima at 331 when excited at 300nm, corresponding to π–π* and n–π* transitions and also due to conjugation in the ligand.

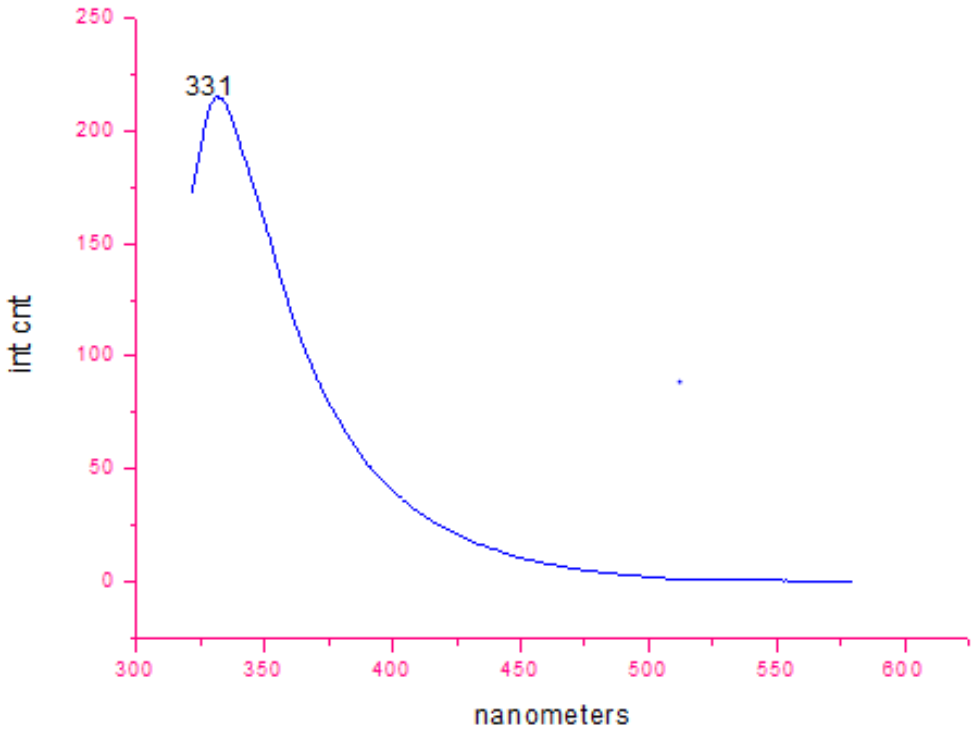

Fig. 7: PL of BDC excited at 300 nm

The emission maxima are observed at 404 nm, 426 nm for Zn/Cu MOF, when excited at 360nm. The emission maxima are observed at 476nm, 503nm and 540nm for Cu/Mn MOF, when excited at 360 nm.

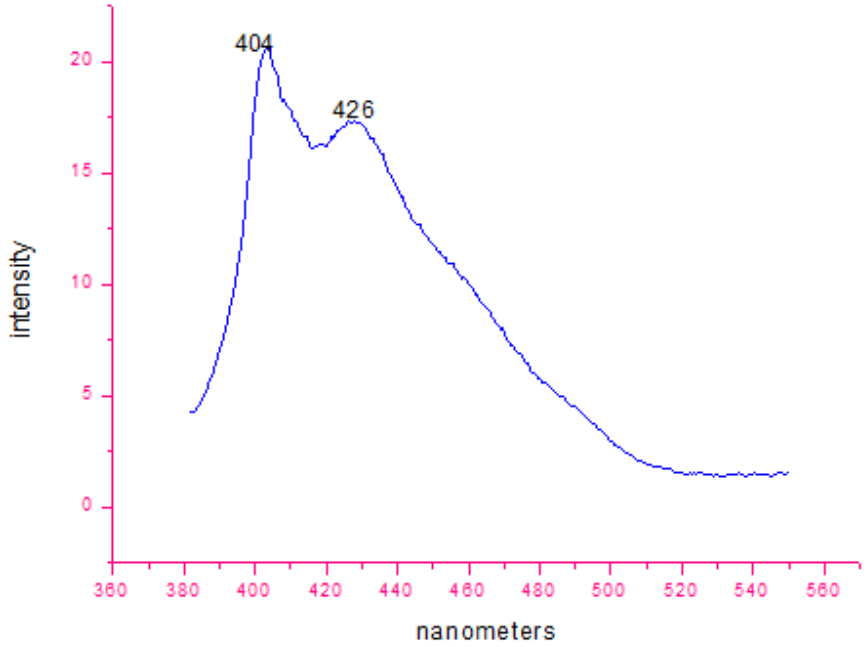

Fig. 8: PL of heterobimetallic Zn/Cu-BDC MOF (Excitation wavelength 360nm)

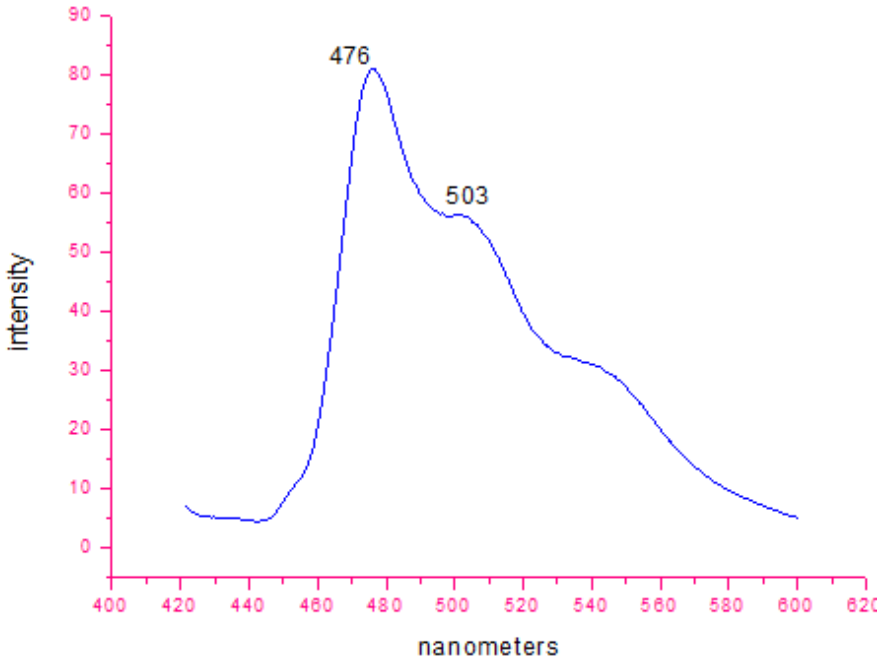

Fig. 9: PL of heterobimetallic Cu/Mn–BDC MOF (Excitation wavelength 360nm)

The PL spectra indicate that the synthesized MOFs are luminescent in nature due to ligand centred emissions. The emission spectra of transition metal complexes are due to ligand centred emissions and charge transfer spectra. The metal ion does not seem to have an effect on the shape of the emission spectra. Detailed investigations on the luminescence lifetimes and quantum yields may provide the missing insights on the luminescence properties of these compounds.

4. CONCLUSIONS

Photoluminescent and nanocrystalline heterobimetallic MOFs; Zn/Cu BDC MOF and Cu/Mn BDC MOF have been hydrothermally synthesized and characterized using FT–IR, PXRD, SEM–EDS and photoluminescent spectroscopy. The applicability of these MOFs as multifunctional materials may be explored in future research.

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Spectral & biological characteristics of a novel Uranyl Proton transfer salt

Shiji Fazil[1]*, Reena Ravindran[2]

Author Affiliations
[1] Dept. of Chemistry, Mannaniya College of Arts & Science, Pangode, Trivandrum
[2] Dept. of Chemistry, S.N College, Chempazhanthi, Trivandrum.

Corresponding Author
***Shiji Fazil,** Dept. of Chemistry, Mannaniya College of arts & science, Pangode, Trivandrum

E-mail: shijifazil@gmail.com

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Abstract

The reaction of $UO2(NO_3)_2.6H_2O$ with the proton transfer compound obtained from 2,2'–dithiobis(benzoic acid) and 4–aminoantipyrine, ($HAAP^+.HTBA^-$), led to the formation of a novel complex of the formulae $[(HAAP)_2UO_2(HTBA)_2(NO_3)_2]$.This complex was characterized using IR, elemental, NMR and magnetic measurement along with the single crystal X–ray study of the ion pair. The anion is a six coordinated complex with an octahedral geometry around the UO_2(II) atom. UO_2(II) complex and ($HAAP^+.HTBA^-$) were screened for antibacterial properties and have exhibited potential activity.

Keywords: 4–aminoantipyrine, Uranyl, 2,2'–dithiobis(benzoic acid), XRD

1. INTRODUCTION

The different aspects of proton transfer systems have been studied by chemists in the recent years. Proton transfer attracts considerable attention because it plays a key role in a wide variety of biological and chemical phenomena [1]. Proton transfer in molecular association between carboxylic acids and Lewis bases confers considerable stability upon the structure making process, resulting generally in more hydrogen bonding associations particularly in systems involving the protonated amine functional groups. Fascinating molecular topologies and crystal packing motifs due to hydrogen bonding interactions are observed in the self assembly of various amines with carboxylic acids [2, 3]. Molecular association between 4–aminoantipyrine and 2–mercaptobenzoic acid, resulted in the formation of one such proton transfer salt, ($HAAP^+.HTBA^-$, Figure 1). In this compound 2,2'–dithiobisbenzoic acid , the oxidized product of 2–mercaptobenzoic acid acts as the proton donor and amino group of 4-aminoantipyrine is the proton acceptor. The flexible 2,2'–dithiobisbenzoic acid , a multifunctional ligand containing both carboxylic and thio groups, can potentially afford various coordination modes and coordination architectures [4, 5].

2. EXPERIMENTAL

2.1. Synthesis of proton transfer salt.

The compound $HAAP^{+}.HTBA^{-}$ was synthesized by refluxing equimolar amounts of 2-mercaptobenzoic acid and 4–amino antipyrine in 50% ethanol–water mixture for 15 h. Brown coloured blocks (M.P 224°C–225ºC), separated after partial room temperature evaporation of the solvent.

2.2. Synthesis of complex, $[(HAAP)_2UO_2(HTBA)_2(NO_3)_2]$

Equimolar solution of UO_2 $(NO_3)_2.6H_2O$ in methanol (20 mL) was added to a stirring solution of proton transfer compound,($HAAP^{+}.HTBA^{-}$) in methanol-acetone mixture and refluxed for about 10h. The pale yellow solid complexes separated on slow evaporation of the reaction mixture was filtered, washed with methanol and dried.

3. RESULTS AND DISCUSSIONS

3.1 Single Crystal Structure of the proton transfer ion pair, $(HAAP^{+}.HTBA^{-})$

The compound, $(HAAP^{+}.HTBA^{-})$ crystallizes in the space group P-1 with triclinic crystal system. The crystal structure reveals the formation of a 1:1 proton transfer compound held together by hydrogen bonds [5].

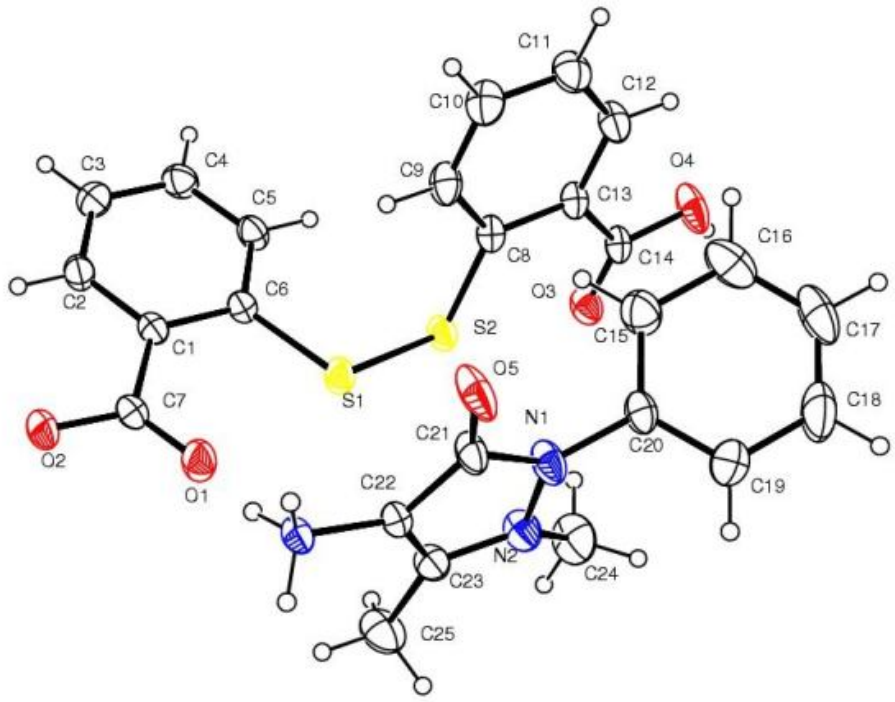

Fig. 1: Ortep diagram of $(HAAP^{+}.HTBA^{-})$

The cation and anion moieties of ligand are held together by a combination of N–H…O and O–H…O hydrogen bonds. The presence of NH_3^{+} is the source of extensive hydrogen bonds in the lattice. These non-covalent interactions form an extended ID supramolecular chain network.

3.2 Characterization of the complex, $[(HAAP)_2UO_2(HTBA)_2(NO_3)_2]$

The infrared spectrum of the ion–pair is characterized by a broad ammonium band centered at 3075 cm^{-1} along with bands at 1608 & 1492 cm^{-1} corresponding to the asymmetric and symmetric stretching vibrations of N–H bands of NH_3^{+}[5]. The C=O stretch of the pyrazolone ring is observed at 1664cm^{-1} which remains as such in the complex also. The band corresponding to the stretching of

carboxylic –C=O, which is observed as a superimpossible band along with the pyrazolone –C=O in the ligand spectrum is red shifted to 1697 cm^{-1} in the complex indicates the coordination of carboxylic –C=O. The presence of NH_3^+ bands without much shift in frequency in the spectrum of the complex confers that of the cation moiety does not involve in complex formation. Hence the coordination of the ion pair ligand to the metal occurs via the anion part only. A new strong peak at 1697 cm^{-1} shows an increase shift of the metal coordinated C=O of –COOH group of the anion moiety and a weak broad band around 3631 cm^{-1} shows the –OH stretching of the COOH group [5,6,7]. Another new band at 1630cm^{-1} confirms the coordinated COO^- group [8]. Also the strong peak at 1582 cm^{-1} in the ligand spectrum corresponding to COO– group is lowered to 1562 cm^{-1} in the spectrum of the complex due to metal coordination. Strong peaks at 1461 cm^{-1} and 1353 cm^{-1} are attributed to asymmetric and symmetric stretching vibrations of coordinated COO^- group [8]. A strong band characteristic of trans UO_2 is observed at 916 cm^{-1} which may be assigned to υ_{as} (O=U=O) and a medium intensity band at 856 cm^{-1} is assignable to υ_s (O=U=O) [6]. Two strong bands at 1220cm^{-1} and 1360 cm^{-1} with a separation of 140cm^{-1} corresponding to υ1 and υ4 and a medium band at 1024 cm^{-1} corresponding to υ2 of the nitrate group indicate the presence of terminal monodentate nitrate group [9].

Table 1: Characterization of ligand & the UO_2(II) complex

Compound	Composition %found (calc)			μ_{eff} (BM)
	Carbon Sulphur	Hydrogen	Nitrogen	
$HAAP^+.HTBA^-$	62.15 (63.90) 10.06 (9.46)	4.13 (4.73)	12.01 (12.43)	–
$[(HAAP)_2UO_2(HTBA)_2(NO_3)_2]$	36.23 (35.50) 14.48 (13.56)	3.71 (3.57)	4.80 (5.60)	0

Table 2: FT-IR bands and tentative assignments of the ligand and UO_2(II) complex

Compound	–C=O of COOH	N–H of NH_3^+	–C=O of pyrazolone	–COO– (asy)	–COO– (sym)	O=U=O (asy)	O=U=O (sym)	υ_{U-N}	υ_{U-O}
$(HAAP^+.HTBA^-)$	1664	3075	1664	1582	1492	–	–	–	–
$[(HAAP)_2UO_2(HTBA)_2(NO_3)_2]$	1697	3064	1664	1560	1392	916	856	576	465

The NMR spectral data of the ligand and the complexes are given in Tables 3 and 4. The ^{1}H NMR spectra of the ligand is dominated by the presence NH_3^+, COO^-, COOH signals and the complex spectrum also shows the respective peaks with a slight shift. The ^{13}C NMR spectra of the complex also shows similarity towards the ligand except that there is a blue shift on the carboxylate –C=O and there is a red shift on the –C=O of carboxylic acid [10] indicates their coordinating to the metal.

Table 3: ^{1}H NMR chemical shifts of ligand and complex

$[HAAP^+.HTBA^-]$	$[(HAAP)_2UO_2(HTBA)_2(NO_3)_2]$	Assignment
2.103	2.231	$C–CH_3$
2.746	2.935	$N–CH_3$
7.222–8.045	7.185–8.558	Aryl protons
10.262	10.356	NH_3^+ of Pyrazolone
12.647	13.103	Carboxylic COOH

Table 4: ^{13}CNMR chemical shift of ligand and complex

$[HAAP^+.HTBA^-]$	$[(HAAP)_2UO_2(HTBA)_2(NO_3)_2]$	Assignment
9.82	10.305	$C–CH_3$
38.18	37.635	$N–NH_3$
119–138	120–141	Aryl carbons
161	160	–C=O of pyrazolone
167	163	–C=O of Carboxylate
170	176	–C=O of COOH

3.3. Antibacterial Studies

The antibacterial activity of the compound, ($HAAP^+.HTBA^-$) was evaluated by means of Disc Diffusion [11, 12] method against two gram positive bacteria namely *B. Cereus* and *S. Aureus* and two gram negative bacteria namely *V. Cholarae* and *S . Thyphimurium* and the results are discussed in the Table 5.

Table 5: Zone of growth of antibacterial activity

Compound	B. Cerus	V. Cholerae	S. Typhimurium	S. Aureaus
($HAAP^+.HTBA^-$)	7 mm	9 mm	1 mm	2 mm
$[(HAAP)_2UO_2(HTBA)_2(NO_3)_2]$	10 mm	9.5 mm	2 mm	2.5 mm

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Isolation and characterization of active compounds from *Jasminum multiflorum* leaves

Afinisha Deepam LS[1,*], Divya S[1], Jisha MJ[1]

Author Affiliations
[1]Department of Chemistry, Christian college Kattakada, Kerala, India–695572

Corresponding Author
*__Afinisha Deepam LS,__ Department of Chemistry, Christian college Kattakada, Kerala, India–695572

E-mail: afinishadpm@gmail.com

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Abstract

Jasminum Multiflorum is a shrub having many medicinal values. Its chemical constituents were extracted with methanol by column chromatography. The components were isolated by TLC and characterized using spectral techniques such as FTIR, C^{13} NMR and H^{1} NMR. The isolated phenolic compounds are tannin and flavonoids.

Keywords: Flavanoid, FTIR, *Jasminum multiflorum*, Tannin, TLC

1. INTRODUCTION

Plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years, and have served humans well as valuable components of seasonings, beverages, cosmetics, dyes, and medicines [1]. Research on medicinal plants has attracted considerable attention since they are rich source of natural compounds. Recently the usage of herbal remedies has been increased among the people. The leaves, roots, and fruits of plants have been used as traditional medicine. Natural products are phytochemicals that occur naturally in plants. These phytochemicals are responsible for colour and organoleptic properties, such as the deep purple of blueberries and smell of garlic. The term is generally used to refer to those chemicals that may have biological significance but are not established as essential nutrients [1, 2]. Jasminum multiflorum is a species of Jasmine in the family Oleaceae. It is an ornamental shrub native to India and Southeast Asia with different biological activities [2–4]. Jasmine flower forms a vital ingredient of almost all ayurvedic medicines owing to its diverse curing qualities [5]. The present study was carried to detect the phytoconstituents, followed by the spectroscopic characterization of *Jasminum multiflorum*.

2. MATERIALS AND METHODS

2.1. Chemicals and methods

Solvents and chemicals used for the present study obtained from Merck, Calbiochem. UV Spectra was taken in DMSO from 200 to 800 nm using UV–1800(Shimadzu). FT–IR Spectra was

taken dissolving in DMSO. H^1 NMR and C^{13} NMR was obtained from National Institute for Interdisciplinary Science and Technology (CSIR), Thiruvananthapuram.

2.2. Preparation of methanolic extract

The leaves of Jasminum Multiflorum were air dried and pulverized to get powder. From the powder 40g is weighed and extracted using methanol in a Soxhlet apparatus. Methanol is evaporated, by using rotary vacuum evaporator (Buchi).

2.3. Column chromatographic separation of compounds

Chromatography is a simple analytical technique used for the separation of the compounds in a mixture. In column chromatography, the sample is adsorbed in silica and passed through the column by eluting with different solvents. Compounds are eluted based on the polar nature of the solvent and the sample component. Silica gel of 100–200 mesh is packed using ethyl acetate. After packing, column is kept for 60 minutes. Methanolic extract of *Jasminum multiflorum* is adsorbed in silica. The column is eluted with solvents petroleum ether, toluene, chloroform, ethyl acetate followed by gradient elution of methanol and water (90:10, 70:30, 50:50 and 30:70).

2.4. Identification of compounds by TLC

The TLC plate coated with silica gel is used for the separation. Solvent system used is Ethyl acetate, Methanol: Water (81:11:8). Developed plate is kept in iodine chamber for the visualization of the compounds.

2.5. Phytochemical analysis

The qualitative analysis for the phytoconstituents such as Tannins, Flavonoids, Saponins, Cardiac glycosides, Steroids and Terpenoids was performed by reported methods [6, 7].

3. RESULTS AND DISCUSSION

3.1. Phytochemical analysis of *Jasminum multiflorum*

The active constituents of plants are responsible for their chemotherapeutic value. The methanolic extract of *Jasminum multiflorum* was subjected to phytochemical screening for various phytoconstituents, which revealed the presence of tannins and flavonoids. Phenolic compounds have been reported to be potential free radical scavengers [8]. The plants rich in tannins have significant activity in cancer prevention and are used in treating intestinal disorders [9, 10]. Flavonoids are known to possess a wide range of biological activities such as antioxidant, antimicrobial, anti–inflammatory and anticancer activities [8, 11–13].

3.2. TLC separation

TLC separation (Fig.1) using Track 13 of Methanol: Water (50:50) shown single spot and R_f value at 0.6206. This fraction is characterized by spectroscopic techniques.

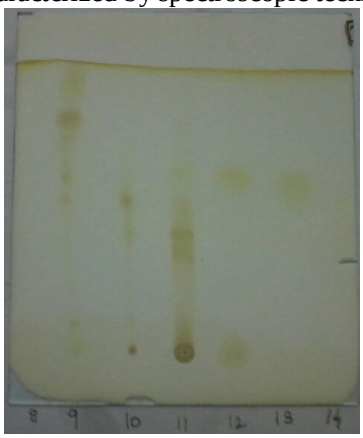

Fig. 1: TLC separation of plant extract

Table 1: Track and Solvent used

Track	Solvent
8	Chloroform–d
9,10	Ethyl acetate
11	Methanol: Water (90:10)
12	Methanol: Water (70:30)
13	Methanol: Water (50:50)
14	Methanol: Water (30:70)

Table 2: TLC Separation of various fraction

Fraction	R_f
9	0.7930, 0.5862, 0.5172
10	0.4137, 0.5172
11	0.6206, 0.4137
12	0.6206
13	0.6206

3.3. UV– Visible Spectra

UV Spectrum of the plant extract was taken in DMSO (Fig.2) at a wavelength range of 200 to 800 nm. A single peak was obtained at 276 nm. The spectra for phenolic compounds (tannins) and flavonoids typically lie in the range of 230–290 nm [14,15]. The result of UV–vis spectroscopic analysis confirms the presence of tannins and flavonoids in the methanolic extract of *Jasminum multiflorum*.

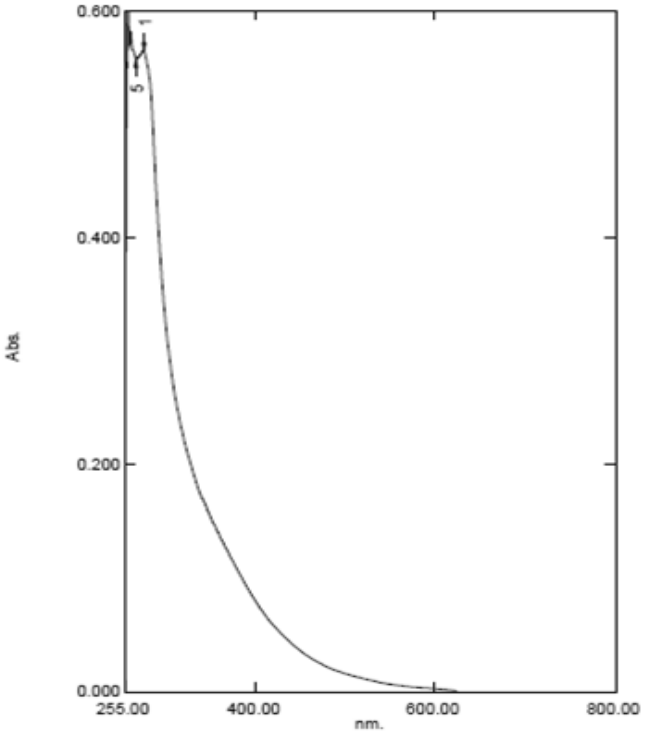

Fig. 2: UV–vis Spectrum of the plant extract

3.4. FT–IR Spectra

The FT–IR spectrum (Fig. 3) was recorded to identify the functional groups present in *Jasminum Multiflorum* based on the peak values in the region of infrared radiation.

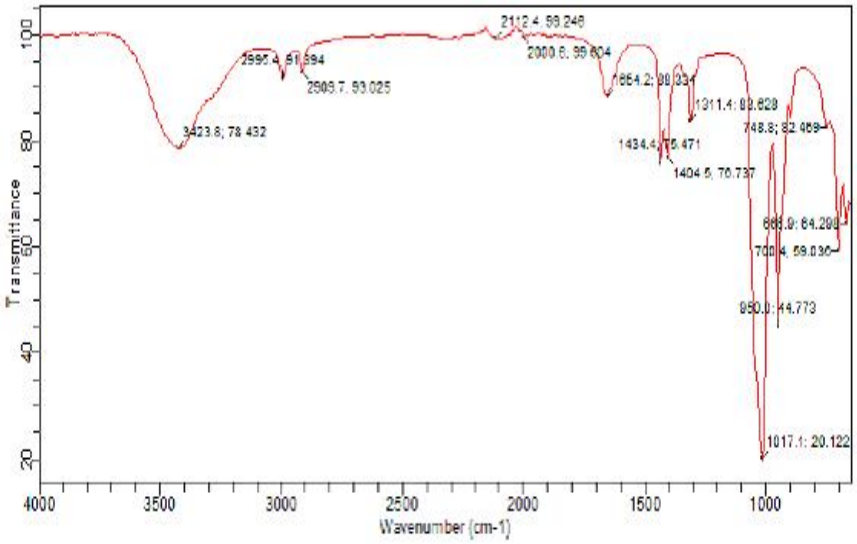

Fig. 3: FTIR Spectrum of the plant extract

FTIR studies enable the identification of the chemical constituents and elucidation of the structures of compounds [8, 9]. The major bands were observed at 3423.8, 1654.2, 1434.4, 1404.5, 1017.1 cm^{-1}. The peak at 3423.8 cm^{-1} indicates the O–H stretching. The bands 1654.2 cm^{-1}, 1434.4 cm^{-1} and 1404.5 cm^{-1} corresponds to the C–C stretch, confirming the presence of aromatic compounds. The peak at 1311.4 cm^{-1} represents C–O stretch which shows the presence of alcohols, carboxylic acids, esters and ethers. In addition, some weak absorption bands were also recorded in the spectra [8,9].

3.5. NMR studies

The C^{13} and HNMR spectra are shown in Fig. 4 and Fig. 5 respectively. The C^{13} NMR spectrum gave peaks at 71, 163, 172, 207, 209 ppm. The peak at 71ppm corresponds to carbon of C–O group and those at 163,172,207 and 209 ppm values correspond to C=O group.

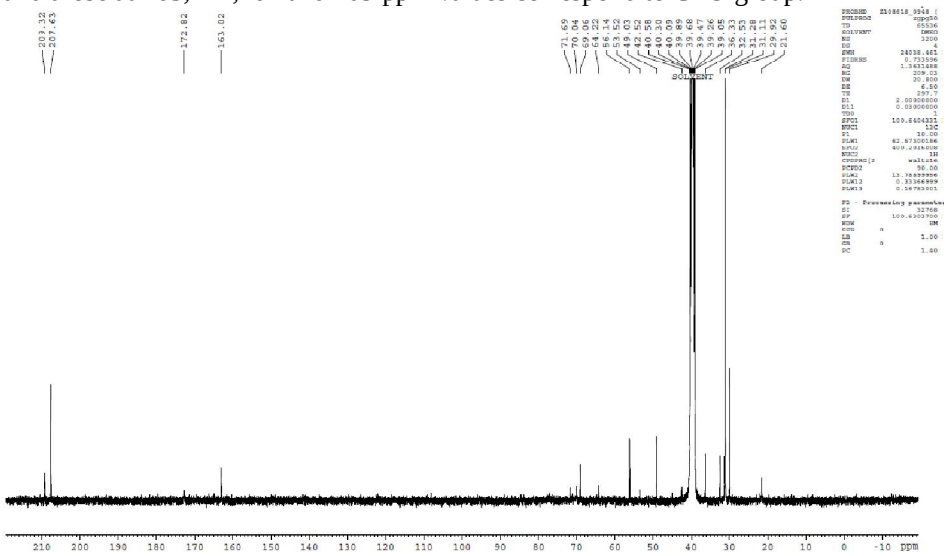

Fig. 4: ^{13}C Spectrum of the plant extract

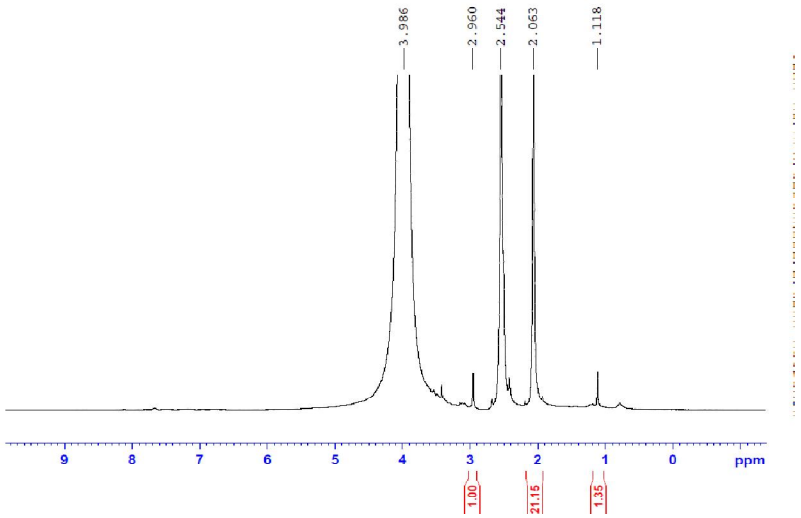

Fig. 5: ^{1}H NMR Spectrum of the plant extract

4. CONCLUSION

The extract obtained from *Jasminum multiflorum* was subjected to phytochemical analysis, which shows the presence of tannin and flavonoids. Using column chromatography and TLC the components were extracted and isolated. Spectral analysis also showed the presence functional groups that may be present in these chemical constituents. Further studies can be carried out regarding its anti–bacterial activity and other medicinal applications.

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National Seminar on

INSIGHTS INTO THE INTERDISCIPLINARY PERSPECTIVESOF CHEMICAL & BIOSCIENCES

26th-28th February, 2018

Organised by
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Dheerpura Society for Advancement of Science and Rural Development

(Reg. No. 01/01/01/16715/06)

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CONTENTS

RESEARCH PAPERS

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Seasonal Variation in the Fatty Acid Composition of Oil from *Mohachao narel,* a Sweet Endosperm Coconut (*Cocos nucifera* L.) Population from Maharashtra

P. AJITHKUMAR[1] AND K. SAMSUDEEN[2]

[1]*Government Arts College, Thiruvananthapuram*
[2]*Central Plantation Crops Research Institute, Kasaragod*
email : ajithkumargck@gmail.com

ABSTRACT

Coconut palm (*Cocos nucifera* L.) is one of nature's greatest gifts to mankind, belonging to Arecaceae. Practically all parts of the plants are useful in one way or the other but the dried kernel (copra) and the oil extracted from it are the most important product of the coconut palm. *'Mohachao Narel',* a coconut variant characterized by sweet and soft endosperm with less fibre content, has been reported from Guhagar taluk of Ratnagiri district of Maharashtra State in India. Farmers of the area get a premium price for sweet endosperm nuts and the sweet kernel is mainly used for raw consumption. Two types of nuts (sweet and normal) were developed in these palms. An experiment was conducted with an objective to analyze the fatty acid composition of oil from sweet and normal endosperm type nut of these palms at various seasons. Saturated and unsaturated fatty acids, ratio of saturated to unsaturated fatty acids were estimated. Short and medium chain saturated fatty acids (SMCSFAs) and long chain saturated fatty acids (LCSFAs) and their ratio was worked out. Profile of mono and poly unsaturated fatty acids were also studied, during three different seasons. Variation in the composition of fatty acid of coconut oil was noticed between these two types. The concentration of SMCSFAs was lower in sweet type. Ratio of SMCSFAs to LCSFAs was also lower in sweet type. Unsaturated fatty acid was higher in sweet type. Composition of Unsaturated fatty acid was also varied between these types. Concentration of Linoleic acid, an essential fatty acid and Oleic acid was much higher in sweet type. Mono unsaturated fatty acids and poly unsaturated fatty acids were higher in sweet type. The ratio of saturated fatty acid to unsaturated fatty acid was much lower in sweet type, compared to that of the normal type. Significant seasonal variations were also observed in both the types of nuts for all these parameters. The results indicate that oil from the sweet type coconut is very unique in terms of fatty acid composition.

Key words *Sweet kernel coconut; fatty acids; GLC; lauric acid; oleic acid; linoleic acid.*

Due to its multifarious uses, coconut (*Cocos nucifera* L.) symbolizes an important plant for the rural communities in developing tropical countries since it provides the basis for food production and by-product utilization in addition to its uses in industrial processing (Persley, 1992). Every part of this 'tree of life' is beneficial to mankind in one manner or other, the most extensively used part being the endosperm and its derivatives. Coconut endosperm, which is hard and white in colour, is rich in proteins, amino acids, sugars, vitamins, minerals and growth factors. The endosperm is mostly used for extraction of coconut oil and culinary purposes.

Certain coconut palms produce nuts containing soft, jelly-like endosperm, called *Makapuno* in Philippines (Torres, 1937), which has been commercially exploited for product diversification especially in confectionary industries. Mutants similar to *Makapuno*-type have also been reported from other coconut-growing regions: Coco Gra (Seychelles), Kopyor (Indonesia), *Thairu* or *Nei Thengai* (India), Dikiri Pol (Sri Lanka), Mapharao Khati (Thailand), Sap (Vietnam), Niu Garuk (Papua New Guinea) and Pia (Polynesia) (Arunachalam and Rajesh, 2008). This *Makapuno* trait results in abortion of embryo and is known to occur because of the effect of lethal recessive gene (Zuniga, 1953). Similar types have been sporadically reported from India (Arunachalam and Rajesh, 2008).

Recently, another variant with sweet and soft endosperm, named *'Mohachao Narel'* has been reported from Guhaghar taluk of Ratnagiri district of Maharashtra State in India (Anitha Karun *et al*., 2010; Samsudeen *et al*., 2010). Studies on fruit component traits of sweet and normal nuts of this population revealed that endosperm thickness was similar in both sweet and normal types, but the endosperm weight was more in normal types. Copra weight and copra recovery was more in normal nuts compared to sweet endosperm types. Oil content of sweet endosperm nuts was significantly lower than normal endosperm. Total soluble sugars (Brix values) were same in both type of nuts, but organoleptic test showed that water in sweet endosperm nuts was poor in taste. Sweet endosperm nuts get a premium price and are mainly used for raw consumption (Samsudeen *et al*., 2010).

Twenty seven palms of *'Mohachao Narel'* were located in Guhaghar taluk of Ratnagiri district (N17° 28'55'' to N17° 29'50'' and E73° 11'03'' to E73°

Table 1. Area percentage of fatty acids in normal and sweet nuts in all seasons

Fatty acid	Area percentage			
	Normal		Sweet	
	Mean	SD	Mean	SD
C6(caproic acid)	0.43	0.07	0.24	0.02
C8(Caprylic acid)	7.60	1.26	4.00	0.33
C10(capric acid)	5.49	0.82	2.66	0.34
C12(lauric acid)	47.99	1.12	34.78	2.70
C14(myristic acid)	18.93	2.11	21.02	0.22
C16(Palmitic acid)	7.28	0.47	13.92	0.99
C18(Stearic acid)	2.20	0.27	2.25	0.40
C18:1(Oleic acid)	4.98	1.04	15.66	0.81
C18:2(Linoleic acid)	1.30	0.24	4.50	0.75
C24(Lignoceric acid)	0.13	0.01	0.00	0.00

19'50''). The number of nuts with sweet endosperm per bunch varied from 10 to 77 percent in different palms (Samsudeen *et al*., 2010).

Coconut oil is the most important product of the coconut palm. Fatty acids are long chain organic acids having usually from 4 to 30 carbon atoms; they have single carboxyl group and a long, non polar hydrocarbon tail; which gives most lipids their hydrophobic and oily or greasy nature. Fatty acids are the basic components of triglycerides (stored fat) and Phospholipids (cell membranes). Three fatty acids esterified in glycerol is triglyceride. Fat is triglyceride in solid form, and oil is triglyceride in liquid form. Fatty acids are classified as saturated (containing only single bonds) and unsaturated fatty acids (containing one or more double bonds). Compound with one double bond is mono unsaturated fatty acid and those with more than one double bond are polyunsaturated fatty acids. Most of the polyunsaturated fatty acids are also essential fatty acids as human body cannot synthesis them. Fatty acids are also classified based on its size or length of carbon chains. Accordingly there are short chain fatty acids(less than 8 carbon atoms), medium chain fatty acids(MCFAs) with8 to 12 carbon atoms and long chain fatty acids(LCFAs) with >12 carbon atoms. MCFAs are quickly and directly absorbed by the human body without getting deposited as fat. Hence they are considered superior to LCFAs in dietary. (Jain *et al.,* 2005) Present study concentrated on a comparative analysis of fatty acid profile of oil from both the sweet type and the normal type nuts in *'Mohachao Narel*', at various seasons.

MATERIALS AND METHODS

A field survey was conducted during summer season in the initial stage of the study, at Guhagar taluk in Ratnagiri district of Maharashtra. A total of 27 mother palms possessing nuts with sweet kernel ('NSD') have been identified in this area. The identified palms were marked for further studies. In this study, as many mature nuts as possible from each of the 27 palms were harvested during three seasons- pre monsoon , monsoon and post monsoon. The collected nuts were transported to CPCRI, Kasaragod.

Nuts were dehusked, broken and categorized

Table 2 Area percentage of fatty acid components of oil from normal and sweet type nuts at various seasons

Fatty acid	Area Percentage							
	Normal				Sweet			
	Premonsoon	Monsoon	Post monsoon	CD	Premonsoon	Monsoon	Post monsoon	CD
C6 (caproic acid)	0.36	0.40	0.51	0.02	0.25	0.23	0.22	ns
C8 (Caprylic acid)	6.52	7.06	9.23	0.43	4.15	3.57	4.27	0.16
C10 (capric acid)	4.60	5.51	6.38	0.66	2.64	2.30	3.05	0.12
C12 (lauric acid)	47.72	46.89	49.34	0.76	33.64	32.43	38.28	0.83
C14 (myristic acid)	21.60	18.23	16.97	0.90	20.95	21.17	20.95	ns
C16 (Palmitic acid)	7.45	7.61	6.79	0.68	14.51	14.58	12.67	0.78
C18 (Stearic acid)	2.32	2.37	1.90	0.35	2.66	1.88	2.21	0.49
C18:1 (Oleic acid)	4.14	6.31	4.49	0.63	15.78	16.45	14.73	0.74
C18:2 (Linoleic acid)	1.08	1.60	1.23	0.15	5.09	4.88	3.52	0.39
C24 (Lignoceric acid)	0.13	0.14	0.12	0.01	--	--	--	--

them as sweet or normal types, on the basis of organoleptic test. Two nuts collected from the individual experimental palms processed to copra for oil extraction. Oil was extracted from the copra, using petroleum ether, in socs plus extractor (Sadasivam and Manikam, 1996). Clear oil was obtained after the evaporation of solvent .Oil from ten nuts, each of both types (sweet and normal) were bulked together for three different seasons and were separately analyzed for fatty acid profile. The analysis was replicated thrice for each type per season and the mean values were reported. The oil samples (200mg) were used for methyl esterification and the gas liquid chromatography (GLC) performed, following the method of Padua Resurrection and Banzon (1979). The identification of the peaks was carried out by retention times. The concentration of each fatty acid was calculated by dividing the area of the fatty acid by the sum of all peak areas of fatty acids.

RESULT AND DISCUSSION

Individual fatty acid concentration was worked out and presented here. Fatty acid compositions were discussed in terms of saturated, unsaturated, mono-unsaturated, poly-unsaturated, short and medium chain saturated fatty acids (SMCSFAs) and long chain saturated fatty acids (LCSFAs). Significant variations in concentration of individual fatty acids between these two types of nuts (fig.1 & 2) and seasons were observed. The data on fatty acid composition of oil from both types of nuts in all three seasons together and those in three seasons separately is presented in table-1and table-2 respectively.

Saturated fatty acids (SFAs)

The data on different fatty acids of the two types showed that, there was significant variation in the area percentage of fatty acids in oils from both. It was clear from the result that coconut oil contained mostly saturated fatty acids. Among the saturated fatty acids, lauric acid (C12) was present in maximum concentration. Myristic acid (C14) and Palmitic acid (C16) were also present in appreciable concentrations. Other saturated fatty acids present were caproic acid (C6), Caprylic acid (C8), Capric acid (C10), stearic acid (C18), and Lignoceric acid (C24). The concentration of all the SMCSFAs (C6 to C12) was significantly higher in the normal type compared to those in the sweet type (Table-1). But the concentration of LCSFAs (C14, C16 and C18) was significantly higher in sweet type than those in the normal type. Concentration of palmitic acid was almost double (13.92%) in sweet type (Table-1). The data showed that Lignoceric acid was present only in normal type. Concentration of lauric acid was the highest (47.99%) in normal type, while it was only 34.78% in sweet type (Table 1). The result of normal nut is in line with Gregorio (2005) and Gopala *et al*. (2010), they reported that coconut oil is rich source of lauric acid. Lauric acid has been reported to have antimicrobial properties (Hoffman *et al.*, 2001; Ouattar *et al*., 2000; Dawson *et al.*, 2002; Alexey Ruzin and Richard, 2000). In addition, Lauric acid has been found to increase high-density lipoprotein (HDL) or good cholesterol and has a more favorable effect on lowering the total to HDL cholesterol ratio than any other fatty acid (Mensink *et al.,* 2003; Thijssen and Mensink, 2005). Lauric acid comprises about half of the fatty acid content in coconut oil. Only other plant source of lauric acid is palm kernel oil. Otherwise it is found in human breast milk, cow's milk and goat's milk.

Concentration of C6, C8 and C10, in normal nut, showed a steady and significant increase from pre monsoon to post monsoon, through the monsoon season. But in sweet type the variation in C6 was not significant; while C8 and C10 showed the highest concentration during post monsoon, followed by pre monsoon and monsoon (Table-2). Concentration of lauric acid, in both type of nuts were highest during post monsoon and the least during monsoon season (Table-2). Concentration of C14 was significantly higher (21.6%) during pre monsoon, compared to monsoon and post monsoon seasons, in normal nuts; while, in sweet type there was no significant variation (Table-2). Concentration of palmitic acid was higher during monsoon season, in both types of nuts, followed by pre monsoon and post monsoon seasons (Table-2). Stearic acid concentration was higher and on par during pre monsoon and monsoon seasons, in normal nut. But in sweet type it showed higher value (2.66%) during pre monsoon and the least (1.88%) during monsoon season (Table-2).

Unsaturated fatty acids (USFAs)

Two USFAs were recorded in both types of nuts. They were oleic acid (C18:1) and linoleic acid (C18:2). Concentration of oleic acid (15.66%) and linoleic acid (4.50%) was higher in sweet type, compared to normal type (4.98% and 1.30% respectively) (Table-1). Compared to the saturated fatty acids, both types showed lower concentration of unsaturated fatty acids. Higher amount (20.16%) of unsaturated fatty

acids was in sweet type, while the normal type had 6.28% only (Table-3). Oil with higher content of unsaturated fatty acids is more vulnerable to lipid peroxidation or rancidity. On the other hand, the unsaturated fatty acids increase the levels of good cholesterol (HDL) by taking the LDL to the liver to be broken down and removed from the body (Mensink R. P., and Katan M. B. 1989). Hence from dietary angle sweet type is desirable. Amount of oleic acid (6.31%) and linoleic acid (1.60%) was significantly higher during monsoon season, in normal nut; while in sweet nut, concentration of oleic acid and linoleic acid was significantly higher and on par during pre monsoon and monsoon seasons (Table-2).

Table 3. Area percentage and ratio of SFAs and USFAs (all seasons)

Nut type	SFAs	USFAs	SFAs/USFAs
Normal	90.05	6.28	14.34
Sweet	78.87	20.16	3.91

Saturated fatty acids to unsaturated fatty acids ratio

Total saturated fatty acids is higher in normal type (90.05%) compared to the sweet type (78.87%). But the sweet type showed higher value of total unsaturated fatty acids (20.16%) compared to the normal type (6.28%) (Table-3). The ratio of saturated fatty acid to unsaturated fatty acid was 14.34% in normal type, while it was only 3.91% in sweet type (Table-3). Amount of total SFAs was higher during post monsoon season, in both types of nuts, followed by pre monsoon and monsoon seasons (Table-6). Concentration of total USFAs was significantly higher during monsoon, in both types of nuts (Table-6). The ratio of saturated fatty acid to unsaturated fatty acid was significantly higher during pre monsoon (17.38), followed by post monsoon (15.92)and monsoon (11.15),in normal type; while it was higher during post monsoon (4.47), followed by pre monsoon (3.78) and monsoon (3.57) seasons (Table-6). Oil with lower ratio of saturated fatty acid to unsaturated fatty acid favorably influence the LDL/HDL cholesterol ratio and is desirable in the diet (Muller *et al.,* 2003). The present study showed lower ratio of saturated fatty acid to unsaturated fatty acid in sweet type and hence desirable for dietary purposes.

Table 4. Area percentage and ratio of SMCSFAs and LCSFAs (all seasons)

Nut type	SMCSFAS	LCSFAS	SMCSFAS /LCSFAS RATIO
Normal	61.51	28.54	2.16
Sweet	41.68	37.19	1.30

Table 5. Area percentage of Mono and Poly Unsaturated fatty acids (all seasons)

Nut type	MUSFAs	PUSFAs	Total
Normal	4.93	1.30	6.23
Sweet	15.66	4.50	20.16

Ratio of SMCSFAs and LCSFAs

The content of SMCSFAs (C12 and less) and LCSFAS (>C12) and the ratio of SMCSFAs to LCSFAS varied in these two types. The area percentage of short and medium chain saturated fatty acids (SMCSFAs) and long chain saturated fatty acids (LCSFAs) and their ratio are presented in the Table-4.The result indicated that short and medium chain saturated fatty acids (SMCSFAs) was higher (61.51%) in normal type and lower (41.68%) in sweet type. Long chain saturated fatty acids (LCSFAs) were higher (37.19%) in sweet type, while it was lower (28.54%) in normal type. The ratio of SMCSFAs/ LCSFAS was higher (2.16) in normal. It was lower (1.30) in sweet type. Amount of SMCSFAs was significantly higher during post monsoon season, in both types of nuts; while those during pre monsoon and monsoon seasons were on par (Table-7). Concentration of LCSFAs was higher during pre monsoon, followed by monsoon and post monsoon seasons, in both types of nuts (Table-7). The ratio of SMCSFAs/LCSFAS was higher during post monsoon, and it was almost similar during the other seasons, in both types of nuts (Table-7). Saturated fatty acids are considered undesirable in the diet mainly because of the presence of LCSFAS. The SMCSFAs are directly absorbed and not deposited as fat and hence are not harmful for human consumption, on the contrary there are some benefits of including medium chain fatty acids in the diet (Temme *et al.*, 1997; Mensink *et al*., 2003). A high ratio of SMCSFAs/ LCSFAS in the diet has health benefits (Hoffman, 2001; Mensink and Katan, 1992). The high ratio of SMCSFAs/LCSFAS in normal type compared to the sweet type is desirable.

Table 6. Area percentage and ratio of SFAs and USFAs during various seasons

Nut type	Season	Saturated Fatty acid (SFAs)	Unsaturated Fatty acid (USFAs)	SFAs/ USFAs
Normal	Pre monsoon	90.71	5.22	17.38
	Monsoon	88.21	7.91	11.15
	Post monsoon	91.23	5.73	15.92
Sweet	Pre monsoon	78.81	20.87	3.78
	Monsoon	76.15	21.34	3.57
	Post monsoon	81.66	18.25	4.47

Mono and poly unsaturated fatty acids

The area percentage of monounsaturated fatty acids (MUSFAs) and polyunsaturated fatty acids(PUSFAs) are presented in table-5. From the analysis it was observed that both MUSFAs and PUSFAs were higher in sweet type. The analysis showed that oleic acid (18:1) was the only monounsaturated fatty acid present in both the types and the polyunsaturated fatty acid present was linoleic acid (18:2). The area percentage of monounsaturated fatty acid (oleic acid) was 15.66% in sweet type and 4.93% only in normal type. The area percentage of polyunsaturated fatty acid (linoleic acid) was 4.50% in sweet type and 1.30% in normal type (Table-5). Concentration of MUSFAs was significantly higher during monsoon (6.31%), followed by pre monsoon

Table 7. Area percentage and ratio of SMCSFAs and LCSFAs during various seasons

Nut type	Season	SMCSFAs	LCSFAs	SMCSFAs/ LCSFAs
Normal	Pre monsoon	59.20	31.50	1.88
	Monsoon	59.86	28.35	2.00
	Post monsoon	65.46	25.78	2.54
Sweet	Pre monsoon	40.69	38.12	1.07
	Monsoon	38.53	37.62	1.02
	Post monsoon	45.83	35.84	1.28

Table 8. Area percentage of mono and poly unsaturated fatty acids during various seasons

Nut type	Season	MUSFAs	PUSFAs	Total
Normal	Pre monsoon	4.14	1.08	5.22
	Monsoon	6.31	1.60	7.91
	Post monsoon	4.49	1.23	5.72
Sweet	Pre monsoon	15.78	5.09	20.87
	Monsoon	16.45	4.88	21.33
	Post monsoon	14.73	3.52	18.25

(15.78%) and post monsoon (14.73%), in sweet type; while in normal type it was higher during monsoon (6.31%), followed by post monsoon (4.49%) and pre monsoon (4.14%) (Table-8). Amount of PUSFAs was higher during pre monsoon (5.09%), followed by monsoon (4.88%) and post monsoon (3.52%), in sweet type. It was higher during monsoon (1.60%), followed by post monsoon (1.23%) and premonsoon (1.08%) in normal type nut (Table-8).The high content of MUSFAs, especially oleic acid is associated with a low incidence of coronary heart disease (CHD) because it decreases total cholesterol (10%) and low-density lipoprotein cholesterol (Dennys *et al*., 2006; Mensink and Katan, 1989; Rickman, 2004). It was reported earlier that oleic acid blocks the action of a cancer-causing oncogene, called HER-2/neu, which is found in about 30% of breast cancer patients (Menendez *et al*., 2005). Present study showed that total concentration of MUSFAs and PUSFAs was almost three times higher in sweet type compared to that in normal type , during all seasons (Table-5 and 8) and hence the sweet type is desirable for dietary purposes.

The results of the present study showed that the coconut oil profile in terms of saturated, unsaturated, mono-unsaturated, poly-unsaturated, short and medium chain saturated fatty acids (SMCSFAs) and long chain saturated fatty acids (LCSFAs) were different in sweet type nut and normal type nut, during all seasons. It was observed that percentage of short and medium chain saturated fatty acids (SMCSFAs) was more in normal type compared to sweet type. Higher percentage of SMCSFAs found in normal type was due to presence of more lauric acid, caprylic acid and caproic acid. Coconut oil is used for human

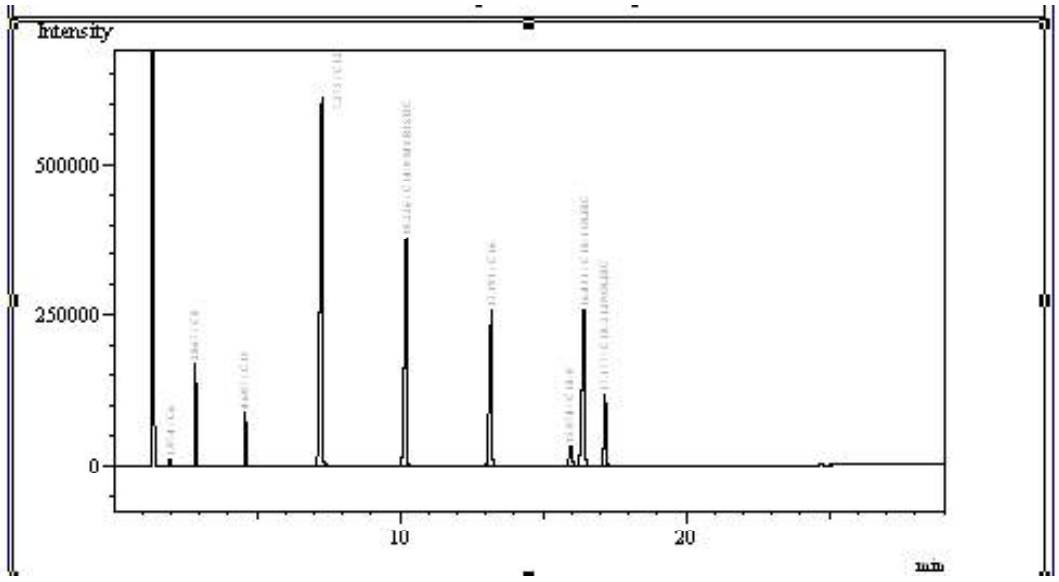

Fig. 1. Chromatogram of oil from sweet kernel

consumption, cosmetic and industrial purposes. Quality required for human consumption is different from that of other uses. Healthy dietary requirement is the major factor deciding the selection of oil for consumption. Coconut oil quality from health angle has been under scrutiny for some time now. It was considered unhealthy because of high saturated fatty acid content around 90% in the coconut oil. But, recent studies have shown that the medium chain fatty acids which constitute more than 65% of saturated fatty acids in coconut oil are good for health (Dayrit, 1995). According to Rajan (2006), coconut oil is the healthiest oil on earth because it contains higher content of short and medium chain saturated fatty acids (SMCSFAs). SMCSFAs are directly absorbed from the intestine to portal veins and transferred to liver for utilization as ready source of energy (B-oxidation in the mitochondria). They are not usually circulated or stored, nor they undergo oxidative stress, and hence SMCSFAs prove to be most protective and safe for human consumption. He also revealed that coconut oil is Cardio protective and prevents degenerative diseases including cancers. The importance of SMCSFAs was also reported by Blackburn *et al*.,(2001) and Enig (2001). According to them SMCSFAs do not contribute to the low- density

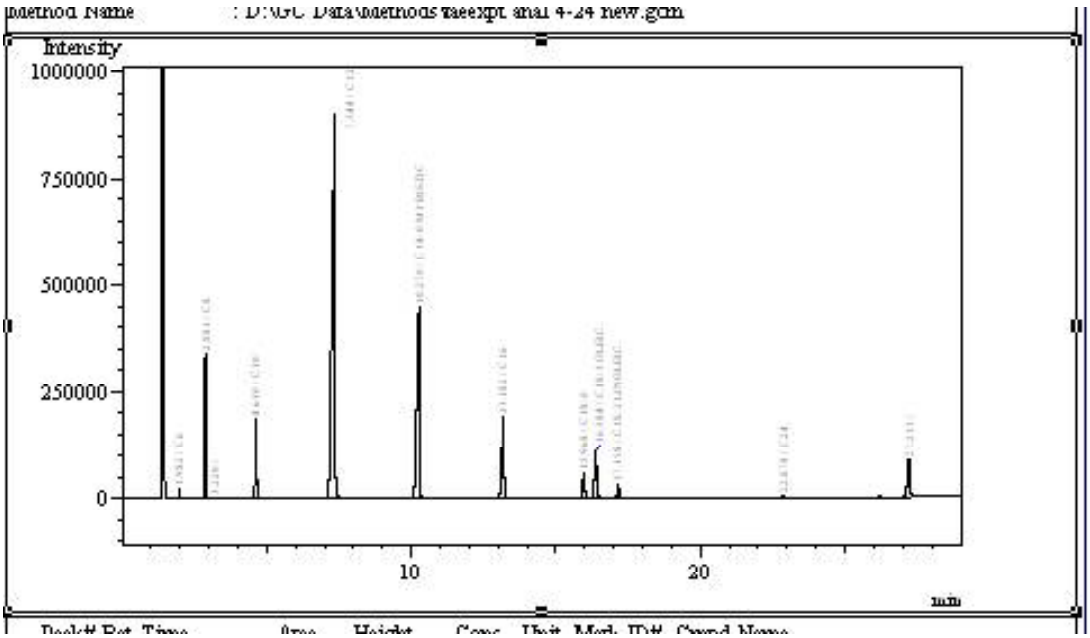

Fig. 2. Chromatogram of oil from normal kernel

lipoprotein (bad cholesterol) in the body. Moreover, Lauric acid content in coconut oil which is approximately 50% lead to increase in high-density lipoprotein (HDL) and has favorable effect on lowering the total to HDL cholesterol ratio than any other fatty acid (Mensink *et al.,* 2003; Thijssen and Mensink, 2005). The result of present study revealed that oil from normal type nut is better in giving health benefit compared to the sweet type. The higher concentration of lauric acid, SMCSFAs and the higher ratio of SMCSFAs/LCSFAS in the oil of normal type nuts makes them more suitable for human consumption.

On the other hand oil from sweet type nuts had lower level of saturated fatty acids, lower ratio of saturated fatty acid to unsaturated fatty acid, higher level of unsaturated, mono and polyunsaturated fatty acids in the oil of sweet nuts, compared to that from the normal nut. Oil with lower ratio of saturated fatty acid to unsaturated fatty acid favorably influence the LDL/HDL cholesterol ratio and is desirable in the diet (Muller *et al.*,2003). This study showed lower ratio of saturated fatty acid to unsaturated fatty acid in sweet type and hence desirable for dietary purposes. The high content of MUSFAs, especially oleic acid is associated with a low incidence of coronary heart disease (CHD) because it decreases total cholesterol (10%) and low-density lipoprotein cholesterol (Dennys *et al*., 2006; Rickman, 2004). It was reported earlier that oleic acid blocks the action of a cancer-causing oncogene, called HER-2/neu, which is found in about 30% of breast cancer patients (Menendez *et al.*, 2005). Present study showed that concentration of MUSFAs and PUSFAs was three times higher (20.16%) in sweet type compared to that in normal type (6.23%). The present study revealed that the fatty acid composition of oil from sweet type nuts contains a healthy mixture of all the types of fatty acids. So coconut palms with sweet nuts, *'Mohachao Narel'* should be efficiently exploited for further breeding programme.

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Identification of Bioactive Compounds Present in the Crude Methanolic Leaf Extract of *Acalypha Fruticosa* Forssk. through GC-MS Analysis

A.M ANADAKUMAR[1]., S. JAMUNA[2], A. VENKATACHALAPATHI[2] AND S. PAULSAMY[2*]

[1]*Department of Botany, NGM Arts and Science College, Pollachi-642001, Tamil Nadu.*
[2]*Department of Botany, Kongunadu Arts and Science College, Coimbatore-641029, Tamil Nadu.*
**email: paulsami@yahoo.com*

ABSTRACT

The aim of the study is to characterize the bioactive chemical compounds present in crude methanolic leaf extract of *Acalypha fruticosa.* Qualitative determination of the different biologically active compounds from crude extracts of *A. fruticosa* using Gas Chromatography–Mass Spectrometry (GC-MS) revealed 15 types of high and low molecular weight chemical entities with varying quantities present in the leaf extracts. These chemical compounds are considered biologically and pharmacologically important. Thus, identification of different biologically active compounds in the methanolic leaf extracts of *A. fruticosa* warrants further biological and pharmacological studies.

Keywords *Acalypha fruticosa; Euphorbiaceae; crude extract; bioactive compounds.*

Medicinal plants are considered as rich resources of ingredients which can be used in drug development either pharmacopoeial, non-pharmacopoeial or synthetic drugs. It plays an appreciable role in the development of human cultures around the whole world. Moreover, some plants are considered as important source of nutrition and as a result of that they are recommended for their therapeutic values (Rooksarr *et al.,* 2017). Isolation of natural products from plants is still a challenging task. Undoubtedly hybrid methods like LC-NMR or GC-MS made on-line structure elucidation possible and provided impressive examples of natural product identification without prior isolation (Ammar *et al.,* 2017).

Acalypha fruticosa Forssk. belongs to the family Euphorbiaceae inhabiting the foot hills of Western Ghats of Coimbatore and Erode districts, Tamil Nadu, India. The leaves are prescribed for digestive troubles such as dyspepsia, colic and diarrhea. The root portion is used in Siddha system of medicine for curing gonorrhea (Pullaiah, 2006). Boiled root decoction is used to cure cerebral malaria (Sahoo, 2001). Despite these huge medicinal values still the medical properties of this plant is unexplored yet. Hence, the present work was aimed at to identifying the therapeutic bioactive compounds from the study species, *Acalypha fruticosa.*

MATERIALS AND METHODS

Collection of plant materials

The clean and healthy leaves of the study species, *Acalypha fruticosa* were collected from Chennimalai (highly arid), Erode district and they were shade dried.

Preparation of plant extracts

To know the presence of major phytochemicals, the shade dried leaves of the population of Maruthamalai were made into a fine powder of 40 mesh size using the pulverizer. Following that, 100 g of the powder was filled in the filter paper and successively extracted using 500 ml solvents *viz.* petroleum ether, hexane, chloroform and finally with methanol using the soxhlet extractor for 8 – 10 hours (Gafner *et al*., 1985). The extract was filtered through Whatman No.1 filter paper to remove all undissolved matter, including cellular materials and other constitutions that are insoluble in the extraction solvents.

Gas Chromatography - Mass Spectrometry (GC-MS) (Joachim and Hübschmann, 2008)

Gas chromatography (GC) analysis was carried out at South Indian Textile Research Institute (SITRA), Coimbatore. It is one of the key techniques generally used for screening/ identification of many groups of plant phytochemicals. The high attainable separation power in combination with wide range of the detectors employing various detection principles to which it can be coupled makes GC an important, often irreplaceable tool in the analysis at trace level of plant phytochemical compounds. Gas chromatographical study includes the important optimization process such as i) introduction of sample extract onto the GC column, ii) separation of its components on an analytical column and iii) detection of target analysis by using mass spectrometric (MS) detector.

Table 1. Chemical components of the methanolic extract of the *Acalypha fruticosa* leaves using GC-MS analysis.

S.No	Component	Molecular formula	Retention time/min.	Molecular weight (m/z)
1.	Benzene, Ethyl (CAS)-	C_8H_{10}	2.230	106
2.	Lycorenan-7-One,1,8,9,10- Trimethoxy-1-Methyl-	$C_{19}H_{25}O_5N$	4.111	347
3.	Hexyl Tiglate	$C_{11}H_{20}O_2$	7.643	184
4.	2H- Indeno1,2-B Oxirene Octahydro-1A.Alpha 1B.B-	$C_9H_{14}O$	8.223	138
5.	Aromadendrene	$C_{15}H_{24}$	10.034	204
6.	1,5 Diazacyclododecan-6-One-	$C_{10}H_{20}ON_2$	10.784	184
7.	Phenol, 2,4,-Bis(1,1- Dimethyl ethyl)	$C_{14}H_{22}O$	12.506	206
8.	1-Dotriocantonal	$C_{32}H_{66}O$	13.987	466
9.	Butenoic acid 3,7 dimethyl 6-octenyl ester	$C_{14}H_{24}O_2$	14.086	224
10.	Vitamin A Aldehyde	$C_{20}H_{28}O$	18.809	284
11.	7, 9 -Di –Tert-Butyl-1-Oxaspiro [4,5] Deca – 6,9 Diene – 2,5-DI	$C_{17}H_{24}O_3$	19.680	276
12.	Cyclopenten-1-one, 4, hydroxyl – 3 methyl -2-(2,4-Pentadienyl)	$C_{11}H_{14}O_2$	19.980	178
13.	Citronellyl propionate	$C_{13}H_{24}O_2$	22.100	212
14.	Piperidine,1-(2-Phenylethyl)-(CAS) -	$C_{13}H_{19}N$	23.121	189
15.	1-Dodecene	$C_{12}H_{24}$	25.263	168

GC-MS analysis

Five ml of methanol extract was evaporated to dryness and reconstituted in 2 µl methanol. The extracts were then subjected to GC-MS analysis. Chromatographic separation was carried out with CE GC 8000 top MSMD 8000 Fyson instrument with Db 35 mr column (10 m x 0.5 mm, 0.25 ìm film thickness). Heating programs were executed from 100 - 250 ^{0}C at 3 minutes by using the helium was used as a carrier gas with a flow rate of 1 ml/min in the split mode (1:50). An aliquot (2 ìl) of oil was injected into the column with the injector heater at 250 ^{0}C.

Analytical conditions

Injection temperature at 250°C, interface temperature at 200°C, quadruple temperature at 150°C and ion source temperature at 230°C were maintained. Injection was performed in split less mode.

Data analysis

The mass spectra of compounds in samples were obtained by electron ionization (EI) at 70 eV, and the detector operated in scan mode from 20 to 600 atomic mass units (amu). Identifications were based on the molecular structure, molecular mass and calculated fragmentations. Resolved spectra were identified for phytochemicals by using the standard mass spectral database of WILEY and NIST.

RESULTS AND DISCUSSION

The methanol extracts of the leaves of *A. fruticosa* were obtained from dry habitat of Chennimalai (highly arid) and subjected to GC-MS analysis. The phytochemical components with their retention time (RT/min.), molecular formula and molecular weight (*m/z*) are presented in Table 1 and Figs. 1 - 15. The analysis by using GC-MS technique revealed the presence of following fifteen compounds: One compound of phenolic group, Phenol, 2,4,-Bis(1,1-Dimethyl ethyl), one compound of aldehyde group, Vitamin A aldehyde, one compound of alkane group, Cyclopenten-1-one, 4, hydroxyl - 3 methyl -2-(2,4-Pentadienyl), five compounds of alkaloid group, 2H-Indeno12-B Oxirene Octahydro-1A.Alpha 1B.B-, Lycorenan-7-One,1,8,9,10- Trimethoxy-1-Methyl-, Benzene Ethyl (CAS)-, Piperidine,1-(2-Phenylethyl)-(CAS) and quinone alkaloids of 1 - Dodecene, one compound of diterpenoid group, 7, 9 -Di –Tert-Butyl-1-Oxaspiro [4,5] Deca – 6,9 Diene – 2,5-DI, four compounds of saturated cyclic hydrocarbon group, Citronellyl propionate, Butenoic acid 3,7 dimethyl 6-octenyl ester, Hexyl Tiglate and 1,5 Diazacyclododecan-6-One, one compound of sesquiterpenoids group, Aromadendrene and one compound of fatty alcohol, 1-Dotriocantonal. They are mainly the constituents of essential oils such as aromatic phenolic compounds, and a single

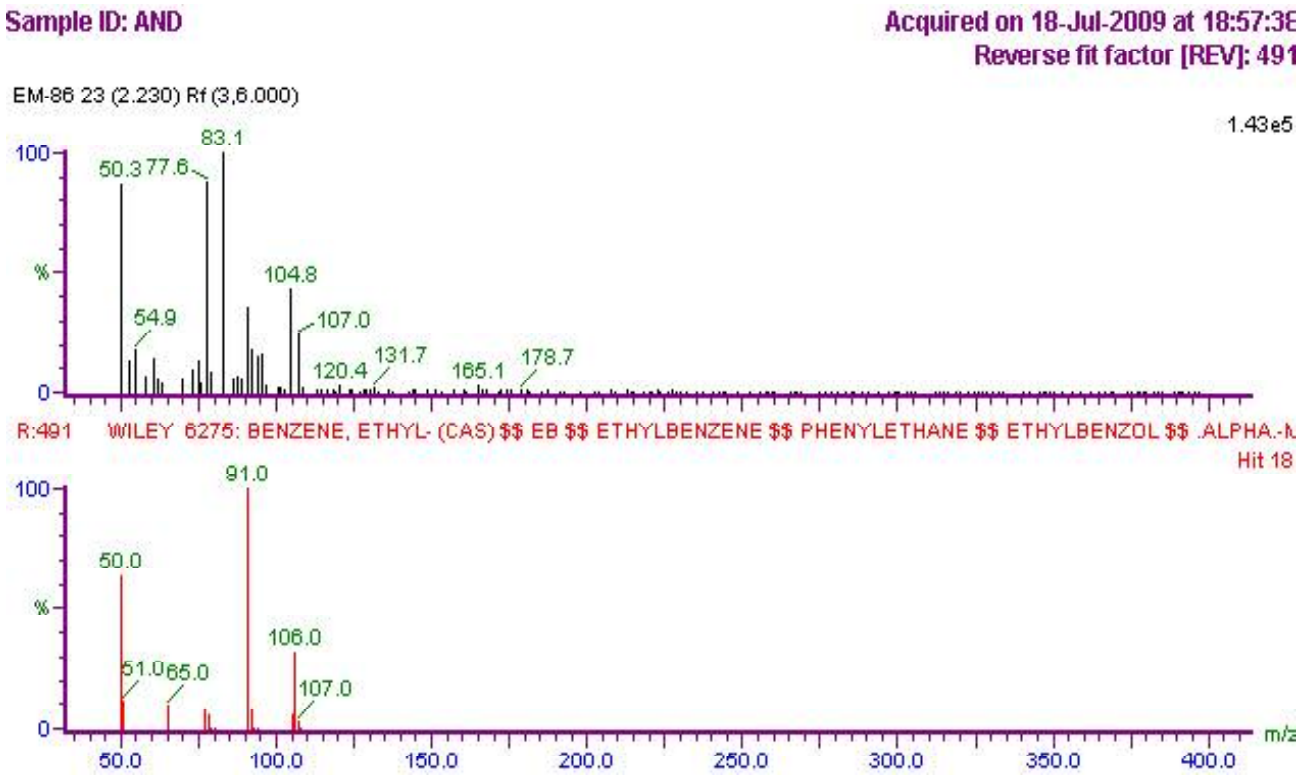

Fig. 1. Mass spectra of the methanolic extract component I of *Acalypha fruticosa* leaves of Chennimalai population.

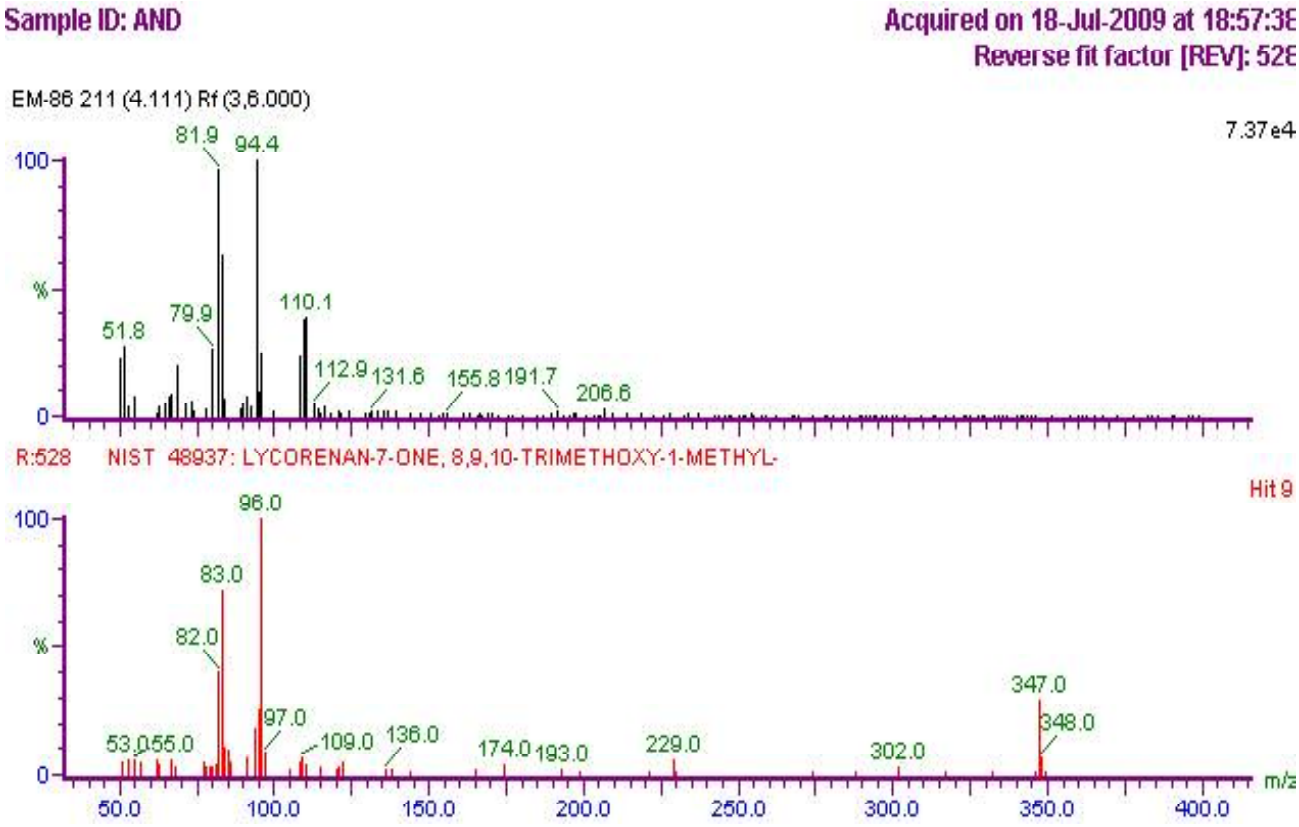

Fig. 2. Mass spectra of the methanolic extract component II of *Acalypha fruticosa* leaves of Chennimalai population.

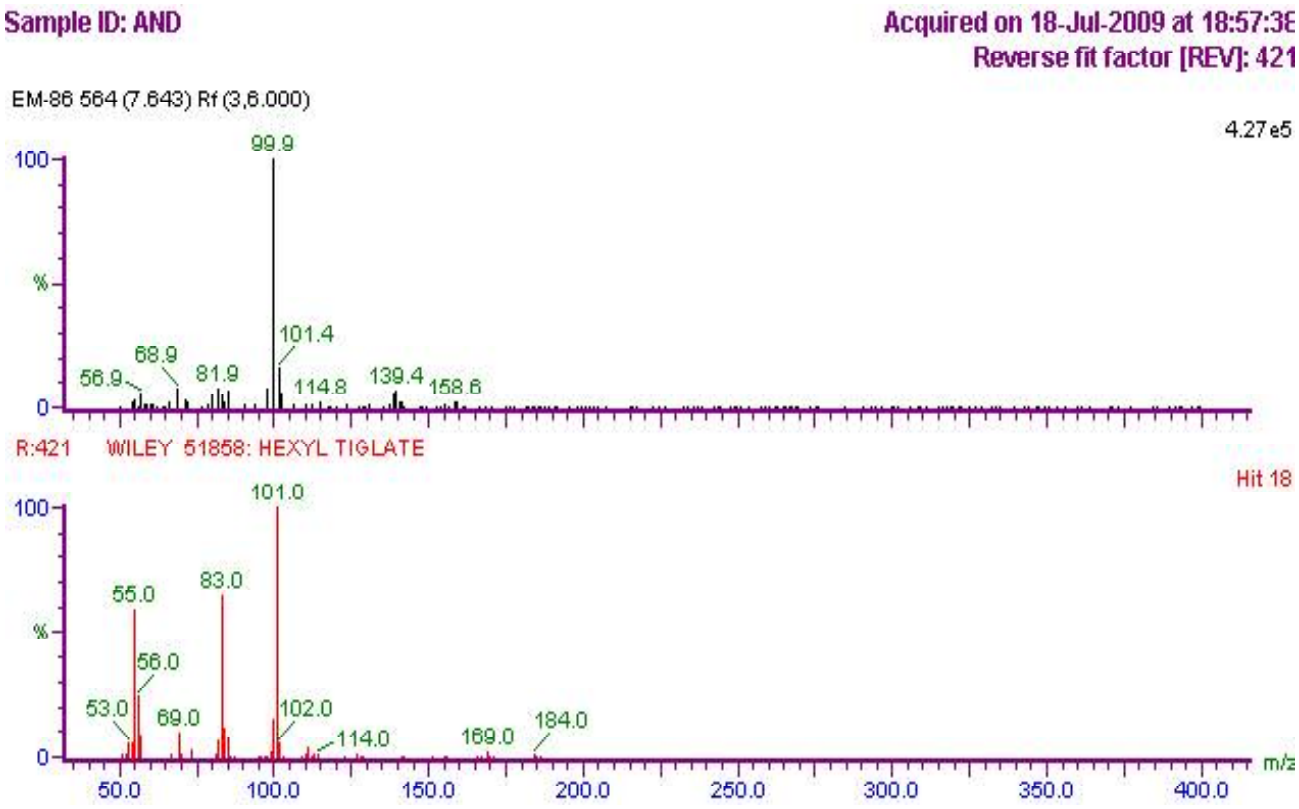

Fig. 3. Mass spectra of the methanolic extract component III of *Acalypha fruticosa* leaves of Chennimalai population.

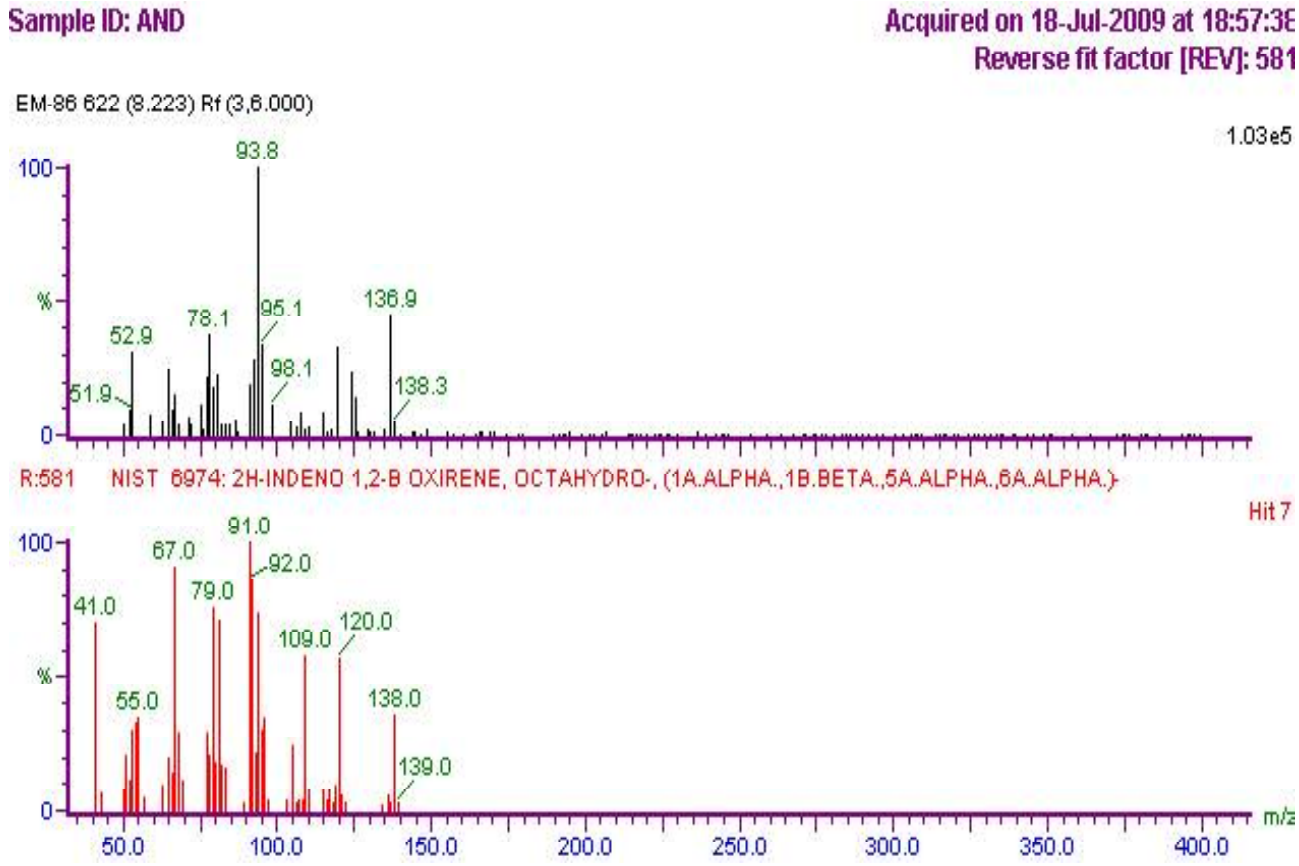

Fig. 4. Mass spectra of the methanolic extract component IV of *Acalypha fruticosa* leaves of Chennimalai population.

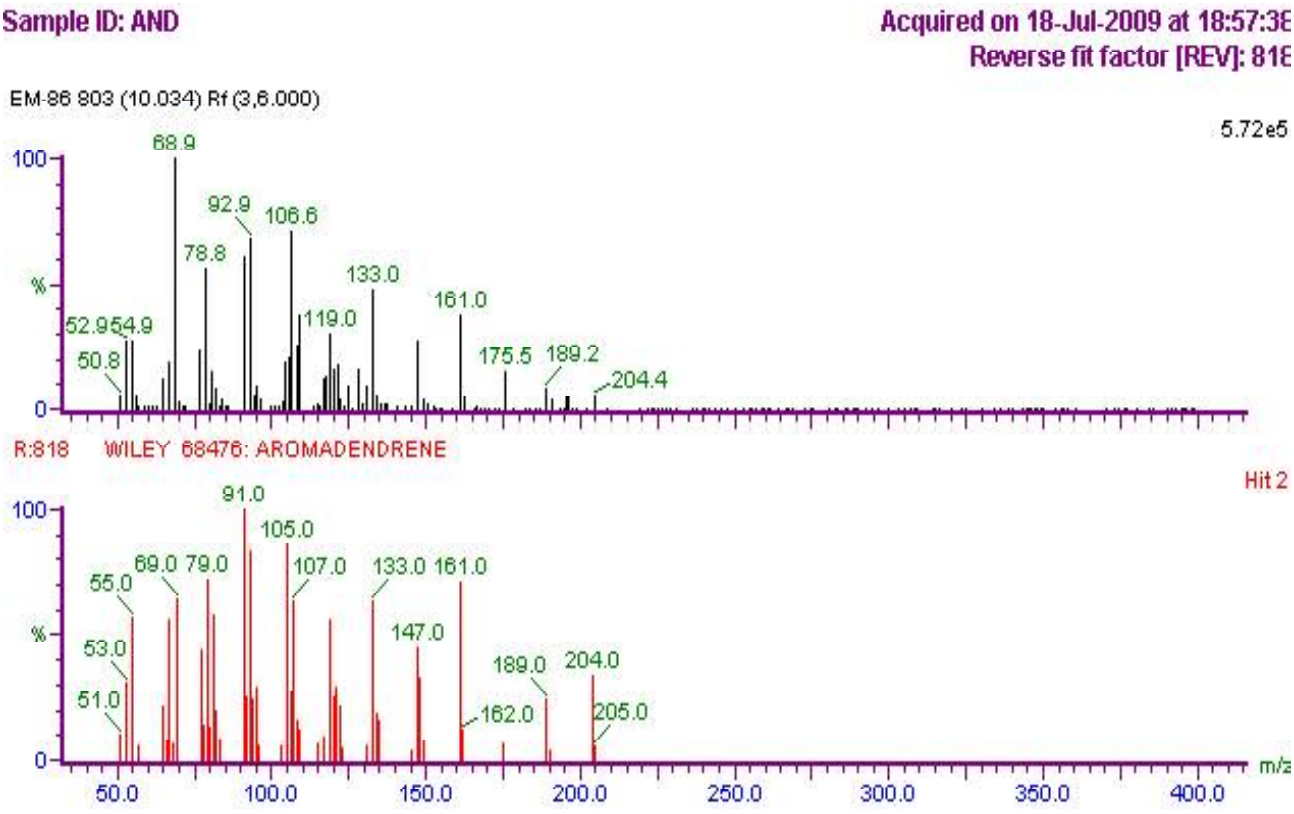

Fig. 5. Mass spectra of the methanolic extract component V of *Acalypha fruticosa* leaves of Chennimalai population.

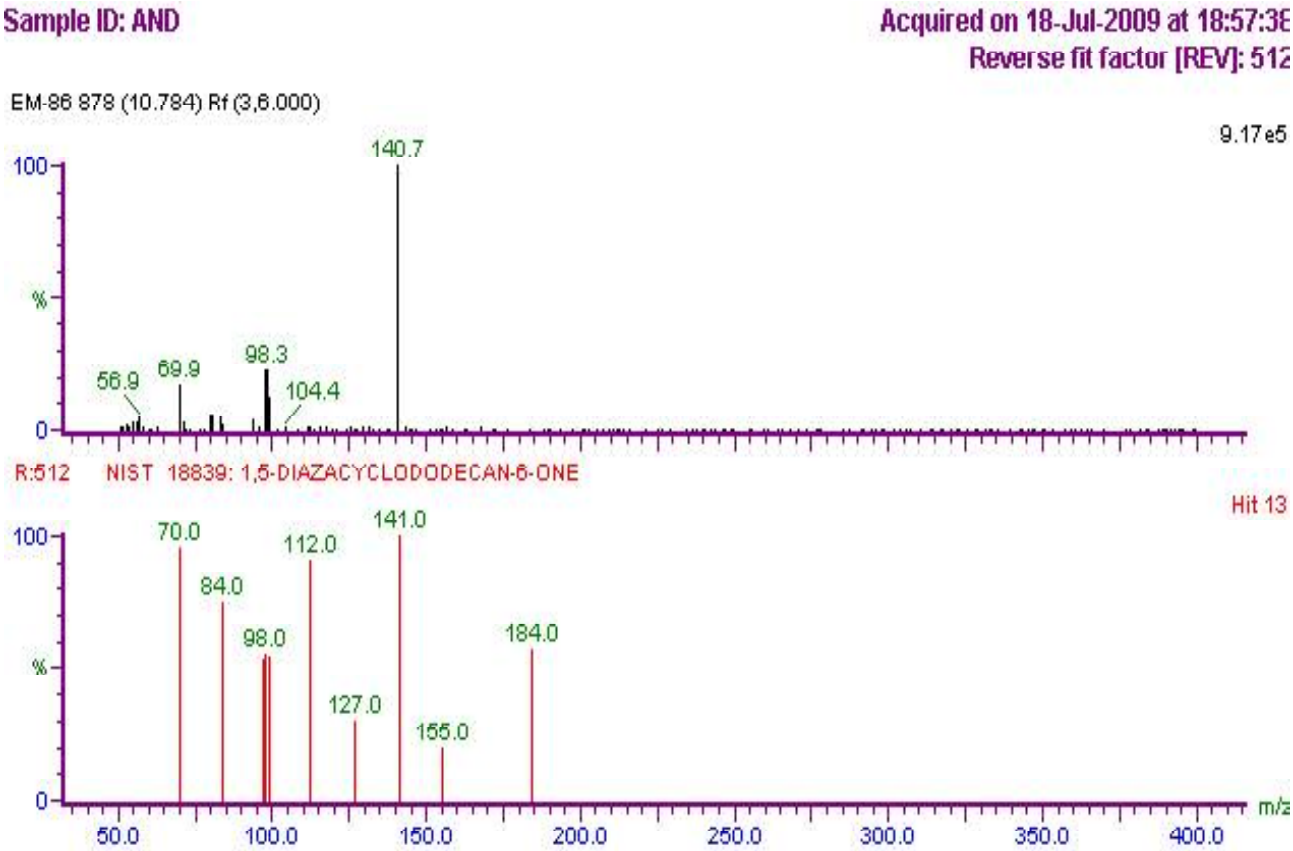

Fig. 6. Mass spectra of the methanolic extract component VI of *Acalypha fruticosa* leaves of Chennimalai population.

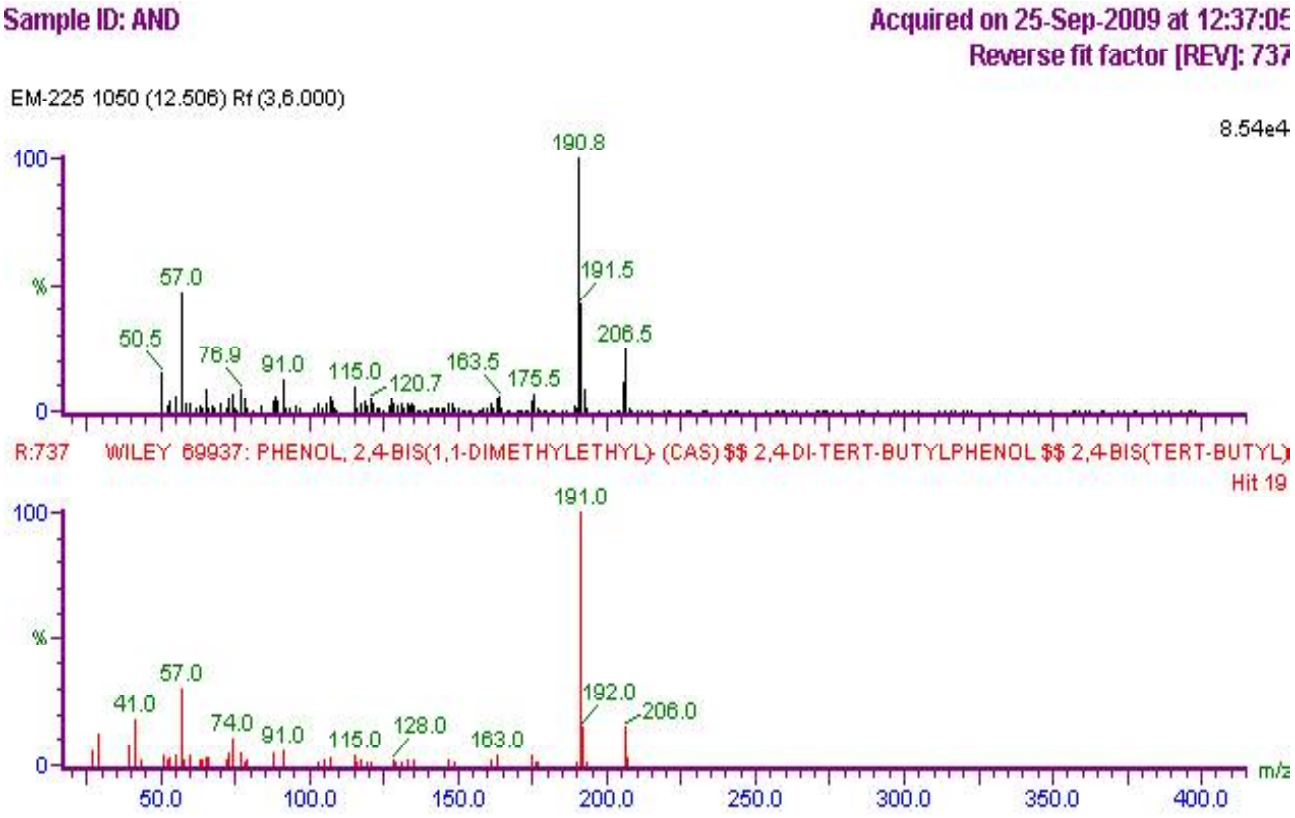

Fig. 7. Mass spectra of the methanolic extract component VII of *Acalypha fruticosa* leaves of Chennimalai population.

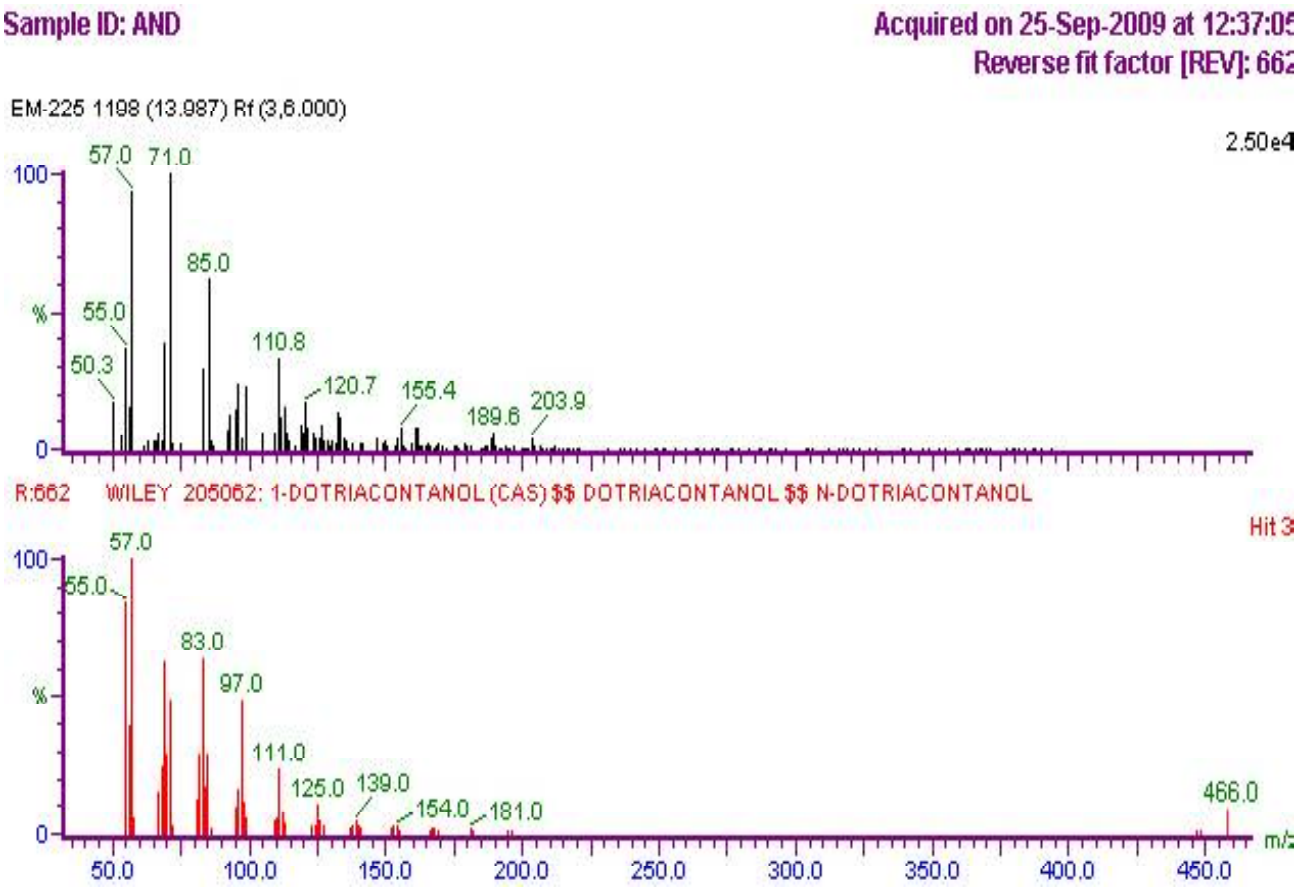

Fig. 8. Mass spectra of the methanolic extract component VIII of *Acalypha fruticosa* leaves of Chennimalai population.

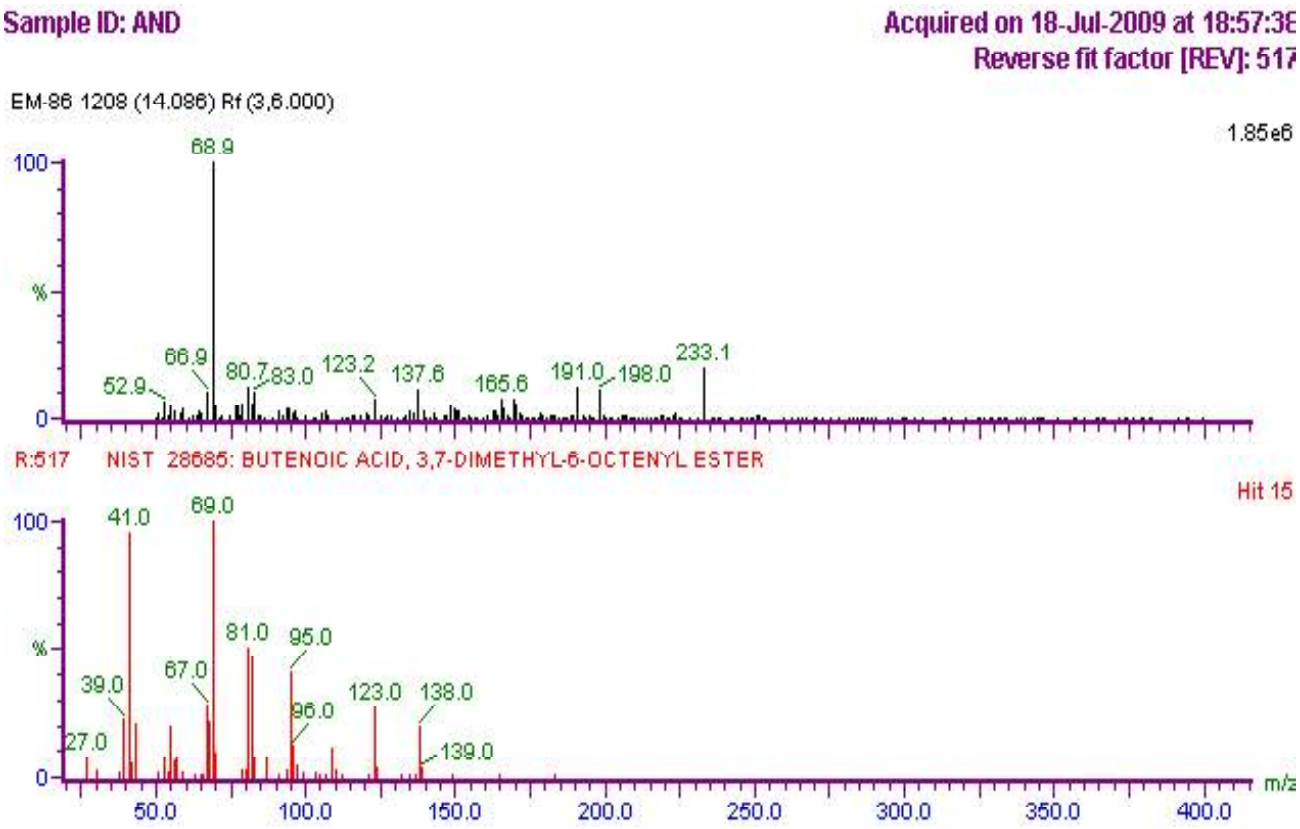

Fig. 9. Mass spectra of the methanolic extract component IX of *Acalypha fruticosa* leaves of Chennimalai population.

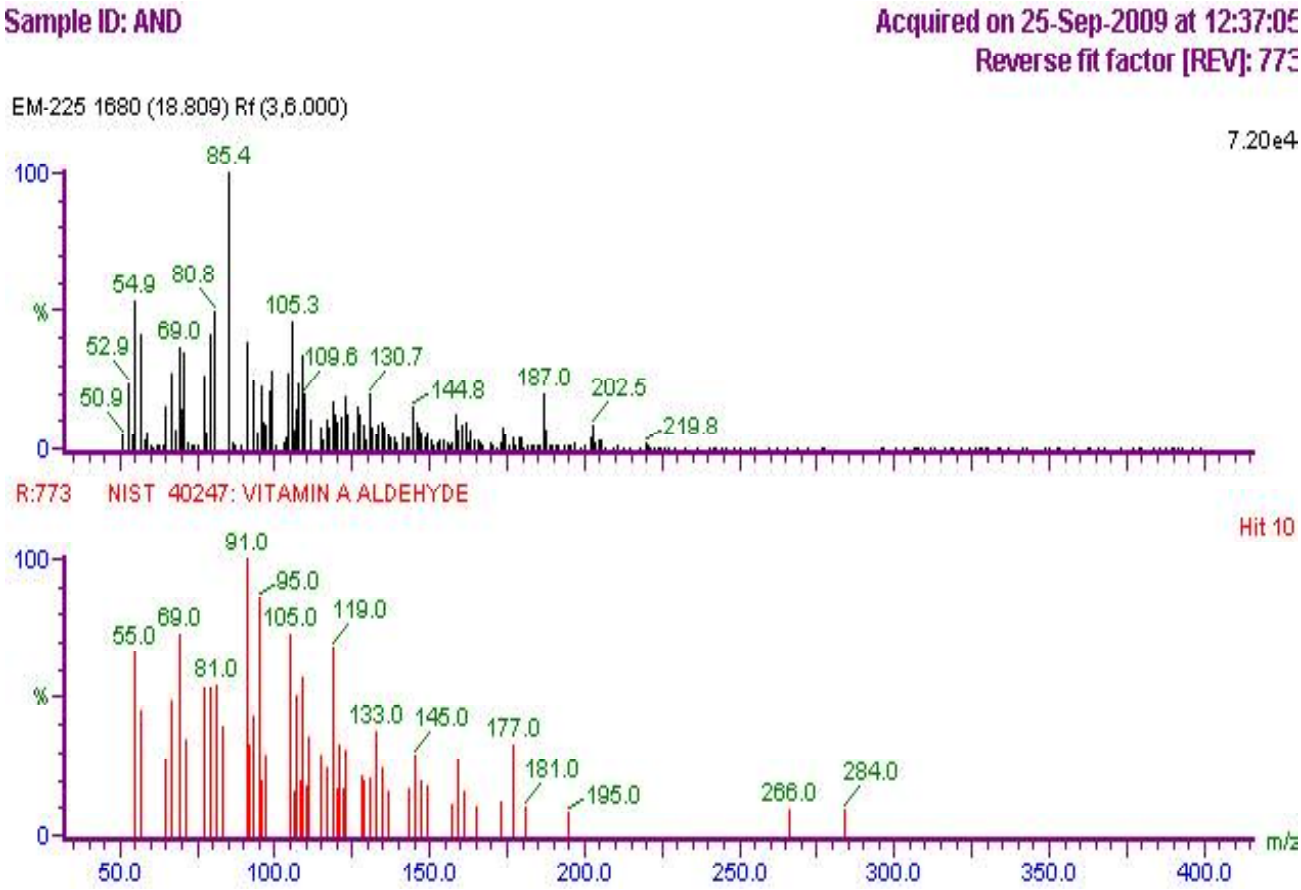

Fig. 10. Mass spectra of the methanolic extract component X of *Acalypha fruticosa* leaves of Chennimalai population.

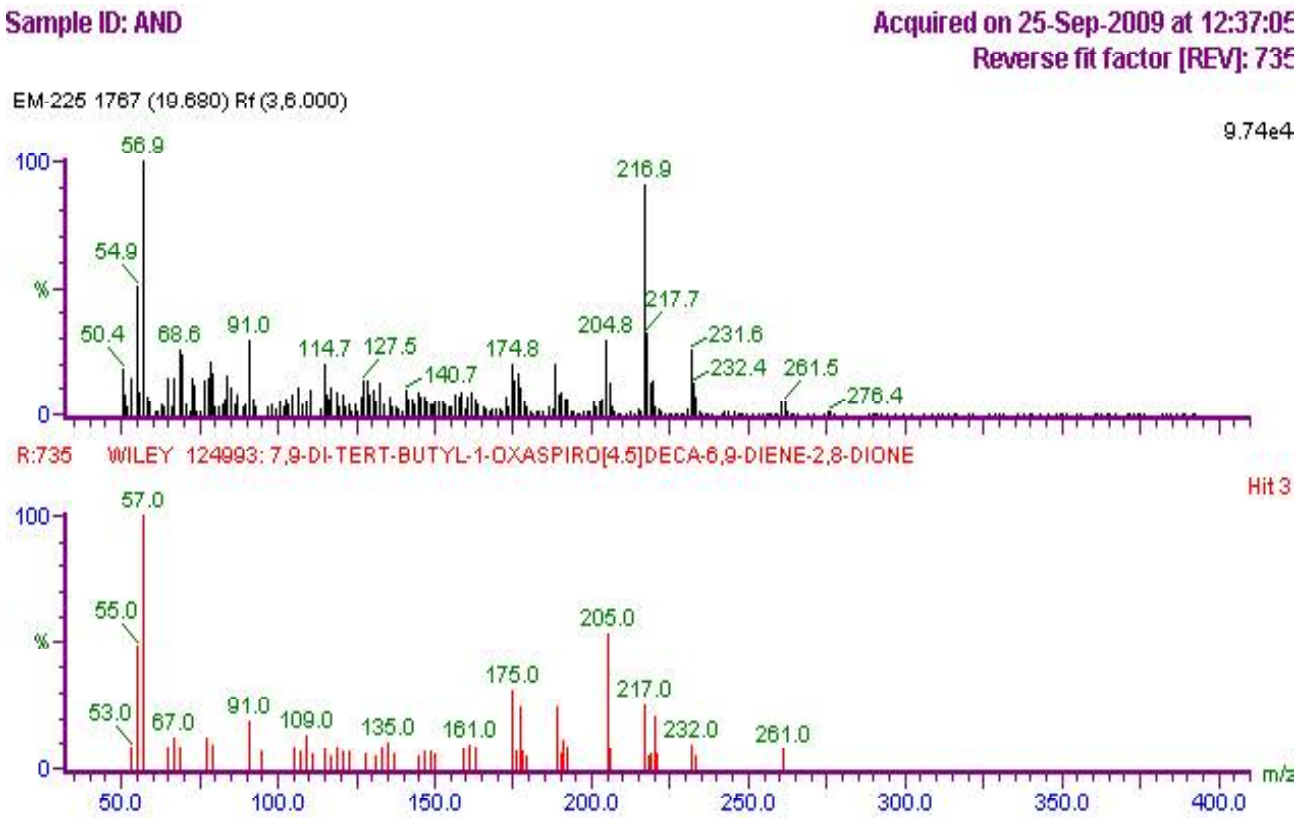

Fig. 11. Mass spectra of the methanolic extract component XI of *Acalypha fruticosa* leaves of Chennimalai population.

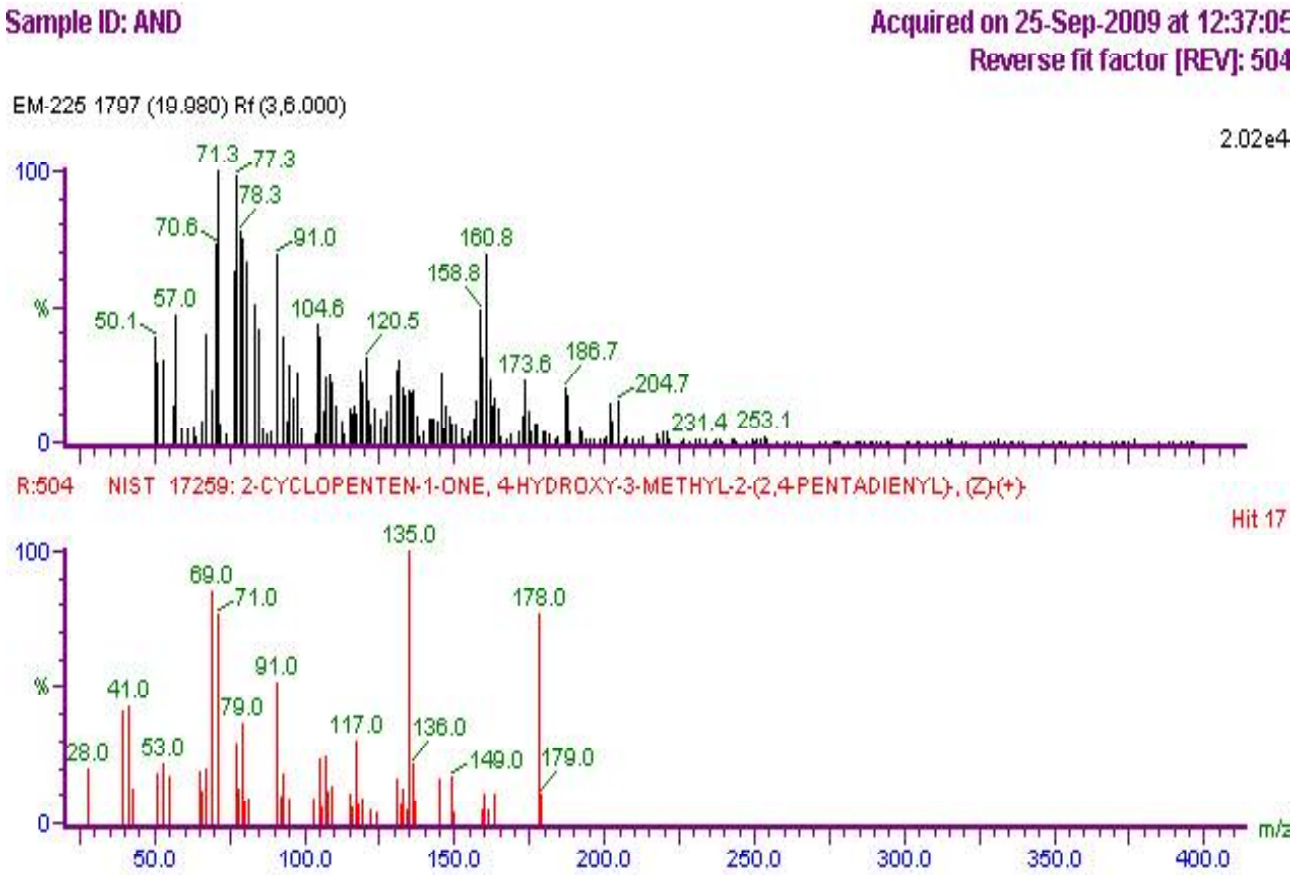

Fig. 12. Mass spectra of the methanolic extract component XII of *Acalypha fruticosa* leaves of Chennimalai population.

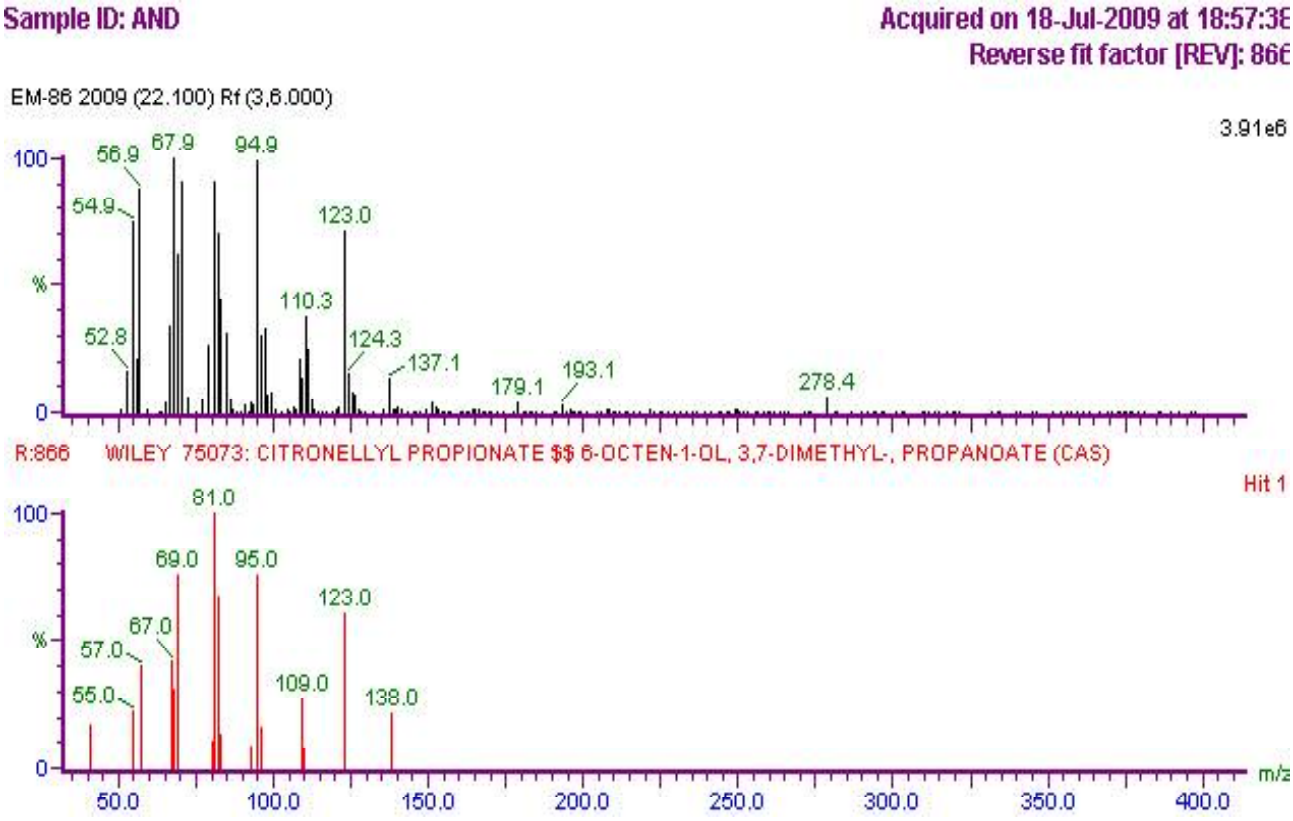

Fig. 13. Mass spectra of the methanolic extract component XIII of *Acalypha fruticosa* leaves of Chennimalai population.

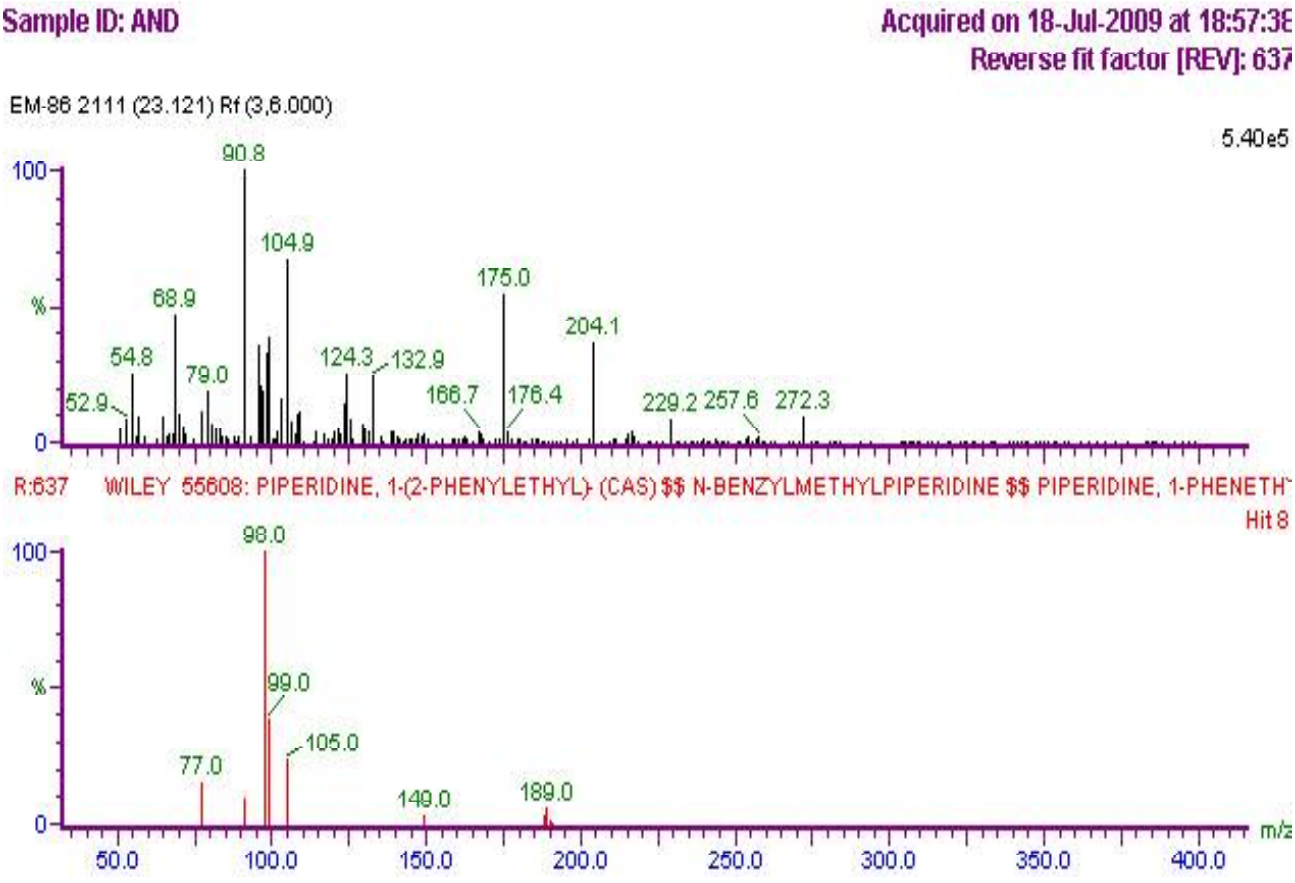

Fig. 14. Mass spectra of the methanolic extract component XIV of *Acalypha fruticosa* leaves of Chennimalai population.

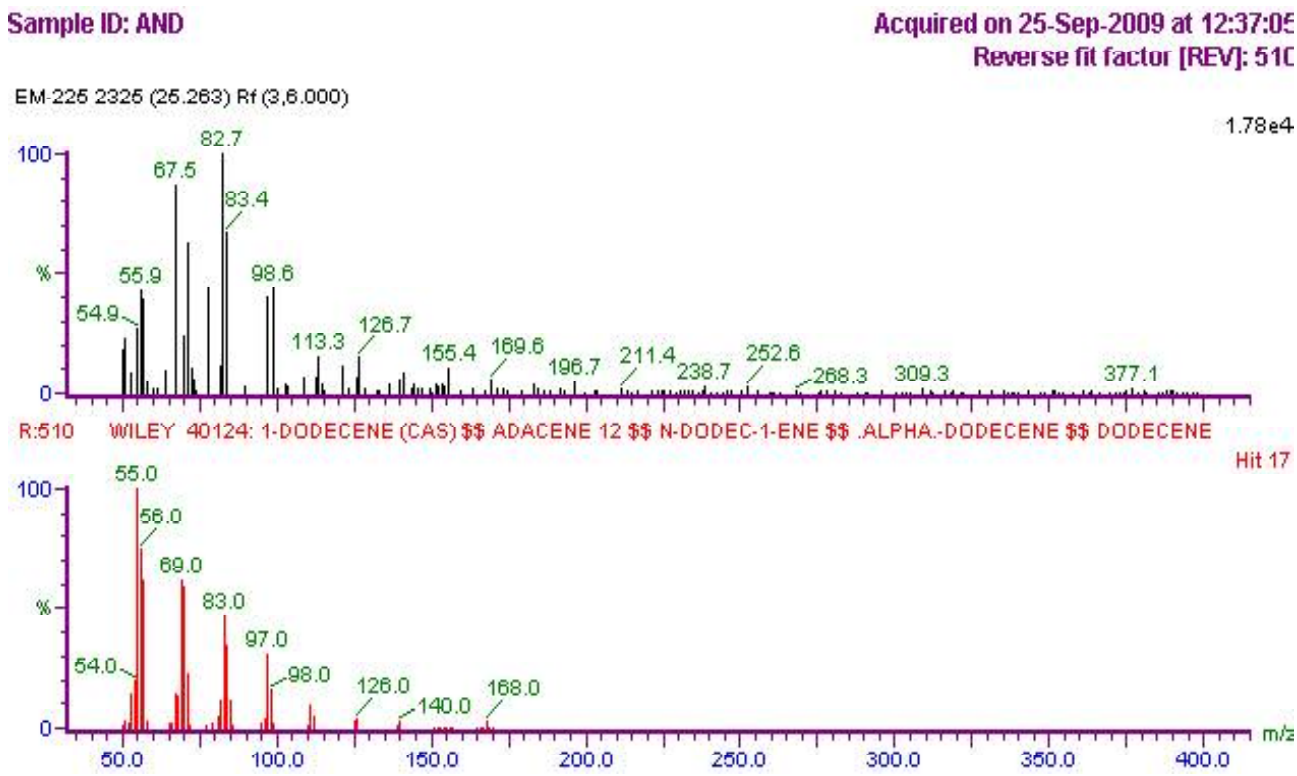

Fig. 15. Mass spectra of the methanolic extract component XV of *Acalypha fruticosa* leaves of Chennimalai population.

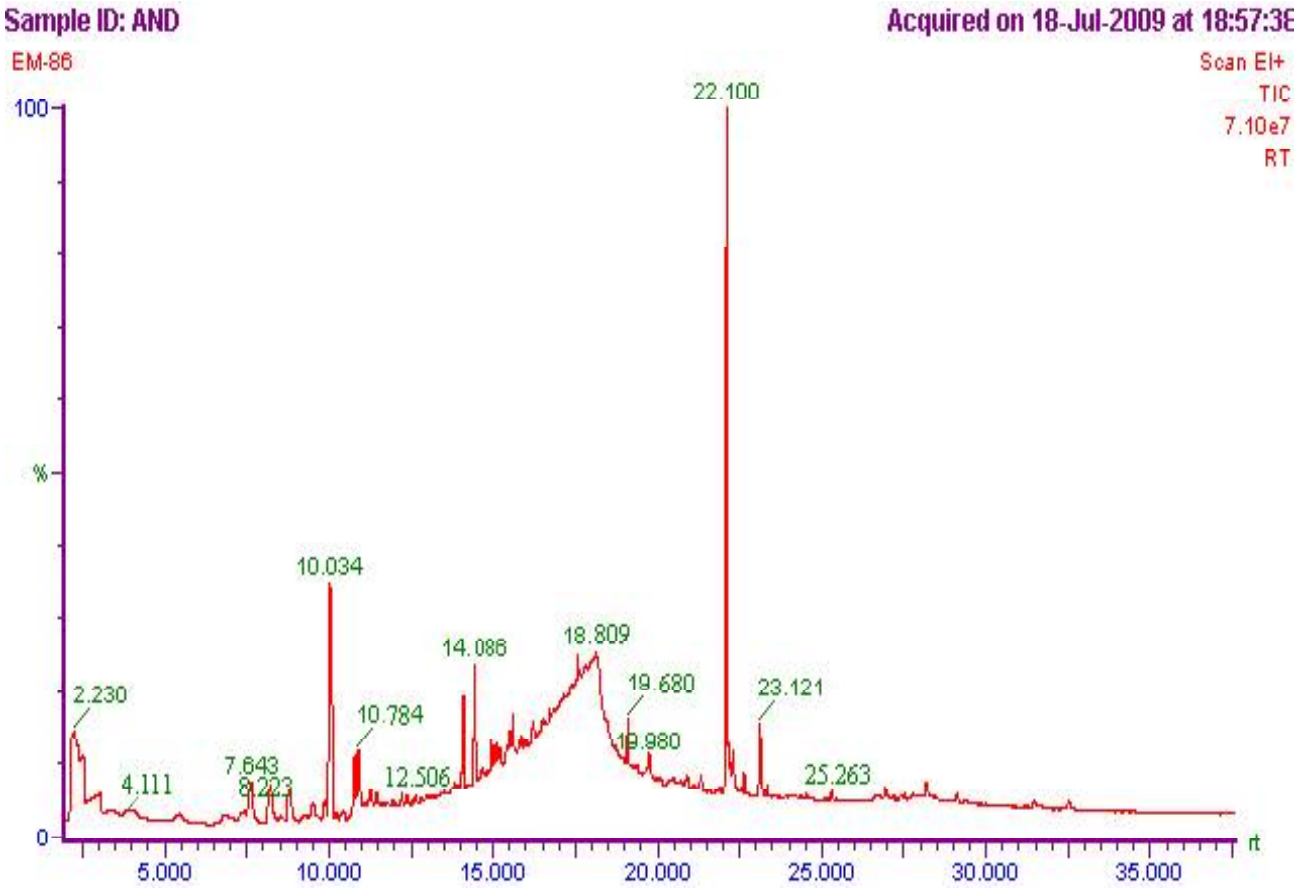

Fig. 16. Gas chromatogram of the methanolic extract of *Acalypha fruticosa* leaves of Chennimalai population.

diterpenoids, 1-Dodecene which has been reported to have many medicinal properties (Alma *et al.*, 2003). (Lei *et al.,* 2008) reported that this Dodecene diterpenoid natural compound have served as anti-tumor promoting agents. Generally terpenoids are an important volatile part of plants and play vital role in traditional herbal remedies. They are used as antibacterial, antineoplastic, anti-carcinogenic, antimalarial, anti-ulcer and hepaticidal and diuretic and other pharmaceutical functions (Rodriguez, 2004; Bertea, *et al.*, 2005).

CONCLUSION

The GC-MS identification studies may give a platform for lightening the traditional knowledge in a perpetual manner. Here we reported the presence of 15 different compounds in methanolic leaf part of *A. fruticosa* for exploring the drug leads in pharmaindustries. Thus, the GC-MS identified phytoceuticals are indeed responsible for eliciting the traditional activity of this study species, *Acalypha fruticosa*. Futhermore, the phytochemical characterization of the extracts, the isolation of responsible bioactive compounds and their biological activity are necessary for future studies.

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Anticarcinogenic Activity of Gold Nanoparticles: An *In Vitro* Approach

S. ANJANA[1], ACHU BABU[2] AND ANNIE ABRAHAM[3]*

[1]*Department of Biotechnology, University of Kerala, Thiruvananthapuram, Kerala, India*
[2]*Department ofBiochemistry, University of Kerala,Thiruvananthapuram, Kerala, India*
[3]*Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India*
**email : annieab2013@gmail.com*

ABSTRACT

The complexity and heterogeneity nature of cancer makes it difficult to successfully diagnose and treat cancer. Advances in cancer research have been focused on studying molecular level of the disease, and nanotechnology plays a critical role in overcoming the obstacles in cancer biology. Gold nanoparticles may offer an advanced non-invasive, site directed therapy against cancer with highly tunable optical and physico-chemical characteristics Gold nanoparticles are widely used in biomedical applications such as imaging, diagnostics, targeted drug delivery and photo-thermal therapeutic applications. Also gold nanoparticle renders several advantages of size-shape tunability, biocompatibility and easy surface modification method that are useful for studying size effect in biomedical applications.The present work we checked the antioxidant and anticancer property of goldnanoparticles. Integrity of the cell is affected by oxidative stress when the generation of oxidants overwhelms antioxidant defense mechanism. Production of free radicals will lead to a disturbance in the antioxidant mechanism of the cell and finally result in cell death. These are by products of biological redox reactions and are involved in various pathological conditions. In this work we checked the stability of gold nanoparticle in DMEM at different time intervals. *In vitro* cytocompatibility of this drug complex was analysed using MTT Assay, Neutral Red Assay, Lactate Dehydrogenase Assay in 3T3L1 adipocytes. Anti cancer activity of this drug complex on MCF7 human breast cancer cell line was studied by morphological analysis using Phase contrast microscopy.

Key words *Anticarcinogenic Activity, Gold Nanoparticles, In Vitro Approach*

Nanotechnology is a multidisciplinary area of research in life sciences, engineering and medicine with broad band applications for molecular imaging, molecular diagnosis and targeted therapy (Misra*et al.,* 2010).Nanotechnology may be used to achieve therapeutic dosing via targeted therapies, sustained release of drug profiles and provide an intracellular sanctuary to protecttherapeutic compounds from efflux or degradation (Panayam and Labhasetwar, 2004).Nanoparticles possess enhanced permeability and retention effects (EPR), which makes them accumulate in tumor tissues to higher extents than in normal tissues due to theleaky tumor blood vasculature (Ferrari*et al*., 2005; Iyer*et al*., 2006). Moreover, the highsurface area of nanoparticles is advantageous to load drugs and thus enhance drug'ssolubility, stability and pharmacokinetic parameters (Ghosh*et al.*, 2008).

Cancer nanotherapies have been implemented to undertake several limitations of the conventional drug delivery systems, which are: nonspecific for distribution and targeting, low water solubility, poor oral bioavailability and lower therapeutic efficacy (Yih and Al Fandi, 2006).In contrast to normal cells tumor anatomical defectiveness along with functional abnormalities such as tumor blood vessels with irregular shape, dilated, leaky or defective and endothelial cells disorganized with large fenestrations, results in extensive leakage of blood plasma components into the tumor. These features help the retention of nanoparticles in tumor site, long enough, to allow local nanoparticle disintegration and release of the drug into tumor's vicinity (Wang and Thanou, 2010; Iyer*et al.,* 2006; Liu *et al.,* 2007) On the other hand, active targeting overcome permeability limitations of the passive targeting since it allows tissue penetration and cellular uptake by cancer cells (Pathak*et al.,* 2007; Peer *et al.,* 2007; Cho *et al.,* 2008). This active targeting involves the functionalization of a carrier system containing the chemotherapeutical agents, which are selectively recognized by the overexpressed receptors existing at the surface of the interest cancer cells (Pathak*et al.,* 2007; Peer *et al.,* 2007; Cho *et al.,* 2008). The surface charge of gold nanoparticles has also been shown to be important in determining particle toxicity, with cationic gold nanoparticles exhibiting moderate toxicity owing to the electrostatic binding of the particles to the negatively charged cell membrane. In contrast, anionic particles have no toxicity as they are repelled from the membrane (Goodman *et al* 2004). Taken together, the size, shape, and surface charge of gold nanoparticles need to be carefully considered when designing gold nanoparticles for human use in order to optimize their therapeutic function, while concurrently decreasing their toxicity profile by minimizing their cellular uptake and interactions. On the other hand, active targeting overcome permeability limitations of the passive targeting since it allows tissue penetration and cellular uptake by cancer cells (Pathak*et al.,* 2007; Peer *et*

al., 2007; Cho *et al.,* 2008). This active targeting involves the functionalization of a carrier system containing the chemotherapeutical agents, which are selectively recognized by the overexpressed receptors existing at the surface of the interest cancer cells (Pathak*et al.,* 2007; Peer *et al.,* 2007; Cho *et al.,* 2008). Since ligand-receptor interactions can be highly selective, the functionalization of the nanoparticle surface will allow a more precise targeting for tissues of interest and will reduce of the toxic effects in the surrounding normal tissues (Pathak*et al.,* 2007; Peer *et al.,* 2007; Cho *et al.,* 2008). As a result, the potential benefits of such delivery vehicles include, controlled and long-term release rates, extended bioactivity, reduced side effects, decreased administrated frequency to the patient, and the ability to co-deliver multiple drugs with synergistic effects to the same target site (Brewer *et al.,* 2011; Pathak*et al.,* 2007; Farokhzad and Langer, 2009).

MATERIALS AND METHODES

All chemicals and reagents used were of analytical grade. All biochemical kits were purchased from M/s Sigma, USA. The cell lines used in the study (MCF-7, breast cancer cells) was procured from Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram and normal cell (3T3L1) was procured from Department of Biotechnology, University of Kerala.

The Gold nanoparticle was procured from Department of Optoelectronics, University of Kerala. All other chemicals and reagents used in the study were of analytical grade quality

STABILITY ANALYSIS BY UV – VISIBLE SPECTROPHOTOMETER

Stability of the AuNPs was studied by dispersing AuNPs in DMEM medium and incubatedat different time intervals. Absorption spectra of samples were recorded. The stability of AuNPs in DMEM was determined using uv – visible spectrophotometer.

ANTIOXIDANT SCAVENGING ASSAYS

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging ability was determined according to the method described by Ruch*et.al*., 1989. Compound was dissolved in phosphate buffer (0.1 M, pH 7.4) at various concentrations and mixed with 600 ìl of hydrogen peroxide solution. Ascorbic acid was used as the reference compound. The concentration of the hydrogen peroxide was measured by reading the absorbance values of the reaction mixtures at 230nm after 10minutes against a blank solution containing phosphate buffer without hydrogen peroxide and the percentage inhibition of hydrogen peroxide was calculated

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity was performed (Ilavarasan et al.,2005) using Griess reagent. Sodium nitroprusside (5mM, 1ml) in phosphate buffer saline (PBS) (0.1 M, 7.4 pH) was mixed with 3 ml of different conc. of the compound and incubated at 25°C for 150 min. 0.5 ml of the samples was mixed with 0.5 ml of Griess reagent and measure the absorbance at 546 nm. The nitric oxide radicals scavenging activity was calculated as percentage inhibition.

DPPH radical scavenging assay

DPPH radical scavenging activity was measured by the method of Blois *et al*., 1958. The free radical scavenging activity of the compound was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. The reaction mixture containing 2.5 ml of DPPH solution (0.1 mM in methanol), 0.2 ml plant extract (adjusted to 3 ml by adding methanol) and absorbance recorded after 30 minutes at 517nm.Quercetin was used as standard.

CELL MORPHOLOGY

Cells were seeded in to a 96 well micro titer plate (5,000 cells/well) in 10% DMEM and incubated for 24hours. Once the cells have attached Gold nanoparticle was added in different concentration (100-300µg/ml) in duplicate and incubated for 24hours at 37°C. Cells treated with 10mM concentration of Cisplatin were taken as positive control and another set maintained without any treatment as control. The cell morphology was observed using phase contrast microscopy.

CYTOTOXICITY ASSAYS

MTT CELL PROLIFERATION ASSAY (Mosmann *et al*., 1983)

Cells were seeded in to 96well microtiterplate (5000 cells/well) in 10%DMEM and incubated for 24 hours. Once the cells have attached, Gold nanoparticle was added in different concentration (100-300µg/ml) in duplicate and incubated for different time intervals (24,48,72hours) at 37°C. Cells treated with 10mM concentration of Cisplatin taken as positive control and

another set maintained without any treatment as control. The outermost wells of the plate were avoided to reduce the dying effects. Instead they were filled with PBS for maintenance of humidity. After incubation at each time interval the medium was removed and equal volumes of fresh medium were added along with 20 ml MTT (5mg/ml) to each well. The plates were kept for 3hours incubation. The yellowish MTT is reduced to dark colored formazan by visible cells only. The fomazan crystals formed were solubilized with MTT lysis buffer (20% SDS in 50% dimethyl formamide). The plate was kept protected from light, overnight at 37°C in an incubator. The color developed was quantitated with ELISA plate reader (BioRad systems, USA)measuring wavelength 570 nm.

The cells survival (CS) expressed as percentage was calculated as follows.

CS = (OD drug exposed cell / Mean OD control wells) x 100

The graph was plotted by taking percentage viability in the y-axis and concentration of drug in the x-axis.

NEUTRAL RED UPTAKE (NRU) ASSAY (Lasarow *et al*., 1992)

Cells were treated with different concentration (100-300 µg/ml) in duplicate and incubated for different time intervals (24,48,72 hours) at 37°C. After incubation, a solution of Neutral red, a vital dye is added to the 96 well plates. The plates were incubated at standard culture conditions to allow neutral red uptake by the cells. After 2 hours incubation, decanted excess neutral red and PBS was added to the wells .The solvent extracts the neutral red dye contained within the cells. The plates were placed on a plate shaker to fully extract the neutral red and evenly distribute the dye in each well and absorbance with a 540 nm was measured using a micro plate reader (BioRad, USA). The absorbance value (optical density) are then used to determine the viability of each well comparing the optical density of the each material treated well compared the negative control well.

LDH CYTOTOXICITY ASSAY (Waterbuck and Meer et al., 2005)

This is a colorimetric assay that quantitatively measures LDH, a stable cytosolic enzyme that is released into the culture medium upon cell damage or lysis occurs during both apoptosis and necrosis (Wolterbeek and Meer, 2005). LDH catalyzes the reduction of NAD+ to NADH and H+ by oxidation of lactate to pyruvate, which in turn catalyze the reduction of a tetrazolium salt to a colored formazan and the absorbance of the formazan developed can read at 490 nm. The amount of formazan produced is proportional to the amount of LDH released into the culture medium.

REAGENTS

1. Assay Buffer: dissolve the cell based assay buffer tablet in 100 ml distilled water. It should be stable for 1 year at room temperature.
2. LDH diaphorase- Reconstitute with 150 ml assay buffer
3. LDH reaction solution

PROCEDURE

Cells were added in 24 well plates, exposed to gold nanoparticle with different concentration (100-300 µg/ml) and incubated for different time intervals (24,48,72 hours). After incubation

the cell suspension was centrifuged at 400 rpm for 5 minutes and transfer 100 ml of the supernatant to each well in a microplate. Then add 100 ml of reaction solution to each well and incubate plate with gentle shaking for 30minutes at room temperature. Read the absorbance at 490 nm using a plate reader (BioRad, USA).

$$A_{test}=(A_{control} + A_{lysed})100$$

LDH activity can be determined by plotting a graph against absorbance in y axis and time interval in x axis.

RESULTS

1 CHARACTERISTICS & PROPERTIES OF GOLD NANOPARTICLES (AuNPs)

1. 1UV-VISIBLE SPECTRUM

The UV-Visible spectrum showed a strong absorption band of AuNPs and exhibited a characteristic peak absorption at 530 nm .The strong absorption band in the visible region is due to the surface plasmon resonance phenomena, a characteristic property of AuNPs, which is the origin of the brilliant red colour of nanoparticle in solution.

1.2DETERMINATION OF THE STABILITY OF THE PARTICLE

TheUV- Visible spectrum analysis shows that, AuNPs are stable in DMEM solution at different time intervals. The results indicate that the particles have good stability in the medium.

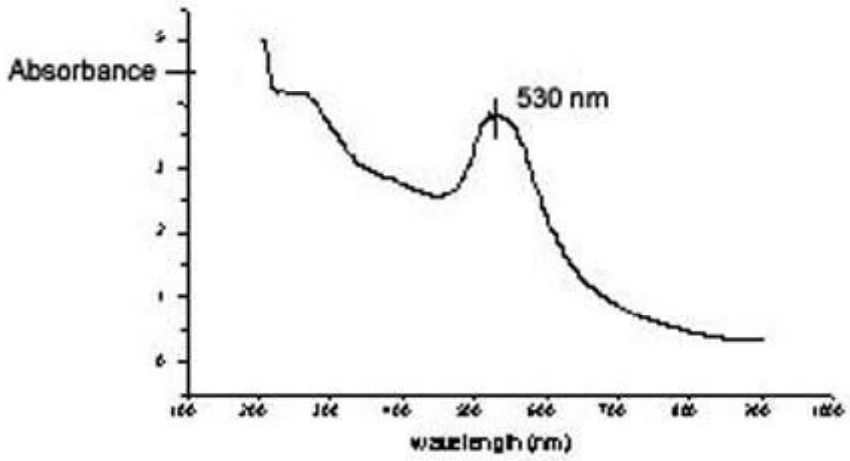

Fig. 1. UV-Visible absorption spectrum analysis

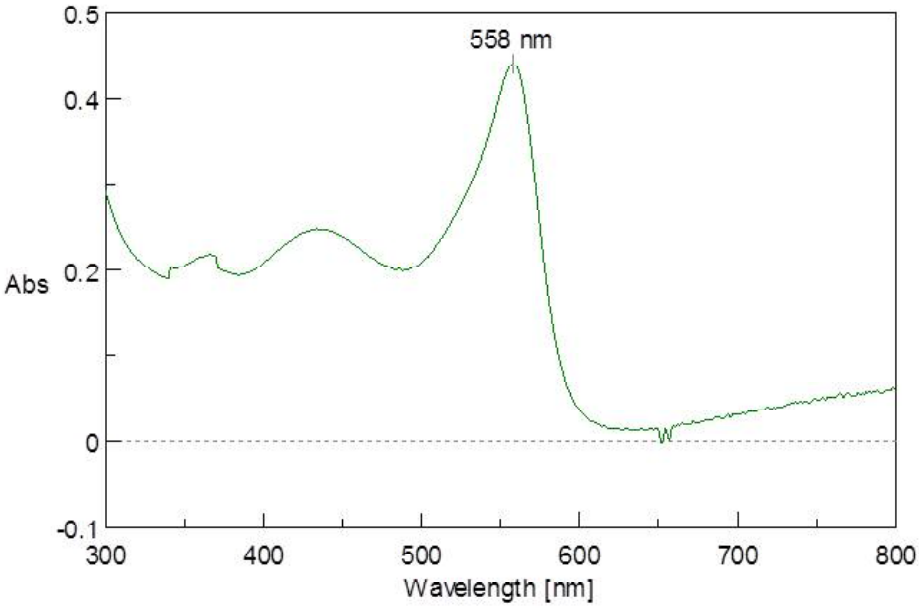

Fig. 2. AuNPs in DMEM (2 hours)

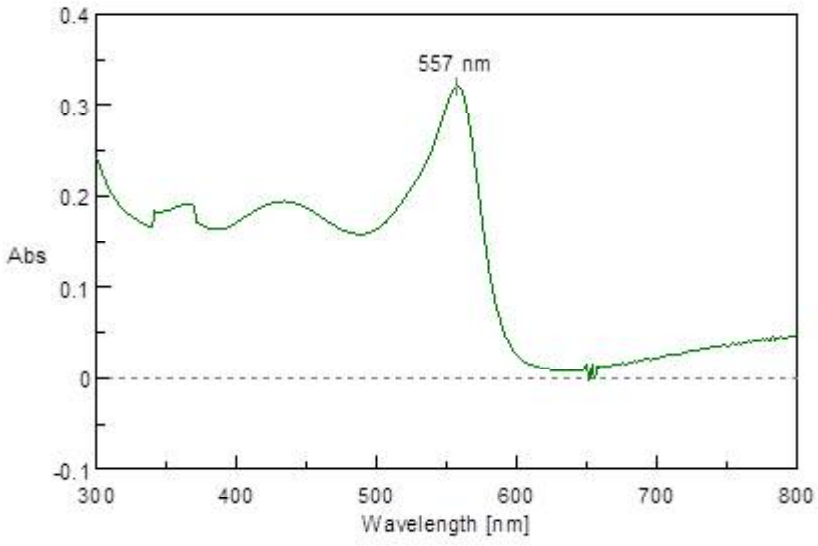

Fig. 7. AuNPs in DMEM (4 hours)

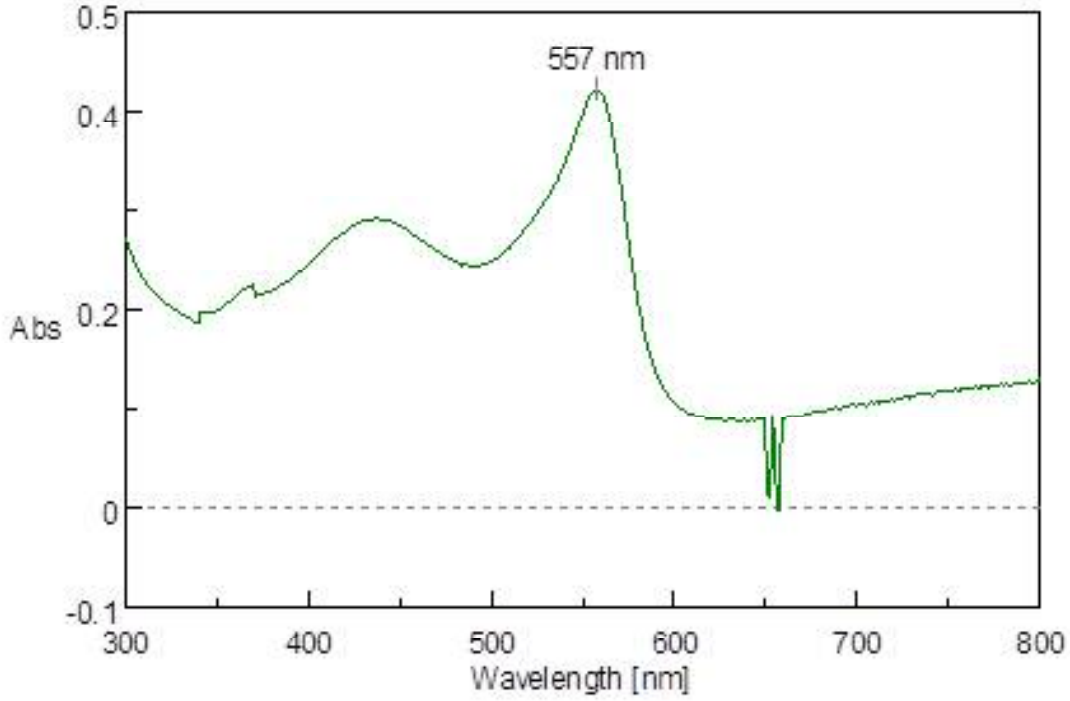

Fig. 3. AuNPs in DMEM (24 hours)

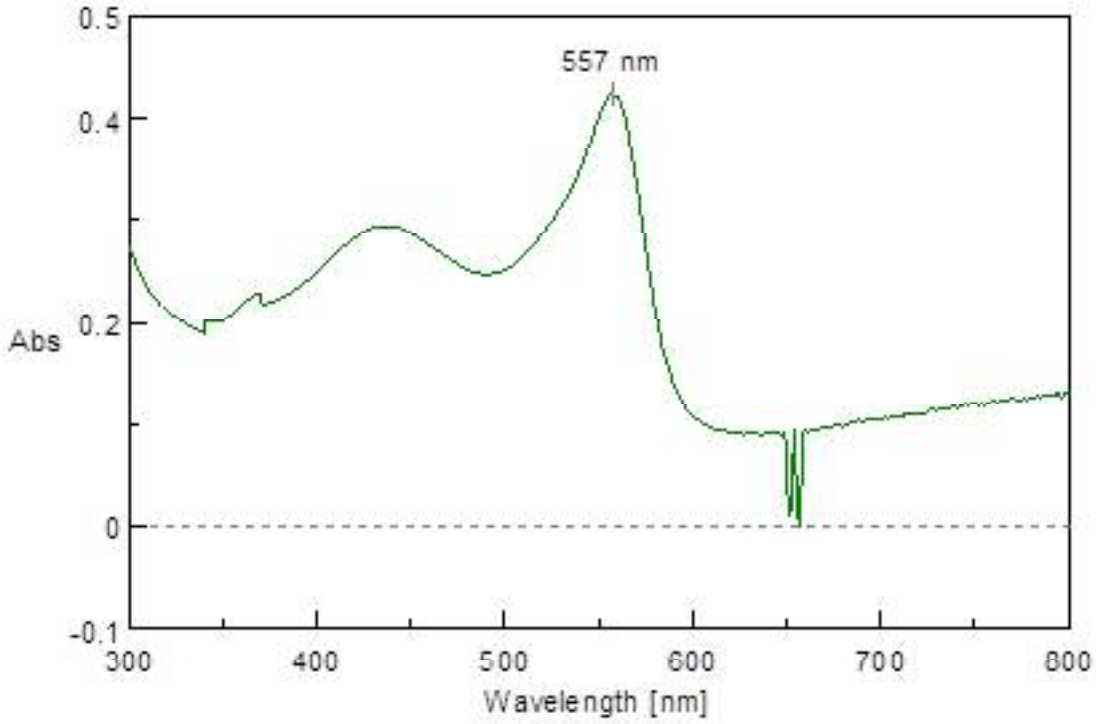

Fig. 4. AuNPs in DMEM (48 hours)

Figure 1-4 show the stability of the particle in DMEM at different time intervals.

2 FREE RADICAL SCAVENGING ASSAYS

Determination of the free radical scavenging activity of AuNPs

A. H_2O_2 ASSAY

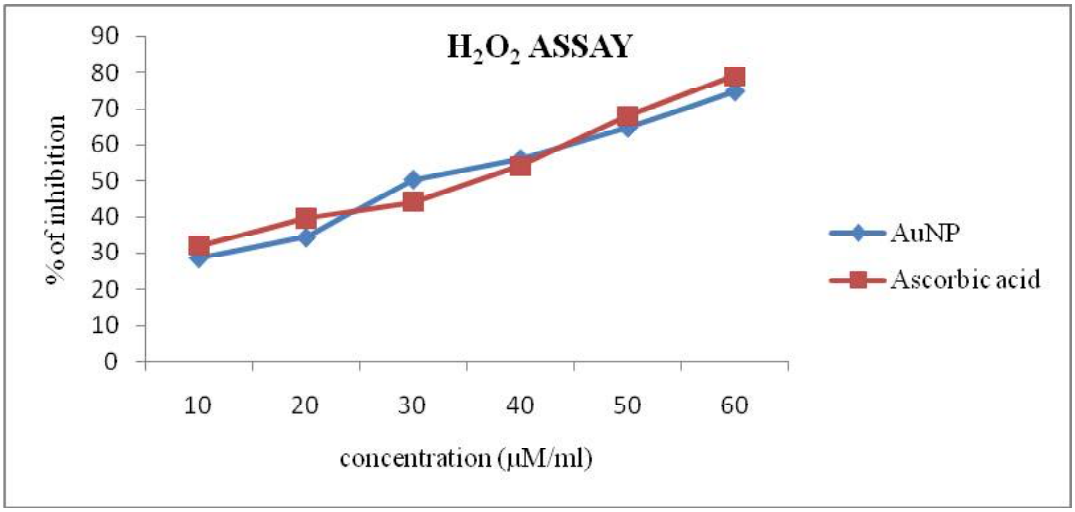

Fig.10. Scavenging activity of different concentration of AuNPs on hydrogen peroxide. Values are means ± SD of six determinations.

B. NITRIC OXIDE ASSAY

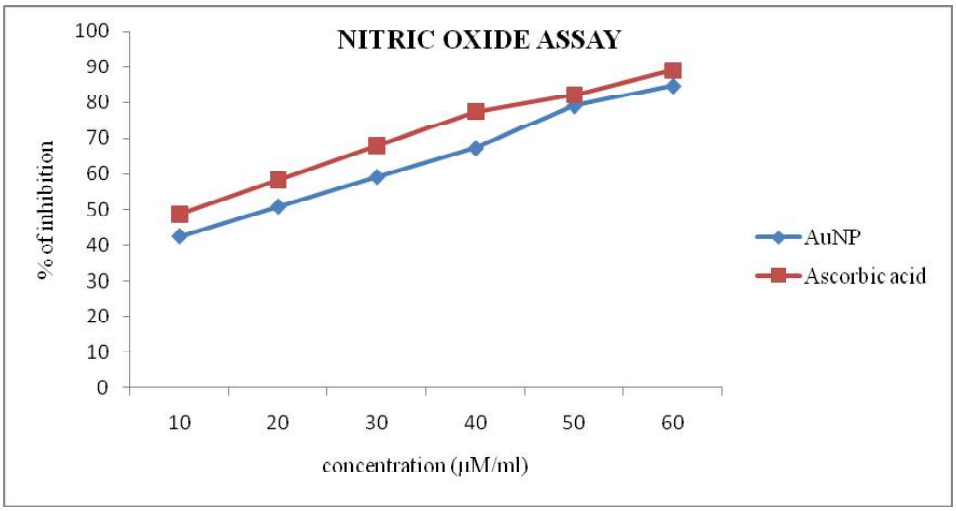

Fig. 5. Scavenging activity of different concentration of AuNPs on NO radicals.Ascorbic acid is used as standard. Values are means ± SD of six determinations.

C. DPPH ASSAY

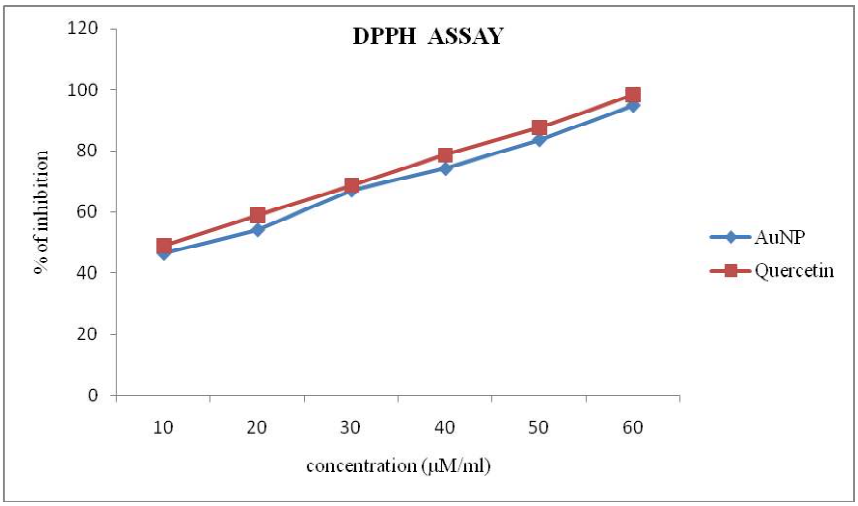

Fig. 6. DPPH,evaluating the antioxidant potential of AuNPs.Quercetin was used as a standard.Values are means ± SD of six determinations.

3CYTOCOMPATIBILITY IN NORMAL CELL

Understanding the interactions of nanosized objects with living cells is of greatimportance for the development of nanobiotechnology because of the increaseddiagnostic and therapeutic applications. Gold nanoparticles (AuNPs) offer a great potential in the diagnosis of cancer. In order to use this forclinical trials, a detailed study oncellular uptake,toxicity evaluation and biocompatibility is necessary.

A) MORPHOLOGY OF NORMAL CELL (3T3L1 ADIPOCYTES)

3T3-L1 cells have a fibroblast-like morphology.

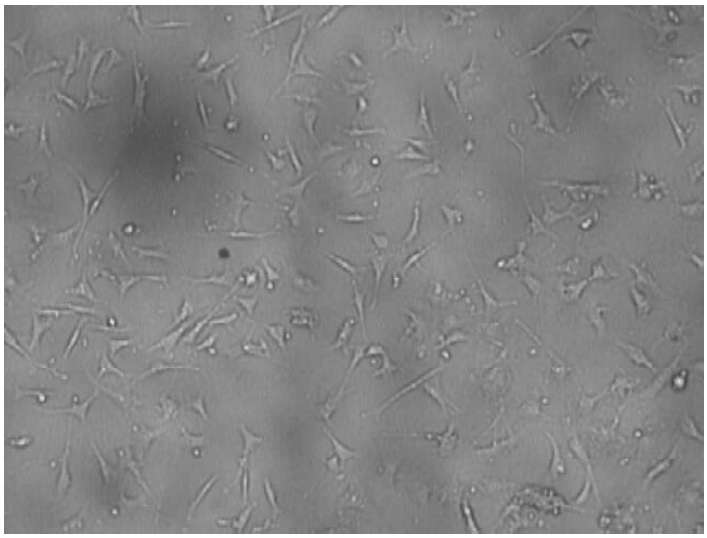

Fig. 7. 3T3L1 CELL

4 CYTOCOMPATIBILITY IN NORMAL CELL.

Toxicity of AuNPs at different time interval was checked in normal cells (3T3L1) by MTT, NRU and LDH leakage assays. The results are shown in the following figures.

a. MTT Assay

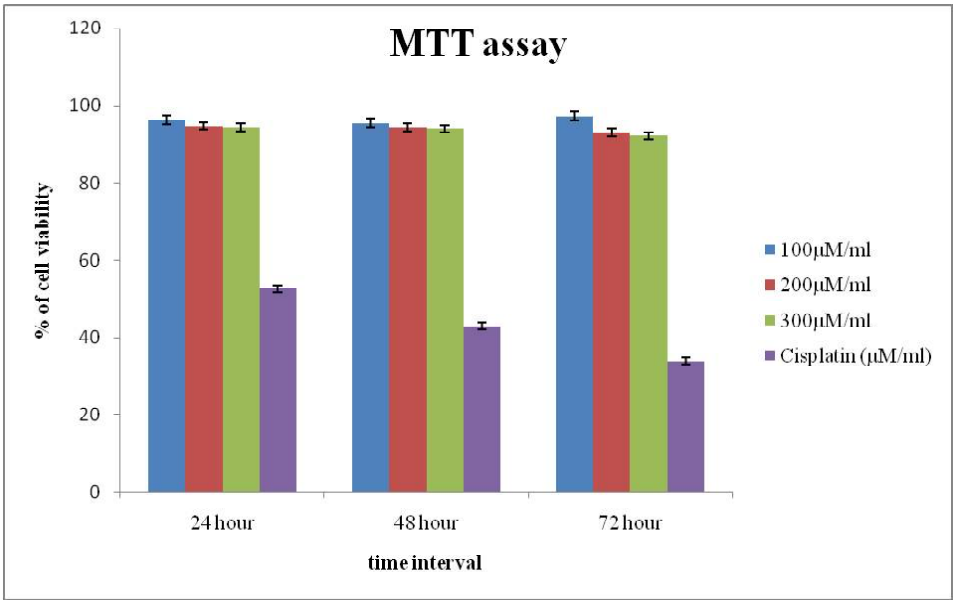

Fig. 8. **Results of MTT assay for AuNPs**: Percentage of viability showed above90% viable cells up to 72 hours treatment even at maximum concentration (300 ìM/ml). Cytotoxicity was found in Cisplatin (10µM/ml) treated group. The results are means ± SD from six independent experiments.

b. NRU Assay

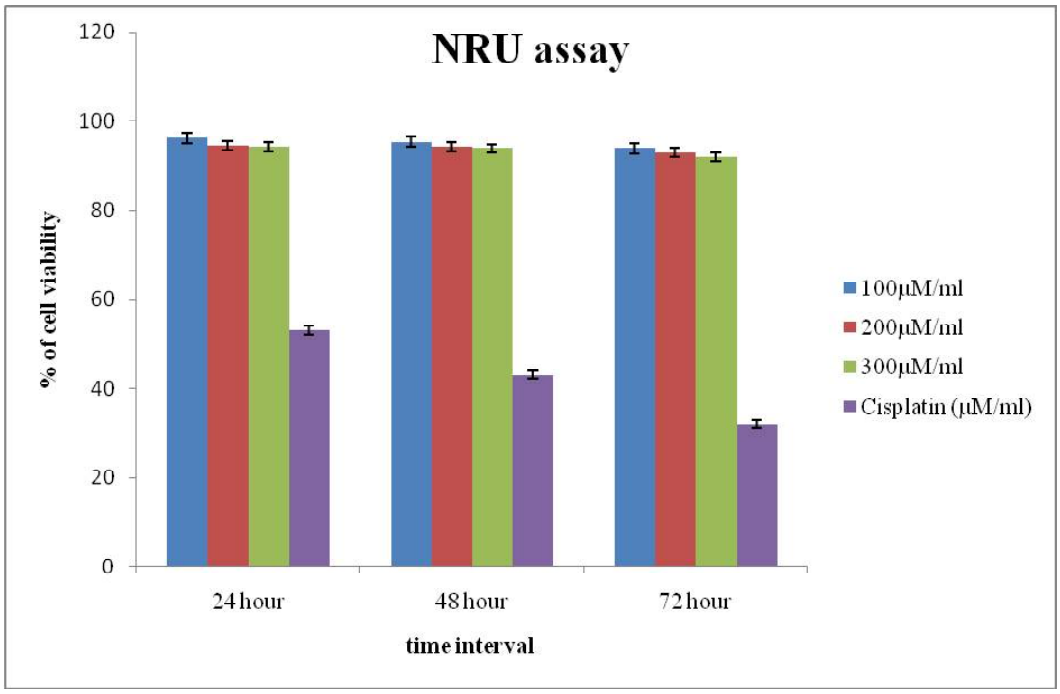

Fig.9. **Results of NRU assay for AuNPs.** Percentage of viability showed above90% viable cells upto 72 hours treatment even at maximum concentration (300iM/ml). Cytotoxicity was found in Cisplatin (10µM/ml) treated group. The results are means ± SD from six independent experiments.

c. LDH Assay

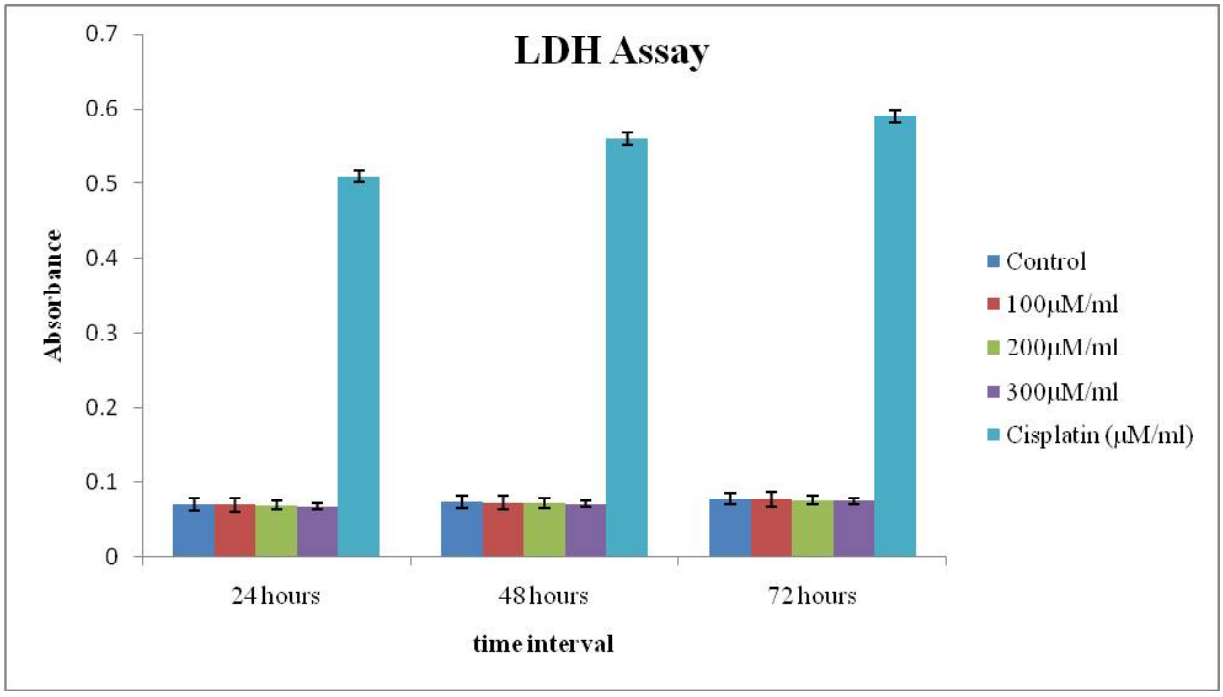

Fig. 10. **Results of LDH leakage for AuNPs:** There is no significant increase in LDH leakage in AuNPstreated group when compared to untreated control. Increased leakage found in Cisplatin(10µM/ml)treated group. The results are means ± SD from six independent experiments.

5 ANTI CANCER PROPERTIES OF AuNPs

5.1MORPHOLOGY IN HeLa CELLS.

The result showed that the cells treated with AuNPs at various concentrationswas irregular confluent aggregates with round and polygonal cell morphology. The result was comparable to that in Cisplatin treated group.

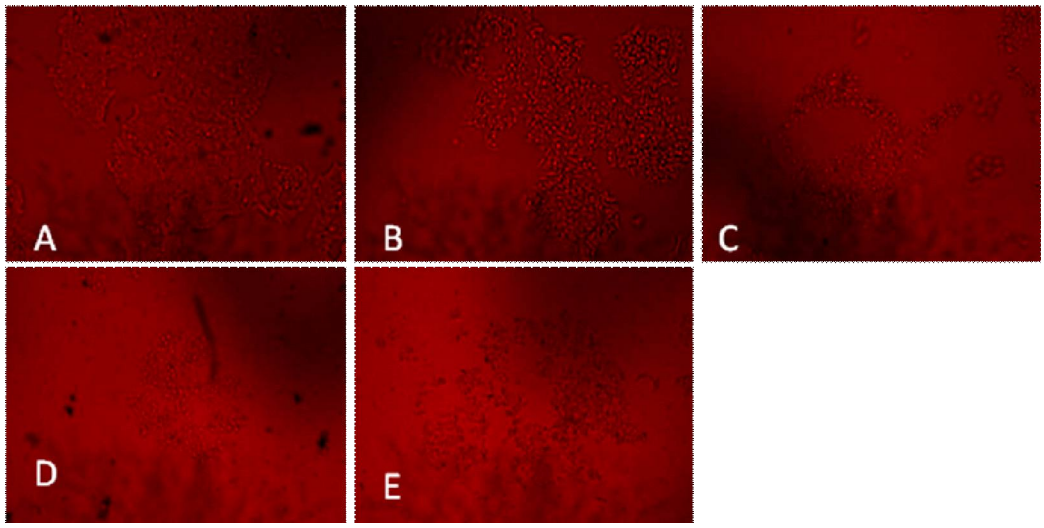

Fig. 11. **Morphological changes of HeLa cell line after 24 hrs incubation of AuNPstreatment:** A:Control cells; B: Cells treated with 100ìM/ml; C: Cells treated with 200 ìM/ml ; D:Cells treated with 300ìM/ml; E: Cells treated with Cisplatin (10µM/ml).

5.2 MORPHOLOGY IN MCF-7 CELLS.

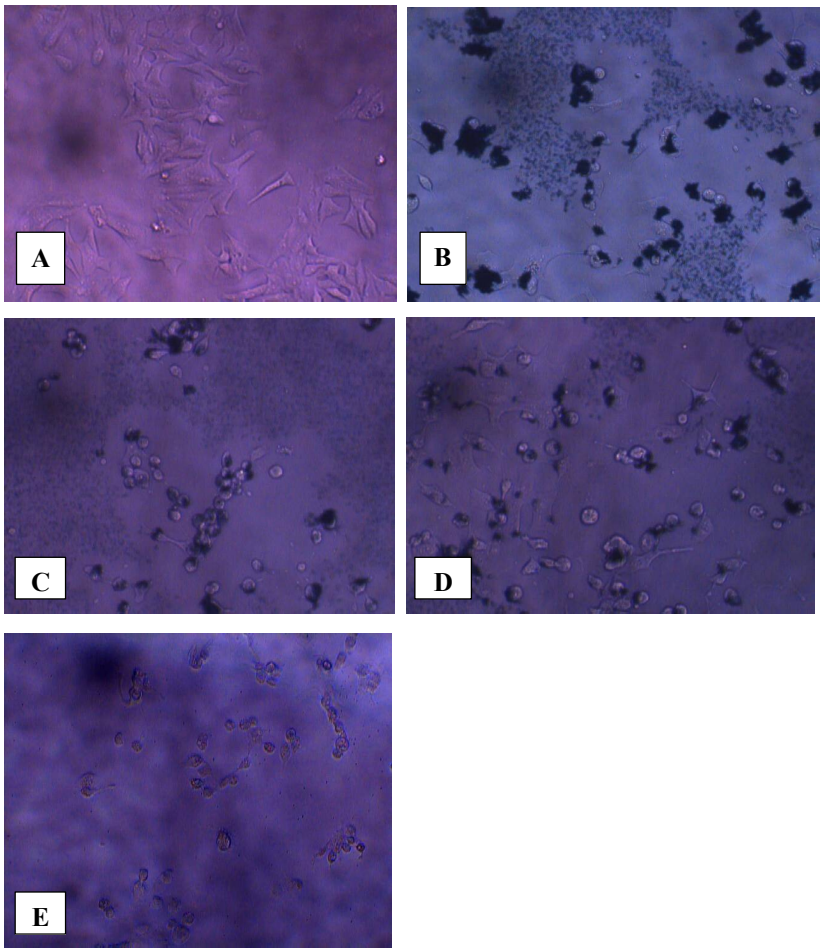

Fig. 12. **Morphological changes of MCF 7 cell line after 24 hrs incubation of AuNPstreatment:** A:Control cells; B: Cells treated with 100ìM C: Cells treated with 200 ìM/ml ; D:Cells treated with 300ìM/ml; E: Cells treated with Cisplatin (10µM/ml).

DISCUSSION

Cancer is a growing health problem around the world, particularly with the steady rise in life expectancy, increasing urbanization, and the subsequent changes in environmental conditions and lifestyle (Ferlay *et al.*, 2010). The distant metastases are regarded as the major reason to cause cancer death (Lacroix *et al.*, 2006). So the cancer treatment at an early stage is highly expected and accurate diagnose of cancer is essential to determine the extent of disease and to plan appropriate therapies (Berois *et al.,2000*).

Gold nanoparticles are widely used in biomedical applications such as imaging, diagnostics, targeted drug delivery and photo-thermal therapeutic applications. Also gold nanoparticle renders several advantages of size-shape tunability, biocompatibility and easy surface modification method that are useful for studying size effect in biomedical applications.

Gold nanoparticles have a long history of medical usage. Red colloidal gold has been used by the Chinese since 2500 B.C. as a drug for longevity (Higby *et al.,* 1982)whereas colloidal gold has been used for rejuvenation medicine(Richards *et al*.,2002). Two properties of AuNPs make them suitable candidates fortherapeutic applications: a) Antibodies and other biomolecules can easily be attached to the surface of AuNPs; b) Plasmon resonances of gold nanoparticles make them to havephoton capture ability that favours the use of nanoparticles superior to photothermal dyes (Loo *et al.*, 2004).

The stability of the gold nanoparticle was studied in DMEM medium at different time intervals (2 hr, 4 hr,24 hr, 48 hr) and the absorption spectra was determined using UV-Visible spectrophotometer. The absorbance peak exhibited a characteristic peak at 530 nm .The strong absorption band in the visible region is due to the surface plasmon resonance phenomena, a characteristic property of AuNPs, which is the origin of the brilliant red colour of nanoparticle in solution (Raji V *et al.,* 2011).

Integrity of the cell is affected by oxidative stress when the generation of oxidants overwhelms antioxidant defense mechanism. Production of free radicals will lead to a disturbance in the antioxidant mechanism of the cell and finally result in cell death. These are by products of biological redox reactions and are involved in various pathological conditions. Cytocompatibility studies such as MTT, NRU and LDH assays were studied on normal cells.

On morphological analysis, control cells did not show any morphological changes but treated cells were in irregular confluent aggregates with round and polygonal cell morphology. In treated MCF 7 and HeLa cells, destruction of monolayer was observed. The treated polygonal cells after 24h of incubation begun to shrink and became spherical in shape. The cell shrinkage increased progressively in dose and time dependent manner. This shrinkage may be due to the growth inhibitory effect of compound.

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A Survey of Spider Diversity on the Bank of River Pampa at Poovathoor, Pathanamthitta District, Kerala

R. ABHILASH[1] AND ANUPRABHA S. KUMAR

Postgraduate Department of Zoology,
Christian College, Chengannur, Kerala
[1]email: greenabhilash@gmail.com

ABSTRACT

A preliminary survey on the diversity of spiders was conducted on the bank of river Pampa at Poovathoor, Pathanamthitta District, Kerala on the first week of February, 2015. A total of 39 species belonging to 17 families were recorded. Among these 17 families, Salticidae represented the most number of spider species (10) which was followed by Araneidae (6). *Fecenia protensa* belonging to family Psechridae spotted in the study was reported for only the third time from India. *Porcataraneus bengalensis,* a rare species belonging to Araneidae was also recorded during the survey.

Key Words. *Spider; Diversity; Wetland Ecosystem; Bio-indicator.*

The spiders operate within the balance of nature and their role in nature's plan is beneficial to man. They are characterised by high within-habitat taxonomic diversity and exhibit taxon and guild-specific responses to environmental change. They are distributed to every continent except Antarctica and have adapted to all known ecological environments, except air and open sea. Spiders serve practical roles as biological agents for the control of crop pests (Breene *et al*., 1993). They prove to be useful indicators of the overall species richness and health of biotic communities (Norris, 1999). Despite this, very little is known about the abundance, distribution and natural history of many species.

About 46,777 valid species belonging to 4,057 genera and 112 families are known globally (World Spider Catalog, 2017), while Indian fauna consists of 1686 valid species belonging to 438 genera and 60 families (Sebastian and Peter, 2009; Keswani *et al*., 2012). Spiders play important roles in the dynamics of a specific habitat and are sensitive to habitat loss, climatic change and environmental upheavals (Daniel, 2002). Though spiders form one of the most ubiquitous and diverse groups of organisms existing in Kerala, their study has always remained largely neglected. They have, however, largely been ignored because of the human tendency to favour some organisms over others of equal importance because they lack a universal appeal (Humphries *et al*. 1995).

In India, most ecological studies on spiders were prevalent in agro-ecosystems mainly in rice ecosystem and coffee plantations (Sebastian *et al*., 2005; Kapoor, 2008). Little is known about the composition of the arachnid communities of natural ecosystems. It was in this circumstance that the present survey of spiders on the bank of river Pampa at Poovathoor was undertaken. The main objective of the study was to get a preliminary data regarding the diversity of spiders on the bank of river Pampa – a wetland body of ecological importance and to assess the ecosystem health of the area based on spider diversity.

MATERIALS AND METHODS

Study area

The study was conducted on the right bank of river Pampa at Poovathoor in Koippuram Panchayath, Pathanamthitta District, Kerala. The 150 sq.m selected was an area with riparian vegetation including different types of grasses and some bamboo plants. In this particular plot, there was a myristica plantation also. The area is located at 9^0 20' 10" North latitude and 76^0 40' 10" East longitudes.

Mode of survey

The survey of spiders was carried out on the first week of February, 2015. The survey was started at 11 Am and lasted for 5 hours. The collection methods (Coddington *et al*., 1991) adopted was:Aerial Hand Collection, Ground Hand Collection and Beat Sheet Method.

Only few species were photographed and identified in their natural habitat. In most cases it was difficult to assess the specimen so that they were captured for further identification and after that they were released in their natural habitat. The identification of spiders was done following 'Handbook of spiders' by Tikader, 1987 and 'Spiders of India' by Sebastian and Peter, 2009.

RESULT

In the present study, 39 species of spiders

Table 1. List of Spiders identified during the survey carried out at Poovathoor on the first week of February, 2015.

Sl. No	FAMILY	SPECIES
1	**Araneidae** Clerck, 1757	*Argiope pulchella*(Thorell,1881)
2	”	*Gasteracantha geminata* (Fabricius, 1798)
3	”	*Cyclosa confraga* (Thorell, 1892)
4	”	*Anepsion maritatum* (O.Pickard-Cambridge,1877)
5	”	*Neoscona mukerjei* (Tikader, 1980)
6	”	*Porcataraneus bengalensis* (Tikader, 1975)
7	**Clubionidae** Wagner, 1887	*Clubiona drassodes* (O.Pickard-Cambridge, 1874)
8	**Gnaphosidae** Pocock, 1898	*Zelotes* sp.
9	**Hersiliidae** Thorell, 1870	*Hersilia savignyi*(Lucas, 1836)
10	**Lycosidae** Sundevall, 1833	*Pardosa sumatrana* (Thorell, 1890)
11	”	*Hippasa greenalliae* (Blackwall, 1867)
12	**Linyphiidae** Blackwall, 1859	*Atypena adelinae* (Barrion & Litsinger, 1995)
13	**Liocranidae** Simon, 1897	*Oedignatha* sp.
14	**Oxyopidae** Thorell, 1870	*Oxyopes shewta* (Tikader, 1970)
15	”	*Oxyopes javanus* (Thorell, 1887)
16	”	*Hamadruas* sp.
17	**Pholcidae** C.L. Koch, 1850	*Pholcus* sp.
18	**Pisauridae** Simon, 1890	*Pisaura gitae* (Tikader, 1970)
19	**Psechridae** Simon, 1890	*Fecenia protensa* (Thorell, 1891)
20	**Salticidae** Blackwall, 1841	*Phintella vittata* (C.L. Koch, 1846)
21	”	*Brettus albolimbatus* (Simon, 1900)
22	”	*Telamonia dimidiata* (Simon, 1899)
23	”	*Hyllus semicupreus* (Simon, 1885)
24	”	*Bavia kairali*
25	”	*Myrmarachne plataleoides* (O.Pickard-Cambridge, 1869)
26	”	*Curubis tetrica* (Simon, 1902)
27	”	*Chalcotropis pennata* (Simon, 1902)
28	”	*Epeus tener* (Simon, 1877)
29	”	*Ptocasius yashodharae* (Tikader, 1977)
30	**Sparassidae** Bertkau, 1872	*Heteropoda venatoria* (Linnaeus, 1767)
31	”	*Thelcticopis* sp.
32	**Tetragnathidae** Menge, 1866	*Tylorida ventralis* (Thorell, 1877)
33	”	*Opadometa fastigata* (Simon, 1877)
34	”	*Tetragnatha viridorufa* (Gravely, 1921)
35	**Theridiidae** Sundevall, 1833	*Meotipa picturata* (Simon, 1895)
36	”	*Theridion* sp.
37	”	*Chrysso angula* (Tikader, 1970)
38	**Trachelidae** Simon, 1897	*Utivarachna* sp.
39	**Uloboridae** Thorell, 1869	*Uloborus* sp.

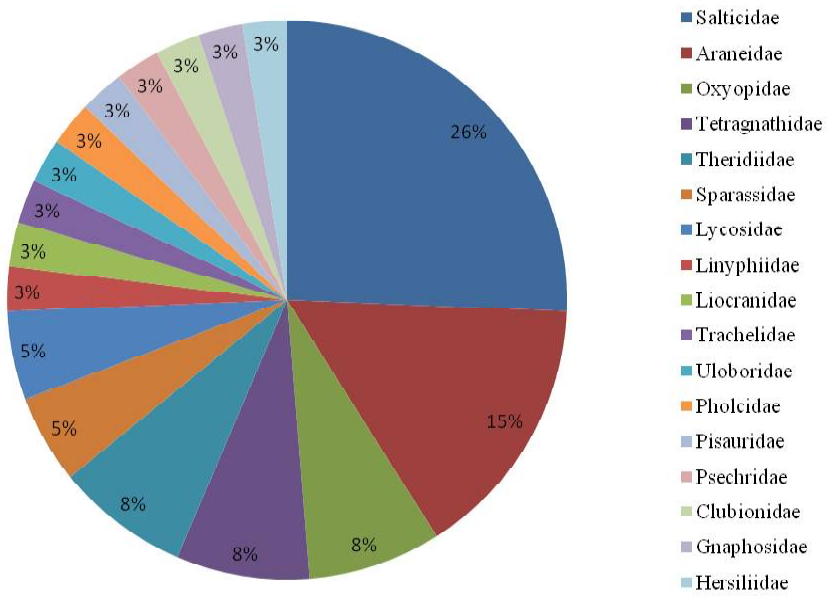

Fig. 1. Percent Occurrence of spider species belonging to different families recorded from Poovathoor on the first week of February 2015

belonging to 17 families were recorded (Table 1). Salticidae was represented by the most number of spider species *i.e.* 10. It was followed by Araneidae with 6 species. Oxyopidae, Tetragnathidae and Theridiidae were represented by 3 species each. Lycosidae and Sparassidae were represented by 2 species. Only 1 species was represented in the case of Linyphiidae, Liocranidae, Trachelidae, Uloboridae, Pholcidae, Pisauridae, Psechridae, Clubionidae, Gnaphosidae and Hersiliidae. Percent occurrence of spider species belonging to different families recorded from the area is given in Fig. 1. Rare species like *Fecenia protensa* belonging to family Psechridae and *Porcataraneus bengalensis* belonging to family Araneidae were also spotted during the survey.

DISCUSSION

The present study revealed that, the right bank of river Pampa at Poovathoor is qualitatively rich in spiders with 39 species coming under 17 families. It indicates that out of the 60 families identified so far from Kerala, nearly 28% families were recognized from the study area. Diversity generally increases when a greater variety of habitat types were present. The study area is endowed with different types of habitats such as small patches of grassland, riparian vegetation and bamboos, myristica plantation and shrubs. This may be the reason for the species richness. Also, the selected spot was an undisturbed patch with no signs of pollution.

In the present study Salticidae represented the most number of spider species which corroborates with the spider survey carried out by Malamel and Padayatti (2014) at Kumarakom Bird Sanctuary. Out of the total 39 species recorded in the study 2 rare species were obtained from the spot. Among them, *Fecenia protensa* is only the third report from India. This sighting has a great importance owing to the fact that their presence in this area supports the existence of Malayan element in the fauna of peninsular India as suggested in the Satpura Hypothesis (Malamel *et al*., 2013). All the four valid species belonging to genus Fecenia are found in Southeast Asia and nearby regions and only one species, *Fecenia protensa,* extends to Sri Lanka and Southern peninsular India. This occurrence of single species supports the existence of Malayan element in the fauna of peninsular India as suggested in Satpura Hypothesis by Hora (1949).

Spiders are extremely sensitive to small changes in the habitat structure. Spiders are often limited to areas within the range of their "physiological tolerances" which make them ideal for land conservation studies (Riechert and Gillespie, 1986). Therefore, documenting spider diversity patterns in this wetland ecosystem can provide important information to justify the conservation of this wetland ecosystem.

CONCLUSION

The species richness of spiders is significantly higher in systems that have not been heavily

manipulated as observed in the present study. Further studies can build upon the present data and continue to catalogue the poorly documented spider fauna and perhaps discover new species along the way. At a time when all the ecosystems are experiencing lot of anthropogenic disturbances, the present investigation emphasizes the urgent need to conserve wetland ecosystems and associated regions of the area. Spiders are well documented as a potential bio-indicator in various ecosystems and their role in the dynamics of insect pest population control is well known, therefore, the data can be used in designing a future Biological Monitoring Program (BMP) on the bank of river Pampa.

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Study on Biodiversity and Physiochemical Parameters of Natural Pond Ecosystem of Adichanallur Village, Kollam District.

P.J. ARCHANA

Post Graduate and Research Department of Botany& Biotechnology
Sree Narayana College, Kollam, Kerala, India. PIN:691001
email : archanapjbiotech@gmail.com

ABSTRACT

An ecosystem is a dynamic complex of plant, animal and microbes such biotic and light, temperature, humidity such abiotic components. Ponds are habitats of great importance as they provide water for domestic, agricultural and industrial use as well as providing food. This freshwater ecosystem have been severely affected by anthropogenic disturbances, it leads to the serious negative effect on the structure and functions of this pond ecosystem. Natural pond ecosystem in Adichanallur Village, Kollam district was selected for present study. In the present study, various biotic components of this natural pond ecosystem were collected , stored and studied. In the pond ecosystem there was an interdependence between its components. There was a transfer of food from plants through a chain of organisms consisting of different levels of consumers and finally through death and decay reached the decomposers. This transfer of food from the producers through a series of organisms with repeated eating and being eaten is transferred to as the food chain. The present study helped to identify various biotic and abiotic components of a natural pond ecosystem and calculated the biodiversity rate of a natural pond ecosystem. Physiochemical parameters are checked during the study.

Key words *biotic; abiotic; food chain*

An ecosystem is a dynamic complex of plant,animal and microorganism communities and the nonliving environment,interacting as a functional unit. A pond are habitats of great human importance as they provide water for domestic,industrial and agricultural use as well as providing food.The study of the relationship between organism and environment is called ecology(Odum,1995). An ecosystem which is described by a habitat,organisms which live in it and interaction between both of them.The interaction of plants ,animals and microbes with the abiotic properties of their environment . The species inhabiting the different habitats belonging to an ecosystem are linked through a food web. All organisms full at least one of these functions: producers, consumers, decomposers (Nebel & Wright,1993).The biotic community together with its non-living environment forms an interacting system called ecosystem. Thus, ecosystem is " *the basic structural and functional unit of ecology* " (Verma,1995) Ecosystem has two structural components (Odum,1995).They are biotic components (living things) and abiotic components (non-living things).The biotic components are divided into three groups they are producers, consumers and decomposers(Agarwal,1997). The consumers are three types – first order consumers, second order consumers and third order consumers. Aquatic ecosystems contain several types of organisms that are classified based on the locality and adaptations. Three groups of aquatic organisms include plankton, nekton and benthos. Planktons are the organisms that float near the surface of the water.Nectons are the free swimming organisms and the benthos is the bottom dwelling organisms. The biodiversity of pond ecosystem is currently threatened condition that is due to the uncontrolled activity of human.

METERIALS AND METHODS

Study area

A pond from the Adichanallur village ,Kollam district, Kerala state was selected for the present study.The water sample collected for study from this pond.

Analysis of physical parameters

The physical parameters like temperature and turbidity were calculated by the following standard filtration methods.

Analysis of chemical parameters

The chemical parameters such as pH, alkalinity, total hardness, dissolved oxygen and BOD were calculated by following Winkler's method.

Abiotic components such as temperature of the atmosphere and pond water were measured with the help of a thermometer. The collected animals and plants from the pond with the help of a net and stored

Table 1.

Sl No:	Producers	First order consumers	Second order consumers	Third order consumers	Decomposers
1.	*Navicula*	*Amoeba*	Hydra	Frog	Bacteria
2.	*Chlamydomonas*	Rotifers	Planaria	Large fish	Fungus
3.	*Volvox*	*Daphnia*	Snail	Fresh water snake	
4.	*Asterionella*	*Vorticella*	Leech	Terrapin	
5.	*Microcystis*	*Actinosphaerium*	*Cyclops*	Pond heron	
6.	*Chara*		Dragon-fly nymph		
7.	*Potamogeton*		Tad-pole larva of frog		
8.	*Vallisneria*		Small fishes		
9.	*Hydrilla*				
10.	*Nymphaea*				
11.	*Sagittaria*				
12.	*Typha*				

them in museum jars. Prepared temporary mounts of various micro-organisms from the pond, and identified them .The specimen collected, were identified and preserved in formalin.

RESULT AND DISCUSSION

The physiochemical character of the pond were analysed The temperature of the pond was 27 ºC .The turbidity is recorded as 9 mg/l in this pond. The pH value of the pond is 7 in this pond water. The dissolved oxygen content is determined as 6 mg/l. Biological Oxygen Demand (BOD) 8 mg/l. (Table 2).The percentage of producers, consumers and decomposers are analysed and these biotic components are represented in Table 1.

The biotic components collected from the pond ecosystem are in the table given below.

The abiotic components such as temperature of the atmosphere and pond water measured are given and the turbidity, pH rate ,Dissolved oxygen,BOD represented the below table.

In the work, various biotic components of a pond ecosystem were collected and studied. The abiotic components such as temperature of water and atmosphere were measured and compared.The producers collected and identified from the pond ecosystem were *Navicularia*, *Chlamydomonas, Volvox, Asterionella, Microcystis, Richteriella, Chara, Potamogetom, Nymphea, Sagittaria and Typha*. From *these Richteriella, Microcystis, Asterionella, Volvox,Chlamydomonasand Navicularia* were microscopic producers and other were rooted hydrophytes.

The first order consumers collected and identified were *Amoeba, Rotifers, Daphnia, Vorticella,* and *Actinophaerium.*These consumers were microscopic. The second order consumers were *Hydra,* Leech, *Planaria,Cyclops,* Dragon-fly nymph,tad-pole larva of frog, small fishes and snail. Third order consumers were frog, large fishes, fresh water snake and terrapins. The decomposers were bacteria and fungi. In the pond ecosystem there was an interdependence between its components.There was a transfer of food from plants (producers) through a chain of organisms

Table 2.

Sl No:	Ecosystem	Atmospheric temperature (ºC)	Water temperature (ºC)	Turbidity(mg/l)	pH	Dissolved oxygen (mg/l)	BOD (mg/l)
1.	Pond	**34**	**27**	**9**	**7**	**6**	**8**

consisting of different levels of consumers and finally through death and decay reached the decomposers. This transfer of food from the producers through a series of organisms with repeated eating and being eaten is referred to as the food chain. Many food chains were interconnected with each other to form a network called food web (Odum, 1975: Verma, 1995; Agarwal, 1997). The present work helped us to collect and study various biotic and abiotic components of a natural pond ecosystem and analysed the physiochemical nature of this pond ecosystem.

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Phytochemical Profiling and the Antibacterial Potential of *Smilax wightii* A.DC. Against Selected Human Pathogenic Bacteria

ATHIRA V ANAND AND T. S. SWAPNA*

Department of Botany, University of Kerala,
Kariavattom Campus, Thiruvananthapuram.
**email: swapnats@yahoo.com*

ABSTRACT

The plant *Smilax wightii* A.DC. belonging to the family Smilacaceae is a medicinal plant in the Western Ghats. This ethnobotanically important plant is very limited in distribution and hence underutilized. The plant is used in Indian and Chinese systems of medicine to cure various ailments. Root is the medicinally important plant part. In Ayurveda, root of *Smilax wightii* is used to cure diseases related to the nervous system and urinary disorders. In Chinese system of medicine the plant is used to cure skin diseases, venereal diseases, swelling, abscesses, dysentery, amoebiasis, gastric complaints and also for the treatment of spermatorrhoea to promote fertility. The aim of the present study is to analyze the phytochemical composition and the antibacterial potential of the root of *Smilax wightii* using different bacterial strains as target microorganisms. Initially the plant material was subjected to Soxhlet extraction using solvents of increasing polarity. The phytochemical profiling was done using standard procedures to analyze the presence of metabolites. The methanolic extract of the root was evaluated for their *in vitro* antibacterial property through disc diffusion method. The target microorganisms selected were the bacterial strains pathogenic to humans. Both Gram positive and Gram negative bacterial strains were used with Amoxycillin as the standard. After incubation, clear zones of inhibition were measured and the activity index was calculated. The root extract showed considerable antibacterial property. The study proved the presence of bioactive compounds in *Smilax wightii* that inhibit the multiplication or growth of the target microorganism. So the plant has the potential to be used against many bacterial infections and further research will help for the development of a potential antibacterial drug from this plant.

Keywords *Antibacterial, Root, Smilax Wightii, Phytochemistry, Methanolic extract*

The plant *Smilax wightii* A.DC. (Smilacaceae), is an endemic plant in the Western Ghats. It is a woody trailing vine which is sparsely spiny in nature with quadrangular branches (Gamble, 2004). The leaves are broadly ovate to suborbicular and round to cuspidate at apex. *Smilax wightii* is a valued medicinal plant in India. The plant is ethnobotanically important, since it is used to cure various ailments in Indian and Chinese systems of medicine. But, because its limited distributionthe plant is underutilized. A decoction of the bulbous roots of this plant is used to promote the healing of sores, swellings and abscesses.In Ayurveda root is used to cure diseases related to the nervous system and the urinary disorders. There are reports regarding the plant being usedto cure skin diseases, venereal diseases, dysentery, amoebiasis,gastric complaints and also for the treatment of spermatorrhoea to promote fertility (Adhikari*et al*., 2010). But sufficient scientific data is not available to support its commercial use.The plant has proved to possess strong antioxidant activity and anti-inflammatory activity (Maheswari*et al*., 2014).The medicinal properties of the plant are not completely known and are yet to be explored. The therapeutic potential of any plant is determined by its phytochemical constitution. Regarding the phytochemistry of *Smilax* species, besides having the common classes of phytochemicals, they show high diversity in its Saponin content. They have been reported to contain phytoconstituent dioscin, plant steroids such as sarsaponin, smilasaponin, diosgenin,asperagenin,smilagenin and sarsapogenin. Steroidal saponins also contribute to the therapeutic potential of the plant. So preliminary phytochemical profiling is the key step in analyzing the medicinal properties of any plant.

Diseases caused by the pathogenic bacteria are a major health hazard in both developed and developing countries. The commonly used synthetic antibiotic drugs have their share of toxic side effects. Also the increasing antibiotic resistance among pathogens is a major issue to be taken care of. So the need of the hour is a better natural therapy to combat these diseases. Screening of medicinal plants for their antibiotic potential is the initial step towards achieving this.Antimicrobial compounds are compounds secreted by microorganisms, or derived from plants or

synthesized in the laboratory which in certain concentration inhibit the growth, multiplication or metabolism of microorganism (Blood, 1990). According to ethnobotanical evidences *Smilax wightii* is being effectively used against some bacterial diseases. So the chances are high that the plant contains bacteriocidal compounds. So the present study focuses on the preliminary phytochemical screening and the investigation of the antibacterial potential of the root of the plant *Smilax wightii*.

MATERIALS AND METHOD

Plant Collection

The plant material was collected from Athirumala forest range of Peppara Wildlife sanctuary, in Thiruvananthapuram district of Kerala, India.

Preparation of Extract

The collected plant material was washed in running water and shade dried. Then it was powdered using a grinder and stored in an air tight container. Soxhlet extraction was done using solvents of increasing polarity. The solvents used were petroleum ether, chloroform, methanol and distilled water. The extract was collected and filtered. The filtrate was evaporated inside an oven at a temperature of 50^0C.

Phytochemical Profiling

Phytochemical profiling was done using standard procedures described by Harborne (1973)and Trease and Evans(1989), to analyse the presence of metabolites.

Test for alkaloids

(i)Mayer's Test

Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.

(ii)Wagner's Test

Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/ reddish precipitate indicates the presence of alkaloids.

(iii) Dragendroff's Test

Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.

Test for flavonoids

(i)Alkaline Reagent Test

Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

(ii)Shinoda test

To 2ml test solution, few fragments of Magnesium ribbon (LobaChemie, India) were added and to it conc. H2SO4 was added drop wise. Pink scarlet or crimson red colour appears.

Test for glycosides

Keller- killiani test

Crude extract was mixed with 2ml of glacial acetic acid containing 1-2 drops of 2% solution of $FeCl_3$. 2 ml of con.H_2SO_4 was poured along the side of the test tube. A brown ring at the junction indicated the presence of Glycoside.

Test for Cardiac glycosides

Keller- killiani test

Crude extract was mixed with 2ml of glacial acetic acid containing 1-2 drops of 2% solution of $FeCl_3$. Then 2 ml of con. H_2SO_4 was poured along the side of the test tube. A blue –green colour indicated the presence of cardiac glycosides.

Test for reducing sugar

Fehling's Test

To 2ml test solution, equal quantity of Fehling's solution A and B was added and solution was heated. A brick red precipitate indicates the presence of glycosides.

Detection of saponins

Foam Test

0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

Test for tannins

Ferric chloride test

A small quantity of the extract was added to 2 ml of water in a test tube. To this mixture 2 to 3 drops of diluted ferric chloride solution was added and the appearance of a green to blue-green colour indicated the presence of Tannins.

Test for phlobatannin

0.5g of the crude extract was dissolved in distilled water and filtered. The filtrate was boiled with 2% HCl. Deposition of red precipitate indicated the presence of phlobatannin.

Test for terpenoids

Salkowski test

To the test solution 2 ml. chloroform was added. Then 3 mL of conc. Sulphuric acid was added and shaken well. Appearance of yellow colour shows the presence of triterpenoids.

Test for steroids

Salkowski test

To the test solution 2ml chloroform was added. Then 3 mL of conc. Sulphuric acid was added and shaken well. Appearance of reddish brown colour at lower layer indicates presence of steroids.

Test for Coumarins

To 1mL of the crude extract add 0.5 mL of 40% NaOH and 1 mL of ethanol. Formation of yellow colour indicated the presence of coumarins.

Antibacterial Activity

Agar-Disc diffusion assay (Kirby et al., 1966) was used to study the antibacterial potential of the methanolic extract of the root of *Smilax wightii* against the target microorganisms.

Target Microorganisms

The microorganisms used were six bacterial strains pathogenic to humans. It included the Gram positive bacteria Bacillus. The Gram negative bacteria selected were Salmonella, Klebsiella, Escherichia coli, Serratia and Vibrio cholerae. The selected bacterial strains are very pathogenic and they can cause many serious diseases.

Reagents

1.Nutrient Agar Medium (1 L)

The culture medium was prepared by dissolving 31.16 g of nutrient agar (HI Media) and10g Agar in 1000ml of distilled water. The prepared medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto 100mm sterile petriplates.

2. Nutrient broth

The nutrient broth was prepared by adding 1.5g of Peptone powder in 100mL distilled water. The medium was poured into sterile culture tubes with 5mL in each tube. This medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes.

Preparation of inoculum

The selected bacterial strains were inoculated into 5 mL of the nutrient broth in sterile condition. This was incubated at 37^0C for two hours.

Preparation of extract

The increasing concentrations of the root methanolic extract namely 250µg/mL, 500µg/mL, 1000µg/mL were prepared using DMSO. The antibiotic drug AmoxycillinTrihydrate was used as the standard. Paper discs with a diameter of 4.5mm made from Whatman No.1 filter paper were impregnated with the different concentrations of the root methanolic extract and 20µg/mL concentration Amoxycillin Trihydrate.

Agar-Disc Diffusion Assay

The selected bacterial suspensions (1.5×10^8) were asceptically swabbed onto the surface of the Agar plates with the help of sterile inoculation loop. The paper discs prepared were placed on the surface using sterile forceps. The petri-plates were incubated at 37^0C for 24 hours. After incubation the zone of inhibition was measured and the activity index was measured using the equation:

$$\text{Activity index} = \frac{\text{Diameter of the zone of inhibition by the plant extract}}{\text{Diameter of the zone of inhibition by the standard}}$$

Table 1. Preliminary phytochemical screening of *Smilax wightii* root extracts

PHYTOCHEMICAL GROUP	ROOT EXTRACT			
	PETROLEUM ETHER	CHLOROFORM	METHANOL	DISTILLED WATER
Alkaloids	- - -	- + +	+ + +	- + +
Flavonoïds	- - -	- + -	+ + +	+ + +
Glycosides	- - -	- - -	+ + -	- - -
Cardiac glycosides	- - -	- - -	- - -	- - -
Reducing sugars	- - -	- + -	+ + +	- - -
Saponins	- - -	- - -	+ + +	+ + +
Tannins	- - -	- - -	- + -	- - -
Phlobatannins	- - -	- - -	- - -	- - -
Terpenoids	+ + +	+ + -	+ - +	- - +
Steroids	- - -	- - -	+ + -	- + -
Coumarins	- - -	- - -	+ + +	- - -

+ + indicate the presence of the phytochemical

- -indicate the absence of the phytochemical

RESULT AND DISCUSSION

All the medicinal and other properties possessed by plants are conferred to them by the diverse class of phytochemicals they contain. Phytochemicals are secondary metabolites and are often found in disposable parts of the plants (Kumar and Singh, 1976). So analysis of the phytochemical constitution of the plant extract is an important step in investigation of the various properties possessed by any plant. So the preliminary phytochemical profiling of the root extract of *Smilax wightii* was done using various standard methods. The Petroleum ether, Chloroform, Methanol and distilled water extracts were analyzed. The results are summarized in the table 1.

In the phytochemical screening the petroleum ether and chloroform extracts showed the presence of trace amounts of terpenoids and alkaloids. The methanolic extract indicated the presence of alkaloids,flavonoid, glycosides, reducing sugars, saponins, tannins,steroids, coumarins etc. The distilled

Table 2. The diameter of the zone of inhibition produced by *Smilax wightii* root extract on the nutrientAgar plates

Bacterial strain	Diameter of the zone of inhibition (in mm)			
	Drug 20 µg/mL	Root extract 250 µg/mL	Root extract 500 µg/mL	Root extract 1000 µg/mL
Salmonella	31	13	17	26
Klebsiella	25	06	13	20
Bacillus	30	20	24	26
Escherichia coli	29	13	18	19
Serratia	25	Nil	Nil	Nil
Vibrio cholerae	31	Nil	19	23

Table 3. The antibacterial activity index of *Smilax wightii*root methanolic extract against the selected bacterial strains.

Bacterial strain	Type of strain	Activity index		
		Root extract 250 µg/mL	Root extract 500 µg/mL	Root extract 1000 µg/mL
Salmonella	Gram(-)	0.41	0.54	0.83
Klebsiella	Gram(-)	0.24	0.52	0.80
Bacillus	Gram(+)	0.66	0.80	0.86
Escherichia coli	Gram(-)	0.44	0.62	0.79
Serratia	Gram(-)	Nil	Nil	Nil
Vibrio cholerae	Gram(-)	Nil	0.61	0.74

water extract too had alkaloids, flavonoids, saponins etc. Among the four solvents used, highest number of phytochemicals was recorded in the root methanolic extract. So the root methanol extract was selected for further analysis.

Antibacterial activity study through Agar – Disc diffusion Assay

There are numerous human pathogenic bacteria in the environment that cause serious health hazards worldwide. They lead to mortality and morbidity in the human population. The development of multidrug resistance among pathogens has become the major challenge in curing these diseases. Most of the drugs used in modern day medicine are originally extracted from plant sources like roots, leaves, barks and seeds (Moore and Winston, 1996). At present there is a need for the development of a potent,plant based antibiotic compound to combat these bacterial diseases.So the screening of *Smilax wightii* for its antibacterial potential was done. The antibacterial activity study of the root methanolic extract of *Smilax Wightii* against six pathogenic bacterial strains was done through Agar –Disc diffusion Assay.The zone of inhibition was measured and recorded in the table 2.

The zone of inhibition represents the inhibition of the microbial growth by the plant extract. The zone of inhibition produced by the different concentrations of the root methanolic extract is comparable with the zone of inhibition produced by the antibiotic drug AmoxycillinTrihydrate. The root extracts with the increasing concentrations like 250µg/mL, 500µg/mL,1000µg/mL showed a concentration dependent increase in the antibacterial activity. The clear zone produced by the 1000 µg/mL concentration of the root methanolic extract was much closer in values to the clear zone produced by the antibiotic. There was good inhibitory activity against the bacterial strains like Salmonella,Bacillus,Vibrio cholerae and Klebsiella. There was no activity recorded against the Gram negative bacteria Serratia. There are reports regarding the *Smilax zeylanica* leaves showing antibacterial activity against many strains. It produces an inhibitory zone of 5.39 to 9.87mm in plates containing E.coli (Aslam et al.,2013). The activity index was calculated and recorded in the Table 3.

The activity index is the measure of the antibacterial activity. The values suggest that *Smilax wightii* root methanolic extract has higher bactericidal activity against the pathogenic strains like Salmonella, Bacillus, Vibrio cholerae, Escherichia coli and Klebsiella. The highest inhibitory activity was recorded against the bacterial strain Bacillus with an activity index of 0.86, followed by Salmonella and Klebsiella with the activity index of 0.83 and 0.80 respectively. So the present analysis has proved that the plant *Smilax wightii* possess antibacterial activity which is comparable with Amoxycillin, a commercially available antibiotic drug. It is obvious that the plant contains some metabolites that has strong bactericidal property.

CONCLUSION

The phytochemical profiling of the root of *Smilax wightii* indicated the presence of the diverse class of phytochemicals. The root methanolic extract contain more diverse class of phytochemicals. The results indicated the presence of compouds like Alkaloids, flavonoid, glycosides, Reducing sugars, Saponins,

Tannins, steroids and Coumarins which may contribute to the antibacterial activity of the compound. Hence the antibacterial activity of the root methanol extract was analysed. This too showed that the root extract has considerable antibacterial activity against the target microorganisms. So there are some metabolites present in the plant *Smilax wightii* that inhibits the multiplication or growth of the target microorganisms. The present investigation has justified the use of the root of *Smilax wightii* against bacterial ailments by traditional healers. So the plant has the potential to be used against many bacterial infections and after further research the plant can be used for the development of a potential antibacterial drug.

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Protein Profiling Alteration in Response to Heavy Metal Accumulation in *Alternanthera sessilis* R Br. With Special Reference to Metallothionein

M. DEVI CHINMAYEE[1], G. SANGEETHA[1], G.S. NIKHILA[1], I. MINI[1], AND T.S. SWAPNA [2 *]

[1]*Department of Botany, University College, Thiruvananthapuram, Kerala*
**Department of Botany, University of Kerala, Kariavattom.*
email: swapnats@yahoo.com

ABSTRACT

Bioaccumulation and biomagnification of heavy metals (HMs) in the environment have become a major threat for all living organisms including plants. Exposure to heavy metals has been documented to induce changes in the expression of plant proteins. Proteins are macromolecules directly responsible for most biological processes in a living cell, while protein function is directly influenced by posttranslational modifications, which cannot be identified through genome studies. Therefore, it is necessary to conduct proteomic studies, which enable the elucidation of the presence and role of proteins under specific environmental conditions like heavy metal stress. The plant selected for present study was *Alternanthera sessilis* belonging to the family Amaranthaceae. In order to assess the role of HMs accumulation towards protein profile of the plants, plants were exposed to increasing of Cd, Cr and Pb for a period of 30 days under heavy metal stress condition. The HMs metal concentration in the bioparts of experimental plant revealed that the treated plants accumulated enormous quantity of lead, cadmium and chromium as compared to control plant in various bioparts. Highest accumulation was shown by Pb treated plants and maximum was reported in Pb treated root. Among three metals, Cd was least accumulated compared to other two metals. The result showed significant HMs absorption and accumulation by *Alternanthera sessilis* root and areal parts. There is a strong positive correlation between concentration of HMs in soil and plant parts. Present study demonstrated that the exposure of plants to heavy metal resulted in the reduction of growth parameters. The total protein content of leaves, roots and stem declined in treated plant in comparison to control. To enhance the understanding of the molecular mechanisms, comparison of protein profile changes due to heavy metal stress is done by SDS-PAGE analysis and found that several proteins were newly synthesized. The work suggests the relationship between HMs stress and alterations of protein patterns which will provide a new insight for better understanding of the molecular basis of stress responses to plants. Metallothioneins (MTs), which widely distributed in eukaryotic and prokaryotic organisms, are a class of cysteine-rich heavy metal-binding proteins with low molecular weights. To date, the molecular mechanisms that regulate heavy metal tolerance have not been fully investigated. Further studies will clarify the roles of *A. sessilis* MT genes in metal homeostasis and scavenging of ROS and assess the applicability of *A. sessilis* MTs to phytoremediation of contaminated soils.

Key words *Metallothioneins; SDS-PAGE; Alternanthera sessilis; phytoremediation; heavy metal.*

Plants experience biotic and abiotic stresses that endanger their survival. In a broad sense, plant stress can be defined as "any unfavorable condition or substance that affects or blocks a plant's metabolism, growth or development" (Lichtenthaler,1996). The metallic elements with atomic weight more than twenty and those with a relative density greater than 5 g cm^{-3} and specific gravity greater than 4 are considered as heavy metals. Heavy metal pollution has emerged as one of the major threats to agricultural crops. Metals like Zn, Ni, Cu, Mo, Mn, Cd, Co, Cr, Pb etc. when present in high concentrations in soil show potential toxic effects on overall growth and metabolism of plants (Schutzendubel and Polle, 2002; Hall and Williams, 2003). Heavy metal ions such as Cu_2+, Zn_2+, Mn_2+, Fe_2+, Ni_2+, and Co_2+ are essential micronutrients for plant metabolism but when present in excess, and other non essential metals such as Cd_2+, Hg_2+, Ag_2+, and Pb_2+ can become extremely toxic (Williams, et al., 2000). The toxic influence of these metals on plants results in strong and fast inhibition of growth processes. The detrimental effects of these heavy metals on the photosynthetic apparatus often correlates with progressing senescence processes (Molas, 2002; Sobkowiak and Dekert, 2003; Alaoui *et al*., 2004).

Heavy metals that have been identified in the polluted environment include As, Cu, Cd, Pb, Cr, Ni, Hg and Zn. The presence of metal may vary from site to site, depending upon the source of individual pollutant. Excessive uptake of metals by plants may produce toxicity in human nutrition, and cause acute and chronic diseases (Garbisu and Alkorta, 2001). Heavy metals selected for present study was Pb, Cr, and Cd. Environmental lead contamination increased substantially in the 20th century with the advent of

motor vehicles and the use of lead in petrol. Children are at particularly high risk of exposure to environmental lead, because of their elevated rates of development, ingestion and metabolism, and because of their developmental and behaviour patterns. Chromium (Cr) is the 24th element of the Periodic table and the most important stable states are 0 (elemental metal), +3 (trivalent) and +6 hexavalent. Chromium induced metabolic disturbance include inhibition of photosynthesis and electron transfer chain (Shanker *et al*., 2003) and disturbance in water relations. Cadmium is a non-essential element that negatively affects plant growth and development. It is released into the environment by power stations, heating systems, metal-working industries or urban traffic. It is widely used in electroplating, pigments, plastic stabilizers and nickel-cadmium batteries (Sanita di Toppi and Gabrielli, 1999). It is recognized as a significant pollutant due to its high toxicity and large solubility in water (Pinto *et al*., 2004).

The exposure of plants to toxic levels of heavy metals triggers a wide range of physiological and metabolic alterations (Villiers *et al.*, 2011). The most widespread visual evidence of heavy metal toxicity is the reduction in plant growth along with leaf chlorosis, necrosis, turgor loss, a decrease in the rate of seed germination, and a crippled photosynthetic apparatus, often resulted in progressing senescence processes and plant death (Carrier *et al.*, 2003; Dalcarso *et al.*, 2010). All these effects are related to ultrastructural, biochemical, and molecular changes in plant tissues and cells brought about by the presence of heavy metals (Gamalero *et al.*, 2009).

The identification of the functional genes or proteins that are involved in responses to heavy-metal stress is a fundamental step in understanding the molecular mechanisms of stress responses; such an understanding could lead to the development of transgenic plants that have an enhanced tolerance to heavy-metal stress or to plants capable of being used in phytoremediation (removal of toxic elements from soils). A large number of physiological and biochemical analyses have been carried out in analyzing the plant response to heavy metals, which has been the subject of some excellent reviews (Sanita di Toppi and Gabrielli, 1999).

In order to alleviate the stress and re-establish cellular homeostasis and antioxidant capacity, plants have developed highly effective mechanisms to regulate the uptake, accumulation, distribution, and detoxification of heavy metal ions (Hossain and Komatsu, 2013). Vital components in these mechanisms are metal transporters responsible for metal uptake and vacuolar transport, chelators for heavy metal detoxification and tolerance, and chaperones which play a significant role in the delivery and trafficking of metal ions (Clemens 2001, Sharma *et al* 2008), all of which are protein molecules. That is why the identification of protein markers could serve as a good starting point for revealing new aspects of heavy metal stress in plants (Bona *et al* 2007). If the toxic metal concentration exceeds a certain threshold inside the cells, an active metabolic process contributes to the production of chelating compounds. Specific peptides such as Phytochelatins (PCs) and Metallothionein MTs are used to chelate metals in the cytosol and to sequester them in specific subcellular compartments. A large number of small molecules are also involved in metal chelation inside the cells, including organic acids, amino acids, and phosphate derivatives (Rauser 1999). Metallothioneins (MTs) are ubiquitous low-molecular-weight, cysteine-rich proteins that can bind metals via mercaptide bonds and first MT was characterized from horse kidneys as cadmium-binding proteins in 1957 (Margoshes & Vallee, 1957).

The positive correlation between MT expression in diverse organisms and the environmental metal concentration suggests that MTs can be effective biomarkers of heavy metal pollution. Such monitoring programs have already gained great potential comprehensively in aquatic and terraneous invertebrates (Chu *et al.*, 2006; Dallinger et al., 2004; Navarro *et al.*, 2009). In plants, MTs are favorable candidates for phytoremediation of heavy metal contaminants, a low-cost, effective, and sustainable plant-based approach for environment governance (Eapen & D'Souza, 2005; Memon & Schroder, 2009). Aim of the present study also includes evaluating stress potential of weed plant *Alternanthera sessilis* in heavy metal contaminated soil. Since these weeds are widespread in many geographical areas and survive many stressed conditions, they are selected for the study and can be used as phytoremediator for heavy metals.

MATERIALS AND METHODS

Bioassay

Pot culture method was adopted for the bioassay. Healthy plants of *Alternanthera sessilis* were collected from its natural habitat. Threshold level of each metal was estimated and three different treatment concentrations (T1, T2, T3) of each metal salt was applied to soil. Garden soil was collected and uniformly saturated with varying concentrations of Lead nitrate (50, 100 and 150 mg Kg^{-1}), Cadmium sulphate (20, 30 and 50 mg Kg^{1}), Potassium dichromate (50, 75 and 100 mg Kg^{-1}) as T1, T2, T3 for each metal. The test plants were grown in pots containing 2kg medium saturated with corresponding concentration of metal. After one month, plants were harvested, washed with double distilled water, blotted and separated leaves and roots were used for the study.

A known quantity of the sample was digested with a mixture of concentrated nitric acid and perchloric acid (4:1) for eight hours and the digested sample was cooled filtered and made up to a known volume. The analysis of trace elements (cadmium, lead and chromium) was carried out following the method of APHA (1992). The estimation of heavy metals were performed by aspirating the digested sample in Atomic absorption spectrophotometer, Perkin Elmer model 2380 and the values expressed in mg Kg^{-1}.

Protein profiling

Total protein levels were determined following the method of Lowry *et al.* (1951) using bovine serum albumin as a reference. SDS-PAGE for total proteins in control and treated plant samples was carried out according to the method of Laemmli (1970). Proteins were extracted with 0.1 M ice-cold phosphate buffer (pH 7.2) and centrifuged. 200 µl of the protein was mixed with 50µl 10% SDS loading buffer containing 1.875M Tris HCl buffer (pH 6.8), 10% SDS, 5% sucrose, 5% mercaptoehanol and 1% bromophenol blue. The samples (50 µl sample + 25µl loading dye) were boiled for 3 min and loaded onto 12% polyacrylamide gel. The gel was run at 200 V and proteins were detected by Coomassie brilliant blue R-250 staining.

MT Content

MT in the leaf tissues after *in vivo* exposures was determined by the concentration of SH-groups following the method of Viarengo *et al.* (1997). Plant tissue samples were homogenized (sucrose 0.5 M, PMSF 0.5 mM, ß-mercaptoethanol 0.01% in Tris–HCl buffer 20 mM, pH8.6), centrifuged (14 000 × g, 20 min, 4æ%C) and cold absolute ethanol and chloroform were added to the supernatant. These samples were centrifuged (7000×g, 10 min, 4 æ%C), cold ethanol was added to the supernatant and the mixture was maintained at "20 æ%C for 1 h. Samples were centrifuged again (7000 × g, 10 min, 4 æ%C) and the pellet was resuspended in the same buffer as used for homogenization, but with 87% ethanol and 1% chloroform. After that samples were dried at 35 æ%C for up to 24 h, the pellet was resuspended (0.25 M NaCl, 1 N HCl, 4 mM EDTA) and Ellman reagent (0.43 mM DTNB buffered with 0.2 M Na-phosphate, pH 8.0) was added to the sample. MT was estimated using reduced glutathione as a reference at 412 nm and expressed as nM MT mg protein"1.

RESULTS AND DISCUSSION

Heavy metal accumulation in *Alternanthera sessilis*

Lead: The concentrations of lead in the soil and plant tissues are given in (Fig 1). Accumulation of lead was high in the roots followed by stem and leaves. More over, the plant was able to withstand a concentration of 150 mg Kg-^{1} without much change in the morphology and functions that indicated its possible utility as an accumulator of lead.. Once introduced in to the soil matrix, lead is very difficult to remove. The metal is strongly bound to organic matter by adsorption, precipitation and complexation. Water soluble and exchangeable lead are the only fractions readily available to plants. Soil pH has a significant effect on the mobility of lead within soil. Lead neither enters into solution nor into the biological cycle as it gets inactivated at high pH and remains immobile in the soil. Lead had very short retention time in water and major portion of lead is bound to the cell walls of plants. The normal range in the concentration of lead in plants is 0.1 to 10 µg g^{-1} (Leeper, 1978). The values reported herein were well above the normal range. The *Alternanthera sessilis* survived under high concentration of lead in the soil with slight variation in the morphological and biochemical parameters.

Cadmium : Phytoaccumulation of cadmium in the roots were comparatively higher than leaf and stem

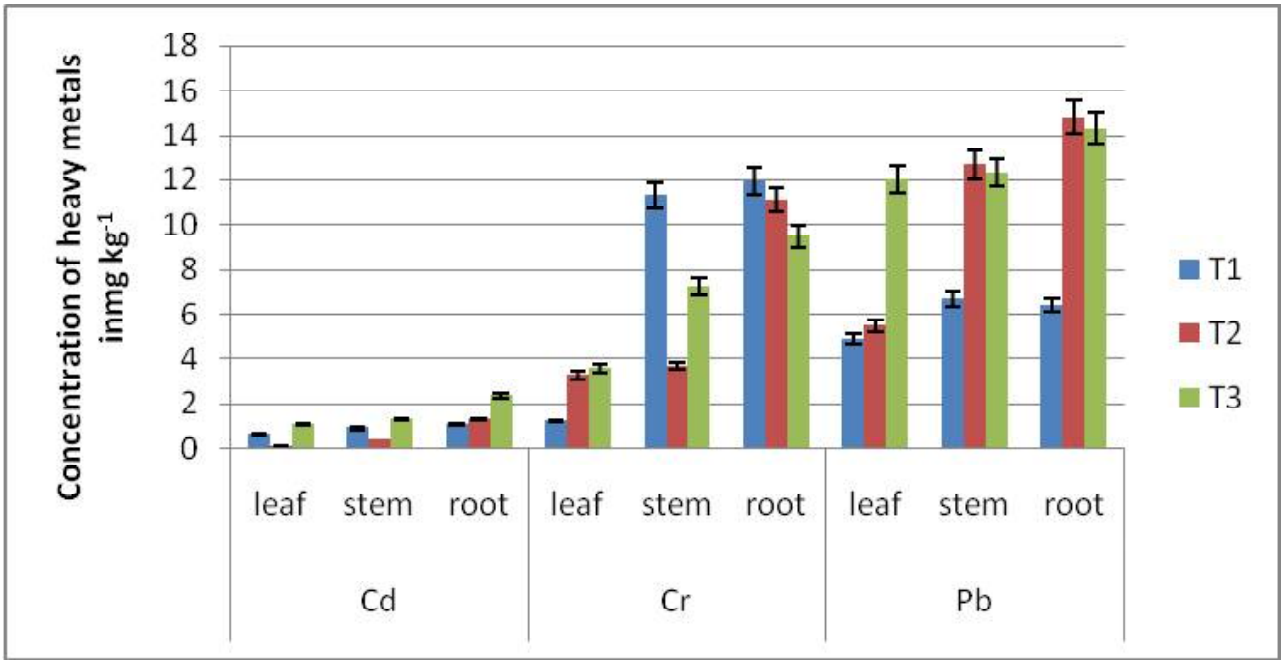

Fig 1. Accumulation of heavy metals(mg kg^{-1}) in *Alternanthera sessilis*

Pb- Lead treated plant Cr- Chromium treated plant Cd- Cadmium treated plant
T1- Treatment 1 T2- Treatment 2 T3- Treatment 3

(Fig 1). Transportation of heavy metals from root to shoot has been studied in several species, including ryegrass (Secale cereale; Jarvis *et al.*, 1976). Movement of Cd from roots to shoots is likely to occur via the xylem and to be driven by transpiration from the leaves. Evidence for this was provided by Salt *et al.*(1995) who showed that ABA- induced stomatal closure dramtaically reduced Cd accumulation in shoots of Indian mustard.

Chromium : The concentration of chromium was high in the roots followed by stem and leaves (Fig 1). There was a gradual decrease in accumulation in leaves as the concentration of treatment increased while increase was displayed in stem and root. Vincent *et al.* (2001) opined that the uptake of the metal depend on the soil pH, drainage status, presence of organic matter, sewage sludge, microbial activity, plant species, chemical form.

The term hyper accumulator is used to describe plants that accumulate more than 1000ìg/gram dry weight in their shoots in the natural habitat (Brooks *et al.*, 1977). Results based on analysis of bioconcentration factor and translocation factor revealed that uptake of these metals was more in the

Table 1. Bioconcentration factor and Translocation factor in *A.sessilis* Under Heavy Metal Stress

Treatment(mgKg?[1])		TF	BCF		
			leaf	stem	root
Cd	T1	1.382	0.031	0.0026	0.0215
	T2	0.327	0.043	0.0115	0.0258
	T3	1	0.0538	0.0431	0.0474
Cr	T1	1.049	0.0243	0.0434	0.355
	T2	0.624	0.2264	0.0491	0.0722
	T3	1.138	0.239	0.148	0.0946
Pb	T1	1.808	0.0974	0.0727	0.1203
	T2	1.221	0.134	0.1265	0.0821
	T3	1.702	0.128	0.1483	0.0954

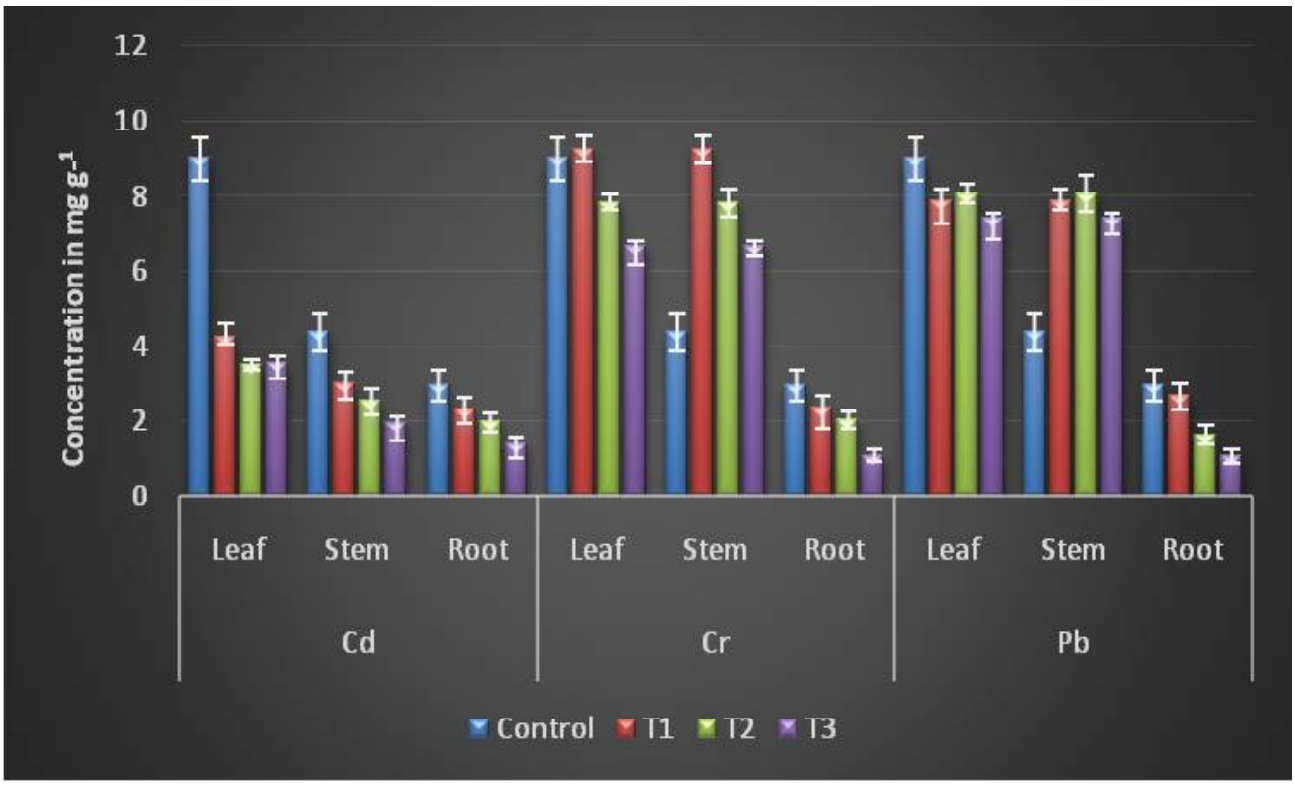

Fig 2. Protein content in *Alternanthera sessilis* under heavy metal stress

Pb- Lead treated plant Cr- Chromium treated plant Cd- Cadmium treated plant
T1- Treatment 1 T2- Treatment 2 T3- Treatment 3

roots than shoots of the plant species investigated in this trial (Table 1). Ogunyemi *et al.* (2003) reported accumulation of Pb and Cd in the shoots and roots of *Amaranthus cruentus* grown in soils from landfill sites with concentration higher than the optimum allowed by FAO/WHO for dietary consumption.

Protein Content and SDS Profile

In comparison with the control, total protein content of root tissue exhibited significant reduction in plants treated with all heavy metals (Table 1).

. Protein content of the stem tissue showed significant reduction in all metal treated plants compared to the control (Fig 2). Protein content of the leaf tissues also was reduced on treatment with all heavy metals more or less uniformly. Reduced protein content may due to inhibition of protein synthesis as reported by Reddy and Prasad (1992) and Orcutt and Nilsen, (2000) or unavailability of essential components like aminoacids (Prasad, 1997) or inhibition of aminoacids mobilization to the site of protein synthesis (Bishno 1993).

Alternanthera sessilis expressed several new polypeptides under heavy metal stress. Several bands were also found to be induced in *Alternanthera sessilis,* in Cd treated *A. sessilis,* five new bands in Cd T1 treatment (molecular weight- 104.138, 83.015, 58.918 ,43.012, 37.94 KDa), 8 new bands in Cd T2 treatment (molecular weight- 106.271, 81.622, 72.859, 58.918, 50.485, 43.012, 23.365, 21.255 KDa) and 9 new bands in Cd T3 treatment (molecular weight- 210.858, 106.271, 63.037, 44.105, 41.25, 37, 22.436, 21.255, 20 KDa) were observed (Fig 3). Four polypeptides present in control were found missing in Cd treatment. Band with Rf value 0.411 and 0.584 were induced due to Cd treatment were seen on Treatment T1 and T2 (Table 2), but disappeared in high concentration Cd treatment (T3).

Eight new bands in Cr treatment T1 (molecular weight- 127.542, 106.271, 72.159, 63.037, 59.489, 42.653, 23.524 KDa), 8 new bands in Cr T2 (molecular weight- 130.155, 06.271, 72.159, 60.649, 52.473, 26.688, 23.052, 21.691, 15.29 KDa), 5 new bands of molecular weight- 104.138, 83.015, 58.918, 43.012, 37.94 KDa were detected in Cr treatment T3 (Fig 3). Two bands with molecular weights 108.447 KDa

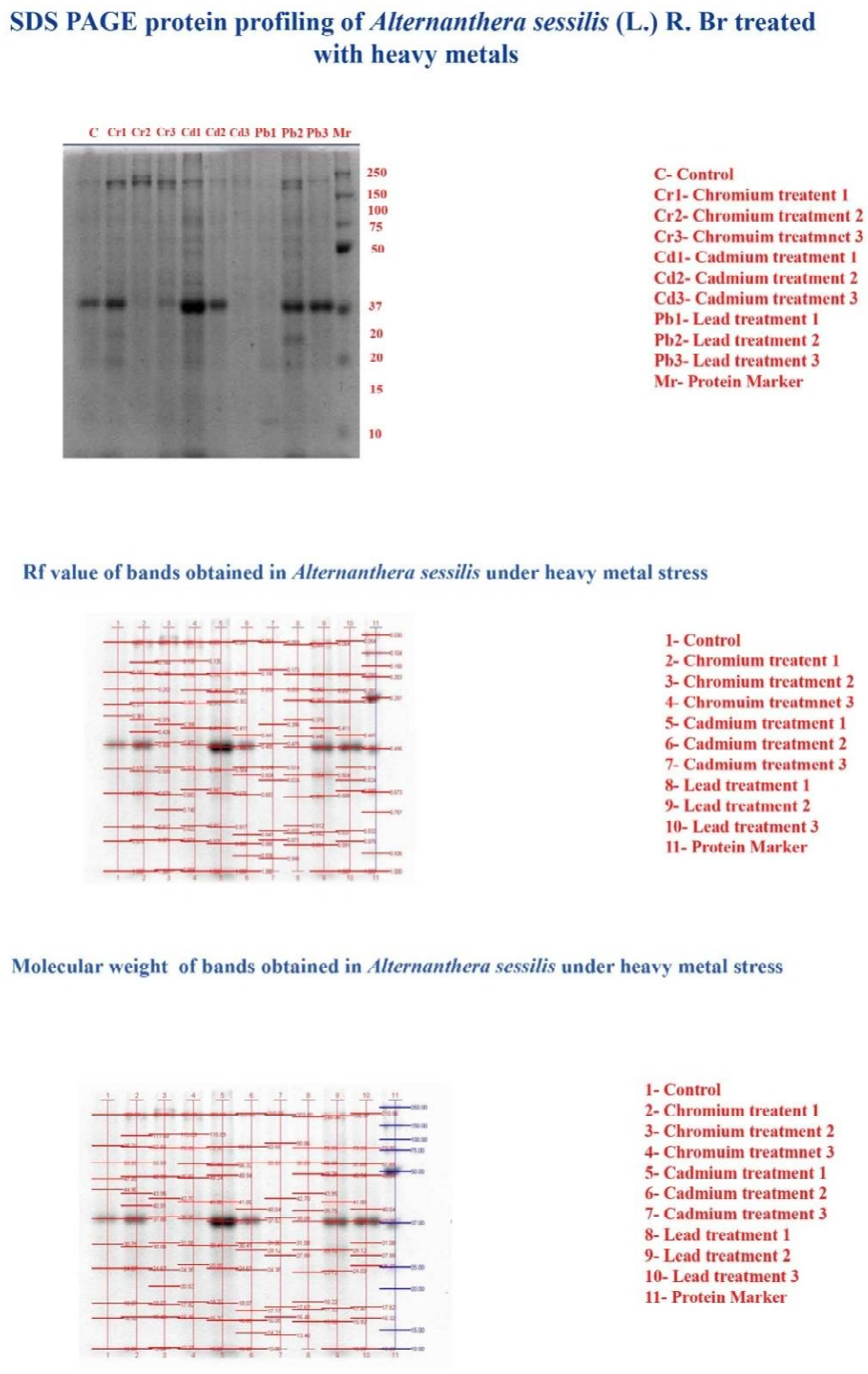

Fig 3

and 68.098 KDa noted in control disappeared due to Cr treatment in *A. sesslis*. Band with Rf value 0.188 and 0.307 were induced due to Cr treatment were seen on Treatment T1 and T2, but disappeared in high concentration Cr treatment (Table 3).

In lead treated *A. sessilis* also many new polypeptide were synthesized and dissociated (Fig 3). Pb treatment induced nine new bands (molecular weight- 117.608, 60.066, 51.969, 44.195, 40.228, 23.844,22.896, 22.285, 18.523 KDa) in treatment (T1), 9 new bands (molecular weight- 190.38, 106.271, 73.566, 55.068, 50.975, 42.653, 33.913, 22.896, 20.969

Table 2. Rf Value and Molecular weight of protein bands in Cd treated *Alternanthera sessilis*

Control			Cd1			Cd2			Cd3		
Band Number	Relative Front	Mol. Wt. (KDa)	Band Number	Relative Front	Mol. Wt. (KDa)	Band Number	Relative Front	Mol. Wt. (KDa)	Band Number	Relative Front	Mol. Wt. (KDa)
1	0.059	203.798	1	0.059	203.798	1	0.059	203.798	1	0.054	210.858
2	0.183	108.447	2	0.193	104.138	2	0.188	106.271	2	0.188	106.271
3	0.252	84.432	3	0.257	83.015	3	0.262	81.622	3	0.252	84.432
4	0.337	68.098	4	0.411	58.918	4	0.302	72.859	4	0.376	63.037
5	0.48	51.469	5	0.48	51.469	5	0.411	58.918	5	0.485	50.975
6	0.579	43.373	6	0.584	43.012	6	0.49	50.485	6	0.569	44.105
7	0.678	36.203	7	0.658	37.94	7	0.584	43.012	7	0.609	41.25
8	0.876	21.399	8	0.876	21.399	8	0.678	36.203	8	0.673	37
9	1	15	9	1	15	9	0.812	23.365	9	0.842	22.436
						10	0.881	21.255	10	0.881	21.255
						11	1	15	11	0.926	20
									12	1	15

KDa) in treatment (T2) and 8 new bands (molecular weight- 203.798, 104.138, 72.859, 55.068, 50.485, 43.737, 40.228, 22.742 KDa) in treatment T3 (Table 4). Protein profiling by SDS PAGE analysis showed several new bands with difference in molecular weight and number under individual metals probably due to difference in their metal binding property or differences in tolerance potential of *Alternanthera sessilis* towards each metal.

MT Content

Metallothionein content varied with increasing concentration of heavy metals and shown in Fig 4. In plants, MTs are induced by various abiotic stresses but are also expressed during development (Rauser 1999). MTs probably have different functions in response to different heavy metals and could also participate in additional antioxidant protection mechanism and plasma membrane repair (Hamer 1986).

Table 3. Rf Value and Molecular weight of protein bands obtained in Cr treated *Alternanthera sessilis*

Control			Cr1			Cr2			Cr3		
Band Number	Relative Front	Mol. Wt. (KDa)	Band Number	Relative Front	Mol. Wt. (KDa)		Relative Front	Mol. Wt. (KDa)		Relative Front	Mol. Wt. (KDa)
1	0.059	203.798	1	0.059	203.798	1	0.059	203.798	1	0.059	203.798
2	0.183	108.447	2	0.144	127.542	2	0.139	130.155	2	0.193	104.138
3	0.252	84.432	3	0.188	106.271	3	0.188	106.271	3	0.257	83.015
4	0.337	68.098	4	0.252	84.432	4	0.307	72.159	4	0.411	58.918
5	0.48	51.469	5	0.307	72.159	5	0.396	60.649	5	0.48	51.469
6	0.579	43.373	6	0.376	63.037	6	0.47	52.473	6	0.584	43.012
7	0.678	36.203	7	0.406	59.489	7	0.574	43.737	7	0.658	37.94
8	0.876	21.399	8	0.48	51.469	8	0.678	36.203	8	0.876	21.399
9	1	15	9	0.589	42.653	9	0.748	26.688	9	1	15
			10	0.678	36.203	10	0.822	23.052			
			11	0.807	23.524	11	0.866	21.691			
			12	0.871	21.544	12	0.995	15.29			

Table 4. Rf Value and Molecular weight of protein bands obtained in Pb treated *Alternanthera sessilis*

Control			Pb1			Pb2			Pb3		
Band Number	Relative Front	Mol. Wt. (KDa)	Band Number	Relative Front	Mol. Wt. (KDa)	Band Number	Relative Front	Mol. Wt. (KDa)	Band Number	Relative Front	Mol. Wt. (KDa)
1	0.069	190.38	1	0.049	218.163	1	0.054	210.858	1	0.015	276.892
2	0.197	77.746	2	0.187	83.544	2	0.187	83.544	2	0.059	203.798
3	0.261	56.333	3	0.246	60.511	3	0.251	59.085	3	0.192	80.593
4	0.31	48.239	4	0.3	48.936	4	0.3	48.936	4	0.256	57.693
5	0.384	43.321	5	0.438	40.036	5	0.374	43.947	5	0.36	44.902
6	0.438	40.036	6	0.483	37.534	6	0.483	37.534	6	0.473	38.076
7	0.493	37	7	0.571	31.083	7	0.567	31.424	7	0.606	28.802
8	0.576	30.747	8	0.65	26.113	8	0.645	26.399	8	0.714	21.659
9	0.65	26.113	9	0.852	16.555	9	0.739	20	9	0.872	16.019
10	0.828	17.249	10	0.985	10	10	0.985	10	10	0.906	15.124
11	0.99	9.733							11	0.985	10

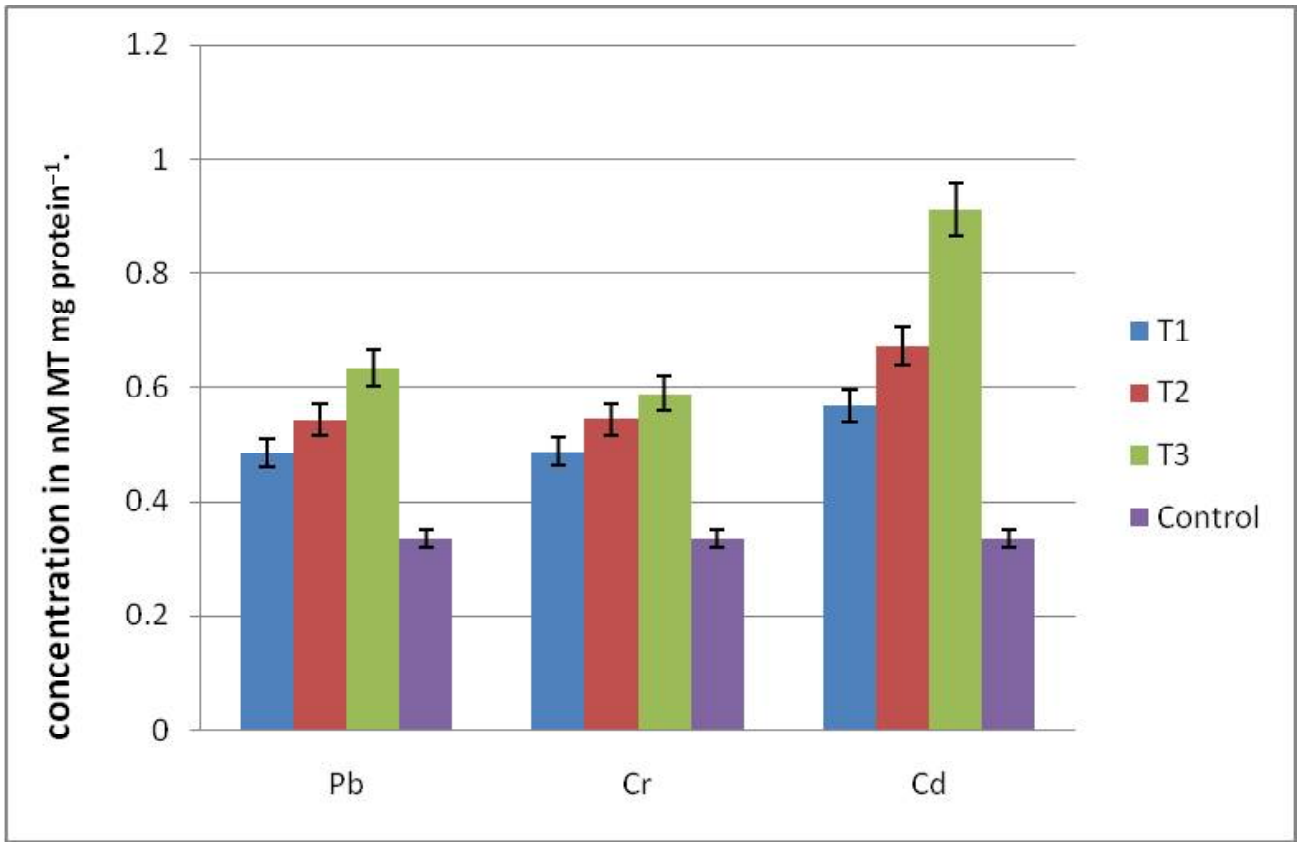

Fig 4. Metallothionein content in *Alternanthera sessilis* under heavy metal stress

Pb- Lead treated plant Cr- Chromium treated plant Cd- Cadmium treated plant

T1- Treatment 1 T2- Treatment 2 T3- Treatment 3

CONCLUSION

The work suggests the relationship between heavy metal stress and alterations of protein patterns which will provide a new insight for better understanding of the molecular basis of stress responses in plants. The result showed significant heavy metal absorption and accumulation by *Alternanthera sessilis* root and areal parts. The

heavy metal concentration in the plant was found in descending order Pb>Cr>Cd. The total protein content of plant declined in treated plant in comparison to control. SDS-PAGE analysis indicated that several proteins were newly synthesized. There is a strong positive correlation between concentration of heavy metals in soil and plant parts. Metallothionein content varied with different heavy metal treatment. Further studies will clarify the roles of *A. sessilis* MT genes in metal homeostasis and scavenging of ROS and assess the applicability of *A. sessilis* MTs in phytoremediation of contaminated soils.

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Nutritional Evaluation of *Plectranthus rotundifolius* Tubers

V.J. DEVI, A.V. ASWATHY AND P G BIJU*

Department of Biochemistry, University of Kerala, Kariavattom Campus, Thiruvananthapuram, Kerala- 695581
**email : pgbiju75@gmail.com*

ABSTRACT

In the present study, the nutritional composition of *Plectranthus rotundifolius* tubers in terms of proximate composition, vitamin and mineral content, and antioxidant enzymes were evaluated. Proximate analysis is the partitioning of compounds in a food into six categories: moisture, ash, protein, lipid, carbohydrate and fibre. *P. rotundifolius* tubers were found to contain 82.81% moisture, 5.85% protein, 5.26% carbohydrate, 1.36% Lipid and fibre and 4.72% ash. Mineral content analysis indicated the presence of Li, Al, Cr, Mn, Fe, Ni, Co, Cu, Zn, Ga, As, Se, Sr, Ag, Ba, Na, K and Ca in P. *rotundifolius* tubers. Significant presence of these minerals indicates that *P. rotundifolius* is a good mineral source. Vitamin analysis revealed the significant presence of antioxidant vitamins, Vitamin A (0.426 ± 0.05 mg/g wet weight, Vitamin C (0.96 ± 0.06 mg/g wet weight) and Vitamin E (9.89 ± 0.15 mg/g wet weight). This potentiates the use of *P. rotundifolius* tubers as a vitamin source. Moreover, due to the antioxidant nature, these vitamins act as a potent scavenger of free radicals. In addition to that, *P. rotundifolius* tubers were found to contain significant amount of antioxidant enzymes Superoxide Dismutase (0.0651 ± 0.06 units/mg protein), Catalase (0.167 ± 0.16 units/mg protein), Glutathione Peroxidase (31.97 ± 0.05 units/mg protein) and Glutathione S Transferase (19.68 ± 0.10 units/mg protein), which highlights the antioxidant nature of the tubers. Hence this study highlights the potential of *P. rotundifolius* tubers as an excellent nutritional source.

Key words *Plectranthus rotundifolius, Tubers, Nutritional evaluation*

Any substance of both plant and animal origin, which is consumed in order to provide nutritional support to the body, is regarded as food. It should contain all the essential nutrients (both micronutrients and macronutrients) in sufficient quantity for maintaining the nutritional status of the body. Balanced diet supplies all nutrients in adequate amount in order to maintain optimal health. Most food has its origin in plants, since they are indispensable constituent of human diet supplying the body with minerals, salts, vitamins and certain hormone precursors, in addition to protein and energy (Amaechi., 2009). Plant tubers, also known as nature's buried treasures have been important sources of food for thousands of years. The main nutritional value of roots and tubers lie in their potential ability to provide one of the cheapest sources of dietary energy in the form of carbohydrates and are vital for economically meeting the quantitative and qualitative nutritional needs of many a nation (Idusigie and Olayide., 1975; Olojede., 2013).

Plectranthus rotundifolius (Fig 1) belongs to the mint family and is a perennial herbaceous plant, commonly known as Chinese potato, coleus potato or Hausa potato. Tubers of this plant is used as a vegetable, most commonly eaten as cooked vegetable. These tubers have medicinal properties and are used as purgative, carminatives and as antihelmintics (Anbuselvi and Hemapriya., 2013). Reports indicate that they are also used to treat nervous and sensory disorders associated with ear and eye problems (Catherine *et al*., 2006). In spite of their importance as a food source, there are only a few published data on the nutritional composition of *Plectranthus rotundifolius* tubers. The present study was therefore initiated to evaluate the nutritive values of *Plectranthus rotundifolius* tubers and to enhance the knowledge on the nutritional implication on feeding these tubers.

Fig. 1. Plectranthus rotundifolius
A : Cultivar, B : Individual Plant C : Tuber (Sample

MATERIALS AND METHODS

Specimen collection

Plectranthus rotundifolius tuber samples were collected from Central Tuber Crops Research Institute (CTCRI), Sreekaryam, Thiruvananthapuram district of Kerala state, India during the months of February and March. The specimen was authenticated by an expert (Dr. Valsaladevi, Curator, Department of Botany, University of Kerala), KUBH-6012.

Proximate analysis

Estimation of the total carbohydrate content was performed by the method of Hedge and Hofreiter., 1962. Carbohydrate is first hydrolysed into simple sugar (glucose) using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxmethyl furfural, which upon reacting with anthrone forms a green coloured product, which is read colorimetrically at 630nm. The crude protein content of the tuber in both PBS and Alkali soluble fraction was estimated by Bradford assay (Bradford., 1976). Tubers were weighed and ground in both PBS and alkali and the protein was precipitated by using 10% TCA. The tubes were centrifuged and the concentration of protein in the pellet was quantified using Bradford reagent. The moisture content of *P. rotundifolius* tuber samples was estimated by hot air oven method. Weighed tuber sample was placed in a preweighed dry crucible, the lid of the dish was loosened and heated in an oven at 105 ± 1p C for 3 days. After 3 days, the dish was removed from the oven, cooled in a desicator and weighed. % moisture content can be calculated by the formula..

$$\% \text{ moisture content} = \frac{\text{Loss in gram of the material on drying} \times 100}{\text{Weight in gram of the material taken for the test}}$$

Ash content was estimated by placing the sample in a high temperature muffle furnace capable of maintaining temperatures of between 500 and 600 ^{o}C for 24 hrs. Water and other volatile materials are vaporized and organic substances are burned in the presence of oxygen in air to CO_2, H_2O and N_2. Most minerals are converted to oxides, sulfates, phosphates, chlorides or silicates. The % ash content can be calculated by the formula..

$$\% \text{ ash content} = \frac{\text{Weight in gram of ash obtained} \times 100}{\text{Weight in gram of the material taken for the test}}$$

Antioxidant vitamin composition

The concentration of the antioxidant vitamins Vitamin A, Vitamin C and Vitamin E was estimated in *Plectranthus rotundifolius* tubers. Vitamin A concentration was determined using UV irradiation method. Ground tuber sample was shaken with equal amount of ethanol and heptane and the two layers were allowed to separate. The heptane layer was divided into two equal parts and one part was irradiated for 3 hours with UV light. The extinction of the non irradiated part was read at 327nm using the irradiated part as blank. The vitamin A concentration was determined from the standard curve by plotting the extinction of standards plotted in the same way. Vitamin C in the tuber sample was estimated by the method of Omaye *et al*., 1962. Ground tuber sample was mixed with distilled water and DTCS reagent. After incubation at 37^{o} C for 3 hours, ice cold 65% Sulphuric acid was added and again incubated at 37^{o} C for 30 min. Series of standards were prepared and processed in the same way. Absorbance was determined at 520 nm and the concentration of Vitamin C was estimated. Concentration of Vitamin E in the tuber sample was estimated by the method of Varley *et al*., 1984. The reaction is based on the conversion of ferric to ferrous ions by tocopherols which then forms a red colour with 2,2'- dipyridyl. Tocopherols and carotenoids in the ground tuber sample was first extracted with xylene, mixed with 2,2' – dipyridyl reagent and the extinction was measured at 460nm to measure carotenoids. A correlation was made for these after adding ferric chloride and reading at 520nm. The amount of vitamin E can be calculated using the formula.

$$\text{Vitamin E } (\mu g/g) = \frac{(\Delta A520nm - \Delta A460nm) \times \text{conc [S]} \times 0.29 \times \text{Total volume}}{\Delta A520nm \times \text{Vol for experiment} \times \text{Weight of sample}}$$

Mineral Analysis

The inductively coupled mass spectrometry system Thermo Scientific ICAP Qc was used for the elemental analysis. The external calibration solutions were prepared from standard certified multielement solution (MERCK).Thermo scientific BRANSTEAD Smart2pure water containing 1% suprapur grade Nitric acid (MERCK) was used to get a range of concentrations 25ppb, 50ppb, 100ppb, 250ppb, 500 ppb for all elements. Samples containing higher

concentration of elements than this calibration range are diluted and analyzed by applying dilution factors. The ion optics was tuned using Thermo scientific Tune-B ICAP-Q solution in standard mode and KED mode. Mass and detector calibration was conducted using Thermo scientific Setup solution ICAPQ. Powdered tuber sample was acid digested with nitric acid and Sulphuric acid and was hold for 25min, zero ramp time at 400 watts and then retained for 30 min, 5 ramp at 500 watts. After cooling, the vessels were opened and the samples are diluted to 100ml with Milli-Q water (Thermo Scientific, Barnstead, Smart 2 pure). The vessels were closed and shaken thoroughly to complete the dissolution. The details of the instrument are given below.

Operating Conditions

Peristaltic pump speed - 40 rpm

Cool flow - 14.00 L/min

Sampling depth - 5

Plasma power - 1550 watts

Auxiliary flow - 0.80 L/min

Nebulizer flow - 0.97

No of main runs – 3

Elements Sodium, Potassium and Calcium were detected by the technique of Flame photometry, which works on the basis of heating the metal, such that the atoms of the metal travel from ground state to their excited state. The atoms then return to their ground state and release their energy as photons of ultraviolet radiation. The wavelength of the UV radiation is then measured. The reagent in this experiment is the standard stock solution of NaCl, KCl and CaCl2 added to deionized H2O to make different concentrations of aqueous Na+ Cl , K+ Cl , Ca2+ Cl, to be analyzed by the flame photometer. The solutions of known concentration and the emission intensities from the flame photometer are used to make a calibration curve from which the concentration of the unknown can be determined.

Activity of antioxidant enzymes

The activity of the antioxidant enzymes Superoxide Dismutase (SOD), Catalase, Glutathione Peroxidase (Gpx) and Glutathione S transferase in the tuber samples were monitored. Activity of Superoxide dismutase was measured by the method of Kakkar *et a.l*, 1984. The assay mixture contained 0.052M sodium pyrophosphate buffer (pH 8.3), 186 µM PMS, 300 µM NBT, 780 µM NADH, appropriately diluted enzyme preparation and water to a final volume of 3 ml. Reaction was started by the addition of NADH, incubated at 30° C for one minute, stopped by the addition of glacial acetic acid followed by n-butanol. The mixture was allowed to stand for 10 minutes, centrifuged, the butanol layer taken out and measured at 560 nm against a butanol blank. A system devoid of enzyme served as the control. Activity of catalase was measured by the method of Aebi H, 1984. Reaction mixture containing enzyme preparation (in 50 mM phosphate buffer, pH 7.0) and 30 mM H_2O_2 (in 50 mM phosphate buffer, pH 7.0) was prepared. A system devoid of the substrate (H_2O_2) served as the control. Reaction was started by the addition of the substrate and decrease in absorbance monitored at 240 nm for 30 seconds at 25°C. The difference in absorbance per unit time was expressed as the activity.

Activity of Glutathione Peroxidase determined by the procedure of Lawrence *et al*, 1976 as modified by Agerguard *et al*, 1982. The reaction mixture contained 0.1M Phosphate buffer (pH 7.4), 20mM Sodium azide, 0.1mM NADPH, and water. To this, enzyme source was added and the mixture was allowed to incubate at room temperature for 5 minutes. The reaction was initiated by the addition of 7.5mM H_2O_2 solution. The oxidation of NADPH was measured using a spectrophotometer at an interval for next 20s for 5min at 340nm. Activity of Glutathione S Transferase was monitored by the method described by Habig *et al*., 1974. Reaction mixture consists of both substrates GSH and CDNB (1-chloro-2,4-dinitrobenzene) in 0.1M Phosphate buffer (pH 6.5). Enzyme source was added to this mixture and the readings were recorded against distilled water blank for a minimum of 3 minutes at 340nm. The complete assay mixture without the sample served as the control to monitor non- specific binding of the substrates.

RESULTS

Proximate composition

P. rotundifolius tubers were subjected to crude carbohydrate, crude protein, crude lipid, moisture and ash content analysis by the methods described in chapter 2 and the result is presented in Fig. 2.

The moisture and ash content of the tuber ranged from 82.81% and 4.72% respectively. The tubers were found to contain 5.85% crude protein and the concentration of protein in PBS and alkali soluble

fractions are presented in Table 1. Carbohydrate content was found to be 5.26% and the other 1.36% was expected to be lipid and fibre. Substantial quantities of minerals are expected as indicated by the ash content. The major content of the tuber was found to be moisture, contributing to the shorter shelf life of this otherwise rich food material.

Table 1. Concentration of protein in both PBS and Alkali soluble fraction of P. rotundifolius tubers. Values are expressed as mean ± SEM of samples analyzed in triplicate.

Protein Fraction	Content (mg/g wet weight)
PBS Soluble Fraction	32.5 ± 0.15
Alkali Soluble Fraction	26.0 ± 0.07

Mineral Analysis

P. rotundifolius tubers were subjected to mineral content analysis. All minerals except Sodium, Potassium and Calcium were analyzed by ICP-MS. Analysis of Sodium, Potassium and calcium were carried out by Flame Photometric Analysis. The result of mineral analysis is illustrated in Table 2. Several essential minerals were found in the tubers. Rich amount of Iron, Sodium and Calcium are of particular nutritional significance and enhance the nutritional importance

Vitamin content Analysis

P. rotundifolius tubers were subjected to antioxidant vitamin analysis by the methods previously discussed. The results are illustrated in Table 3. Significant amount of vitamin A (0.426 ± 0.05 mg/g wet weight), Vitamin C (0.96 ± 0.06 mg/g wet weight) and Vitamin E (9.89 ± 0.15 mg/g wet weight) were obtained and the tubers were especially found to be a rich source of vitamin E (alpha-tocopherol) in particular. This indicates the vitamin abundance and the antioxidant nature of *P. rotundifolius* tubers.

Table 3. Concentration antioxidant vitamins in *P. rotundifolius* tubers. Values are expressed as mean ± SEM of samples analyzed in triplicate.

PARAMETERS	ESTIMATED QUANTITY (mg/g wet weight)
VITAMIN A	0.426 ± 0.05
VITAMIN C	0.96 ± 0.06
VITAMIN E	9.89 ± 0.15

Antioxidant enzyme assay

As a conformation of the antioxidant nature of P. rotundifolius tubers, the activities of the antioxidant enzymes Superoxide dismutase, Catalase, Glutathione peroxidase and Glutathione s transferase were monitored. The results are indicated in Table 4. The tubers were found to have significant activities of the enzymes superoxide Dismutase (0.0651 ± 0.06 units/mg protein), Catalase (0.167 ± 0.16 units/mg protein), Glutathione Peroxidase (31.97 ± 0.05 units/mg protein) and Glutathione S Transferase (19.68 ± 0.10 units/mg protein), which highlights the antioxidant nature of the tubers.

Table 2. Mineral content analysis of P. rotundifolius tubers f *P. rotundifolius* tubers.

Parameter	Estimated Quantity (ppm)		Parameter	Estimated Quantity (ppm)
Li	0.6		Ga	1.9
Be	0.0		As	0.1
Al	392.3		Se	1.1
Cr	2.2		Sr	9.7
Mn	7.7		Ag	2.2
Fe	**267.7**		Cd	0.0
Ni	5.9		Ba	10.3
Co	0.9		**Ca**	**166.27**
Cu	15.8		**Na**	**201.89**
Zn	12.9		**K**	**99.67**

Table 4. Activity of antioxidant enzymes in *P. rotundifolius* tubers. Values are expressed as mean ± SEM of samples analyzed in triplicate.

Antioxidant Enzyme	Activity (Units/mg protein)
Superoxide Dismutase (SOD)	0.0651 ± 0.06
Catalase	0.167 ± 0.16
Glutathione Peroxidase (GPx)	31.97 ± 0.05
Glutathione S Transferase (GST)	19.68 ± 0.10

DISCUSSION

In the present study, proximate composition, mineral and vitamin analysis and activities of antioxidant enzymes of Plectranthus rotundifolius tubers were evaluated. The partitioning of compounds in a food into six categories viz. moisture, ash, crude protein, crude lipid, crude fiber and nitrogen free extracts (digestible carbohydrates) based on the chemical properties of the compound is termed as proximate analysis. Generally plant tubers are rich in starch and indeed they are often considered solely as a source of carbohydrate for diets or of industrial use (Peter, R. S., 2003). Tubers do contain protein, but their levels are substantially lower than that of seeds (Peter, R.S., 2003). In our study, *P. rotundifolius* tubers were found to contain substantial quantity of carbohydrate and protein, while slightly deficient in lipids and fibers.(fig.2)

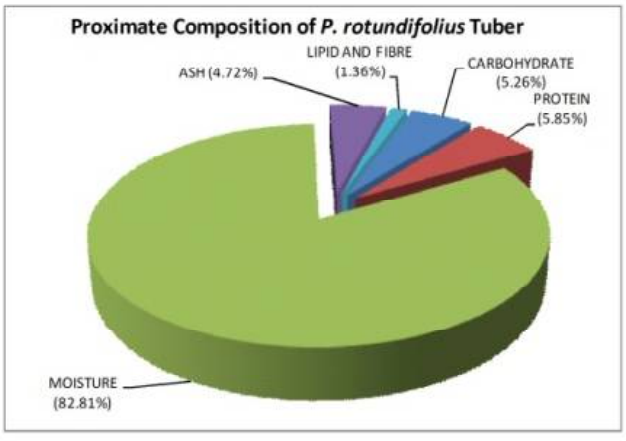

Fig 2. Proximate composition of *P. rotundifolius* tubers

Ash content in food is obtained as the residue remaining after all the moisture has been removed as well as the organic material (fat, protein, carbohydrates, vitamins, organic acid etc) have been incinerated at a high temperature. It is generally taken to be a measure of the mineral content of the original food (Onwuka, 2005). Presence of significant ash content in *P. rotundifolius* reveals the mineral abundance in the tuber sample. Moreover mineral analysis also revealed the presence of significant minerals like iron, sodium and potassium in significant amount (Table 2). Moisture content of a feed sample is related to the shelf life of the sample (Olaitan *et al*., 2015). High moisture content in feed samples often leads to spoilage from increasing microbial action when stored (Onyeike *et al*., 1995). The major content of the tuber was found to be moisture, contributing to the shorter shelf life of this otherwise rich food material.

Vitamins are the organic compounds that are required in minute quantities to sustain life and prevent disease. In the present study, the amount of antioxidant vitamins like Vitamin A, Vitamin C and Vitamin E was found in appreciable quantity (Table 3). Vitamin A is required by human for normal growth of body cells, skin, and for proper vision and its deficiency can result in night blindness and reduced resistance to diseases. Vitamin C aids wound healing and also help in resisting infection. Its deficiency can cause scurvy, poor wound healing and low resistance to infection. The recommended dietary allowance of vitamin C is 45mg per day (WHO, 1991). Vitamin E is a powerful, fat-soluble antioxidant that helps protect cell membranes against damage caused by free radicals and prevents the oxidation of LDL cholesterol. It is necessary for structural and functional maintenance of skeletal, cardiac, and smooth muscle and it also assists in the formation of red blood cells and helps to maintain stores of vitamins A and K, iron, and selenium. In the present study, vitamin analysis of P. *rotundifolius* tubers revealed the presence of substantial quantities of Vitamin A, C and E. This potentiates the use of these tubers as a food rich in vitamins with potent free radicals scavenging activity.

Antioxidant enzymes have been considered to be of particular importance for the scavenging of free radicals. Superoxide Dismutase (SOD), Catalase, Glutathione Peroxidase (GPx) and Glutathione S Transferase (GST) plays important roles in the removal of superoxide, hydrogen peroxide and hydroxyl radicals. In our study, the activities of these enzymes

were monitored, and significant activity was found in the sample (Table 4). This is a confirmation of the antioxidant nature of *P. rotundifolius* tubers.

This work is focused on the nutritional and antioxidant potential of *P. rotundifolius* tubers, which revealed the potential nutritive value of these moderately consumed tubers. They can be used as an alternative source against hunger and malnutrition, which is currently one of the major problems in many developing countries. The outcome of this work is aimed at spreading the knowledge on the nutritive value of these tubers and promote their utilization as a valuable food source.

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Micropropagation of Five Ecotypes of *Musa paradisiaca cv. nendran* and Media Optimization for Selected Ecotype, *Chaganasseri Nendran.*

P.V. JAYASREE, STEEPHAN MATHEW*, M.S. SHEEBA*

Post Graduate and Research Department of Botany, Government College for Women, Thiruvananthapuram, Kerala, India
**email : sheebamadhavan@gmail.com*

ABSTRACT

Plant tissue culture technology is being widely used for large scale plant multiplication. Bananas and plantains constitute the fourth most important global food commodity. India's fertile soils support hundreds of banana varieties, but farmers only cultivate roughly 12 local and 30 exotic varieties commercially. Nendran (AAB) is a popular variety in Kerala where it is relished as a fruit as well as used for processing. In this study, five Nendran ecotypes cultivated in Kerala such as Attunendran, Changanasseri nendran, Chengazhikodan, Manjeri nendran and Mettupalayam nendran were used for micropropagation. The cultures of the collected ecotypes were successfully initiated and established through meristem culture in MS medium containing BAP (3 mg/L). Media optimization for multiplication of selected ecotype, Changanessry nendran, was done by using different concentrations of BAP and combinations of BAP (0.5, 0.1, 1, 2, 3,4 and 5 mg/L) and NAA (0.5 and 0.1 mg/L) in MS medium. Number of shoots and growth parameters were measured for finding out optimal media. Combination of BAP and NAA at a concentration of 0.5mg/L and 0.1 mg/L respectively was found to be optimal media for culturing Changanasseri Nendran. Established cultures of five ecotypes were successfully hardened.

Key words *Nendran; Micropropagation; MS medium; BAP; NAA; Ecotype*

Plant cell culture has been hailed as one of the most significant potential adjuncts to plant improvement (Brown and Thorpe; 1995). The composition of a medium, preferable to a certain plant species, is nearly the main task for the establishment of a successful plant cell and tissue culture technique. Shoot-tip cultures of *Musa* cultivars (both banana and plantain) are induced by culturing small excised shoot apices on modified MS semisolid medium supplemented with various concentrations and combinations of auxins and cytokinins (Nirmalya B. and Edmond de L., 1985). The different types of banana have not been fully exploited since Cavendish types mainly dominate the market place globally (Sumalatha A., 2016).

In tissue culture, plant growth regulators (PGR) are critical media components in determining the developmental pathway of the plant cells. Cytokinins such as benzylaminopurine (BAP) and kinetin are generally known to reduce the apical meristem dominance and induce both axillary and adventitious shoots formation from meristematic explants in banana. Concentration of BAP is an important factor to increase the multiplication rate for plantlets and concurrently control its mutagenic effect so as to decrease the percentage of morphologically abnormal plantlets formation (U. P. Bhosale, 2011).

The majority of edible bananas are (Musa L.) are triploid (2n = 3x = 33) cultivars known as deserts, plantains or cooking bananas with predominant AAA, AAB and ABB genomes (Aish M. et.al., 2007). The procedure for commercial micropropagation of banana plantlets consists of four stages: Culture initiation, bud multiplication, plantlet regeneration and acclimatization in the nursery. TTC plantlets' uniformity of growth makes it possible to control the time of flowering and harvesting, and give a significant increase in yield and fruit quality (Shin-C. H. and Hong-Ji S., 1998).

India's fertile soils support hundreds of banana varieties, but farmers only cultivate roughly 12 local and 30 exotic varieties commercially. Each of these types varies subtly in their taste, appearance, and even aroma. In Kerala, the variety, Nendran ranks first in commercial value. The cultivators of the Agasthiamalai ranges call this variety "King of Banana". The shelf-life of the fruits of Nendran is more, compared to that of others. So, the fruits of Nendran have been exported to the Arabian and European countries. There are many varieties of banana cultivated in the Agasthiamalai ranges. Medicinally inflorescence axis of Nendran is a good lithontryptic; its fruit is laxative and the flower is for menstrual disorder. Fruit pulp contains vitamins B1, B2, B3, vitamin C, amino acids, iron, calcium, phosphorus and proteins in substantial amount which are the daily need diet for human beings (J. Lohi das, 2010).

Nendran is known to display considerable diversity in plant stature, pseudostem colour, presence or absence of male axis, bunch size, etc. Bunch has 5-6 hands weighing about 12-15 kg. Fruits have a

distinct neck with thick green skin turning buff yellow on ripening. Fruits remain as starchy even on ripening. Nendran is highly susceptible to Banana Bract Mosaic Virus (BBMV), fungal attack and nematodes(Sheela and Ramachandran Nair,2001).

Fewer studies in micropropagation were done in ecotypes of nendran. It is very essential to optimize the culture media of these ecotypes in large scale production. So this study is focused to initiate and establish the cultures of five selected varieties of nendran such as Attunendran, Changanasseri nendran, Chengazhikodan, Manjeri nendran and Mettupalayam nendran and media optimization of selected ecotype, changanassery nendran.

MATERIALS AND METHODS

Explants

Sword suckers of selected elite plants of banana cultivars (Attunendran, Changanassrei nendran, Chengazhikodan, Manjeri nendran and Mettupalayam) were collected from fields at Mannarkkad and the pseudostem containing the meristem was selected as explant. The sheathing leaf bases were removed from the pseudostem, leaving the young leaves around the meristem.

Chemicals and instruments

Chemicals for MS media, sucrose, myo-inositol and plant hormones such as indole-3-butyric acid (IBA), Naphthalene acetic acid (NAA), benzyl aminopurine (BAP) used were tissue culture grade. Aseptic operations (inoculations were carried out in a laminar air flow hood (Frendz Inc). The instruments were frequently glass bead sterilized during manipulation.

Culture initiation

The explant material was washed thoroughly in running tap water for 15-20 minutes. Then the explants were treated with 0.1% Carbendazime (a fungicide) and Cefotaxime (an antibiotic) in a shaker for about 20 minutes and transferred to laminar air flow cabinet for aseptic manipulation. Subsequently they were briefly rinsed with distilled water followed by treatment with 0.1% mercuric chloride solution for 10 minutes and then washed 5-6 times in sterile distilled water. Intact shoot apex and one or more pairs of leaf primordial together with 2-3 mm in size including the rhizomatous base were selected for inoculation. The explants are cultured in nutrient medium (MS with 3 mg/L BAP) (Murashige T., 1978). Multiplication of shoots is done at regular intervals to increase the number of plants.

Media optimization for Changanassery nendran

From the cultures initiated, Changanassery nendran was selected for studies on medium optimization. Cultures of banana (Changanassery) cultivar raised from shoot tip culture were used for the study. Initiated cultures were multiplied and maintained in MS medium with 3mg/L BAP solidified with 10 g/L Agar Agar. For shoot multiplication, clumps with shoots were transferred to fresh medium regularly. By regular subculturing the stock plants were multiplied to sufficient quantities for further experiments. In order to find out a better media for multiplication of Banana cv. Changanassery, explants were cultured in different combinations of auxin & cytokinin. The explants were cultured in, different concentrations of BAP(cytokinin), i.e, 0-5 mg/L and combinations of BAP & NAA (auxin) shown in Table 1. The cultures were maintained 4 weeks and the data about the length of shoots, girth of shoots, number of leaves, and length of leaves were taken and has been recorded.

Table 1. Concentrations of BAP and NAA used in media optimization studies

Sl.No.	BAP (mg/L)	NAA (mg/L)
1	0.1	0.1
2	1	0.1
3	2	0.1
4	0.1	0.5
5	1	0.5
6	2	0.5

Rooting

After obtaining the required number of shoots by multiplication they are transferred to rooting medium. The medium used for rooting is MS+0.5 mg/L IBA. Roots develop in two week's time.

Hardening

After rooting phase banana plantlets were removed from containers, the gel like medium is gently washed off from developed roots. Pre-hardened plantlets with well developed roots were taken to green house for hardening. Soils with well mixed dried leaves

Table 2. Response of explants during culture initiation of the five cultivars of Banana

Variety	No. of shoot tips initiated	Percentage of survival	Browning	Time taken for shoot bud for establishment	No. of shoots developed after 17 weeks of initiation
Attunendran	10	60%	moderate	6-7 weeks	50
Changanasseri nendran	12	83.3%	moderate	5-6 weeks	52
Chengazhikodan	10	50%	moderate	5-6 weeks	30
Manjeri nendran	10	30%	moderate	7-8 weeks	18
Mettupalayam	10	30%	moderate	7-8 weeks	30

were taken as potting media.

RESULTS AND DISCUSSION

Culture initiation of nendran ecotypes

Chengazhikodan, Manjeri and, Mettupalayam nendran showed considerable variation in percentage of contamination and time taken to develop into shoot clumps (Table 2). The frequent subculturing (5-7) days in the beginning reduced the rate of browning and contamination to a great extent. After establishment, the interval between subcultures was increased to 2 weeks and then to 4 weeks. The highest percentage of survival is shown by cv. Changanasseri nendran (83.3%) and least survival rate (30%) by Manjeri and Mettupalayam nendran. The major contamination was due to fungi. Bacterial contamination was also observed in some. After 2 weeks greening of the leaf sheaths occurred and the meristem became enlarged. Meristems of cv. Changanasseri and cv. Chengazhikkodan showed early response compared to others. After 7-8 transfers new shoots developed from the axillary buds. The meristems of cv. Attunendran became multiplying in 6-7 weeks while the cv. Manjeri nendran and cv. Mettupalayam took about 7-8 weeks to become multiplying cultures.

Optimization of multiplication media for Changanasseri nendran

BAP has a marked effect in stimulating the growth of axillary and adventitious buds and foliar development of shoot tip cultures. Medium containing 3 mg/L BAP was used for initiation. Various concentrations of BAP ranging from 0-5 mg/L (0, 1, 2, 3, 4, and5mg/L) and also combination of BAP and NAA at different concentrations were tried out (Table 1 & Figure 1). Response in shoot production and growth parameters was recorded after four weeks (Table 3). No significant increase in number of shoots up to 5 mg/L BAP. Parameters like number of leaves, girth of shoots were found to show an increase at higher concentrations of BAP. Length of shoots and leaf tend to increase at lower concentrations of BAP.

Table 3. Response of Changanassery nendran on MS medium supplemented with different concentrations of BAP.

Sl. No.	BAP mg/L	No.of shoots (Mean±SD)*	Length of shoots in cm (Mean±SD)*	Girth of shoots in cm (Mean±SD)*	No. of leaves (Mean± SD)*	Length of leaf in cm (Mean±SD)*
1	0	1a	4.4±1.43b	0.26±0.05	2.2±0.45a	3.3±1.12cd
2	1	1a	9±0.29c	0.24±0.05	3.4±0.55b	2.84±0.59cd
3	2	1a	7.7±0.44d	0.28±0.08	3±0.71ab	4±0.71e
4	3	1a	2.7±0.29a	0.26±0.05	2.8±0.84ab	1.4±0.36ab
5	4	1.25±0.50ab	3.5±0.70ab	0.32±0.10	3.8±0.84bc	2.2±0.44bc
6	5	1.50±0.55b	2.8±0.57a	0.30±0.14	4.6±1.34c	0.8±0.44a

(*values followed by the same alphabet are not significantly different by Duncan's Multiple range test at Pe"0.05 within a column)

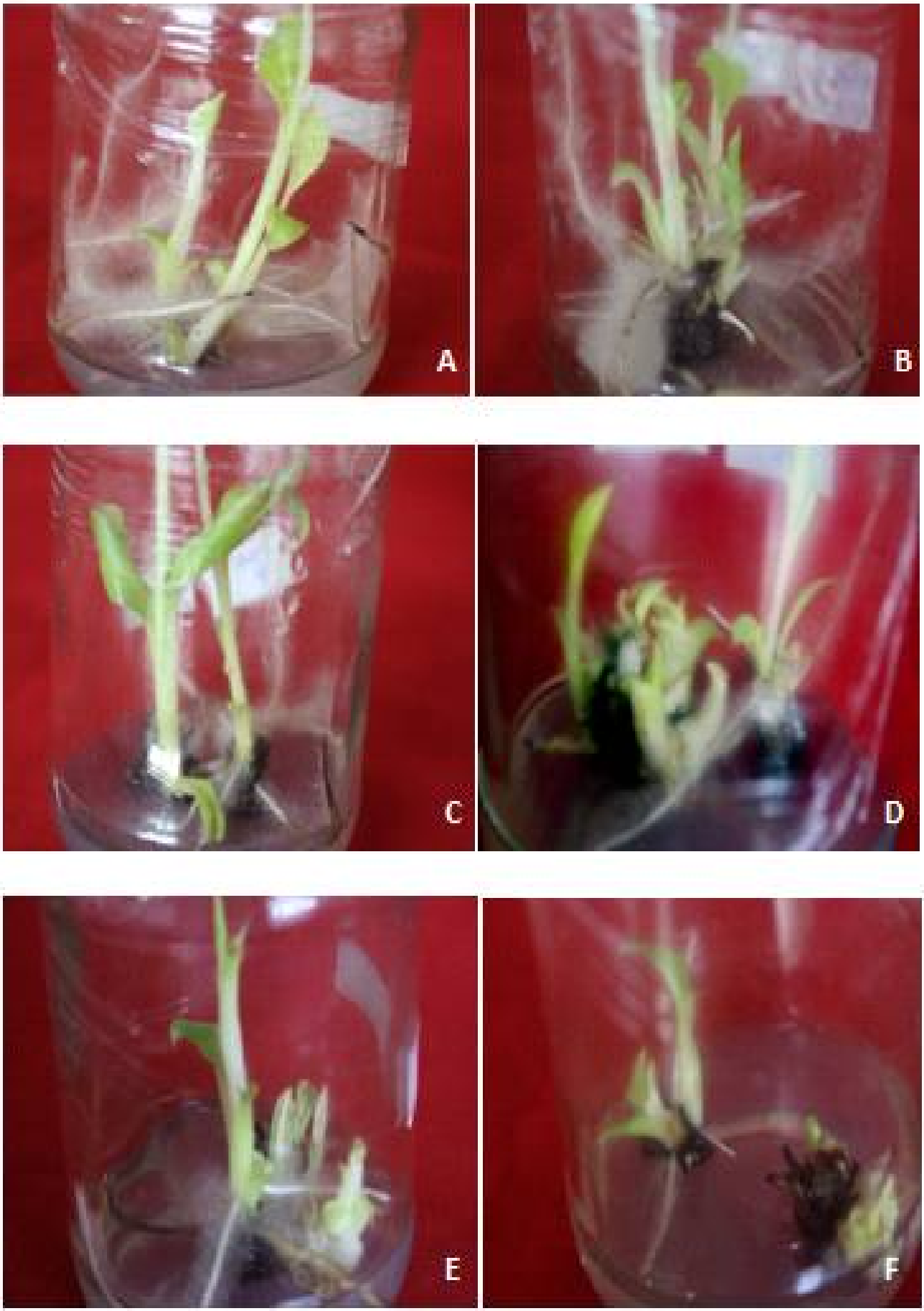

Fig.1. Response of Changanassery nendran in MS+ BAP
(A-Basal medium, B- 1 mg/L , C- 2 mg/L, D- 3 mg/L, E- 4 mg/L, F- 5 mg/L)

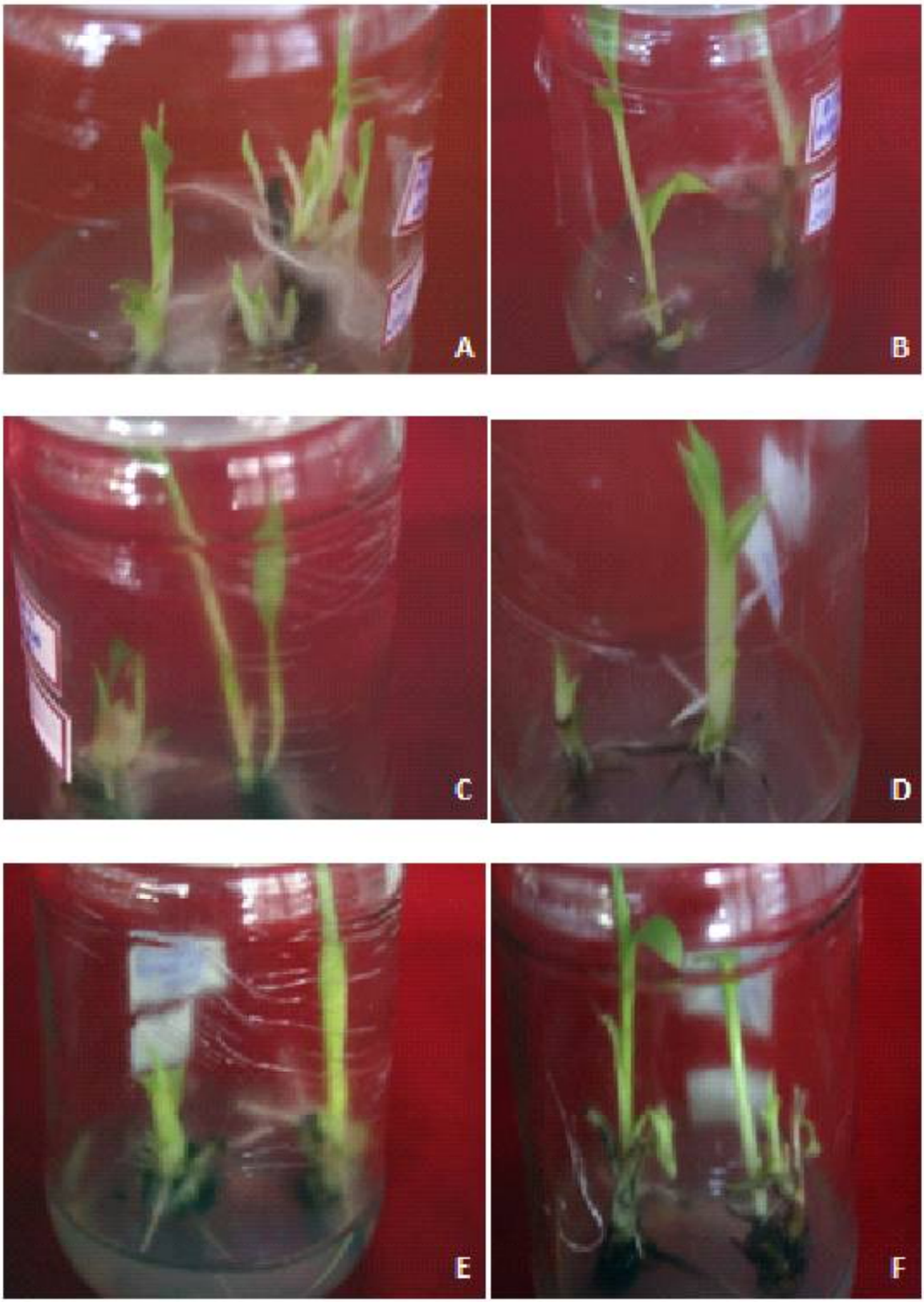

Fig. 2. Response of Changanassery nendran in MS+ BAP+NAA
• 0.5 + 0.1; B- 0.5+ 0.5;C-1+ 0.1; D-1+ 0.5; E- 2+ 0.1; F- 2+ 0.5)

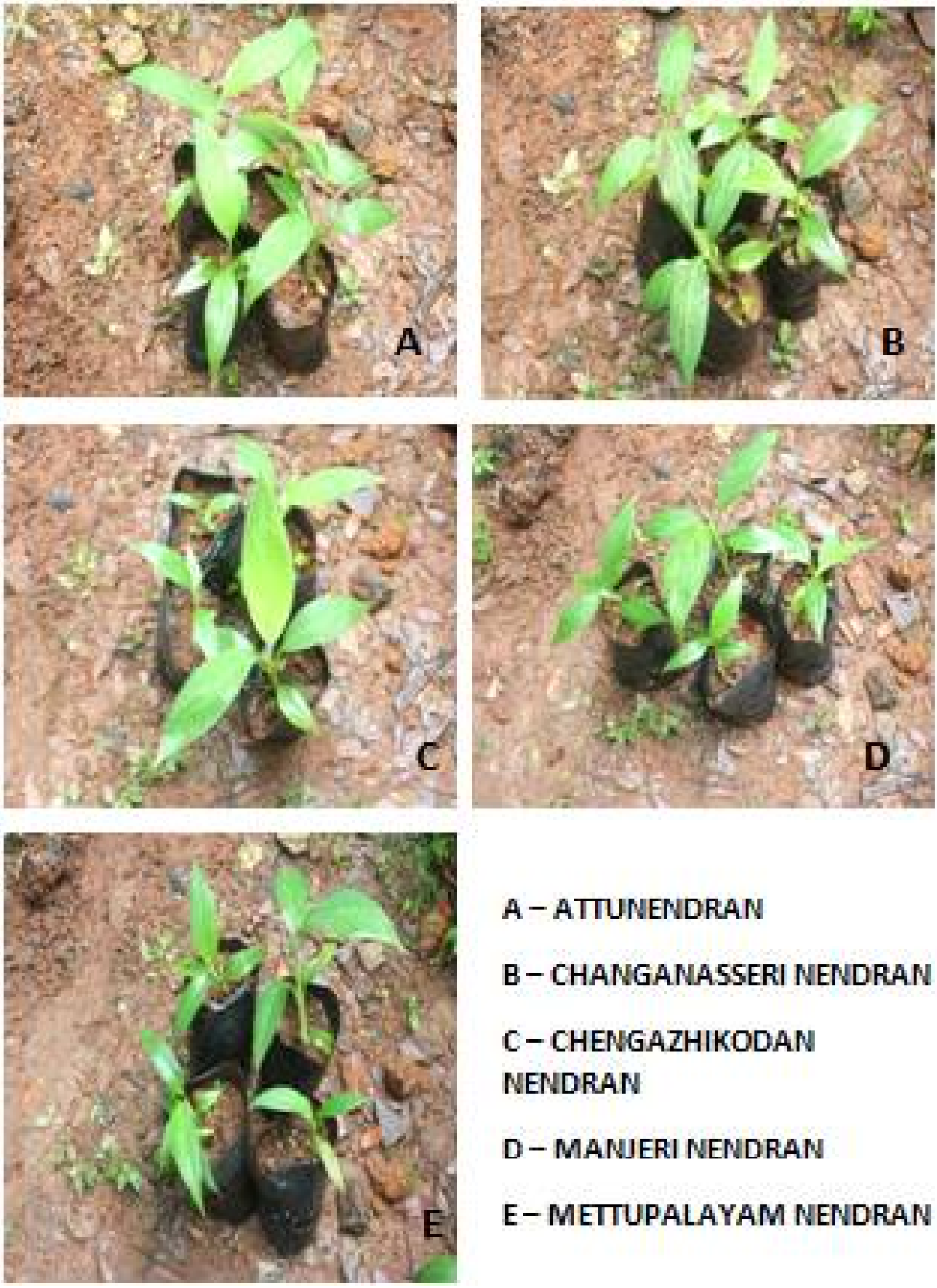

Figure 3: Hardened plants of banana nendran ecotypes

Table 4. Effect of various concentrations of BAP and NAA in the growth and shoot proliferation of banana cv. Changanasseri.

Sl. No.	BAP	NAA	No. of shoots (Mean±SD)*	Length of shoots in cm (Mean±SD)*	Girth of shoots in cm (Mean±SD)*	No. of leaves (Mean± SD)*	Length of leaf in cm (Mean±SD)*
1	0.5	0.5	1.20±0.45a	5.6±0.63b	0.24±0.55a	3.4±0.55b	1.98±0.36b
2	0.5	0.1	4.20±1.10a	3.3±0.27a	0.26±0.55a	2.4±0.89a	0.92±0.26a
3	1	0.5	1ab	3.3±1.15a	0.3a	2.6±0.55ab	1.06±0.49ab
4	1	0.1	1.60±0.55ab	4.1±0.96ab	0.3±0.10a	0.33±0.10ab	2.4±1.10b
5	2	0.5	1.40±0.55c	3.0±0.71a	0.28±0.04a	0.28±0.04a	2ab
6	2	0.1	2.20±0.84b	3.7±2.39a	0.44±0.09b	0.44±0.09a	2.2±0.09ab

(*values followed by the same alphabet are not significantly different by Duncan's Multiple range test at Pe"0.05 within a column).

Effect of BAP and NAA on shoot proliferation

Three levels of BAP (0, 1, and 2mg/L) were tried in combination with two levels of NAA (0.1, 0.5 mg/L) in factorial combination. The data are presented in Table 4 & Figure 2. The highest number of shoots was observed in combination of 0.5mg/L BAP and 0.1 mg/L NAA. 4-5 shoots per explants were obtained in these combinations. This response was higher to that observed in treatment of 5 mg/L BAP alone (Table 3). . Higher concentration of BAP and kinetin beyond optimum levels were also reported to cause necrosis and reduction in shoot formation during in vitro multiplication of banana cv. Nendran (Madhulatha et al., 2004). NAA at a concentration of 0.5 mg/L had a negative effect on shoot number. The number of shoots decreased to 1-3 shoots per explants.

Incorporation of NAA leads to an increase in the number of rootable shoots when compared to the addition of BAP alone. The increase in concentration of NAA from 0.1mg/L to 0.5mg/L resulted in the increase in the number of leaves, and number of shoots. All the parameters like number of leaves, length of leaf, girth of shoot, and length of shoot decreased with the addition of NAA compared to treatment where BAP was used alone. At the same time it resulted in a significant increase in the number of shoots. The results of this experiment show that the incorporation of NAA at 0.1mg/L along with 0.5mg/L BAP can significantly improve shoot number. Basal medium (MS) supplemented with moderate amounts of BAP and NAA is effective in shoot multiplication and growth (Lohidas and Sujin, 2015). The ideal level of BAP for this combination may have to be decided after repeated subculture in same medium as discussed earlier since all explants came from medium containing 3mg/L BAP.

Rooting and hardening

All the multiplied shoots were kept in rooting media (MS+ 0.5 mg/L IBA). To initiate rhizogenesis, IBA or NAA are commonly used in the proliferation medium (Strose et al., 2005). Rooted shoots were hardened successfully (Figure 3).

CONCLUSION

Five selected ecotypes of *M. paradisiaca cv. Nendran* were initiated in the MS medium with 3 mg/L BAP. Each ecotype has different initiation period. Media optimization studies were conducted in Changanassery nendran using MS containing BAP alone and BAP and NAA combinations. Number of shoots and other growth parameters were analyzed to find out optimal media. From the studies, a combination of BAP and NAA in MS medium at concentration of 0.5 mg/L and 0.1 mg/L respectively found to be the optimal media for multiplication of ecotype, Changanassery nendran.

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Ecological Role of Ponds in Urban Area

T.G. DHANALEKSHMY

Department of Zoology,
All Saints' College, Thiruvananthapuram-695007.
email : lekshmydhana@yahoo.com

ABSTRACT

Ponds are an important freshwater habitat having richness and rarity in the population of plant and animal species. The ecosystem services they offer include mainly the water management and nutrient retention. A Pond indicates blends of three diverse nourishment web parts, one based upon cyanobacteria and green growth, another based upon expansive plants, and another based upon rotted plants (Boon E et al, 2013). Maintaining clean ponds is one of the simplest and most effective ways to protect fresh water wildlife. The pond selected for the study was the Mithranandapuram fresh water pond with an area of 100 cents located in Thiruvananthapuram Corporation limits on the western side of Sri Padmanabhaswamy Temple which has ritualistic and historical importance and is used by the temple priests to take ritualistic bath prior to performing poojas. Sampling was done in the month of March and November, 2016. The physico-chemical properties and bacteriological analysis of the water samples were done using standard methods. Water samples were in the alkaline range in both seasons. Temperature varied from 30.2-34.4^{0}C; pH, 8.1-8.6; conductivity, 34-270 µmol/cm; total hardness, 35-240 mg/L; total alkalinity, 24-72 mg/L; chloride, 30-42 mg/L; sulphate, 15.04-32.6 mg/L; ammoniacal nitrogen, 0.03-1.8; DO, 2.4-2.8 mg/L; BOD, 0.8-1; and Chemical Oxygen Demand, 14-16 mg/L, all showing high value in the summer season. Coliform count was far above the safety limit of WHO and had the highest number of bacterial count in summer. Groups of phytoplankton encountered were Chlorophyceae, diatoms Bacillariophyceae and Euglenophyceae. Rotifers and Copepods were also seen. High count in both the seasons indicates poor water quality. Presence of rotifers and copepods indicate the presence of decomposed organic matter and the high level of pollution. The present study shows the need of maintenance and conservation of a temple pond in an urban area, a symbol of our heritage and culture, rich in biological diversity, in a sustainable and holistic manner, which can create a better habitat and environment for all, thus bridging the gap between science and practice.

Keywords *Biological diversity, Conservation, Ecosystem services, Physico-chemical parameters, Holistic*

Ponds are an important freshwater habitat having richness and rarity in the population of plant and animal species. The ecosystem services they offer include mainly the water management and nutrient retention. A Pond indicates blends of three diverse nourishment web parts, one based upon cyanobacteria and green growth, another based upon expansive plants, and another based upon rotted plants[1]. Maintaining clean ponds is one of the simplest and most effective ways to protect fresh water wildlife. A large pond that will not dry out in the dry season along with the active involvement of the community can provide safe drinking water supplies[2]. The greenery of Kerala is maintained because of the moderate amount of annual rainfall we get but it is observed that in the urban area of Thiruvananthapuram District, there is scarcity of drinking water which is mainly due to improper management of the available fresh water sources, especially the ponds which help in percolating the rain water to the ground and increase the water table. Maintaining clean ponds is one of the simplest and most effective ways to protect freshwater wildlife. Planktons dominate the limnetic zone of the pond and they play a crucial role in the food chain. Plankton has short life spans and when they die, they fall into the deep-water part of the pond. Ponds are among the maximum various freshwater habitats and were recently determined to help extra species, in addition to extra unusual, rare, and threatened species in comparison to lakes, rivers, and streams[3]. Physico-chemical parameters and plankton diversity are an important criterion for evaluating the suitability of water for drinking and other purposes. The pond selected for the study was the Mithranandapuram fresh water pond with an area of 100 cents located in Thiruvananthapuram Corporation limits on the western side of Sri Padmanabhaswamy Temple which has ritualistic and historical importance and is used by the temple priests to take ritualistic bath prior to performing poojas. The cleaning and maintenance works of this pond was started in 2011 and is still on its half way due to various technical and financial constraints. In November, 2014, a private company "Crystal" along with "Biocel Company" cleaned the pond using some enzymes which is supposed to destroy the sludge and dirt in the water. The details of the process were not revealed by the Companies. Pond water is being used for the rituals but the overall aesthetic appearance of the pond is not satisfactory.

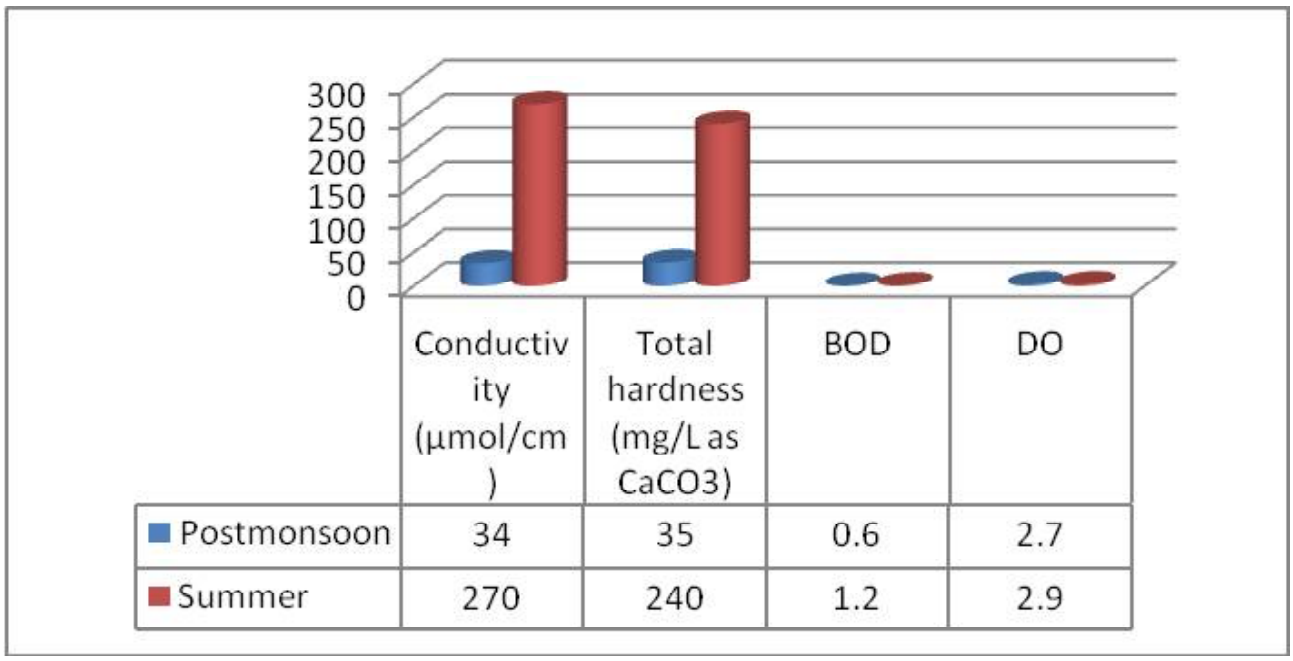

Fig. 1. Graph showing the physicochemical parameters of water samples

MATERIALS AND METHODS

The present study was conducted to understand the physico-chemical and biological characteristics of the temple pond water seasonal wise to assess the quality of water and the pond biodiversity. The water samples were analyzed for the eleven physico-chemical properties. Sampling was done in the summer and post-monsoon seasons during morning hours between 7:00 A.M. to 11:00 A.M. Water samples from the pond were collected separately in polyethylene bottles, BOD bottle and for bacteriological analysis in sterilized bottles. All the precautions were taken during the sampling. The collected water samples were analyzed for different physico-chemical parameters such as temperature, pH, electrical conductivity, total hardness, alkalinity, chloride, sulphate, ammoniacal nitrogen, dissolved oxygen, biological oxygen demand and chemical oxygen demand by the standard methods[4]. Plankton was identified using standard keys[5,6] and photographs were taken using binocular microscope with image analyser.

RESULTS AND DISCUSSION

The difference in value during the post monsoon and summer seasons is shown in Figs.1&2. The values were within the permissible limits[7] but showed notable difference with high value in the summer season. pH ranged from 8.1-8.6 (Table.1). According to Umavathi et al., pH range of 5.0-8.5 is best for planktonic growth[8]. Electrical Conductivity is a useful tool to evaluate the purity of water[9]. High value observed may be due to an abundance of dissolved salts from the minerals from rain water runoff, or other discharges which points to the pollution status[10] as well as trophic level of the aquatic body (Fig.1). A study by Shrivastava and Kanungo has also reported a high range of Electrical Conductivity[11]. High values of calcium and magnesium hardness observed during summer can be due to low water levels and organic matter in the pond (Fig.1). Increase in calcium and magnesium concentrations during pre-monsoon may be the effect of bacterial decompositions[12]. Estimation of dissolved oxygen is a key test in water pollution. In the present study dissolved oxygen did not show significant seasonal variation (Fig.1). High alkalinity of the pond in both the seasons point to the poor water quality (Fig.2). Chemical Oxygen Demand value (Table.1) was seen to be higher than the Biochemical Oxygen Demand value in both the seasons (Fig.1) which points to the existence of organic substances in the pond which in the coming period can affect the quality of water. Coliform count was far above the safety limit and had the highest number of bacterial count in summer. Occurrence of pathogens or indicator organisms in ground or surface water mainly depends on the range of human activities and animal sources that release pathogens to the environment[13].

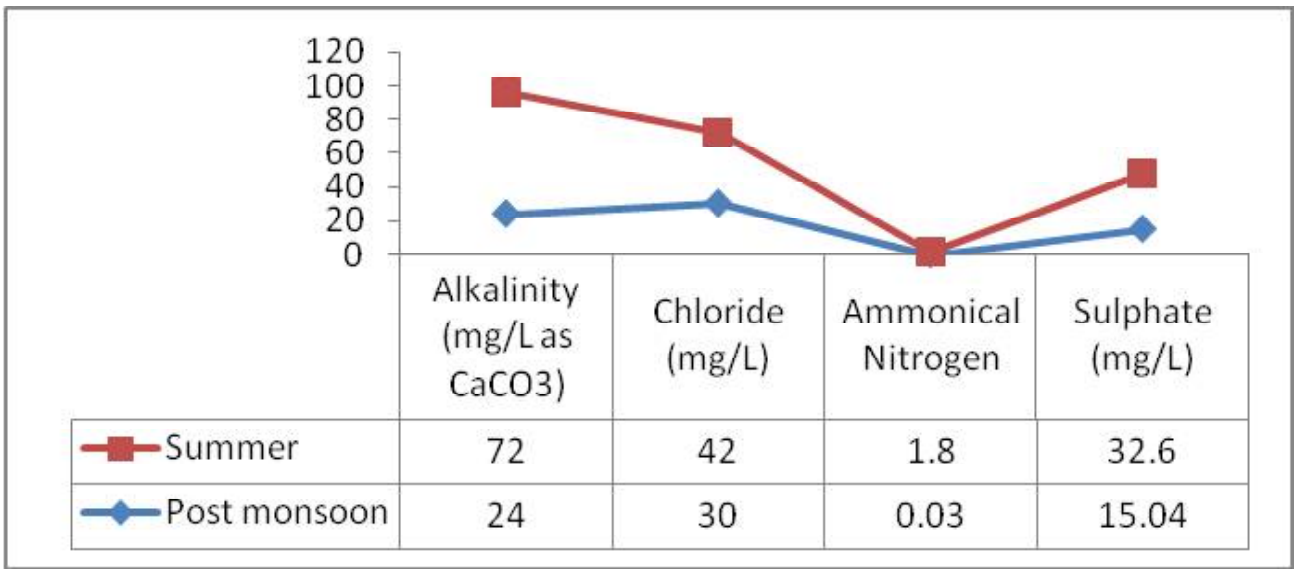

Fig. 2. Graph showing the physicochemical parameters of water samples

Table 1. Physicochemical parameters & Bacteriological Results of water samples

Parameters	Post Monsoon	Summer
pH	8.1	8.6
Temperature (^{0}C)	30.2	34.4
COD	14	16
Total Coliform (MPN/100ml)	75	110
Faecal Coliform (MPN/100ml)	45	38

Phytoplankton and Zooplankton are considered as bio-indicators (Figs.3-8) which are useful in predicting the level and degree of pollutants. Chlorophyceae sps pediastrum, oocystis, oedogonium, cladophora was seen more followed by Bacillariophycea sps, Navicula, Fragillaria and Euglenophyceae sps, euglena and phacus. Diatoms remain as benthic or epiphytic forms and can serve as good indicators of organic pollution. Diatoms are ubiquitous in aquatic habitats, where they are important primary producers and are an important food source for zooplankton and are useful in biomonitoring. Presence of navicula, a motile diatom in both the seasons, considered to be indicator of organic pollution[14] points to the abundance of decaying organic material. Presence of Euglenophycea in both the seasons is a good indicator of availability of rich organic nutrient in the pond. Brachionus sps were seen to be dominating in the pond which can be due to more alkaline nature of this water body. Density of zooplankton, especially rotifers increase significantly with increase in nutrient concentration[15]. Copepod is also an ecologically and economically important group of zooplankton. Copepods recorded from the pond in both the seasons, point to the abundant availability of the phytoplankton in the pond as food source.

CONCLUSION

The Temple Pond was seen to be least maintained. Wastes were seen dumped on either sides of the pond at the entry point and aquatic weeds, decaying plant parts and solid wastes were seen floating in the water surface. High value of several parameters in both seasons indicates poor water quality. High count in both the seasons indicates water unfit for consumption. Groups of phytoplankton and zooplankton in the pond indicate the high nutrient content and enhanced freshwater biodiversity.


Fig.3. Copepod

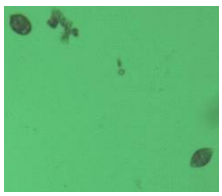
Fig.4. Phacus


Fig.5. Navicula


Fig.6.Brachionus


Fig.7. Pediastrum

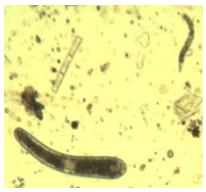
Fig.8. Euglena

Biological monitoring can be used as a valuable method for protecting and conserving the biological integrity of a natural ecosystem. Bio-indicators along with physico-chemical quality can provide valuable information about the health status of the pond.

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Exploring the Potential of Coconut Based Beverages as Functional Foods

G. AMEETHA[1], MAYA MADHAVAN[2] AND T. RAJAMOHAN[3]

[1]*Post Graduate Central University of Punjab,*
[2]*Dept. of Chemistry, University college Trivandrum*
[3]*Former Professor & Head, Dept. of Biochemistry, University of Kerala*
email : ameethaammu355@gmail.com[1] ; drmayamadhavan@gmail.com[2] ; trmohanbio@gmail.com[3]

ABSTRACT

Functional foods enclose a wide range of food and its ingredients with a variety of bioactives responsible for their efficiency in health promotion and disease prevention. Coconut Palm is considered very auspicious in Indian system of medicine and culture with several medicinal properties. In Kerala due to the over usage of soft drinks, use of natural coconut products is diminishing day by day. For these reasons we carried out an analysis of natural coconut products such as Tender coconut water, Toddy and Neera and their significant beneficial effects in health and disease by comparative evaluation of the major nutritional components as well as the microbiological qualities during refrigerated storage.Fresh coconuts of tender stages (4-6 months), Neera marketed by Kaipuzha Agricultural producers, Kollam and fresh Toddy from Sicillipuram were taken. Qualitative analysis of carbohydrates & amino acids, quantitative analysis of carbohydrates by Nelson-Somogyis method and Roe-Pappado Paulose method, estimation of Magnesium by Atomic Absorption Spectrophotometric method, Sodium and Potassium by Flame Photometric method were carried out. P^H was determined using P^H paper. Isolation and enumeration of microorganisms were also done using pour plate and spread plate method. Analysis of minerals revealed that the concentration of potassium was higher in all the three natural coconut drinks, while sodium and magnesium were present in lower concentrations. Higher concentration of potassium found in these natural coconut drinks particularly the Tender Coconut Water suggests that it is beneficial for cardiovascular disease and hypertension. In microbial analysis no growth was observed in any dilution of Neera and Tender coconut water. We conclude that all the natural drinks from coconut palm are equally good and contain appreciable amounts of bioactive components. But most studies have shown that Tender Coconut Water is the best choice for health promotion and disease prevention and it is beneficial for patients suffering from cardiovascular disease and hypertension.

Keywords *Functional foods; Tender Coconut Water; Neera, Toddy; Hypertension; Diabetes; Cardiovascular diseases*

Functional foods emerge as the strongest positive determinant of promptness to compromise on taste. According to the International Food Information Council (IFIC), functional foods are foods or dietary components that may provide a health benefit beyond basic nutrition. Functional foods represent one of the most intensively investigated and widely promoted areas in the food and nutrition sciences today. Diet is only one aspect of a compendious approach to good health.

Coconut water with its many applications is one of the world's most versatile natural products. This refreshing beverage is consumed worldwide because it is nutritious and beneficial for health. *Cocos nucifera* is one of the highest nutritional and medicinal value plants with various fraction of proteins which play a major role in several biological applications such as antimicrobial, anti-inflammatory, anti-diabetic, anti-neoplastic, anti-parasitic, insecticidal and leishmanicidal activities (Selvaraj Mohana Roopan, 2016). The wide application of coconut drinks can be justified by its unique chemical composition of sugars, vitamins, minerals and amino acids. The total sugar content and acidity of coconut water did not change with maturity (Prakruthi Appaiah et al, 2015).

Overwhelming evidence from epidemiological, in vivo, in vitro and clinical trial data indicates that, a plant based diet can reduce the risk of chronic disease, particularly cancer. Tender Coconut Water is a natural isotonic beverage with almost the same level of electrolyte as we have in our blood (Prathapan A, Rajamohan T, 2011) The richness of macro and micro nutrients in Tender Coconut Water are reported to have hypolipidemic, cardioprotective and hepatoprotective effects (Sandhya et al, 2008). Innovative fermented functional beverages using coconut water as the main ingredient is being used for providing the intrinsic health properties. Coconut water is a non diary substrate that can be introduced as a new vehicle for the consumption of functional cultured beverages especially by vegetarians.

Sodium, Potassium, Phosphorous, Chloride and

Table I. Qualitative Analysis of Carbohydrates

TEST FOR CARBOHYDRATES	Tender Coconut Water	TODDY	NEERA
Carbohydrates Molisch's test	+	+	+
Starch Iodine test	–	–	–
Reducing sugar			
Fehling's Test	+	+	+
Benedict's Test	+	+	+
Picric acid Test	+	+	+
Fructose Phloroglucinol Test	+	+	+
Ketohexose Seliwanoff's test	+++	++	++
Pentose sugar Bial's Test	-	-	-

+++ - present +- slightly present

++ - moderately present - absent

Table II. Qualitative Analysis of Amino acids.

TEST FOR AMINOACIDS	Tender Coconut Water	TODDY	NEERA
Amino acids Ninhydrin Test	+	+	+
Aromatic amino acids			
Xanthoproteic Test	+	+	+
Folin's Test	+	+	+
Tyrosine Millon's Test	+	+	+
Tryptophan			
Hopkins- Cole Test	–	+	+
Aldehyde Test	–	+	+
Ehrlisch's Test	–	+	+
Arginine Sakaguchi's Test	+	+	+
Cysteine and Cystine Basic lead acetate Test	+	–	–
Cysteine Nitroprusside Test	+	–	–
Proline Isatin Test	+	+	+
Test for Methionine	–	+	+
Hystidine and Tyrosine Pauly's test	+	+	+
Histidine Biuret Test	+	+	+

Table-III. Quantitative Analysis of Carbohydrates

CARBOHYDRATE	Tender Coconut Water(g/dl)*	TODDY(g/dl)*	NEERA(g/dl)*
GLUCOSE	4.400	3.500	1.500
FRUCTOSE	1.600	1.200.	0.600

*values are means of triplicate determination

Magnesium are the major minerals found in coconut water, besides Vitamin C and sugars (Magda 1992; Campos et al, 1996; Nadanasabapathy and Kumar,1999). Functional activities as anticarcinogenic (Sylianco et al, 1992) and intravenous hydrating solution used in severe dehydration cases have been reported for coconut water (Magat and Agustin 1997; Falck et al, 2000). Hypertension is the most important known risk factor for stroke. Clinical, experimental and epidemiological evidence suggests that a high dietary intake of potassium is associated with lower blood pressure. High intake of potassium from food sources may protect against health problems associated with stroke. Modern human diet is both excessive in NaCl and deficient in fruits and vegetables which are rich in K^+ and HCO_3^- yielding organates like citrate. With the modern diet, the K^+/Na^+ ratio and HCO_3^-/Cl^- ratio have both become reversed. Dietary potassium modulates both the pressure and hyper calciuric effects of the modern dietary excess of NaCl. Evidence shows that reduced sodium intake lowers blood pressure and can prevent hypertension. According to systematic reviews and meta-analyses, sodium reduction does not affect fasting glucose levels or consistently affect insulin resistance, (Patel S M et al, 2015) nor does it affect glycated hemoglobin levels in patients with diabetes (Suckling RJ et al,2010).Analysis of the National Health and Nutritional Examination Follow-Up Study (NHEFS) found that dietary sodium intake was either inversely or directly associated with increased risk of cardiovascular disease.

MATERIALS & METHODS

This was a study which was carried out at the Coconut Research and Development Centre, Laboratory East Pattom, Trivandrum, Kerala, India for a period of 6 months from December 2015 to May 2016. The study included samples of Tender Coconut Water, Toddy and Neera received from appropriate places and brought to the lab in a sterile container with adequate cooling and used for each day experiment.

The presence of carbohydrates was detected using qualitative analysis and the main sugars present in it, viz. glucose and fructose were estimated by Nelson-Somogyi's method and Roe- Pappado Paulose method respectively. Using Atomic Absorption Spectrophotometer method, Magnesium was quantitatively analysed. Through Flame Photometric Method, Sodium and Potassium were analysed. P^H of the three samples was determined on the first, third and fifth day using P^H paper. Isolation and enumeration of microorganisms were done. For isolating bacteria and fungi, pour plate and spread plate method were used.

RESULTS

From the qualitative analysis it was observed that all the three natural coconut drinks contains reducing sugars namely glucose and fructose in appreciable amounts, while starch and pentose sugars are absent. (Table-I)

Qualitative analysis of Tender Coconut Water reveals that it contains amino acids such as Arginine, Cystine, Proline and Histidine. In addition to these amino acids, Tryptophan and Methionine were detected in Neera and Toddy.(Table-II)

Quantitative analysis of glucose by Nelson-Somogyi method indicate that Neera has lower amount of glucose than either Tender Coconut Water or Toddy. When fructose was estimated by Roe-pappado paulose method, it was found that all the three beverages contained lower amounts of fructose than glucose while Neera contains the lowest amount of fructose among the three natural drinks. (Table-III)

Quantitative analysis of minerals present in coconut drinks revealed a higher concentration of

Table IV. Quantitative Estimation of Minerals

MINERAL	TCW(mg/dl)	TODDY(mg/dl)	NEERA(mg/dl)
Magnesium(Mg+)	14.50	4.75	5.60
Sodium(Na+)	32	12	14
Potassium(K+)	176	132	145
K+/Na+ ratio	5.5	11	10.3

Potassium ; while the amount of Sodium and Magnesium were lower. Among the minerals present, the amount of Potassium was higher in all the three natural drinks. When the Potassium/Sodium ratios were looked at, it was found that Tender Coconut Water has the minimum ratio compared to Toddy and Neera. (Table-IV)

An analysis of the P^H levels of fresh samples of Tender Coconut Water, Neera and Toddy showed that the values were 4.0, 4.0. and 2.0 respectively. These observations indicated that all the drinks were acidic in nature. Among the three, Toddy is highly acidic. Tender Coconut Water was clear and colourless. Toddy was turbid and less viscous while the Neera was partially colourless and less viscous**.** It was also found that there was no change in the pH between the first , third and fifth days. (Table-V)

Microbial analysis of the samples indicated that among the three samples namely Neera, Toddy and Tender Coconut Water, only Toddy was found to contain microbes after pour plate method with serial dilutions. The microbe was identified to be Gram positive. Colonies were not observed after 3rd day and 5th day of plating in Neera and Tender Coconut Water. Tests showed positive results for Toddy which may be its general microbial characteristic responsible for the biochemical properties.(Table-VI)

DISCUSSION

Over the past few years, the disease spectrum across the world has undergone a classical epidemiological transition shifting its focus to non-communicable diseases- including cardiovascular diseases, hypertension, diabetes mellitus and chronic kidney diseases. While these diseases may have a genetic predisposition, there is a strong association with environmental influences, suggesting that they are lifestyle related.

Natural coconut drinks are good sources of minerals namely sodium, potassium, calcium, manganeese and magnesium. Sodium and potassium are two critical minerals that have consistently been identified as nutrients of concern in the diet . These two cations have an inverse relation in the body and hence it is important not only to look at the individual mineral intakes but also the ratio of these two minerals in the diet. Recent data suggest that the dietary sodium to potassium ratio is more strongly associated with an increased risk of hypertension and cardiovascular disease-related mortality than the risk associated from either sodium or potassium alone. The effect of a chronic high salt intake is a gradual increase in blood pressure throughout life. The INTERSALT study (International Study of Salt and Blood Pressure) suggested a strong relation between salt intake and a progressive increase in blood pressure with age is, 0.4 mm Hg per year for a 6 g/day salt intake. (Elliot P et al, 1996)

Among the minerals present the amount of potassium was higher in all the three natural coconut drinks. Potassium was identified as a shortfall nutrient

Table V. Determination of pH

P^H	TCW	TODDY	NEERA
1st day	4	2	4
3rd day	4	2	4
5th day	4	2	4

Table VI.Microbial Analysis

	Tender Coconut Water	TODDY	NEERA
FRESH SAMPLE(1st day)	No colonies	45 CFU/10^{-1} dilution	No colonies
Observation after 3rd day	No colonies	50 CFU/10^{-1} dilution	No colonies
Observation after 5th day	No colonies	50 CFU/10^{-1} dilution	No colonies

by the Dietary guidelines for Americans 2010 advisory committee. Increased intake of potassium is beneficial in patients with coronary heart disease and stroke (Suter,1999) The committee concluded that there was a moderate body of evidence of the association between potassium intake and blood pressure reduction in adults, which in turn influences the risk of stroke and coronary heart disease. Evidence is also accumulating of the protective effect of adequate dietary potassium on age related bone loss and reduction of kidney stones. Low potassium-to-sodium intake ratios are more strongly related to cardiovascular disease risk than either nutrient alone. Western diets have led to a decrease in potassium intake with reduced consumption of fruits and vegetables with a concomitant increase in sodium consumption through increased consumption of processed foods. (Weaver CM, 2013).

In this study it was observed that all the three natural coconut drinks contain reducing sugars namely glucose and fructose in appreciable amounts. Other benefits of increasing potassium consumption may include improved glucose control, glucose intolerance and insulin resistance becoming a concern for hypertensive individuals prescribed to potassium wasting diuretics (Michael S. Stone et al, 2016). Both glucose and fructose content was higher in Tender Coconut Water compared to Toddy and Neera. Glucose intolerance can often be a result of severe hypokalemia due to a deficit in potassium balance that may occur in primary or secondary aldosteronism or prolonged treatement with diuretics. Studies show that high potassium intake may be associated with a decreased risk for developing type 2 diabetes mellitus in women with a BMI of 29 or less (Vasanti.S.Malik, Frank B.Hu, 2012).This relationship also extends to the kalemic effects of insulin. Higher plasma insulin levels are associated with increased potassium absorption into cells.

Several studies have shown an increased risk of cardiovascular disease or death among people consuming less than 3.0 g of sodium per day, as compared with average intake (Graudal N et al, 2014; P Fister R et al, 2014) but many of these studies included people at high cardiovascular risk, (O'Donnell M.J et al, 2011; Thomas MC, Moran J et al, 2011) who were not representative of the general population. The association between sodium intake and cardiovascular disease is complex and may be modified by other dietary factors, such as potassium intake, which has also been associated with cardiovascular risk (Aburto N.J et al, 2013; Cook NR et al, 2009). It is clearly evident from the study that the ratio of Na^+/k^+ is lesser in these natural coconut drinks. Tender Coconut Water shows lesser amount of Na^+/k^+ ratio than Toddy and Neera. The antihypertensive effect of increased potassium intake is related to numerous mechanisms. Acutely, increased plasma potassium is associated with endothelium dependent vasodilation via stimulation of Na+-K+ ATPase pumps and the opening of potassium channels in vascular smooth muscle cells and adrenergic nerve receptors (Anne R. Crecelius et al, 2014). In addition to enhanced vasodilation, other possible mechanisms in which potassium is proposed to lower blood pressure and improve vascular outcomes include increases in sodium excretion, modulation of baroreceptor sensitivity, reduced sensitivity to catecholamine related vasoconstriction, improved insulin sensitivity and decreases in oxidative stress and inflammation. (Graudal N.A et al, 2012)

Qualitative analysis of Tender Coconut Water reveals that it contains amino acids such as Arginine, Cystine, Proline and Histidine. In addition to these amino acids, Tryptophan and Methionine were detected in Neera and Toddy. Amino acids are essential for healing wounds and repairing tissue, especially in the muscles, bone, skin and hair as well as for the removal of all kinds of waste products

produced in connection with the metabolism, Studies revealed that Tender Coconut Water is a resource of amino acid L.Arginine (Anthony Loperito Loki and T.Rajamohan, 2003). There are reports that L.Arginine possesses hypertensive and cardioprotective properties (Anurag and Rajamohan 2013)

The P^H levels of fresh samples of Tender Coconut Water, Neera and Toddy was 4.0, 4.0 and 2.0 respectively. P^H level plays an important role in maintaining the day-to-day function and overall health. Natural juices from plant sources have a low P^H ,which means it is acidic. Similarly the present observations indicate that all the drinks are acidic in nature. Among the three, Toddy is highly acidic. Tender Coconut Water was clear and colourless. Toddy was turbid and less viscous while the Neera was particularly colourless and less viscous.

Microbial analysis indicate that there was no microbes in Neera and Tender Coconut Water, only Toddy was found to contain microbes. The microbe identified was Gram positive. Fungal contamination was also absent in the three samples as there was no growth in SDA plates. Test showed positive results for Toddy are general characteristics and morphology, microbial analysis and biochemical characteristics. Since Tender Coconut Water and Neera contains no microbial growth after the refrigerated storage at regular intervals of time it is beneficial for the promotion of health and disease prevention.

As pointed by the results of the present study, we suggest that the coconut based beverages have a high potential as functional foods and hence effective steps should be taken to popularize the health benefits of these drinks. They can be used as a perfect food supplement for persons having hypertension, diabetes and cardiovascular diseases.

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Pharmacognostic Studies of *Asystasia chelonoides var.chelonoides*, Nees.

GEETHA R. NAIR[1], S.R. SUJA[2] AND M. JAYASREE[3]

[1,3]Department of Botany, Mahatma Gandhi College,Thiruvananthapuram.
[2]Division of Ethnomedicine and Ethnopharmacology, JNTBGRI, Palode.
email:geethasgma@gmail.com

ABSTRACT

***Asystasia chelonoides var.chelonoides*, Nees (Acanthaceae) is an under shrub distributed throughout India. It is extensively used as a relief for Asthma and Jaundice. The leaves have good hypoglycemic and hypolipidemic effect. Pharmacognostic studies of the plant were carried out following the WHO guide lines on the establishment of quality standards for medicinal plants. Other physiochemical parameters were also determined. Pharmacognostic investigations include macro and microscopic studies on fresh and powdered leaves, organoleptic study, stomatal index, physiochemical constants like total ash and extractable values to facilitates the correct identification.**

Key words *Asystasia chelonoides, pharmacognosy, hypoglycemic, hypolipidemic.*

Asystasia chelonoldes var.chelonoides, is a member of the family Acanthaceae. It is commonly called "Kattumanikulukki pachila". Tribal people use the leaves and flowers with honey for giddiness and Jaundice. Studies are going on throughout the world for the search of protective molecules that would provide maximum protection to the liver, kidney as well as other organs and practically very little or no side effects would be exerted during their function in the body (Montilla *et al.*; 2005; Mansour *et al.*; 2006). A number of herbs are traditionally used in different countries in response to drug or toxin induced hepatic and renal disorders. (Beshbishy *et al.*; 2005). Good medicinal practices in neutraceutical and pharmaceutical industries warrants to correct identification of the dried plants or powdered drug which detects and prevents the adulterations if any. The adulteration is done deliberately, but its may occur accidently in some cases (Kokate *et al.*; 2008) According to WHO (1988), the macroscopic and microscopic description of a medicinal plant is the first step towards establishing the identity and the degree of purity of such materials and should be carried out before any tests are undertaken. (Anonymous 2002) and Khandelwal 2003).

MATERIALS AND METHODS

The plant specimens for the proposed study were collected from Kollam and Thiruvananthapuram District of Kerala. The specimens were identified and authenticated by The Flora of Presidency of Madras (Gamble, 1935). Care was taken to select healthy plants and for normal organs. The stem, petiole, root and leaf were subjected to pharmacognostic studies (Evans 1996). The external morphology of the plant parts and other structural peculiarities were studied in the macroscopic observation. The moisture content, ash test, leaf microscopy, stomatal index were identified.

Stomatal Index: Take leaf fragments of about 5x5mm in size in a test tube containing about 5ml of chloral hydrate solution and heat in a boiling water bath for about 15minutes or until the fragments become transparent. Transfer a fragment to a microscopic slide and prepare the mount with lower epidermis uppermost in chloral hydrate solution and put a small drop of glycerol-ethanol solution in one side of the cover glass to prevent the preparation from drying. The number of stomata and total number of epidermal cells are counted.

$$\text{Stomatal index} = \frac{S}{E+S} X\ 100$$

RESULTS AND DISCUSSION

Macroscopic Studies

Macroscopic characters of the stem shows branched, cylindrical, solid, slight woody and glabrous stem. Leaves are lightly coloured when young and dark green at maturity. Leaves with

reticulate venation is an important diagnostic features of the drug plant. Inflorescence paniculate, corolla pale yellow about 0.75inch long .Fruit is an elliptical capsule with a collateral solid base, opening widely in a reflexed curve. Seeds compressed, angular, glabrous and tuberculate.

Microscopic studies:

Stem – The stem is four angled 2-3 mm thick. It consists of a thin epidermis, prominent cuticle, broad cortex, discrete vascular bundles and large pith. Epidermal cells are small and rectangular. Beneath

Table 1. Physico chemical parameters of leaf samples of *Asystasia chelonoides var.chelonoides*, Nees.

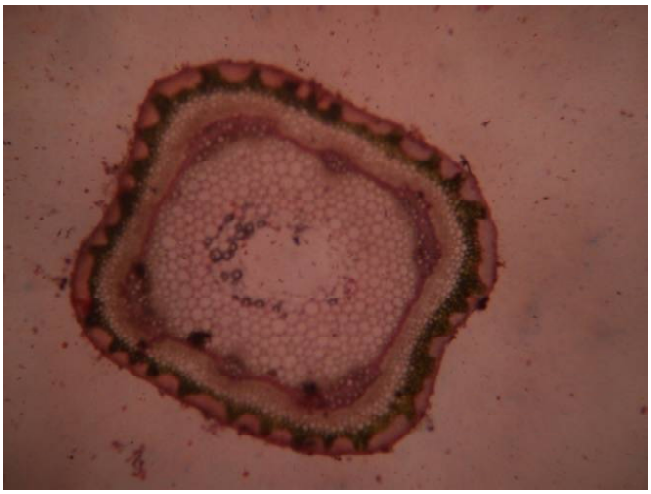
Sl No	Factors	Value
1	Moisture content	52.52%
2	Total Ash	7.47%
3	Water Soluble Ash	3.78 %
4	Acid Insoluble Ash	0.48 %

the epidermis single row of collenchyma cells followed by chlorenchymatous cortex. Vascular bundles have prominent, circular bundle cap, rectangular mass of phloem and xylem tissue. Xylem has thick walled vessels embedded with parenchymatous cells and fibres. Phloem has sieve elements and phloem parenchyma. The pith is wide and parenchymatous.

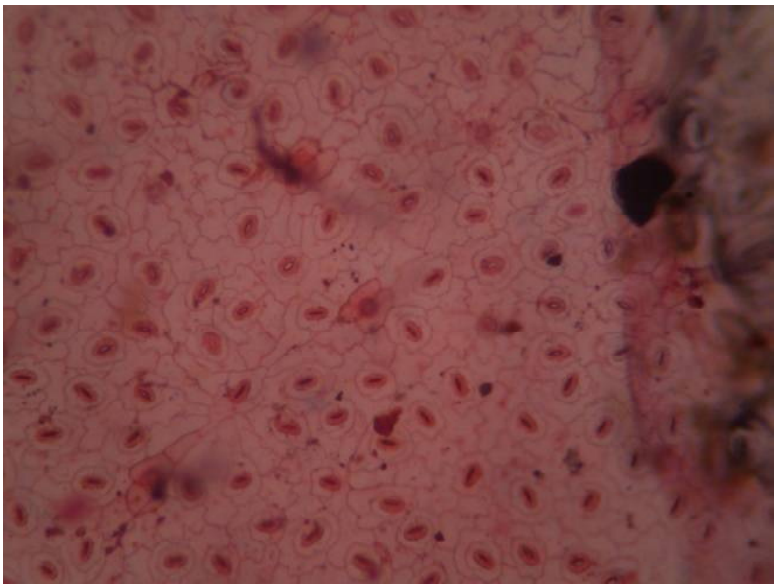
Leaf – Leaves opposite, entire on the margins, elliptic, ovate, glabrous, the base always narrowed, 1-2.5 inch long, 1-1.5 inch broad. Leaf has smooth and even lamina with a midrib. The adaxial epidermis is thick with elliptical cells and prominent cuticle. The abaxial epidermis is with rectangular cells with thick cuticle. The vascular bundles are collateral with thick walled xylem elements. The ground tissue beneath the vascular bundle is parenchymatous. There are three or four layers of rectangular cells forming a narrow band in between the adaxial epidermis and the vascular bundle on the adaxial part. The mesophyll tissue is differentiated into one or two layers of compact palisade cells and spongy tissue having loosely arranged cells.

Stomata and stomatal index – For studying stomatal morphology and the index calculation, epidermal peelings were prepared. stomata are diacytic

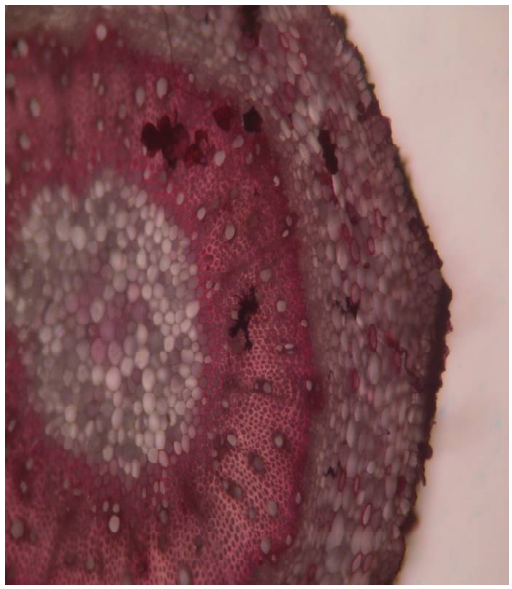
Habit: *Asystasia chelonoides var.chelonoides, Nees*



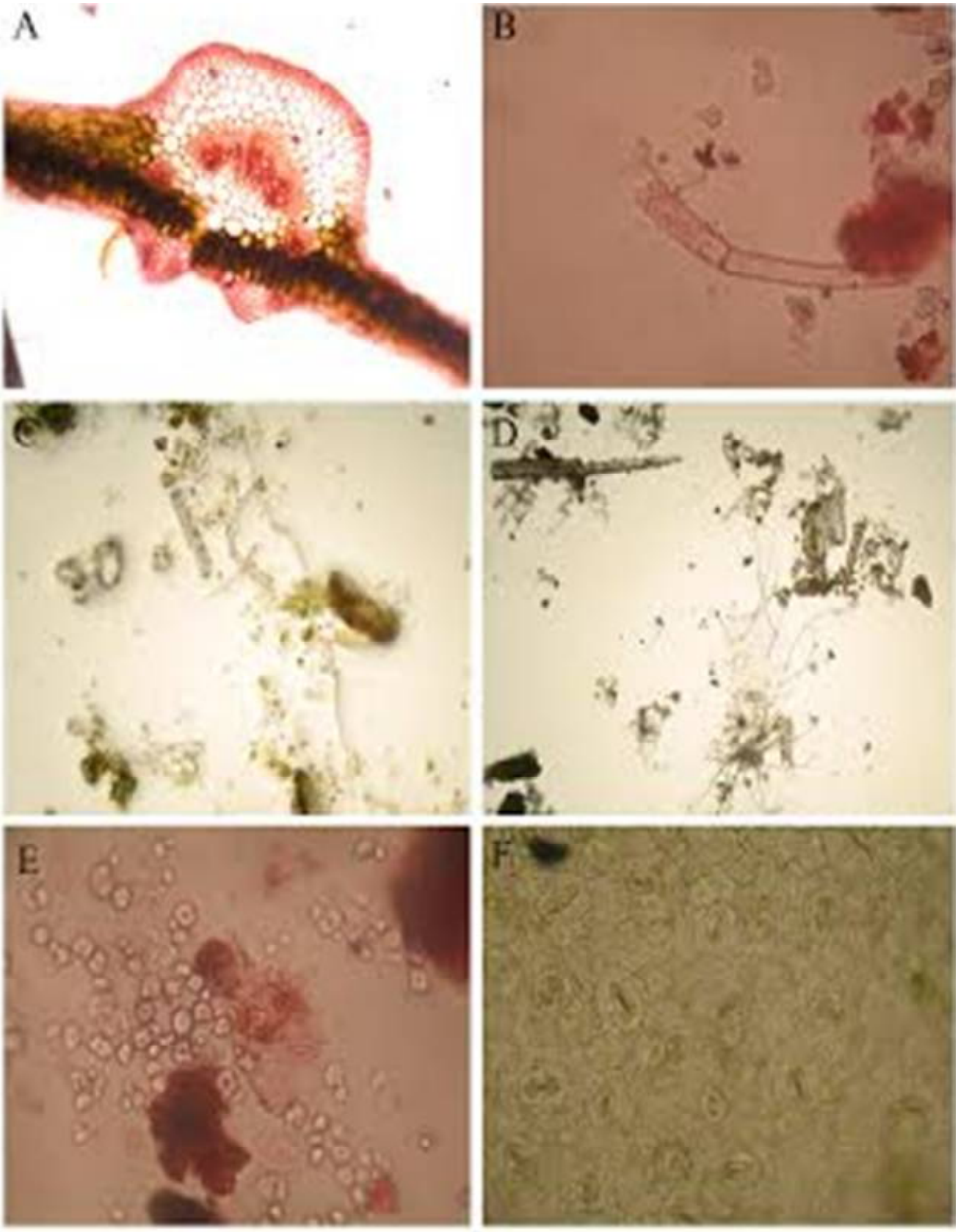
TS of stem



Stomata



T S of root



type, which is surrounded by two subsidiary cells.

The stomatal index was calculated by counting the number of stomatal cells as well as upper and lower epidermal cells. The stomatal index was found to be 28.15.

Powder Microscopy

In the powder, leaf fragments and bark elements were observed. Hairs, bristles and Trichomes were present. Xylem trachieds and vessels of varying length. Thin walled xylem fibres were present.

Physico Chemical Parameters

Various Physico- chemical parameters like moisture content, total ash, water soluble ash, acid soluble etc were also calculated. (Table 1)

Pharmacognostic analysis of Asystasia showed many important features useful for the authentification of the drug plant. Macroscopic characters of the stem are glabrous and quadrangular. Massive sclerenchymatous thick walled fibres and vessels are also unique to Asystasia. Diacytic type of stomata is a useful identification mark of the plant. The microscopical studies of the transverse section showed the presence of simple and glandular trichomes, which is characteristic of the family Acanthaceae. The extractive values are useful to evaluate the chemical constituents present in the crude drug and also help in the estimation of specific constituent soluble in a particular solvent (Ozarkar, K.R 2005). Pharmacognostic studies of Asystasia also help in the identification and preparation of a monograph of the plant.

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Mechanism of Desiccation Tolerance in Forked Fern *Dicranopteris linearis* (Burm.f Underw): Some Observations

C.H. KAVITHA[1] AND K. MARUGAN[2]

[1]*Department of Botany, St Johns College, Anchal*
[2]*Department of Biotechnology and Botany,*
Govt. Arts College, Trivandrum, 695 014, Kerala, India
email : kavithach1979@gmail.com

ABSTRACT

Drought tolerance mechanism in plants still remains a biological challenge to scientists because of multiple of factors. Most of the works were carried in lichens, bryophytes, the resurrection plant *Selaginella* and a few dicot flowering plant species. Limited works were undertaken among ferns related with desiccation tolerance. The present study was carried to unravel the molecular and biochemical specificities of the forked fern – *Dicranopteris linearis* against desiccation under *in vitro* conditions. *In vitro* experiments were conducted against five different desiccation regimes such as 2, 4, 6, 8 and 10 d using poly ethylene glycol (PEG) treatments under controlled conditions and also subsequent rehydration. A set of control was also maintained under optimal conditions. All the experiments were conducted following standard protocols and the results were statistically analysed using two way ANOVA. Primary metabolic analysis revealed varied pattern in the levels of sugars, free amino acids and proteins. The higher concentration of sugar and amino acid proline in the tissues confirm their role as osmolytes. Enhanced level of protein concentration and total free amino acid suggests its role as stress protein to mitigate metabolic pathways. Flavonoid content relates its role as antioxidant in the fern. Optimal level of RWC was maintained during different regimes of desiccation. Trehalose level further substantiates the resurrection nature of the plant. FTIR analysis reveals desiccation stress influenced carbohydrate, protein, lipid, and cell wall pectin synthesis pathway from day 2 to 10. Chlorophyll fluorescence and photosynthetic pigments analysis revealed that the ferns withstand desiccation *via* maximum quantum yield of PSII and photochemical quenching. The amount of total phenolic compounds and the fractionated phenolic acid level increase significantly up to 4 day of desiccation and subsequently maintained their level till 10th day. Changes in the dynamics of antioxidants and antioxidant (AOX) enzymes provide an insight of defense system in the fern against various degrees of oxidative stress, and the integrated stress tolerance of plants. The subcellular localization of desiccation induced H_2O_2 synthesis and its relation to H^+ATPase of the desiccated fronds was studied. The role of NO in ameliorating desiccation stress coupled with DNA damage in the fronds subjected to desiccation at different regimes was also carried. ABA level also substantiate the desiccation tolerance potential of the fern.Four different MAPK pathways were activated in the fronds in response to desiccation. The overall results revealed the desiccation tolerance capacity of the fern under induced desiccation stress conditions.

Keywords *Desiccation tolerance; Dicranopteris linearis; osmolytes; antioxidants; chlorophyll fluorescence; phenolic acids; MAPK pathways.*

Desiccation tolerance was reported widely in the plants like mosses, ferns, their reproductive structures, seeds of higher plants and rarely in the whole flowering plants (Alpert and Oliver, 2002). Drought and desiccation tolerance are correlated with physiological and biochemical events in the cell system. The population explosion, climate change and the need of water for agriculture are regularly increasing. Therefore, the researchers have initiated their efforts to analyse efficacy of water-usage tolerance against drought and targeting to apply these information into a biotechnological mode for crop improvement (Leprince and Butnik, 2010). The resurrection plants are unique source for tolerant genes and also possess novel osmolytes. Ferns are widely distributed along the tropical parts of the world and mostly tolerant to drought. *Dicranopteris linearis* commonly known as forked fern was chosen for the present study to undravel the mechanism of desiccation tolerance using analytical, biochemical and molecular tools.

MATERIALS AND METHODS

Dicranopteris linearis (Burm.f.) Underw. was collected from the wild habitats of Ponmudi Hills, Thiruvananthapuram, Kerala. *In vitro* experiments were conducted against five different desiccation regimes such as 2, 4, 6, 8 and 10 d using poly ethylene glycol (PEG) treatments under controlled conditions and also subsequent rehydration. A set of control was

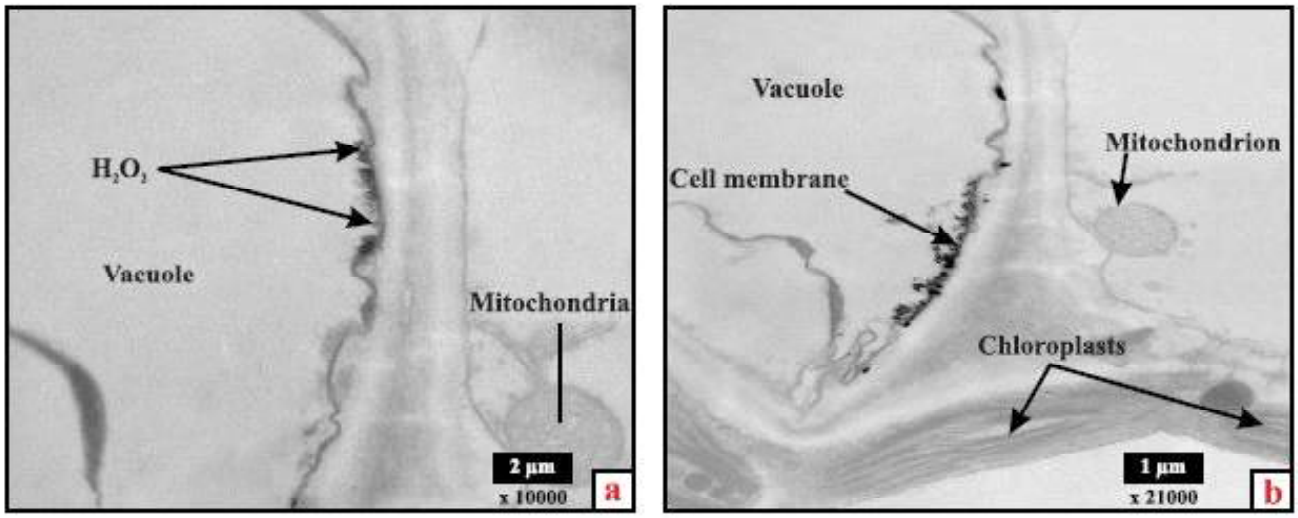

Fig 1 a& b. TEM images of 4 and 6 day desiccated leaf cells showing cerium per hydroxides deposited along the membrane.

also maintained under optimal conditions. All the experiments were conducted following standard protocols and the results were statistically analysed using two way ANOVA.

RESULT AND DISCUSSION

As an initial part of the study, RWC of the desiccated and rehydrated fern was analysed. 60 to 85.5% of RWC was noticed in the fronds of the desiccated ferns up to 8 days of desiccation. Similarly, the RWC recovery reached approximately 90% during the fully hydrated condition. Thus, suggests that the frond was capable of storing water and able to recover from continuous cycles of desiccation *via* rehydration.

Metabolic profile of the forked fern against desiccation rehydration treatment was further analysed. The level of total sugar change significantly during the treatment periods and also, the total proteins suggests its role as stress related proteins. Similarly, the amount of free amino acids also increased concomitant with desiccation periods. Increase in the level of phenylalanine – the amino acid crucial for initiating secondary metabolite synthesis was noted. Proteomics studies of the resurrection fern *Polypodium virginianum* have revealed the production of specific proteins in connection with rehydration after desiccation i.e., rehydration-specific polypeptides which were instrumental in helping the plant to recover completely during rehydration (Reynolds and Bewely, 1993). Proline, the osmolyte which provide tolerance to stress was also enhanced. The data were statistically significant at 1% level. Agduma and Dionisio-Sese (2015) reported highest proline content in the desiccated *S. tamariscina* compared to its control plant. In contrast, rehydrated *S. tamariscina* was showed lower proline content relative to the desiccated samples. Similarly, desiccated fronds of *Selaginella bryopteris* showed an

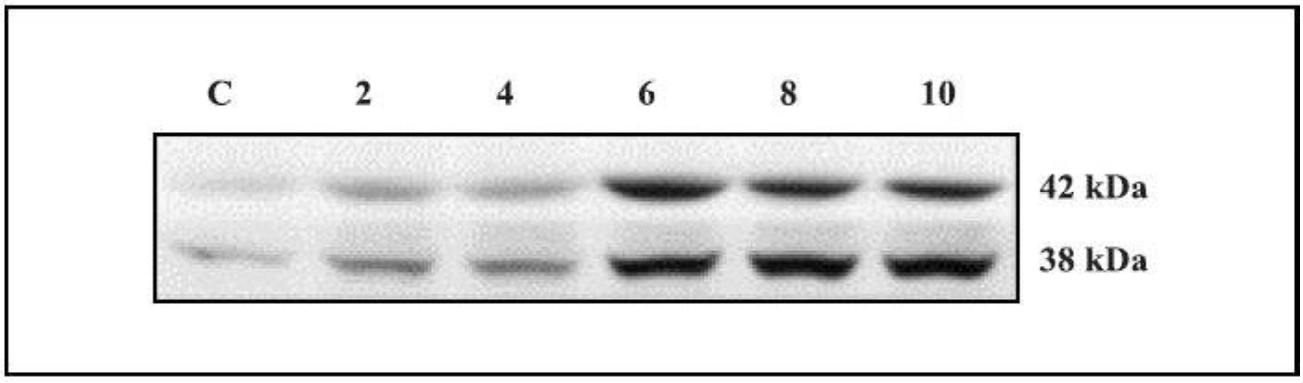

Fig 2 a. Desiccation induced activation of 42 and 38kDa protein kinase of *D. linearis* exposed to 0,2,4,6,8,10 days of desiccation.

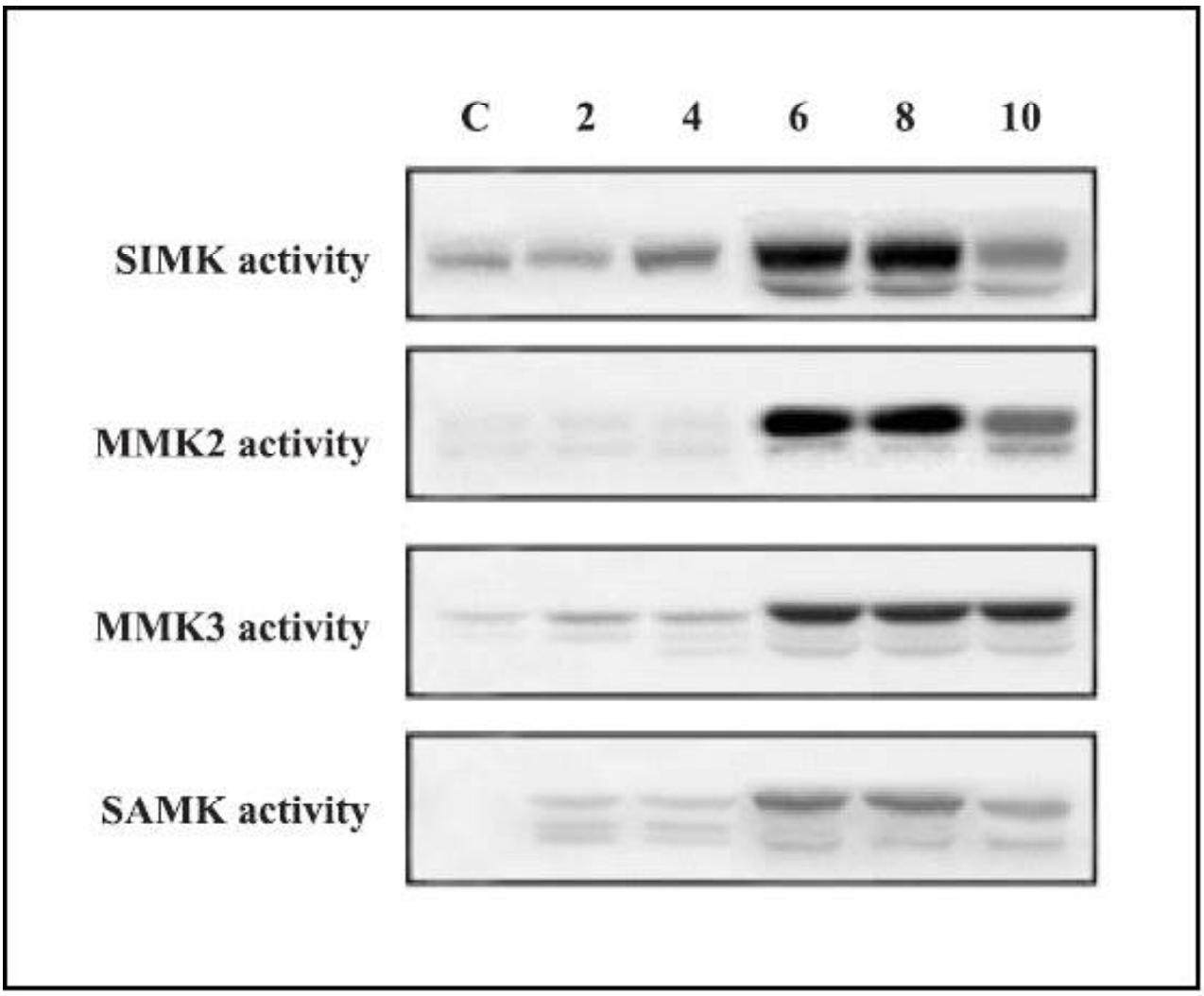

Fig 2b. Differential activation of MAPKs by desiccation treatment (2-10d) in *D.linearis*.

increased proline content when compared to control. The rehydrated fronds showed reduced proline content though it was still approximately 4 times higher than that of control fronds (Pandey et al., 2010). The increase in the concentration of flavonoids subjected to desiccation treatment indicates its positive role as an effective antioxidant. Trehalose, the disaccharide also showed a positive response with desiccation as revealed by HPLC chromatograms. In *Selaginella lepidophylla* high trehalose level (ÃÙ than 130 mg/g DW) was noticed during dry as well as hydrated conditions and was considered as one of the reason for acquisition of desiccation tolerance by the species (Pampurova and Dijck, 2014).

Desiccation treatments influence the physiological and biochemical process and thereby regulating the biomass of plants. Fourier transform infrared spectrometry (FTIR) was attempted to evaluate the changes of functional groups in the desiccated fronds. The obtained IR spectra were further processed by de-convolution and curve fitting to examine the chemistry of the molecules and its structural changes. 2 d desiccation stress changed the peak areas of proteins, lipids and cell wall pectin. Almost all the compounds were changed significantly compared to the control at 10 d of desiccation. The FTIR method provides remarkable way to analyze the structural conformation of the proteins. The C-O, NH_2, and C-N bonding of the peptide linkage absorb wavelength of light in between 1800 and 1200 cm^{-1} region. The absorption band of C-O stretching vibrations of the amide group depends on the nature of hydrogen bonding between C-O and N-H moieties and was particularly applicable for detecting the secondary models of polypeptides. The experimental results suggest that the FTIR technique was effective in analyzing the environmental stress response of the plants.

Table 1. Influence of desiccation (2-10days) and rehydration stress on Activity of enzymes (U/mg protein) - NADPH oxidase (NOX), Superoxide dismutase (SOD),Catalase(CAT), Ascorbate peroxidase (APX), Monodehydro ascorbate reductase (MDHAR), Dehydro ascorbate reductase (DHAR) and Glutathione reductase (GR) and in *D.linearis* . Values were the means of five replicates and significance of F were denoted by ** ($p < 0.01$).

Days	NADPH oxidase (NOX)		Superoxide dismutase (SOD)		Catalase(CAT)		Ascorbate peroxidase (APX)		Monodehydro ascorbate reductase (MDHAR)		Dehydro ascorbate reductase (DHAR)		Glutathione reductase (GR)	
Control	211.104		5.634		3.72		158.22		12.66		50.93		143.1	
	D	R	D	R	D	R	D	R	D	R	D	R	D	R
2	405.37	314.78	7.06	7.31	8.36	5.56	248.98	195.87	60.97	35.02	58.17	32.91	240.99	258.76
4	860.11	820.22	7.69	5.67	17.11	16.29	281.86	259.44	80.08	31.81	237.90	232.82	414.37	382.83
6	1294.03	888.51	12.78	10.34	17.64	16.18	281.99	250.51	84.42	52.21	221.70	239.24	385.04	322.14
8	1330.64	988.26	14.77	8.29	30.17	28.39	482.23	460.63	91.84	62.09	413.13	370.57	481.84	649.82
10	1536.87	1314.73	13.86	8.72	21.17	17.95	504.32	387.20	94.10	64.83	281.77	222.98	320.89	266.86
F ratio D	1933.36**		32.37**		531.91**		1699.44**		66.30**		356.12**		183.56**	
H	723.72**		65.57**		42.44**		391.47**		495.99**		15.22**		0.86	
CD D	26.079		1.246		0.986		7.917		4.736		18.618		25.694	
H	16.497		0.788		0.624		5.007		2.995		11.775		16.250	

Exploring the mechanism of desiccation tolerance is crucial in order to unravel the position of ferns in tropical region of the earth. Pigment and fluorescence responses of the forked fern against desiccation and rehydration stress were analyzed. Chlorophyll a and b pigments were increased (2nd day) marginally indicating the physiological tolerance of the stressed plants with duration of desiccation. Similarly, the carotenoids also showed a steady increase. The quantum yield potential of photosystem II (F_v/F_m) was 0.61, 0.77, 0.79, 0.80 and 0.76 respectively, when subjected to 2, 4, 6, 8 and 10 days of desiccation. The F_v/F_m ratio, Fm, Fv, Fo the potential parameters of chlorophyll fluorescence can be used for early detection of desiccation stress in the fern. The present results revealed that desiccation stress resulted an initial reduced F_v/F_m which may be due to the decreased efficiency of energy transfer from the antennae molecules to the reaction centers and / or inhibition of the activity circumscribed around PSII reaction centers (Abdeshahian et al., 2010). Further, photosynthetic quenching and ETR and non-photochemical quenching were maintained remarkably in the fronds till the 8 d of desiccation stress.

The amount of phenolics and its fractionation in the frond during successive periods of desiccation and its localization using transmission electron microscope (TEM) was also carried. Phenolics distribution at the cellular level was localized in the parenchyma cells. The content of phenolics showed positive correlation with desiccation and subsequent rehydration as compared to control and was substantiated by TEM. Phenolics were noticed in the vacuoles of all cells and also along the cell walls. Fractionation of phenols by RP-HPLC reveals the waxing and waning pattern of phenolic acid such as ferulic, hydroxy benzoic, phloroglucinol and others in the tissue during the different regimes of desiccation. The *in situ* location of phenolics reveals the protective chemical defense of the compound against environmental constraints. Zivkovic et al., (2010) reported induction of high amount of phenolics in rustyback fern (*Asplenium*

ceterach) following short term desiccation followed by alterations in phenolic acid metabolism guided by enzymes peroxidases and poly phenol oxidases.

Free radical-induced peroxidation leads to damage membranes, enzymes and nucleic acids and is likely to be the major causes of injury during desiccation of plants. Protective mechanisms to scavenge the peroxidatively produced free radicals and peroxides have evolved within plants, keeping these reactive molecules to minimum. The hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\cdot-}$), showed a remarkable increase in the treated groups when compared to control revealing its role as signaling cascade. Peroxidation of membrane lipids is a sensitive diagnostic index of oxidative stress. Lipid peroxidation levels in the analysis showed an initial increase (2d) followed by a decrease and maintained at par with the control indicating the active anti-oxidative machinery in the fern.

The differential responses of antioxidants may help to understand the functional interplay of them in the defense system. Reduced glutathione and ascorbate content exhibited increase (up to 6 days) followed by a decrease with duration of desiccation (ÃÙ 6 days) and further the quantities were maintained till the 10th day of stress when compared to control. Among the antioxidant enzymes, ascorbate peroxidase was up regulated to 3 folds (10th d) and superoxide dismutase to 2 folds. Similarly, NADPH oxidase (NOX) was increased to 1536.87 (6th d) from 211.1 U/mg protein of control. Catalase, peroxidase and glutathione reductase activities were also increased remarkably under desiccation stress (Table 1). Efficiency of the antioxidant system was also supported by the membrane damage assay of lipid peroxidation. The biochemical parameters were strongly supported by the amounts of H_2O_2 and $O_2^{\cdot-}$ contents. Pandey et al., (2010) in the study on desiccation induced biochemical changes in *Selaginella bryopteris* during dehydration noticed increased activities of AOX enzymes SOD, APX and CAT.

The subcellular localization of desiccation induced H_2O_2 synthesis and its relation to H^+ATPase of the desiccated fronds was studied. Desiccation induced H_2O_2 accumulation was noticed initially on the plasma membrane, cell wall and subsequently continued into the cytoplasm and cell organelles like chloroplast, mitochondria (Fig 1a and b). Desiccation treatment does not inhibit H^+ATPase activity i.e., a positive correlation between H_2O_2 synthesis and H^+ATPase activity. This in turn favors plant's adaptation in maintaining transportation. The desiccation induced stress in the cytosol leads to the formation of NO that trigger DNA damage. The DNA migrations (comet assay) i.e., tail DNA percent showed marginal increase with duration of desiccation. The overall study reveals that the NO induces the antioxidant machinery and thereby ameliorates the cellular oxidative stress by scavenging the radicals.

Desiccation induced oxidative stress was exhibited as significant rise in the level of hydrogen peroxide (H_2O_2) and it correlated positively with duration. Subsequently, the activity of peroxidases (POX) enzyme (both cytosolic and cell wall bound) was also examined. The activity of cytosolic peroxidase was significantly higher throughout the periods of desiccation compared to wall bounded POX. Further, the medium level of cell wall peroxidase activity suggests its involvement in lignification of cell walls during stressed conditions. Zivkovic et al., (2010) reported an increased POX activity due to dehydration stress in the rusty back fern *Asplenium ceterach* and also suggested its role to efficiently scavenge H_2O_2 from the cells using phenolics and reduced ascorbate.

Abscisic acid (ABA), a signaling molecule was also evaluated by HPLC against different regimes of desiccation. Interestingly, a duration dependent positive correlation was noticed. Vishwakarma et al., (2017) substantiated the role of abscisic acid signaling *vs* abiotic stress tolerance in plants.

Finally, to elucidate MAPK signal transduction events leading to the cellular response was analyzed initially by protein phosphorylation induced by increased durations of desiccation. Exposure of forked ferns fronds to desiccation activated four distinct mitogen-activated protein kinases (MAPKs): SIMK, MMK2, MMK3, and SAMK as evident by immunokinase assay. Comparison of the kinetics of MAPK activation revealed that SIMK, MMK2, MMK3, and SAMK are activated effectively. SIMK and SAMK were more activated than MMK2 and MMK3.Overall the present results suggest that ferns respond to desiccation by induction of several distinct MAPK pathways (Fig 2 a and b).

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Teak Phenylalanine Ammonia-lyase, its Purification, Characterization and Kinetics

GREESHMA MURUKAN[1*] AND K. MURUGAN[2]

[1]*Plant Biochemistry and Molecular Biology Laboratory, Dept. of Botany, University College,*
[2] *Department of Biotechnology & Botany, Govt. Arts College, Thiruvananthapuram, 695 014, Kerala, India.*
Email: harimurukan@gmail.com

ABSTRACT

Anthocyanins are a group of flavanoid pigments with immense therapeutical values. Teak leaves show sound variation in the anthocyanin content between young to mature leaves. Phenylalanine ammonia-lyase (PAL), the key enzyme regulates the secondary metabolic pathway in plants. In this juncture, the present study was targeted to isolate, purify and characterize the enzyme. Phenylalanine ammonia-lyase was isolated and purified to homogeneity from the young leaf of teak using Sepharose 4B - L-phenylalanine affinity column. The purified PAL displayed a single band on SDS–polyacrylamide gel electrophoresis. The specific activity of purified PAL enhanced about 156.6 fold with 4.03 % yield. The molecular mass of the purified enzyme was estimated as53 kDa by size elution chromatography. Optimum activity as a function of pH and temperature was determined using phenyl alanine as substrate. Optimum pH and temperatures values ranged between the pH 8.0–9.0 and 40–50 °C. At the optimum pH and temperature, the K_m values were determined by Lineweaver–Burk method. The lower K_m value with phenyl alanine (1.88 m M) reveals that PAL has significant reactivity towards the substrate. The enzyme showed varied responses towards Mg^{2+}, Zn^{2+}, K^+, but inhibited with KCN, NaN_3, Hg^{2+}, Pb^{2+}, Cu^{2+}, Co^{2+}, *p*-CMB, iodoacetamide and NaBH. The enzyme inhibitor kinetics was also analyzed. Future studies are warranted to sequence the enzyme protein and also its functional expression.

Key words *Teak; phenylalanine ammonia-lyase; purification; chromatography; kinetics.*

Phenylalanine ammonia-lyase (PAL), is the starter enzyme involved in the deamination of phenylalanine to trans-cinnamic acid of phenylpropanoid pathway (PPP). The activation of PAL is associated with the synthesis of secondary products like phenols, flavonoids and lignins (Dikilitas, 2012). The properties, regulation, expression and cellular distributions of PAL have been analyzed in many species. The number of PAL genes ranges from 1 to 5 in the plant species reported so far (Angelika et al., 2009). The different proteins encoded were assumed to play role in diverse secondary metabolic pathways such as the formation of structural components or defence. In the French bean PAL1 was expressed in roots, leaves and shoots, while PAL2 was expressed in roots, shoots and petals, and PAL3 only in roots. Tyrosine ammonia lyase (TAL), another allied enzyme involved in catalyzing L-tyrosine to *p*-coumaric acid, with the release of ammonia. This is involved in many physiological reactions, including antioxidant, antimicrobial, antimutagenic, anxiolytic, analgesic, sedative, and immunoregulatory activities (Sarina et al., 2014). For induction of hyperpigmentation by plant tyrosine ammonia lyase was massively used. PAL is regulated co-ordinated and environmentally by transcriptional regulation through MYB, LIM and NTS transcription factors (Zhao and Dixon 2011). The regulation of PPP is channelling in plants. For example, in tobacco, metabolic channelling towards lignin synthesis occurs, via NtPAL1. This regulates the coupling with trans-cinnamate 4-monooxygenase (NtC4H), which catalyses the subsequent reaction in the pathway towards lignin synthesis (Achnine et al., 2004). de Jong et al., (2015) characterized the willow phenylalanine ammonia-lyase gene family and revealed its differential expression. PAL is an example for amino acid-transforming enzymes that do not possess the cofactor pyridoxal 5'-phosphate. Instead, it contains the unusual dehydroalanine. The synthesis of the prosthetic groups can be through autocatalysis of an inactive precursor, as is the case for histidine decarboxylase.

Many studies among different plant species showed that the induction of PAL activity was correlated with the biotic and abiotic stresses. The resistance of plants may depend on the rate and the extent of PAL synthesis. Therefore, PAL provides the level of resistance in the species against stress. For example, *Citrus* species showed less growth at high salinity with lower PAL activity and as a result of that they became highly susceptible to nematode infection

(Dunn et al., 1998). Partial purification and characterization of the enzyme was reported from many plants and further to investigate their properties. Teak plants are known for their wood. Young coloured leaves of the plant were used by the locals for curing many disorders (Greeshma Murukan and Murugan, 2017). Previously, Greeshma et al., (2015) confirmed that the young leaf of teak was rich in anthocyanin, an important phytochemical of phenyl propanoid pathway. In this scenario, the present study was carried out includes purification, characterization and kinetics of the PAL enzyme from the young leaf of *Tectona grandis* L f.

PLANT MATERIAL

Fresh leaf samples of *Tectona grandis* L f. was collected from its natural habitats, Thiruvanathapuram, Kerala.

Isolation and Assay of Phenylalanine ammonia lyase (PAL)

Isolation of PAL was done by the method of Gao et al., (2008). 1 g of finely chopped fresh tissues was homogenized in 10 ml 0.1 M Tris-HCl buffer containing 0.5 % PVPP at pH 8.8. The activity of PAL was estimated by the method of Havir (1981). The activity was related to the amount of ammonia formed by the action of PAL on the substrate. The initial absorbency was read at 290 nm. The reaction was allowed to proceed for 30 min at room temperature, and was stopped by the addition of 0.5 ml 10 % Trichloro acetic acid (TCA), then the final absorbancy was recorded. The difference between initial and final absorbancy was used for the estimation of PAL. One unit of enzyme activity was defined as amount of enzyme that increases the absorbance by 0.1/min under standard assay condition.

Purification and characterization of PAL

All the steps of PAL purification was carried out at 4°C ±1°C. Cunha (1988) purification protocol was adapted with some modifications. 200 g fresh young leaves of teak was chopped finely and homogenized with 250 ml 0.1 M, pH 8.8 Tris-HCl extraction buffer, containing 20 mM 2-mercaptoethanol and 0.5 % PEG.

The crude extract was continued with $(NH_4)_2SO_4$ precipitation (20 - 80 %). $(NH_4)_2SO_4$ precipitated crude enzyme fraction was dialyzed against 0.001 mM Tris-HCl buffer for 12 h. The resulted $(NH_4)_2SO_4$ precipitate was subjected to DEAE cellulose ion exchange chromatography. The enzyme protein was eluted with a linear gradient of NaCl (0.1to 0.4M) in Tris-HCl buffer. The 0.16 to 0.32 M active fractions were subjected to $(NH_4)_2SO_4$ precipitation for 120 min. The precipitate was centrifuged and dialyzed against 0.001 mM Tris-HCl buffer for overnight.

The active DEAE cellulose enzyme fraction was loaded on Sephacryl S-300 gel column which was pre-equilibrated with 20 mM Tris - HCl at pH 7.5. 3 ml fractions were collected with a flow rate of 1 ml/10 min using 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM $NaHSO_3$. The 16-20 fractions were pooled and was salt precipitated for 120 min. The salt precipitate was centrifuged and the pellet was subjected to dialysis against 0.001 mM Tris-HCl buffer for 12 h.

Affinity column comprises sepharose 4B L-phenyl alanine matrix and was equilibrated with acetic acid (pH 6) at room temperature for 6 to 12 h. PAL protein was eluted with 20 mM sodium borate containing 5 mM L-phenylalanine (pH 6.0) at 24°C. 4 ml of fractions were eluted at the flow rate of 1 ml/ min. Purified protein fractions were salt precipitated, dialyzed and evaluated for the PAL activity. Protein content was assayed by Bradford method, (1976).

Analysis of molecular weight by size elution chromatography

Sephacryl S-300 gel matrix was used for measuring the molecular mass in the size elution chromatography. Ovalbumin (43 kDa), lysozyme (14.3 kDa), ATPase (100 kDa) and bovine serum albumin (68 kDa) in 0.1 M Tris buffer containing 10 % sucrose were used as markers and was loaded gently. The elution volume (*Ve*) was calculated. A calibration curve for the size elution column was drawn by plotting log molecular mass against the partition coefficient (K_{AV}). Lastly, the purified PAL was loaded to the column and the elution volume was recorded. The molecular mass was calculated from the K_{AV}, compared with the calibration curve.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Laemmli (1970) and Fairbanks et al. (1971)

protocol was used to carry out SDSPAGE. Concentration of acrylamide used for separating gel and stacking gel were 10 % and 4 % respectively.

pH and temperature optima

The optimal pH of PAL activity was analyzed in the assay mixture with different pH ranging from 4-10 using acetate buffer (4.0-5.5), phosphate buffer (6-8) and boric acid borax buffer (8.5-10.0). The optimal temperature was evaluated by incubating the assay mixture at pH 7 for 30 min at a temperature ranging from 10 to 90°C. The residual enzyme activity was also calculated.

$$RA\ (\%) = \frac{\text{Activity (U/mL) of the treated enzyme solution}}{\text{Activity (U/mL) of the untreated enzyme solution}} \times 100$$

Thermo stability and pH stability

PAL enzyme protein was pre-incubated at temperatures in the range of 45-85°C for 30 min at optimum pH for thermo stability evaluation. For pH stability, enzyme extracts were pre-incubating at pH values in the range of 4.0-9.0 for 30 min at optimum temperature. The residual enzyme activity was determined as previously described.

Effect of inhibitors

Potassium cyanide (KCN), sodium azide, *p*-chloromerucuricbenzoate *(p*-CMB), sodium borohydride, iodoacetamide, salicylic acid monovalent and divalent cations such as K^+, Cu^{2+}, Hg^{2+}, Pb^{2+}, Ca^{2+}, cobalt and Mn^{2+} were evaluated for their effectiveness as inhibitors of PAL activity, using phenylalanine as the substrate. The results were reported as % of inhibition. The compounds showing highest inhibitions were further assessed by studying the kinetics of interaction of PAL with inhibitors. In separate experiments, inhibitors at various concentrations were added and were monitored at time intervals of 2 min (up to 10 min). First and second order rate constants were calculated.

Enzyme kinetics and substrate specificity

PAL activity was assayed using the substrates phenylalanine, tyrosine, alanine, coumarate and cinnamic acid at optimum pH. The *K*m value and maximum velocity (*V*max) were determined by the Lineweaver-Burk plot. Substrate specificity (*V*max/*K*m) was calculated by using the data obtained from the above (Lineweaver and Burk, 1934).

RESULTS AND DISCUSSION

PAL catalyses the deamination of phenylalanine in to trans-cinnamic acid, the first committed step regulates the phenylpropanoid pathway in plants (Reichert et al., 2009). The production of phenylpropanoid compounds is important in various processes such as plant development, defense and plant-microbe signaling (Calabrese et al., 2004; Pilbak et al., 2006).

Correlation of anthocyanin level with PAL activity

Sound variation in the anthocyanin level was noticed between the young (35.2 mg/g) and mature teak leaves (17.6 mg/g). Interestingly, a positive correlation was seen between PAL activity and anthocyanin level in the young teak leaves ($r^2 = 0.734$, $P > 0.1$). However, the correlation between mean PAL activity with the anthocyanin concentration of mature leaves was insignificant ($r = 0.14$, $P > 0.1$). Anthocyanin plays multiple roles in plants including defense. Similarly the compound was used by the locals as curative to many disorders like skin, inflammation, microbicidal etc (Greeshma Murukan and Murugan, 2017). Therefore, PAL was subjected to purification and characterization.

Purification data

PAL from young teak leaves was purified to homogeneity using ammonium sulphate ($(NH_4)_2SO_4$) precipitation (20 – 80 % saturation), ion exchange (DEAE - cellulose), gel filtration (sephacryl S – 300) and finally affinity chromatography (sepharose 4B - L-phenylalanine). The crude leaf extract of PAL revealed a specific activity of 4.61U / mg protein. After $(NH_4)_2SO_4$ precipitation and dialysis, the enzyme exhibited a specific activity of 249U / mg protein with a purification fold of 54. Subsequently, the enzyme was subjected to different chromatography. The 80 % ammonium sulphate protein precipitate was loaded gently on ion exchange column and linearly eluted with 0.1 – 0.4 M NaCl in Tris –HCl buffer (0.001 M pH 6.5). The concentrated 0.16 to 0.32 M NaCl buffer enzyme fractions was further purified by sephacryl S

Table 1. Purification profile of the purified PAL from teak leaves

Purification steps	Enzyme activity (Units/g tissue)	Total protein (mg)	Specific activity (Units/mg protein)	Fold purification	Yield (%)
Crude extract	4120	893	4.61	1.0	100
Ammonium sulfate 80%	1743	7.0	249	54	42.3
DEAE - cellulose	927	2.5	370.8	80.43	22.5
Sephacryl S – 300	478	1	478	103.7	11.6
Sepharose 4B - L-phenylalanine	166	0.23	721.7	156.6	4.03

– 300. The elution profile from gel column 16 to 20 were pooled and revealed a specific activity of 478 U / mg protein. The enzyme was purified to homogeneity by sepharose 4B - L-phenylalanine affinity column. The resultant showed a single peak with remarkable PAL activity. Thus, the overall protocol yielded a purified PAL with specific activity 721.7 units with a low protein of 0.23 mg. The specific activity of purified PAL enhanced about 156.6 fold with 4.03 % yield (Table 1). This is remarkable as compared to other purified PAL from sweet potato (Tanaka and Uritani, 1977), soybean cell suspension cultures (Havir, 1981) and tulsi plant (Hao et al., 1996).

Physical properties

Gel filtration chromatography was used to elucidate the molecular mass of teak PAL. The molecular mass of the purified PAL is 53 kDa estimated from the markers, suggesting that the Teak PAL exists as a monomer in the native form (Fig.1). The molecular mass of *Rhizoctonia* PAL was the sum of 70 and 90 kDa. Maize PAL was reported as heterodimer. In fact, Teak PAL differs from the PALs reported from *Rhizoctonia*, and maize in terms of their heterodimeric or homotetrameric nature (Kalghatgi and Rao, 1975; Bevan et al., 1989). Similarly, the molecular mass of beans PAL was 83,000 Da (Bolwell and Rodgers, 1991). Native and subunit molecular mass of loblolly pine PAL was 280,000 Da and 74,000 Da respectively (Whetten and Sederoff, 1992).

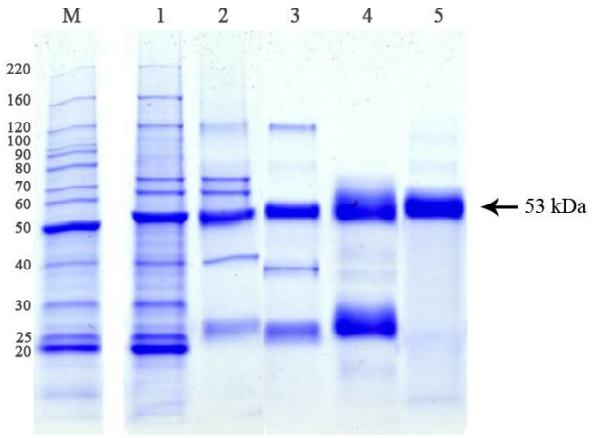

Fig. 1. SDS-PAGE of the purified phenylalanine ammonia lyase (PAL) of teak leaves

Lane 1 Marker, lane 2- crude PAL, lane -3 ($(NH_4)_2SO_4$) precipitation, lane 4 ion exchange (DEAE - cellulose), lane 5 gel filtration (sephacryl S – 300) and lane 6 affinity chromatography (sepharose 4B - L-phenylalanine).

pH optima and stability

The residual activity of PAL after 24 h of pre-incubation at 9 was 98.7% which is different from that of *Rhizoctonia solani,* maize (pH optima 8.7 and 7.7) by Kalghatgi and Rao (1975) and Havir et al.,

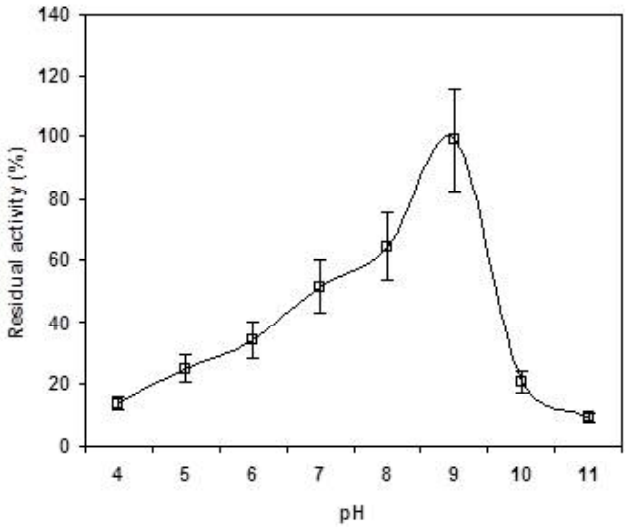

Fig. 2. pH stability of the purified PAL from teak leaves after a pre-incubation period of 24 h at the indicated pH

(1981) respectively. In phenyl propanoid metabolism studies, the enzyme PAL was routinely assayed using phenyl alanine to form trans-cinnamic acid at an optimum pH 8.5. Further, it is found that PAL in teak is reasonably stable over a range of alkaline pH 8 – 9 (Fig 2). In this aspect, the enzyme was similar to that of other reported PALs (Hanson and Havir, 1981). The low profile of the enzyme activity at acidic and pH above 9.0 may be due to the possible structural changes of the enzyme and/or the protein may undergo Maillard reaction and/or Strecker destabilization process.

Thermal optima and stability

Temperature sensitivity of PAL was analyzed by incubating the enzyme at temperatures 10 - 90°C. The teak PAL showed a peak activity at 50°C, indicating the stability of the protein at this temperature which was higher than the optimal temperature for cotton PAL (Dubery and Smit, 1994). The enzyme expressed a decreased activity from 60°C.

The thermal stability of the enzyme was also evaluated by incubating the protein from 45 to 85°C temperatures for 30 min, and after cooling, the residual enzyme activity was calculated. Teak PAL lost nearly 60 % of the activity at 65°C and about 29.5 % of it at 75°C after 30 min (Fig 3). These thermo labile properties were superior to PAL of *Phaseolus vulgaris* (Cunha, 1988) and yeast (Fritz et al., 1976). The reduction of residual activity with duration at higher temperatures may be due to the unfolding of the protein structure.

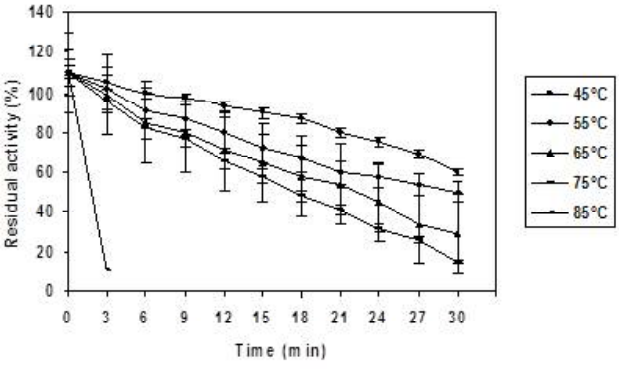

Fig. 3. Thermal stability the purified PAL from teak leaves at varying temperatures (45°C – 85°C) versus time

Influence of inhibitors

The teak PAL assay was fortified with different concentrations of cations, anions and inhibitors like sodium azide (NaN_3), KCN, Salicylic acid, iodoacetamide, *p*-chloro-mercury-benzoate (*p*-CMB) and sodium borohydride (NaBH)(Table 2 and 3). Teak PAL activity was optimal with Mn^{2+}, Ca^{2+}, K^+, but inhibited with KCN, NaN_3, Hg^{2+}, Pb^{2+}, Cu^{2+}, Co^{2+}, *p*-CMB, iodoacetamide and NaBH. In fact, teak PAL was comparable with maize and potato, as the Pal was deactivated by NaBH, at varying concentrations (6 - 30 ìM) (Havir et al., 1981). Further, PAL activity was less affected by nickel. The impact of metal ions on PAL activity was contradictory in plants (Monge et al., 1995). However, commonly the Na^+, K^+ and nitrates have no influence on PAL activity, while Pb^{2+}, Ni^{2+} and Zn^{2+} were inhibits the enzyme effectively.

Kinetics of interaction of NaBH, *p*- CMB, KCN, iodoacetamide and Hg^{2+}

Inhibitor enzyme kinetics was presented in the Table 4 that NaBH, KCN, p- CMB, iodoacetamide and Hg^{2+} inhibit PAL activity significantly. It is of immense importance to note the kinetics of interaction of inhibitors with enzyme by monitoring the change of activity. The PAL enzyme was incubated for 10 min with various doses of NaBH, KCN, p- CMB, iodoacetamide and Hg^{2+}. Regularly, at 2 min intervals, the residual enzyme activity was monitored. The enzyme was assayed when both the inhibitor and the substrate were added together and assayed for 5 min for any activity loss during the enzyme inhibitor interaction assay. The value was normalized to 100 % in each case and measured to calculate in % of remaining activity when the inhibitor inhibited the enzyme alone. At various doses of the inhibitor, the ln % remaining activity was plotted against the duration of incubation. The slopes of the curves gave the first order rate constants summarized in Table 4. Increase in the extent of inhibition of enzyme activity was reflected by increased value of rate constants. The log of the first order rate constant was plotted against log KCN, log NaBH, log *p*-CMB, log Hg 2+ , iodoacetamide and concentration and a second order rate constant was calculated as 97.5 M^{-1} sec^{-1}, 89.7 M^{-1} sec^{-1}, 79.6 M^{-1} sec^{-1}, 74.6 M^{-1} sec^{-1}, and 69.8 M^{-1} sec^{-1} respectively. The data reveals that KCN was a potent inhibitor of PAL (Figs.5 a, b c, d and e).

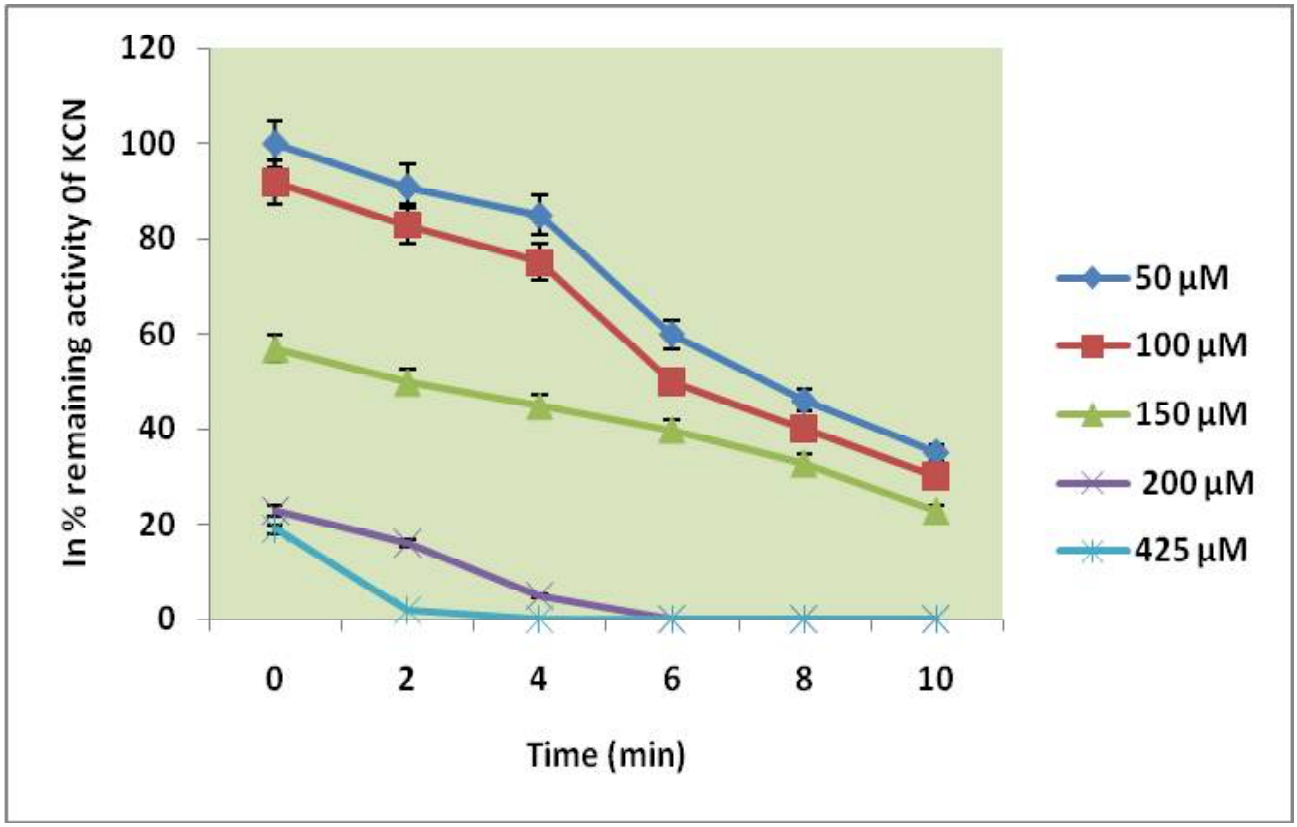

Fig. 5a. Purified teak PAL against varying concentrations of KCN. (The concentrations of KCN used were 50 to 450 µM *50 µM, 100 µM, 150 µM, 200 µM, 425 µM)

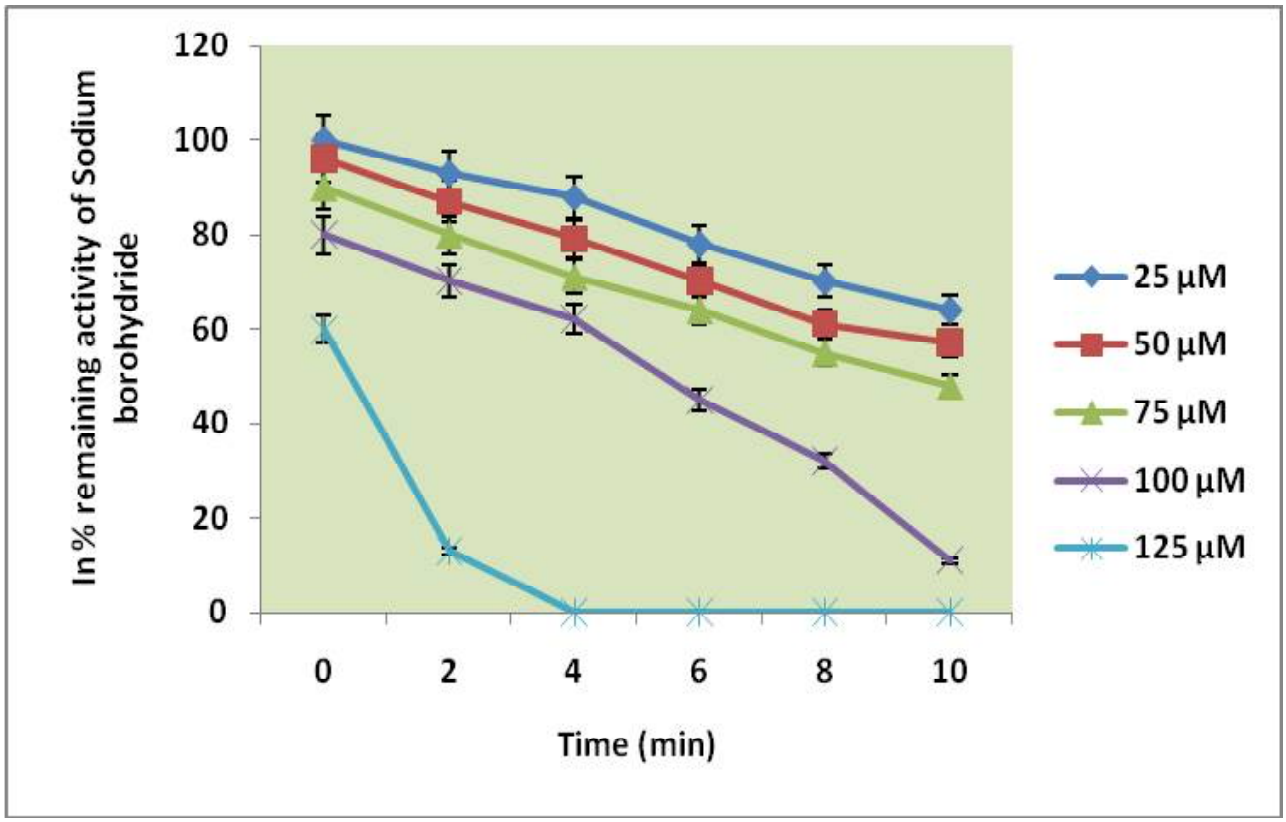

Fig. 5b. Purified teak PAL against varying concentrations of sodium borohydride (NaBH) against time. (The concentrations of NaBH used were 25-125 µM, • -25 µM, 50 µM, 75 µM, 100 µM, 125 µM)

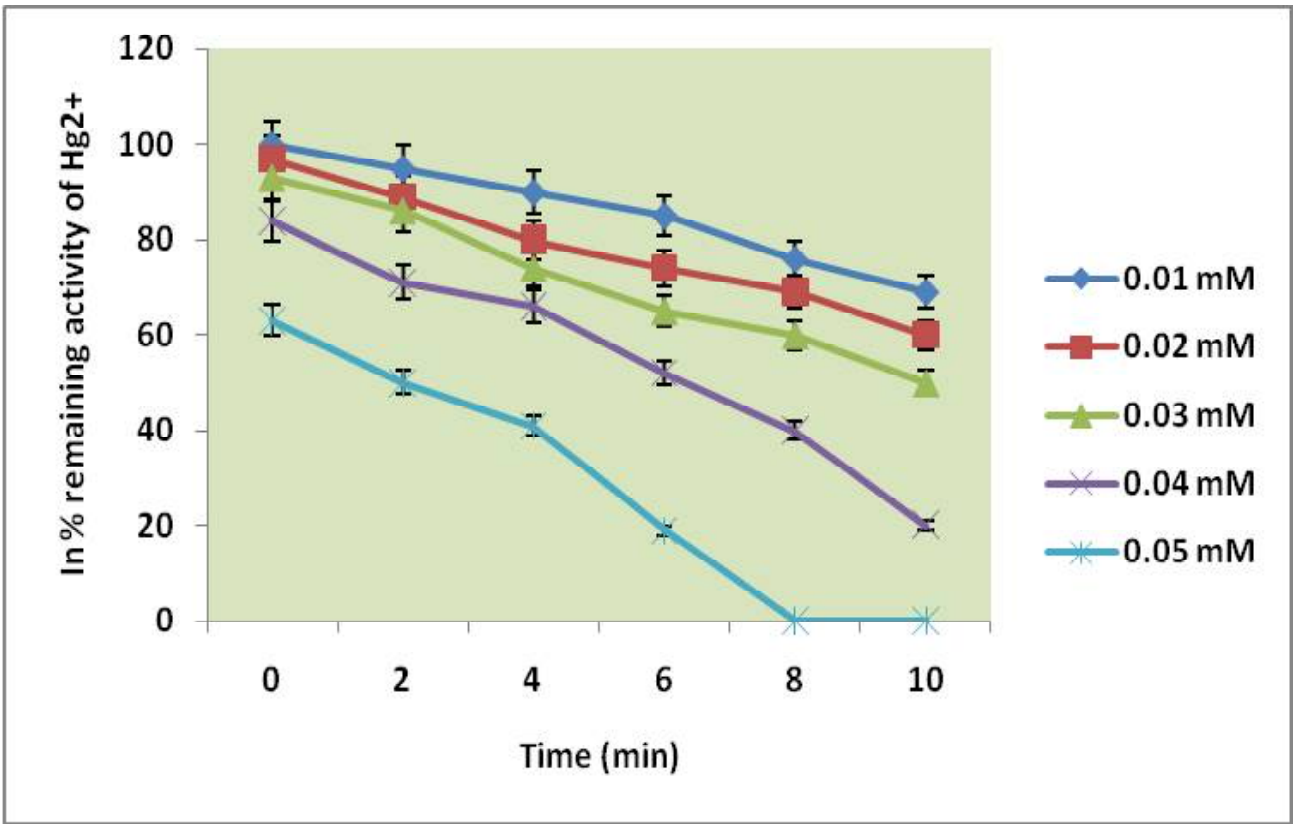

Fig. 5c. Purified teak PAL with varying concentrations of Hg^{2+} and time. (The concentrations of Hg^{2+} used were 0.01 to 0.05 mM • 0.01mM, 0.02 mM, 0.03 mM, 0.04 mM, 0.05 mM)

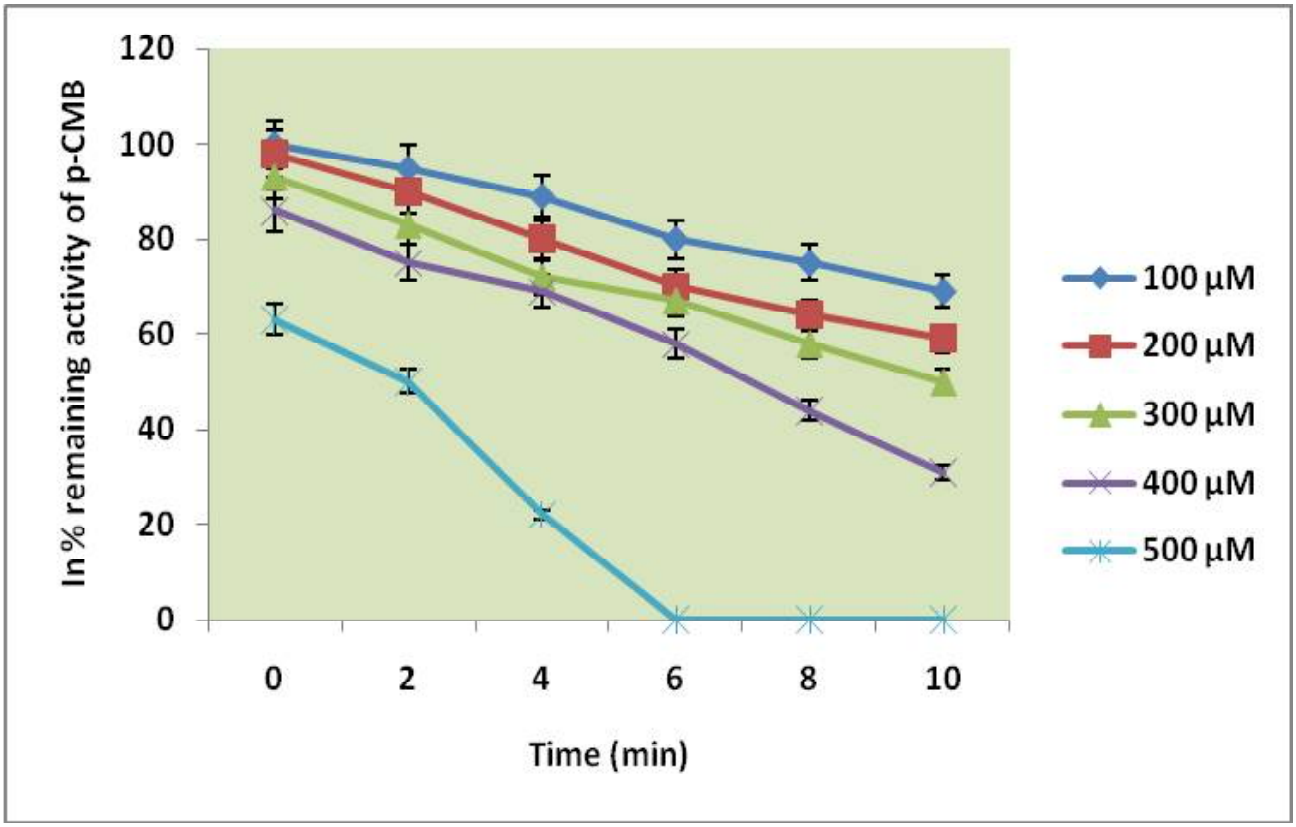

Fig. 5d. Teak PAL with varying concentrations of Benzoic acid (p- CMB) and time. (The concentrations of p-CMB used were 100-500 µM, •-100 µM, 200 µM,-300 µM, 400 µM, 500 µM)

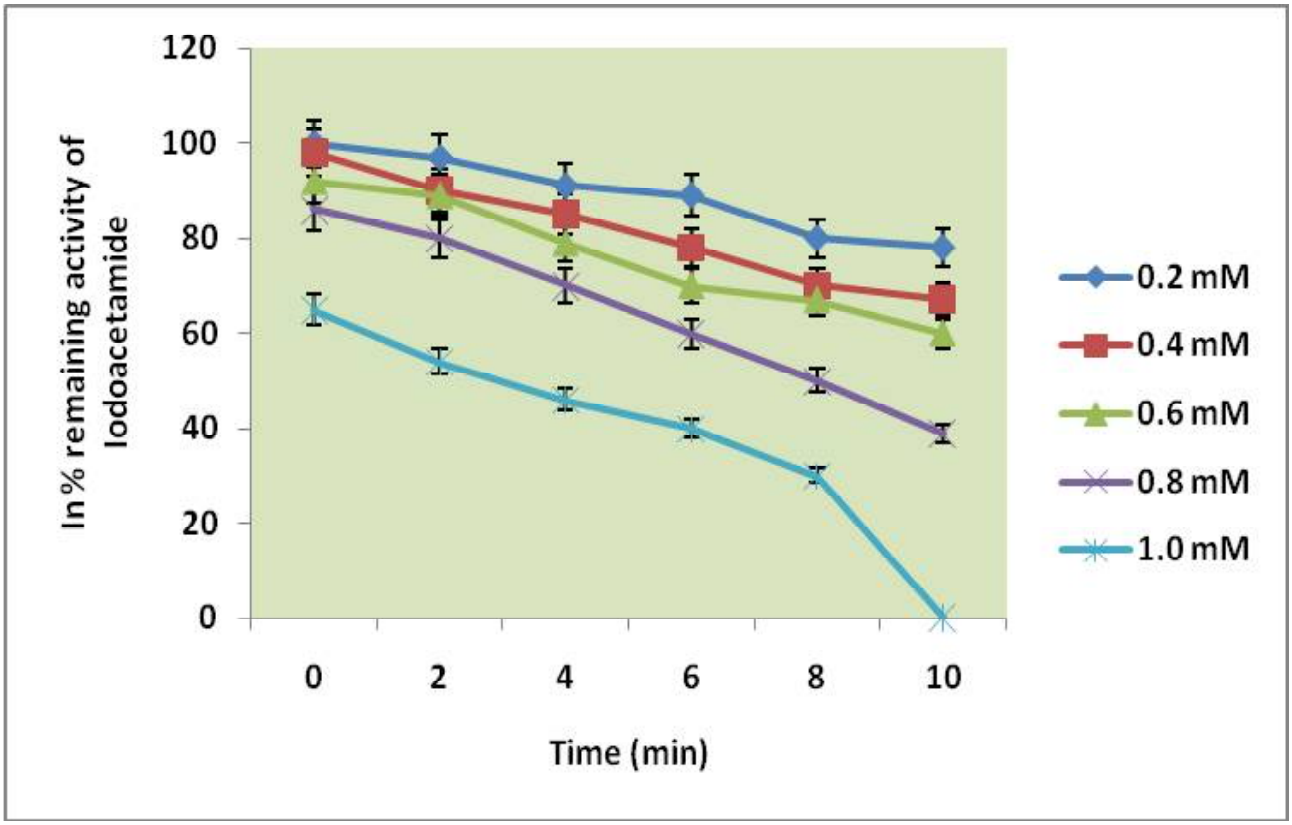

Fig. 5c. Purified teak PAL with varying concentrations of iodoactamide and time. (The concentrations of iodoactamide used were 0.2 to 1 mM • 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM, 1 mM)

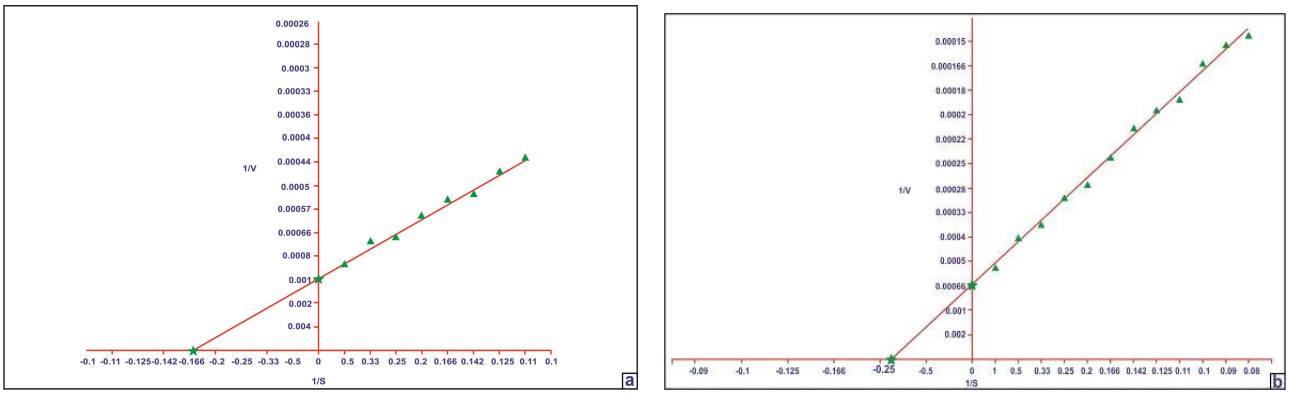

Fig. 6. a&b. Double reciprocal plot of teak PAL activity using the substrates a-phenyl alanine, b-tyrosine

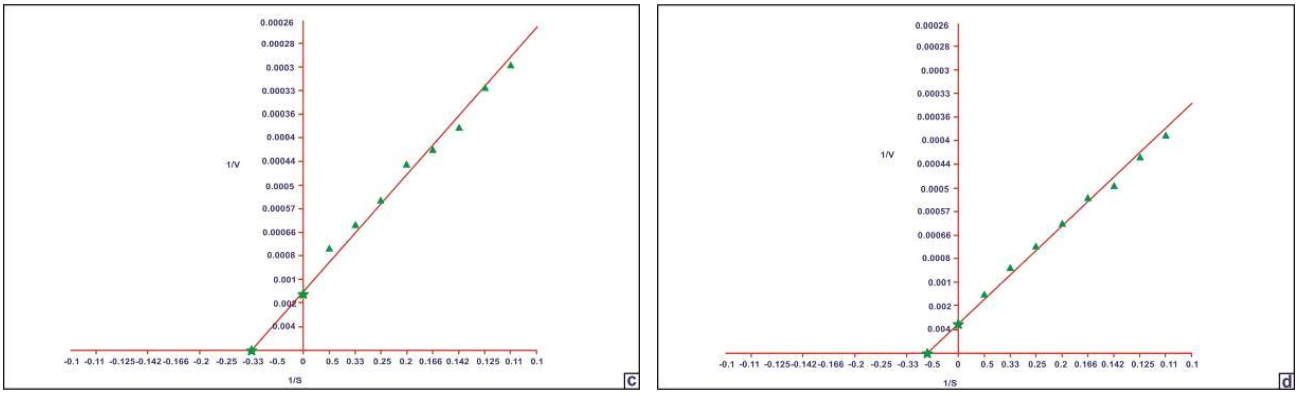

Fig. 6.c&d. Double reciprocal plot of teak PAL activity using the substrates c-alanine, d-cinnamate

Table 2. Effect of inhibitors on teak PAL activity: Samples were incubated with the inhibitor for 5 min at 25°C. Assays were done in triplicates

Inhibitors	Conc. (mM)	Percentage of Inhibition (%)
Potassium	0.1	16.9 ± 0.23
	0.5	43.7 ± 0.04
	1	64 ± 0.67
Mercury	0.01	46 ± 0.87
	0.05	85 ± 0.01
Lead	0.5	39 ± 0.88
	1	66 ± 0.02
Copper	0.5	51.8 ± 0.07
	1	65 ± 0.70
Cobalt	0.5	60 ± 0.34
	1	71 ± 0.06
Manganese	0.01	13.2 ± 0.01
	0.1	19.6 ± 0.08
	0.5	32.5 ± 0.56
Calcium	10 µM	15.6 ± 0.07
	50 µM	50.5 ± 0.25
	100 µM	61± 0.29
Nickel	0.1	18.8 ± 0.04
	0.5	37.6 ± 0.27
Zinc	10 µM	31 ± 0.09
	50 µM	59.5 ± 0.17
	100 µM	79.5 ± 0.98

F = 874,** CD = 7.943, SE= 0.972

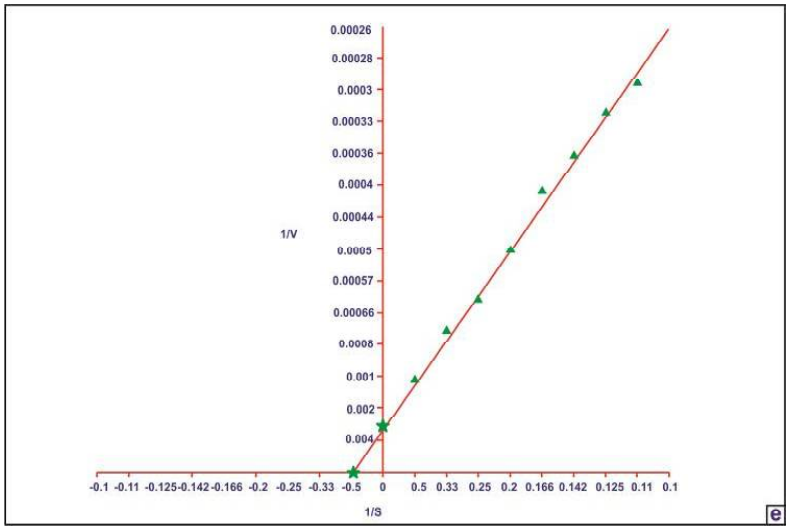

Fig. 6.e. Double reciprocal plot of teak PAL activity using the substrate coumarate

Table 3. Effect of inhibitors on teak PAL activity: Samples were incubated with the inhibitor for 5 min at 25°C. Assays were done in triplicates

Inhibitors	Conc. (mM)	Percentage of Inhibition (%)
Sodium azide	0.1	18.6 ± 0.45
	0.5	44.5 ± 1.9
	1	64 ± 2.3
Sodium borohydride	25 µM	49 ± 0.23
	50 µM	80 ± 3.1
	125 µM	90 ± 3.7
Salicylic acid	100 µM	21 ± 1
	500 µM	38.9 ± 1.7
	1 mM	71 ± 2.5
p-CMB	100 µM	30 ±4.8
	300 µM	62 ± 5.6
	500 µM	85.9 ± 0.08
KCN	50 µM	32 ± 3.8
	100 µM	83 ± 6.4
	250 µM	96.9 ± 7.1
Iodoacetamide	0.1	12.4 ± 0.07
	1	81 ± 0.18

F = 2598,** CD = 3.126, SE= 0.543

Substrate specificity and Kinetics

The substrate specificity was studied, using the major substrates like tyrosine, phenylalanine, coumarate, cinnamate, and alanine. Differential activity was displayed by PAL against the substrates. The *K*m value was calculated through double

Table 4. First order rate constant for inhibition of activity of teak PAL by *p*-CMB, Hg^{2+}, NaBH and KCN. *p*-CMB - *p*-chloromerucuricbenzoate, Hg^{2+}-Mercury, NaBH-Sodium borohydride, KCN - Potassium cyanide and iodoacetamide - IA

Inhibitor conc. (mM)	1 (Ks^{-1})	2 (Ks^{-1})	3 (Ks^{-1})	5 (Ks^{-1})	10 (Ks^{-1})
p-CMB	1.4×10^{-2}	1.5×10^{-2}	2.3×10^{-2}	3.0×10^{-2}	3.2×10^{-2}
Hg^{2+}	1.5×10^{-2}	1.7×10^{-2}	2.4×10^{-2}	3.3×10^{-2}	3.6×10^{-2}
NaBH	1.2×10^{-2}	1.6×10^{-2}	2.0×10^{-2}	2.2×10^{-2}	2.7×10^{-2}
KCN	1.2×10^{-2}	1.3×10^{-2}	1.9×10^{-2}	2.3×10^{-2}	2.4×10^{-2}
IA	1.6×10^{-2}	1.9×10^{-2}	2.5×10^{-2}	3.3×10^{-2}	3.9×10^{-2}

reciprocal plot. The *K*m value is lower for phenylalanine (1.88 mM) as compared to other substrates like tyrosine (4.67 mM), alanine (7.9mM), cinnamate (9.8 mM) and coumarate (10.6 mM). The values were higher than maize PAL (Havir et al., 1981), but comparable with pine PAL (Whetten and Sederoff, 1992). Further, the *K*m value for sweet potato PAL was 30 ìM (Tanaka and Uritani, 1977). The *K*m value of PAL from tulsi plant was 329 ìM (Hao et al., 1996). All these substrates seem to be reversible competitive inhibitors for PAL. Tyrosine, cinnamate, coumarate and alanine were documented as substrates for PAL, although the rate of reaction was less, but the amount of activity detected was remarkable over an increased duration of incubation. Havir et al., (1981) have analyzed the potato PAL kinetics of cinnamate and reported that it could not be classified as competitive or non competitive inhibitor. Similarly, all the above said compounds were known inhibitors of maize PAL (Havir et al., 1981).

CONCLUSION

PAL, the marker enzyme of secondary metabolic pathway was purified through salt precipitation to affinity chromatographic technique. Fold of purified PAL was 156.6. The molecular mass was detected by gel elution chromatography (53 k Da). The enzyme was characterized for pH, temperature, inhibitors and substrates. The *K*m value is lower for phenylalanine (1.88 mM). Further studies are planned to characterize the PAL gene and to up regulation of its activity.

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Mechanism of Anti-Inflammatory Potential of Terpenoids from *Thuidium tamariscellum*(C. Muell.) Bosch. & Sande-Lac. A Moss Using Animal and Macrophage Models

G.M. GREESHMA[1]* AND K. MURUGAN[2]

[1]*Dept. of Botany, University College*
[2]*Dept. of Botany & Biotechnology, Govt. Arts College, Thiruvananthapuram, Kerala, India*
**email: greeshmagm1990@gmail.com*

ABSTRACT

***Thuidium tamariscellum* is a creeping epiphytic moss growing on trunks and branches of woody trees of moist shady rocky surface at high altitudes regions. The present study was aimed to evaluate the cyclooxygenase (COX) and lipoxygenase (LOX) inhibitory activity of terpenoids from *Thuidium tamariscellum*. Initially, GC-MS analysis of the purified terpenoid extract revealed the following components such as Dodecanal, Tetradecanal, Hexadecanal, Phytol isomer and 4,8,12,16-tetramethylheptadecan-4-olide similarly the purified *in-vitro* anti-inflammatory activity of terpenoid extracts was assessed in carrageenan induced paw edema model in Albino Wistar rats. Remarkable inhibition of paw edema was noticed as compared to control and the activity was comparable to ibuprofen. Further, the purified terpenoid was evaluated for COX-2 and LOX-5 inhibitory activity to determine the possible mechanism of their anti-inflammatory potential. Antioxidant power was also analyzed by DPPH scavenging potential. The results of the study demonstrated that terpenoids from *T. tamariscellum* significantly inhibited COX and LOX activity like indomethacin (synthetic drug). Different concentrations viz. 10, 25, 50, 75 µg/ml of the extract, and indomethacin (25µg/ml) were showed remarkable percentage inhibition of COX- 2. Similarly optimal inhibition was also noticed for LOX activity. The treatment significantly decreased the levels of NO and myeloperoxidase. 25 µg/ml terpenoid extract revealed 60.25 % of DPPH radical inhibition and there by pinpointing the antioxidant potential of the molecule. Thus, the overall results justify the local use of the moss as preventive of inflammation.**

Key words *Thuidium tamariscellum, cyclooxygenase, lipoxygenase, purified terpenoid, antioxidant, paw edema*

Macrophages are the main immune cells, involved in tissue damage in a number of inflammatory conditions such as acute and chronic. ROS are synthesized by activated macrophages like $\bullet O_2^{"}$, H_2O_2, and the highly reactive hydroxyl radical •OH; NO•, arachidonic acid metabolites, and lysosomal enzymes are necessary to perform their phagocytic function. These cells also play an important role in the development of various chronic diseases such as cancer and allergies (Manish Mittal et al.,2014). Traditional medicine has got an important role in modern drug discovery, the use of plant based medicines or their active components represents a promissory alternative for the treatment of various illness, including inflammatory disorders (Si-Yuan Pan et al.,2013). Based on ethnopharmacological uses, many medicinal plants have attracted considerable interest, particularly in the treatment of various disease conditions including chronic inflammatory diseases. The research into medicinal plants with alleged folkloric use as analgesics, anti-inflammatory agents, should therefore be viewed as a fruitful and logical research strategy in the search for new analgesic and anti-inflammatory drugs. Currently, in many countries natural products are used in traditional medical systems as they are considered safer. But due to limited knowledge of their biological activities & limited scientific evidence natural bioactives has limited its incorporation into clinical practice. (Choi et al.,2003).

Prostaglandins (PGs) and leukotrienes (LTs) are two hormone-like substances released by the body which is involved in the formation of inflammation. These inflammatory inducing agents are produced from arachidonic acid (AA) by the enzymes cycloxygenase (COX) and lipoxygenase (LOX). COX is the first enzyme in the pathway for producing PG and thromboxane (Tx) from arachidonic acid and can occur as three isoforms, COX-1, COX-2, COX-3. LOX is the first enzyme in the pathway for producing leukotriene (LT) from arachidonic acid. Isoenzymes of LOX include 5-LOX, 12-LOX and two 15-LOX isoforms (15 LOX-1 and 15 LOX-2). The current strategy includes the development of dual LOX/COX inhibitors with a higher safety profile, particularly medicinal plants of folkloric use as pain relievers and anti-inflammatory agents (Jenny et al., 2015). Pharmacological validations on Indian medicinal plants are limited and the enormous potential of the different plants used in Ayurveda and tribal folklore has not been exploited for their effective use in medicinal

therapeutics. Thus, the present study aims to evaluation of anti-inflammatory potential of terpenoid extract from *Thuidium tamariscellum* and its mode of action.

MATERIALS AND METHODS

Thuidium tamariscellum belonging to thuidaceae is a slender, yellowish-green epiphytic moss growing on branches & trunks of trees at high altitude areas. The moss were collected and identified by authenticating with the herbaria of University of Calicut, Kerala.

Fractionation was done by silica gel Column Chromatography (CC) using petroleum ether:ethyl acetate as solvent combinations. The purified fraction was quantified for the presence of terpenoid as described by Ferugson(1956) and further analyzed by GC-MS.

GC-MS analysis

For GC-MS analysis, the sample was injected into a HP-5 column (30 m X 0.25 mm i.d with 0.25 ìM film thickness), Agilent technologies 6890 N JEOL GC Mate II GC-MS model. Helium was used as carrier gas with a flow rate of 1 mL/min; the injector was operated at 200 °C and column oven temperature was programmed as 50 - 250 °C at a rate of 10 °C / min injection mode. The following MS conditions were used: ionization voltage of 70 eV; ion source temperature of 250 °C; interface temperature of 250 °C; mass range of 50 - 600 mass units. A chromatogram was obtained and the mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

Carrageenan induced paw edema

The present study was conducted to evaluate the anti-inflammatory effect of natural product on carrageenan induced paw edema in rats. The terpenoid extract was administered at doses of 200 and 400 mg/kg to Wistar albino rats as the study dose and Indomethacin at dose of 10 mg/kg as a standard drug before an hour of administration of carrageenan (0.1 ml of 1% w/v into sub plantar region of rat paw). The paw volume was measured using mercury displacement technique with the help of plethysmograph at 0 and 3h after carrageenan injection. The difference between 0 and 3h reading was taken as the volume of edema and percentage of edema inhibition was calculated for each group.

Animal study

Experimental study was carried out using Swiss albino mice weighing 30-35g. The animals were procured from Biogen, Bangalore. The animals were housed in polypropylene cages. The cages were maintained clean and hygienic. The mice were fed with commercial pelleted feed and water *ad libitum*. The animal caring and handling were done according to the CPCSEA guidelines.

Murine macrophages (RAW 264.7 cell line)

Murine macrophage was obtained from National Centre for Cell Science (NCCS), Pune. RAW 264.7 cells were grown to 60% confluence followed by activation with 1 ìL lipopolysaccharide (LPS) (1ìg/mL). LPS stimulated RAW cells were exposed with different concentration of sample solution. Indomethacin, a standard anti-inflammatory drug in different concentration corresponding to the sample were also added and incubated for 24 h. After incubation the anti-inflammatory assays were performed using the cell lysate.

Cycloxygenase (COX) activity

The COX -2 activity was assayed by the method of Walker and Gierse (2010). COX activity was determined by reading absorbance at 632 nm.

5-Lipoxygenase (LOX) activity

The determination of 5-LOX activity was as per Axelrod et al (1981). The LOX activity was monitored as an increase of absorbance at 234 nm (Shimadzu), which reflects the formation of 5-hydroxyeicosatetraenoic acid.

Myleoperoxidase (MPO) activity

Cell lysate was homogenized in a solution containing 50 mM potassium phosphate buffer and 0.57% hexadecyltrimethyl ammonium bromide (HTAB). Bradley et al., (1982) protocol was adapted for the assay. The change in absorbance at 460 nm was measured. MPO activity was presented as units per mL of cell lysate. One unit of MPO activity was defined as that degrading 1 ìM of peroxide per minute at 25°C.

Cellular nitrite levels

Cellular nitrite level was estimated by the method of Lepoivre et al (1989). The absorbance was read at 540 nm against a Griess reagent.

Antioxidant assay

DPPH Radical Scavenging Assay

2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) antioxidant method as described by Blois(1958) was used to analyze the free radical scavenging ability of the terpenoids extract from the moss and the absorbance was measured at 517 nm. Ascorbic acid was used as the standard.

Statistical analysis

All the data were mean SD of six independent experiments. Significant level was noted as $p < 0.05$.

RESULTS AND DISCUSSION

The crude methanolic extract of the moss was purified by silica gel column chromatography using petroleum ether:ethyl acetate as solvent combinations. The fraction eluted from the column was further quantified for the presence of terpenoids. The fraction eluted using 95:5 solvent combination showed significant amount of terpenoids i.e 5.12 mg/g. Subsequently, the fraction was subjected for GC-MS analysis. Table 1 shows the terpenoid composition of the moss as detected by using GC-MS spectra technique. Retention time and the relative abundance of each compound were recognized. The GC-MS analysis of the purified fraction revealed the presence of 5 peaks of terpenoids such as Dodecanal, Tetradecanal, Hexadecanal, Phytol isomer and 4,8,12,16-tetramethylheptadecan-4-olide Out of which Dodecanal showed the highest peak followed by4,8,12,16-tetramethylheptadecan-4-olide. Similarly, the filamentous green algae *Spirogyra longata* also showed the presence of 8 terpenes including 4,8,12,16 tetramethyl heptadecan-4-olide. (Eman et al., 2015)

Effect of terpenoids extract on Carrageenan induced paw edema

After administration of study drug, terpenoid extract and carrageenan the paw edema of the animals was measured using mercury displacement technique with the help of plethysmograph at 0 and 3h after carrageenan injection. The terpenoid extract from the moss reduced carrageenan induced paw edema at all doses i.e a dose dependent anti-inflammatory effects was noticed (Table 2). The smallest dose 200mg/kg effectively reduced carrageenan induced paw edema at the 3rd h after administration. Similarly, 400mg/kg significantly reduced carrageenan induced paw edema at the 2nd h after administration as compared to the standard drug indomethacin.

Antioxidant assay

Results of DPPH assay of the purified terpenoid extract revealed remarkable IC_{50} value of 16 µg/ml. The standards quercetin and ascorbate under similar conditions of the experiment was also analyzed. The IC_{50} values for scavenging DPPH radical for quercetin and ascorbate were 5.7 ± 0.24 and 28.3 ± 0.36 µg/ml respectively (Table 3).

Table 1. GC-MS analysis showing the terpenoid composition in *Thuidium tamariscellum*

Sl no	Compound(IUPAC Name)	Molecular formula	Retention time
1	Dodecanal,	$C_{12}H_{24}O$	18.65
2	Tetradecanal,	$C_{14}H_{28}O$	23.63
3	Hexadecanal,	$C_{16}H_{32}O$	28.11
4	Phytol isomer	$C_{20}H_{40}O$	34.82
5	4,8,12,16-tetramethylheptadecan-4-olide	$C_{21}H_{40}O_2$	41.05

Table 2. Anti- inflammatory potential on Carrageenan induced paw edema in Wistar albino rats by purified fraction of terpenoid

Groups	Paw volume in ml at different time interval				
	0 h	1st h	2nd h	3rd h	%inhibition
Normal	0.27±0.04	0.30±0.04	0.32±0.02	0.32±0.02	-
Positive control	0.45±0.05	0.60±0.04	0.75±0.02	0.97±0.06	-
Standard	0.17±0.02	0.65±0.02	0.55±0.02	0.32±0.08	73.3
200mg/kg	0.42±0.04	0.55±0.04	0.50±0.08	0.47±0.02	63.5
400mg/kg	0.40±0.05	0.50±0.04	0.47±0.02	0.45±0.02	69.9

Table 3. AOX potential of the terpenoid extract of the moss *T. tamariscellum*

Concentration (µg/ml)	DPPH scavenging effect (%)
10	37.7 ±4.2
20	56.4 ±3.9
30	72.94 ±6.2
40	86.16 ±5.8

COX-2 & LOX-5 inhibitory activity of indomethacin (standard drug) and purified terpenoid

Dose dependent inhibition of COX-2 was exhibited by the synthetic drug indomethacin and also the purified terpenoid. Indomethacin showed an inhibition at 25 µg/ml (79.2%) with an IC_{50} value of 17.8 µg/ml. Concentration dependent percentage inhibition of COX-2 was exhibited by various concentrations (10, 25, 50 and 75 µg/ml) of purified terpenoid. The IC_{50} value was 30.6 µg/ml (Table 4).

Maximum LOX-5 inhibitory effect of Indomethacin (25 µg/ml) was found to be 72.6% with an IC_{50} value of 14.5 ìg/ml. Dose dependent inhibition of LOX-5 was demonstrated by purified terpenoid i.e., increased percentage inhibition was exhibited by purified terpenoid with different concentration (10, 25, 50 and 75 µg/ml), (Table 4).

Lipoxygenases (LOXs) are non-heme iron-containing dioxygenases catalyzing the production of leukotrienes. Leukotrienes function as inflammation initiators and their activity is considered to be partly responsible for the anti-inflammatory activity (Shailasree sekhar et al., 2013). The percent inhibitory activity against COX-2 and LOX -5 by the terpenoid extracts at 10, 25, 50 and 75 µg/ml indirectly reveals the percent inhibition of prostaglandins and leukotrienes production.

The significance of the dual inhibition of LOX and COX lies in the effective reduction of chronic inflammatory disorders. COX inhibition has been documented to be connected with protection of the gastrointestinal integrity and thereby preventing the formation of gastric ulcers (Hawkey 2001). The gastrointestinal integrity level remains safe guarded with the dual blocking of the enzymes as is clinically proved in other studies (Hawkey 2001). The occurrence of cardiovascular disorders in selective COX-2 inhibition is also reduced in such situations of dual inhibition (Afshin et al.,2011). The use of herbals for the treatment of chronic inflammatory conditions has existed for a long time but the validation for such use has been of interest in the pharmaceutical industries only recently. Most of the studies reported were from higher plant groups. In the present study, the purified terpenoids of the moss *Thuidium tamariscellum* exhibits effective anti-inflammatory potential in terms of dual inhibitions. The moss species *Thuidium tamariscellum* was reported for traditional use as curative but with no studies for correlation of this effect on LOX/COX inhibition. The results presented in this study have conclusively established the presence of bioactive molecules in the extracts capable of dual inhibition of LOX/COX enzymes involved in biosynthesis of pro-inflammatory leukotrienes and prostaglandins.

Table 4. Cycloxygenase and 5-Lipoxygenase inhibition by standard and terpenoid extract

(µg/ml)	COX-2 % Inhibition	LOX-5 % Inhibition
Control	-----	----
Standard Control		
10	32.5 ± 1.3	44 ±0.765
25	79.2 ±2.4	72.6 ±2.65
50	90 ±5.9	94.5 ±1.98
Terpenoid extract		
10	23.6 ±0.98	27 ±0.432
25	45.2 ±1.13	39.5 ±1.54
50	60.7 ±3.67	61.4 ±3.76
75	74.5 ±2.89	71.2 ±5.12

Values expressed are mean± SD; n=6

Table 5. Estimation of myeloperoxidase and cellular nitrite levels in RAW 264.7 cell lines by standard and terpenoid extract.

(µg/ml)	Myeloperoxidase Enzyme (u/ml)	Cellular nitrite level
Control	-----	----
Standard Control		
10	0.048 ±0.002	322.3 ±10.2
25	0.034 ±0.001	219.7 ±21.7
50	0.01 ±0.001	142.2 ±10.8
Terpenoid extract		
10	0.059 ±0.005	410.5 ±16.3
25	0.045 ±0.002	300.1 ±9.78
50	0.030 ±0.002	199.4 ±15.3
75	0.016 ±0.003	133.7 ±2.76

Values expressed are mean± SD; n=6

Nitric oxide (NO) and Myeloperoxidase level

Inflammation is a reaction occurring in many types of tissue injuries, infections or immunological induction as a defense against foreign or altered endogenous molecules. The process of inflammation comprises a chain of reactions of the terminal tissues, which tend to eliminate the injurious and to repair the damaged region. NO is a short-lived free radical produced from L-arginine by nitric oxide synthase (NOS) that mediates diverse functions by activating on diverse cells through interactions with various molecular targets. Although NO acts as an essential multifunctional mediator in many biological systems, over synthesis of NO by inducible nitric oxide synthase (iNOS) is involved in different types of inflammation and multistage carcinogenesis at inflammatory sites (Ulrich et al., 2012). In the present study, a concentration dependent and gradual enhancement in the percentage inhibition was observed with terpenoid extract of the moss. The NO level was reduced to133.7 ±2.76 at 75 ìg/mL concentration. Indomethacin at a concentration range of 10-50 ìg/mL was also employed and served as reference standard. The lowest dose (10 ìg/mL) produced 322.3 ±10.2 nitric oxide levels and the highest concentration produced 142.2 ±10.8. Like terpenoid extract dose dependent increase in the percentage of inhibition was observed in all the concentrations tested. The results reveal that the terpenoid extract inhibits the production of NO as stimulated by LPS in mouse macrophage cell line RAW 264.7. The results obtained are presented in Table 5.

Myeloperoxidase level estimation showed that the inhibition occurs in a concentration dependent manner with the synthetic drug and terpenoid extract. Maximum inhibition was seen with 75 µg/ml terpenoid extract i.e., 0.016 ±0.003 activity.

Recently many biological studies on crude plant extracts revealed potent antioxidant and free radical scavenging activities as well as ideal anticancer activity (Mantovani et al., 2008). For example, many *Indigofera* species exhibited effective anti-inflammatory, analgesic and lipoxygenase inhibitory activities (Bhagavan et al., 2013). Reactive Oxygen or nitrogen Species (ROS/RNS) participate in normal cell function as well as in pathological disorders such as inflammation. Many studies have demonstrated the participation of ROS and RNS in connection with inflammation models.

Preliminary phytochemical and quantitative analysis of *Thuidium tamariscellum* indicated the presence of rich amount of terpenoid compounds. Anti-inflammatory and antinociceptive activities as well as antioxidant potential of many plants have been attributed to their polyphenols (Gonzalez et al., 2011; Sharififar et al., 2009) or to their high triterpene. Polyphenols are known as important secondary plant products in many medicinal plants. They have fascinated the attention in relation to their wide range of activities in the prevention of malignancy, inflammation and heart diseases (Garcia-Mediavilla et al., 2007). Research documents suggest that these molecules may inhibit diverse enzymes, which are activated during inflammatory conditions. Prostaglandins and NO production is involved in

inflammation. In addition, inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2) and lipooxygenase -5 are responsible for the synthesis of huge quantities of these pro-inflammatory mediators (Garcia-Mediavilla et al., 2007). Many results confirm that quercetin and kaempferol remarkably reduce the levels of iNOS, COX-2, lipoxygenase and reactive C-protein (RCP) in a dose dependent manner (Tunon et al., 2009). Similarly, flavonoids like kaempferol, quercetin and daidzein blocks the activation of the signal transducer and activator of transcription 1 (STAT-1), another unique transcription factor for iNOS. Luteolin 7-glucoside, kaempferol and quercetin effectively inhibit lipopolysaccharide (LPS)-induced PGE2 production. Many flavonoids significantly inhibit LPS-induced COX-2 expression, while mPGES-1 expression was down regulated by them (Hamalainen et al., 2011). Chu-Wen Li analyzed anti-Inflammatory property of the ethanol extract of the root and rhizome of *Pogostemon cablin*. Araújo et al., (2013) evaluated the cytotoxic and anti-inflammatory activities of extracts and lectins from *Moringa oleifera* Seeds. Senthil Kumar et al., (2016), studied the beneficial effects of methanolic extract of *Indigofera linnaei* on the inflammatory and nociceptive responses in rodent models . Castro et al., (2014) documented *in vivo* and *in vitro* anti-inflammatory activity of *Cryptostegia grandiflora*. Sekhar et al., (2013) analyzed *in vitro* antioxidant activity, lipoxygenase, cyclooxygenase-2 inhibition and DNA protection properties of *Memecylon* species. Bindu et al., (2016) screened *in vitro* anti-inflammatory screening of stem bark of *Cordia obliqua* in RAW 264.7 cell lines. Similarly, Jacob et al., (2015) studied the dual COX/LOX inhibition: screening and evaluation of effect of medicinal plants of Kerala as antiinflammatory agents. The observed beneficial effects of terpenoid extract of *Thuidium tamariscellum* on inflammatory responses in paw edema and macrophages substantiate their ethnic usage by the local people.

CONCLUSION

On the basis of the above results, it may be summarized that the terpenoid extract of *Thuidium tamariscellum* the bryophyta exhibited anti-inflammatory activity by COX/5-LOX, myeloperoxidase and nitric oxide inhibition. The results were substantiated by paw edema data. This shows the multiple mechanism of action of the phytochemical in anti-inflammatory activity. The comparable antioxidant activity expressed by the extract indicates that there is an antioxidant component, which is also explained by the fractionation of terpenoids by GC-MS analysis

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Angiosperm Diversity and Phytogeographical Affinities of Riparian Vegetation along Kanjirapuzha Basin, Kerala, India

KUNNAMKUMARATH JISHA, MUHAMMED ISMAIL MOOSA SHANID AND MAYA CHANDRASHEKARAN NAIR

Post Graduate and Research Department of Botany,
Govt. Victoria College, Palakkad, Kerala, India- 678001
email: balu jisha@gmail.com

ABSTRACT

The present investigation focuses on the Angiosperm diversity along the Kanjirapuzha River, which is one of the major tributary of river Thutha, which in turn drains into Bharathapuzha, the second longest river of Kerala state. Kanjirapuzha basin is located between 10°57' and 11°00' North latitude and 76°26' and 76°32' East longitude, at northern lip of Palghat gap of southern Western Ghats, and it covers a total length of 32km. The present paper summarizes the angiosperm diversity along the riparian system of Kanjirapuzha along with phytogeographical affinities. Documentation of riparian vegetation along Kanjirapuzha basin recorded 196 species of flowering plants under 62 families. Among these, majority of plants belongs to the family Fabaceae (14 members) followed by Euphorbiaceae (13), Asteraceae (13), Poaceae (12) and Cyperaceae (11). The vegetation mainly consists of herbaceous members (127), followed by shrubs (37) and trees (30). From the data it was found that, there is a gradual reduction in the number of native and strict riparian elements and a massive inhabitation of invasive elements. In the riparian vegetation of Kanjirapuzha, number of strict riparian elements is below 10. Eight taxa recorded from the Kanjirapuzha basin are endemic to Western Ghats and at the same time, broad scale phytogeographical affinities of the reverine flora extend to African, Australian, Holarctic, Indo-Pacific and South American floristic kingdoms. Inter-specific diversity is much evident in taxa like *Fimbristylis*, *Ficus*, *Ipomoea* and *Lindernia* and two members showed intraspecific diversity viz. *Lindernia rotundifolia* and *Lindernia crustacea*. This study also summarizes the major threats encountered in the vegetation as grazing, construction of dams and check dams, presence of invasive species, timber harvesting, destruction of riparian elements, sand mining and tourism and land encroachment for agriculture.

Key words *Riparian vegetation, Kanjirapuzha , Regulated river, Southern Western Ghats, Palghat Gap, Thutha river*

Rivers represent the most rapidly replenishing and most continuing geomorphic features on the surface of the planet. Riparian zones are the interface between aquatic and terrestrial ecosystems. As ecotones, they encompass sharp gradients of environmental factors, ecological processes and plant communities (Gregory *et al*., 1991). Depending on the characteristics of the watercourse, riparian zones range in size from narrow strips to extensive areas associated with large, winding rivers or floodplains. Riparian landscapes are highly threatened ecosystems as they are inherently rare habitats, occupying a mere one-thousandth of the earth's surface (Hynes, 1970).

Rivers have been an integral part of human development throughout history and they have been considered as the pillars of human civilization as they formed the nuclei for human settlements from the very origin of mankind (Sunil *et al*., 2010). The unique characteristic features of rivers and riparian ecosystem along with their interaction with immediate environment have gained more focus in the past two decades (Naiman and Decamps 1993, 1997). There are few studies in the Indian region dealing with riparian vegetation analysis (Singh and Joshua, 1989; Bachan, 2003; Sreedharan, 2005; Sunil *et al.*, 2010; Nandhakumar *et al*., 2012; Vyas *et al*., 2012; Joby Paul, 2013; Vincy *et al.,* 2014; Bhaskar and Karthik 2015; Sankhwal *et al*., 2015; Shah *et al*., 2015) which reports that riparian fragments of tropical regions are composed of evergreen, semi evergreen and deciduous matrix due to the edaphic and climatic specificities. Its ecological uniqueness depends on multiple factors viz., its origin, geological past, climate, impact of monsoon and anthropological activities.

The Indian sub-continent is traversed by a large number of rivers, which have played a pivotal role in shaping the history of human civilizations. At the very same time, they have been greatly exploited for meeting the increasing demand of the human and are therefore, now considered to be the most threatened ecosystem (Malmqvist and Rundle, 2002). Healthy riparian forests are essential to the maintenance of water quality and biological integrity in surface water

Table 1. Study sites of sample plots

Sl. No	Location	Latitude	Longitude
1.	Varmankode	11°00'00"N	76°32'32"E
2.	Kanjirapuzha Dam	10°59' 22"N	76°32' 5"E
3.	Unnammedu	10°59'09"N	76°32'06"E
4.	Thodamkulam	10°58'57"N	76°30'53"E
5.	Pullatta	10°58'39"N	76°30'45"E
6.	Chooriyodu	10°58'27"N	76°29'54"E
7.	Kilirani	10°57'06"N	76°28'49"E
8.	Mothikkal	10°57'03"N	76°26'49"E

systems, and their destruction often leads to subsequent degradation of adjoining aquatic ecosystems (Naiman and Decamps, 1997). In this context, the present study has been conducted to assess the flowering plant diversity in the riparian zones of Kanjirapuzha in the northern lip of Palghat gap. This is the first attempt to discern the river bank vegetation of this riparian system.

MATERIALS AND METHODS

Study Area

Kanjirapuzha is the major rivulet of Thutha river, which is the main tributary of Bharathapuzha and the present investigation focuses on the angiosperm diversity and phytogeographical affinities of the riparian systems of Kanjirapuzha. The Kanjirapuzha basin is located between 10°57' and 11°00' North latitude and 76°26' and 76°32' East longitude. The river draining has a length of 32km with an area of 117km^2. A masonry earth dam, Kanjirapuzha dam is located in this river and it has a catchment area of 7,000 hectares, which lies entirely within the state boundary. It is built for providing irrigation to a Cultural Command Area (CCA) of 9,713 hectares of Palakkad district, Kerala. The study area experiences a humid tropical climate and an average rainfall of 2135 mm. The temperature of this area ranges from 20^0 C to 38^0 C.

Methodology

The present study was carried out between February 2017 to July 2017. For effective vegetation analysis, eight different sample plots were selected on a 32km stretch of Kanjirapuzha basin (Table 1). These sample plots were selected based on various factors such as proximity to the river, accessibility, vegetation density, disturbance gradients, etc. All species occurring in the study area were identified with the help of regional floras, including Flora of Palghat (Vajravelu, 1990), Flora of Presidency of Madras (Gamble and Fischer, 1915-1936) along with reference to local herbaria as MH and CALI. Collected taxa were classified on the basis of APG IV (Chase *et al.,* 2016). The phytogeographical affinities (Cox, 2001) and biological life form spectrum (Raunkier, 1934) of vegetation were analysed. Nomenclature of the plants was made up to date with reference to IPNI, the plant list (www.theplantlist.org) and Flowering Plants of Kerala (Sasidharan, 2002). The elements were photographed using digital camera of Nikon D810.

RESULTS AND DISCUSSION

Floristic analysis

Analysis of the riparian flora of Kanjirapuzha basin revealed the presence of 196 angiosperms belonging to 153 genera under 62 families. Of the taxa recorded, 158 belong to dicotyledonae and 38 were monocots. The present study recorded the dominance of Fabaceae (14 members) followed by Euphorbiaceae (13), Asteraceae (13), Poaceae (12), and Cyperaceae (11). The vegetation mainly consists of herbaceous members (112), followed by trees (31), shrubs (29) and climbers (24) (Fig. 1).

In this study, it was found that the numbers of endemic taxa are comparatively less and those present are distributed all along the riparian zone of Kanjirapuzha. *Dioscorea alata, Naregamia alata, Mussaenda frondosa, Ochreinauclea missionis, Heliotropium rottleri, Ipomoea marginata, Lindernia tamilnadensis* and *Ruellia prostrata* are

Table 2. Alien and invasive species recorded from Kanjirapuzha basin

Sl. No	Name of species	Family
1	*Ageratum conyzoides* L.	Asteraceae
2	*Alternanthera tenella* Colla, Mem.	Amaranthaceae
3	*Antigonon leptopus* Hook. & Arn.	Polygonaceae
4	*Calotropis gigantea* (L.) R.	Apocynaceae
5	*Catharanthus pusillus* (Murr.) G.	Apocynaceae
6	*Chromolaena odorata* (L.) King & Robins.	Asteraceae
7	*Cleome rutidosperma* Wight & Arn.	Cleomaceae
8	*Cleome viscosa* L.	Cleomaceae
9	*Cuscuta chinensis* Lam.	Convolvulaceae
10	*Eclipta prostrata* (L.) L.	Asteraceae
11	*Evolvulus nummularius* (L.) L.	Convolvulaceae
12	*Grangea maderaspatana* (L.) Poir.	Asteraceae
13	*Hyptis suaveolens* (L.) Poit.	Lamiaceae
14	*Ipomoea carnea* Jack.	Convolvulaceae
15	*Ipomoea obscura* (L.) Ker-Gawl.	Convolvulaceae
16	*Ipomoea pes-tigridis* L.	Convolvulaceae
17	*Lantana camara* L.	Verbanaceae
18	*Melochia corchorifolia* L.	Malvaceae
19	*Mikania micrantha* Kunth.	Asteraceae
20	*Mimosa pudica* L.	Fabaceae
21	*Monochoria vaginalis* (Burm. f.) Presl.	Pontederiaceae
22	*Passiflora foetida* L.	Passifloraceae
23	*Peperomia pellucida* (L.) Kunth	Piperaceae
24	*Physalis angulata* L.	Solanaceae
25	*Saccharum spontaneum* L.	Poaceae
26	*Scoparia dulcis* L.	Plantaginaceae
27	*Senna alata* (L.) Roxb.	Fabaceae
28	*Senna tora* (L.) Roxb.	Fabaceae
29	*Sida acuta* Burm.	Malvaceae
30	*Synedrella nodiflora* (L.) Gaertn.	Asteraceae
31	*Tridax procumbens* L.	Asteraceae
32	*Urena lobata* L.	Malvaceae

Table 3. Phytogeographic affinities of the plants of Kanjirapuzha

Distributional range	Number of species	%
Endemic	8	4.08
Indo-Pacific	67	34.18
Indo-Pacific and South American	24	12.24
Indo-Pacific, African and South American	25	12.75
Indo-Pacific and African	34	17.34
Indo-Pacific and Australian	14	7.14
Indo-Pacific and Holarctic	6	3.06
African	1	0.51
Indo-Pacific, South American, Australian and African	14	7.14
Indo-Pacific, African and Australian	3	1.53

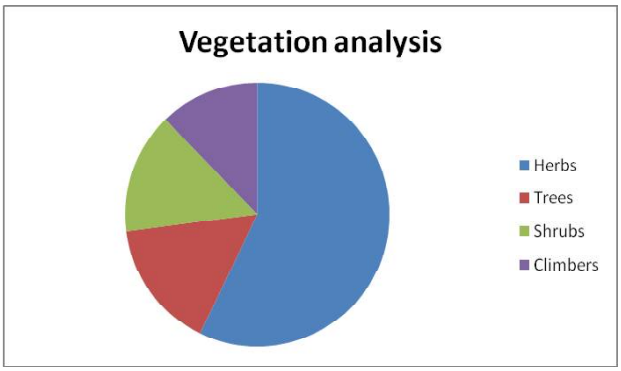

Fig. 1. Vegetation analysis of the study area

the endemic taxa found along the riparian zones of Kanjirapuzha. The percentage of occurrence of RET taxa is very less and is represented by *Cayratia pedata* and *Ochreinauclea missionis*.

According to *bsienvis.nic.in* (2017), the analysis of the floristic elements of Kanjirapuzha showed that 32 plant species of the riparian elements of Kanjirapuzha basin are invasive (Table 2). World's worst invasive alien species like, *Lantana camara, Ageratum conyzoides, Mikania micrantha, Sida acuta, Synedrella nudiflora* and *Chromolaena odorata* are widely distributed in this region. The human activities and other disturbances cause the disappearance of native species and resulted in the dominance of invasive elements. From the data it can be inferred that most of the invasive elements belong to the family, Asteraceae followed by Convolvulaceae.

Plant wealth of Kanjirapuzha showed phytogeoraphical affinities with African, Australian, Holarctic, Indo-Pacific and South American floristic kingdoms (Table 3). Majority of the vegetation include Indo-Pacific elements.

Raunkiaer's life form spectra analysis of the vegetation (Fig. 2) revealed that the biological spectra consist of 8 Chamaeophytes, 3 Cryptophytes, 82 Phanerophytes and 103 Therophytes. The therophytes (52.55%) dominated in the biological spectrum of riparian vegetation followed by phanerophytes (41.83%), chamaeophytes (4.08%) and cryptophytes (1.53%).

Threats to riparian vegetation

The riverine vegetation along Kanjirapuzha basin encounters many threats and it eventually affects the diversity and species richness. One of the major threats found in the riparian zone of Kanjirapuzha basin is the dam construction and flow regulation. The changes in flow regime can have a substantial effect on both riparian plants and aquatic biota and this will lead to the decline in riparian vegetation. Another major cause of degradation of riparian vegetation is the introduction of exotic and invasive species. These invasive species reduce the native riparian vegetation and reduce the species diversity. Other major threats to the riparian ecosystem of Kanjirapuzha basin are grazing, land use for agriculture, timber harvesting, cutting of riparian elements and pollution by solid waste disposal.

CONCLUSION

The present floristic treatment restricted to watershed of the river Kanjirapuzha revealed the presence of 196 angiosperms belonging to 62 families. Earlier floristic studies conducted in many parts of Western Ghats recorded the dominance of Poaceae members, but the present study showed the

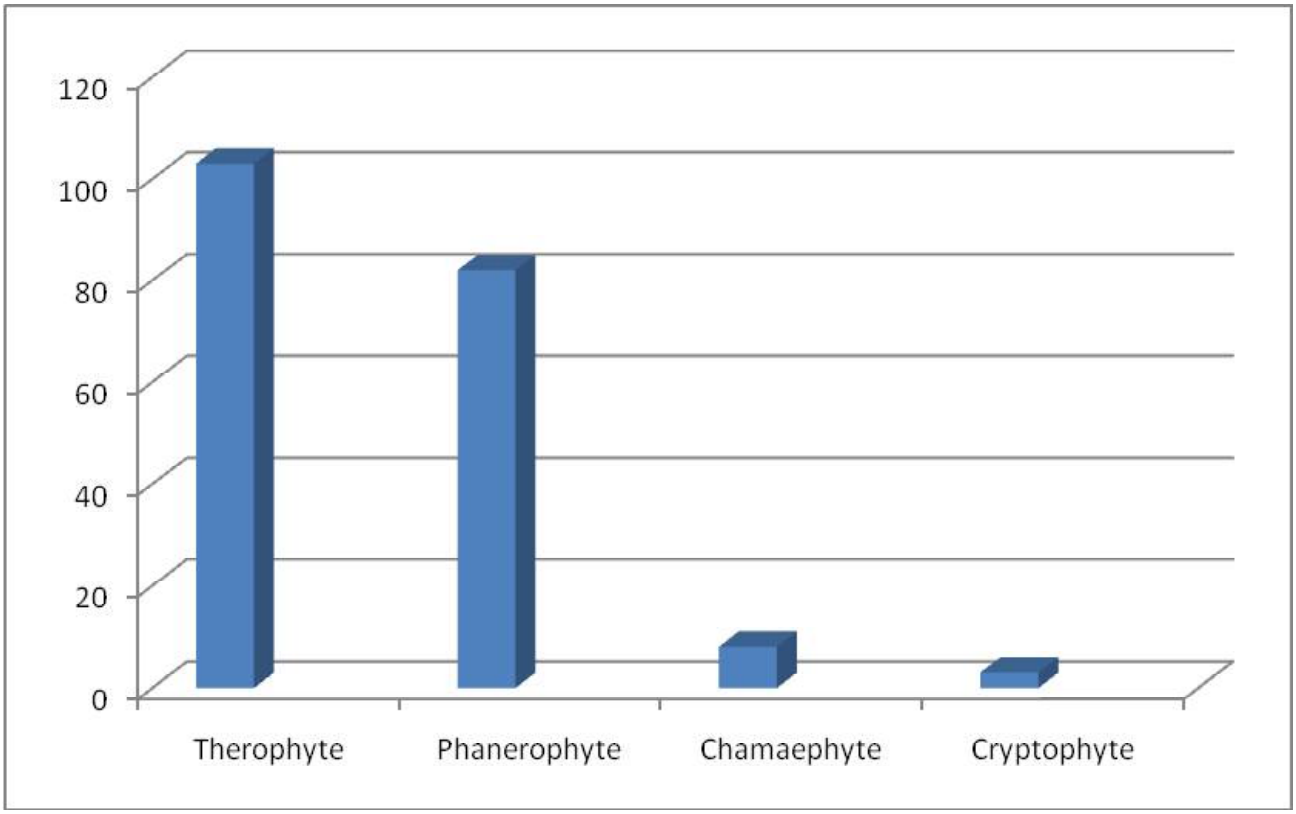

Fig. 2. Biological spectra of riparian vegetation along Kanjirapuzha basin

dominance of Fabaceae members and Poaceae has been replaced back in the third position, due to frequent floodplain disturbances.

The regulation of river flow directly impacts floodplain wetlands. The construction of a dam or check dams permanently submerges part of the upstream floodplain, whereas the reduction or absence of downstream flow disrupts the flood cycle, as variable amounts of water may be released at different times and for different periods than in the natural cycle (Chauhan and Gopal, 2005). As Kanjirapuzha is a regulated river with control in water inflow from the reservoir, the vegetation along the riparian bed experiences differential water inflow in different seasons. Hence, much of the herbaceous vegetation faces the flood plain effect in most of the seasons and this in turn affects the frequency of occurrence of these taxa.

The analysis of angiosperm diversity along Kanjirapuzha basin for a short period revealed that intense anthropogenic activities around the basin have made a negative impact on the native riparian vegetation. Most of the natural riparian vegetations are replaced by alien invasive species and are extremely fragmented also. Further documentation and long term observations are essential to formulate effective management strategies for the preservation of this riverine ecosystem.

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Non Toxic Biopolymeric Network for Future Bone Tissue Engineering

JISSY JOSEPH[1, 2], ARUN A RAUF[1], BOBY T EDWIN[2]

[1]*Department of Biochemistry, University of Kerala, Kariavattom Campus, Trivandrum, Kerala.*
[2]*Department of Botany, T.K.M College of Arts and Science, Kollam, Kerala.*
**email : bobytedwin2003@gmail.com*

ABSTRACT

Scaffolds are highly porous 3-D structures made up of biopolymers used as support in tissue engineering. They are cell friendly, biodegradable and contain functional molecules for the attachment of cell, proliferation and differentiation. In the present study the scaffolds was fabricated by freeze – drying technique. The cross linked scaffolds was subjected to further studies such as physical and biological characterization. Morphological characterisation of the scaffold was done by SEM. Swelling ratio and degradation properties of the scaffolds were also examined. The scaffolds found to be non-toxic and cytofriendly in L929 culture studies.

Keywords *tissue engineering; freeze – dryer; SEM; swelling ratio; degradation rate*

In recent years, Tissue Engineering has become an excellent alternative in organ transplant, to replace diseased or damaged organs. Tissue engineering is a multidisciplinary field, which allows construction of 3D structures to restore or maintain the lost function of a tissue or organ. Tissue engineering use scaffolds, cells, growth factors in ambient physical conditions for constructions a transplant. Three dimensional structures called scaffold promotes the regeneration of the tissue, in order to reconstruct, replace or repair living tissues and organs (1). The scaffold provides the initial structural integrity, cell proliferation and controls the shape of the functioning of the formed tissue.

In tissue engineering, biopolymers have a pivotal role in fabricating porous, biodegradable scaffolds. It supplies the necessary support for cell attachment, proliferation and differentiation for correct tissue regeneration. Parallel to the formation of the new tissue, the scaffold must undergo a biocompatible degradation. In the present study, an effort was done to find suitable biomaterial for scaffolds in tissue engineering application. In this study two different biopolymers, gelatin and pectin were used as they are naturally derived ones. Gelatin is a derivative of collagen which is the major constituent of skin, bones and connective tissue (2, 3). It is one of the most convenient proteins to use as it is much cheaper than collagen and does not exhibit antigenicity (4). Pectin is a naturally occurring biopolymer found in the cell walls of plants. Pectin is a water-soluble carbohydrate substance (5) .The advantages of these biopolymers are in terms of material reproducibility, good process ability and are easily available. These two polymers have attracted increasing attention in pharmaceutical and medical fields. The aim of this research work is study the physical and biological properties of scaffolds including morphology, swelling ratio, degradation rate, cell attachment and cell proliferation.

MATERIALS AND METHODS

Materials

The materials were purchased commercially. Gelatin (Fisher Scientific) and Pectin (Sisco Research Laboratories) were used; analytical grade chemicals were used in this work.

Preparation of the scaffolds

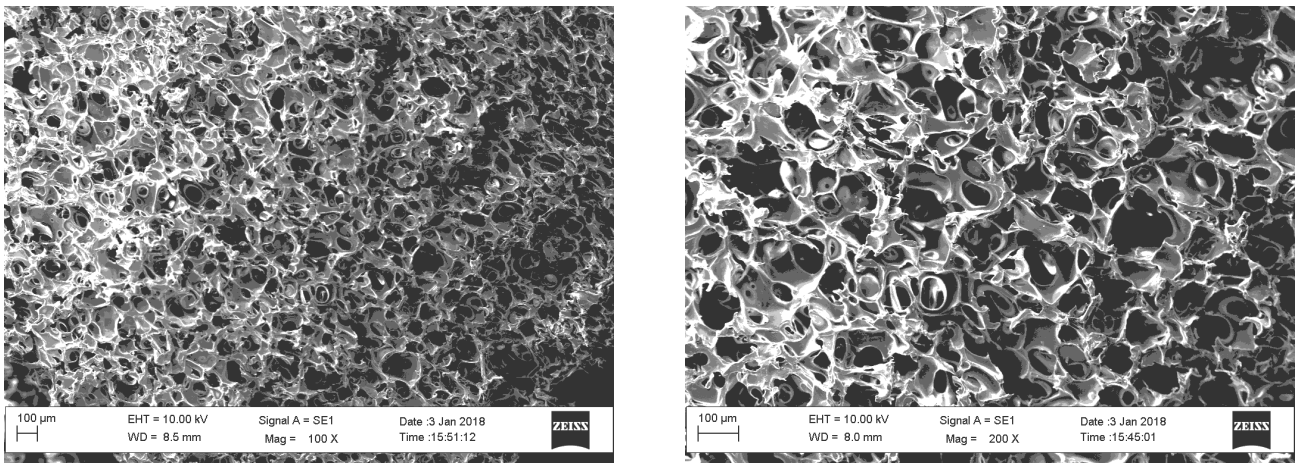
Gelatin and Pectin were dissolved at 37ºc under continuous agitation to obtain homogeneous solution. The solutions were then poured into mould and frozen overnight prior to lyophilisation. The resulting freeze dried scaffolds were cross linked and subjected to following studies.

Morphological observation

The morphology the scaffolds inner structure was investigated by scanning electron microscopy (Hitachi S-2400, Japan) at 20kV. The cross sections of the scaffolds were coated with gold prior to SEM observation.

Swelling property

The water up taking capacities of the scaffolds were determined by using phosphate buffered saline (PBS). Known weights of the scaffolds were placed in the PBS (pH 7.4) solution. The wet weights of the scaffolds were taken by blotting the scaffolds to



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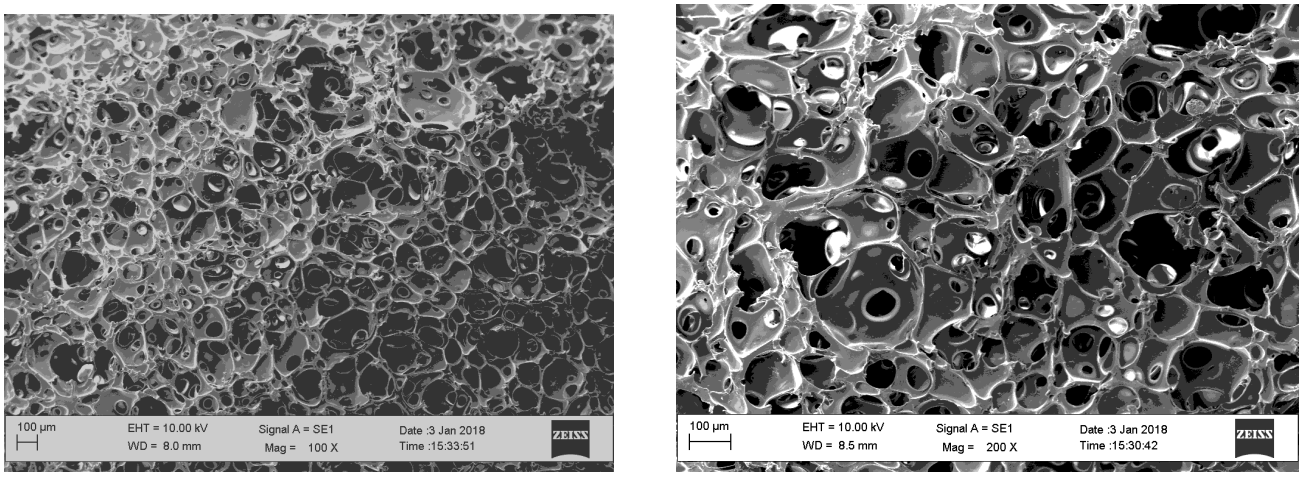

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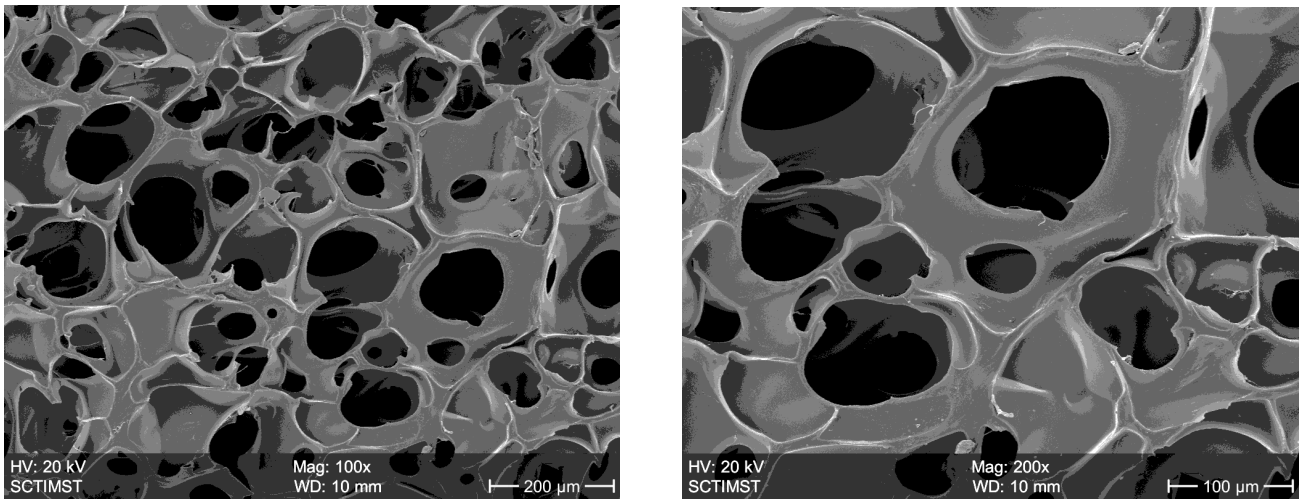

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Fig.1. Showing the SEM images of cross section of fabricated scaffolds at various magnifications (100x, 200x) pectin (a) gelatin (b), gelatin-pectin (c).

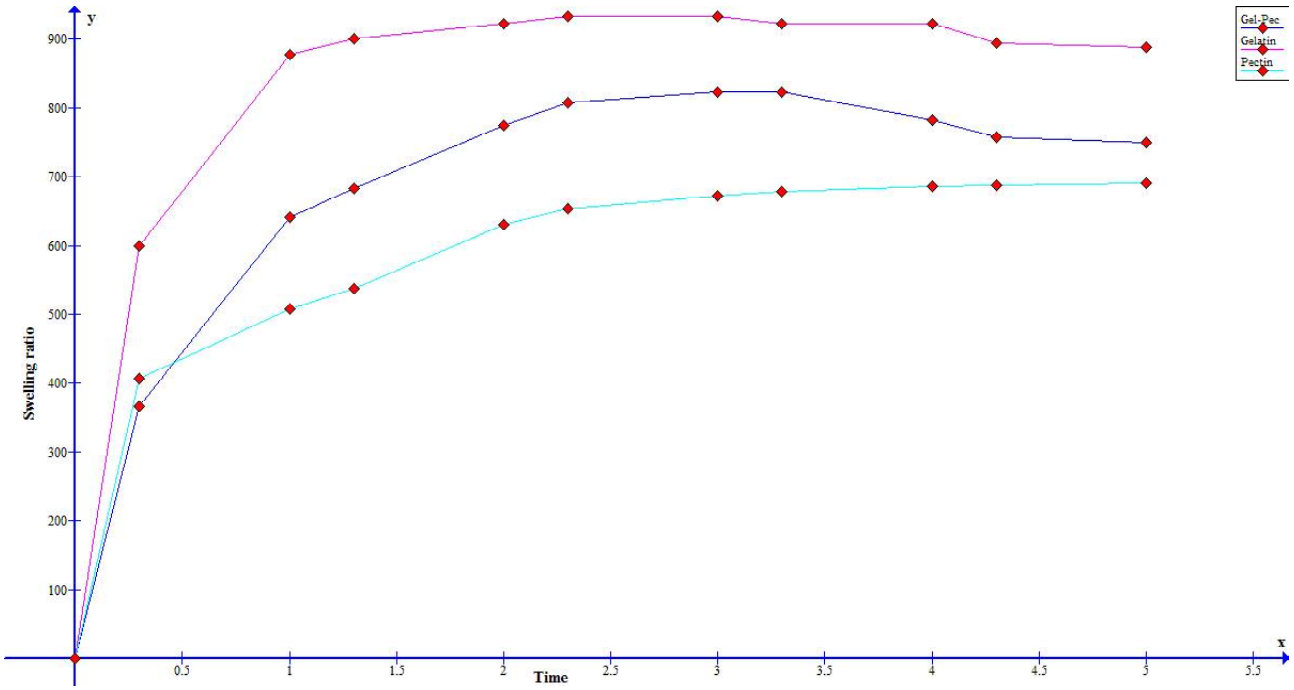

Fig.2. The graph showing swelling ratio of various scaffolds at different time interval.

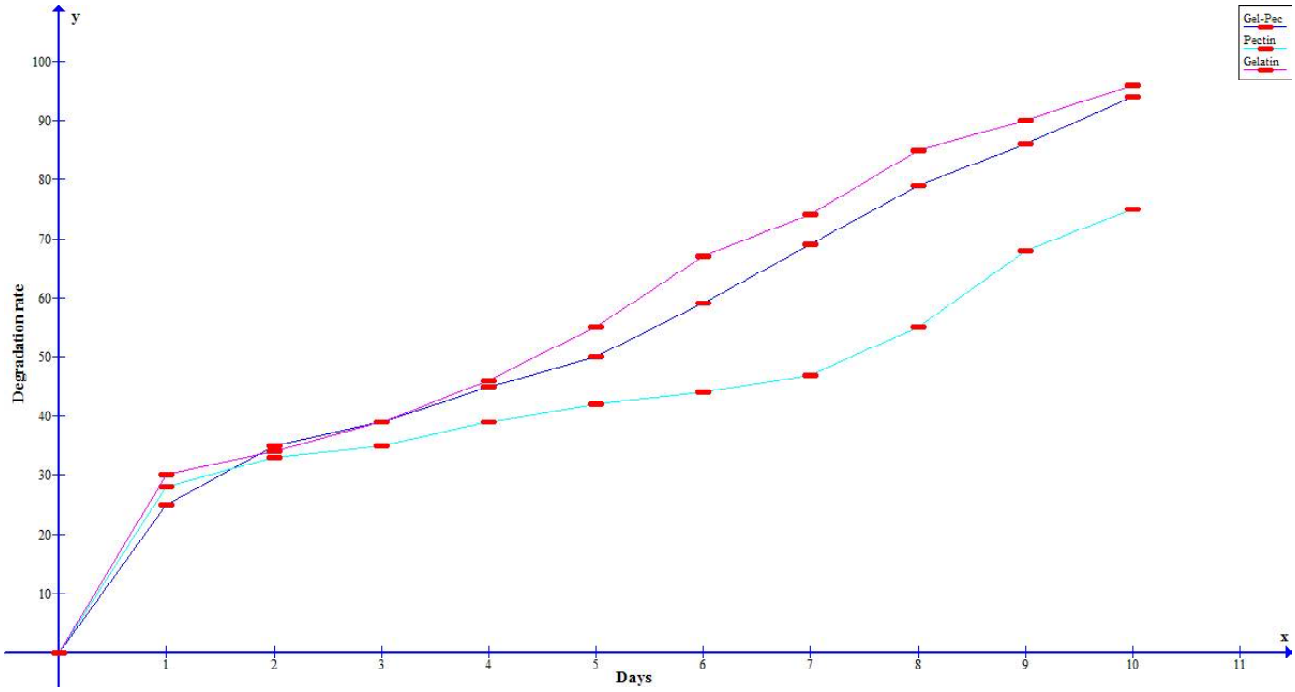

Fig.3. Degradation rate of various scaffolds at different days.

remove excess water and then weighed immediately. Then the swelling ratios of the scaffolds were calculated.

In vitro biodegradation rate

The biodegradation of scaffolds was investigated using stimulated body fluid solution (SBF, pH 7.4). The samples of known weight were immersed in SBF solution. Every other day, samples were taken from the medium and weighed. The experiment was done triplicates for each different scaffold. The extent of in vitro degradation was expressed as a percentage.

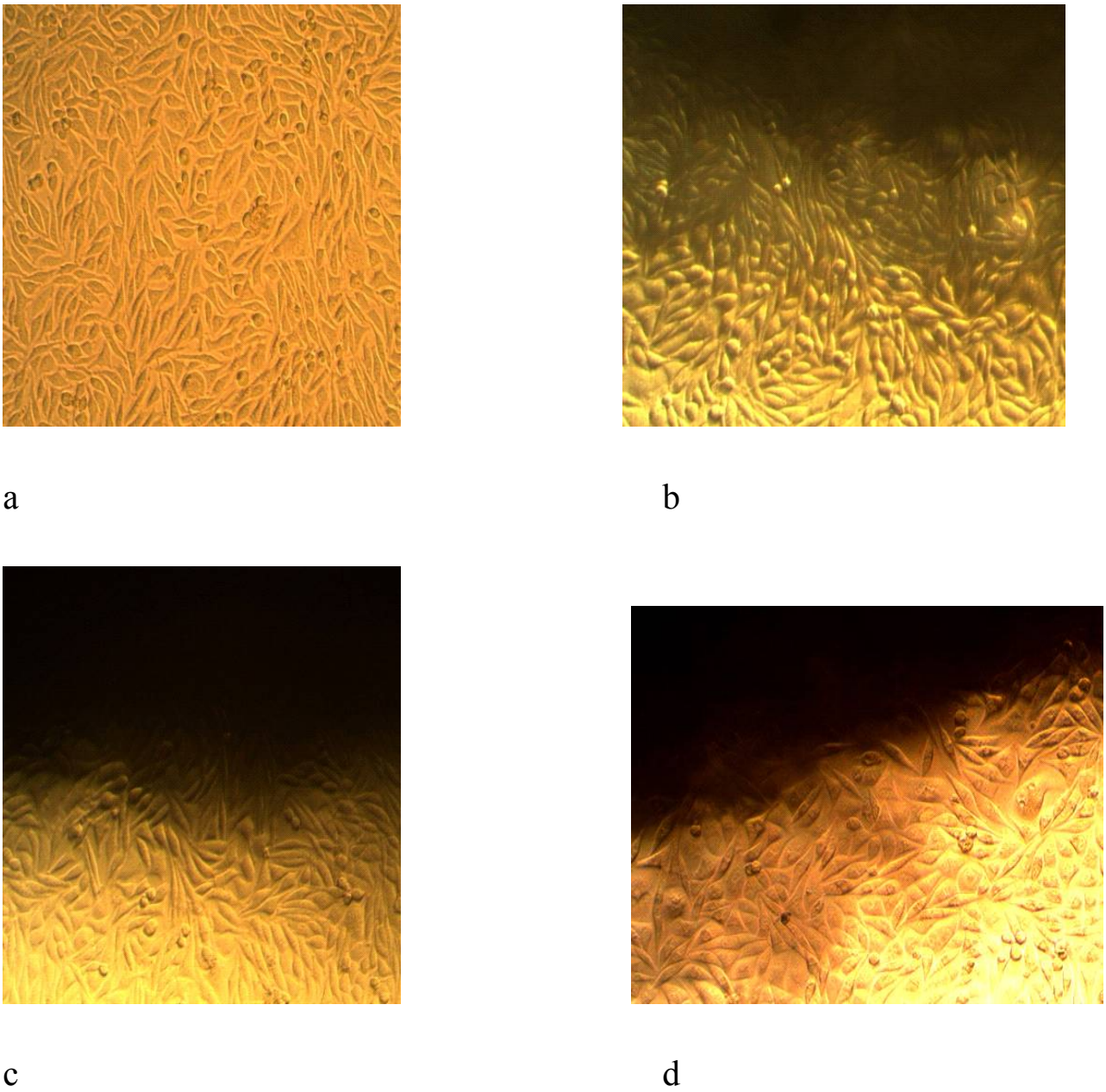

Fig.5. Viability of L929 cell adhesion on different scaffolds. Phase contrast microscopic image of various scaffolds control (a) gel-pec (b), gelatin (c) pectin (d)

In vitro cytotoxicity test

Cytotoxicity of scaffolds was evaluated using fibroblast L929 cells. For cell attachment, cells were seeded onto the scaffolds in tissue culture plates containing DMEM and are observed by phase contrast inverted light microscopy. For cell proliferation, after the culture, scaffolds were rinsed with PBS and 350 µl of MTT (0.5 mg/ml) was added and incubated at 37°C in 5% CO2 incubator .DMSO was added to elute the fromazan crystals of MTT and the absorbance of the solution was measured at 570 nm using a spectrophotometer. The extent of in vitro cell viability was expressed as a percentage of cell adhesion and proliferation. All experiments were run in duplicate.

RESULTS AND DISCUSSION

Morphological Characterisation of the scaffolds

The microstructure of the gelatin and gelatin–pectin scaffold is shown in Figure 1. The SEM images shows that scaffold are highly porous and have smooth surface structure .The pore size found in the range of 100- 400 µm and in case of pectin and 100-700 µm

gelatin and 150- 500µm in the case of gelatin – pectin scaffolds. The pores are uniformly distributed in the solid matrix and are vary in size. The difference in pore size is due to incorporation of cross linker which imparts good interaction with the polymers. The interconnected pores help in migration, proliferation and differentiation of cells and provide nutrient supply (6). The difference in structure of the scaffolds might be due to the nature of material. In general, porosity and pore size were important elements of biological activity of porous scaffold biomaterials.

Swelling Property

The swelling ratio of various scaffolds was shown in Fig.2. Among the different samples gelatin - pectin scaffold showed minimum swelling ratio. This is attributed due to good interaction between polymer and incorporated organic fillers. The highest rate of hydrophilicity was observed among gelatin alone scaffolds. Gelatin is widely known for its hydrophilicity, which absorb water up to 10 times of its dry weight. The rate of water uptake was found to increase in time and reaches in equilibrium state. The maximum water uptake corresponding to the maximum hydration ability of the scaffold. The swelling ability helps in the uptake of nutrients to the interior of porous scaffold during in vitro culture. During in vitro culture, the increase in diameter of pore size facilitate the cells not only to just attach but also to migrate inside the scaffolds and grow in a three dimensional fashion (4).

Degradation of the scaffold

The *invitro* degradation rate of scaffold was shown in Fig .3. The rate of degradation was slightly less in gelatin - pectin compared to gelatin scaffold. This is due to better interaction between polymers and cross linker. As the time increase the degradation rates of scaffold found to increase. The aim of tissue engineering is the regeneration of new tissues with controlled rate of degradation of biomaterials. Among different porous scaffold gelatin -pectin scaffold showed minimum and controlled degradation rate. Physiological environment plays an important role in degradation behaviour of biomaterials. During the degradation the surface of biomaterial is renewed, which provides a better environment for the growth of cells. Degradation rate may affect many cellular processes including cell attachment, cell proliferation, tissue regeneration, and host response (7).

Cell viability

Figure 4 revealed the number of fibroblast cells proliferation on each scaffolds at 48hr after the culture. It should be pointed out that there was no significant difference in initial cell proliferation on gelatin – pectin, gelatin and pectin scaffolds comparing to control. The images showed the number of fibroblast cells proliferated on each scaffolds after 48 hr of the culture, respectively. The results showed that the morphology of cells in contact with all scaffolds were not significantly different. Furthermore, it could be concluded that gelatin scaffolds could induce cell proliferation as good as the gelatin -pectin scaffold. Reduction of MTT reagent is determined by mitochondrial redox activity. MTT reagent is a pale yellow substance, which is reduced to form a dark blue formazan product when incubating with viable cells by mitochondrial succinate dehydrogenase. Therefore, the productions of formazan can reflect the level of cell viability on the material (7). Figure 4 shows the spectroscopic absorbance of formazan produced at 520nm by viable cells after 2 days culture. The results indicate that a slight variation in proliferation of cells found on gel-pec scaffold as compared to gelatin and pectin scaffold. This may be due to abundance of amino acids, such as arginine, glycine, aspartic acid, which positively effects cell adhesion, viability and growth. The result, there was no significant reduction in cell viability observed in tests comparing to the control.

CONCLUSION

In the present experiment, the intercalated scaffold is developed as a biomaterial for tissue engineering application. Data reveal that the scaffolds were successfully fabricated using lyophilisation/freeze drying technique. Four different characteristic features have significant influence on the composite scaffolds; they are pore size, hydrophilicity, degradation rate and cytotoxicity. The results proved that these scaffolds possessed admirable properties, especially the cell viability. The disadvantage of gelatin scaffolds is the rapid degradation rate. Therefore, the gelatin scaffolds should be further modified by blending with other materials to improve their degradation rate. The composite scaffold had a pore size in the range of 100-500µm respectively. The scaffolds are highly porous with interconnected pores, which helps diffusion

of nutrients and cell proliferation, it also demonstrated controlled rate of swelling and degradation and high cell viability. All these results suggest that novel scaffold will be ideal polymeric matrix for the healing of bone, to deliver bone growth factors and tissue regeneration.

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Assessment of Lead Contamination in Common Tubers collected from Local Markets of Thrissur District, Kerala, India

M.V. LAKSHMI[1] AND NEETHU GOPALAKRISHNAN[2]

St. Joseph's College (Autonomous), Irinjalakuda, Thrissur
**email : lakshmibotania@gmail.com*

ABSTRACT

The present study was carried out in eight different local markets of Thrissur District (Thrissur, Cherpu, Kunnamkulam,Irinjalakuda, Chalakudy, Valapad, Kodungallur, Guruvayur) to check the concentration of Lead in tubers (Potato, Tapioca and Yam). The tubers were also collected from farmers and Home gardens. All the collected samples were shade dried, powdered and digested. The concentration of Lead was found out using AAS (Atomic Absorption Spectrophotometer). Lead is a highly toxic metal which causes serious threats on renal, reproductive and nervous system. Exposure to Lead may lead to severe diseases in humans and animals. It accumulates in environment with increasing hazards due to its non-biodegradable nature. Tapioca had significant concentration of Lead (15 ppm) than other samples and the content in samples were exceeded the permissible limits of WHO and therefore it may cause deleterious effects to the human health. Lowest Lead concentration was found in Yam (1.9 ppm). Mean± SD concentration of each heavy metal was calculated and data were given in ppm for each sample. The tubers show highest metal contamination because of the area that may polluted with various industrial activities. Highest level of Lead was reported in Thrissur market. The study concluded that the atmospheric depositions and marketing systems of vegetables plays a major role in increasing the levels of heavy metal content. So the consumers are at greater risk of using these vegetables with increased levels of heavy metals beyond permissible limits as defined by WHO.

Keywords *Heavy metals, Atomic Absorption Spectrophotometer, Lead, WHO.*

Heavy metals are very harmful because of their non-biodegradable nature and their potential to accumulate in different body parts. Excessive accumulation of heavy metals can develop systematic health problems in human body. Accumulation of heavy metals in agricultural soil result in increased metal uptake by crops, which affects food quality and safety. Heavy metal contamination of the food items is one of the most important aspects of food quality assurance (Marshall, 2004; Radwan and Salama, 2006). The analysis of heavy metal contamination contributes to the understanding of the potential health risk in environmental studies.

Tubers play a significant role in the human diet and they are the global sources of carbohydrates. Tubers are richest sources of a number of compounds such as phenolic compounds, saponins, phytic acids, glycoalkaloids and carotenoids. Potatoes and yams are tubers whereas cassava and sweet potatoes are storage roots. A number of species and varieties are consumed but cassava, potatoes and sweet potatoes consist of 90% global production of root and tuber crops (Chandrasekara and Josheph Kumar, 2016).

The present study shows the information on the concentration of Lead in some selected tubers sold in different markets of Thrissur district. It is hypothesized that the use of untreated waste water may increase the levels of heavy metals in the vegetables. The observed concentration of Lead was compared with WHO standards. Therefore this work was conducted to reveal if the content of heavy metals in vegetables is within the permissible limit or not. The main purposes of the present study were to investigate the concentration of Lead in the selected edible tubers and compare the concentration of Lead in organic and inorganic vegetables.

MATERIALS AND METHODS

The present study was carried out in eight different markets of Thrissur district during the period, April to June 2017. Tubers like Potato, Tapioca, and Yam have been selected for the present study.

Study area and sampling locations

Thrissur district is located in the central part of Kerala; it is an important historical city known as the cultural capital of Kerala. There were a number of markets located in this district. Our study was carried out in the selected markets of Thrissur district. The major ones are as follows: ThrissurSakthanThampuran market, Cherpu market, Valapad market, Irinjalakuda market, Chalakudy market, Kodungallur and Kunnamkulam market. The organic vegetables are collected from Velangalloor and Thrissur.All these tubers were brought to the laboratory for further analysis.

Fig. 1. Thrissur Map

Preparation and treatment of samples: In the laboratory, the collected samples were washed with tap water, and with distilled water, cut into small piecesand then dried in an oven at 80º (Larry and Morgan, 1986). At the end of the drying, the oven was turned off and left overnight to enable the sample cool to room temperature. Then each sample was grounded into fine powder using a commercial blender, sieved and finally stored in an aluminium foil, until used for acid digestion.

Digestion procedure: Tri-acid mixture (Nitric acid 69% , Perchloric acid 70% , Sulphuric acid 98% in the ratio 5:1:1) was added to a beaker containing 1g dry sample. The mixture was then digested at 80° till the transparent solution was achieved. After cooling, the digested samples were filtered using Whatman no.42 filter paper and filtrate was diluted to 50 ml with deionised water. This was then transferred into a screw capped glass bottle.

Instrumental analysis: Determination of Lead content in filtrate of the samples was achieved by Atomic Absorption Spectrometer (AAS).

Statistical analysis: Statistical analyses were carried out using SPSS software version 20. The Mean and standard deviation of each heavy metal were calculated and the data were given in ppm for each sample. ANOVA and t-tests were used for comparing the mean concentration between the vegetables. The criterion for significance in the procedures was set at $p < 0.05$ (significant).

RESULTS AND DISCUSSION

The concentration of Lead was found higher in Tapioca than other samples as given in table 1.

The level of lead in tubers is in the range -1.9 to 15 ppm. Tapioca shows highest concentration (upto 15ppm) and lowest concentration was found in Yam (-1,9 ppm). High level of lead content was obtained in the vegetables collected from Thrissur andCherpu market. Lowest values are obtained in the tuberscultivated in home garden.

The roots can absorb heavy metals easily and less transported to the other edible parts such as leaves and shoots of many plants (Wild, 1996). As tubers are grown under the soil they are more contaminated that the other vegetables. They can absorb heavy metals more readily than the other plants (Divya*et al.,*2015). Proper washing of vegetables is necessary to reduce a small amount of heavy metals. The required standard conditions of WHO have been given in Table 2. It

Table1. Bioaccumulation of Lead in selected vegetables of different markets

Sl.No.	Selected vegetables	Plant part	TCR(Pb)	CRP	KNK	IJK	CKL	Organic	Home	Average	S.D
1	Potato	Tuber	5.7	8.35	4.65	8.7	7.3	2.5	2	5.6	2.494136
2	Yam	Tuber	10.8	10.25	5.85	6.85	-1.9	8.25	-3.1	5.285714	5.190081
3	Tapioca	Tuber	15	6.55	12.55	6	2.4	0.65	5.95	7.014286	4.75978

was clearly seen that almost Lead was found in greater amounts than the maximum safe limits given by FAO/ WHO. The chief source of toxic metals is the discharge from industries and domestic waste into sewage. Generally, agricultural soils have low background levels of heavy metals; contaminations are mainly through fertilizer application, irrigation with partially treated or untreated sewage (Farooq et al. 2008).

The increase of Lead in the vegetables of Thrissur market was attributed to heavily traffic in this area which leads to the accumulation of Lead emitted from car exhaustions. The recent results obtained by Divya*et al*. (2015) reported that the Lead content in Tubers such as Tapioca, Sweet Potato, Yam, Colocasia, Elephant Yam, Potato Collected from different markets of Ernakulam district ranges from 2.4-26.4 mg/Kg. The lowest value was for elephant yam collected from Ernakulam market and highest value for yam collected from Tripunithara market. In the present study, Tapioca shows the highest conecentartion of Lead (15 ppm) and the highest Lead content in Tapioca were found out by Divya*et al*. (2015) is 14 ppm. There is lower concentration of Lead in Yam and Potato collected from Thrissur district when compared to the Lead contcentartion in the same tubers collected from Ernakulam district.

Lead has been shown to have toxic impact on a variety of metabolic processes essential to plant growth and development, including photosynthesis, transpiration, DNA synthesis, and mitotic activity (Pehlivana *et al*., 2008).So these toxic elements transferred to our body by daily consumption that can create problems in various biological systems. People consuming them are in high health risks of toxic metal exposure. It is therefore suggested that continuous analysis of heavy metals in vegetables should be done to avoid maximum levels of these metals in the living beings. The ultimate solutions should be carried out in order to prevent maximum contamination of heavy metals and all vegetables should be washed properly before consumption, as washing can remove appropriate amount of pollutants from the vegetable surface. The world health organization (WHO) has reported that 15-18 millions are affected by lead in blood.

CONCLUSION

The present study provides the information on heavy metal contamination in selected tubers of Thrissur district. The concentration of Lead in the selected tubers and permissible levels needed for safe consumption stated by WHO were compared. The samples collected from Thrissur market shows more concentration of heavy metals than those collected from other markets and lowest concentration was obtained in the vegetables collected from farmers and those were cultivated in the Kitchen garden. The high concentration may be due to the application of polluted water into the agricultural field and the application of fertilizers.

Vegetable species differ widely in their ability to take up and accumulate heavy metals, even among cultivars and varieties within the same species (Zhu *et al.,*2007 ; Saumel*et al.,*2012). In this study, significant differences were found in the concentrations of Lead in different markets; the concentrations decreased in the order of

Table 2. WHO maximum permissible level of heavy metals in vegetables found in mg/kg

Sl.No.	Heavy metals	Maximum permissible level of heavy metals according to WHO(mg /kg)
1	Lead (Pb)	0.3
2	Cadmium (Cd)	0.1

Thrissur>Cherpu>Irinjalakuda>Kunnamkulam>Chalakudy.

Lead is a serious cumulative body poison, which enters into the body system through air, water and food and cannot be removed by washing fruits and vegetables (Chove *et al.,* 2003). Lead has been shown to have toxic impact on a variety of metabolic processes essential to plant growth and development, including photosynthesis, transpiration, DNA synthesis, and mitotic activity (Pehlivana *et al*, 2008).The Potato and Tapioca samples had Lead content that was higher than the FAO standard of 0.3 mg/kg. The high concentration of Lead in these tubers may probably due to the use of untreated industrial waste water for irrigation, pollutants from soil or due to cultivation of the vegetables near the industrial area. It can be stated that the field of cultivation noticeably influences the heavy metal uptake by vegetables.

This study further confirms the increased danger of growing tubers on soils irrigated with contaminated industrial and domestic wastewaters. However, the levels of the metals are currently within the FAO/WHO safe limits guidelines. But, if the practice of treating the soils in the irrigation gardens with contaminated waters is not controlled, it may lead to health hazard on the part of consumers of the vegetables on the long term. Therefore, there is the need to continually monitor, control and take necessary policy decisions so as to limit and ultimately prevent these avoidable problems. However, in the meantime, farmers from the study areas are hereby encouraged to use well water for irrigation in their gardens instead of contaminated streams.

Urbanization and industrialization are the main causes of heavy metal pollution. It concludes that transportation and marketing of tubers shows a considerable role in heavy metal contamination. The usage of purified water from various industries for irrigation purposes may also elevate the levels of heavy metals in the samples. The prolonged consumption of these tubers leads to dangerous accumulation of toxic heavy metals in the kidney and liver and ultimately leads to many diseases in human. The results of the present study showed that consumers are at greater risk of purchasing fresh vegetables with high levels of heavy metals beyond permissible limits as defined by WHO. It will negatively influence the health systems of human. So the government and industries should take necessary actions to avoid the heavy metal pollution in our environment.

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Studies on the Anatomy and Histochemistry of *Eichhornia crassipes* (Mart.) Solms

LATHA SADANANDAN[1], SOJA S.[2] AND THUSHARA V.[3]

[1]*PG and Research Department of Botany, Sree Narayana College, Kollam - 691001*
[2] *PG and Research Department of Botany, University College,Thiruvananthapuram-695034*
[3]*PG Department of Botany, Sree Narayana College, Cherthala, Alappuzha – 688 582*
email: sadananandanlath@gmail.com

ABSTRACT

The anatomical studies using hand sections of all parts of *Eichhornia crassipes* (Mart.) Solms were supported by micrometry. The histochemical localization of primary metabolites and secondary metabolites were carried out using suitable stains. The anatomical features of the plant showed absence of cuticle in all parts except leaf, epidermis on all parts consisted of a single layer of rectangular cells which is a characteristic feature of this species, presence of gas filled chambers and passages in roots, leaves and rhizome, Raphide crystals (Calcium oxalate) in parenchyma cells of roots, petioles, rhizomes and leaves. The type of stomata on the leaves were of anomocytic type. The stomatal indices and their frequency on the upper and lower epidermis were almost the same *i.e* 47 and 1.6mm^2 respectively. The palisade ratio on the leaves was 4 palisade cells per epidermal cell. The histochemical localization for primary and secondary metabolites showed the presence of protein, reducing sugar and starch in all plant parts while lipid was found only on leaves. Among the secondary metabolites studied, only phenols were present while alkaloids and flavonoids were absent. Proteins were localized on the vascular tissues while starch on the palisade tissues in leaf and cortex in other parts. Reducing sugar were present in the cortex. Lipid was present as thick cuticle on the leaf surface. Phenols were localized in most tissues of all parts of the plant. A preliminary understanding about the anatomical characters and histochemistry of *Eichhornia crassipes* (Mart.) Solms helps one to find out any variations that occurred in them by environmental or anthropogenic factors such as pollution.

Key Words *Eichhornia crassipes*, Anatomy, Histochemistry

Eichhornia crassipes (Mart.) Solms, commonly known as water hyacinth, belonging to the family Pontederiaceae is an aquatic plant (Plate 1A) native to the Amazon basin (Brazil), and is often considered a highly problematic invasive species outside its native range. One of the fastest growing plants known, *E. crassipes* reproduces primarily by way of runners or stolon, which eventually form daughter plants. Each plant can produce thousands of seeds each year, and these seeds can remain viable for more than 28 years. When not controlled, water hyacinth will cover lakes and ponds entirely. This dramatically impacts water flow, blocks sunlight from reaching native aquatic plants, and starves the water of oxygen, often killing fishes. The plants also create a prime habitat for mosquitoes, the classic vectors of diseases, Water hyacinth is often problematic in man-made ponds if uncontrolled (Villamagna and Murphy, 2009). Utilization of this plant to produce eco-friendly products can help to reduce such environmental problems to some extent. A preliminary knowledge on the chemistry of the plant paves way to the discovery of new potential drugs in pharmaceutical companies, eco-friendly biofuel, or eco-friendly products in textile or paper industries. With this in view, the study aims to understand the anatomy and histochemistry of the plant.

MATERIALS AND METHODS

Anatomy of all parts of *Eichhornia crassipes* (Mart.) Solms viz., root, rhizome, petiole and leaf sections were studied using hand sections stained with Safranin and measured by micrometry. Microscopic characters of the leaf such as stomatal type, stomatal index on the upper epidermis and lower epidermis, stomatal frequency, palisade ratio and features such as Calcium crystals, sclerids and trichomes were studied. The values were found out using the formulae (Wallis, 1997):

$$\text{Stomatal Index} = \frac{S \times 100}{E+S}$$

Stomatal frequency = Stomatal frequency was calculated by multiplying the number of stomata by the field area of 0.0346 mm^2.

Palisade ratio = Average number of palisade cells beneath one epidermal cell, using four contiguous epidermal cells for the count.

The localization of various phytochemicals such as primary and secondary metabolites in the various parts of plant tissues were carried out by taking the hand sections of the plant tissues viz., root, rhizome, petiole and leaf, followed by their staining in suitable stains viz., Coomassie Brilliant Blue (Protein), Dragendorff's reagent (alkaloids), Fehling's reagent (reducing sugar), 5% alcoholic Ferric chloride

(phenols), Iodine solution (starch), Lead acetate (flavonoids), Sudan III (oil, cuticle and suberin)

RESULTS AND DISCUSSION

ANATOMY STUDIES

ROOT

Root epidermis consisted of single layered compactly arranged rectangular cells (4.0µm). There is no cuticle on the outside of the root epidermis. Hypodermis is composed of 1 – 2 layers of thick walled cells (3.5 µm). Beneath the hypodermis cortex is differentiated into outer and inner cortex. The outer cortex is composed of 3 – 4 layered parenchymatous cells (4.0 µm). Each air space (7.0 µm) has trabeculae. The inner cortex consists of 6~10 layers of parenchymateous cells. There is no sclerenchyma cell in the cortex. The stele is surrounded on the outside by single layered endodermis (3.5 µm). Endodermis is followed by a single layered pericycle. The stele consists of 7 to 10 xylem bundles alternating with phloem bundles (3.5 µm). Each vascular bundle consist of a single metaxylem vessel (7.0 µm), surrounded by smaller vessels. The root center is occupied by sclerified parenchyma cells (4.0 µm)(Plate 1B).

RHIZOME

The single layered epidermis is made of compactly arranged rectangular cells (4.0 µm). The cortex beneath the epidermis consists of 4 – 6 layered outer cortical cells (3.8 µm) having different size vascular bundles with patch of sclerenchyma to the outside of each bundle. Air space (3.4 µm) is also present in cortical region. Raphides are present in air spaces. Xylem (5 µm) is V shaped. Phloem is present in between the arms of xylem. There is an inner portion of large air spaces separated from each other by a single cell layer of parenchyma. Air space are spherical. Vascular bundles are also present in the center. (Plate 1C).

PETIOLE

Single layered epidermis composed of parenchyma cells (3.5 µm). Cuticle is absent. Vascular bundles are embedded in outer parenchyma cells. Each vascular bundle has a bundle cap of sclerenchyma cells. Hexagonal air spaces (47 µm) are surrounded by bands of single layered parenchyma cells. Raphides are present in aerenchyma. (Plate 1D)

LEAF

Epidermis consist of cuticle. Trichomes are absent. The mesophyll is differentiated into palisade and spongy mesophyll. Palisade layer is present in both upper and lower side of the epidermis. The mesophyll consist of a large number of air spaces surrounded by thin walls of chloroplast. Smaller and larger vascular bundles are present in both upper and lower epidermis side. Each vascular bundle is collateral with xylem towards the lower epidermis side and phloem towards the upper epidermis side (Plate 1E). Vascular bundles are surrounded by bundle sheath of parenchyma cells. Numerous raphides and sclereids are observed in the palisade layer (Plate 1H and 1I).

The stomatal type of *Eichhornia crassipes* was Anomocytic, i.e. the size of the subsidiary cells and epidermal cells are equal (4 µm) . The average size of guard cells was calculated to be 7 µm×4 µm, while the average size of the stomatal pore was 4 µm×4 µm on both upper and lower epidermis (Plate 1F) .Comparison of average Stomatal Indices on the lower and upper epidermis of the leaves showed that the leaf is Amphistomatic i.e. there is equal distribution of stomata on both epidermis (approx. 47) with an average of 47.15 on lower epidermis and 47.09 on upper epidermis. This is due to the upright position of the leaves above the water level, allowing equal distribution of sunlight on both surfaces of the leaf. The frequency of stomata on the upper and lower epidermis are almost the same, i.e., 1.6 mm^2 (1.631 mm^2 and 1.629 mm^2on lower epidermis and upper epidermis respectively). The average number of palisade cells per epidermal cell (palisade ratio) is four (Plate 1G).

HISTOCHEMICAL LOCALIZATION

ROOT

Presence of protein in the epidermis and stelar region. Reducing sugar was concentrated in the endodermis and inner cortex while starch was concentrated in the outer cortex and outside metaxylem. Presence of phenol was confirmed throughout the section viz., epidermis, hypodermis, outer and inner cortex and stele. Alkaloid was present in the hypodermis. Flavonoids and lipids were totally absent (Plate 2 A-G).

RHIZOME

The stelar region showed the presence of protein and Reducing sugar was observed throughout. Starch was concentrated to the ground tissue inner to cortex. Among the secondary metabolites phenol was present in the vascular tissues while the others studied viz.,

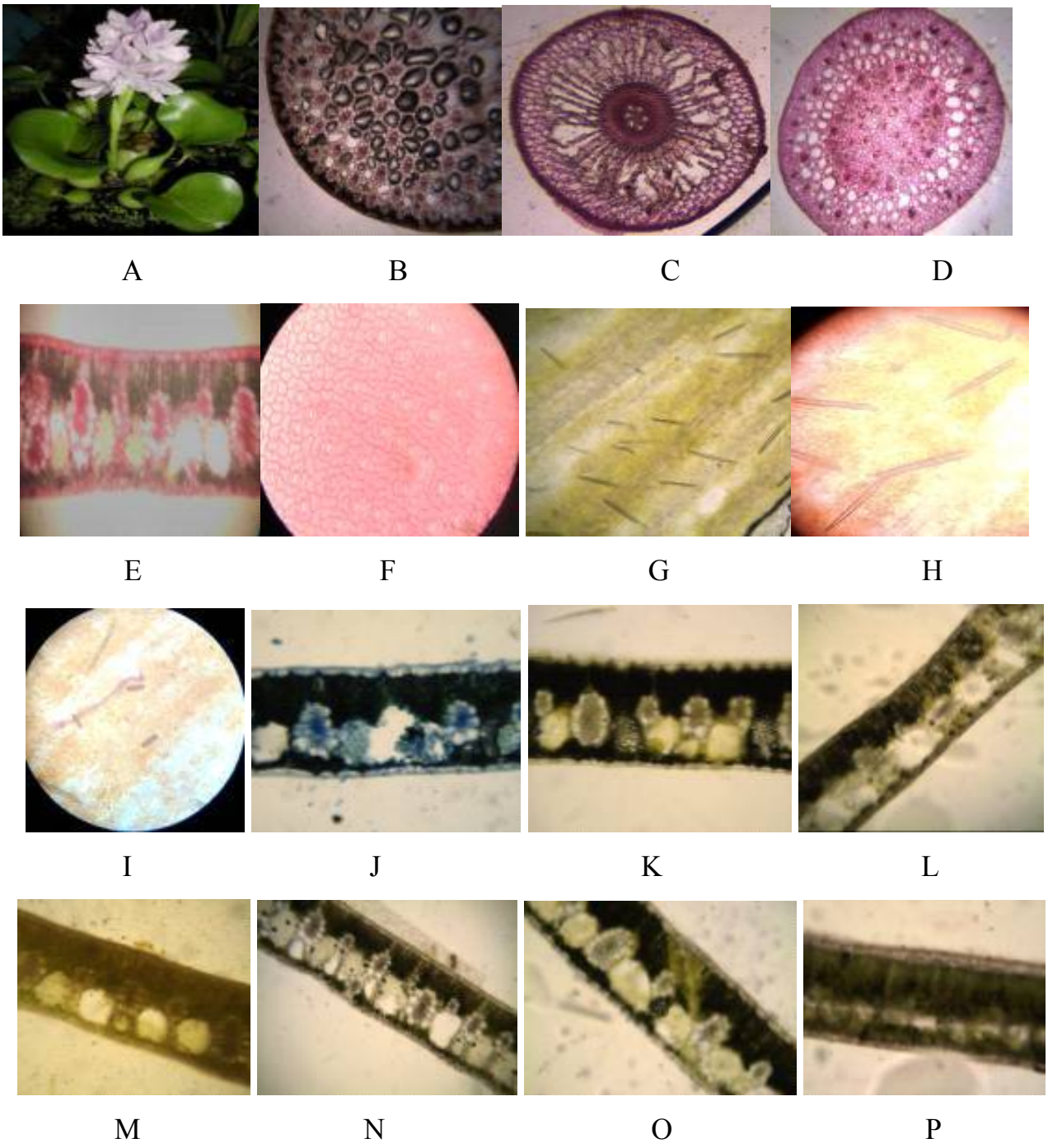

Plate 1.*A-* *Eichhornia crassipes* (Mart.) Solms, B- Root T.S., C- Rhizome T.S., D- Petiole –T.S, E- Leaf- T.S, F- Anomocytic stomata, G – Palisade tissue, H – Raphides, I- Sclerides, J-P:Histochemical localization in leaf

alkaloids, flavonoids and lipids were absent (Plate 2 H-N).

PETIOLE

The presence of protein was found in stellar region of the petiole. Reducing sugar was observed in the outer and inner aerenchyma while starch was found in the hypodermis. Among the secondary metabolites studied, only phenol was observed in epidermis while alkaloids, flavonoids and lipids were absent (Plate 2 O-T).

LEAF

Presence of protein in the upper and lower epidermis, stele and palisade tissues. Reducing sugar was concentrated in mesophyll parenchyma while starch was observed in the upper and lower palisade tissues. Phenol was concentrated in the palisade tissues. Lipid was present in high concentration on the upper and lower epidermis in the form of cuticle. Flavonoids and alkaloids were absent (Plate 1 J-P).

The type of stomata on the leaf was of

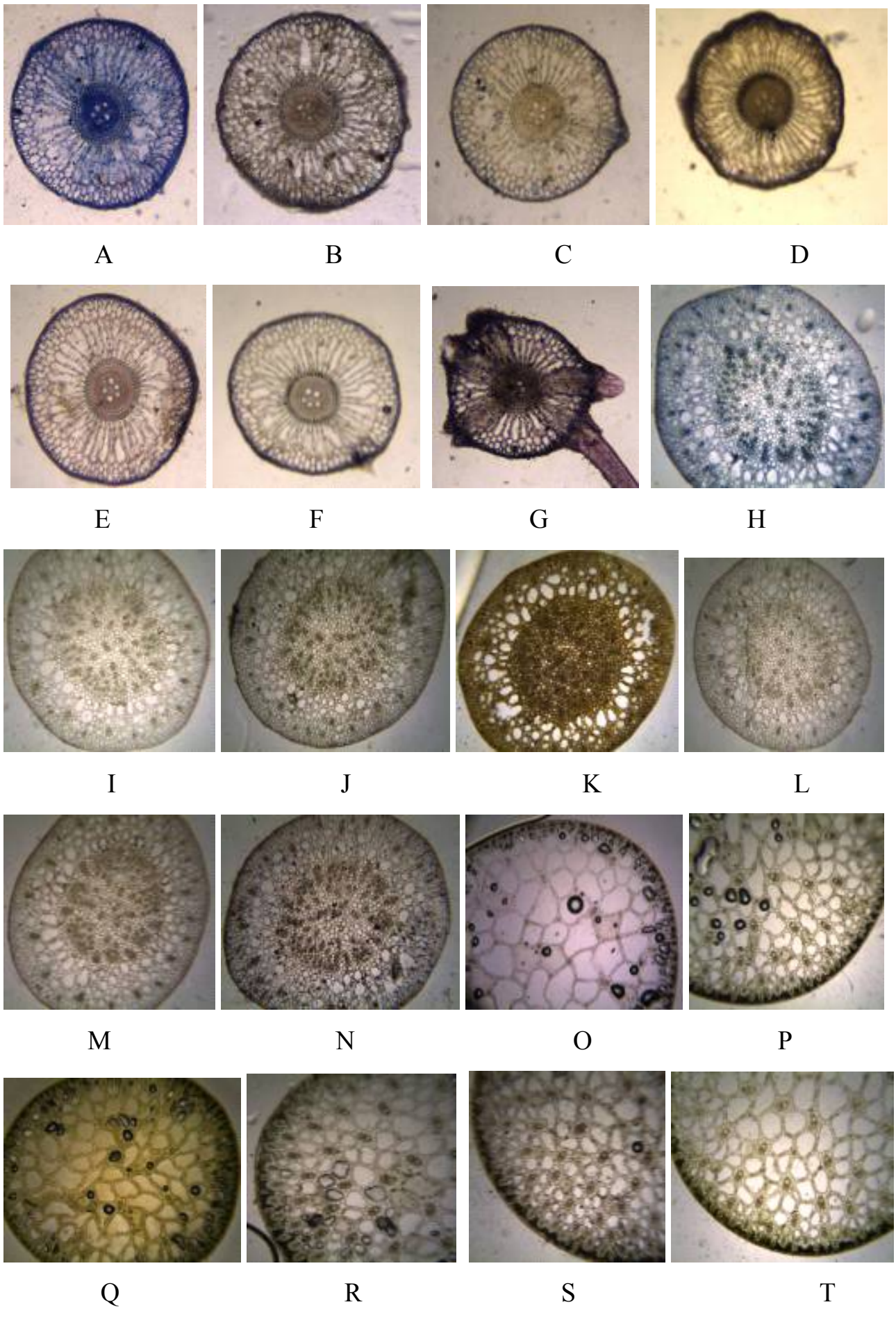

Plate 2. Histochemical Localization A-G : Root, H- N: Rhizome, O – T : Petiole

Anomocytic type though it was reported to be Paracytic type by Mahmood *et al*., 2005. Anatomical studies are supported by earlier workers (Nasir and Ali, 1977, Barnabas, 1996). The absence of alkaloids, anthraquinones, tannins and saponins in this study is supported by the Ph.D work of Nehal (1985), while they found the presence of sterol. The presence of terpenoids, flavonoids, phenols and cardiac glycosides are supported by Kurup and co-workers (2013).

SUMMARY AND CONCLUSION

Anatomical characterization of all parts of *Eichhornia crassipes* (Mart.) Solms were carried out using hand sections of fresh material with Saffranin aided with Micrometry. Histochemical localization of primary and secondary metabolites were carried out using suitable stains in all parts of the plant. The anatomical features of plant studied showed many hydrophytic adaptations such as absence of cuticle in all parts except leaf. Epidermis on all parts of water hyacinth consisted of a single layer of rectangular cells which is characteristically a constant feature of this species. A very thin cuticle and thin cellulose walls of epidermal cells in a typical hydrophyte facilitate steady absorption from surrounding water. The most pronounced anatomical feature of this plant is the presence of gas filled chambers and passages in roots, leaves and rhizome. There were many Raphide crystals (Calcium oxalate) in parenchyma cells of roots, petioles, rhizomes and leaves. The type of stomata on the leaves were of Anomocytic type. The Stomatal Indices and their frequency on the upper and lower epidermis were almost the same i.e 47 and 1.6mm^2 respectively. The Palisade Ratio on the leaves was 4 palisade cells per epidermal cell. The histochemical localization for primary and secondary metabolites showed the presence of protein, reducing sugar and starch in all plant parts while lipid was found only on leaves. Among the secondary metabolites studied, only phenols were present while alkaloids and flavonoids were absent. Proteins were localized on the vascular tissues while starch on the palisade tissues in leaf and cortex in other parts. Reducing sugar were present in the cortex. Lipid was present as thick cuticle on the leaf surface. Phenols were localized in most tissues of all parts of the plant. The work serves a basic knowledge about the plant and paves way for it's exploitation in many ways for the production of eco-friendly products thus reducing the environmental problems caused by them.

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Effects of Industrial Effluent on Soil Characteristics, Plant Growth and Selected Nutritional Factors in *Amaranthus Sps.*

D.S. LEKSHMI AND V. RAJANI

Post Graduate Department of Environmental Sciences, All Saints' College, Thiruvananthapuram, Kerala, India[2]
email : lekshmids1@gmail.com

ABSTRACT

For the existence of life the nature recourses are essential. Soil and water is essential natural resource like other resources. The excessive and untreated release of industrial effluents results major environmental issues such as the pollution, health hazards to plants and animals etc. In India the main source of soil pollution is the effluent discharge from industries. Generally, industries discharge huge amount of effluent to the open land or to the nearby water resources. The composition of effluent differs according to the source of production. The macro and micro nutrients present in the effluent increases the soil fertility; along with it the heavy metals and toxic substance present in the soil also can be increased in concentration. The present study is dealing with the effect of industrial effluent on soil characteristics, plant growth and selected nutritional factors in the *Amaranthus* species. In this study, the industrial effluent was collected and the soil sample sets with *Amaranthus* species were treated with 10%, 20%, 30%, 40% and 50% of effluents and some important soil parameters were analysed along with the control (effluent free soil). A control was also observed simultaneously. The result showed that the treatment of soil with the effluent led to noticeable changes in the soil characters such as the pH, electrical conductivity, organic carbon , iron, copper, manganese, potash, phosphorous and zinc, plant morphology and nutritional factors. The periodic addition of effluent also made drastic changes in the growth rate of the *Amaranthus* plant species. In the first week of effluent addition, the shoot length and the number of leaves of plant in the control set were 5.1cm and 6 respectively but in the 50% was 5.1cm shoot length and 7 leaves. After the treatment of five weeks the control set plant had the highest shoot length of 21.7cm and 15 leaves, it gets decreased in the 50% by 17.3cm shoot length and 9 leaves. From this preliminary observation it was very clear that the effluent releasing from the industries is capable to make desired changes in the normal growth rate of the plants and it is not suitable for the irrigation purposes. The nutritional factors such as the carbohydrate and protein get reduced in concentration as the treatment concentrations increased but the concentration of tannin, â-carotene, and phenol were got increased. So the untreated releases of industrial effluents are capable to cause serious environmental issues such as the soil pollution, effect on the growth rate and on nutritional factors of plants.

Keywords *Industrial Effluent, Nutritional Factors, Phenol, Amaranthus sps, soil parameters*

The industrial revolution plays an important role in the development of developing nations. But the main issue facing by the India was the high cost industrial waste disposal in limited land area. However industrial revolution plays an important role in the development on many other countries around the world. Generally, most of the industrial wastes are disposed untreated to the nearby water bodies, water or directly emitted to the air. Even at certain companies which are provided with these treatment facilities are not being operated properly. Due to this direct emission of untreated waste water to the nearby water bodies or land ultimately results in the pollution of water resources and agriculture land. Generally the industrial effluents are discharged to the nearby water bodies as treated or untreated condition, so the effluent releasing to the water bodies is the main source of water pollution. The industries release the toxic substance as suspended solids, hazardous chemicals, oils, dyes, thermal pollutants etc. Discharge of highly toxic wastes to the water bodies' results in increase in BOD, COD, TDS, and TSS etc. This change in the water quality parameter makes it unsuitable neither for drinking or other uses. The usage of industrial effluents for irrigation leads to reduction in the plant growth, yield and soil fertility. The industries like pesticide industry, leather industry, pharmaceuticals, mines and quarry industry etc release a huge amount of toxic heavy metals through their effluents discharging out. It leads to the natural processes such as bioaccumulation, biomagnifications etc. The accumulation of heavy metals makes physiological and biochemical changes in plants and animals. The quality of dissolved minerals in water depends upon the source

of water and its path before use (Ahmed *et al.*, 1993). Soil ecosystems throughout has been contaminated by various anthropogenic activities results in health hazards to animals and plants through food chain (Tu *et al.*, 2000; Dahmani-Mueller *et al.*, 2001; McGrath *et al.*, 2002). So in order to reduce these effects the treatment of the effluent before the release to the open land area or to the water bodies are very necessary.

MATERIALS AND METHODS

The present study is based on the effect of industrial effluent on the soil parameters, growth rate and nutritional factor in Amaranthus sps. The Amranthus sps (red spinach) is a leafy vegetable with high nutritional value. It is a short- lived perennial plants. It is a summer annual plant mainly considered as a leafy vegetable. It has a life span of three months.

Fig.1. *Amaranthus sps*

Amaranthus sps

Classification of Amaranthus sps

Kingdom: Plantae

Class: Eudicots

Order: Caryophyllales

Family: Amaranthaceae

Genus: Amaranthus

EXPERIMENTAL SETUP

The experiment was done in two stages, stage 1 and stage 2 respectively. in stage one the seedlings of spinach was sawed in five sets of grow bags (figure 2) and treated by the industrial effluent of four different concentration as 100%, 75%, 50%, 25%respectively and the remaining one was kept separate as control and treated by normal water. The treatment of effluent was done twice in a week and weekly observation was done. After three days of the first addition of the effluent only the seedlings sawed in the control sample set was germinated. One day later the seedlings in the 25% effluent treated sample were observed as germinated and the remaining sets had been seeing unchanged. The number of seedlings and the health of seedlings germinated in the 25% treatment sample (figure 3) was less when compared with the control sample set. In stage 2, the germinated seeds from the control set were split to five sets and the treatment concentration was reduced to 10%, 20%, 30%, 40%, and 50% respectively. And the effluent treatment was reduced by once in a week. Shoot length, no of leaves present are observed periodically once in a week.

The following plant nutrients and soil parameters were analysed.

Estimation of Protein

Took 2g of sample. Grinded thoroughly in phosphate buffer. Filtered it out by using cheese cloth. Centrifuged the filtrate at 5000rpm for 10 minutes. Took the supernatant and make it to known volume by using buffer .Took 1ml of the above solution .Added 1ml of 10% TCA .Shaked thoroughly .Keep in freezer for 15 minutes .Centrifuge at 10,000 rpm for 10 minutes . Decant the upper layer .Take the pellet .Dissolve it in known volume of 0.1 N NaOH. Take 0.1 ml of aliquot. Make up to 1ml by using 0.1 N NaOH. Add 5ml of reagent C .Keep it for 30 minutes .Take the OD at 670nm.

Estimation of phenol

Took 1gm of sample. Refluxed it in 80% methanol for 20 minutes. Grind thoroughly and filtrate taken. The filtrate was subjected to centrifugation at 1000 rpm for 10 minutes .Collect the supernatant. Make it to known volume by using methanol. Take an aliquot 0.1 ml. Make it to 3ml using methanol. Add 0.5ml folin reagent .Add 2ml 20% Na2CO3.Keep in boiling water bath for 5minutes .A white precipitate is formed. Centrifuge at 5000rpm for 5minutes .Take the OD at 650nm against the blank.

Estimation of beta carotene

Took 2gm of sample. Added 20ml 60% KOH

and kept it for 3hours in darkness. Transfer the mixture to separating funnel .Wash it with petroleum ether 6 times .Collected ether layer after each washing. Pool the ether layer and wash with water. Makes the ether layer to known volume. Read the absorbance at 429 nm.

Estimation of tannin

Extract 1gm of plant sample in 50 % methanol. Mix occasionally by swirling. After 20-28hours, centrifuge and collect the supernatant .Pipette out 1ml of the supernatant .Quickly add 5ml of vanillin hydrochloride reagent. Read in a spectrophotometer at 500 nm after 20 minutes. Prepare a blank with vanillin hydrochloride reagent alone. Prepare a standard graph with 20-100ìg catechin using the diluted stock solution.

Estimation of soil pH using pH meter

Weighed 20 gm of 2.0 mm air dried soil into a beaker and added 50 ml of distilled water and stirred with a glass rod thoroughly for about 5 minutes and kept it for half an hour. Calibrating the pH meter, pH was recorded.

Estimation of electrical conductivity

The soil water suspension of 20 gm: 50 ml ratio was prepared. Allowed the soil water suspension in the beaker to settle for additional half an hour (the total intermittently shaking period is 1 hr.) After the calibration conductivity was recorded.

Estimation of organic carbon

Weighed 1 gm of 0.5 mm sieved soil into dry 500 ml conical flask and added 10 ml of K2Cr2O7 into the flask with pipette. 20 ml conc. H2SO4 is added with a burette and swirled gently until soil and reagents are mixed then more vigorously for one minute. Allowed the reaction to precede for 30 min. Added 200 ml of distilled water, 10 ml of concentrated orthophosphoric acid and about 0.2 gm NaF (one small teaspoon) and allowed the sample to stand for 1.5 hrs. The titration end point was clear in a cooled solution. Just before titration 1 ml ferroin indicator was added into the conical flask. Titrated the excess K2Cr2O7 with 0.5 N ferrous ammonium sulphate till the colour flashed from yellowish green to greenish and finally brownish red at the end point. Simultaneously blank test was run without soil.

Determination of phosphorous in soil (Olsen's method)

Weighted 2.5 gm of soil sample in 150 ml plastic conical flask, added a pinch (0.3 gm) of phosphate free activated charcoal AR grade. 50 ml of Olsen reagent was added and shaken for 20 minutes. Filtered the content out and 5ml of aliquot was transferred into 25 ml volumetric flask and 5 ml of filtrate was pipette out into 25 ml volumetric flask. 4 ml of the freshly prepared ascorbic acid and ammonium molybdate solutions were added to it. Shaken well and kept it for 30 minutes then made up to the volume. Prepared the standard curve using 0, 1, 2, 3, 4 & 5 ml of 5 ppm standard phosphorous solution into 25 ml volumetric flask and developed the colour using the same procedure as above. The corresponding phosphorous concentration was 0, 0.2, 0.4, 0.6, 0.8 & 1ppm. The absorbance and colour intensity at 882 nm were observed.

Determination of available potassium

25 ml of NH4 extracting solution was added to the conical flask containing 5 gm air dry soil sample. Shake the mixture for 5 min and filter. Determine potassium as indicated in preparation of standard curve, dilute if necessary.

Determination of available iron, manganese, zinc and copper

Weighed 12.5g of soil sample in 100ml iodine value flasks. 25 ml DTPA solution is added to each. Shaked the mixture for two hours on the shaker at 70-80 oscillation per minute filtered out the mixture through Whatman No. 1 filter paper and the filtrate is collected. The micronutrients are determined on atomic absorption spectrophotometer

RESULTS

EFFLUENT PARAMETERS

Table 1. Effluent Parameters

EFFLUENT PARAMETERS	
Ph	3
Fluride(ppm)	1
Residual chlorine(ppm)	0
Phosphate(ppm)	0
Iron(ppm)	5
Ammonia(ppm)	3

Table 2. Morphological observations of plant at different treatment concentration

OBSERVATION PERIODS	TREATMENT CONCENTRATIONS					
	Control		10%		20%	
	Shoot Length(Cm)	No Of Leaves	Shoot Length(Cm)	No Of Leaves	Shoot Length(Cm)	No Of Leaves
1st week	5.1	6	5.2	6	5.2	6
2nd week	8.6	7	8.4	7	8.1	7
3rd week	14.3	12	14.1	8	12.2	7
4th week	18.1	13	17.5	11	14.1	9
5th week	21.7	15	20	13	18	12

The analysis of effluent parameters reveals that the effluent is acidic in nature and the iron content in the effluent is high to make desirable changes to the soil parameters, it is capable to affect the sensitive plant crops and also its nutritional factors.

EFFECT OF INDUSTRIAL EFFLUENT ON PLANT MORPHOLOGY

From the above results, it was observed that, after the five weeks of effluent treatment at concentrations of 10%, 20%, 30%, 40% and 50%, the control set of plants showed the highest growth with a shoot length of 21.7cm 15 leaves and the lowest growth rate was observed in the plant set of 50% effluent treatment with a shoot length of 17.3cm and 9 leaves. Hence, the present results are highly significant. The rate of reduction was found to increase with time and increase in concentration of effluent. The distinguishable change to the morphology (shoot length and number of leaves) may be due to the effect of effluent added. The highest no of leaves is observed in the control set of plants and it got reduced as the effluent concentration increases from 10% to 50%, the no of leaves present in the control set is 15 and it got reduced by 9 in the 50% set.

EFFECTS OF INDUSTRIAL EFFLUENT ON PLANT NUTRIENTS

From the above observations the highest protein was present in the 10% and lowest amount of protein was present in the 50%. The highest amount of â-carotene was present in the 50% sample set and the least amount was present in the control. The highest amount of phenol was present in the 10% and the least amount of phenol was present in the 50%. The highest amount of carbohydrate was present in the 50% set and the highest in the control set. The amount of tannin was highest in the 50% and the least amount

Table 3. Morphological observations of plant at different treatment concentration

OBSERVATION PERIODS	TREATMENT CONCENTRATIONS					
	30%		40%		50%	
	Shoot Length	No Of Leaves	Shoot Length	No Of Leaves	Shoot Length	No Of Leaves
1st week	5.1	6	5.1	6	5.1	7
2nd week	8.3	7	7	7	7	7
3rd week	10.2	7	9.6	8	7.3	6
4th week	12.9	8	12.5	9	9.8	8
5th week	17.8	10	15	10	17.3	9

Table 4. Effect of on nutritional factors at different treatment concentrations

Nutritional factors	Treatment concentrations					
	Control	10%	20%	30%	40%	50%
Protein(mg/l)	0.1159	0.1215	0.118	0.107	0.091	0.083
β-carotene (mg/l)	0.0020	0.0023	0.0024	0.0026	0.0028	0.0034
Phenol(mg/l)	0.00186	0.00187	0.00189	0.00193	0.00195	0.00201
Carbohydrate (mg/l)	9.860	8.570	8.470	7.660	6.630	1.407
Tannin (mg/l)	0.022	0.030	0.031	0.038	0.039	0.045

is present in the control set. From the above observations it is very clear that the industrial effluent were capable to cause desired changes in the amount of nutritional factors in the plant body.

EFFECTS OF INDUSTRIAL EFFLUENT ON SOIL PARAMETERS

As per the test results, various soil parameters showed fluctuations from the normal range. The pH of soil showed changes in their values. The pH value of the control was 6.7 and in case of the soli treated with 10% effluent, the pH showed an increase in value, 6.9. After that, it showed a gradual decrease in pH values- 6.8, 6.2, 4.5, and 4.1 as the effluent concentration increased. The data revealed that the soil pH is affected due to application of effluent. The highest pH value was showed by the 10% (6.9) and the lowest pH value was for 50% (4.1). There are studies which also observed that the pH decreases with the increase in effluent concentration (Vimaldeep *et al*., 2014, Kansal *et al.,* 1999; Yadav *et al.,* 2002). The electrical conductivity of the control was found to be 0. 23 and it's got increased by 0.81, 0.84, 0.87, 0.91 and 1.09 as the effluent concentration increased (10%, 20%, 30%, 40% and 50%). The highest electrical conductivity is to 50% (1.09) and lowest for the control

Table 5. Analysis of Soil parameters in control and samples.

Soil Parameters	Treatment concentrations					
	Control	10%	20%	30%	40%	50%
pH	6.7	6.9	6.8	6.2	4.5	4.1
Electrical conductivity (ds/m)	0.23	0.81	0.84	0.87	0.91	1.09
Organic carbon (%)	0.16	0.29	0.43	0.45	0.64	0.88
Available phosphorus (ppm)	123.02	145.57	159.32	160.23	168.3	186.27
Available potassium (ppm)	143	242	275	275	356	429
Available iron(ppm)	31.5	27.9	28.3	30.2	66	89.3
Available manganese (ppm)	4.9	4.8	5.2	8.5	11.3	20.2
Available zinc(ppm)	0.6	0.4	0.5	1.2	1.8	2
Available copper (ppm)	1.1	0.8	0.9	1.3	2.1	2.7

(0.23). This change of electrical conductivity from normal to marginal range is harmful for the survival of sensitive crops. The high rate of electrical conductivity indicates the presence of excess nitrogen or high range of exchangeable sodium (Mir Tariq Ahmad *et al.,* 2012). The excess accumulation of sodium in soil results poor tilt and low permeability making them unfavourable for plant growth. The electrical conductivity of soil also affects the crop yield, top soil depth, pH, salt concentration and increases water holding capacity.

As the treatment concentration increases the amount of organic carbon present in the soil got decreased. The amount of organic carbon present in the soil was 0.16% and as the treatment concentration increases from 10%, 20%, 30%, 40%, and 50% the amount of organic carbon also changes from 0.29, 0.43, 0.45, 0.64 and 0.88% respectively. The highest amount of organic carbon was present in 50%set (0.88%) and the lowest was recorded in the control set (0.16%). In the study of The effluent treatment also showed slight fluctuations in the amount of soil potash, copper, zinc, manganese, potassium and iron. The amount of available phosphorous in the soil getting increased by 123.02, 145.57, 159.32, 160.23, 168.3 and 186.27ppm as the effluent concentration got increased, likely the amount of potash present in the soil had been increased from the control, 143ppm by 242, 275, 275, 356 and 429ppm as the treatment concentration increased. The available manganese in the control set was 4.9ppm as the treatment concentration increased from 10% to 50 %, the amount of manganese also changed as 4.8, 5.2, 8.5, 11.3 and 20.2ppm. The presence of zinc in the soil is essential for the growth of plants and it has a vital role in the metabolism of human body but the increased presence of heavy metal in the soil results to bio-magnification.

The amount of zinc in the control set or the effluent free set was 0.6ppm, as the treatment concentration increased the amount of zinc showed slight increase in the rate as 0.4, 0.5, 1.2, 1.8, and 2ppm. The amount of iron had been decreased from control value 31.5ppm to 27.9ppm and 28.3ppm as the treatment concentration increased by 10% and 20% respectively. While at the concentrations 30%, 40% and 50%, the amount of iron showed an increase in values as 30.2, 66, and 89.3ppm respectively. The amount of copper also showed a gradual decrease in values from the control (1.1ppm) to 0.8ppm and o.9ppm as the concentration increased by 10% and 20% respectively. But as the concentrations increases again as 30%, 40% and 50% the value got increased by 1.3, 2.1 an2.7ppm respectively. The highest concentration of available phosphorous was in the 50% set (186.27ppm) and the lowest was recorded in the control (123.02ppm). Available potash was recorded highest in the 50% (429ppm) and the lowest in the control set (143ppm). The value of available iron was recorded highest in the 50% set (89.3ppm) and the lowest value in the 10% (27.9ppm). Available manganese was highest in the 50% (20.2ppm) and lowest in the 10% treatment set. The concentration of available zinc was recorded highest in the 50% (2ppm) and the lowest value was recorded in the 10% (0.4ppm). The concentration of available copper was highest in the 50% (2.7ppm) and the lowest was recorded in the 10% (0.8ppm).

DISCUSSION

Katepogu Raju *et al.,* 2015 also observed that the addition of industrial effluent is capable to cause desired changes to the growth rate o the plant. As the treatment period increases the growth rate of the plant gets decreases with decreased shoot length. And also he observed that the higher concentrations of effluent were found to inhibit the germination and growth of paddy.

M.O. Islam *et al*., 2006 observed that plant height of rice was decreased in the contaminated soils as compared with the normal agricultural soils. The reduction of plant height by different treatments over normal agricultural soil (Reduction Over Control) ranged between 7 to 19, 25 to 37 and 50 to 65% during 30, 60 and 85 days of treatment.

Giese AC *et al*., 1964 said that one of the major molecules severely affected by stress is protein. With reference to Giese, Kour J *et al*., 2017 analysed the total protein content in leaf of tomato and capsicum which is treated with the industrial effluent. The results revealed that the reduction in protein content was very obvious with an increase in the concentrations of effluent, when compared to the control plants. Suresh *et al*., 2014 also found that the increased concentration of effluent decreases the plant protein level. Robbercht. R *et al.,* 1983 said that Phenol is one of the major chemical constituents, providing good

protection against stress injury in plants. Stafford HA *et al*., 1965 observed that, the synthesis of phenol triggered only under stressful environments. With reference to Robbercht. R and Stafford H A, Kour J *et al*., 2017 analysed the impact of distillery effluent on the total phenol content was analyzed. It was found that there was more prominent increase in the phenol content of the distillery effluent treated capsicum than that of the effluent treated *Lycopersicum esculantum*. Khan and Jain *et al*., 1995 observed that the plants exposed to the industrial effluents results in the reduction carbohydrate present in the plant body. Lakshmi *et al*., 1999 also observed that the industrial effluents are capable to cause desired reduction in the concentration of carbohydrate in the plant body as the treatment concentration increases. Himabindu and Reddy (2005) observed that the increased concentration of effluent treatment results in the increased â-carotene concentration in the plant. Likely in the present study the higher concentration of carotene is present in the 50% and the least concentration of carotene is present in the control set of sample *Amaranthus* species.

Mir Tariq Ahmad *et al.,* 2012 also studied the effect of effluent on the physico chemical properties of soil. He observed that the EC increased with the application of effluent as irrigation water having high concentration of salts, particularly Na^+ and Cl^- has significantly increased the salinity as compared to the uncontaminated soil. He also observed that the higher concentration of cations such as Na and K in waste water led to an increase in EC and exchangeable Na and K in soil irrigated with waste water.

Mir Tariq Ahmad *et al*., 2012 also observed that the pH of effluent irrigated soil ranged from 7.76 to 8.7 while pH of uncontaminated soil was ranged from 6.90 to 7.31. In sample Site 1 and 2, the contaminated soil pH increased with the application of effluent as compared to uncontaminated soil. The increase of soil pH is due to addition of various soluble salts in industrial effluent also reported.

Dr. Deepti Sahare *et al*., 2014 also identified that the value of organic carbon increases with the addition of effluent. It is being also reported that increase in organic carbon facilitates the accumulation of available nutrients and metals in the soil.

Dr. Deepti Sahare *et al*. , 2014 also studied the impact of irrigation of industrial effluents on soil-plant health, in that the study, the soil samples were collected under three categories, first which comprised of those soil samples which are continuously receiving industrial effluent discharges and second, which had those soil samples which are totally unaffected from industrial effluent discharges but are still in the nearby area and third which comprised of those soil samples which are very far. Again different parameters of these soil samples were studied namely pH, EC (electrical conductivity), OC(organic carbon), N, P, K, Na+, Ca2+, Mg2+, Zn, Cu, Fe, Mn and Pb and it was found that many of these parameters were considerably higher in case of first set of samples. So, from the study it is very clear that the effluent treatment it capable to cause noticeable changes in the soil parameters. Except the pH all the parameters got increased with the increase in treatment concentrations.

CONCLUSION

The industrial effluents are one of the major pollutant sources for the water pollution and soil pollution. Majority of industries in India is facing a serious issue of disposal of waste water or the effluent produced. The treated as well as the untreated effluent release to the nearby water bodies' results in the water pollution and also the unscientific disposal of effluent to the open land area also results in the soil pollution.

- The observations shows that as the treatment concentrations increased, the growth rate of the plant got reduced and reduction in the number of leaves occurred
- The nutritional factors such as the carbohydrate and protein got reduced in concentrations as the treatment concentrations increased, but the concentration of tannin, â-carotene, and phenol were got increased
- The soil was also severally affected by the effluent treatment. Most critical soil parameters like pH and electrical conductivity of the soil were increased as the treatment concentrations increased from 10%, 20%, 30%, 40% and 50%.
- The present study reveals the effect of industrial effluents on the soil characteristics, plant morphology and nutritional factors.

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Antibacterial Potential of Methanolic and n-hexane Extracts *of Lobelia alsinoides* Lam.

P. RAJASREE, A K. ANIL KUMAR, M. S . SHEEBA,

Post Graduate and Research Department of Botany, Government College for Women, Vazhuthacadu, Thiruvananthapuram-14, Kerala)
email : sheebamadhavan@gmail.com

ABSTRACT

India has enormous resources of medicinal and herbal plants. Antimicrobial activity of *Lobelia alsinoides* Lam. (methnol and n- hexane extracts) were studied in four selected bacterial strains such as *Escheritia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiella* by disc diffusion assay and found out the minimum inhibitory concentration (MIC) of these extracts. Both n-hexane and methanol extract have significant antibacterial activity against all the tested microorganisms. In the case of activity against *E. coli* and *S. aureus*, both methanolic and n- hexane extracts showed significant activity in a dose dependant manner. A dose of 0.3 mg/ml of both n-hexane and methanol extracts showed highest activity against the above said bacterial strains. The activity can be compared with the inhibition zone produced by positive control, cefotaxime. Nine concentrations of methanolic extracts were selected to find out the MIC of *L. alsinoides*. It was shown that among five selected strains of bacteria two of the strains showed MIC at 0.41 mg and rest showed 1.22 mg as inhibitory concentration. N-hexane extracts inhibited the growth of the micro organisms at a concentration of 1.2 mg against most of the micro organisms.

Key words *Anti-microbial activity, Disc diffusion assay, MIC, methanol, n-hexane extract*

India has a rich culture of medicinal herbs and spices, which includes about more than 2000 species and has a vast geographical area with high potential abilities for Ayurvedic, Unani, Siddha traditional medicines but only very few have been studied chemically and pharmacologically for their potential medicinal value (Gupta *et al.*, 2005; Sandhu and Heinrich, 2005).

Traditional use of medicine is recognized as a way to learn about potential future medicines. Researchers have identified number of compounds used in mainstream medicine which were derived from "ethnomedical" plant sources (Farnsworth, 1976; Gilani and Rahman, 2005). Plants are used medicinally in different countries and are a source of many potent and powerful drugs (Ebadi M., 2007; Kumari *et al*., 2011).

Lobelia alsinoides Lam.is a herb belonging to the family Lobeliaceae, widely distributed in South and Southeast Asia. Research has demonstrated that many chemical constituents dominated by piperidine alkaloids and flavonoids, such as lobelanidine, lobeline, and lobelanine, have been obtained from related species, *Lobelia chinensis* and the plant possess several possesses a number of pharmacological activities (Chen M W *et.al* 2014; Ma and Wink, 2008 Neugebauer N. M, 2007). The aim of the present study is to evaluate the antimicrobial potential of methanolic and n-hexane extracts obtained from *Lobelia alsinoides Lam.*

MATERIALS AND METHOD

Whole plant of *L. alsinoides* Lam were collected from Pattambi, Cheruthuruthi, Kottayam and authenticated. Shade dried plants were used to prepare plant extracts both in methanol and n- Hexane. In vitro antibacterial screening was carried out against one gram positive bacteria: *Staphylococcus aureus* (ATCC 25923) and three gram negative bacteria: *Klebsiella pneumonia* (ATCC 22736), *Pseudomonas aeruginosa* (ATCC BAA-427), *Escherichia coli* (ATCC 9637). Bacterial strains were procured from P.S.G College Coimbatore.

Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Serratia marcescens and *Klebsiella* were cultured as lawns on Muller Hinton Agar. The disc (4 mm diameter) was prepared from the Whatmann no. 1 filter paper, stored at 4^0C and sterilized by autoclaving at 121^0C. These discs were used for the impregnation of plant extracts for testing the antibacterial potential.

The antibacterial activity of methanolic and n-hexane extracts was determined by standard disc diffusion method at concentrations of 100 µg, 200 µg, , 250 µg and 300 µg plant extracts which were prepared in DMSO. Antibiotic Cefotaxime was used as positive control for comparing antibacterial activity. Further Minimum inhibitory concentration of each extract was tested to evaluate the antimicrobial potential. The data was subjected to statistical analysis using One-way analysis of variance (ANOVA) by SPSS software. P value of less than 0.05 was considered to indicate

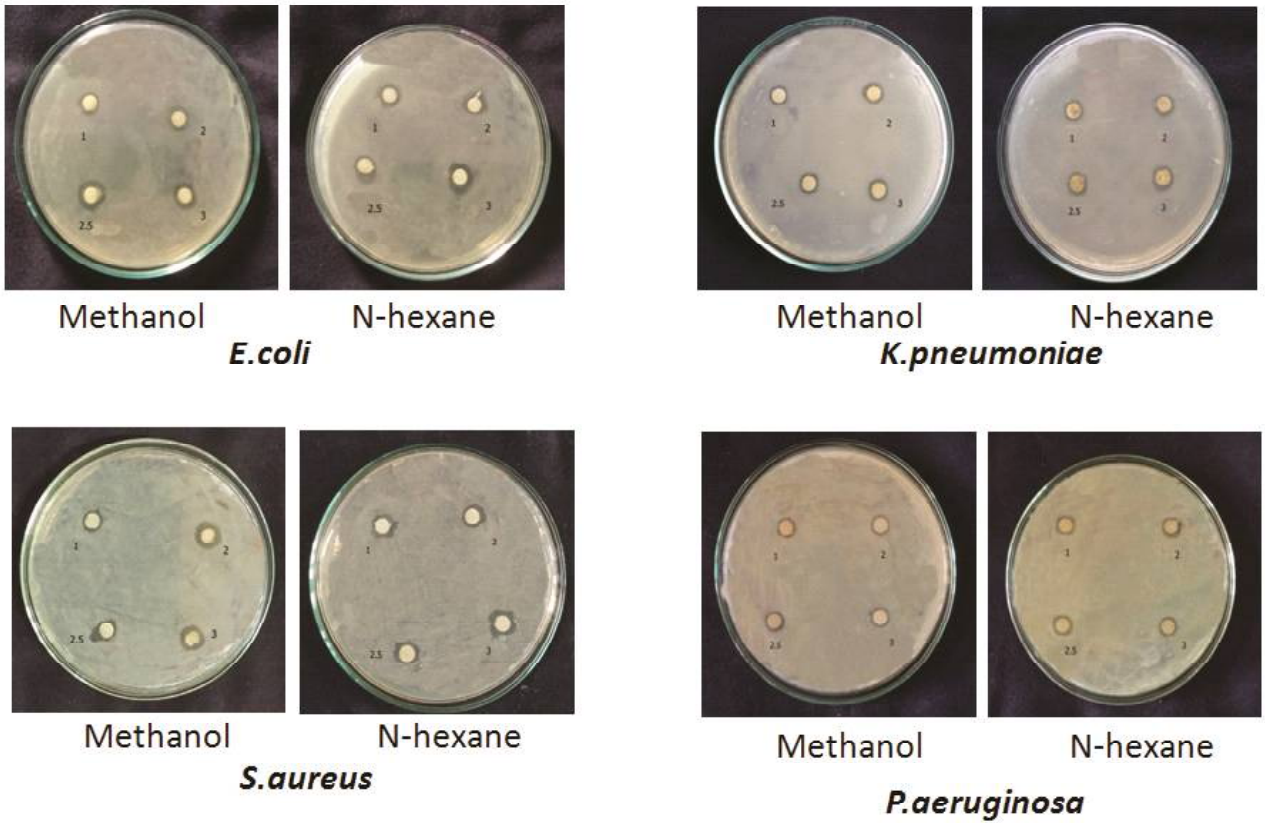

Fig. 1. Inhibition zones of Methanol & N-Hexane extract of *Labelia alsinoides*

statistical significance.

RESULTS AND DISCUSSION

In vitro antibacterial activity of two plants *Lobelia alsinoides* (whole plant) in two different solvents (methanol and n-hexane) were analyzed for antimicrobial activity against four different bacterial strains *Escherichia coli, Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* in four different dosages (0.1mg, 0.2mg, 0.25mg and 0.3 mg).

Antibacterial assay has been done by disc diffusion method and further the minimum inhibitory concentration (MIC) assay required to inhibit the growth of microorganism was determined. Disc diffusion assay done in *L. alsinoides* showed that both

Table 1. Inhibiton zone (mm) produced by the extracts (Methanol and N-Hexane) of *Labelia alsinoides.* The data presented as mean S.E.

SLNo.	EXTRACT	DOSAGE	INHIBITION ZONE (in mm)			
			E.coli	*S.aureus*	*P.aeruginosa*	*K.pneumonie*
1	METHANOL	0.1mg	8.2±0.09	8.08±.03	7.1±0.069	8.08±0.05
2	METHANOL	0.2mg	10.3±0.18	12.9±0.03	7.14±0.06	9.02±0.02
3	METHANOL	0.25mg	13.1±0.09	13.4±0.04	8.14±0.024	10.08±0.04
4	METHANOL	0.3mg	15.1±0.04	14.1±0.11	8.5±0.04	12.02±0.03
5	HEXANE	0.1mg	8.1±0.09	8.06±0.04	7.08±0.04	9.16±0.10
6	HEXANE	0.2mg	13.1±0.09	12.4±0.04	7.5±0.06	9.5±0.06
7	HEXANE	0.25mg	14.1±0.10	13.06±0.04	8.16±0.24	10.06±0.06

Table 2. Effect of positive control, cefotaxime against tested bacterial strains. The data presented as mean ±S.E.

	Positive Control	Dosage	INHIBITION ZONE (in mm)			
			E.coli	*S.aureus*	*P.aeruginosa*	*K.pneumoniae*
1	CEFOTAXIME	0.1mg	16.04±0.02	16.04±0.03	27.9±0.10	27.8±0.09
2	CEFOTAXIME	0.2mg	18.8±0.07	16.9±0.02	28.9±0.02	28.9±0.10
3	CEFOTAXIME	0.2.5mg	19.9±0.10	23.9±0.02	29.9±0.02	30.0±0.03
4	CEFOTAXIME	0.3mg	22.0±0.09	27.8±0.12	32.9±0.02	32.2±0.12

n-hexane and methanol extract have significant antibacterial activity against all the tested microorganisms (Table 1). The antibacterial study of *L. alsinoides* was conducted for the first time and it showed antibacterial activity against all the tested four bacterial strains (Fig 1)

In the case of activity against *E.coli* and *S.aureus*, both methanolic and n- hexane extracts showed significant activity in a dose dependant manner. A dose of 0.3 mg/ml of both n-hexane and methanol extracts showed highest activity against the above said bacterial strains. The activity can be compared with the inhibition zone produced by positive control, cefotaxime (Table 2).

Against *P.aeruginosa* both extracts of *L. alsinoides* showed moderate antimicrobial activity and both the results were not much significant when compared with the positive control, cefotaxime(Fig:3). For *K.pneumonia*, methanolic extract have higher activity when compared to n- hexane extract at higher concentration and it showed somewhat similar results at 0.25mg concentration.

Nine concentrations of methanolic extracts were selected to find out the MIC of *L. alsinoides*. It was shown that among five selected strains of bacteria two of the strains showed MIC at 0.41 mg and rest showed 1.22 mg as inhibitory concentration. N-hexane

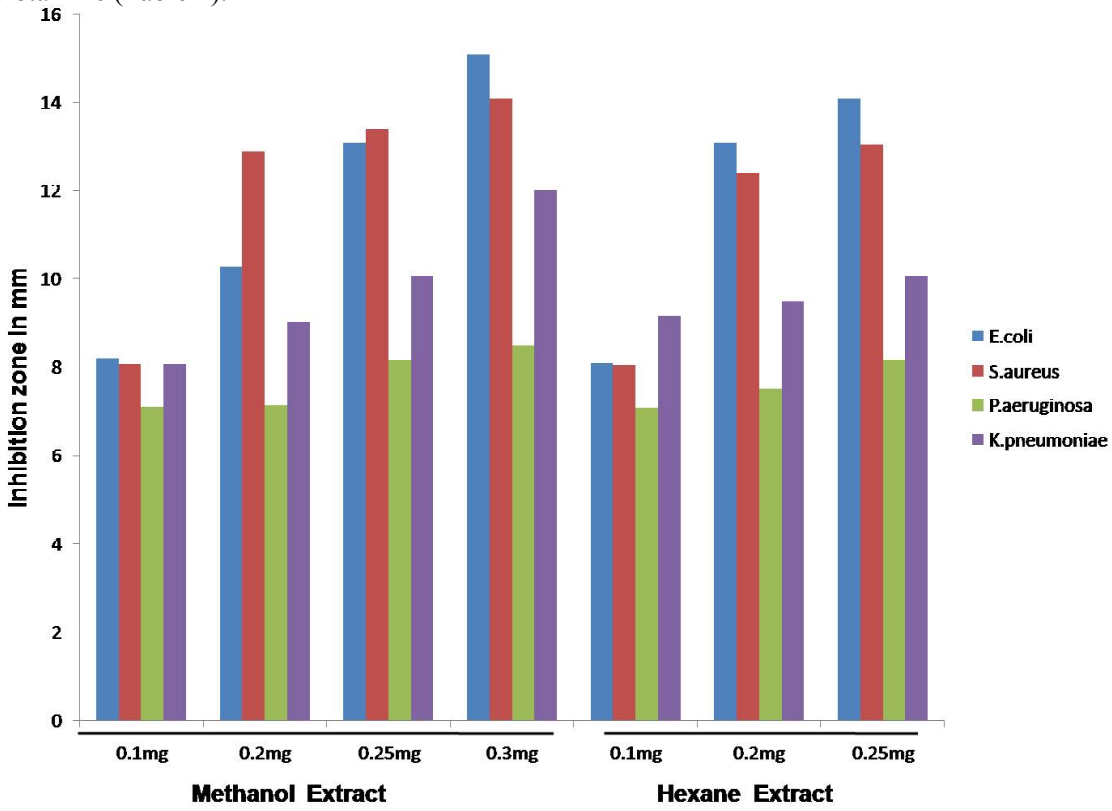

Fig. 2. Inhibition zone produced by the *L. alsinoides* methanol and n-hexane extracts

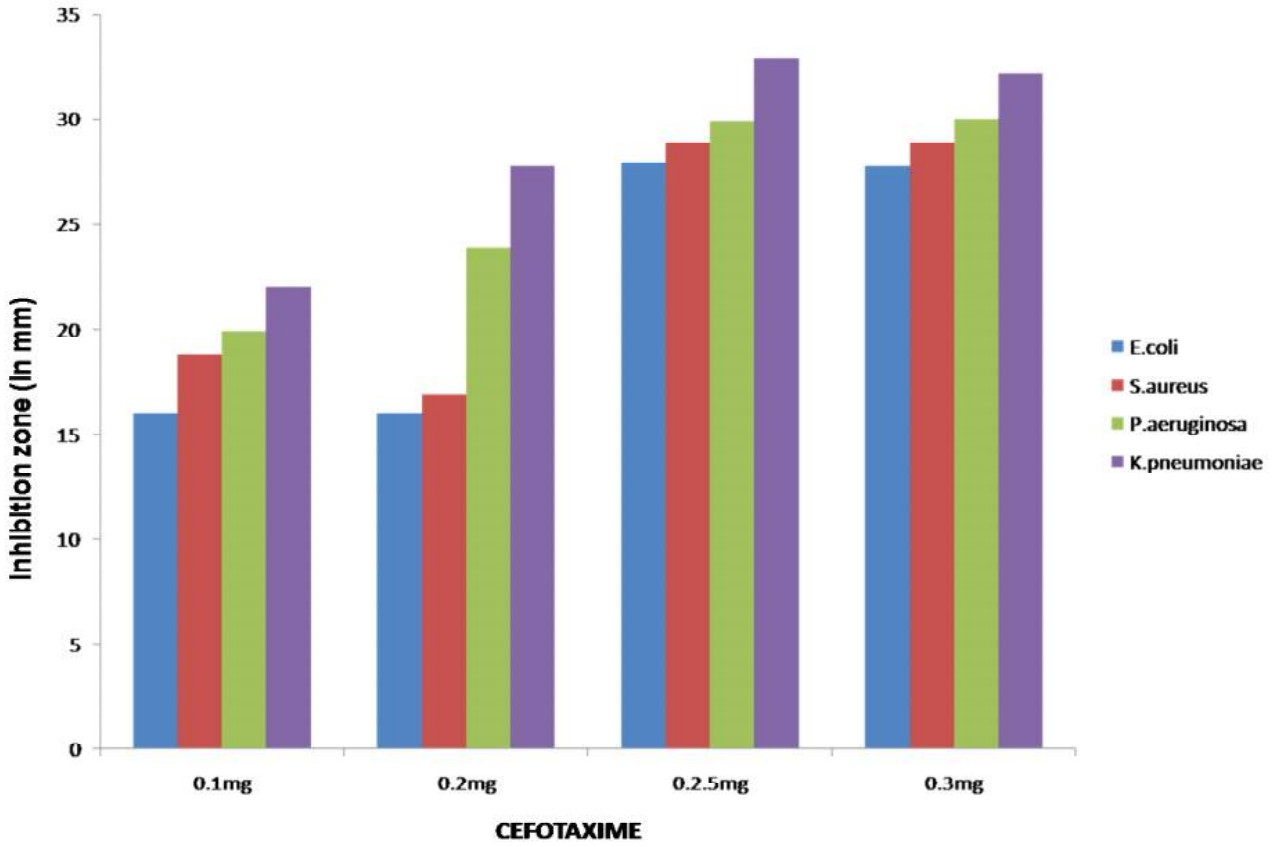

Fig. 3. Inhibition zone produced by cefotaxime (positive control)

extracts inhibited the growth of the micro organisms at a concentration of 1.2 mg against most of the micro organisms.

CONCLUSION

L.alsinoides extract showed significant antimicrobial activity against all the tested microorganisms. The extract showed highest activity in *E.coli* at the 0.3mg concentration for both methanol and n-hexane extracts.

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In vitro Phytochemical Screening and Free Radical Scavenging Activity of Extracts of *Mollugo cerviana*

ROBINA ANTONY AND P.G. BIJU*

Department of Biochemistry, University of Kerala, Kariavattom Campus, Thiruvananthapuram, Kerala- 695581.
**email: pgbiju75@gmail.com*

ABSTRACT

***Mollugo cerviana,* belonging to the family Molluginaceae, is a flowering plant known for its hepatoprotective, antiproliferative, antimicrobial and other bioactivities. Oxidative stress reflects an imbalance between the systemic appearance of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates. ROS are a known cause for the onset of several diseases such as cancer, inflammatory diseases and heart diseases etc. The present study deals with the phytochemical constituent screening of methanolic and ethyl acetate extract of the plant, *in vitro* antioxidant activity evaluation of the extracts by diphenyl-picrylhydrazyl (DPPH) assay, ABTS assay, nitric oxide radical scavenging, hydroxyl radical scavenging, superoxide radical scavenging, ferrous iron chelation, total antioxidant and reducing capacity assay. Results obtained in this study indicate that methanolic extract possess more phytochemicals when ethyl acetate extract. Methanolic extract of *Mollugo cerviana* exhibited significant potency in antioxidant activity as indicated by the radical scavenging assays.**

Key words *Antioxidant; ROS; Radical scavenging; Phytochemicals; Mollugo cerviana*

Plants have always been utilized as a source of drug and many of the drugs have been directly or indirectly obtained from plant sources (Gurib Fakem *et al*., 2006). Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions. Plants are used medicinally in different countries and are a source of many potent and powerful drugs (Srivastavaj *et al*., 1996; Mahesh etal., 2008). Traditional medical systems like Siddha and Ayurveda are largely based upon herbal and herbomineral preparations and have specific diagnostic and therapeutic principles (Tripathi *et al*., 2009).

Oxidative stress reflects an imbalance between the systemic accumulation of reacrtive oxygen species (ROS) and a biological system's ability to detoxify the reactive intermediates or to repair the resulting damage. A large number of medicinal plants have been investigated for their antioxidant properties. Natural antioxidants are very effective in the prevention of the destructive processes caused by ROS induced oxidative stress (Zengin *et al*., 2011).

Mollugo cerviana (Fig.1) commonly known as —ÞThreadstem carpetweed" in English and "Parpadakam" in Malayalam, is found on most continents growing as a weed in many types of dry, sandy habitats. The plant is used in Indian traditional systems of medicine for several medicinal properties, including hepatoprotective, antiproliferative, antimicrobial and antipyretic activities. Although previous studies have reported the phytochemical screening of the whole plant extracts, and its antimicrobial activity (Valarmathi *et al*., 2012), no systematic study has been carried out so far on the radical scavenging potential and antioxidant efficiency. In this study we have investigated the phytochemical constituents and *in vitro* antioxidant activity of the methanolic extract of *Mollugo cerviana*.

Fig. 1

MATERIALS AND METHODS

Chemical reagents

DPPH and ABTS were purchased from Sigma-Aldrich Co, USA. All other biochemicals, chemicals and solvents were purchased from SRL, Ranbaxy and Spectrochem, India.

Collection of plant material and extraction

Mollugo cerviana (L.) Ser. samples were collected from Alappuzha district of Kerala state, India during the months of February and March. The specimen was authenticated by an expert (Dr. Valsaladevi, Curator, Department of Botany, University of Kerala) by comparing fresh specimen with an existing herbarium voucher submission at the Herbarium of Department of Botany, University of Kerala (KUBH-7184).

The aerial parts of the *Mollugo cerviana* were cleaned, air dried and powdered with mechanical blender for extraction. 100 g of the dried powdered extract were filled in a soxhlet extraction apparatus and extracted with 2000 ml of methanol and ethyl acetate respectively. The apparatus was adjusted with the respective boiling points of the solvents (64^0C for methanol and 77^0C for ethyl acetate). The extract was then transferred to a conical flask, concentrated and evaporated to dryness in a thermostat water bath set at boiling points and obtained greenish gummy exudates (Harborne, 1998). The final residue was weighed and used for further dilution at the required for phytochemical, antioxidant and free radical scavenging assay.

Qualitative Phytochemical

Detection of Alkaloids

a. Wagner's Test: Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids

b. Hager's Test: Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow colored precipitate.

Detection of Saponins

a. Froth Test: Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

b. Foam Test: 0.5 g of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

c. Emulsion test: 2 g of powdered sample is boiled together with 20ml distilled water in a water bath and filtered. 10ml of filtered sample is mixed with 5ml of distilled water in a test tube and shaken vigorously to obtain a stable persistent froth. The frothing is then mixed with 3 drops of olive oil for the formation of emulsion which indicates the presence of saponins.

Detection of Tannins

a. 0.1% Ferric chloride test: 0.5g of powdered sample is boiled in 20ml of distilled water in a water bath and filtered. 0.1% $FeCl_3$ is added to the filtered samples and observed for brownish green or blue black coloration which shows the presence of tannins.

Detection of Flavonoids

a. Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

b. Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids

c. A few chop of 1% ammonia solution is added to the aqueous extract in a test tube. A yellow coloration is observed if flavonoid compounds are present.

Detection of Terpenoids

a. Copper acetate Test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes.

b. 5 ml of aqueous extract of each plant is mixed with 2ml of $CHCl_3$ in a test tube. 3ml of conc. H_2SO_4 is carefully added to the mixture to form a Layer. An interface with a reddish brown coloration is formed if terpenoid constituent is present.

Detection of Glycosides

a. The extract was hydrolysed with HCl solution and neutralized with NaOH solution. A few drops of Fehling solution A and B are added. The presence of glycosides is confirmed by the formation of red precipitate.

b. A small amount of extract was dissolved in 1ml of water and aqueous NaOH solution was added. Formation of yellow color indicated the presence of glycosides

Detection of Phenols

a. 5% Ferric Chloride Test: Extracts (dissolved in distilled water) were treated with 3-4 drops of 5% ferric chloride solution. Formation of bluish black/dark green colour indicates the presence of phenols.

Detection of Coumarins

a. To the extract in alcohol, a few drops of sodium hydroxide solution were added. Dark yellow colour formation indicates the presence of coumarins.

Detection of Carboxylic acid

a. Extract dissolved in water is treated with sodium bicarbonate. Brisk effervescence indicates the presence of carboxylic acid

Detection of Anthraquinones

a. To 1ml of the extract add 1ml of 10% FeCl3 and 0.5ml of conc. HCl. Boil in a water bath for few minutes. Appearance of a pink or deep red colour indicates the presence of anthraquinones.

Detection of Steroids

a. Libermann- Burchard Test: To the extract was dissolved n chloroform, 1ml of acetic acid and 1ml of acetic anhydride were added, then heated on a water bath and cooled. Few drops of concentrated sulphuric acid were added along the sides of the test tubes. Appearance of bluish green colour indicates the presence of steroids.

b. Salkowski test: The extract was dissolved in chloroform and equal volume of concentrated sulphuric acid was added. Formation of bluish red to cherry red colour in chloroform layer and green fluorescence n the acid layer indicates the presence of steroids.

In vitro Antioxidant studies

Determination of 2,2 –diphenyl-1-picrylhydrazy (DPPH) radical scavenging activity

DPPH radical scavenging activity was measured by the method of Blois (1958). The free radical scavenging activity of plant extract and active components are measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. 0.1 mM solution of DPPH in ethanol was prepared and 0.1 ml of this solution was added to 3.0 ml of test solution at different concentrations. Thirty minutes later, the absorbance was measured at 517 nm. Lower the absorbance of the reaction mixture, higher the free radical scavenging activity. A system devoid of the compound served as control.

$$\text{DPPH scavenging (\%)} = \frac{A\,(\text{cont}) - A\,(\text{test})}{A\,(\text{cont})} \times 100$$

Hydroxyl radical scavenging assay

The effect of hydroxyl radical was assayed by using 2-deoxyribose oxidation method (Chung *et al.,* 2005). 2-deoxyribose is oxidized by the hydroxyl radical that is formed by fenton reaction and degraded to malondialdehyde. The reaction mixture contained 0.2M sodium phosphate (PH -7.6), 10mM 2-deoxyribose, 10 mM H_2O_2, 10mM FeSo4 – EDTA, 100µM Ascorbate and different concentrations of sample in a test tube. The reaction was started by the addition of H_2O_2. After incubation at 37°C for 1 hour, reaction was stopped by adding 2.5% (W/V) TCA and 1% (W/V) TBA. The mixture was boiled for 10 minutes, cooled in ice, and then measured at 520nm. The reaction mixture not containing test sample measured as control. Mannitol was used as standard antioxidant. The scavenging activity of hydroxyl radicals were expressed as

$$\text{\% Scavenging} = \frac{A(\text{cont}) - A(\text{test}) \times 100}{A(\text{cont})}$$

ABTS radical scavenging assay

Trolox equivalent antioxidant capacity (TEAC) was estimated as 2,2'-Azino-bis(3-ethylbenzthiazoline)- 6- sulfonic acid (ABTS) radical cation scavenging according to the method of Re *et al*., 1999. Reagent solution consists of 7 mM ABTS and 2.45mM Potassium persulphate in 100 mM Phosphate buffer solution (pH 7.4) and was left to stand for 12 – 16 hr at laboratory temperature in the dark for the formation of ABTS radical cation. A working solution was diluted to absorbance values 0.7 at 734 nm with 100 mM Phosphate buffer solution (pH 7.4). 10µl of the sample of different concentrations was mixed with working solution (990 µl) and absorbance was measured at 734 nm after 5 min. Ascorbic acid and Quercetin were used as the standards.

$$\text{ABTS scavenging (\%)} = \frac{\text{A (cont)} - \text{A (test)} \times 100}{\text{A (cont)}}$$

Ferrous ion chelating potential

The chelating of ferrous ions by methanolic extract was estimated by the method of (Singh and Rajini, 2004). The different concentrations of methanolic extracts were mixed with 100il of 2.0 mM ferrous sulphate solution and 300il of 5.0 mM ferrozine. The mixture was incubated at room temperature for 10 minutes. The absorbance of the solution was measured at 562 nm. Different concentrations of EDTA were used as standard. Percentage of inhibition was calculated by using this formula,

$$\text{Percentage of inhibition} = \frac{\text{A(cont)} - \text{A(test)} \times 100}{\text{A (cont)}}$$

Nitric oxide radical scavenging Assay

Nitric oxide (Green *et al*., 1982) generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions which are measured by given reaction. The reaction mixture containing 10 mM sodium nitroprusside in PBS and test material was incubated at 25 ^{0}C for 15 minutes. After incubation 0.5 ml of the reaction mixture was added to 0.5 ml of griess reagent (1% sulphanilamide, 2% H_3PO_4 & 0.1 % Naphthyl ethyne diamine hydrochloride). The absorbance of the chromophore formed was determined at 546nm. The antioxidant potential can be calculated from the formula,

$$\text{\% Scavenging} = \frac{\text{A(cont)} - \text{A(test)} \times 100}{\text{A(cont)}}$$

Determination of Superoxide radical scavenging activity

Superoxide radical scavenging activity was measured by the method of Liu *et al.,* (1997) with slight modifications.

Reagents:

a. Tris – HCl buffer (16 mM, pH 8.0)
b. NBT (50 mM)
c. PMS (10 mM)
d. NADH (78 mM)

Superoxide radicals are generated in PMS - NADH systems by oxidation of NADH and assayed by the reduction of NBT. In this experiment, the Superoxide radicals are generated in 3.0 ml of Tris - HCl buffer (16 mM, pH 8.0) containing 1.0 ml of NBT (50 ìM), 1.0 ml NADH (78 ìM) solution and sample (50-300 ìg/ml) in water. The reaction is started by the addition of 1.0 ml of PMS (10 ìM) to the mixture. The reaction mixture is incubated at 25^{0}C for 5 minutes and the absorbance measured at 560 nm against a blank. Decreased absorbance of the reaction mixture indicates increased Superoxide anion scavenging activity. A system devoid of compound served as the control. The % inhibition of Superoxide anion generation was calculated as

$$\text{\% scavenging} = \frac{\text{A (cont)} - \text{A (test)} \times 100}{\text{A (cont)}}$$

Estimation of Total Antioxidant Potential

The total antioxidant capacity of the extracts was evaluated by the phosphomolybdenum method according to the procedure described by Prieto *et al*. A 0.3 mL of extract was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95 ^{O}C for 90 min. Then, the absorbance of the solution was measured at 695 nm using a UV-VIS spectrophotometer against blank after cooling to room temperature. Methanol (0.3 mL) in the place of extract was used as the blank. The total antioxidant activity is expressed as the number of gram equivalent of ascorbic acid. The calibration curve was prepared by mixing ascorbic acid with methanol.

Reducing power assay (total reducing power)

The reducing power of extracts was determined as per the method of Oyaizu (1986). Different concentrations of test sample (1 ml)was mixed with 2.5 ml phosphate buffer (0.2 M p^H 6.6) and 2.5 ml potassium hexacyanoferrate (0.1 %) followed by incubation at 50^{0}C in a water bath for 20 minutes. After incubation, 2.5 ml of TCA (10%) was added to terminate the reaction. The upper portion of the solution (2.5 ml) was mixed with 2.5 ml distilled water and 0.5 ml $FeCl_3$ solution (0.01%) was added. The reaction mixture was left for 10 minutes at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. All the tests were

performed six times. A higher absorbance of the reaction mixture indicated greater reducing power. The extract concentration providing 0.5 of absorbance (EC50) was calculated from the graph of absorbance at 700 nm against extract concentration.

Statistical Analysis

Values are expressed as mean ± SEM of triplicate values of each sample within a parameter. Graphical representation and statistical analysis was carried out on Graphpad Prism 5.0 software (San Diego, CA, USA)

RESULTS AND DISCUSSION

In the present study, the methanolic and ethyl acetate extracts prepared from the dried aerial parts of plant material was subjected to preliminary phytochemical screening, and *in vitro* antioxidant studies.

Phytochemical analysis

Medicinal plants contain secondary metabolites, which are organic compounds that are not directly involved in the normal growth, development of organism. However, they often play an important role in plant defences (Harborne *et al*., 1973). Preliminary phytochemical screening of methanolic (ME) and ethyl acetate (EAE) extract of *Mollugo cerviana* revealed the presence of active phytoconstituents (Table 1). The standard procedures showed that ME contains alkaloids, tannins, flavonoids, terpenoids, coumarins, anthraquinones and steroids. EAE showed the presence of terpenoids, tannins, alkaloids, flavonoids and caumarins. Based on the phytochemical content, ME was selected for further *in vitro* radical scavenging activity assay.

Antioxidants assays

Antioxidants exert their mode of action by inhibiting the formation of reactive oxygen species either by inhibition of enzymes or by chelating trace elements. DPPH is widely used to evaluate the free radical scavenging effect of natural antioxidants. An imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage (Siddique *et al*., 1997). DPPH is a preliminary free radical that shows a characteristic absorbance at 517 nm. The extract concentration necessary for the 50% inhibitory concentration was compared in the study and the value is inversely propotional to the activity. The results were compared with the reference standard drug ascorbic acid. The results obtained were comparable with studies carried out on Pleurotus flabellatus (Dasgupta *et al*, 2013) and Amanita vaginata (Paloi *et al*., 2014). The results show an excellent effect for the methanolic extract in scavenging DPPH radicals (Fig: 2).

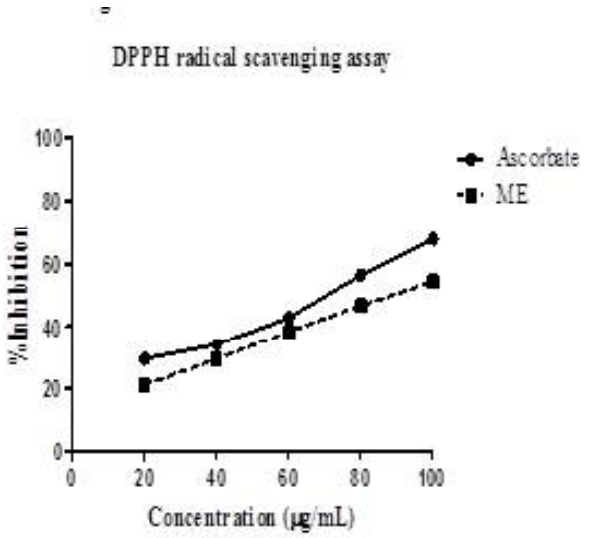

Fig. 2

Hydroxyl radical scavenging assay (Fig: 3) shows that the ME exhibited an IC_{50} value of 79.03µg/ml where as manitol exhibited an IC_{50} value of 59.83µg/ml. The methanolic extract showed maximum hydroxyl radical scavenging activity which was comparable with free radical scavenging activity of mannitol. ABTS, a stable free radical with the characteristic absorption at 734nm was used to study the radical scavenging effect of *M. cerviana* ME. ME was observed to exhibit significant ability to scavenge ABTS radicals (Fig:4) and this activity was comparable with that of ascorbate. ABTS assay is an excellent tool for determining the antioxidant activity of phytochemicals (Jagadish *et al*, 2008). Prieto *et al* have described phosphomolybdenum method for assessing the total antioxidant capacity of plant extract. The antioxidant activity was expressed as the number of gram equivalents of ascorbic acid.

The ability to deactivate and or chelate Fe^{2+} is the main mechanism of the ferrous ion chelating activity which helps in promotion of Fenton reaction and hydroperoxide decomposition. The ferrous ion chelating ability of ME was effective and the IC_{50} value was found to be 75.72µg/ml (Fig: 5). Nitric oxide is a diffusible free radical which is an important effecter molecule in diverse biological systems. It is generated by the endothelial cells and macrophages,

which are mediators of various physiological processes (Hagerman *et al*., 1998). ME was observed to exhibit significant ability to scavenge Nitric Oxide radicals (Fig:6). ME and ascorbate exhibited an IC_{50} value of 76.08and 54.65µg/ml, respectively.

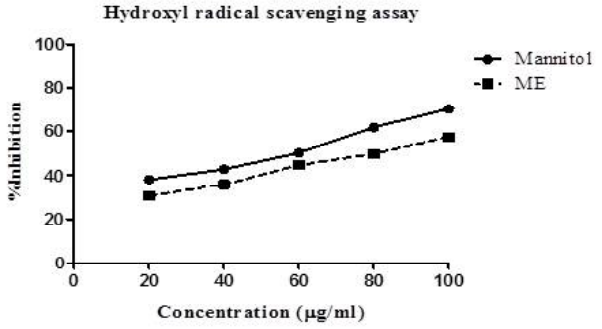

Fig. 3

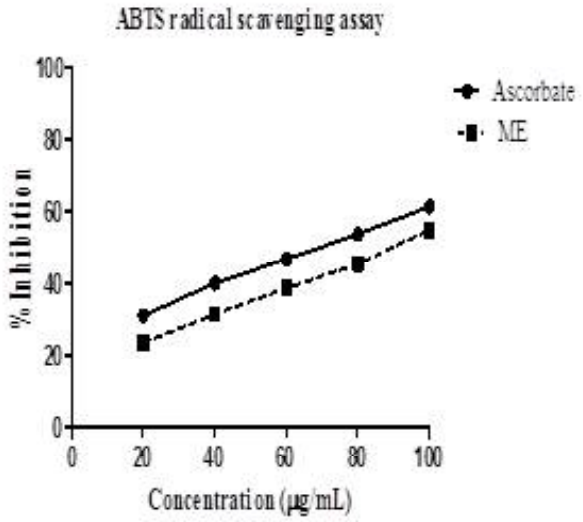

Fig. 4

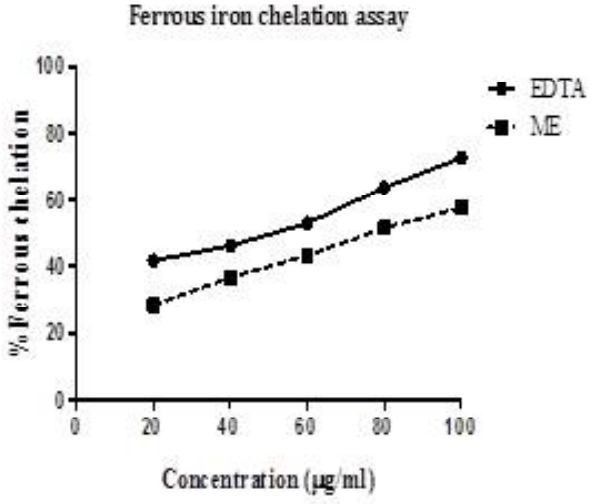

Fig. 5

Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive species. One risk of the superoxide generation is related to its interaction with nitric oxide to form peroxinitrite which is a potent oxidant that causes nitrosative stress in the organ systems (Lee *et al*., 2007). Methanolic extract showed a potent superoxide radical (Fig: 7) scavenging activity. The total anti-oxidant capacity (Fig:8) of ME reveals that it exhibited a very strong antioxidant potential. Methanolic extract was observed to exhibit significant antioxidant activity and was found to have a dose dependent effect.

The reducing power of the extract was determined through the transformation of Fe^{3+} to Fe^{2+}, whereby the yellow colour of the test solution changes to various shades of green and blue, depending on the

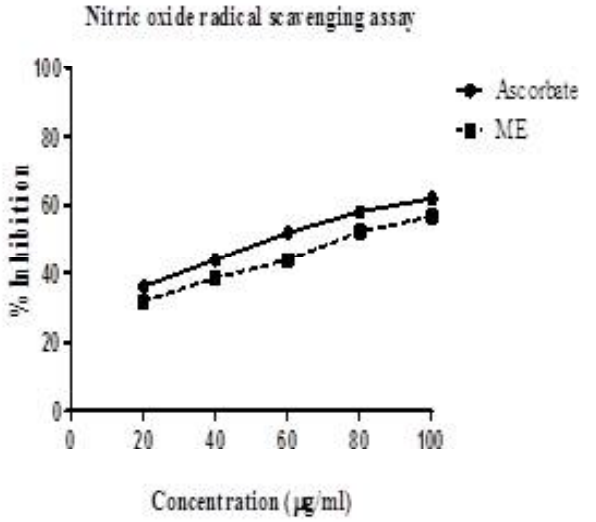

Fig. 6

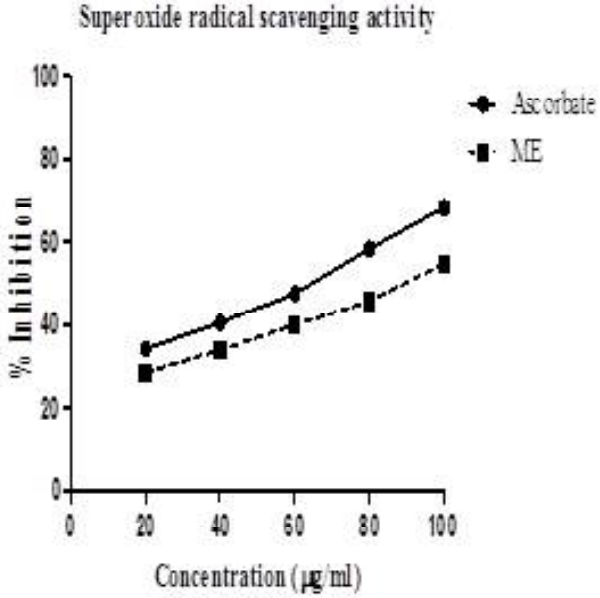

Fig. 7

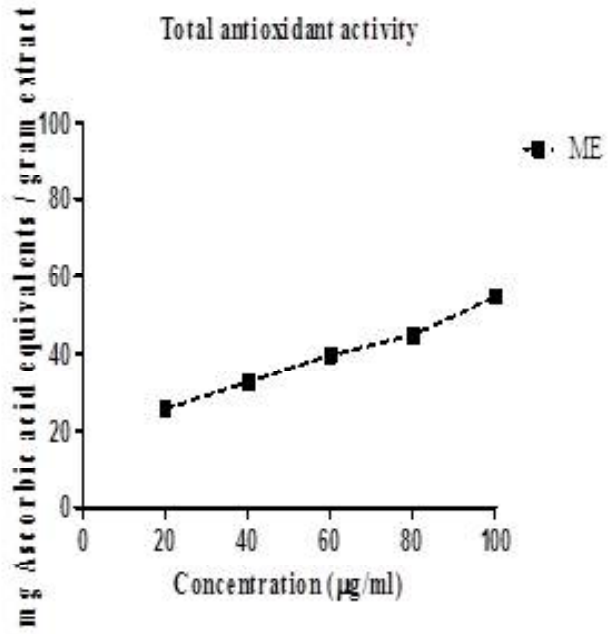

Fig. 8

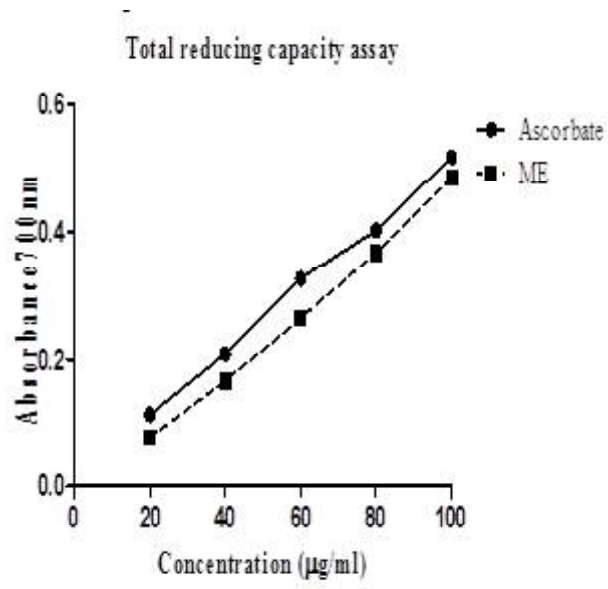

Fig. 9

reducing power of the sample. The results of reducing power assay are provided in Fig: 9. Absorbance is increased with increasing the concentration of the extracts. All the extracts showed potent reducing power ability. Ability of the extract to scavenge these wide varieties of free radicals marks usage of this extract as a potent antioxidant.

ME was observed to exhibit significant ability to scavenge free radicals and exhibited appreciable activity when compared with standard reference antioxidants. In vitro anti-oxidant activity (Fig: 2 to 9)

Table 1. Phytochemical analysis

Test	ME	EAE
Alkaloids	+	+
Saponins	+	-
Tannins	+	+
Flavonoids	+	+
Terpenoids	+	+
Glycosides	-	-
Phenols	-	-
Coumarins	+	+
Carboxylic acids	+	-
Anthraquinones	-	-
Steroids	+	-

ME methanolic extract, EAE ethyl acetate. (+) indicate ´presence, (-) indicate —absence.

shows the DPPH, Hydroxyl radical, Ferrous iron chelating, Nitric oxide, superoxide radical scavenging assay, total antioxidant, total reducing and ABTS radical scavenging activity of ME of *Mollugo cerviana*. ME was able to effectively scavenge the free radicals in different concentrations in a dose dependant manner in all the assays. Antioxidants play an important role in scavenging free radicals and provide protection against degenerative diseases [Liu G T, 1989 and Anandjiwala *et al*., 2008]. This study proves that ME of *Mollugo cerviana* is a promising source of novel antioxidants. Hence the phytoconstituents of ME need to be further explored for their individual role in antioxidant potential and protection against oxidative stress.

CONCLUSION

This study supports the traditional use of *M. Cerviana* and revealed the phytochemicals present in it. In addition, all the antioxidant assay methods showed that the methanol extracts of *M. cerviana* contains more antioxidant activities. This study also demonstrated that the methanolic extract is an important source of phytochemicals such as terpenoids, alkaloids and tannins, which are a good source of antioxidant activity. Based on above results it is concluded that methanolic extract of *M. Cerviana* show strong free radical scavenging activity and is a source of potent antioxidants.

Table 2. Invitro freeradical scavenging assays

Assays	IC_{50} Ascorbate (µg/ml)	IC_{50} *M.Cerviana*(µg/ml)
DPPH	72.39	89.39
Hydroxyl radical scavenging	59.83	79.03
Ferrous iron chelating assay	51.69	75.72
Nitric oxide radical scavenging assay	54.65	76.08
Superoxide radical scavenging assay	63.90	90.86
Total antioxidant assay	62.78	91.59
ABTS radical scavenging assay	70.90	90.86

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Antibacterial and Anti-biofilm Assay of Methanolic Extract of *Mollugo Pentaphylla* Linn.

R. JIJIN AND P.G. BIJU*

Department of Biochemistry, University of Kerala, Kariavattom Campus, Thiruvananthapuram 695581, Kerala, India.
**email: pgbiju75@gmail.com*

ABSTRACT

The present study investigates the antibacterial and anti-biofilm activity on the methanolic extract of aerial parts of *Mollugo pentaphylla*. Antimicrobial activity of methanolic extract was evaluated in both gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) and gram negative (*Escherichia coli and Psuedomonas aeruginosa*) bacterial strains by disc diffusion method. Methanolic extract exhibited potent activity against gram negative and a moderate activity against gram positive bacteria. Anti-biofilm activity of Methanolic extract was examined by Crystal violet method. Methanolic extract exhibits high anti-biofilm activity against Gram negative bacterial strains and no activity were observed on gram positive strains. Anti-biofilm and anti-bacterial efficacy of *Mollugo pentaphylla* plays a vital role over biofilm producing pathogens and act as a good source for controlling microbial population.

Keywords *Antibacterial,Anti-biofilm, gram positive bacteria, gram negative bacteria, Mollugo pentaphylla.*

Medicinal plants play a vital role in disease prevention, and their promotion and use fit into all existing prevention strategies. Traditional medical systems like Siddha and Ayurvedic medicines are largely based upon herbal and herbomineral preparations and have specific diagnostic and therapeutic principles (Tripathi 2001) Antimicrobial studies have shown that gram-negative bacteria show a higher resistance to plant extracts than gram-positive bacteria. This may be due to the variation in the cell wall structures of gram-positive and gram-negative bacteria. Antibiotics are the mainstay of bacterial treatment (Ronald 2004). The goal of these drugs is to kill the invading bacteria without harming the host. Antibiotic effectiveness depends on mechanism of action, drug distribution, site of infection, immune status of the host, and resistance factors of bacteria (Ronald 2004, Roden 2001)

Biofilms are defined as microbially derived sessile communities that are characterized by the cells enclosed within the self-produced polymeric matrix (Hassan *et al*., 2011). Biofilms are formed with interaction among microbial aggregates, filamentous bacterial strains, organic and inorganic particles, which are held together by EPS (Extra cellular polymeric substance) (Slobodníkova *et.al* 2016, Subramanian *et.al* 2010). The biofilms acts as a protective barrier and provides resistance against antibiotics, degrading enzymes and host immune response (Sahwany et.al 2016, Novak *et.al* 2001). Plant extracts and other biologically active compounds isolated from leaves, stems and roots have gained interest in anti-biofilm activity (Chanda *et.al* 2011, Sedigheh *et.al* 2005, Bazzaz *et.al* 2010).

Mollugo pentaphylla (Fig.1) commonly known as "Five stem carpetweed" in English and "Parpadakam" in Malayalam is found on dry as well as moist areas. The whole plant is used as a mild laxative medicine, also as a stomachic, antiseptic and antipyretic.

The aim of this study is to investigate the antibacterial and anti-biofilm activity of *M.pentaphylla* methanolic extract against different bacterial strains.

Fig.1. *Mollugo pentaphylla* Linn

MATERIALS AND METHODS

Chemicals and Reagents

Luria Bertani Broth, Nutrient Broth, Luria Bertani Agar and Mueller Hinton Agar were purchased from HIMEDIA. All other biochemicals, chemicals and solvents were purchased from SRL, Ranbaxy and Spectrochem, India. *E.coli* (HiMedia Laboratories, India) and *B. Subtilis, S.aureus and P.aeruginosa* (Department of Biotechnology, University of Kerala, Thiruvananthapuram) were used for this study.

Plant Material

Mollugo pentaphylla.Linn samples were collected from Thiruvananthapuram district of Kerala state, India during the months of April. The specimen was authenticated by an expert (Dr. Valsaladevi, Curator, Department of Botany, University of Kerala) by comparing fresh specimen with an existing herbarium voucher submission at the herbarium of Department of Botany, University of Kerala (KUBH – 5919).

Preparation of Extracts

The aerial parts of the *Mollugo pentaphylla* were cleaned, air dried and powdered with mechanical blender for extraction. The dried powdered extract were filled in a soxhlet extraction apparatus and extracted with methanol. The apparatus was adjusted with the boiling point of 65^{0}C. The extract was then transferred to a conical flask, concentrated and evaporated to dryness in a thermostat water bath set at boiling points and obtained greenish gummy exudates (Harborne 1973). The final residue was weighed and used for further dilution at the required concentrations for further experiments.

Antibacterial Activity Assay by Disk Diffusion Method

Antimicrobial activity of the crude methanolic extract was conducted by Disc diffusion method (Bauer *et.al* 1959, Bauer *et.al* 1966). Nutrient agar was melted in hot water bath and allowed to cool. On cooling, it was poured to sterile petri dishes and allowed to solidify in horizontal position. Crude extracts were dissolved in PBS to get a final concentration of 30mg/ml. UV sterilized Whattmann no 1 filter paper disks were impregnated with 5µl extract and was allowed to dry. Sterile 24 hr Cell suspensions (*E. coli) prepared* in LB broth and *B. subtilis*, *S. aureus* and *P. aeruginosa* in Nutrient broth were used to inoculate Luria Bertani (LB) and Mueller Hinton (MH) agar plates by streaking swabs over the entire agar surface followed by the application of the respective plant extract discs. Plates were then incubated for 24 h at 37 °C. Testing was done with filter paper disks impregnated with 5µl Gentamycin (30mg/ml) and Ampicillin (30 mg/ ml) as standard antimicrobial agent controls, and sterile filter paper disks impregnated with 5µl sterile PBS served as negative control. The plates were examined after 24 hrs incubation. The zone showing inhibition was determined by gross virtual examination, and the diameter of the zone to the nearest mm was recorded with a ruler. Inhibition diameter is obtained after taking off the value of negative control from that of respective concentrations.

Anti-biofilm Activity Assay by Crystal Violet Method

Crystal Violet staining was used as an indicator of bacterial biofilm biomass. (Stepanovic *et.al* 2000). After the growth of biofilm, the wells were washed with 200 µl of 150 mM NaCl to remove weakly adherent cells. 200µl of 99% methanol were added to each well for 15 min. Methanol was removed, and the plates were allowed to dry at 25^0 C. Then 200 µl of 0.1% crystal violet were added the excess of crystal violet was removed after 5 min, and the plates were washed twice with water. Finally 33% acetic acid was added. The optical density was measured at 620 nm on a microtiter plate reader (Thermo scientific multi scan EV).

Results

Antibacterial Activity

The antimicrobial potential of *M. pentaphylla* methanolic extract (ME) was evaluated according to their zone of inhibition against various pathogens and the results were compared with the activity of the standards viz, Ampicillin and Gentamycin. 30 mg of ME of *M. pentaphylla* were found to be more effective against Gram negative strains when compared with the standards (Fig 2 and 3, Tables 1 and 2).

Anti biofilm Activity

The anti-biofilm activity of *M. pentaphylla* was evaluated by crystal violet method and the results were

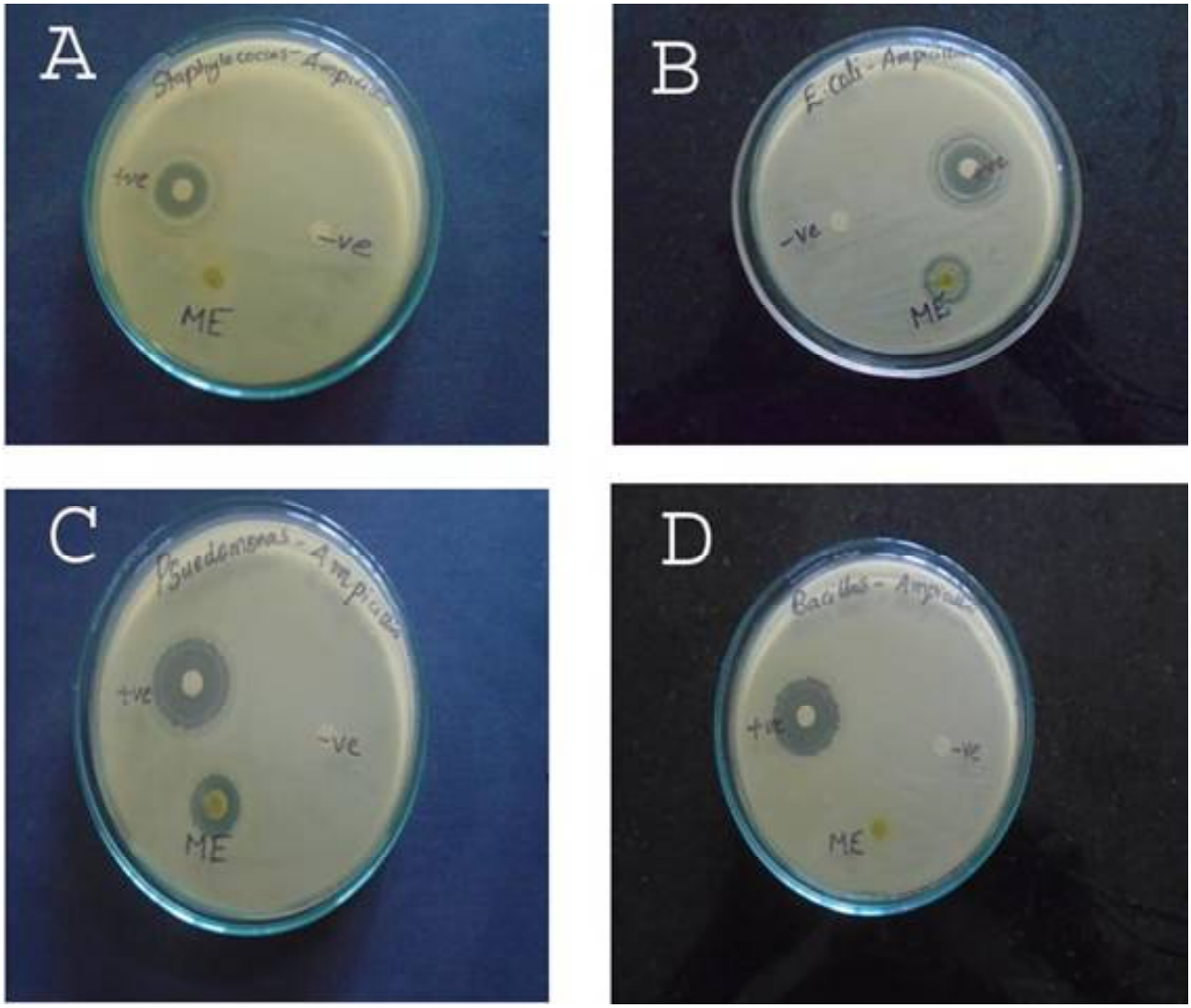

Fig. 2. Antibacterial activity of *Mollugo pentaphylla* against Gram positive and Gram negative bacteria (Standard- Ampicillin) **A:** ME of *M.pentaphylla* against *S.aureus* **B:** ME of *M.pentaphylla* against *E.Coli* **C:** ME of *M.pentaphylla* against *P.aeruginosa* **D:** ME of *M.pentaphylla* against *B.subtilis.*

compared with the activity of the standards viz, Ampicillin and Gentamycin. A dose dependent increase in the activity was observed against Gram negative strains when compared with the standards Insert. (Fig 4, 5, 6 and 7).

DISCUSSION

The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. Chemical compounds in plants mediate their effect on the human body through processes identical to those already well understood for the chemical compounds in conventional drugs; thus herbal medicines do not differ greatly from conventional drugs in terms of how they work. This enables herbal medicines to be as effective as conventional medicines, but also gives them the same potential to cause harmful side effects (Tapsell *et al.,* 2006, Lai P K, Roy J, 2004). Much recent attention has been given to traditional medicines and natural products with potential and promising antimicrobial properties (Gautam R, Jachak S M 2009, Fürst R, Zündorf I.2014, Gu R *et al., 2*014).

In this study, the antimicrobial activity of ME of *Mollugo pentaphylla* is evaluated in both gram positive and gram negative bacteria by disc diffusion method. Disc diffusion assay method of ME exhibited

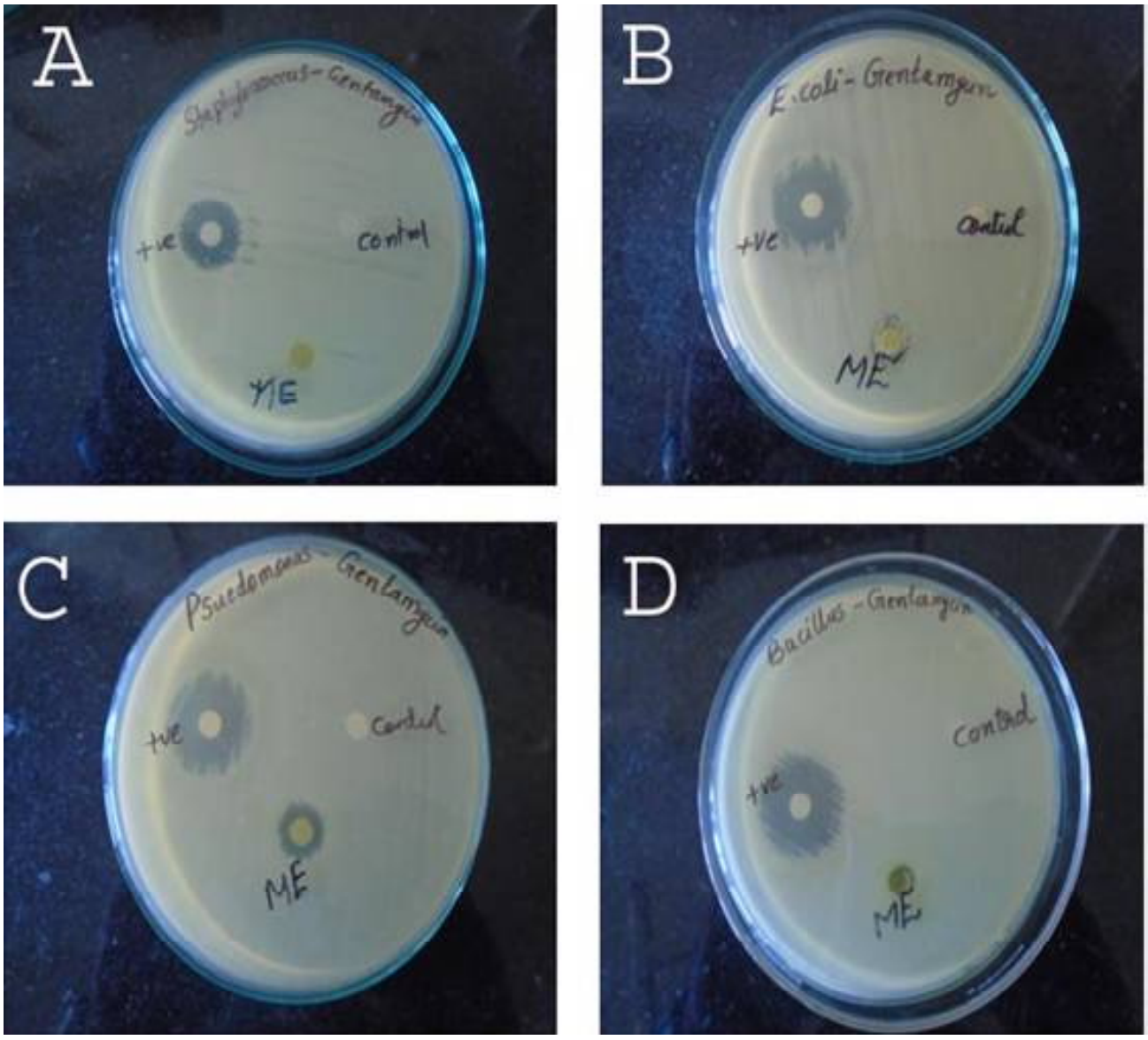

Fig. 3. Antibacterial activity of *Mollugo pentaphylla* aganist Gram positive and Gram negative bacteria (Standard- Ampicillin) **A**: ME of *M.pentaphylla* aganist *S.aureus* **B:** ME of *M.pentaphylla* aganist *E.Coli* **C:** ME of *M.pentaphylla* aganist *P.aeruginosa* **D:** ME of *M.pentaphylla* aganist *B.subtilis.*

a potent antimicrobial activity against the gram negative bacteria *E. coli* and *P. aeruginosa* and a moderate antimicrobial activity against the gram positive bacteria *B. subtilis and S. aureus*. Ability of ME to inhibit the gram negative bacteria is a clear indication of the usage of the plant extract as a gram negative antimicrobial agent. Further studies are required to reveal how these extracts exert their activity against gram negative but not positive bacteria.

Two standard antibiotics of with different mechanisms of activity were selected as positive controls. Ampicillin, a beta-lactam antibiotic is included in the Penicillin group. Mode of activity of Ampicillin is by irreversible inhibition of the enzyme transpeptidase, which is needed by bacteria to make their cell walls. Gentamycin, an aminoglycoside antibiotic composed of a mixture of related gentamycin components and fractions is used to treat many types of bacterial infections, particularly those caused by Gram-negative organisms (Moulds *et al.,* 2010). The mode of action of Gentamycin is at the 30S ribosomal level by binding irreversibly and inhibiting protein synthesis.

The Biofilm inhibition potential is a desired property that expected from herbs extracts. Anti-biofilm activity of *M. pentaphylla* against different bacterial strains was examined. Among the different doses of plant extracts selected, 70 mg/ml showed

Table 1. Zone of inhibition of *M.pentaphylla* against bacterial strains (Standard Ampicillin)

Bacterial strain	*Treatment type*	*Diameter(mm)*
E. coli	Ampicillin (+ve control)	20
	ME	13
	Vehicle(-ve Control)	0
P. aeruginosa	Ampicillin(+ve control)	20
	ME	14
	Vehicle (+ve control)	0
S.aureus	Ampicillin (+ve control)	16
	ME	0
	Vehicle(-ve control)	0
B.subtilis	Ampicillin(+ve control)	20
	ME	0
	Vehicle(-ve control)	0

maximum activity. Results indicated that *M. pentapylla* extract was more active against Gram negative bacteria than Gram positive bacteria. The similar results were also reported in *Citrus bergamia* (Mandalari et.al 2007) *Woodfordia fruticosa* (Parekh. J, Chanda. S 2007) and *Artocarpus lakoocha* (Mahalakshmi et.al 2016) showing that Gram negative bacteria were more susceptible to some plant extracts than Gram positive. This is in contrast to previous studies showed that plant extracts were more active against Gram positive bacteria than Gram negative bacteria (Vlietinck 1995, Rabe et.al 1997). The Gram negative bacteria are considered to be more resistant due to their outer membrane acting as a barrier to many environmental substances including antimicrobial substances.

Table 2. Zone of inhibition of *M.pentaphylla* against bacterial strains (Standard- Gentamycin)

Bacterial strain	*Treatment type*	*Diameter(mm)*
E. coli	Gentamycin (+ve control)	22
	ME	12
	Vehicle(-ve Control)	0
P. aeruginosa	Gentamycin (+ve control)	22
	ME	12
	Vehicle (-ve control)	0
S.aureus	Gentamycin (+ve control)	16
	ME	0
	Vehicle (-ve control)	0
B.subtilis	Gentamycin (+ve control)	22
	ME	2
	Vehicle (-ve control)	0

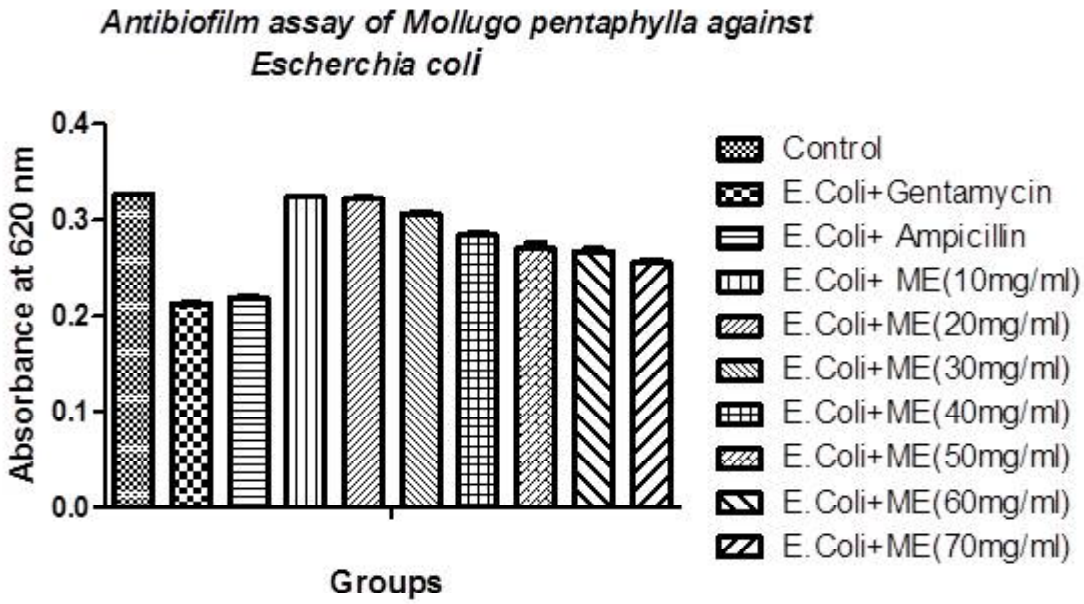

Fig.4. Anti-biofilm assay of *M. Pentaphylla* against *E. Coli*

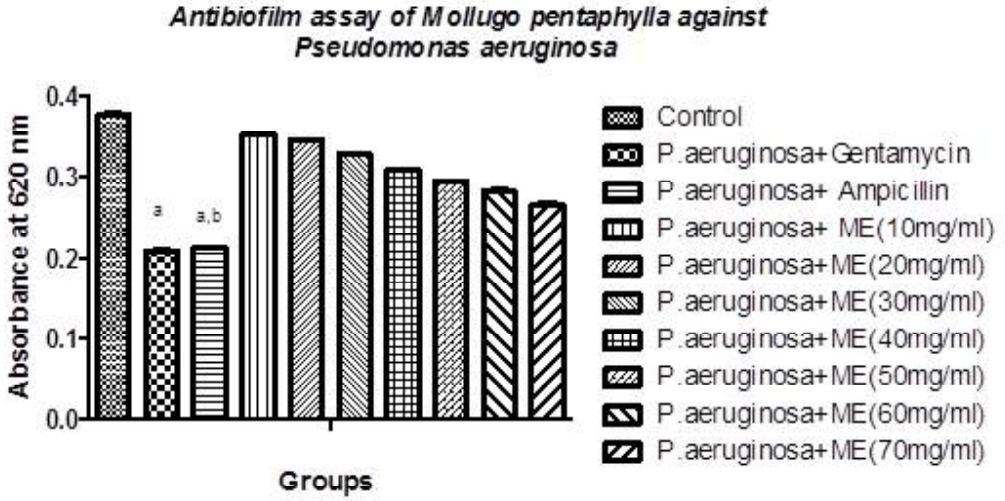

Fig.5. Anti-biofilm assay of *M. Pentaphylla* against *P. aeruginosa*

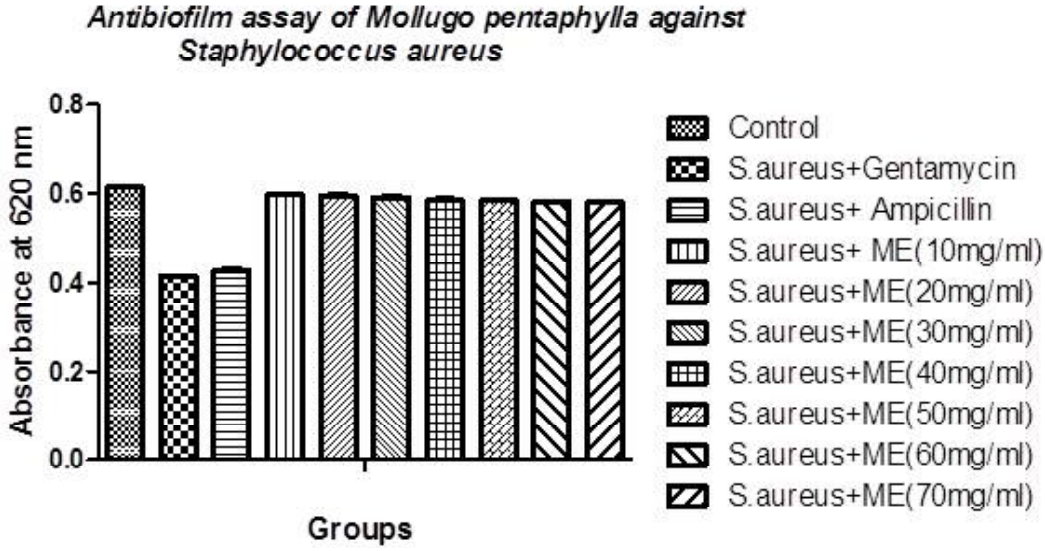

Fig.6. Anti-biofilm assay of *M. Pentaphylla* against *S. auerus*

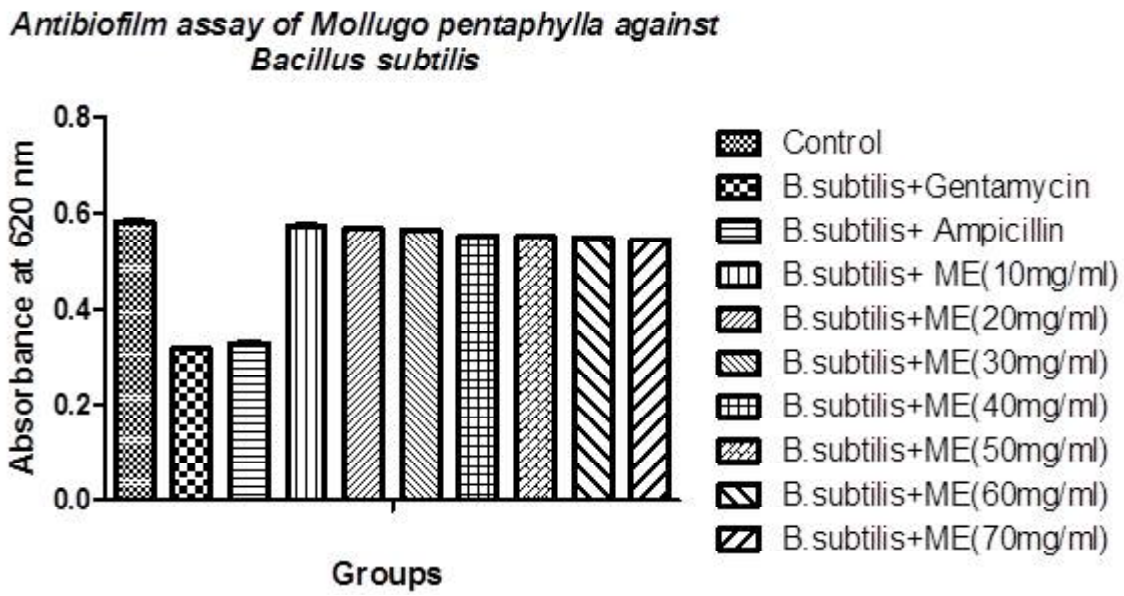

Fig.7. Anti-biofilm assay of *M. pentaphylla* against *B. subtilis*

Values are expressed as mean ± SEM of triplicate readings.

Based on the observed results, ME of *M. pentaphylla* was found to be a promising source of potent antimicrobials. Future fractionation and characterization of this fraction and identification of individual phytochemical for antimicrobial activity needs to be carried out for the unravelling of novel antimicrobial lead molecules.

CONCLUSION

The study suggests that Methanolic extract of *M. pentaphylla* possess compounds with potential antimicrobial properties. To conclude, the methanolic extract of *M. pentaphylla* showed high anti-biofilm and antibacterial activity against gram negative bacterial strains. Further research is needed to elucidate the antimicrobial agents and their mode of activity from *Mollugo pentaphylla.*

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Study on Heavy Metal Content in Water and Selected Freshwater Fish Tissues of Two Ponds Around an Industrial Area in Kollam, Kerala, India

D. MEERA[1*], SHERLY P ANAND[2] AND SANALKUMAR MG[3]

[1]*P.G. and Research Department of Zoology,*
S.N. College, Kollam- 691001, Kerala, South India
[2]*TKMM College, Nangiyar Kulangara , Haripad, Kerala, South India*
[3]*Department of Zoology, N. S. S. College, Pandalam, Kerala, South India*
email:meeradivakaran@gmail.com[1] ; drsherlypanand@gmail.com[2]

ABSTRACT

The present study explains about the heavy metal pollution of two urban ponds in Panmana, Chavara, Kollam district. The heavy metal content of the water system was studied seasonally during December 2013 to November 2014 using AAS. From the analysis, it was observed that permissible limit of metals like Mn, Fe, Pb and Ni was found to be high in the water bodies when compared to the allowable limit prescribed by Indian standards. Metal distribution pattern in fish tissue (gill and Muscle) was also analysed using AAS. Heavy metals such as Fe, Mn, Cr and Ni in gill and liver tissue of Anabas from station 1 and station 2 were above the permissible level set by IAEA-407. This study detects the presence of various heavy metals in water and its effect on selected fish species inhabiting there. Gills and muscle tissues of the fish were selected to study the effect of metal toxicity and its accumulation.

Keywords *Heavy metal, fish tissue, gill, liver, accumulation.*

The environmental contaminations of nature all around the world, especially in urban areas are frequently high due to the pollution discharge (White and Rasmussen, 1998). Industries release their effluents into the environment devoid of any pollution treatments procedures. Hence, these effluents are the source of different chemical contaminants that will pollute that environment (Rank and Nielsen, 1998). The concentration of heavy metals in natural water is now increasing several folds because of severe industrial and agricultural actions (Jordao et al., 2002). The burning of fossil fuels, domestic wastes runoff, and mining are the other sources that add the heavy metals to the water systems. These toxic chemicals can be accommodated merely into the food chain because of their long persistence and later undergo bioaccumulation and biomagnifications processes (Ashraf et al., 2012). Hence, the status of any aquatic ecosystem can be improved only through systematic screening of water systems by examining the factors that impair the natural environmental conditions of that watershed.

Chemical analysis of toxic compounds from water alone is not adequate to illustrate the undesirable property of the various chemical combinations on polluted water systems. Some portions of these pollutants assimilate into the aquatic biota when they are in the bioavailable form. Accumulation of these chemical compounds in tissue or organs of an organism using all directions (from water, food, and sediment) accessible to the organism is termed as bioaccumulation (Landrum, 1989). In a body, the bioaccumulation studies reveal the type of metal entry, the amount of metal uptake, and their distribution pattern in different tissues (Murugan et al., 2008). Organic or inorganic pollutants when entered into the aquatic ecosystems, the aquatic biota especially fishes of that ecosystem are probably under contamination because of its bioaccumulation ability (Adeyeye E 1993). Also, it is unpredictable to assess the probable effect of this toxic chemical to the inhabiting organisms and their consumers (Van der Oost et al., 2003). Using biomarkers are proficient in distinguishing the effects of environmental contaminant exposure by persistent or severe intensity, without former awareness of the toxicants there in the aquatic system. Thus, the possible benefit of using biomarkers for examining together the quality of aquatic ecosystems and physical condition of aquatic inhabitants in the contaminated water bodies now seeks attention by researchers in past years (Mdegela et al., 2006).

MATERIAL AND METHODS

Study area and sampling procedure

For heavy metal analysis, water samples from two freshwater ponds labelled as station 1 (S1) and station 2 (S2) were collected seasonally during pre-monsoon, monsoon, post-monsoon and summer from December 2013 to November 2014. Water samples were collected in clean, white coloured polyethene one-litre cans. After labelling, the samples were

Table 1. Showing the Permissible level of metals in water and fish tissue by various organisation

Indian standard (mg/l) (BIS)	**PL of Heavy metals in water**					
	Zn	Mn	Fe	Cd	Pb	Ni
	15	0.3	0.3	0.01	0.05	0.02
IAEA-407 (mg/kg)	**PL of Heavy metals in tissue**					
	Zn	Mn	Fe	Cd	Pb	Ni
	67.1	3.52	146	0.19	0.12	0.60

PL= Permissible limits (In dry wt. in mg/kg),

transported to the laboratory for more analysis.

Gill and muscle tissue of *Anabas testudineus* were selected for studying the intensity metal accumulation in the selected water systems. The two pond systems are of freshwater nature and are seen around the Panmana panchayat in Chavara taluk, southwest coast of India. Fishes of identical size and similar weight were collected from the local fisherman from both the ponds. Collected fishes were directly preserved in ice box and transported to lab and preserved in -10 ^{o}C advance analysis

Metal determination technique

From water samples, heavy metals like Zn, Mn, Fe, Cd, Pb, Ni were analysed according to the standard procedures of APHA using atomic absorption spectrometer. The accumulation pattern of metals like Zn, Mn, Fe, Cd, Pb, Ni in gill and muscle tissue of *Anabas testudineus*, was also analysed according to the standard procedure of APHA using Atomic Absorption Spectrophotometer (APHA, 2012)

STATISTICAL ANALYSIS

Seasonal variation of heavy metal content in water samples were statistically examined, and the result was interpreted using Two Way Analysis of variance (ANOVA). One way ANOVA was used to point out whether there is any significant difference in heavy metal concentration and with different fish tissues. Entire Statistical analysis was performed using SPSS 16.0

RESULT AND DISCUSSION

Heavy metal content in water

The concentrations of heavy metals such as Zn, Mn, Fe, Cd, Pb and Ni from two different freshwater pond systems collected in 4 seasons are given in Table 2 to Table 7. Between the two stations, the highest concentration of Zn was observed in S1 during summer season (2.78mg/l) and lowest in S2 (0.07mg/l) (Table 2). Two way ANOVA shows that Zinc had no significant variation between stations and between season at($P>0.05$). The concentration of Zn was found to be below the permissible level in both the station in

Table 2. Two way ANOVA showing Seasonal variations of Zinc(mg/L) in different stations in pond water ecosystems during December 2013 to November 2014

Site	Summer	Pre monsoon	Monsoon	Post monsoon	Total	F		p	
						season	Site	season	Site
Site 1	2.78 ± 0.18	1.77 ± 0.05	0.94 ± 0.04	1.86 ± 0.6	1.84 ± 0.73	27.71	3.89	p> 0.05	p> 0.05
Site 2	0.7 ± 0.01	0.37 ± 0.03	0.07 ± 0	0.73 ± 0.02	0.47 ± 0.28				

Table 3. Two way ANOVA showing Seasonal variations of Manganese (mg/L) in different stations in pond water ecosystems during December 2013 to November 2014

Site	Summer	Pre monsoon	Monsoon	Post monsoon	Total	F		p	
Site 1	1.57 ± 0.09	0.74 ± 0.04	0.08 ± 0.01	1.76 ± 0.11	1.03 ± 0.7	season	Site	season	Site
Site 2	0.92 ± 0.04	0.42 ± 0.01	0.17 ± 0.05	0.74 ± 0.09	0.56 ± 0.31	4.035	5.39	p> 0.05	p> 0.05

all seasons. Allowable limit of Zn in water was shown in Table 1. Low concentration of Zinc in natural water might be due to its restricted mobility in water systems (Nazir et al., 2015)

In the case of Manganese, among the two stations, the highest concentration of Mn was observed in S1 during post-monsoon (1.76 mg/l) and lowest in the monsoon from S2 (0.17mg/l). Two way ANOVA (Table 3) shows that Mn had no significant variation between stations and between season at (P >0.05). According to IS bureau, except in monsoon season, the concentration of Mn in both the station was found to above the permissible limit.In monsoon due to high rainfall, dilution of water will results in decreased level of Mn in water samples. The high concentration of Mn in both stations could be a clear indication of industrial pollution in this area. In animals, highest concentration of Mn will develop neuronal issues (Abbasi et al.,1998)

In the case of Iron, both stations showed the comparatively high concentration of Fe in water. The highest level of Fe was observed in S1 during summer (17.16 mg/l) and lowest in the monsoon from S2 (5.5mg/l). Two-way ANOVA (Table 4) shows that Fe showed a significant variation between stations and between season at(P <0.05). The concentration of Fe was found to be above the permissible limit prescribed by BIS in both the locations. The presence of high level of iron in water is substantial evidence for industrial pollution of this area.

In the case of Cd, both stations showed a comparatively low concentration of Cd in water. The concentration of Cd was found to be uniform in S1.

Table 4. Two way ANOVA showing Seasonal variations of Iron(mg/L) in different stations in pond water ecosystems during December 2013 to November 2014

Site	Summer	Pre monsoon	Monsoon	Post monsoon	Total	F		p	
Site 1	17.16 ± 0.6	14.78 ± 0.49	10.59 ± 0.75	15.06 ± 0.55	14.4 ± 2.54	season	Site	season	Site
Site 2	11.2 ± 0.06	10.16 ± 0.03	5.5 ± 0.06	11.65 ± 0.11	9.63 ± 2.55	79.11	30.17	P <0.05	P <0.05

Table 5. Two way ANOVA showing Seasonal variations of Cadmium (mg/L) in different stations in pond water ecosystems during December 2013 to November 2014

Site	Summer	Pre monsoon	Monsoon	Post monsoon	Total	F		p	
Site 1	0.02 ± 0	0.02 ± 0	0.01 ± 0	0.02 ± 0	0.02 ± 0	season	Site	season	Site
Site 2	0.01 ± 0	0.01 ± 0	0 ± 0	0.01 ± 0	0.01 ± 0	2.21	5.53	p> 0.05	p> 0.05

Table 6. Two way ANOVA showingSeasonal variations of Lead (mg/L) in different stations in pond water ecosystems during December 2013 to November 2014

Site	Summer	Pre monsoon	Monsoon	Post monsoon	Total	F		p	
						season	Site	season	Site
Site 1	0.12 ± 0	0.08 ± 0	0.04 ± 0.02	0.08 ± 0	0.08 ± 0.03				
Site 2	0.03 ± 0	0.02 ± 0	0.01 ± 0	0.01 ± 0	0.01 ± 0	25	2.70	P <0.05	p> 0.05

In S2 Cd was found to be at the very low level. Two-way ANOVA (Table 5) shows that Cd does not have a significant variation between stations and between season at(P >0.05). In S1 except for monsoon season, the concentration of Cd was found to be high in all other seasons and this could be due to the dumping of high industrial effluent in this area. But in S2 the observed concentration of Cd was below the permissible limit set by Indian standard (Table 1). Cd when entering into an aquatic system will remain for a very extended time. Metal processing industry, refineries are the primary sources for Cd pollution (WHO,2011)

In the case of Pb, the station showed a comparatively low concentration of Cd in water. The concentration of Cd was found to be uniform in most of the seasons in S1. In S2 Cd was in very low level. Two-way ANOVA (Table 6) shows that Cd does not have a significant variation between stations and between season at(P >0.05). In S1except in monsoon season, Pb was found to be above the permissible limit in all another season, and this could be due to pollution aspect. But in S2 the concentration of Pb was found to be below the allowable limit set by Indian standard (Table 1). Many researchers documented that in water systems the high level of Pb could be mainly from chemical fertilisers, battery wastes etc (Aboud et al.,2009)

In the case of Ni, a comparatively high concentration of Ni was observed in station one. In S1the concentration of Ni was found to be high in post-monsoon (1.08 mg/l) followed by summer (1.07 mg/l). In S2 Ni concentration was found to be high in post-monsoon (0.35mg/l). In both the stations, in all seasons the concentration of Ni was found to be above the permissible limit prescribed by BIS (Table 1). The most common source of Nickel in water bodies likely due to the nickel alloy manufacturing units (ATSDR). The high concentration of Ni in water samples could be due to the discharging of nearby industrial wastewater into this area Two way ANOVA (Table 7) shows that Ni shows a significant variation between seasons (P < 0.05) but had no such considerable variation between site at(P >0.05).

Bioaccumulation status of different heavy metals such as Zn, Mn, Fe, Cd, Pb and Ni was observed in the gill, and muscle tissue of Anabas from station one and two was showed in Table 8 & 10. From the analysis, it was found that in both sites (S1 and S2) the concentration of Zn in gill and muscle tissue was below the permissible limit prescribed by IAEA. The concentration of Mn, Fe and Ni in gill and muscle tissue of S1 and S2 was found to be above the permitted limit specified by IAEA. In the case of Cd, the measured concentration of Cd was found to be below the permissible threshold in both gill and muscle

Table 7. Two way ANOVA showingSeasonal variations of Nickel (mg/L) in different stations in pond water ecosystems during December 2013 to November 2014.

Site	Summer	Pre monsoon	Monsoon	Post monsoon	Total	F		p	
						season	Site	season	Site
Site 1	1.07 ± 0.01	0.77 ± 0.02	0.09 ± 0	1.08 ± 0.05	0.75 ± 0.42				
Site 2	0.27 ± 0.02	0.16 ± 0.01	0.06 ± 0	0.35 ± 0.03	0.21 ± 0.12	9.57	2.77	p> 0.05	p> 0.05

Table 8. Scheffe Multiple Comparison test showing the variation in Heavy metals concentration between gill and liver from site 1

Site	Metal	Tissue		Scheffe Multiple Comparisons			No
				Pair	F`	p	
Site 1	Zinc (ppm)	Gill (A)	14.41	A & B	489**	0.000	10
		Muscle (B)	9.66				
	Manganese(ppm)	Gill (A)	7.22	A & B	2007.4**	0.000	10
		Muscle (B)	5.21				
	Iron (ppm)	Gill (A)	236.10	A & B	28.5**	0.000	10
		Muscle (B)	227.03				
	Cadmium (ppm)	Gill (A)	0.03	A & B	165.1**	0.000	10
		Muscle (B)	0.01				
	Lead (ppm)	Gill (A)	0.37	A & B	685.4**	0.000	10
		Muscle (B)	0.12				
	Nickel (ppm)	Gill (A)	2.06	A & B	11.1**	0.000	`10
		Muscle (B)	1.83				

**: - Significant at 0.01 level

tissue. The level of lead was found to be high in gill and liver of S1. However, for S2 Pb was below the permissible limit set by IAEA in both the tissues.

Researchers have conducted various bioaccumulation studies to understand the possible effects, mechanisms, and abnormalities due to bioaccumulation of heavy metal in aquatic biota.Bioaccumulation and the exposure of metals will always result in trophic transmission. Many research documented the bioaccumulation status of heavy metals in diverse fish tissues (Khan et al.,2012,). Accumulation of metals in aquatic biota always causes metal toxicity (Marsden et al., 2004). In this study, the very high concentration of Fe was obtained in both gill, and Muscle tissue of fish analysed. A similar finding was reported by Censi et al. (2006).

Heavy metal like cadmium (Cd), lead (Pb), nickel (Ni) are considered as non-essential elements. They do not have any recognised biological role (Chang et al.,1996, Türkmen et al ., 2005). Heavy metals when present in an incredibly narrow concentration can be toxic to all living forms. Excess concentration of Ni can induce respiratory distress by damaging the gill and weaken the branchial gas exchange ultimately leading to normal breathing impairment and suffocation (Kennedy,2011). Ni acts as a competitive inhibitor of Fe because of its comparable ionic radii (Chen et al., 2005). Lead is a toxic heavy metal determined by various authors in many bioaccumulation studies (Goyer et al., 1995).

Various research works showed that cells when exposed to metals and metalloid species, different signalling pathways were being evicted (Davidson et al., 2009). Two-way ANOVA table (Table 9) shows that the metals concentration from S1 does not indicates a significant variation at ($P > 0.05$) but had considerable variation between tissues at($P > 0.01$). Scheffe, Multiple Comparison tests, was also

Table 09. Scheffe Multiple Comparison test showing the variation in Heavy metals concentration between gill and liver from site 2

Site	Metal	Tissue		Scheffe Multiple Comparisons			No
				Pair	F`	p	
Site 2	Zinc (ppm)	Gill (A)	1.44	A & B			10
		Muscle (B)	0.64		934.2**	0.000	
	Manganese(ppm)	Gill (A)	3.78	A & B			10
		Muscle (B)	3.63		10.8**	0.000	
	Iron (ppm)	Gill (A)	232.19	A & B			10
		Muscle (B)	207.21		428.1**	0.000	
	Cadmium (ppm)	Gill (A)	0.01	A & B			10
		Muscle (B)	0.00		33.9**	0.000	
	Lead (ppm)	Gill (A)	0.05	A & B			10
		Muscle (B)	0.01		406.3**	0.000	
	Nickel (ppm)	Gill (A)	0.86	A & B			``10
		Muscle (B)	0.63		646.7**	0.000	

**: - Significant at 0.01 level

conducted to find the difference of each metal between gill and muscle tissue from site 1 (Table 8). Scheffe, Multiple Comparison tests, shows that there exists significant variation between each metal between selected tissues (gill and Muscle) at $p<0.01$.

In the case of S2 also the two way ANOVA table (Table 11) does not show a significant variation between the selected metals at $p>0.05$ but show substantial variation between tissues at($P >0.01$). Scheffe Multiple Comparison tests (Table 10) was used to prove this variation in metal between the fish tissue and reveals that there exist a significant change of each metal between liver and gill at $p<0.01$.

Bioaccumulation mainly depends on the uptake and retention of heavy metals in the body of the organism from its environment. It is always better to analyse specific tissues for bioaccumulation studies than considering the whole organism since it will accurately provide the exact result of metal accumulation process in a body. Examining particular tissues for bioaccumulation studies also offer the ability of each organ to extract the pollutant to accumulate in it.

In the fish body, gill is the principal site for uptake of metals because of the presence of negatively charged gill membrane proteins on the gill array. This peculiar anatomical nature makes them highly sensitive to bind with cations. Other properties make the gill a dominant site of metal accumulation are; the high countercurrent flow of blood and water, upper captivating surface area, and the minute diffusion space flanked by blood and water (Hayton and Barron, 1990). Metals often targeted to ion transporters in the gill stricture and found their way of entry into the animal body (McDonald and Wood, 1993). During the rapid and sharp toxic metal exposure, gill's normal histological nature gets damaged to result in declining the gas exchange efficiency and exchange of essential ions results in the death of that organism. Bioaccumulation of heavy metals (Cr, Mn, Fe, Co, Ni, Cu, Zn, Cd and Pb) in vital organs (gill, liver and muscle) of Mastacembelus armatus from river Ganga, in Varanasi, was reported by Pandey et al., (2017) and found out that metal pollution index was highest in gill followed by muscle.

Muscles are the main fish tissue consumed by humans. Metals when gathering in fish muscles can induce an alteration in histo architectural level, dropping of glycogen content. Muscles are one of the recessive sites for metal accumulation (Alhashemiet al., 2012). Analysis of muscle tissue is one of the important pollution screening agenda that is proficient in noticing the tendency of measuring the concentration of metal contaminants in the aquatic systems. Muscles tissue

of Nile tilapia was collected from drainage canals of Saudi Arabia for measuring the level of zinc (Zn), cadmium (Cd), cobalt (Co) and lead (Pb) seasonally. Cd was found to be high in the fish muscle samples (E.M. Younis et al., 2014)

CONCLUSION

The result of the present study showed that the two-water body selected for the study is highly polluted by various heavy metals. Elevated concentrations of heavy metal in water samples provide the clear indication of the metal pollution aspect from this area. Fish tissue analysis also indicates the bioaccumulation status of heavy metals in this study sites. Bioaccumulation of heavy metal in fish tissue is mainly due to water pollution in that system. Most of the heavy metal detected from these water samples is beyond the desirable limit of drinking water standards prescribed by BIS. Heavy metal concentration in Gill and muscle tissue of Anabas was also above the desirable limit of standards set by IAEA. The possible reason for the presence of heavy metals in the water and fish tissue could be due to the discharge of untreated industrial wastewater. Hence, it is recommended that public awareness programme should be given to the local peoples for avoiding consumption of fish from this area and using this water for duck rearing or cattle-rearing purposes. The governing bodies should implement sustainable steps for monitoring secure and proper waste disposal strategies to avoid more pollution.

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Changes in Moisture Content and Phytochemicals During Embryogeny in Desiccation-Tolerant *Vigna unguiculata* and Desiccation-Intolerant *Syzygium cumini*

PARVATHY S NAIR*, AJITH KUMAR K.G., GAYATRI G.P, SUNIL KESAVA DETH G., DINESH BABU, K.V.,

**P G Department of Botanyand Reasearch centre, Government College for Women, Vazhuthakaud, Trivandrum.695014, Kerala, Kollam*
email : snairparvathy92@gmail.com

ABSTRACT

***Syzygium cumini* is a tropical endangered evergreen tree species known for its edible fruits and medicinal properties. Seed recalcitrance is a major challenge for the natural regeneration and germplasm conservation of this species. The present work was carried out with a view to understand the seed physiology during embryogeny of this desiccation-intolerant species and to compare it with the desiccation-tolerant *Vigna unguiculata.* The results indicate that the seeds of *Syzygium cumini* maintained higher moisture content in the embryonal axis and cotyledonary tissues during embryogeny. The seeds were dispersed with higher moisture content and the seeds were desiccated very quickly under natural desiccation indicating the lack of innate mechanism to prevent water loss. The critical water level of the embryo was found to be 58.86% and the seeds lost viability for about 8 days under natural desiccation. However, the water content in the embryonal axis and cotyledonary tissues of desiccation-tolerant *Vigna unguiculata* decreased sharply during embryogeny. Changes in phytochemicals were also observed during embryogeny in the seeds of both species.**

Key words *Orthodox seed, Recalcitrant seed, Desiccation intolerant, Desiccation tolerant, Fourier Transform Infrared Spectrometry, prerequisite, Critical Water Level, Syzygium cumini, Vigna unguiculata,*

Syzygium cumini (L) Skeels., commonly known as Jamun or Indian black plum is an evergreen tropical tree belonging to family Myrtaceae. It is native to the Indian Subcontinent. The tree starts flowering during March- April and set seeds in the May – June. Mature fruit is a fleshy, purplish berry, 20mm in diameter and 25 mm long containing a single seed(Arathi *et al.,* 1996). Some fruits have 2 to 5 seeds tightly compressed within a leathery coat and some are seedless (Mortan, 1987). The seeds are recalcitrant and viability is lost very quickly within a few days of their dispersal (Srimathi *et al*., 2001). This poses great challenge to their natural regeneration and conservation as the seeds cannot be stored for a longer period of time. Seed recalcitrance is a major threat in the regeneration of many tropical species and many efforts have been made to understand the exact mechanism of seed recalcitrance. The present investigation was mainly aimed at understanding the seed physiology and change in phytochemicals during the embryogeny of desiccation-intolerant seeds of *Syzygium cumini* and its comparison with the desiccation-tolerant seeds of *Vigna unguiculata* var *sesquipedalis*.

MATERIALS AND METHODS

The species *Syzygium cumini* was selected for the study, as it is highly recalcitrant and sensitive to desiccation. Fruits at different stages of maturity such as immature, mature, and fully mature are collected and de-pulped. Fully mature fruits are collected and allowed to natural drying. Desiccation had been given for four days and eight days. Hundred healthy seeds were taken randomly and dissected to remove embryos for FTIR analysis. From the mature seed lot, twenty seeds were randomly taken for viability test using tetrazolium salt (fig 2). Ten seeds were randomly taken for the determination of moisture content. Fifty mature seeds were arranged in five rows in a plastic tray containing sterile coir pith and the tray was kept in a growth chamber for germination studies (fig 1). The same methodology was adopted for just fallen seeds also. The remaining mature seeds were allowed to desiccate under natural conditionsfora period of eight days. Every two days, desiccated seeds were randomly taken for the determination of moisture content. Fifty desiccated seeds were taken for germination studies and ten seeds were dissected to remove embryos for FTIR analysis.

A. Viability test(Tetrazolium test)

For determining seed viability, tetrazolium test

(fig 2) was employed. This test provides an indication of whether the seed is alive or dead. Ten seeds were cut open to expose embryos and were soaked in 1% solution of tetrazolium salt(2, 3, 5-Triphenyltetrazolium chloride) purchased from Sigma Chemicals, USA, for 12 hours and kept at 4°C. Generally the chemical form a red insoluble compound in contact with living tissues.

B. Moisture Content Analysis

For determining the moisture content in the embryos, five seeds were taken randomly from the seed lot and were oven dried at 100°C. The dry weight was taken periodically to get consistent result. The moisture content was calculated in wet basis using the following equation.

By deducting the moisture content percentage we can find out the biomass of the seeds also.

MC= (WW-DW)/(WW x 100): MC -The percentage of moisture content; WW -Fresh weight of sample/initial weight of sample (gm); DW -Dry weight of sample/final weight of sample (gm)

D. FTIR Spectroscopic analysis

For FTIR analysis, the seeds were thoroughly washed in distilled water and the seed coat was removed. The embryo was chopped and ground in methanol using mortar and pestle. The homogenized sample was centrifuged and the supernatant was taken. The methanol was evaporated in a rotary evaporator and the sample was dispersed in dry potassium bromide. The mixture was mixed in a mortar and pressed at pressure of six bars to form a KBr thin disc. The disc was placed in a sample cup of a diffuse reflectance accessory. The IR spectrum was obtained using Perkin Elmer 2000 Infrared spectrophotometer. The sample was scanned from 400 to 4000cm-1 for sixteen times to increase the signal to noise ratio. Samples were run in triplicate and data were collected within one day (Manigandan *et al*., 2015; Marimuthu and Gurumoorthi, 2013).

RESULTS AND DISCUSSION

a. Change in moisture content during embryogeny and embryo desiccation in the desiccation-intolerant seeds of *Syzygium cumini*

Significant changes have been observed in the moisture content of the embryonal axis and cotyledonary tissues of *Syzygium cumini* during embryogeny and embryo desiccation (fig. 3). The immature embryonal axis had higher moisture content of 73.47% while the cotyledonary tissue had only 69.85%. As the seed attained maturity, the moisture content in the axis and cotyledons slightly dropped to 72.72% and 65.71% respectively. There was an increase in the level of moisture content from 72.72%

Fig 1. Germination trial of *Syzygium cumini* seeds in sterilized coir pith.

Fig 2. Tetrazolium test to show the viability of the embryos of Syzygium *cumini*

to 83% in the embryonal axis just before seed dispersal while the cotyledons showed a sudden drop in moisture content from 65.71% to 60%. The maintenance of higher moisture content in the embryonal axis and also the filling up of more than half of the volume of seed with embryonic tissue are unique to recalcitrant seeds.

When the seeds were allowed natural desiccation for two days, there was a rapid loss of water from both embryonal axis and cotyledons indicating the lack any innate mechanism to counter water loss due to dehydration. The rate of dehydration was much quicker for cotyledons as it dropped sharply to 33%

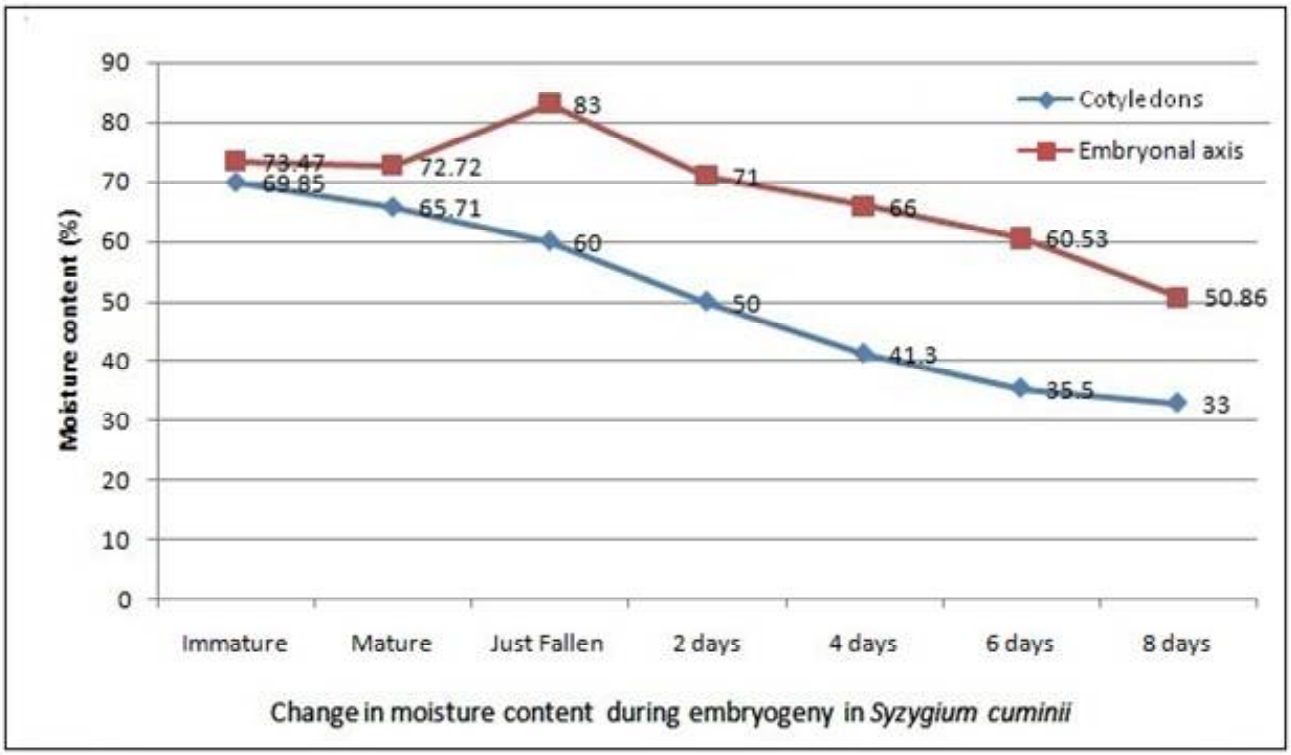

Fig 3. Changes in moisture content in the embryonal axis (red) and cotyledonary tissues (blue) of *Syzygium cumini* during embryogeny and embryo desiccation.

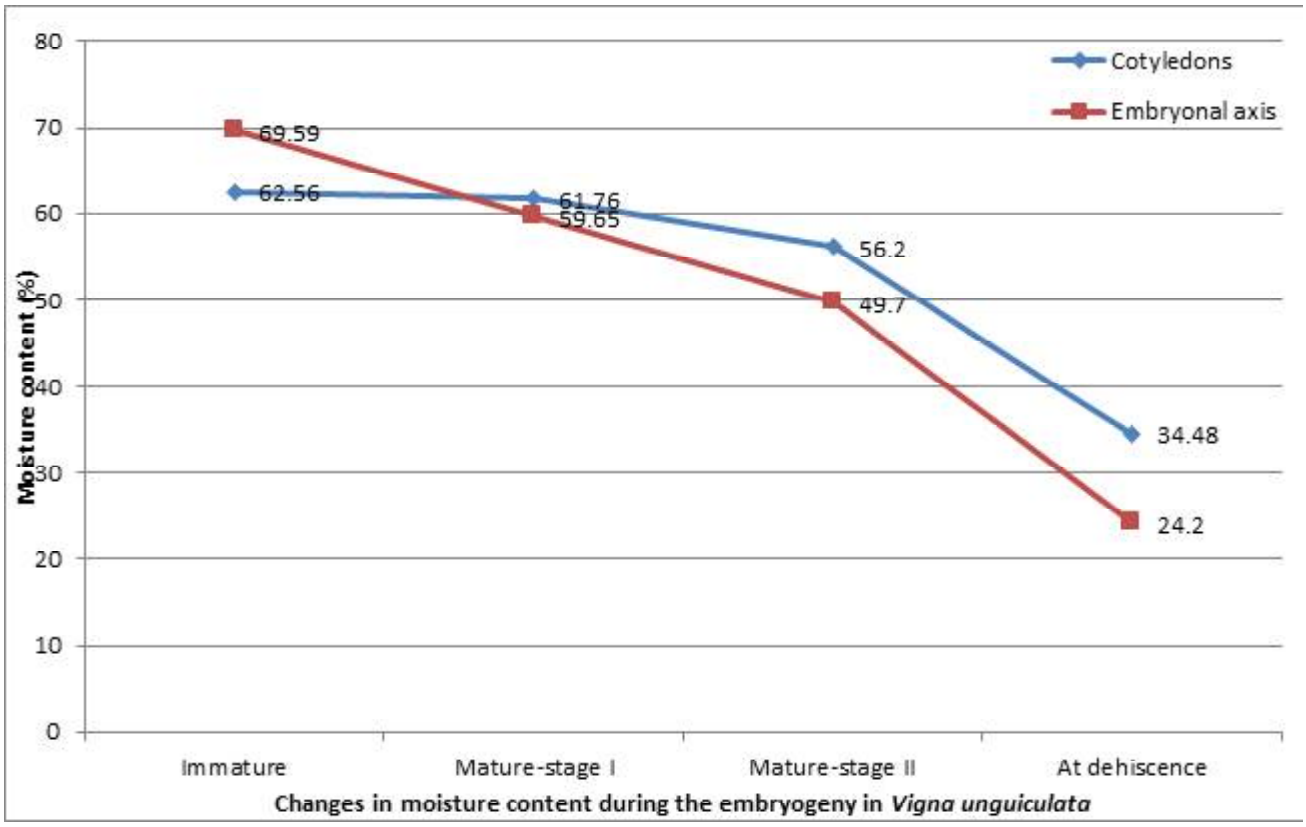

Fig 4. Changes in moisture content in the embryonal axis and cotyledonary tissues of *Vigna unguiculata* during embryogeny.

after 8 days of desiccation. However, the drop in moisture content was slower in the embryonal axis with a reduction from 83% to 50.86% indicating the role of cotyledonary tissue in protecting embryonal axis from desiccation water loss in order to maintain the viability.

b. Change in moisture content during embryogeny in the desiccation-tolerant seeds of *Vigna unguiculata* var *sesquipedalis*

Changes in the moisture content during embryogeny of desiccation-tolerant *Vigna unguiculata* have been studied in order to make comparison with desiccation-intolerant *Syzygium cumini* and the results are shown (fig. 4). In *Vigna unguiculata*, there was a sharp decline in water content in the embryonal axis and cotyledons during embryogeny. At the time of dehiscence, the embryonal axis had vey lower water content (24%) compared to desiccation-intolerant *Syzygium cumini.*

However, cotyledon maintained higher water content of 34.48%. Maintenance of lower water content in the axis and higher water content in the cotyledons at the time of seed dispersal is contrary to what has been observed in desiccation-intolerant *Syzygium cumini* in which the cotyledon had lower watercontentthan the axis.

c. Biomass accumulation during embryogeny in desiccation-tolerant *Vigna unguiculata* and desiccation-intolerant *Syzygium cumini*

The biomass accumulation during embryogeny in the desiccation-tolerant *Vigna unguiculata* and desiccation-intolerant *Syzygium cumini* has been studied in order to understand any variation exists between these two types of seeds during embryo maturation. According to the results (fig. 5), there was a tendency for the desiccation-tolerant *Vigna unguiculata* seeds to increase biomass in the embryonal axis and cotyledons right from immature stage to maturity stage II. The apparent increase in biomass from mature stage II to dehiscence was due to maturation drying, a prerequisite for the germination in desiccation-tolerance of seeds. The desiccation-intolerant *Syzygium cumini* also showed a similar response during the initial phases of embryogeny but the biomass was found to be accumulating in the cotyledons during the later stage of embryo maturation while it was almost maintained in the embryonal axis.

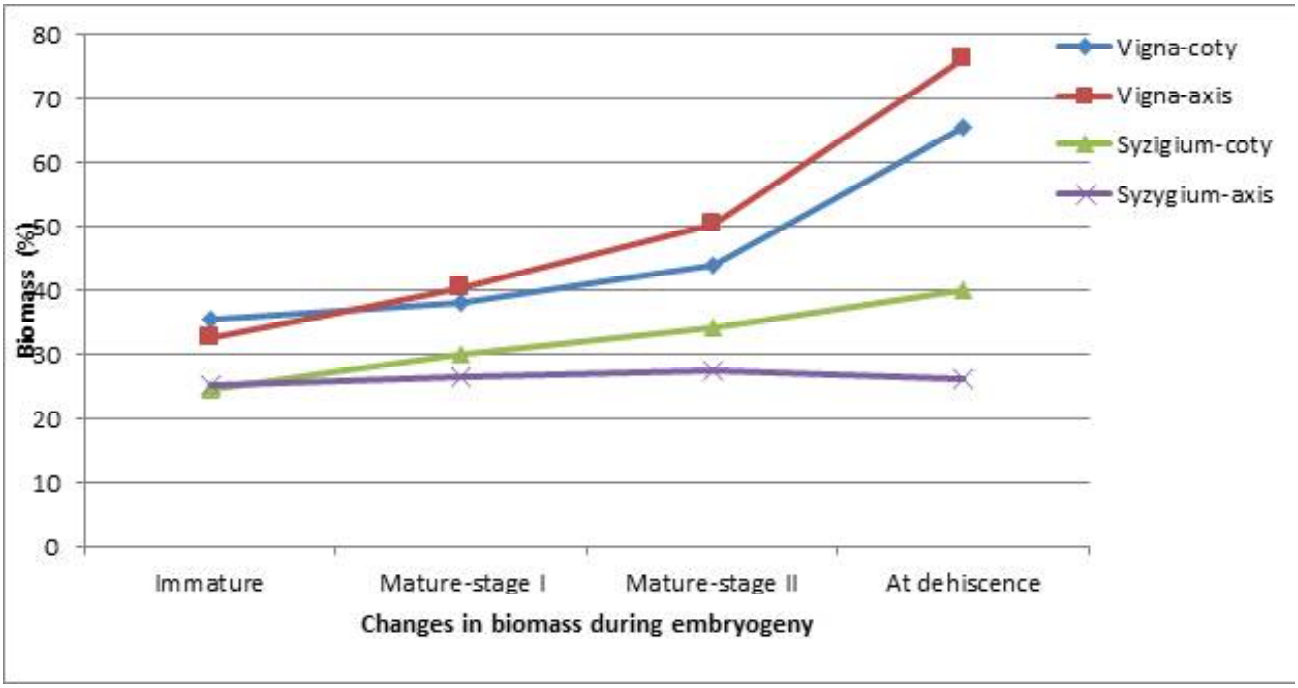

Fig 5. Changes in biomass in the cotyledons and embryonal axes of desiccation-tolerant *Vigna unguiculata* and desiccation-intolerant *Syzygium cumini*

d. Change in phytochemicals during embryogeny in desiccation-tolerant *Vigna unguiculata* and desiccation-intolerant *Syzygium cumini*

Molecular fingerprinting helps to understand the change in molecular composition during embryo development. There are several classes of molecules which are crucial in one way or other way influencing germination process. The present study was intended to make a molecular profiling of biomolecules during different stages of embryogeny using FTIR spectroscopy.

The chemicals screened for the presence in the seed included alkenes, aromatic rings, aldehydes, quinones, esters/lactones, carboxylic acids and their salts, ammonium salts, alcohol, primary/secondary/ tertiary alcohols, phenols, carbohydrates, ethers, primary/secondary amines, nitriles, sulphides, polysulphides, sulphur oxy compounds, peroxides, organic phosphates, aromatic phosphates, aliphatic phosphates, aromatic nitro-compounds, halides such as C-F, C-Cl, C-Br and C-I, silicon oxy compound, aromatic nitro compound, aliphatic nitro compound, aliphatic secondary amines, hetro cyclic amines, antheroquinone, terpenes, Cardiac glycosides, saponins, sulphonamides, alkaloides, tannins, steroides, flavanoides, anhydride, sulphonic acid, Thioamide and thioureas, Phosphine oxide, Thiocarbonyl, Benzaldehyde Hex -1 -yne data are presented .

The young seed of *Vigna unguiculata* contained all phytochemicals except, aldehydes, polysulphides, primary, secondaryand tertiary alcohols, hetero cyclic amine, aliphatic sec amines, tannins, flavanoids, sulfonamide, alkaloides, sulphonic acid, thioamide and thiourea. The phytochemicals detected in the young seeds were alkenes, aromatic rings, quinones, esters and lactones, carboxylic acid and their salts, ammonium salts, alcohols, phenols, carbohydrates, ether, sulphides, sulphur oxy compound, peroxides, organic phosphate, aromatic phosphate, aliphatic phosphate, halides, aromatic and aliphatic nitro compound, antheroquione, terpenes, cardiac glycosides, saponins, steroids, anhydrides, phosphine oxide, thiocarbonyl, phosphoramide, benzaldehyde, hex 1 yne .Many compounds detected in the young seeds were largely undetected in the mature stage I and mature stage II seed. In mature stage I these include alkenes, aldehydes, quinones, esters/lactones, , primary, secondary and tertiary alcohols and polysulphides, silicon oxy compounds, hetero cyclic amines, terpenes, tannins, flavanoids, sulphonic acids, phosphine oxide, phosphine, benzaldehyde. Compounds such as hetero cyclic amine and sulphonic acid were detected in the mature stage II seed and these were absent in the

Fig 6. The class of photochemical identified by FTIR analysis.

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	RECALCITRANT SEED SYZYGIUM CUMINI			ORTHODOX SEED VIGNA UNGUICULATA		
			YOUNG	MATURE STAGE I	MATURE STAGE II	YOUNG	MATURE STAGE I	MATURE STAGE II
1	ALKENES	C-H STRETCH	-	-	3078.39	3084.18	3016.67	3088.03
		C=C STRETCH	-	-	-	1622.13	-	-
		=C-H BEND	-	-	-	1303.88	-	-
2	AROMATIC RING	=C-H BEND	1039.63	1043.49	1047.36	999.13	1047.35	1101.35
		C-H STRETCH	-	-	3078.39	3084.18	3086.11	3088.03
		C=C STRETCH	-	-	-	1454.33	1508.33	-
3	ALDEHYDE	C-H STRETCH	-	-	2731.20	-	-	-
		C=O STRETCH	1726.29	1728.22	1728.22	1730.15	-	1726.29
4	QUINONES	C=O STRETCH	1608.63	1602.85	-	1622.13	-	1608.63
5	ESTERS AND LACTONES	C=Od STRETCH	1726.29	1728.22	1728.22	1730.15	-	1726.29
		C-O STRETCH	1039.63	1043.66	1047.36	1047.35	1047.35	1232.51
6	CARBOXYLIC ACIDS AND THEIR SALTS	O-H STRETCH	2906.73	3047.53	2731.20	2854.65	2854.65	2733.13
		C=O STRETCH	-	1228.66	1219.01	1303.88	1255.66	1231.51
		C-O STRETCH	-	-	-	1730.15	-	-
7	AMMONIUM SALTS	NH_4 SRETCH	3259.70	3047.53	3049.46	3084.18	3086.11	3088.03
		C=O STRETCH	1444.68	1444.68	1444.68	1454.33	1452.40	1396.46
8	ALCOHOLS	O-H STRETCH	3259.70	3259.70	3236.55	3261.63	3261.63	3512.37
9	$1^0,2^0,3^0$ ALCOHOLS	C-O STRETCH	-	-	1139.93	1147.65	1116.78	1143.79
		O-H STRETCH	-	-	3602.73	-	-	-
10	PHENOLS	C-O STRETCH	1039.63	1043.49	1047.36	1047.35	1047.35	1143.79
		O-H STRETCH	3259.70	3259.70	3236.55	3261.63	3261.63	-
		O-H BEND	1338.60	1228.66	1444.68	1454.33	1255.66	1232.51

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	RECALCITRANT SEED SYZYGIUM CUMINI			ORTHODOX SEED VIGNA UNGUICULATA		
			YOUNG	MATURE STAGE I	MATURE STAGE II	YOUNG	MATURE STAGE I	MATURE STAGE II
11	CARBOHYDRATES	O-H STRETCH	3259.70	3259.70	3078.39	3084.18	3086.11	3088.03
		C-O –C STRETCH	2906.73	3047.53	2827.64	2854.65	2854.65	2856.58
		C-H Stretch	1209.37	1228.66	1219.01	1411.89	1452.40	1232.51
12	ETHER	C-O STRETCH	1039.63	1043.49	1047.36	1288.45	1047.35	1232.51
13	SULPHIDES	C-S STRETCH	615.29	559.36	-	563.21	707.88	555.50
14	POLYSULPHIDES	S-S STRETCH	487.99	-	-	-	-	-
15	SULPHUR – OXY COMPOUND	SO_2 STRETCH	1338.60	1348.24	1342.46	1346.31	1350.17	1396.46
16	PEROXIDES	C-O STRETCH	-	999.13	993.34	933.55	927.76	923.90
17	ORGANIC PHOSPHATE	P=O STRETCH	1338.60	1348.24	1342.48	1288.45	1255.66	1101.35
18	AROMATIC PHOSPHATES	P-O-C STRETCH	1209.37	1228.66	993.34	933.55	927.76	1232.51
19	ALIPHATIC PHOSPHATES	P-O-C STRETCH	1039.63	1043.49	1047.36	1047.35	1047.35	-
20	HALIDES	C-F	1039.63	1043.49	1139.93	1047.35	1047.35	1101.35
		C-Cl	758.02	758.02	-	713.66	767.67	754.17
		C-Br	615.29	-	-	626.87	624.94	626.87
		C-I	532.35	559.36	540.07	534.28	534.28	555.50
21	AROMATIC NITROCOMPOUND	N=O	1444.68	1444.68	1444.68	1454.33	1452.40	1396.46
22	ALIPHATIC NITROCOMPOUND	N=O	1444.68	1444.68	1444.68	1454.33	1452.40	1396.43
23	ALI. SEC. AMINE	N-H STRETCH	-	-	-	-	3340.71	-
24	HETEROCYCLIC AMINE	N-H STRETCH	-	-	3464.15	-	-	3431.36

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	RECALCITRANT SEED SYZYGIUM CUMINI			ORTHODOX SEED VIGNA UNGUICULATA		
			YOUNG	MATURE STAGE I	MATURE STAGE II	YOUNG	MATURE STAGE I	MATURE STAGE II
25	ANTHEROQUINONE	C-O STRETCH	1039.63	1043.49	1047.36	1047.35	1047.35	1232.51
26	TERPENES	C-H STRETCH	-	-	2886.51	2854.65	2854.65	2856.58
		C=C STRETCH	-	-	-	1622.13	-	-
27	CARDIAC GLYCOSIDES	C-O STRETCH	1039.63	1043.49	1047.36	1047.35	1047.35	1232.51
		C=O STRETCH	1608.63	1602.85	1444.68	1454.33	1452.40	1446.61
28	SAPONINS	C=O STRETCH	1608.63	1602.85	1444.68	1454.33	1452.40	1396.46
		C-O STRETCH	1039.63	1043.49	1047.36	1047.35	1047.35	1101.35
29	SULPHONAMIDES	N-H STRETCH	-	-	-	-	3340.71	-
		S-O STRETCH	758.02	758.02	927.76	871.82	927.16	923.90
30	ALKALOIDES	N-H STRETCH	-	-	-	-	3340.71	-
		C-N STRETCH	1039.63	1043.49	1047.36	1047.35	1047.35	1232.51
		N-H BEND	1608.63	1602.85	1596.13	1454.33	1452.40	1608.63
31	TANNINS	O-H STRETCH	-	-	3618.46	-	-	3537.45
		C-O STRETCH	1039.63	1043.49	1047.36	1047.35	1047.35	1232.51
32	STEROIDES	C-O STRETCH	1039.63	1043.49	1047.36	1047.35	1047.35	1232.51
		C=O STRETCH	1444.68	1444.68	1444.68	1454.33	1452.40	1446.61
33	FLAVANOIDES	O-H STRETCH	-	-	3618.46	-	-	3537.45
		C=O STRETCH	1444.68	1444.68	1444.68	1454.33	1452.40	1446.61
34	ANHYDRIDE	C-O STRETCH	1039.63	1043.49	1047.36	1047.35	1047.35	-
		C=O STRETCH	1444.68	1444.68	1444.68	1454.33	1452.40	1396.46
35	SULPHONIC ACID	O-H STRETCH(SO_3H)	-	-	3618.46	-	-	3537.45
36	THIOAMIDE& THIOUREAS	N-H STRETCH	-	-	3618.46	-	3340.71	-
		C=S STRETCH	1039.63	1043.49	1047.36	1047.35	1047.35	1232.51
37	PHOSPHINE OXIDE	P=O STRETCH	1209.37	-	-	1288.45	-	-
		P-H BEND	867.97	999.13	1047.36	1047.35	927.76	923.90

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	RECALCITRANT SEED SYZYGIUM CUMINI			ORTHODOX SEED VIGNA UNGUICULATA		
			YOUNG	MATURE STAGE I	MATURE STAGE II	YOUNG	MATURE STAGE I	MATURE STAGE II
38	THIOCARBONYL	C=S	-	1228.66	1139.93	1288.45	1116.78	1143.79
39	ARENES	C-H	758.02	758.02	-	933.55	667.37	754.17
		C=S	-	-	1596.13	-	1508.33	-
40	PHOSPHORAMIDE	P=O	1209.37	1228.66	1219.01	1244.09	1255.66	1232.51
41	BENZALDEHYDE	C=O STRETCH	1608.63	1602.85	1729.22	1622.13	-	1608.63
42	1-HEXENE	C-H STRETCH	3018.60	3047.53	3049.46	3010.88	3016.67	3088.03

immature seed indicating that these are synthesized as a result of dehydration.

Certain compounds viz., ammonium salts, carbohydrate, ether, sulphur oxy compound, peroxides, organic phosphate, aromatic phosphate, aliphatic phosphate, antheroquinones, cardiac glycosides, steroids, phosphoramides, hex 1 yne, thiocarbonyl aromatic ring, phenol, alcohol, primary/secondary/ tertiary alcohols, sulphides, sulphur oxy compounds, peroxides, organic phosphates, aromatic nitro-compounds, and the halides C-F, C-Cl, C-Br, C-I were common in both mature stage I and immature seeds.

Completely dehydrated mature stage II seed showed the presence of esters/lactones, quinones, ammonium salts, alcohol, ether, sulphides, peroxides, sulphur oxy compounds, organic phosphates, aromatic phosphate, aromatic and aliphatic nitro-compounds, benzaldehyde hex 1 yne, phosphoramide, thiocarbonyl, sulphonic acid, flavonoids, steroids, saponins, cardiac glycosides, hetero cyclic amines, tannins andhalides such as C-F, C-Cl, C-Br and C-I. carboxylic acids and their salts were produced only in the young seeds.

The class of compound such esters and lactones present in the youngseed were reappeared in the mature stage II seed .

As we compare with recalcitrant seed the chemicals screened for the presence in the young seeds of S.cumini included, quinones, esters and lactones, ammonium salts, alcohol, phenols, carbohydrates, ethers, sulphides, sulphur oxy compound polysulphides, aliphatic phosphate, organic phosphates, aromatic phosphates, aliphatic and aromatic nitro-compounds, cardiac glycosides, saponins, steroids, anhydrides, phosphine oxide, phosphoramide, benzaldehyde, hex 1 yne halides such as C-F, C-Cl, C-Br and C-I are presented .But in the mature stage I seeds shows slight variation have no, phosphine oxide, Halides such as C- Br are absent.But all these compounds are reappeared in the matured stage IIseed and it helps for germination

The phytochemicals, identified based on the characteristic stretch and bend of their functional groups.

The mature satge II seeds showed the presence of aldehyde, esters and lactones, ammonium salts, alcohol, esters and lactones, primary/secondary/tertiary alcohols, phenols, carbohydrates, ethers, sulphur-oxy compounds, peroxides, organic phosphates, aromatic phosphates, aliphatic phosphates, C-F and C-I compounds, aliphatic and aromatic nitrocompounds, heterocyclic amine, antheroquinone, cardiac glycosides, saponnins, tannins, steroids, flavanoides, anhydrides, sulphonic acids, thioamide and thioureas, thiocarbonyl, phosphoamide, benzaldehyde. These compounds are assumed to be vital for faster germination and also for preventing microbial infection due to their fully hydrated state.

Aldehydes, primary/secondary/tertiary alcohols, peroxides, aliphatic nitro compound, heterocyclicamines and tannins, flavanoides, sulphonic

Fig 7. The class of phytochemicals identified by FTIR analysis

SL NO	COMPOUND	RECALCITRANT SEED *SYZYGIUM CUMINII*			ORTHODOX SEED *VIGNA UNGUICULATA*		
		YOUNG	MATURE STAGE I	MATURE STAGE II	YOUNG	MATUR STAGE I	MATUR STAGEII
1	ALKENES	-	-	-	+	-	-
2	AROMATIC RING	-	-	-	+	+	-
3	ALDEHYDE	-	-	+	-	-	-
4	QUINONES	+	+	-	+	-	+
5	ESTERS AND LACTONES	+	+	+	+	-	+
6	CARBOXYLIC ACIDS AND THEIR SALTS	-	-	-	+	-	-
7	AMMONIUM SALTS	+	+	+	+	+	+
8	ALCOHOLS	+	+	+	+	+	+
9	$1^0,2^0,3^0$ ALCOHOLS	-	-	+	-	-	-
10	PHENOLS	+	+	+	+	+	-
11	CARBOHYDRATES	+	+	+	+	+	+
12	ETHER	+	+	+	+	+	+
13	SULPHIDES	+	+	-	+	+	+
14	POLYSULPHIDES	+	-	-	-	-	-
15	SULPHUR –OXY COMPOUND	+	+	+	+	+	+
16	PEROXIDES	-	+	+	+	+	+
17	ORGANIC PHOSPHATE	+	+	+	+	+	+
18	AROMATIC PHOSPHATES	+	+	+	+	+	+
19	ALIPHATIC PHOSPHATES	+	+	+	+	+	-
20	HALIDES C-F	+	+	+	+	+	+
	C-Cl	+	+	-	+	+	+
	C-Br	+	-	-	+	+	+
	C-I	+	+	+	+	+	+
21	AROMATIC NITRO COMPOUND	+	+	+	+	+	+
22	ALIPHATIC NITROCOMPOUND	+	+	+	+	+	+
23	ALI. SEC. AMINE	-	-	-	-	+	-

23	ALI. SEC. AMINE	-	-	-	-	+	-
24	HETEROCYCLIC AMINE	-	-	+	-	-	+
25	ANTHEROQUINONE	+	+	+	+	+	+
26	TERPENES	-	-	-	+	-	-
27	CARDIAC GLYCOSIDES	+	+	+	+	+	+
28	SAPONINS	+	+	+	+	+	+
29	SULPHONAMIDES	-	-	-	-	+	-
30	ALKALOIDES	-	-	-	-	+	-
31	TANNINS	-	-	+	-	-	+
32	STEROIDES	+	+	+	+	+	+
33	FLAVANOIDES	-	-	+	-	-	+
34	ANHYDRIDE	+	+	+	+	+	-
35	SULPHONIC ACID	-	-	+	-	-	+
36	THIOAMIDE&THIOUREAs	-	-	+	-	+	-
37	PHOSPHINE OXIDE	+	-	-	+	-	-
38	THIOCARBONYL	-	+	+	+	+	+
39	ARENES	-	-	-	-	+	-
40	PHOSPHORAMIDE	+	+	+	+	+	+
41	BENZALDEHYDE	+	+	+	+	-	+
42	HEX-1-YNE	+	+	+	+	+	+

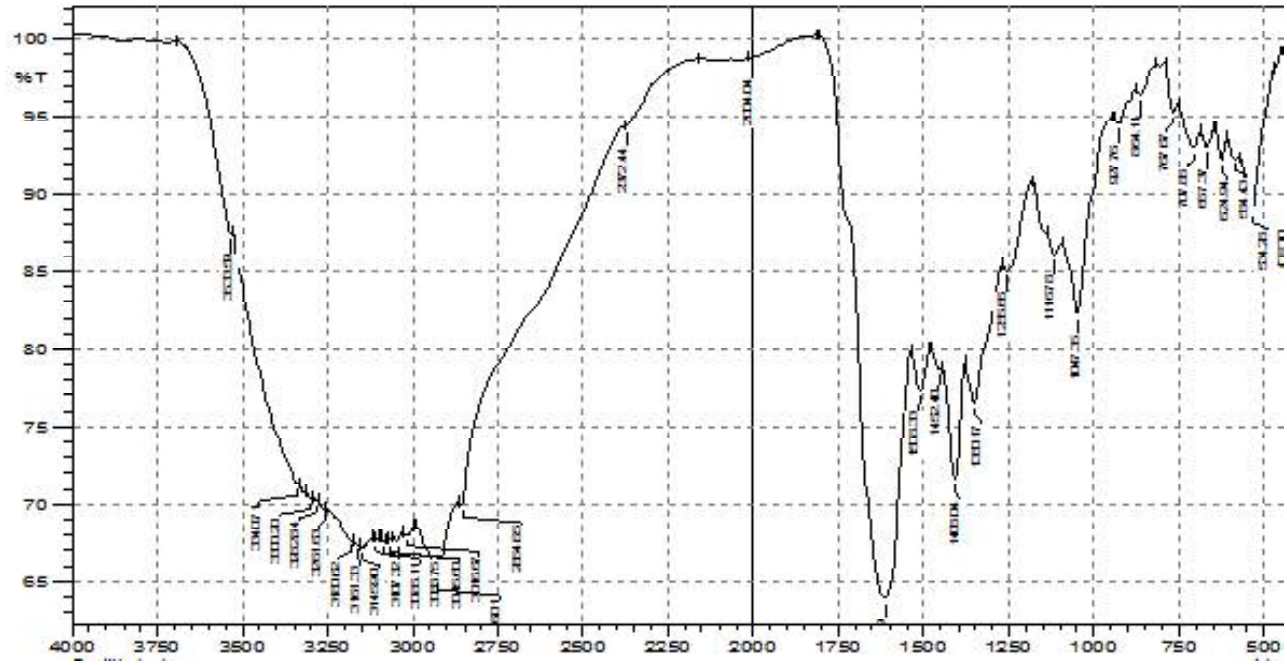

Fig 8. FTIR spectrum of young seed of *Vigna unguiculata(methanolic extract)*

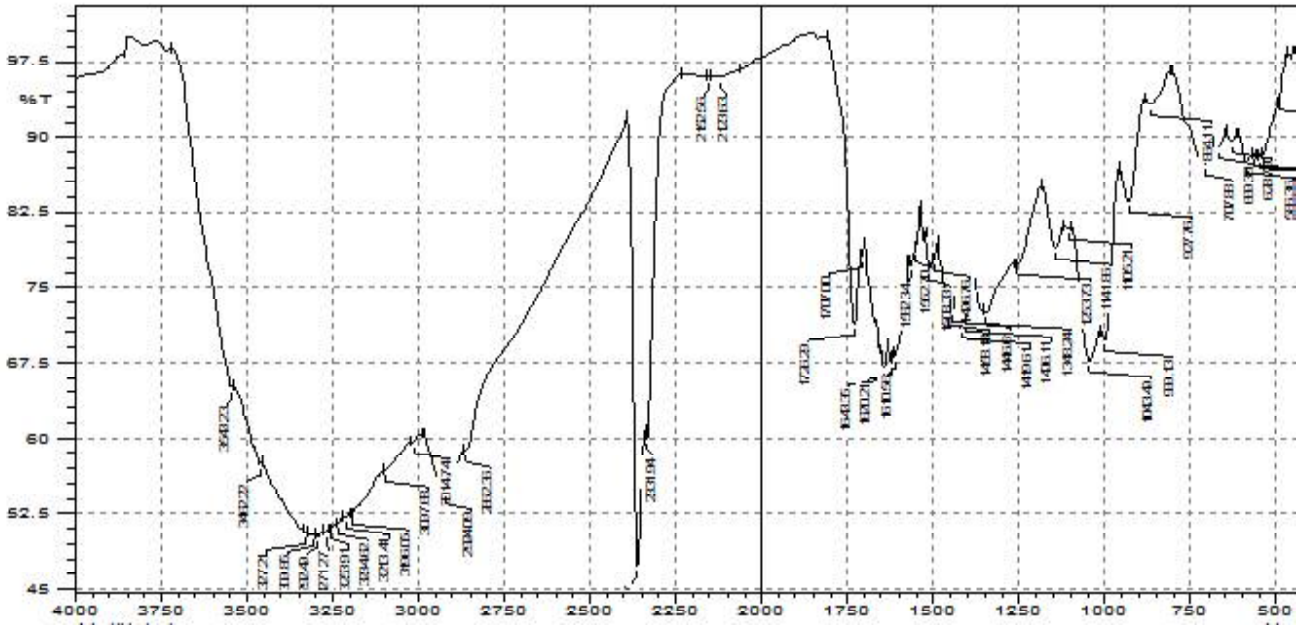

Fig 9. FTIR spectrum of mature stage I seed of *Vigna unguiculata(methanolic extract)*

acid, thioamide and thiourea and thiocarbonyl were detected only in the mature stage II seeds and these could not be detected in youngseed.

As we compare the young stageseeds of *Syzygium cumini* and *Vigna ungiculata* certain compounds were present such as quinones, esters and lactones, ammonium salts and alcohols, phenol, carbohydrate, ether, sulphides, sulphur oxy compound, antheroquinones, terpenes, cardiac glycosides, saponins, steroids, anhydries, phosphine oxide, phosphoramaide and benzaldehyde were detected .

Mature stage II seed comparison with *Syzygium cumini* and *Vigna ungiculata*showed the presence of esters and lactones, ammonium salts and alcohols,

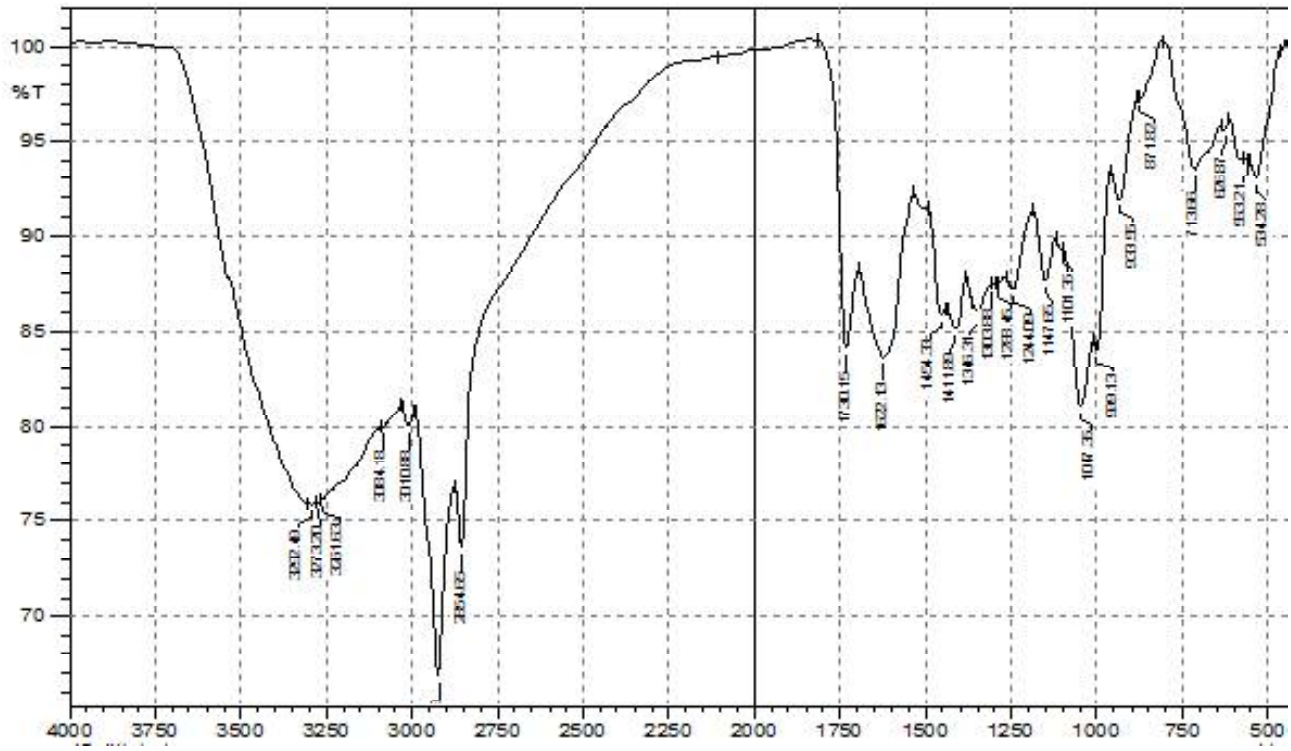

Fig 10. FTIR spectrum of mature stage II seed of *Vigna unguiculata(methanolic extract)*

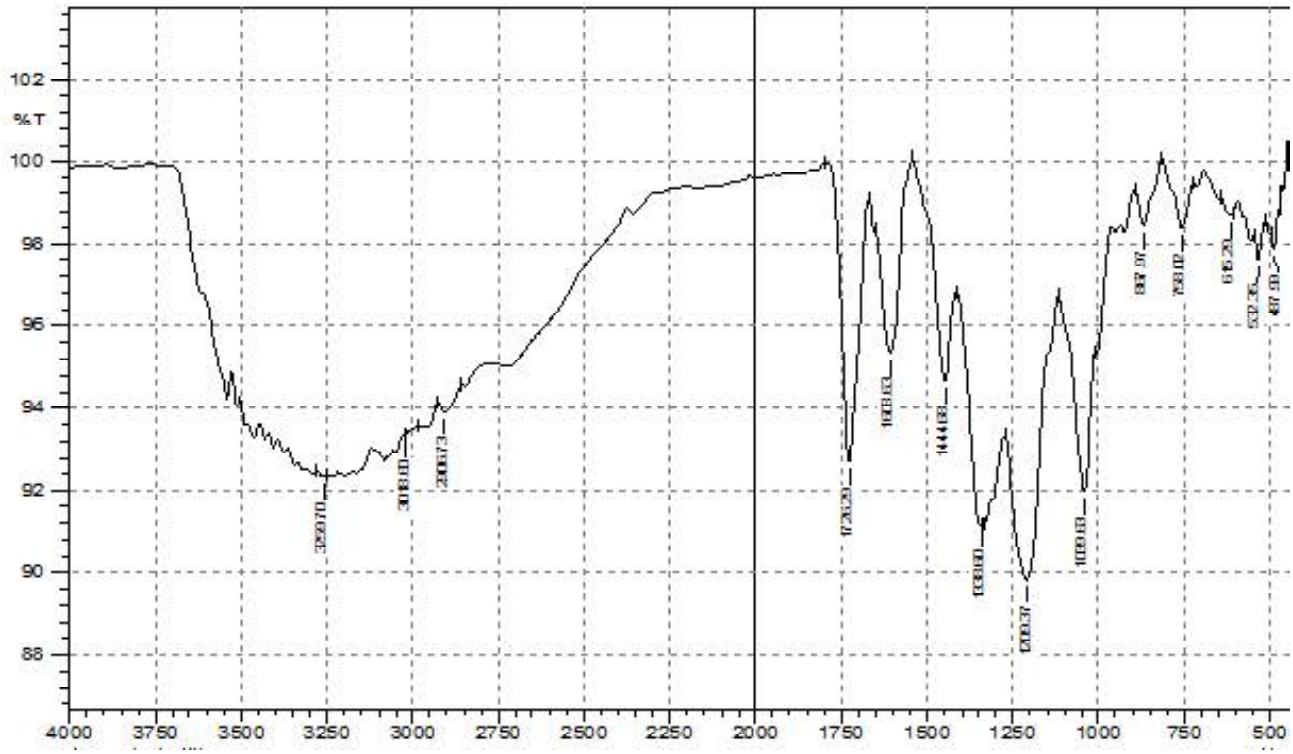

Fig 11. FTIR spectrum of young seed of *S.cumini(methanolic extract)*

phenol, carbohydrate, ether, sulphides, sulphur oxy compound, antheroquinones, peroxides, cardiac glycosides, saponins, steroids, flavonoids, sulphonic acids, thiocarbonyl, phosphoramide, benzaldehyde and hex 1 yne were present and these are synthesized as a result of maturation.

CONCLUSIONS

Significant changes have been observed in the moisture content of the embryonal axis and cotyledonary tissues of *Syzygium cumini* during embryogeny and embryo desiccation.Seeds of

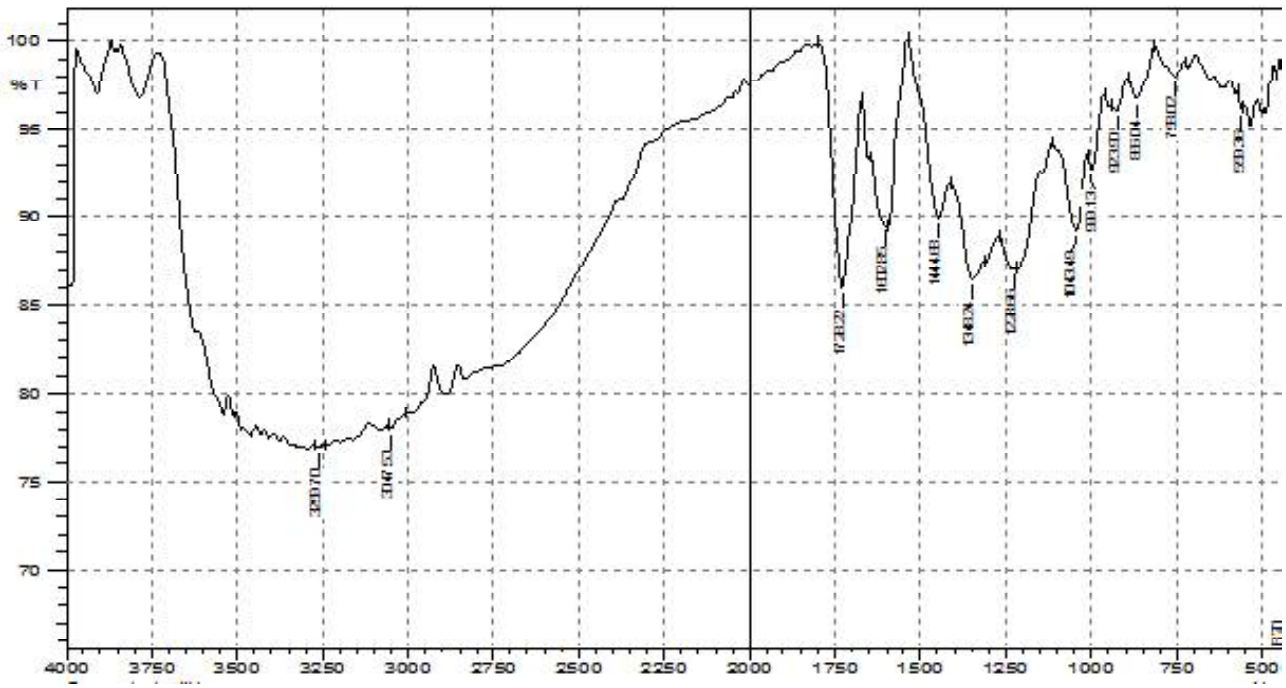

Fig 12. FTIR spectrum of mature stage I seed of *S.cumini(methanolic extract)*

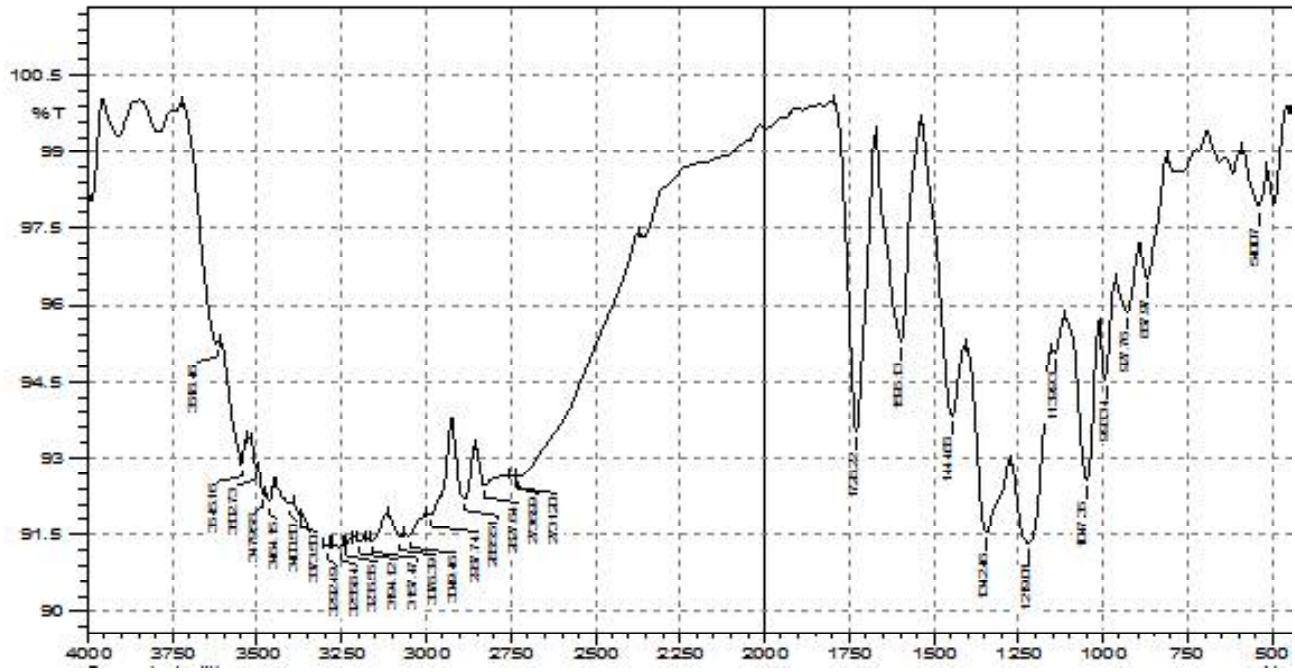

Fig 13. FTIR spectrum of mature stage II seed of *S.cumini (methanolic extract)*

Syzygium cumini maintained higher water content in the embryonal axis and cotyledons during embryogeny, a characteristic of recalcitrant seeds.The seeds do no exhibit maturation drying and germinate immediately after dispersal.The desiccation-tolerant seeds of *Vigna unguiculata*, show a sharp decline in water content in the embryonal axis and cotyledons during embryogeny.There is an increase in biomass accumulation in the embryonal axis and cotyledons of both desiccation-tolerant seeds of *Vigna unguiculata* and desiccation-intolerant seeds of *Syzygium cumini.* The seeds of *Syzygium cumini* do not have any innate mechanisms to counter water loss due to desiccation.The critical threshold water level of the embryo was found to be 58.86%. The seeds remain viable for about 8 days under natural conditions of desiccation, after that viability is sharply reduced.FTIR Spectroscopy showed variations in the phytochemicals during embryogeny of both desiccation-tolerant *Vigna unguiculata* and desiccation-intolerant *Syzygium cumini.*

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Screening of Antifungal Activity of a Lectin Isolated from Marine Sponge *Axinella donnani*

S. RATHEESH AND ARUN. A. RAUF*

Dept. of Biochemistry, University of Kerala, Trivandrum-695581
**arunar@rediffmail.com*

ABSTRACT

The present study has been taken to explore the screening of *in vitro* antifungal activities of lectin from marine sponge *Axinella donnani* . The lectin was subjected to evaluation for inhibition of microbial growth based on the disc diffusion method against four phytopathogenic fungi. The results showed that the ADL(1mg/ml) had a potent antifungal activity against *Penicllium crysogenum* and *Aspergillus flavus*. A comparison of the antifungal activity of ADL against antibiotics such as Amphotericin B was also carried out. These findings indicate that the lectin may be of importance to clinical microbiology and have therapeutic applications.

Key words *Axinella donnani, lectin,Disc diffusion method, Antifungal activity*

Marine natural products are exceptional in their structural/chemical features and consequently in their pharmacological properties than the terrestrial natural products (Ibrahim and Mohamed, 2016; Kiuru et al., 2014). Marine sponges, belong to the phylum Porifera (Andersen,2017; Anjum et al., 2016), are gaining more attention by researchers and industrial sectors from all over the world due to their ability to produce a variety of bioactive secondary metabolites that have many applications including drug discovery (Garcia-Vilas et al., 2015; Kobayashi, 2016; Mioso et al., 2017) . Marine sponges are a promising source of bioactive lectins, which are structurally diverse, many of them in the form of glycoproteins (Kawsar et al., 2009; Takahashi et al., 2008; Moura et al., 2006, Molchanova et al., 2005). Their bioactivity extends from simple anti microbial action to complex anti tumour properties (Leung et al., 2008,Yan et al., 2009., Matsumoto et al.,2012, Rabelo et al.,2012).

The aim of this work was to analyze the comparative effects of the antifungal properties of lectins from the marine sponge *Axinella donnani* against fungal strains.

MATERIALS AND METHODS

Chemicals

Agar, dextrose, peptone, beef extract were purchased from SRL Ltd.,and Amphotericin B from HImedia.,India.

Source of material

Lectin from the marine sponge *Axinella donnani* (ADL) was extracted with PBS buffer, fractionated by ammonium sulphate precipitation and purified by DEAE-Cellulose ion exchange chromatography.Different concentrations of ADL (0.1-1mg/ml) were prepared with PBS(P^H 7.4) for the screening studies.

Test organisms

The fungal strains used in this study were obtained from the, Department of Biotechnology, University of Kerala, Trivandrum. The fungal pathogens were *Penicillium crysogenum,Candida albicans,Aspergillus flavus,Collectrichum corchori.*

Screening of Antifungal Activity

The *in vitro* antifungal activity of the purified lectin was determined by disc diffusion method (Bauer et al., 1996). SDA medium was used for the culture of fungi. After preparation of the media, SDS was melted in hot water bath and allowed to cool. On cooling, it was poured to sterile petri dishes and allowed to solidify in horizontal position. The culture colonies from the stock cultures were selected and 100 µl was transferred to the culture medium. A sterile spreader was dipped into the properly diluted inoculums and spread evenly. Sterile paper disc of diameter 5mm (made from Whatmann No-1 filter paper) impregnated with ADL (0.1-1mg/ml) were placed on the surface of the SDA plates seeded with test organisms. They were touched down with a sterile forceps to ensure complete contact with surface. The plates were incubated at 37°C for about 24 hrs. Control disc impregnated with PBS was also used along with the test disc in each experiment as negative control. After incubation, the test cultures were plated on air dried nutrient SDA plates using a sterile glass spreader. Using a clean forceps, the sterile discs loaded with the lectin was plated on the surface of SDA plates seeded with the test fungal strains. Commercially available Hi combs of Amphotericin B (Himedia, Mumbai) were used as the positive control. PBS were

used as the control. The plates were then incubated at35±2°C for 72 hours. The zone of bacterial growth inhibition was observed and its diameter was measured in millimeters.

RESULTS AND DISCUSSION

In vitro antifungal susceptibility by ADLwas determined against four phytopathogenic fungi with antifungal antibiotic Amphotericin B as positive control. ADL (1mg/ml in SDA medium) showed significant inhibition (10mm) of mycelial growth against *Penicllium crysogenum* and *Aspergillus flavus* among tested fungi (Fig1&2). On the other hand, the growth of all the four fungi was totally inhibited by antifungal antibiotic Amphotericin (10 µg/mL SDA).Similarly antifungal effects of a 30 kDa D-Galactoside-Specific Lectin from the demosponge, *Halichondria okadai* lectins have reported and showed strong antifungal activity(Kawser et al.,2011). Antifungal lectins have been drawing the attention of many researchers because of their ability to deter pathogenic fungi from invading agricultural crops and causing diseases in animals. The inhibition of fungi growth can occur through lectin binding to hyphae resulting in poor absorption of nutrients as well as by interference on spore germination process. The results of the present study indicate that the ADL may be of importance to clinical microbiology and have therapeutic applications.

ANTIFUNGAL ACTIVITY OF THE PURIFIED LECTIN (ADL) AGAINST PHYTOPATHOGENIC FUNGI

Name of fungus	Percentage inhibition of fungal mycelia growth(1mg/ml ADL) (mm)	Percentage inhibition of fungal mycelia growth Amphotericin* (mm)
Penicllium crysogenum	10±0	10.33±0.58
Aspergillus flavus	10±0	10 ± 1.15
Candida albicans	Nil	13 ± 0.41
Colletotrichum corchori	Nil	12 ± 0.53

*Standard antifungal antibiotic, growth measured-radial growth in cm.

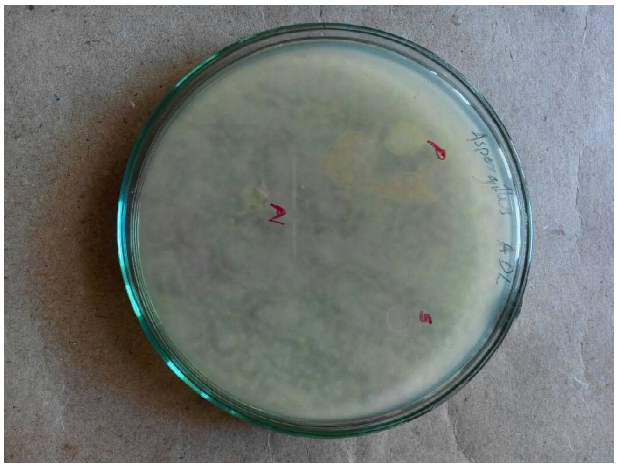

Fig. 1

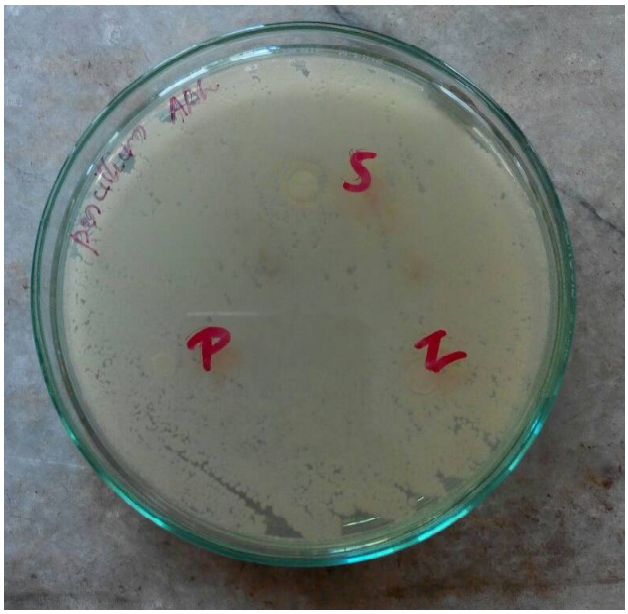

Fig. 2

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Effect of Tea Waste Extract on Germination and Growth of Green Gram Seeds: Some Observations

REMYA KRISHNAN*, SUMAYYA A, ARUNIMA SUDHEESH, ASWATHY ASOKAN AND JERRY C. JACSON

Department of Botany, St. John's College, Anchal, Kollam
**krishnar07@mail.com*

ABSTRACT

Recycling of food waste is one of the big challenges of our time. Converting organic waste to useful products is wiser application rather than they have been land filled. The present study is an attempt to recycle food waste especially tea waste, i.e., an attempt was made to study the effectiveness of tea waste as a fertilizer in the germination and growth rate of *Vigna radiata* (Green gram). Non-polar and polar solvent extracts of tea waste were made by hot continuous soxhlet extraction. The extract was lyophilized and made up to known volume by DMSO and water. Various poly phenol contents in the extracts were quantified and the results revealed significant amounts of phenols, flavonoids and tannins. Next phase of the study was analysis of effect of both the extracts on seed germination and growth. Effect of 1% and 5% concentration of both extracts and controls were examined in germination and growth of seeds. Highest percentage of seed germination was observed in 5% aqueous extract (88%) followed by 1% aqueous extract and control on 3rd day. Petroleum ether extract showed comparatively low germination rate. After 7th day the growth rate of seedlings, length of leaf lamina, total protein content and growth of root system were analyzed. 5% aqueous extract treated seedlings showed highest height, profuse roots, highest protein content (21.17 mg/g) supporting the role of extract as fertilizer. From the results it can be concluded that the tea waste can be used as a potent fertilizer. Furthermore it is an effective way of recycling a bio waste in a useful manner. Further studies, experiments and awareness are recommended for large scale recycling of tea waste as fertilizer.

Key words *Bio waste, green gram, poly phenols, protein, recycling, tea waste.*

Bio-fertilizers are eco-friendly fertilizers, which are being used to improve the quality and fertility of the soil. Excess use of chemical fertilizers has led to the pollution and contamination of the soil, polluted water basins, destroyed micro-organisms and friendly insects, made the crop more prone to diseases and reduced soil fertility. Farmers are now looking for more eco-friendly fertilizers. Bio-fertilizers are identified as plant extracts, composted urban wastes and various microbial mixtures and chemical formulations supplemented with organic compounds. They ensure the well-being of the nutrients present in the soil and make the soil more fertile with time.

Biodegradable waste include green waste, food waste, paper waste, and biodegradable plastics, human waste, manure, sewage, sewage sludge and slaughter house waste. Egg shells, banana skins and tea waste are inevitable food waste. Home composting is a great way to stop this sort of waste ending up in landfill. Also it can be recycled into a good quality soil improver or fertilizer. Production of organic fertilizer from food wastes is an effective environmental sanitation method. Consequence of synthetic fertilizer use that is not always taken in to consideration is its cost. Organic fertilizer produced from food waste is a measure of promoting waste to wealth program in developing countries (Oladapo et al., 2014). Sharma and Mitra (1990) reported the application of organic materials to increased grain and straw yield of rice. Ranganathan and Selvaseelan (1997) also found that application of spent mushroom and rice straw compost are comparable with FYM (Farm Yard Manure) and grain yield increased by 20 per cent over NPK fertilizer. Larkin et al., (2008) stated that the appropriate use of bio fertilizers produce important and positive effects on soil quality, disease reduction, and yield.

Food waste has high energy content and it seems ideal to achieve dual benefits of energy production and waste stabilization. Tea waste is considered as agricultural waste. Encouraging tea waste recycling/ composting is an effort to inspire and empower the next generation to live more sustainably. It only takes a little effort, from recycling kitchen waste to honing your gardening skills with a composting heap, to play an important part in creating change. The present work emphasized on the mechanism of food waste composting especially recycling tea waste, as a fertilizer.

MATERIALS AND METHODS

Study materials:

Tea waste

The material used for current study is the tea waste. i.e., the tea powder collected after making black tea.

Vigna radiata (Green gram)

The seeds of *Vigna radiata* (L.)Wilczek) (Green gram) is used for seed germination and growth studies. It is a member of the legume family.

Hot continuous soxhlet extraction

20g tea waste is dried in shade and extracted with a non-polar and polar solvents (petroleum ether and water respectively) using hot continuous soxhlet extraction for six hours. The extract is lyophilized and made up to known volume using distilled water and DMSO (Dimethyl sulphoxide). The extracts were stored at 4^0C for further studies.

Analytical studies

Total phenols content were estimated by the method of Mayr *et al.,* (1995). Total flavonoids content of the extracts were determined by $AlCl_3$ method with slight modification. Total flavonoids were expressed as mg quercetin equivalent/g of weight (Mervat, 2009). Estimation of total terpenoids was done according to the method of Ferguson (1956). Total tannin content was measured using the method of (Wang et al., 2011). Following Bradford method (1976) total protein content was estimated.

Study of percentage of seed germination

25 healthy seeds were sown in soil collected in a petri dish containing sand and red soil (1:1 ratio) and properly irrigated. 1 and 5% extracts of both the solvents were applied to the seeds regularly and germination rate and other growth parameters were calculated. Controls such as tap water and DMSO treated seeds were also maintained. The petri dishes were maintained at room temperature. Germination rate was calculated with Ellis and Robert equation on 3rd day.

$$\% \text{ of seed germination} = \frac{\text{No of germinated seeds X 100}}{\text{No of seeds sown}}$$

Other growth parameters like height of seedlings and width of leaf lamina were calculated on 7th day. All the experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Preliminary phytochemical analysis:

The hot continuous soxhlet extraction of tea waste was done using a non-polar and polar solvents i.e. petroleum ether and water respectively. As first phase of the study various phytochemicals in both the extracts were screened qualitatively, which revealed the presence of a pool of polyphenols like flavonoids, phenols and tannins. Further the quantitative analysis was carried out. The two solvent extracts showed marked variability in the amount of phytochemicals (Table 1). Interestingly it has been noticed that terpenoids were absent in both the extracts. All the polyphenols were found in higher amounts in aqueous extracts than in petroleum extract. Flavonoids were found in higher amounts followed by tannins and phenols in aqueous extracts. But in petroleum ether extracts tannins were found in higher amount followed by flavonoids and phenols (Table 1).

Table 1. Amount of various polyphenol content in the tea waste extract

Polyphenols	P. ether extract (mg/g)	Aqueous extract (mg/g)
Phenol	1.05	3.85
Flavonoids	2.27	12.41
Tannin	4.35	5.55

The chemical composition of tea includes hundreds of compounds, i.e., polyphenols methylxanthines carbohydrates, proteins, free amino acids, vitamins, organic acids, tannins, volatile compounds and many others. Plants exhibit allelopathic activity due to release of allelochemicals of different chemical classes like polyphenolic compounds (flavonoids and tannins) (Einhelling, 1995). Phenolic compounds at higher concentrations could cause root and seedling injury to plants (Nassar et al., (2014). If the level of phenols is reduced to one percent, raw plant waste would be safe as manure. At 66.5mg/g concentration phenols acts as an allelo chemical,

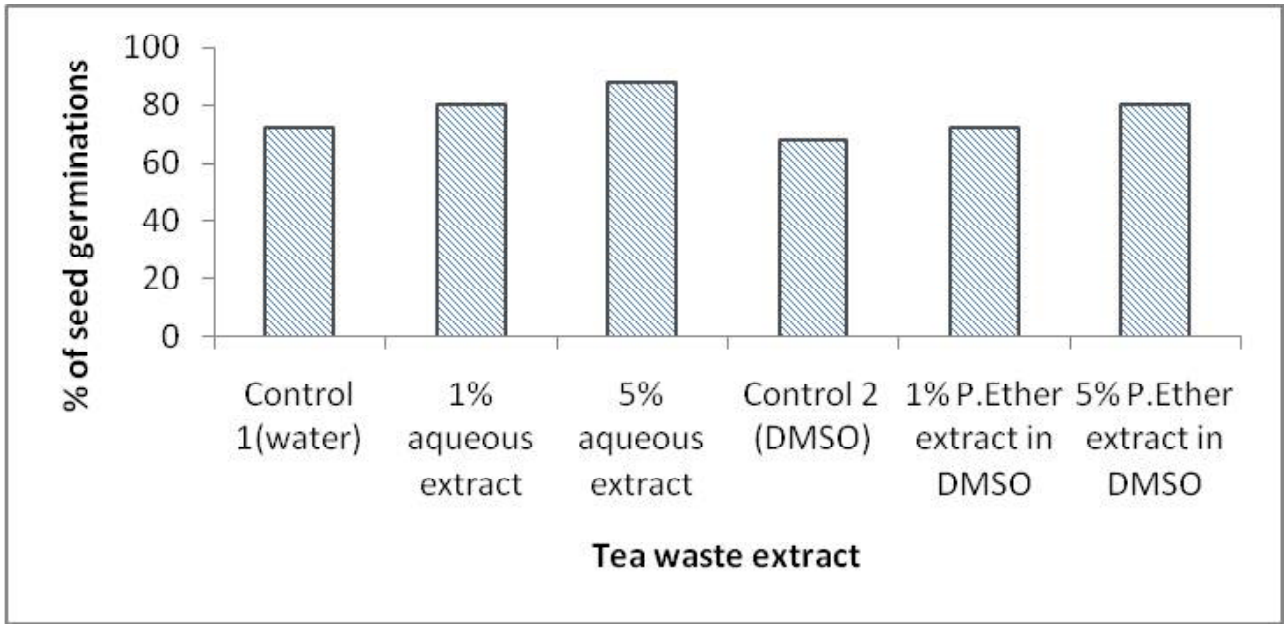

Fig.1. Seed germination of green gram against various extracts of tea

similarly at higher concentrations tannins (118mg/g) were also reported to have growth suppressor effect (Nassar et al., (2014). In the present study concentration of phenol and tannins were comparatively very low (3.85mg/g and 5.55mg/g).

Seed germination and growth of *Vigna radiata:*

The effect of tea waste extract on seed germination and growth of *Vigna radiata* seedlings were analyzed. Highest percentage of seed germination was observed in 5% aqueous extract (88%) followed by 1% aqueous extract and 5% petroleum extract in DMSO (80%). In control (water only) 72% germination rate was obtained while in DMSO control germination rate was 68 %. From the results it can be stated that as concentration of aqueous extract increases from 1% to 5%, the rate germination of the seeds of green gram also increases. The rate of germination is influenced by the concentration of extracts because the germination rate in control is comparatively low. In the case of petroleum extract in DMSO, a similar trend in activity was also observed (Fig. 1).

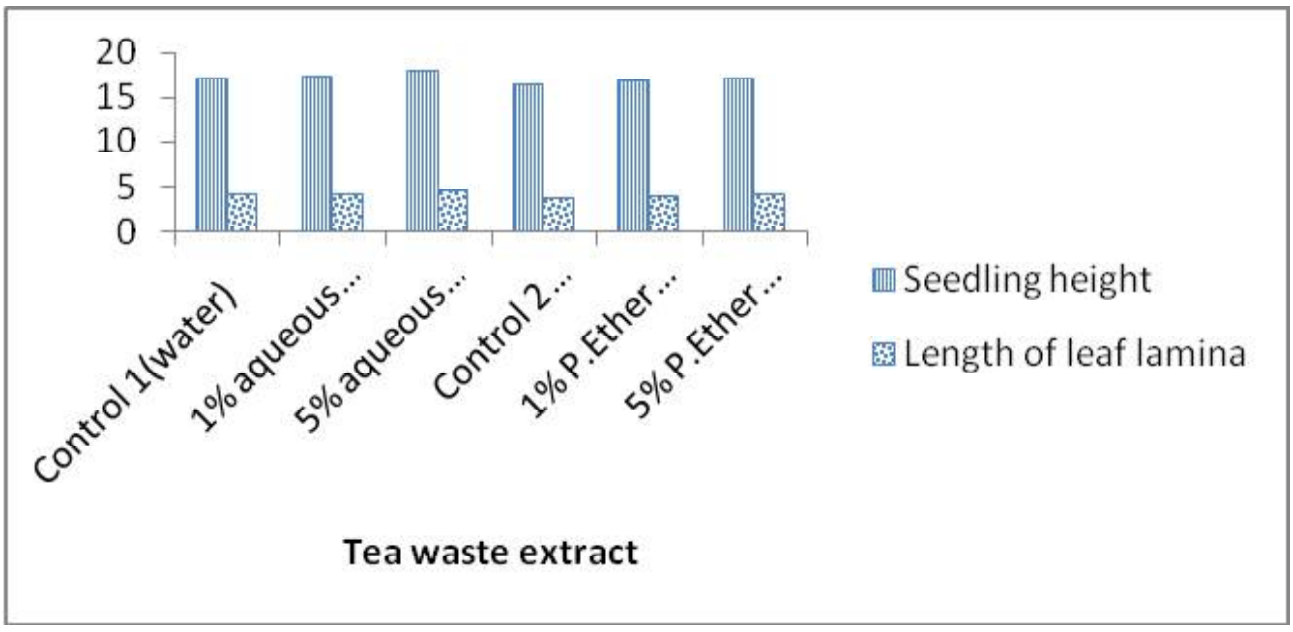

Fig.2. Morphological parameters of green gram against various extracts of tea

Fig. 3a & b. Root development in green gram treated with tea extracts 3a. Aqueous extract, 3b. PE extract in DMSO

The rate of seedling growth and length of leaf lamina was also analyzed in the 7th day from the date of germination. The data showed similarity with germination rate. 5 % aqueous extract treated seedlings exhibited about 18 cm seedling height and 4.5 cm length of leaf lamina. In 5% petroleum ether in DMSO treated green gram seeds it was 17 and 4 cm respectively. It was equal to control 1 (water). In 1% aqueous extract treated plants seedlings showed 17.2 cm length and 4 cm lamina length. In control 2 (DMSO treated) the seedling height was 16.5 cm while leaf lamina has only 3.6 cm length. The results also suggest the positive role of tea waste extract in growth promotion of green gram seedlings. Aqueous extract was better than both the control and petroleum extracts in DMSO (Fig. 2).

In Fig.3 a & b the effect of different solvent extracts and their concentration in rooting of green gram seedlings were clearly visible. Profound rooting pattern was observed in 5% aqueous extract treated seedlings. Here also the result showed similarity with germination rate of seeds and seedling growth rate. From the results it could be noted that the root length, number of main roots, root volume and number of root nodes were significantly increased with 5 % aqueous extract treatment (Fig.4 a& b). Initially, the number of second roots was not significantly different among the treatments, as number of days increased the rooting pattern also showed significant variation.

Yield enhancement in many plants, with various phenolic compounds were also reported by Datta and Nanda (1978) (Triticales), Tayal and Sharma (1982) (green gram), Malik et al., (1986) (ground nut) and Setia et al., (1989) (Lentil). Phenols were also known for modifying endogenous levels of phytohormones and thereby influence various plant processes (Kefeli and Dashek, 1984). From the present preliminary investigation, it can be concluded that all the polyphenols exhibited remarkable negative allelopathic potential by significantly affecting the germination and

Fig. 4 a & b. Root growth in green gram treated with tea extracts 4a. Aqueous extract, 4b. PE extract in DMSO

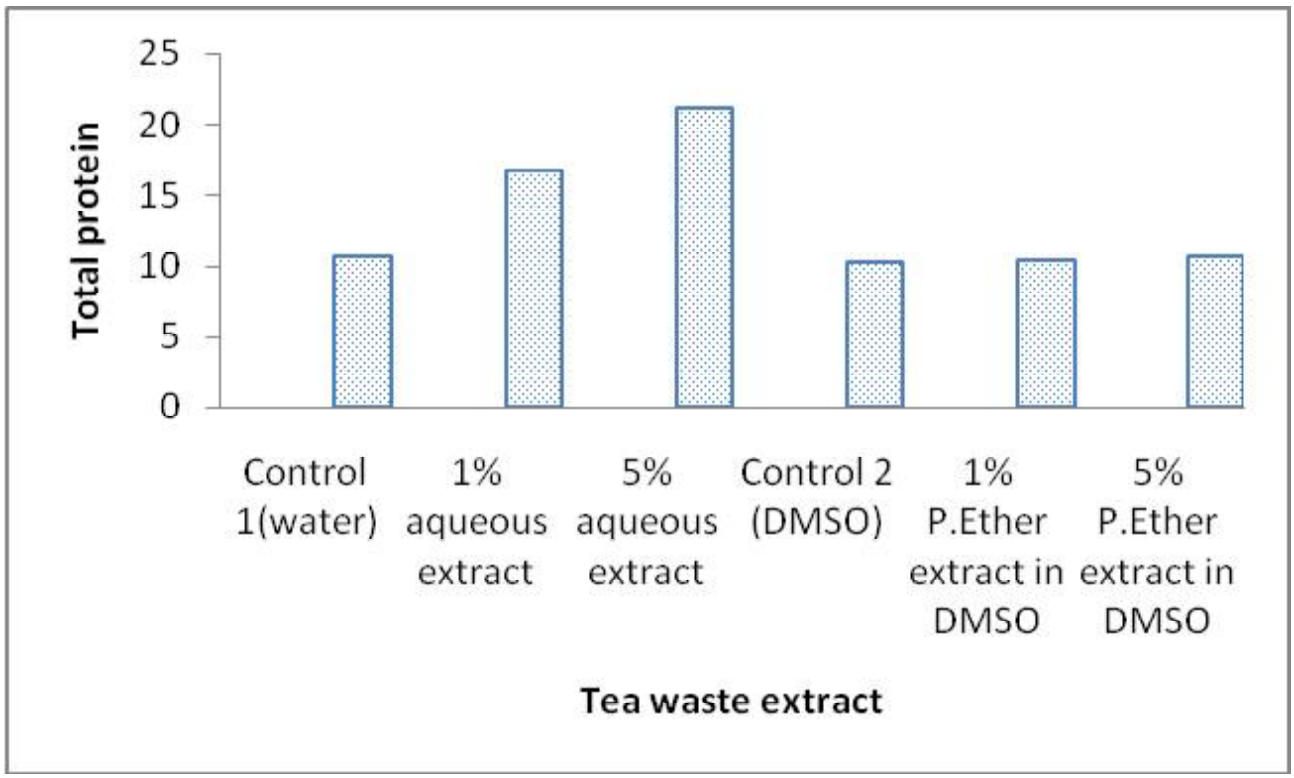

Fig. 5. Total protein in green gram seedlings against the tea waste extracts

hypocotyle and root growth of *Vigna radiata.* Similar studies were reported by Devkota et al., (2013) by screening the allelopathic potential of *Ageratum conyzoides, Cannabis sativa, Eclipta prostrata* and *Woodfordia fruticosa*.

Murugalakshmi Kumari et al., (2002) reported that the treatment of *Gracilaria corticata* fertilizer on black gram had increased the growth parameters such as shoot length and root length with optimum concentration of seaweed extract. Sunarpi et al., (2010) observed similar surveillance in *Oryza sativa*. Similarly in the present study also we observed the enhancement of seed germination and seedling growth of *Vigna radiata* in lower concentrations of tea waste extract. Thambiraj et al., (2012) studied the influence *of Sargassum wightii* and *Hypnea musciformis* seaweed liquid fertilizer on the growth and biochemical constituents of *Cyamopsis tetragonoloba* (L). The seeds of *C. tetragonoloba* treated in SLF performed better growth and certain biochemical attributes than water soaked seeds. Enhancement of seed germination might be attributed to the role of tea extract in increasing the availability of macro and micronutrients in the soil and making it available to the germinating seeds with positive enhancement in the metabolic activity resulting in higher germination (Cooper, 1979).

Quantification of total protein content

Green gram seeds are rich sources of protein. The protein profile of the germinating seeds are strongly corroborating with the physical parameters. Total protein content of the control and treated seedlings of green grams were analyzed. The results showed significant variation in the total protein content of the seedlings (Fig. 5). In 5% aqueous extract treated seedlings the total protein content was 21.17mg/gm followed by 1% aqueous extract treated seedlings. All other seedlings showed more or less similar protein content (ranged between 10.29-10.64mg/g). From the results it was clear that tea waste extract has a significant role in enhancement of seed gerimination, seedling growth and protein content of *Vigna radiata* in lower concentrations.

Vigna seeds treated with biofertilizer showed significant increase in all parameters such as number and length of leaves, length of plants, shoot length, root length, and bio-chemical parameter such as carbohydrate content and protein content (Fernandes Peter and Bhalerao Satish, 2015). Sridhar and Rengasamy (2002) observed the rate of enhancement in the total protein content at lower concentration of seaweed liquid fertilizer (SLF) from *Ulva lactuca.* Venkataraman and Mohan (2003) also reported that

the SLF treated seedlings showed a marked increase in soluble protein and soluble sugar contents. Similar to the previous observations, in the present study also the protein profiles varied with reference to the concentrations of tea waste extract treatment. Similar results i.e. increase in total protein and carbohydrate content of green grams supplemented with agro waste (viz, Wheat Bran, Mustard Oil Cake, Cicer Brown Husk, Peanut Shell, Tea Waste and FYM) as fertilizer is reported by Saima Ibrahim and ErumMumtaz (2014). The germination rate and seedling growth rate are comparable with the results of SLF treated green gram seeds (Paul and Shridevi, 2014). In SLF treated seedlings the rate of germination was 100% while the height of seedlings was lesser than the tea waste extract treated seedlings.

CONCLUSION

The collection and utilization of bio-waste is a central component in the development of a sustainable society. For agriculture it may be attractive to use recycled bio-waste, mainly as a fertilizer. The results of present study revealed that the tea waste can be effectively used as fertilizer. The petroleum ether and aqueous extracts of tea waste showed a pool of polyphenols. In optimum concentrations they promoted seed germination and seedling growth of *Vigna radiata.* The analysis of total protein content in the seedlings also showed remarkable increase when compared with control plants suggesting the positive effect of tea waste extracts in growth and metabolism of green gram. The present results also give clear directions for recycling tea waste from home kitchen and also from restaurants. It can be use directly on plants or as aqueous extractions along with proper irrigation. So that low cost bio fertilizers are obtained for farming and dumping of tea waste can be avoided. Further studies, experiments and awarness are recommended for large scale recycling of tea waste as fertilizer.

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In Vitro Anthelmintic Activity of the Ethanolic Extracts from the Leaves of *Azadirachta indica* A. Juss

SHEEJA GEORGE E

Department of Botany, S. N. College for Women, Kollam, Kerala, South India
email : sheejajebi@yahoo.co.in

ABSTRACT

Helminthiasis or parasitic worm infection is a foremost degenerative disease distressing a large proportion of world's population. The problem associated with chemical control of helminths is the development of drug-resistance in worms. Use of plants and plant products to cure helminthiasis is advisable as they are easily available, cheap and are free from side-effects. *Azadirachta indica* A. Juss, commonly known as Neem, is one of the most useful traditional medicinal plants in India and has been used as a house-hold remedy against helminthiasis in our country from time immemorial. A study was carried out to analyze the *in vitro* anthelmintic activity of the leaves of *A. indica* so as to authenticate its traditional use against helminth parasites of human beings and animals. Adult Indian Earth worm, *Pheritima prosthuma*, being anatomicaly and physiologically similar with the round worm parasites of human beings, was used as the experimental animal. Three concentrations (15, 30 and 50 mg/ml) of the ethanolic extract solution of shade-dried leaves of *A. indica* were used as test solutions; Albendazole (50 mg/ml) was used as reference standard and normal saline as control group. Two equi-sized worms each were subjected to the test solutions, reference standard and the control. Each treatment was with three replications. The results were expressed in terms of average time taken for paralysis and death of the two worms in each solution. The ethanolic extracts from the leaves of *A. indica* were found to be more effective than the reference drug in paralyzing and killing the worms. The leaf extracts showed dose-dependent activity and the best result (paralysis in 15 minutes and death in 35 minutes) was observed with 50 mg/ml leaf extract solution. The experimental evidence obtained in the present study could provide a rationale for the traditional use of this plant as anthelmintic.

Key words *Azadirachta indica;* helminthiasis; *Pheritima prosthuma;* Albendazole

Helminthiasis or parasitic worm infection is a foremost degenerative disease distressing a large proportion of world's population. The parasites that infect the gastrointestinal tract of animals and human beings include pinworm, roundworm and tapeworm. These worms alter their hosts' nutrient absorption while receiving nourishment from them and thereby cause anaemia, malnutrition, eosinophilia and pneumonia (Bundy, 1994). Helminths also harm the hosts by secreting some toxins, causing serious diseases. The gastro-intestinal helminths become resistant to currently available synthetic anthelmintic drugs on continuous use. Henceforth it is important to screen medicinal plants for their anthelmintic activity to develop alternative strategies against gastrointestinal nematodes.

A large number of medicinal plants are claimed to possess anthelmintic properties and are utilized by ethnic people worldwide. As plants and plant products are free from side-effects, they are preferable than synthetic drugs in curing diseases. Moreover, plant-based drugs are easily available and are least expensive. There are reports on the efficacy of a number of medicinal plants in curing intestinal parasitism in human beings (Sarkar *et al*., 2000; Vidya *et al*., 2012; Ganesh *et al*., 2013; Ullah *et al*., 2013). In South India, a large number of medicinal plants are traditionally used as anthelminths. *Azadirachta indica* A. Juss, commonly known as Neem, is one of the most useful traditional medicinal plants in India (Chhibber & Sharma, 2014) and has been used as house-hold remedy against various human ailments including helminthiasis in our country. A study was carried out to analyze the *in vitro* anthelmintic activity of the ethanolic extracts of the shade-dried leaves of *A. indica* so as to authenticate its traditional use against helminth parasites.

MATERIALS AND METHODS

Plants were collected locally from Manrothuruthu village of Kollam District, Kerala, South India and were identified and authenticated in the Department of Botany, SN College for Women, Kollam, Kerala, South India. Fresh young leaves (Plate 2) were taken out and washed well under running tap water and the moisture left on the leaf was removed by wiping with a clean cloth. The leaves were then shade-dried for 14 days. The shade-dried leaves were crushed into coarse powder (Plate 3) in an electric blender and subjected to Soxhlet extraction using ethanol as solvent. The extracts were concentrated by rotary evaporator and used for testing anthelminthic activity.

Adult Indian Earthworm, *Pheritima prosthuma*

(Plate 1) - due to its anatomical, morphological and physiological resemblances with the intestinal round worm parasites of human beings (Mali & Mehta, 2007) - was used as the helminth representative. Equi-sized worms with an average length of 6-7 cm were collected from moist soil and were identified in the Department of Zoology, SN College for Women, Kollam, Kerala, South India. The worms were washed with water to remove all fecal matters and kept in normal saline solution. Albendazole with trade name Zentel (Glaxo SmithKline Pharmaceuticals Ltd (Mumbai, India) was used as the reference drug. In all the experiments 50 mg/ml albendazole was used as the reference standard and distilled water was used as the control. To prepare a concentration of 50 mg/ml albendazole, 500 mg of albendazole was accurately weighed out and dissolved in 10 ml distilled water.

The anthelmintic assay was performed *in vitro* as per the method of Ekeanyanwu *et al*. (2012). Effect of three different concentrations of *A. indica* leaf extracts on the experimental animal, *P. posthuma* was analysed. Three different concentrations (15, 30 and 50 mg/ml) of the leaf extract were prepared as test samples by dissolving required quantity of the extract in distilled water. Earthworms were divided into five groups, each containing two earthworms. Three groups were used for the leaf extract test solutions, one group was applied to reference standard (50mg/ml albandazole) and another to control (distilled water). The worms were placed in petri dish each containing 25 ml of above test solutions and reference standard. For each treatment, triplets (three petri dishes) were used and each petri dish contained two equal sized earthworms. All the test solution and standard drug solution were prepared freshly before starting the experiments. Observations were made for the time taken to paralyze and / or death of individual worms. Mean time for paralysis was noted when no movement of any sort could be observed, except when the worm was shaken vigorously; Death was concluded when the worms completely lost their mobility followed with fading away of their body color. The test results were compared with Reference compound Albendazole (50 mg ml^{-1}) treated samples. Data were represented as mean ± SE

RESULTS AND DISCUSSION

The results of the experiments conducted to analyze the *in vitro* anthelmintic activity of the alcoholic extract of the leaves of *A. indica* are given in Table 1. Death and paralysis of the worms were observed with all the three tested solutions of the leaf extract of *A. indica* (Plate 5). The leaf extracts showed dose-dependent activity and the least time taken for paralysis (15 minutes) and death (35 minutes) of the worms was observed with 50 mg/ml leaf extract solution (Table 1). The average time taken for paralysis and death of the worms in 30 mg/ml solution of the leaf extract were 17 minutes and 37 minutes respectively. It took an average of 20 minutes for paralysis and 40 minutes for death of the worms with 15 mg/ml of the leaf extract solution. The ethanolic extracts from the leaves of *A. indica* were found to be more effective than the reference drug (Albandazole 50 mg/ml) which took an average of 36 minutes and 50 minutes for paralysis and death respectively (Table 1). The results revealed that, the leaves of *A. indica* possess considerable anthelmintic activity; hence can be used as an easily-available, cheap alternative for curing helminthiasis.

The present study is in line with Chhibber and Sharma (2014) who have reported that, *A. indica* is an efficient medicinal plant which can be utilized for curing a number of diseases. Ullah *et al.* (2013) have reported the *in vitro* anthelmintic activity *Curcuma longa, Citrullus colocynthis* and *Peganum harmala* against *P. prosthuma.* The same methodology followed in the present study revealed similar activity of *A.*

Table 1. Anthelmintic activity of *A. indica*

Sl No.	Group	Treatment	Paralysis time (minutes)	Death time (minutes)
1	Group I	Normal Saline	--	
2	Group II	Albendazole (50 mg ml^{-1})	36 ± 3.61	50 ± 2.87
3	Group III	Leaf extract (15 mg ml^{-1})	20 ± 2.64	40 ± 3.85
4	Group IV	Leaf extract (30 mg ml^{-1})	17 ± 3.35	37 ± 3.81
5	Group V	Leaf extract (50 mg ml^{-1})	15 ± 3.49	35 ± 3.45

Mean±SD; n= 3 in each group

indica too. Dose dependant activity of *A. indica* leaf extracts against *P. prosthuma* as revealed in the present study also supports the findings of Asha *et al.* (2001), Bazh and Bahy (2013) and George *et al.* (2014) who have reported dose-dependent anthelmintic activity of *Ocimum sanctum*, *Curcuma longa* and *Nymphoides macrosperma* respectively.

CONCLUSION

The experimental evidence obtained in the present study could provide a rationale for the traditional use of *A. indica* as anthelmintic. The plant may be further explored for its phytochemical profile to recognize the active constituent accountable for anthelmintic activity.

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Plate 1 *P. prosthuma*

Plate 2 Leaves of *A. indica*

Plate 3 Dried & Powdered leaves of *A. indica*

Plate 4 *P. prosthuma* on normal saline

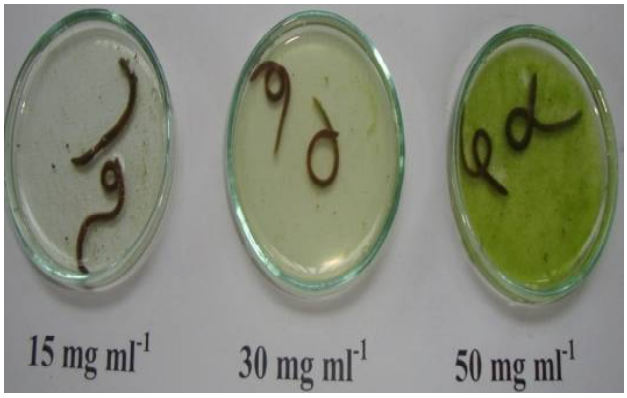

Plate 5 *P. prosthuma* on different concentrations of test solution

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Physico-Chemical and Phytochemical Evaluation of a Medicinal Herb *Hyptis capitata* Jacq. (Lamiaceae)

V. SUMITHA, K. MURUGAN AND I. MINI

Post graduate and Research Department of Botany, University College, Thiruvananthapuram, Kerala, India – 695 034
email : sumithasiva.v@gmail.com

ABSTRACT

Medicinal plants are rich sources of therapeutic phytochemicals and are widely used by traditional healers all over the world.Lamiaceae or mint family is well known for medicinal properties. *Hyptis capitata* is a slightly aromatic folk medicinal perennial herb belongs to Lamiaceae, commonly called buttonweed.The plant has some folkloric advantages such as anti-cough, anti-spasmodic, anti-inflammatory and wound healing properties. The present study aims atflourescence analysis of dried powder, physico-chemical parameters (moisture, acid insoluble ash, water soluble ash, alcohol soluble extractive and water soluble extractive) followed by extraction, detection and estimation of phytochemicalsin the leaf and stem of *Hyptis capitata*.The fluorescence characteristics of leaf and stem powder revealed variable colours when treated with solvents of different polarity and chemical reagents.The crude drug powder showed significant physicochemical values of moisture and total ash. Water soluble extractive values were found significant (3.04% and 4.56%) in leaf and stem of the plant.The percentage of yield of crude extract increased in various solvents in leaf (ethyl acetate>petroleum ether> chloroform> methanol>distilled water) andstem (methanol>distilled water>chloroform>ethyl acetate> petroleum ether). Majority of phytochemicals were detected in leaf extracted in petroleum ether and methanolic extractof stem.Preliminary phytochemical screening revealed alkaloids, coumarins, glycosides, flavonoids, phenols, quinines, tannins, terpenoids, steroids, iridioids and saponins were present in leaf extracted in ethylacetate.The total phenols (840mg/g&341 mg/g) and total flavonoids (1.45 mg/g & 2.27mg/g) were high t in ethyl acetate and methanolic extract of leaf and stem.The study revealed that ethylacetate and methanol were suitable polar solvents for the extraction of phytochemicals for further pharmacological studies.

Key words *Hyptis capitata,physicochemical analysis, fluorescence analysis, solvent extract, phytochemicals*

Medicinal plants are immense source of therapeutic phytochemicals used by traditional healers all over the world which may lead to the discovery and development of new drug.Plants have the ability to synthesize variety of chemical compounds that are used to perform important biological functions. The development of formulation of drugs of plant origin involves botanical identification of the drug, cultivation and post harvestmethods as well as extraction and standardization procedures. Lamiaceae or mint family is well known for medicinal properties. *Hyptis capitata* is a slightly aromatic folk medicinal perennial herb belongs to Lamiaceae, commonly called buttonweed, knob weed, false ironwort and is a native to Florida, Mexico, Central America and South America but naturalized in Australia,South east Asia and some tropical islands.The plant has some folkloric advantages such as anti-cough, anti-spasmodic, anti-inflammatory and wound healing properties.The plant is also known to contain the chemical constituents responsible for cytotoxicity and anti-HIV. The present study aims at physico-chemical analysis, extraction and estimation of phytochemicalsin the leaf and stem of *Hyptis capitata*.

MATERIALS AND METHODS

The study material *Hyptis capitata* was collected from natural habitat identified by the Department of Botany,Kariavattom,University of Kerala with an accession number KUBH-6166 and voucher specimen was deposited in the herbarium of the Department.

The plants were thoroughly washed and leaf and stem were separated, shade dried, powdered and kept in a desiccator for further studies.The successive extraction of the samples from non polar to polar solvents was done using different solvents such as petroleum ether (PE),chloroform(CHL),ethyl acetate (EA) methanol (ME) and distilled water (Aq) using soxhlet method.The liquid extract obtained from each solvent was evaporated to dryness at 55^0-60^0C.The solidified dried extract was weighed and calculated the yield in terms of percentage and colour of the extract were also evaluated(Harbone, 1998).

A small quantity of dried leaf and stem powder was placed separately on grease free clean microscopic slide and 1-2 drops of freshly prepared reagent solution was added, mixed by gentle tilting the slide for few minutes. The slide was viewed inside the UV chamber and observed the colour in day light and UV light. The colour in different reagents was recorded **(**Kokate, 1997). Physico-chemical parameters such as, moisture content, total ash, water soluble ash,acid insoluble ash, water and alcohol soluble extractive were determined in leaf and stem powder (WHO,2002).

The crude extracts of leaf and stem (PE, CHL, EA, ME and Aqueous)were subjected to preliminary qualitative screening for phytochemicals viz., alkaloids, coumarins, glycosides, flavonoids,phenols, tannin, quinines, terpenoids,steroids,iridioids, saponins and anthraquinones (Harborne,1998).Quantitative estimation of total phenols(Mayr *et al*.,1995)and total flavonoids (Chang *et al*.,2002) were carried out.

RESULTS AND DISCUSSION

Physico-chemical analysis

Physico-chemical parameters are constant for the drugs which showed significant values and used for specific identification of the plant. The requirement of moisture content for herbal drug is less than 14%(Anonymous, 1980). The loss on drying indicated the moisture content of the drug powder (Table- 1)to be 7.35% and 10.5 % in leaf and stem of *H. capitata*respectively.Low moisture content recorded in the present study indicated less chance of microbial contamination during storage.Ash value is a criterion to assess the identity of the crude drug especially in the powder form (Patnia *et al*.,2012).Moreover, the total ash of a crude drug also reflected the care taken in drug preservation and the purity of crude and the prepared drug.Total ash value gives an idea of earthen materials or inorganic salt usually consists of carbonate,oxides,phosphates,silicates and silica in the drug or adhering to it.The total ash value of leaf and stem of *H. capitata*were 15.5 % and 16.75 % respectively.The results obtained in the present study remained in agreement with the reports on *Leucas cephalotes* (Priyank *et al*., 2011).The acid insoluble ash is a part of total ash and it measures the amount of silica present, especially as sand and siliceous earth.Water soluble ash is the water soluble portion of the total ash.The acid insoluble ash and water soluble ash were 1.7%,1.5%,7.9% and 4.55% respectively. The acid insoluble ash value of a crude drug less than the total ash value of the same drug indicated that very small amount of earthy matters present in it.More water soluble ash in both leaf and stem denotedthat the plant powder ash is more soluble to water compared to other.It is useful for the evaluation of crude drug as it gives an idea about the nature of the chemical constituents present in it and is useful for the estimation of chemical constituents,soluble in that particular solvent for extraction(Joseph and George,2011).Water soluble extractive values of leaf and stem showed significant amount (3.04%. and 4.56%) which denoted that water permeates the cells of aerial parts.The water soluble extractive value was more than alcohol soluble extractive value indicated that the leaf and stem powder contained more amount of water soluble chemical constituents.These results were in agreement with the reports on *Hyptis suaveolens* (Pachkore and Dhale, 2011).

Table 1. Physico-chemical Analysis of leaf and stem of *Hyptis capitata* Jacq.

Parameters	Leaf	Stem
Moisture	81%	91%
L.O.D	7.35%	10.5%
Total Ash	15.5%	16.75%
A.IS.A	1.7%	1.5%
W.S.A	7.9%	4.55%
Alc.S.E	2.8%	2.32%
W.S.E	3.04%	4.56%

Flourescence Analysis

Behaviour ofcrude drug powder of leaf and stemof *H.capitata* upon treatment with different reagents showed different colours both in day light and UV light(Table-2).Fluorescence seen in UV light mainly exhibit the flourescent nature of many natural products which is lacking in natural day light.If the substance themselves are not fluorescent,they may often be converted into fluorescent derivatives or decomposition products by applying different reagents. Hence this method can be used to assess the crude

Table 2. Flourescence Analysis of Leaf and stem powder of *Hyptis capitata* Jacq.

Reagents	Day light		UV light	
	Leaf	**Stem**	**Leaf**	**Stem**
Powder+1N NaOH	Dark	Brown	Black	Black
Pd+50% H_2SO_4	Dark	Dark green	Dark	Black
Pd+Methanol	Dark	Greenish yellow	Green	Flourescent green
Pd+Benzene	Dark green	Dark green	Red orange	Bluish dark
Pd+P.E	Dark	Dark	Dark	Dark
Pd+E.A	Dark	Dark	Orange red	Orange red
Pd+ Iodine	Yellowish green	Yellowish brown	Dark	Black
Pd+Ammonia	Brown	Dark green	Dark	Black
Pd+Picric acid	Yellowish green	Yellow	Dark	Black
Pd+Acetic acid	Dark green	Yellow	Orange	Orange

drugs qualitatively and it is an important technique of pharmacognostic evaluation (Janchen and Issaq, 1998).Variable colours were obtained when the crude drug powder of leaf and stem were treated with solvents of different polarity and chemical reagents.On treating powdered drug of leaf and stem separately with the reagents such as 1N NaOH, methanol, ethyl acetate and acetic acid showed identical colours which indicated similar compounds present in both parts.

Successive Extraction

The premier step to utilize the biologically active compound and processing of the bioconstituents from plant resource are extraction which is the separation of medicinally active portion of the plant using selective solvent through standard procedures. Appropriate extraction methods and solvents are very important to promote the quality and quantity of the therapeutically important active component of the study material.The ethyl acetate and methanolic

Table 3. Preliminary phytochemical screening of *Hyptis capitata*Jacq. in various solvents

Phytochemicals	Name of test	Leaf Extract					Stem Extract				
		PE	**CHL**	**EA**	**ME**	**DW**	**PE**	**CHL**	**EA**	**ME**	**DW**
Alkaloids	Dagendroff re-agent	+	+	+	+	_	+	+	+	+	+
Coumarins	Alcoholic NaOH	+	+	+	+	_	+	+	+	+	+
Flavonoids	Ammonium	+	+	+	_	+	+	+	+	+	+
Glycosides	Keller-Killiani	+	+	+	+	+	+	_	+	+	_
Phenols	$FeCl_2$	+	+	+	+	+	_	_	_	_	+
Tannins	$FeCl_2$	+	+	+	+	+	_	_	_	+	+
Quinones	Concentrated H_2SO_4	_	_	_	+	+	+	_	+	+	_
Terpenoids	Salkowski	+	_	+	+	+	+	_	+	+	+
Steroids	Salkowski	+	+	+	+	-	_	+	+	+	_
Iridioids	$CuSO_4$	+	+	_	_	_	_	_	_	_	_
Saponins	Foam test	+	_	+	+	+	_	_	_	+	+

(+ Present ; - Absent)

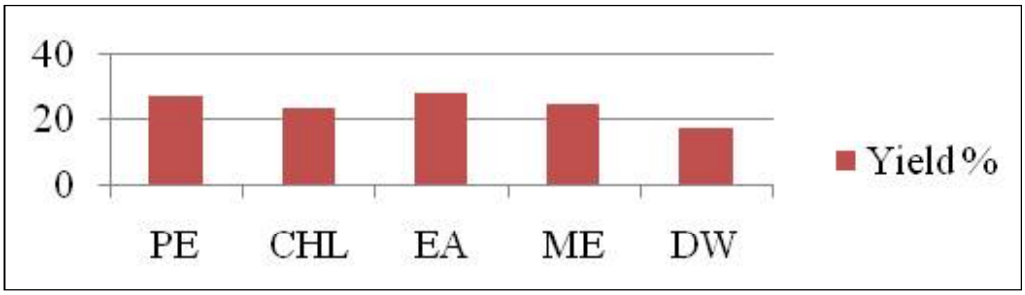

Fig. 1. Yield of Leaf Extracts from various solvents

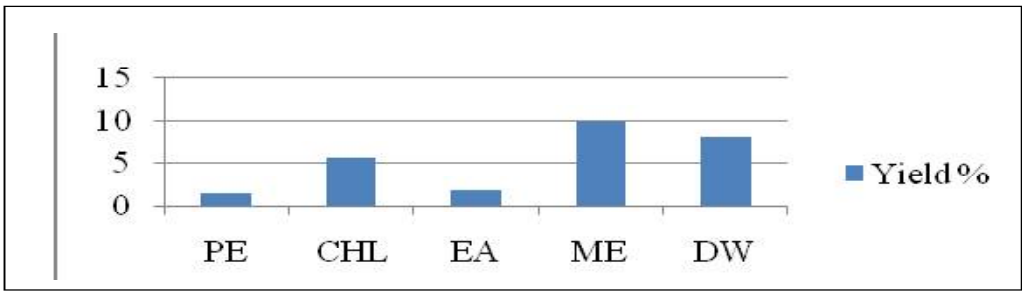

Fig. 2. Yield of Stem Extracts from various solvents

extract of leaf (28.34%) and stem (9.96%) revealed maximum yield (Fig.1&2). The variation in percentage of yield of extract production might be due to the solubility of various ingredients in different solvents used**.**During extraction, solvents diffuse into the solid plant material and solubilized compounds with similar polarity. Dehkharghanian *et al.,* (2010) opined that difference in the polarity of the solvent determines the type, composition, and antioxidant activity of phytochemical.

Qualitative Phytochemical Analysis

Traditionally used medicinal plants depict variety of therapeutic properties mainly attributed to its secondary metabolites. The crude extracts obtained by the soxhlation method was subjected to qualitative detection of twelve phytochemicals (Table-3). Petroleum ether extract of leaf revealed maximum number of phytochemicals. The phytochemicals such as glycosides, phenols, tannins and steroids were common in all extracts. Comparatively very few phytochemicals were present in aqueous extract. More number of phytochemicals were eluted in methanolic extract of stem which decreased in the order of ME>EA>PE>Aq>CHL. This observation highlighted that the leaf and stem of *H.capitata*possess enormous phytochemicals which may be successfully extracted by polar solvents.

Table 4. Phytochemicals in various extracts of leaf and stem of *Hyptis capitata*Jacq.

Phytochemicals (mg/g)	PE		CHL		EA		ME		Aq	
	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem
Total phenols	780 ± 55.67	270 ± 45.29	98.33 ± 68.98	83.33 ± 20.82	840 ± 133	128 ± 62.92	822 ± 52.04	341 ± 28.22	552 ± 46.46	273.3± 95.69
Flavonoids	1.06 ± 0.653	0.406 ± 0.28	0.464 ± 0.19	0.854± 0.05	1.45 ± 0.33	3.03 ± 0.20	0.078 ± 0.03	4.8 ± 0.10	0.661 ± 0.33	2.27 ± 0.49

Results are expressed as mean with SD

Quantitative Phytochemical Analysis

The results of quantitative estimation of phytochemicals were presented in the Table-4.Total phenols and flavonoids showed high amount in ethylacetate and methanolic extract of leaf and stem which highlighted that the ethylacetate is a semipolar solvent that could dissolve phenols and steroids and was effective in extracting alkaloid and glycosidic compounds (Houghton and Raman,1998).Yu Lin *et al*.(2010)reported that phenolic compounds have low molecular and medium polarity.Effectivity of phenolic compounds depended on type, structure,number and position of hydroxyl group of benzene ring (Wong *et al*., 2006 ;Widyawati *et al*.,2012). The flavonoids were major phenolic compound which constituted around 80% concentration in plant cells (Aberoumand and Deokule,2008).The phytochemicals extracted by methanol can donate hydrogen atom so that these compounds could form complex with aluminium ion of total flavonoid assay.Total phenols and flavonoids of leaf and stem extracts could associated with antioxidant activity.Leopoldini *et al.*(2011)reported the capability of phytochemical compounds in scavenging free radical involved donating hydrogen atom or electron.

CONCLUSION

The present study revealed that the *Hyptis capitata* Jacq is a valuable medicinal plant which contained many important phytochemicals.The physicochemical characteristics and fluorescence analysis indicated the identity and purity of the crude drug powder. The successive extraction and estimation of phytochemicals revealed maximum yield of crude extract. High amount of total phenols and flavonoids were recorded in polar solvents such as ethylacetate and methanol.The present study revealed that the polar solvents were suitable for extracting therapeutically important phyochemicals for further pharmacological studies. This study warrants the isolation and characterization of active principle present in the medicinal herb to elucidate its bioactivity.

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Study on Pigment Variation of Selected Varieties of *Ixora coccinea* L.

A. RESMI, S. SURABHI AND M. ARADHYA

Department of Botany, Sree Narayana College for Women, Kollam
**email : resmiasokan@gmail.com*

ABSTRACT

Natural dyes are the colours derived from plants or animals without chemical processing. Environmental issues in the production and application of synthetic dyes revived consumer interest in natural dyes during the last decades. So the demand for natural colorants for foods, drugs, cosmetics and textiles are increasing enormously. The present study was an attempt to extract and quantify colouring pigments from selected varieties of *Ixora coccinea*. The pigments were isolated from fresh flowers and leaves of five varieties of *Ixora* such as Red, Petite Red, Petite Pink, Maui Yellow and Kampong White. Anthocyanin and carotenoid contents were high in Red variety followed by Petite Red. Total chlorophyll content was also high in the Red variety followed by Petite Pink variety. From the results it could be assumed that Red, Petite pink and Petite pink varieties of *Ixora* are potent sources of natural dyes. These plants could be used as a good source of natural dye. Further studies are recommended to analyse the bio-potentialities and role as food or fabric colourants.

Key words *Ixora; anthocyanin;pigments; natural dye.*

Use of natural dyes started to decline after the invention of synthetic dyes in the second half of the nineteenth century. Natural dyes are considered eco-friendly as these were renewable and biodegradable. Natural dyes are the colours derived from plants and animals or insect matter without any chemical processing (Rakhi et al., 2006). Environmental issues regarding the production and application of synthetic dyes revived consumer interest in natural dyes during the last decades. Apart from their application in textiles, natural dyes are also used for the coloration of food, medicines, handicraft items, toys and leather processing. Many of the dye-yielding plants are also used as medicines in various traditional medicinal systems (Saxena and Raja, 2014).

Ixora coccinea known as Jungle Geranium or Flame of the Woods is a common flowering shrub of Rubiaceae, native to Asia. There are about 500 species of *Ixora* (Nayak et al., 1999). Extensive literature surveys revealed that *Ixora* is important medicinal plant with diverse pharmacological spectrum. The plant is traditionally used for their hepatoprotective, chemoprotective, antimicrobial, anti-oxidant and anti-inflammatory activities. The plant shows the presence of many chemical constituents which are responsible for varied pharmacological and medicinal properties. The flowers of *Ixora* contain anthocyanin and have antidiarrheal (Maniyar et al., 2010) and cytotoxic properties (Latha et al., 1998). In this background the present study attempts to extract and quantify different colouring pigments from five selected varieties of *Ixora coccinea*.

MATERIALS AND METHODS

Plant materials

The plant materials used for the present study were leaves and flowers of five varieties of *Ixora coccinea* (Rubiaceae). Different varieties used in the study were Red, Petite Red, Petite Pink, Maui Yellow and Kampong White (Fig 1a-e).

Quantification of total chlorophyll

Total chlorophyll, chlorophyll a, chlorophyll b and carotenoids present in the leaves of *Ixora* were quantified by following the methodology of Arnon (1949).

Quantification of anthocyanin

Total anthocyanin content present in the flowers of different *Ixora* varieties were quantified using the methodology of Tatsuzawa et al., (2004).

RESULTS AND DISCUSSION

Total chlorophyll and carotenoid content

Chlorophylls and carotenoids are essential pigments of higher plants and responsible for variations of colour from dark-green to yellow. Total chlorophyll and carotenoid contents in selected varieties of *Ixora* have no significant variation among them (Fig.2). Carotenoid concentration was higher in Red variety (35.192 mg/g tissue) followed by 'Petite Red' (34.198 mg/g tissue), and lowest in Kampong White. Interestingly all varieties showed almost same range of carotenoid concentration, i.e., above 30 mg/g. While amount of chlorophyll a in varieties were low compared to chlorophyll b. In Maui Yellow, Kampong White and

Fig.1a-e. Different varieties of *Ixora coccinea* (a-Red, b-Petite Red, c-Petite Pink, d-Maui Yellow and e-Kampong White)

Petite Pink also carotenoids were abundant compared to other pigments (Fig.2).

Anthocyanin content

Flowers exhibit practically every colour and shade that we could imagine. Anthocyanins are responsible for red, blue, and purple colours in higher plants. In the present study anthocyanin content of the selected varieties of *Ixora* showed significant variation (Fig.2). Anthocyanin content of 'Red' variety was higher (22.710 mg/g) followed by 'Petite Red', other varieties showed comparatively lower amount of anthocyanin. Concentration of anthocyanin in Kampong White was 5.176mg/g.

Addition of small amounts of other pigments with anthocyanins causes mixing or blending of colours. When co-pigmentation takes place, a chemical interaction occurs between the anthocyanin and other flavonoids (Goodwin, 1965). Darker colours are usually caused by anthocyanins. Anthocyanins are bioactive flavonoid compounds beneficial against many chronic diseases when consumed in the form of food (Winny and Valerie, 2011).

Usually the fruits that contain higher amounts of anthocyanins (Wu et al., 2006). However nowadays anthocyanins from flowers are also using as a source of food or as dyes. In the present study also comparison of anthocyanin and carotenoids confirmed the finding of Alkema, et al. (1982), i.e., varying petals colours are obtained due to the presence and interaction of different carotenoids and flavonoids. Red variety showed deep red coloured petals due to higher content of anthocyanin and a medium level of carotenoids. Similarly, Petite red with high levels of

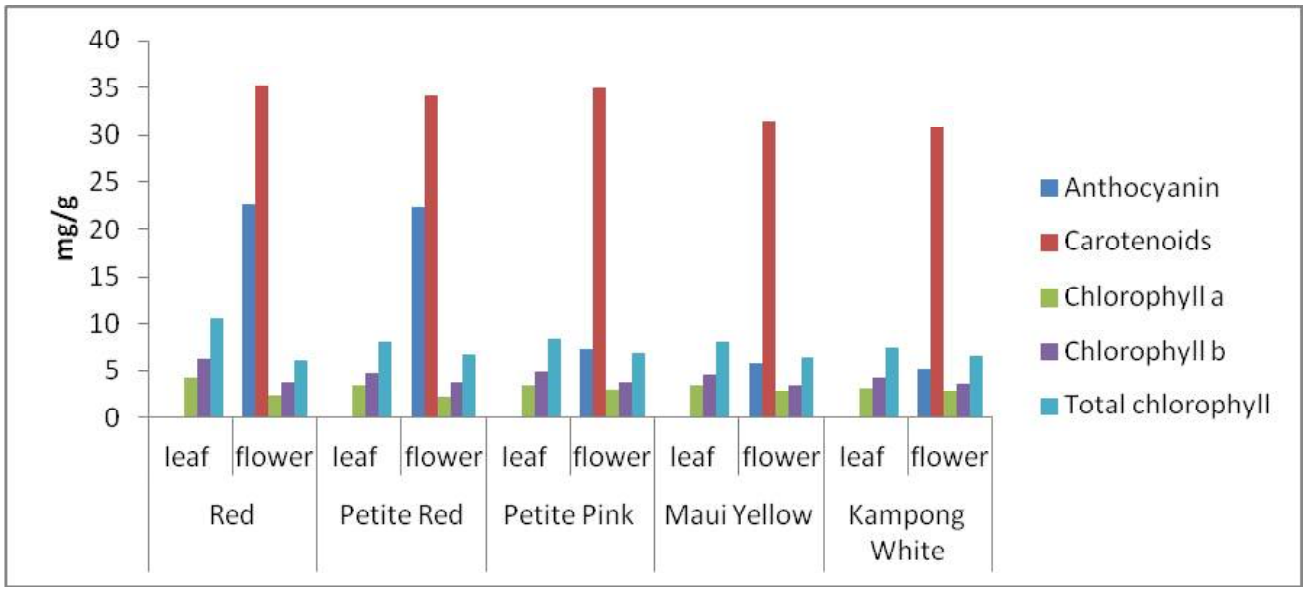

Fig. 2. Pigment variation in different varieties of *Ixora coccinea*

carotenoids and anthocyanins showed orange red coloured petals. Consequently, it was confirmed that the pigments responsible for the petals colours are found in most cultivars and their pigment components affect the flower colours of the cultivars (Chang et al., 2015).

CONCLUSION

The pigments present in different varieties of *I. Coccinea* differ. Pigments such as anthocyanin and carotenoids can be used commercially as nontoxic and eco-friendly substitutes to synthetic dyes. The present study provides an insight into the pigment richness of *Ixora* varieties. It could be concluded that each pigment is responsible for the petal and leaf colour and the compositions of the pigments affect their flower colours and that the cultivars could be a good source for pharmaceutical, floriculture and pigment industries.

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Anti-bacterial Activity of *Andrographis elongata* (Vahl) T. Anderson

SHEEJA YB [1], OOMMEN P SAJ[2] AND LAIJA S NAIR[3*]

Department of Botany, University College, Trivandrum-695034, Kerala, India
**email: sjkattakada@gmail.com*

ABSTRACT

Andrographis elongata* is an endemic medicinal plant belonging to the family Acanthaceae. *A. elongata* is distributed to the Western Ghats and used in the treatment of snake bite, hyperglycemia, skin disease, and also veterinary medicines. The plant is used by the tribal village herbalist, village dwellers, local medicine men and other traditional healers as a panacea in old Travancore. *A. elongata* is a best medicine for diabetes. The medicinal qualities of the plant lie in their active principles. The main objective of this work was to evaluate the anti-bacterial activity of the methanol extracts of the leaves of *A. elongata* by using different bacterial strains such as *Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumoniae* and *E.coli.* The findings of the present study showed that among the different gram positive and gram negative bacterial strains studied, *Pseudomonas aeruginosa* is more sensitive to methanol leaf extract of *A. elongata.

Key words *Andrographis elongata,* Anti-bacterial activity, *Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumoniae* and *E.coli*

Herbal medicines have an important role in prevention and curing of ailments. In India, there are 500 traditional communities depending on plants for their health care needs (Lalitha *et al*., 2015). According to World Health Organization (WHO), 80% of the rural people depend on plants for their primary health care needs (Sakarkar and Deshmukh, 2011). The active principles derived from plants possess diverse pharmacological activities (Kumar *et al.,* 2009) such as antioxidant, anti-proliferative, antibacterial *etc* (Roja and Heble,1994). *Andrographis elongata* is an endemic medicinal plant (Ahmedulla and Nayar, 1986) belonging to the family Acanthaceae, distributed in the Western Ghats of South India. It is used for the treatment of snake bite, hyperglycemia, skin disease, and also in veterinary medicines (Cinnappan Alagesaboopathi, 2010) in old Travancore. The present investigation aims to provide a scientific basis of the anti-bacterial potentiality of the plant that may account on its traditional practice.

MATERIALS AND METHODS

Plant material collection

Andrographis elongata (Vahl) T Anderson was collected from the home garden in Parassala, Thiruvananthapuram district, Kerala.

Preparation of extracts

The plant material were collected, weighed and dried under the shade for 10 days. The dried leaves were powdered and 50 gm of powdered samples were packed in a thimble and kept in soxhlet apparatus. Methanol was used as the solvent. Five hundred ml of methanol was taken in the round bottom flask. The whole apparatus was kept over a heating mantle and heated continuously for 8 hrs at boiling point. The extracts were concentrated to dryness (at 37^0C - 40^0C in oven) and weighed, the residues were transferred to a pre weighed sample bottles and stored in desiccator for further studies (Harborne, 1973).

Tested microorganisms

Microorganisms used in this study were *Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumoniae* and *E.coli*.

Sterilization of Glasswares

All glassware including petriplates, test tubes, conical flasks etc. were sterilized by autoclaving at 121 ^{0}C for 10 minutes.

Preparation of nutrient agar medium

The nutrient agar medium was prepared by adding 28 g of nutrient agar in 1000 ml distilled water. Culture media was autoclaved at 121^0 C for 15 minutes. Required volume of autoclaved medium was poured into sterile petridishes under aseptic condition (Pelezcar, 1957).

Inoculation of Bacteria

From pure culture, bacteria were transferred to test tubes containing peptone water. Then this was

Table 1. Anti-microbial activity of methanol leaves extract of *A. elongata*

Bacterial species	Gram +/-	Minimum inhibitory concentration (MIC) in mm				
		25 µg / disc	50 µg / disc	100 µg / disc	Standard (Streptomycin) 50 µg / disc	Control
E.coli	+	Nil	1.0	1.0	3.0	Nil
Enterococcus faecalis	+	1.1	1.6	1.8	3.0	Nil
Pseudomonas aeroginosa	_	1.8	2.0	2.2	4.8	Nil
Staphylococcus aureus	_	1.2	1.5	1.8	3.8	Nil
Klebsiella pneumonia	_	Nil	1.0	10.0	3.8	Nil

incubated for one hour at 37^0 C for regeneration. After one hour this was taken out and swabbed into the nutrient agar plate. Then it was kept aside. Inoculation was done under flame in high aseptic condition, under laminar air flow chamber.

Test for Antibacterial Activity (NCCLS, 1993)

Different bacterial strains such as *Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumoniae* and *E.coli* was used used for analysis. Wells of approximately 10mm was bored using a well cutter and sample of 25, 50, and100µl concentrations were added. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well (NCCLS, 1993). Streptomycin was used as a positive control.

RESULT AND DISCUSSION

The antibacterial potential of *Andrographis elongata* was evaluated to identify the effects against the bacterial strains according to their zone of inhibition .The leaf extract of *A. elongata* showed inhibition zone diameter of 1.0 cm, 1.8 cm, 2.2 cm, 1.8 cm and 1.1 cm at a concentration of 100 mg/ml against *E.coli, Enterococcus faecalis, Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella pneumoniae* respectively (Table: 1). The methanol extract of *A.elongata* possessed higher antibacterial activity against *Pseudomonas aeruginosa* than other strains. The extract showed the inhibition zone diameter of 1.8 cm, 2.0 cm and 2.2 cms at a concentration of 25 mg/ml, 50 mg/ml and 100 mg/ml respectively against *Pseudomonas aeruginosa*. At lower concentration (25mg/ml) no activity was exhibited against *E.coli* and *Klebsiella pneumoniae.*

Thus from the result it is revealed that there is a dose dependent increase in antibacterial activity of plant extract against different strains of bacteria. The maximum antibacterial activity was exhibited by the methanol leaf extract against *Pseudomonas aeruginosa* followed by *Staphylococcus aureus* and *Enterococcus faecalis*. The antibacterial activity of the methanol extract of *A.elongata* might be due to the presence of flavonoids, terpenoids and polyphenols. Among the three gram negative bacteria tested, the extracts showed more antibacterial activity against *Pseudomonas aeruginosa* followed by *Staphylococcus aureus.* Antimicrobial resistance is a threat to mankind because most of the infection causing bacteria has become multidrug resistant.

Antibacterial activity of leaf extracts *A. paniculata* revealed that petroleum ether extract inhibited *Shigella sp., Proteus vulgaris* and *Klebsiella pneumoniae* with maximum inhibition zone being 5mm at 1mg/ml. HPLC analysis revealed the presence of andrographolide in this plant which may be responsible for antibacterial activity. During this study it was confirmed that standard andrographolide inhibited all the bacterium except E. coli. (Anitha and Rayunukaa, 2013). Similarly in the present study resistant nature of *E.coli* and *Klebsiella pneumoniae* may be due to the presence of andrographolide. The extract obtained from the leaf of *Andrographis macrobotrys* using methanol showed highest activity against pathogen like *Pseudomonas aeruginosa* (Alagesaboopathi, 2014). The present findings also correlated with the earlier studies of Tan Yee Kuan (2015), Nagaraja and Chandrashekhar Biradar (2014), and Suparna Deepak *et al.,* (2014).

According to Shuddhalwar, et al 2014, greater than 16.00mm zone of inhibition represents the sensitive nature of bacterium. Accordingly in the present study *Pseudomonas aeruginosa,*

Staphylococcus aureus and Enterococcus faecalis were reported to be sensitive against the leaf extract of *A. elongata*

CONCLUSION

Antibiotic resistant bacteria may keep people sick longer, and sometimes people are unable to recover at all. Because of the concern about the side effects of conventional medicine, the use of natural products as an alternate to conventional treatment in healing and treatment of various diseases has been on the rise in the last few decades. This study partially explained the use of plant extract against some human pathogenic microbes. Of the different strains of bacteria studied, this plant extract is found to be more susceptible to *Pseudomonas aeruginosa*. The antibacterial activity of the methanol extract of *A.elongata* might be due to the presence of flavonoids, terpenoids and polyphenols. The active plant extract may be further subjected to biological and pharmacological investigation for isolation of antibacterial and therapeutic compounds.

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Bioassay Guided Fractionation and Identification of Bioactive Compounds of Mushroom *Termitomyces mummiformis*

G. SHIRMILA JOSE[1*] AND P.M. RADHAMANY[2]

[1]*Department of Botany, All Saints College, Thiruvananthapuram-695007*
[2]*Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram -695581, Kerala, India.*
**email: shirmijo@ gmail.com*

ABSTRACT

Bioactive substances produced by mushrooms show many medicinal effects including antimicrobial and antioxidant properties. Both fruiting body as well as the mycelium contains compounds with wide range of antimicrobial activity. Objective of this current study is to screen and report the antimicrobial compound of *Termitomyces mummiformis* by adopting bioautography method. The human fungal pathogen of *Aspergillus terreus, Mucor sp., Fusarium sp.,* and bacterial pathogens *Pseudomonas sp., E.coli*, were scanned for this current work. Out of three fungal strains scrutinized the human pathogen *Aspergillus terreus, Fusarium sp.,* and one bacterial strain *pseudomonas sp.,* show sensitivity with extract. Three active bands were observed from the bioautography. But only one antimicrobial bioactive band (Rf value-0.76) of the mushroom *T. mummiformis* was identified as tannic acid by HPLC analysis. Therefore, it appears reasonable to surmise that additional agents, thus far undiscovered, also exist.

Key words *Mushroom, Termitomyces mummiformis, Antimicrobial activity, Bioautography, HPLC, Tannic acid.*

Mushroom has proven to produce a wide range of secondary metabolites with high therapeutic values. They were verbosely appreciated, not only for their texture, flavor, but also for their nutritional properties. Bioactive substances produced by mushrooms show many medicinal effects (Grace *et al.,* 2006; Harhaji *et al.,* 2008) including antimicrobial (Kitzberge *et al.,* 2007; Barros *et al.,* 2007) and antioxidant properties (Tseng *et al.,* 2008; Wu *et al.,* 2009). The commonly used Asian medicinal mushrooms (*Ganoderma lucidum, Termitomyces reticulates, Agaricus bisporus , Chlororhyllum rhacodes, Macrolepiota procera var. procera. Amanita rubescens var. rubescens, Pleurotous dryinus, Armillaria ostoyae, Pleurotous ostreatus Polyporus squamosus Boletus edulis Boletus pseudosulphureu, Leccinum scabrum, Suillus luteu, Lepista nuda, Lepista personata, Hydnum repandum, Lactarius deliciosus, Lactarius piperatus, Lactarius salmonicolor, Lactarius volemus, Russula delica, Russula integra var.integra, Russula nigricans, Russula vinosa, Boletus erythropus var. erythropus, Trametes versicolor* has a wide range of biological activities including, antioxidant, antimicrobial, antiviral, antitumor, and immuno modulatory effects (Chu *et al.,* 2002; Loganathan *et al.,* 2010 and Keleº *et al.,* 2011).

Nowadays the microbial deceases are to be a serious problem due to the emergent antibiotics resistance. The association between multi-resistant microorganisms and hospital infections certainly highlighted the problem and the urgent need for solutions. Therefore, the research of new antimicrobial substances effective against pathogenic microorganisms resistant to current drugs is crucial. This situation provided the impetus to the search for new antimicrobial substances from various natural sources (Cordell, 2000). In mushroom both fruiting body as well as the mycelium contain compounds with wide range of antimicrobial activity and their compounds could be isolated from many mushrooms species and could be of benefit for human. A number of medicinal mushrooms, such as *Aleurodiscus, Coprinus, Clitocybe, Daedalea, Marasmius, Merulius, Pleurotus, Polyporus, Poria, Psathyrella, and Tricholoma spp.,* are rich sources of s-glucan, lectin, phenolic compounds, flavonoids, polysaccharides, triterpenoids, diatery fibre, lentinan, schizophyllan, lovastatin, pleuran, steroids, glycopeptides, terpenes, saponins, xanthones, coumarins, alkaloid, kinon, fenil propanoid, kalvasin, volvotoksin, flammutoksin, porisin, eryngeolysin etc., (Smania, 2003; Wang *et al.,* 2004). There is a renewed interest in traditional medicine and an increasing demand for more drugs from natural sources, with the nationwide growth of interest in health and the expanding health food market, research into the industrial uses of secondary metabolites has also increased.

Moreover the antimicrobial characterization of our present wild mushrooms *T. mummiformis,* there is a lack of research work has been observed. Herein, the aim of this current study is to report the screening

and identification of antimicrobial compounds by adopting bioautography method. As far as our literature survey could ascertain, a few information was available on the in vitro antimicrobial activities of Indian wild mushrooms and it is the first time that the wild mushrooms *T. mummiformis* was submitted .

MATERIALS AND METHOD

Sampling site and collection of sample

The mushrooms were collected from Thiruvananthapuram, Kerala (India) during the period of May-December. The collected material was transported to the laboratory in Department of Botany, University of Kerala. All the macro and micro characters including habit and morphological features were recorded. The specimen cited in the article (*Termitomyces mummiformis*, (Fig 1) was kept in the herbarium of Department of Botany, University of Kerala, Kariavattom (KUBH-5802).

Preparation of extract

The mushrooms were shade dried and powdered in an electric mixer grinder. The powdered mushroom, 200g was extracted with methanol as a solvent by using soxhlet apparatus. After extraction the extract was evaporated to dryness under reduced pressure in a rotary evaporator. This dried extract (1.8g) was used for following experiments.

The human fungal pathogen of *Aspergillus terreus, Mucor sp., Fusarium sp.,* and bacterial pathogens *Pseudomonas sp., E.coli*, from the Aravind Eye Hospital (Madurai) were collected and used for this current work.

Fungal stock preparation

The strains were maintained in potato dextrose agar (PDA) medium. A potato dextrose broth was prepared with distilled water and autoclaved at 121 ^{0}C for 15 minutes. After 15 minutes, the broth was inoculated with the strains of test organism that were previously lyophilized. The media was allowed to incubated for 48hr before inoculation with the respective strains of fungal pathogen.

Bacterial stock Preparation

Nutrient broth was prepared with distilled water and autoclaved at 121 ºC for 15 minutes. After 15 minutes, the broth media was inoculated with the strains of test organism that were previously lyophilized. The media was allowed to incubate for overnight before inoculation with the respective strains of bacteria.

Bioautography guided identification of antimicrobial constituents

About 2µg of extracts of mushroom was loaded on TLC plates (Merck, 20 x 20 cm^2). The plates were developed in Hexane: methanol: acetic acid (3:9:2) to separate the various constituents of the extracts. The developed plates were air dried for complete removal of solvents. Cultures were grown on nutrient broth for bacteria and potato dextrose broth medium for fungi were prepared in 250 ml conical flask. The developed TLC plates were inoculated with a fine spray of concentrated suspension containing approximately 10^9 organisms per ml of actively growing microorganism. The plate was incubated for 24 h at 35^{o}C in a clean chamber at 100% relative humidity in the dark, and then sprayed with an aqueous solution of 2 mg/ml solution of *p*-iodonitrotetrazolium violet (Sigma) (INT). Inhibition zones were observed as clear spots against purple background and their Rf values were compared with the reference plate. We tried to use digital photography to record results but it did not work well. Good results were obtained after using a scanner (HP scanjet).

Activity guided isolation and identification of compounds

HPLC analysis

The active partial purified (antimicrobial) compound zone from the reference TLC plates were scribed and dissolved with HPLC grade methanol (1.0 mg/mL) was filtered through a cellulose acetate membrane filter prior to HPLC (Simadzu corporation-Kyoto, Japhan) analysis. Separation was achieved on a reverse phase C_{18} column (250 x 4.6mm, 5micron) temperature at 24^{o}C. The mobile phase of the optimized chromatographic method consisted of solvent A (methanol) and solvent B (0.5% (v/v) acetic acid in water). The elution profile was as follows: 0 min 10% A in B, 28.6 min 60% A in B, 30 min 10% A in B. All gradients were linear. The flow rate was 1ml/min, and injection volume was 20µL. Absorption was measured at 290 nm. Detection was carried out in UV-visible detector. The eluted components were identified (System controller-CBM-20A/20Alite) based on the retention time by comparison with retention time of reference standard. The compounds present in the samples were characterize according to their UV-vis spectra and identified by their retention

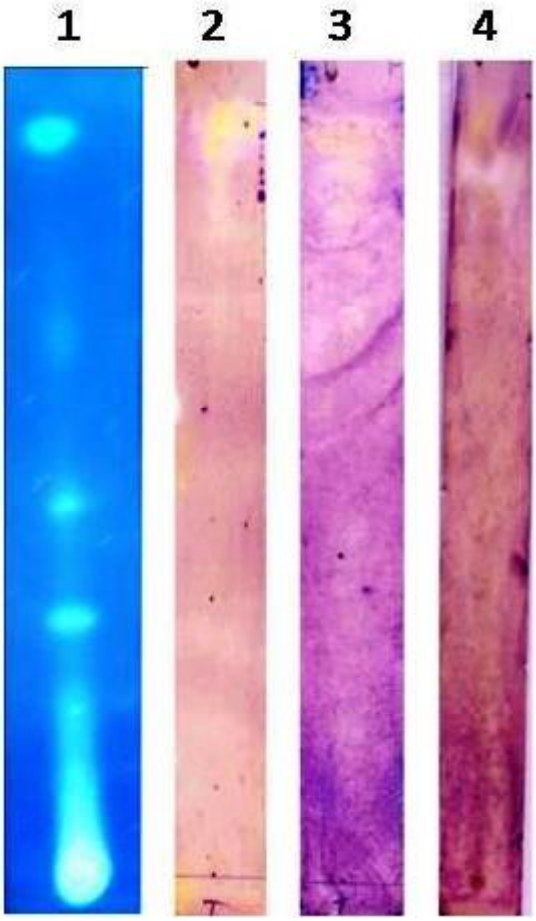
times in comparison with those of commercial standards.

RESULTS

Usually, pure active compounds reveal more activity than crude extracts. Searching wild sources may bring new natural products with antimicrobial properties that provide good protection against the infectious diseases. Bioautography was used to separate antibacterial and antifungal compounds to obtain more information on the diversity of antimicrobial compound present in *T. mummiformis* (Fig. 1) mushroom methanolic extract.

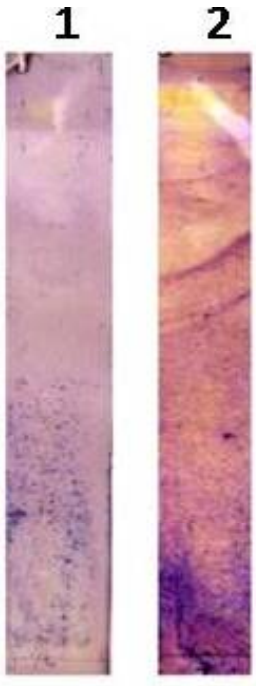
Fig. 1. Wild edible mushroom *Termitomyces mummiformis.*

Inhibition zones of antimicrobials are observed as white- creme zones on a purple –red background (Fig. 2 & 3). The zone areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compound that inhibited the growth of tested microbes. Many of these were most likely due to the same compounds. A representative bioautograms of *T. mummiformis* methanolic extract against the different bacteria and fungi is shown in (Fig. 2 & 3).



1- Plate under long UV; 2- *Aspergillus terreus,* 3- *Fusarium sp.,* 4- *Mucor sp.,*

Fig. 2. Bioautography of methanolic extract of *T. mummiformis,* antifungal constituents.



1- *E. coli* ; 2- *Pseudomonas sp.,*

Fig. 3. Bioautography of methanolic extract of *T. mummiformis,* antibacterial constituents.

Out of three fungal strain tested, the human pathogen *Aspergillus terreus, Fusarium sp.,* and one bacterial strain *Pseudomonas sp.,* show sensitivity with extract. The observed results infer that the *T. mummiformis* methanolic extract gave active bands against both bacterial and fungal organism (Fig: 2 &3). According to the inhibition bands, the bands with 0.68, 0.72 and 0.77 Rf value constituents shows antimicrobial activity (Table- 1).

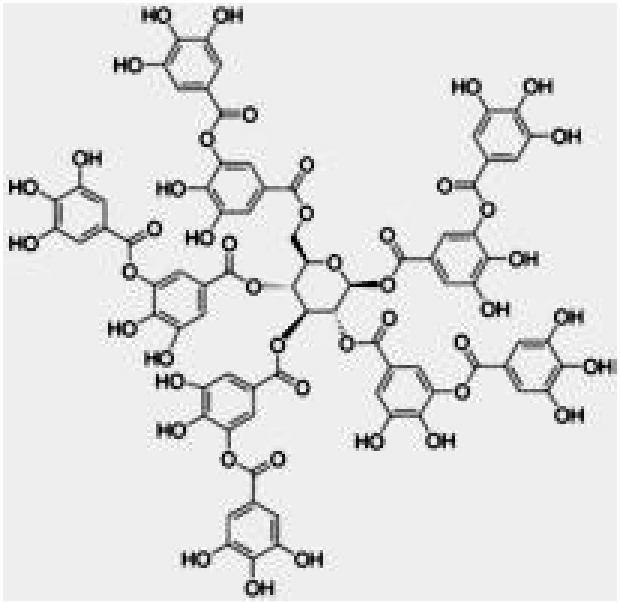
Table 1. The biochromatogram (Rf) values of antimicrobial constituent.

Mushroom	Rf value	Antibacterial activity			Antifungal activity	
		Pseudomonas sp.,	*E.coli,*	*Aspergillus terreus,*	*Fusarium sp.,*	*Mucor sp.,*
T. mummiformis	0.28	-	-	-	-	-
	0.33	-	-	-	-	-
	0.37	-	-	+	-	-
	0.41	-	-	-	-	-
	0.58	-	-	-	-	-
	0.61	-	-	-	-	-
	0.68	+	-	+	+	-
	0.72	+	-	+	+	-
	0.77	+	-	+	+	-

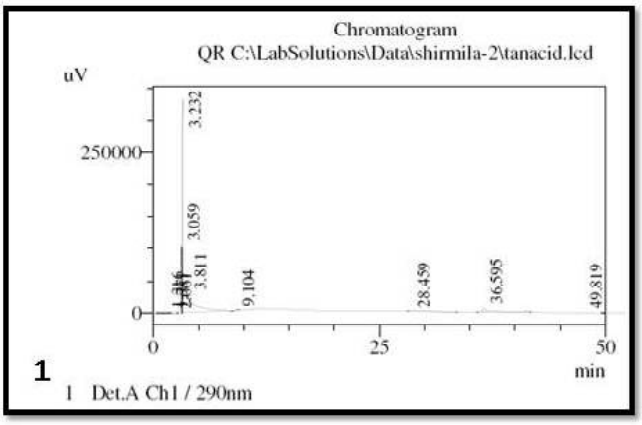
Identification of antimicrobial compound by HPLC

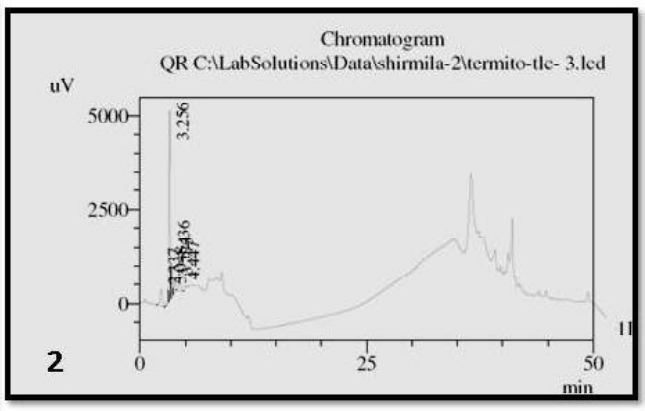
The antimicrobial active band areas were scraped from the plates, and they eluted from the silica with methanol. Eluted samples were further purified using the above preparative chromatography method. Finally, the components were identified by HPLC (Fig 4).

Three active bands were observed from the bioautography. But only one antimicrobial bioactive band (Rf value-0.76) of the mushroom *T. mummiformis* was identified as tannic acid (Fig. 5).



Tannic acid - Antimicrobial compound of *T. mummiformis* .





- HPLC chromatogram of standard chemicals

2- Antimicrobial compound of *T. mammiformis* . The mushroom active bands with Rf value 0.77- tannic acid.

Fig. 4. HPLC identification of antioxidant and antimicrobial compound of *T. mummiformis*. The mushroom active bands with Rf value 0.77- tannic acid.

DISCUSSION

Antimicrobial activity

Infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat to public health, despite the tremendous progress in human medicine. Antimicrobial activity of natural extracts and pure compounds can be detected by observing the growth response of various microorganisms to samples that are placed in contact with them. Several methods for detecting antimicrobial activity are available, but since they are not equally sensitive or not based upon the same principle, results will be profoundly influenced by the method (Cos *et al.,* 2006). The antibacterial and antifungal test methods are classified into three main groups: diffusion, dilution and bioautographiy methods.

A fourth and upcoming test method is the conductimetric assay, detecting microbial growth as a change in the electrical conductivity or impedance in the growth medium (Sawai *et al.,* 2002). Therein, we will discuss the bioautography method that has been applied in the present work.

Bioautography belongs to microbiological screening methods commonly used for the detection of antimicrobial activity. The screening can be defined as the first procedure, which is applied to an analyzed sample, in order to establish the presence or absence of given analyses. Basically speaking, it is a simple measurement providing a "yes/no" response. Quite often, screening methods give higher sensitivity than any other methods. Moreover, they are simple, cheap, time-saving and do not require sophisticated equipment. Among the all bioautographic methods, the most widely applied is direct bioautography. The principle of this method is that a developed TLC plate is dipped in a suspension of microorganisms growing in a proper broth and then incubated in a humid atmosphere. A silica surface of the TLC plate covered with the broth medium becomes a source of nutrients and enables growth of the microorganisms directly on it. However, in the places where antimicrobial agents were spotted, the inhibition zones of the microorganism growth are formed. Visualization of these zones is usually carried out using dehydrogenase activity-detecting reagents; the most common are tetrazolium salts. The dehydrogenase of living microorganisms converts tetrazolium salt into intensely colored formazan. As a result, cream-white spots appear against a purple background on the TLC plate surface, pointing the presence of antibacterial agents.

In the present study we follow the direct bioautographic method and the results show cream to white zones with respect to antimicrobial activity of the compounds (Fig. 2 & 3). Baroty et al., (2010) worked out characterization of antioxidant and antimicrobial compounds of cinnamon and ginger essential oils by TLC- bioautography. Yamac and Bilgili (2006) reported antimicrobial activities of fruit bodies and mycelia cultures of mushroom.

According to Sule *et al.,* (2011); Nalina and Rahim, (2007) once an activity has been located at the TLC plate, the sample can be analyzed by LC–MS or HPLC to establish, whether known or new compounds and/or substance classes are involved. Here in we identified the activity guided antimicrobial compound tannic acid by HPLC (Fig. 4 &5). The current identification was supported by Colak *et al.,* (2010). He report the antimicrobial efficacy of tannic

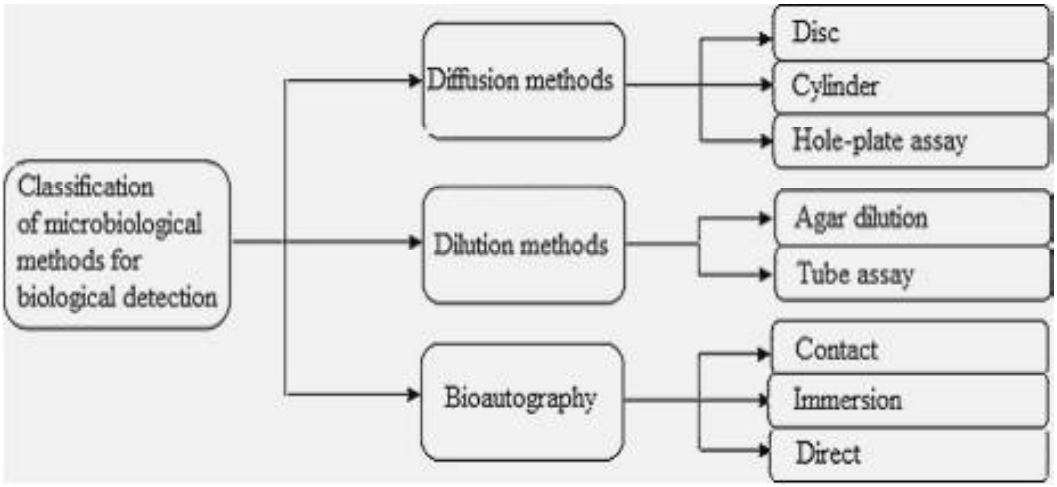


Fig. 5. The classification of microbiological methods for biological detection.

acid on sixteen test microorganisms -*Bacillus cereus* (ATCC 11778*), Salmonella typhimurium* CCM 5445*, Proteus vulgaris* (ATTC 6889*), Enterobacter aerogenes (*ATCC 13048), *Pseudomonas aeruginosa* (ATTC 27853), *Escherichia coli* (ATTC 25922*), Staphylococcus aureus (*ATCC 6538), *Neisseria canis, Aspergillus niger, A. fumigatus, A. flavus, Penicillium granulatum P. granulosum, Geotricum candidum, Yarrowia lypolitica and Rhodotorula rubra* (DSM 70403) and surmise that 3% of tannic acid exhibited antimicrobial activity against all bacteria, moulds and yeasts tested.

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Preliminary Phytochemical Screening of *Sida rhombifolia*, L., Using Different Solvents.

R.V. SUSHAMA RAJ* AND L. RAJESWARI

**Department of Botany, H.H.M.S.P.B. N.S.S. College for Women, Neeramankara, Thiruvananthapuram, Kerala, India.*
**email : drsushamarajrv@gmail.com*

ABSTRACT

Plants and plant extracts have important role in modern medicine as their chemical and medicinal constituents are found in natural form. The secondary metabolites represent a large reservoir of structural moieties which work together exhibiting a wide range of biological activities. Plants and plant based products are bases of many modern pharmaceuticals that are currently in use for various diseases. *Sida rhombifolia*, L. commonly called bala or atibala is a perennial or sometimes annual plant in the family *Malvaceae*. Present study involved phytochemical screening of leaf and root of *Sida rhombifolia*, L. using six different solvents viz; distilled water, acetone, alcohol, chloroform, petroleum ether and benzene. The extracts prepared from powdered plant parts were subjected to qualitative phytochemical screening using standard procedures. Of the 15 phytochemicals tested, 14 were found in various solvent extracts of *Sida rhombifolia*, L. By this study, it was confirmed that the selected plant species is a potent source of useful drugs. However, further studies are required in this direction for its comprehensive analysis including qualitative or semi qualitative analysis, characterize its chemical structure and assess its biological activities.

Key words *Sida rhombifolia*, leaves, root, phytochemical screening

The use of plants as medicines goes back to early man. Certainly the great civilisations of the ancient Chinese, Indians, and North Africans provided written evidence of man's ingenuity in utilising plants for the treatment of a wide variety of diseases. In ancient Greece, for example, scholars classified plants and gave descriptions of them thus aiding the identification process. Theophrastus, the father of botany, has been recorded many medicinal plants. Phytochemicals are bioactive chemicals of plant origin. They are regarded as secondary metabolites because the plants that manufacture them may have little need for them. They are naturally synthesized in all parts of the plant body, such as bark, leaves, stem, root, flower, fruits, seeds etc. i.e. any part of the plant body may contain these active compounds (Cowan, 1999). The quantity and quality of phytochemicals present in plant parts may contain active compounds. The quantity and quality of phytochemicals present in plant parts may differ from one part to another. In fact, there is lack of information on the distribution of the biological activity in different plant parts essentially related to the difference in distribution of active compound which are frequent in some plant parts (Mojab, 2003; Parekh and Chanda, 2008; Parekh and Chanda, 2009).

Medicinal plants contain some organic compounds which provide definite physiological action on the human body and these bioactive substances include tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids (Edoga, 2005; Mann, 1978). These compounds are synthesized by primary or rather secondary metabolism of living organisms. Secondary metabolites are chemically and taxonomically extremely diverse compounds with obscure function. They are widely used in the human therapy, veterinary, agriculture, scientific research and countless other areas (Vasu, 2009). A large number of phytochemicals belonging to several chemical classes have been shown to have inhibitory effects on all types of microorganisms in vitro (Cowan, 1999).

Sida rhombifolia, L. commonly called bala or atibala is a perennial or sometimes annual plant in the family *Malvaceae*. The species is native to tropics and subtropics and usually confined to waste ground such as roadsides and rocky areas. Synonyms include *Malva rhombifolia*. It is used in ayurvedic and siddha medicine, where it is known as Kurumthotti. The stems are erect to sprawling and branch growing 50 to 120 cms in height, with the lower sections being woody. The dark green diamond shaped leaves are arranged alternatively along the stem, 4 to 8 cms long with short petiole. They are pale below, with short greyish hairs. The leaves have toothed or serrate margins. The

petioles have small spring stipules at their bases. The moderately delicate flowers occurs singly on flower stalks that arise from the area between the stems and leaf petioles (axillary position). Flowers are pentamerous with creamy to orange – yellow petals and monadalphous androecium. Gynoecium is pentacarpellary with five capitate stigma.

MATERIALA AND METHODS

Collection of plant material

The leaves and root of *Sida rhombifolia,* L. were collected from five different locations of Thiruvananthapuram. Collected plant parts were shade dried for one to two weeks. Constant monitoring was carried out to avoid microbial contamination. The dried plant material was taken and ground using motor and pestle to obtain a fine powder. The powder was further passed through a 2 mm sieve to obtain finer particles. The powdered samples were stored in a clean glassware container and stored in low temperature until needed for analysis (Das *et al.,* 2010).

Preparation of plant extracts

5 gram of dried and powdered sample was taken. It was put separately in acetone, petroleum ether, chloroform, ethyl alcohol, benzene and distilled water. Mixed and extracted for 24 hours on a stirrer with continuous stirring. After extraction, the extracts were filtered through Whatman No.1 filter paper, centrifuged the filtrate for clarification and stored for further phytochemical investigations (Das *et al.,* 2010).

Preliminary phytochemical investigations

Analysis on the presence of different phytochemical constituents such as proteins, amino acids, carbohydrates, alkaloids, saponins, phytosterols, glycosides, phenols, tannins, flavonoids, steroids, terpenoids, and vitamin C were analysed according to the standard procedures, as described below, were used (Harborne, 1998).

Test for Proteins

Biuret Test:- Test solution was treated with equal volume of 10% sodium hydroxide solution and two drops of 1% copper sulphate solution, mixed well and observed for the formation of violet/ pink colour. If it is so, presence of proteins was detected.

Millon's Test:- Two ml of crude extract when mixed with 2ml of Millon's reagent, if a white precipitate appeared which turned red upon gentle heating and disappeared on cooling confirmed the presence of protein.

Xanthoproteic Test:- Two ml of extracts were treated with few drops of conc. Nitric acid. Mixed well. Formation of light to dark yellow colour was noted which indicates the presence of proteins.

Test for Free Amino Acids

Ninhydrin Test :- Test solution when boiled with 0.2% solution of Ninhydrin. Formation of purple color suggests the presence of free amino acids.

Test for Carbohydrates

Benedict's test:- Test solution was mixed with few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) and boiled in water bath, observed for the formation of reddish brown precipitate to show a positive result for the presence of carbohydrate.

Fehling's Test: - Filtrates were hydrolysed with dil. HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

Molisch's Test :- Filtrates were treated with 2 drops of alcoholic á-naphthol solution in a test tube. Formation of the violet ring at the junction indicates the presence of Carbohydrates.

Test for Alkaloids

Wagner's Test :- A fraction of extract was treated with 3-5drops of Wagner's reagent [1.27g of iodine and 2g of potassium iodide in 100ml of water] and observed for the formation of reddish brown precipitate (or colouration) which indicates the presence of alkaloids.

Mayer's Test:-Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.

Test for Saponins

Foam Test :- Test solution was mixed with water and shaken and observed for the formation of froth, which should be stable for 15 minutes. This result indicates the presence of Saponins.

Froth Test:- Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

Test for Phytosterols

Salkowski's Test :- Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

Test for Glycosides

Liebermann's test :- Crude extract was mixed with each of 2ml of chloroform and 2ml of acetic acid. The mixture was cooled in ice. Carefully concentrated H_2SO_4 was added. A colour change from violet to blue to green indicated the presence of steroidal nucleus, i.e., glycine portion of glycoside.

Salkowski's test:- Crude extract was mixed with 2ml of chloroform. Then 2ml of concentrated H_2SO_4 was added carefully and shaken gently. A reddish brown colour indicated the presence of steroidal ring, i.e., glycone portion of the glycoside.

Keller Killiani Test:- Test solution was treated with few drops of glacial acetic acid and Ferric chloride solution and mixed. Concentrated sulphuric acid was added, and observed for the formation of two layers. Lower reddish brown layer and upper acetic acid layer which turns bluish green would indicate a positive test for glycosides.

Test for Phenols

Ferric Chloride Test:- Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Test for Tannins

Gelatin Test:- To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

Braymer's test:- 2ml of extract was treated with 10% alcoholic ferric chloride solution. Formation of blue or greenish colour solution shows the presence of Tannins.

Test for Flavonoids

Shinoda test :- Crude extract was mixed with few fragments of magnesium ribbon. Con. HCl was added drop wise. Pink scarlet colour appeared after few minutes which indicated the presence of flavonoids.

Alkaline reagent test:- Crude extract was mixed with 2ml of 2% solution of NaOH. An intense yellow colour was formed which turned colourless on addition of few drops of diluted acid which indicated the presence of flavonoids.

Test for Steroids

Liebermann Burchard test :- Crude extract was mixed with few drops of acetic anhydride, boiled and cooled. Concentrated sulphuric acid was then added from the sides of the test tube and observed for the formation of a brown ring at the junction of two layers. Green coloration of the upper layer indicate a positive test for steroids.

Test for Vitamin C

DNPH Test :- Crude extract was treated with Dinitrophenyl hydrazine dissolved in Con. H_2SO_4. The formation of yellow precipitate would suggest the presence of Vitamin C.

Test for Phlobatannins

Precipitate test :- Deposition of a red precipitate when 2mls of extract was boiled with 1ml of 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatannins.

Test for Quinones

HCl Test :- A small amount of extract was treated with concentrated HCL and observed for the formation of yellow precipitate (or coloration).

Test for Oxalate

Acid Test:- To 3ml portion of extracts were added a few drops of ethanolic acid glacial. A greenish black coloration indicates presence of oxalates.

Table 1. Result of phytochemical screening of *Sida rhombifolia*, L. (+ indicates presence and - indicates absence).

Phytochemicals	Extract											
	Distilled Water		Acetone		Alcohol		Chloroform		P. Ether		Benzene	
	Leaves	Root	Leaves	Root	Leaves	Root	Leaves	Root	Leaves	Root	Leaves	Root
Proteins												
Biuret Test	+	+	–	–	+	+	+	+	+	+	+	+
Xanthoproteic Test	+	+	–	–	+	+	+	+	+	+	+	+
Millon's Test	+	+	–	–	+	+	+	+	+	+	+	+
Amino Acids												
Ninhydrin Test	+	+	–	–	+	+	+	+	+	+	+	+
Carbohydrates												
Benedict's test	+	+	–	–	+	+	+	+	+	+	+	+
Molisch's Test	+	+	–	–	+	+	+	+	+	+	+	+
Fehling's Test	+	+	–	–	+	+	+	+	+	+	+	+
Alkaloids												
Wagner's Test	–	–	–	–	–	–	–	–	+	+	+	+
Mayer's Test	–	–	–	–	–	–	–	–	+	+	+	+
Saponins												
Foam Test	–	–	–	–	+	+	–	–	+	+	+	+
Froth Test	–	–	–	–	+	+	–	–	+	+	+	+
Phytosterols												
Salkowski's Test	–	–	–	–	+	+	–	–	–	–	–	–
Glycosides												
Liebermann's test	–	–	–	–	–	–	–	–	+	+	+	+
Salkowski's test	–	–	–	–	–	–	–	–	+	+	+	+
Keller Killiani Test	–	–	–	–	–	–	–	–	+	+	+	+
Phenols												
Ferric Chloride Test	–	–	+	+	+	+	+	+	–	–	–	–
Tannins												
Gelatin Test	+	+	–	–	–	–	+	+	–	–	+	+
Braymer's test	+	+	–	–	–	–	+	+	–	–	+	+
Flavonoids												
Shinoda test	–	–	+	+	–	–	–	–	–	–	–	–
Alkaline reagent test	–	–	+	+	–	–	–	–	–	–	–	–
Steroids												
Liebermann Burchard test	–	–	–	–	+	+	–	–	–	–	–	–
Vitamin C												
DNPH Test	–	–	–	–	–	–	–	–	–	–	–	–
Phlobatannins												
Precipitate test	–	–	–	–	+	+	–	–	–	–	+	+
Quinones												
HCl Test	–	–	–	–	+	+	–	–	–	–	+	+
Oxalate												
Acid Test	–	–	–	–	+	+	–	–	–	–	+	+

RESULTS AND DISCUSSION

Results obtained for qualitative screening of phytochemicals in different parts of *Sida rhombifolia*, L. is presented in Table 1. Of the 15 phytochemicals, 14 were found in various solvent extracts. Phytochemical screening showed presence of proteins, amino acids and carbohydrates in distilled water, alcohol, chloroform, petroleum ether and benzene extract and all these are absent only in acetone extract. Alkaloids present in petroleum ether and benzene extract and was absent in other extracts. Phytosterols only present in alcohol extract. Glycosides present in petroleum ether and benzene extract and absent in distilled water, alcohol, chloroform and acetone extract. Phenols present in acetone, alcohol and chloroform extract tannins present in distilled water, chloroform and benzene extracts. Flavonoids are present only in acetone extract and is absent in all other extracts. Steroids show presence only in alcohol extract. Vitamin C is completely absent in all the six extracts of *Sida rhombifolia*, L. Phytochemicals phlobatannins, quinones and oxalate were shows presence in alcohol and benzene extract . Among the extracts tested maximum result was observed in alcohol and benzene extract of *Sida rhombifolia,* L. followed by chloroform, distilled water, petroleum ether and acetone. Acetone extract of *Sida rhombifolia,* L. shows least result. It shows the presence of phenols and flavonoids.

According to Tiwari *et al*., the factors affecting the choice of solvent are; quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process, potential health hazard of the extractant. The logic in using different solvents when screening for phytochemicals in plant materials was clearly validated in present study. For instance, the results shows that alkaloids were exceptionally present in petroleum ether and benzene extracts but absent in all other extracts. Steroids showed their presence in alcohol extract. This corroborates the reports of Misra *et al*. Proteins and carbohydrates showed their presence in all extracts irrespective to the solvents and plant parts.

Phytochemical screening of the extracts of *Sida rhombifolia,* L. revealed the presence of alkaloids, steroids, flavonoids, aminoacids, phenols and quiones (Table1). These compounds have significant application against human pathogens, including those that cause enteric infections (El Mahmood *et al*.,). The result indicates that *Sida rhombifolia,* L. hold promises as source of pharmaceutically important phytochemicals. Alkaloid which plays some metabolic role and control development in living system is also present in petroleum ether and benzene extracts. They are also involved in protective function in animals and are used as medicine especially the steroidal alkaloids. Tannins are known to inhibit pathogenic fungi, is present in distilled water, chloroform and benzene extracts. The flavonoids and phenolic compounds in plant have been reported to exert multiple biological effects including antioxidant, free radical scavenging abilities, anti-inflammatory, anti-carcinogenic etc.

CONCLUSION

It is very necessary to introduce new and biologically safe and active drugs for an eco friendly life style. Phytochemicals found present in the *Sida rhombifolia*, L. indicates their potential as a source of principles that may supply novel medicines. However, further studies are required in this direction for its comprehensive analysis including qualitative or semi qualitative analysis, characterize its chemical structure and assess its biological activities. Exploration of maximum potential of this valuable plant species is necessary, in medicinal field and pharmaceutical sciences, for their appropriate application.

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Pest Infestation Status of Selected *Musa* Cultivars in Relation to *Odoiporus longicollis* [Olivier]

AJITHA T, KAVITHA K.J , SHABITH RAJ K AND EVANS D.A

**Department of Zoology, University College,*
Thiruvananthapuram 695034
email : drevansda@gmail.com

ABSTRACT

Distribution of total phenols (TP), total flavonoids (TF) and activity of enzymes such as Phenylalanine ammonia lyase (PAL), Polyphenol oxidase (PPO), and Peroxidase (PO) in different cultivars of *Musa* showed high variation and have great influence on the pest status of cultivars in relation to infestation by *Odoiporus longicollis* [Olivier]. Under field condition the pest showed extreme specificity in selection of host plants and they selectively avoided those cultivars which possessed very high activity of PAL, PPO, PO and high contents of TP and TF. Pest susceptible cultivars possessed very low activity of the three enzymes and very low amount of TP and TF. Pest status and genome constitution did not show any correlation in the present study using 26 cultivars. As the cultivar *Amrithsagar* (AAA) was susceptible to infestation by *O. longcollis*, another cultivar *Yangambi* (AAA) was highly resistant to pest. While *Bonda bathesa* and *Cuba* (both ABB) showed susceptibility to this pest, *Karpooravally*, *Peyan* and *Saba* (all ABB) showed non preference by this pest. Rearing of larvae of *O. longicollis* in cultivars possessing very high activity of PAL, PPO and PO and very high contents of TP and TF has resulted mortality of larvae in seven days. Mother weevils are highly judicious in selecting host plants for oviposition and their selective avoidance of resistant cultivars (R) may be by sensing the cultivar specific volatile molecules, which needs detailed study.

Key words *Odoiporus longicollis; Musa cultivars; pest resistance; total phenol; total flavonoids; Phenylalanine ammonia lyase; Polyphenol oxidase; Peroxidase;*

Banana and plantain are clumped perennial herbs. India is one of the major biodiversity centres of plantain and contributes 15% of the banana production in the world (Alagesan *et al*., 2016). In India 70% of banana production is contributed by small scale farmers (Frison *et al.,* 1998). It can be cultivated throughout the year and it can be used both as vegetable and fruit. Some rare cultivars have cultural importance in Kerala, such as *Kadali* (AA), in temple worship, *Kappa* (AAA), in marriage ceremony (Kavitha *et al*., 2015 b) and certain cultivar of certain places are so unique and thereby they got GI tag and one such banana is a unique *Nendran* (AAB) cultivar and is called as *Kazhchakula* from Guruvayoor, Kerala , which has special place in worship in Guruvayur temple..

One of the reasons for extreme diversity of *Musa* cultivars in India is postulated as natural hybridization of *Musa acuminata* triploid (hybrid) with *Musa balbisiana*, which has resulted preponderance of diversity particularly in cultivars with AAB and ABB genome constitution (Price,1995). India is endowed with considerable diversity for edible banana cultivars in the North Eastern and Western Ghats region. Diversity among *Musa* cultivars in India is vast owing to diverse agro-climatic conditions from dry arid zones to humid tropical and cold temperate zones. The local cultivar diversity is more conspicuous in South India, especially in the states of Kerala, Tamil Nadu and Karnataka and cultivar variability decreases from extreme South towards the North (Amalraj, 1992). The South Indian states especially Kerala and Tamil Nadu are endowed with delicate edible diploid cultivars (AA) which can be nurtured only in sheltered humid environments (Uma & Sathiamoorthy, 2002). The wealth of banana cultivars in the coast of Kerala is unequalled by any other banana growing regions in the world (Nayar, 1962).

One of the major constraints in banana production is infestation by pests. It is attacked by different insect pests among which banana pseudostem borer *O. longicollis* is a key pest limiting the production and productivity of banana and plantains (Ostmark, 1974; Visalakshi.*et al.,* 1989). It is a monophagous pest, and both the adults and larvae cause severe damage to banana plants (Shukla and Kumar, 1970). Long life span of adults and larval endophytic behaviour are the major factors limiting the efficiency of conventional methods of control, especially chemical control using insecticides (Shanmugam *et al.,* 2013). Almost all common edible cultivars are affected by the weevil which showed extreme specificity to some cultivars and extreme non preference to some others (Prasad, 1987; Gawel, 1991; Kavitha *et al*., 2015a). The severity of loss is greater when infestation occurs at early vegetative stage (Visalakshi *et al.,* 1989) and cultivars such as *Nendran, Palayankodan* and *Red Kappa* are highly susceptible (HS) to infestation by *O. longicollis* (Kavitha *et al., 2015 a&b*).

Organic compounds generated from primary metabolites that do not have any direct function in growth and development are categorized as secondary metabolites (SM). These SM are specific in one species or a taxonomically related group of species. Biosynthesis and degradation of secondary metabolites play important roles in ecology and physiology of organism in which they occur (Waller and Nowack, 1978; Waller and Dermer, 1981). Chief ecological function is to defend host plants against herbivory or infestation (Harborne, 1982). So the quantity and pattern of distribution of important SM obviously indicate the status of host plant resistance.

Field study conducted to identify the pest resistant *Musa* cultivars in different sites of Kerala has resulted in the identification of 24 cultivars in a remote village and among them nine were highly resistant to attack by insect pests (Kavitha *et al.,*2015 a & b) and one of the reason for pest resistance was identified as presence of extremely high content of Phenols, Flavonoids and high activity of related enzymes such as Phenylalanine ammonia lyase (PAL), Peroxidase (PO) and Polyphenol oxidase (PPO) (Kavitha *et al.,*2017a). Further study in various agro ecosystems of Kerala has resulted in the identification of another 26 cultivars and their genome constitution and pest status in relation to *O. longicollis* under field condition was studied (Ajitha *et al*., 2017). Studies done by the investigators showed that farmers are not interested in cultivating *Musa* cultivars with long duration to set flower, small fruit bunch, less palatable fruits and those with low market value due to non familiarity among the public (Kavitha *et al*., 2015b). The above 26 cultivars are all with low commercial viability and hence they were rare in the agro ecosystem but at the same time many of them did not show attack by *O. longicollis* in field condition. The defense mechanism behind the non preference shown by *O. longicollis* on these 26 cultivars was reported in this communication.

MATERIALS AND METHODS

Collection of *Musa* cultivars:

Kerala state has banana procuring centers in all Grama panchayaths, which ensure reasonable market value to the farmers. Regularly visited these quasi governmental organizations, which are under the Department of Agriculture, Government of Kerala and interacted with farmers, about the different types of *Musa* cultivars in their local place and procured suckers of cultivars, which are not common or rare in their locality. Suckers were planted in the campus of University College, Thiruvananthapuram and maintained with organic manure. No synthetic fertilizers were provided. The cultivars such as *Cuba*, *Bondabathesa, Lady's finger* and *Yangambi,* were collected from an enthusiastic farmer of Kerala (Mr. Vinod Sahadevan, Parassala, Thiruvananthapuram District) who maintains more than 150 cultivars of *Musa* collected from various Agricultural Universities of India. All other cultivars are indigenous to Kerala.

Leaf sample collection:

Tender cigar leaf, possessing 20 to 30 cm length was cut from the tip and kept in ice cold condition till weighing and processing.

Assay of enzymes, Phenols and Flavonoids

All estimations were done as described in the standard techniques. Total phenols (Mayr *et al.,* 1995), Total flavonoids (Chang *et al.,* 2002) and assay of enzymes such as Phenylalanine Ammonia lyase (Whetten and Sederoff, 1992), Polyphenol oxidase (Mayer *et al.,* 1965), Peroxidase (Hammer Schimist *et al*., 1982). Activity of enzymes was expressed as units/mg protein.

Rearing of *O. longicollis* larvae in *Musa* cultivars

Cultivars which never showed infestation by *O. longicollis* in field conditions and those possessed very high activity of PAL, PO, PPO and bearing very high contents of TP and TF were used for this study. Those cultivars possessed very low contents of TP and TF, and very low activity of PAL, PO and PPO were used as control. Four month old cultivars with pseudostem of 25 to 30 cm circumference, whose crown was chopped down at a height of 100cm above the ground. A small depression was made on the free cut end of live pseudostem and seven *O. longicollis* larvae (third instar) were put in to it (third instar larvae are voracious feeders than younger instars, moderately large in size and easy to handle). The larvae were allowed to bore into the pseudostem and cut end was covered with a piece of mosquito net. In order to prevent the entry of rain water, the cut end was closed by a piece of plastic, if there is rain. On the seventh day the live pseudostem (live stump) was cut 15 cm below the first cut and the larvae were carefully dissected out. Those cultivars which caused complete mortality of larvae within seven days were called

Resistant (R) and those cultivars on which no mortality was observed were designated as Susceptible (S).

Statistical analysis:

Five leaf samples from each cultivar were evaluated. The data was analyzed by one way analysis of variance (ANOVA).The values with level of significance pÃ0.05.

RESULTS AND DISCUSSION

Musa cultivars numbered as 1-5 showed very low content of TP when compared to cultivars 15-26 (Fig.1). The content of TP in cultivars 6-14 was slightly but significantly higher than (1-5) but lower than 15-26 groups. The quantitative distribution of TF in the 26 cultivars also showed a same pattern as that of TP., viz. cultivars 1-5 showed lowest amount of TF and cultivars 15-26 showed highest amount (Fig.2).

Activity of PAL showed very high variation among the 26 *Musa* cultivars (Fig.3). Activity of PAL was very low in cultivars 1-5, but cultivars 15-26 have maintained highly elevated activity of this enzyme. *Musa* cultivars 6-14 showed only a moderate activity of PAL, which was higher than that of the first category of cultivars included in 1-5 and lower than the cultivars 15-26 (Fig.3).

Activity of PPO was very low in *Musa* cultivars which are susceptible (1-5) to infestation by *O. longicollis*. Slightly elevated activity of PPO was observed in cultivars sporadic attack (6-14) by this pest and the activity of PPO was very high in cultivars (15-26) which did not show any infestation by this pest. The pattern of activity was almost identical in case of another related enzyme PO (Fig.5).

Rearing of *O. longicollis* larvae in *Musa* cultivars which showed very high activity of PAL, PPO and PO and very high content of TP and TF has resulted mortality of larvae after 7th day and were termed as Resistant (R). Under the field condition cultivars (15-26) showed no infestation by *O. longicollis*. Cultivars 1-5 showed infestation by this pest and were termed as Susceptible (S) and cultivars 6-14 showed sporadic attack by this pest and are designated as Non preferred (NP). The NP cultivars which are existing in the agro ecosystem as lone clones and are surrounded by abundance of commercially viable cultivars (CVC) which are all highly susceptible (HS), the pest showed no interest on attacking them. Rearing of larvae in NP cultivars did not result mortality after seven days.

Genome constitution of all the 26 *Musa* cultivars discussed in this paper has been studied and their pest status in relation to *O. longicollis* under the field condition has been documented (Ajitha *et al.,* 2017). No commercial cultivation was done on any of the 26 cultivars and were located as lone clones in a neglected state. Most of the farmers in Kerala who are fully engaged in *Musa* cultivation are small scale farmers and they do not have their own land for cultivation. So they cultivate *Musa* cultivars in a land on lease for one year contract with owner of the land, and hence they are very particular to plant *Musa* cultivars which can give harvest within one year (Kavitha *et al*., 2017c).

Agro ecosystem of Kerala are with abundance of *Musa* cultivars and farmers are very particular in selecting commercially viable cultivars (CVC) only for their sustenance and they are very much interested in CVC which are able to give harvest within one year. Other traits preferred by farmers are large fruit bunch, high market value, palatable taste and high demand among the public. So agro ecosystems of Kerala are with abundance of CVC such as *Nendran* (AAB), *Kappa* or *Red Banana* (AAA), *Palayankodan* (AAB) and *Njalippovan* (AB) and the pest status of *Njalippovan* is NP and other three are S. (Kavitha, *et al.,* 2017 a,b&c, 2015a and b). *O.longicollis* exhibited extreme specificity in selecting the host plant for oviposition (Padmanabhan and Sundararaju, 1999), which was confirmed through field study by the investigator's team and *Nendran*, *Kappa* and *Palayankodan* were found to be the most preferred cultivar by this pest (Kavitha *et al*.,2015a). In the agro ecosystem the three cultivars are abundant and *O. longicollis* showed extreme preference on them and can be referred them as highly susceptible (HS). In presence of HS cultivars the pest showed no interest on other cultivars. Quantitative estimation of TP, TF, and activity of PAL, PO and PPO in the above three cultivars proved that the above constituent were least minimum in these CVC (Kavitha *et al*., 2017b). In the present investigation, cultivars numbered as 1-5 showed very low content of TP, TF, PAL, PPO and PO, but their distribution in agro ecosystem was either scanty or rare. So no aggressive infestation by *O. longicollis* was noticed on these plants. Field study in Kerala proved that selective cultivation of HS cultivars which are also CVC have resulted many of the agro ecosystems as true breeding sites of *O. longicollis* (Kavitha *et al*., 2017 c). Rearing of *O.*

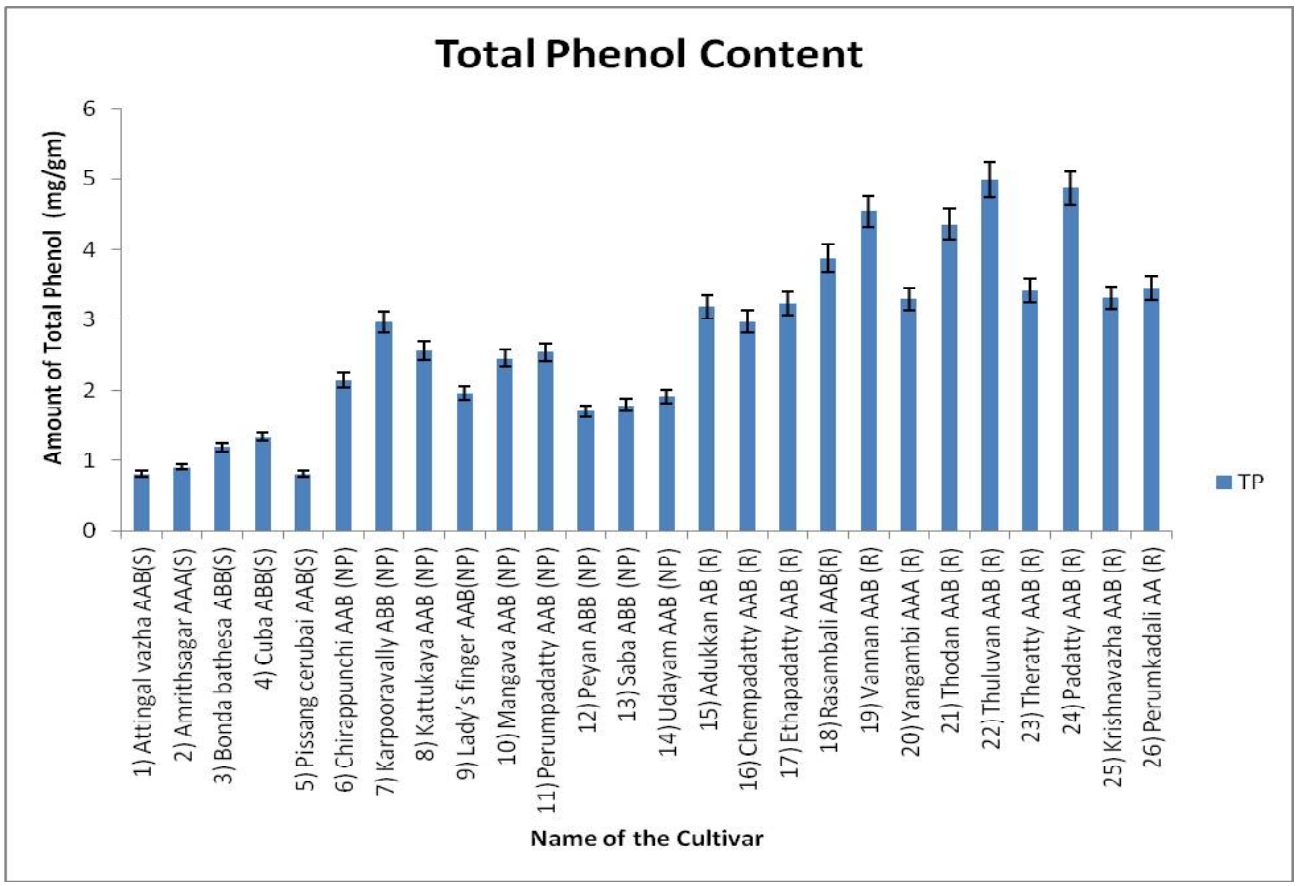

Fig. 1. Amount of total phenols in different cultivars of *Musa* and their significance in relation to pest status.

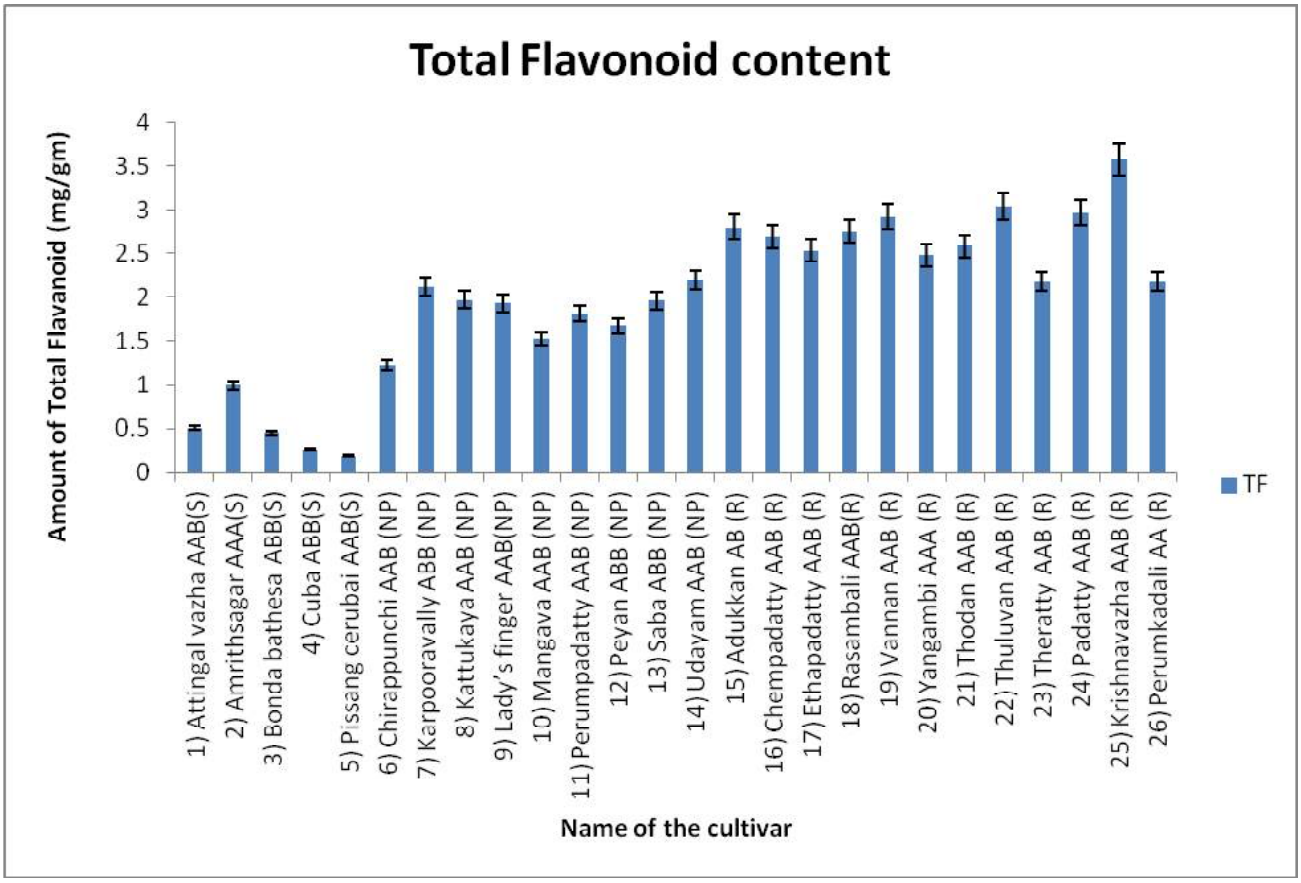

Fig. 2. Amount of total flavonoids in different cultivars of *Musa* and their role in categorizing the cultivars as Susceptible, Non-prefered and Resistant to infestation by *O. longicollis*.

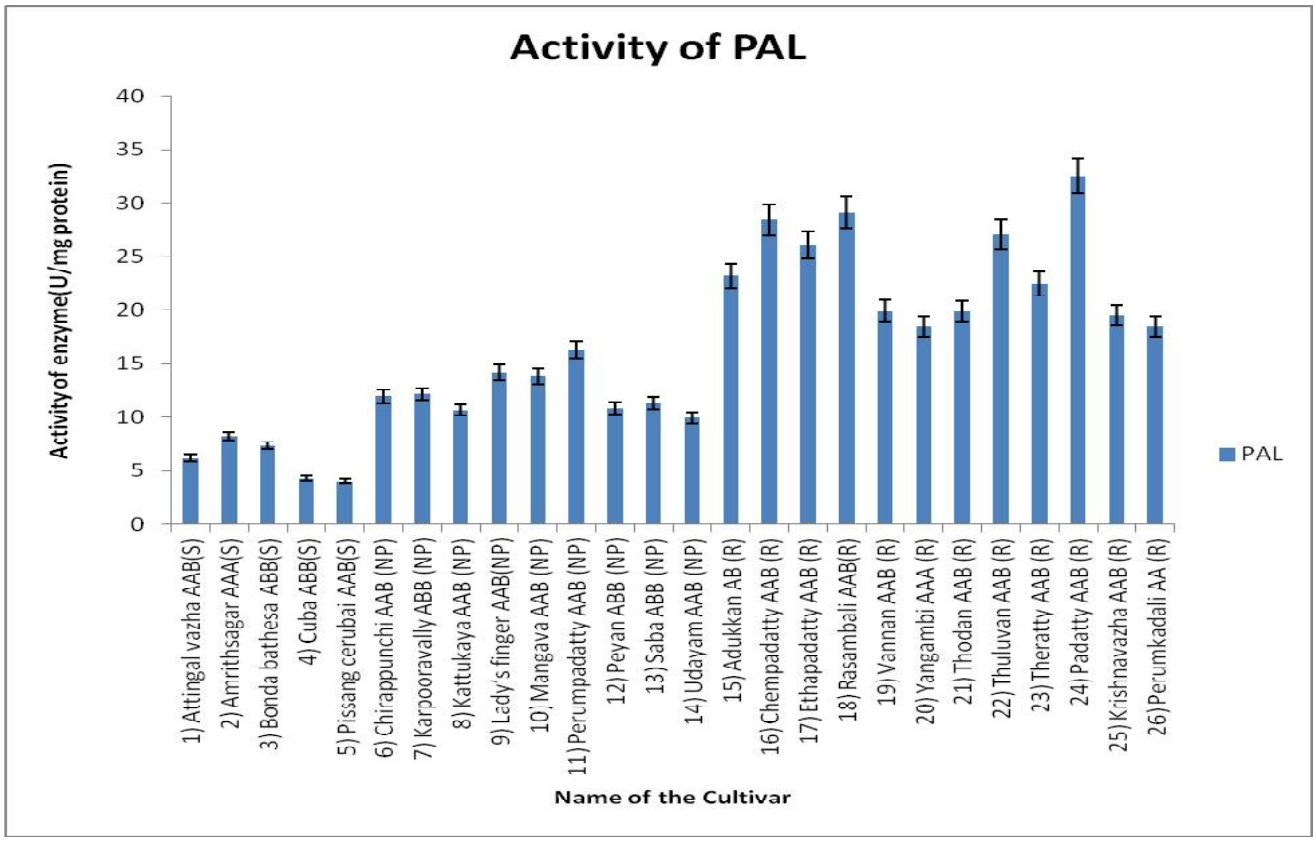

Fig. 3. Activity of Phenyl alanine ammonia lyase in different cultivars of *Musa* and their pest status in relation to *O. longicollis*.

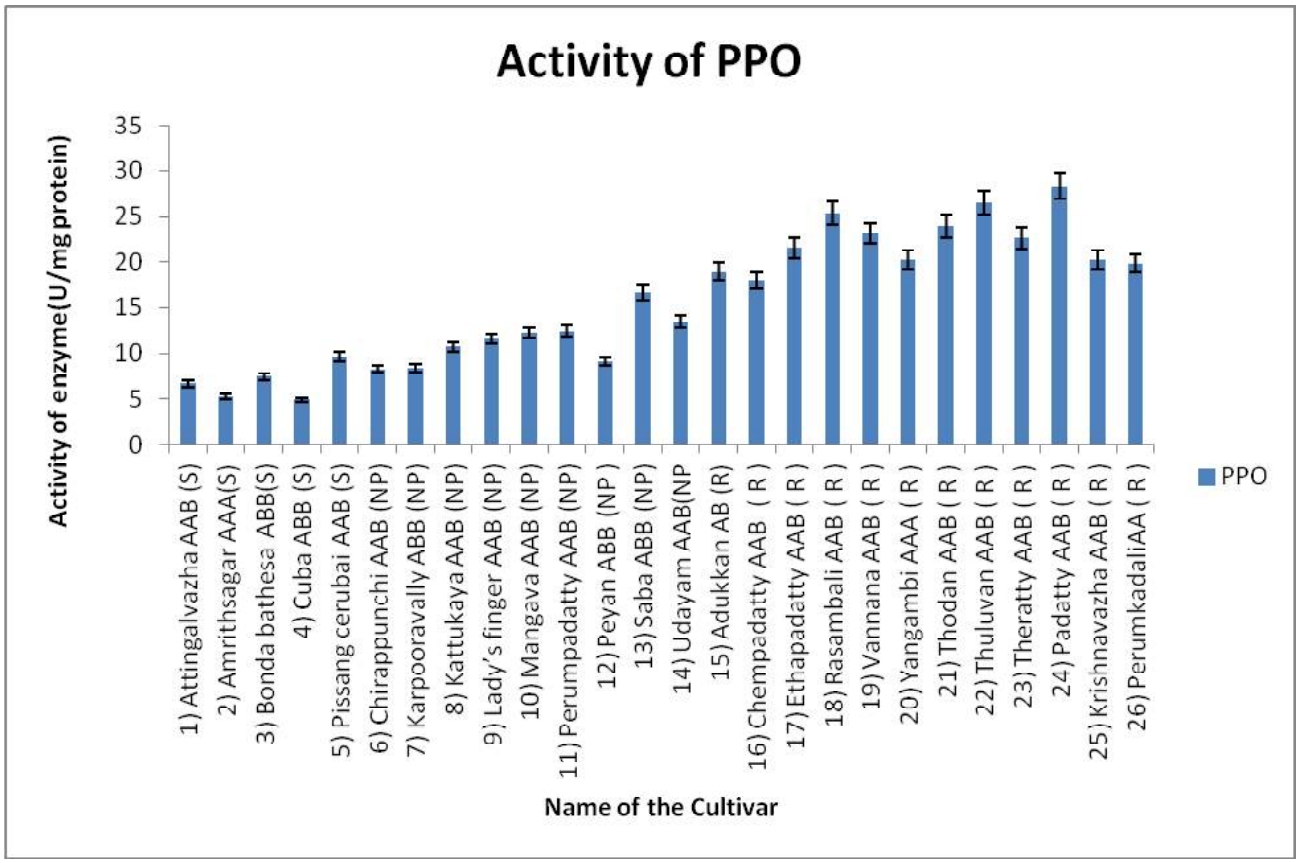

Fig. 4. Activity of Polyphenol oxidase in *Musa* cultivars and the pest status in relation to infestation by *O. longicollis*.

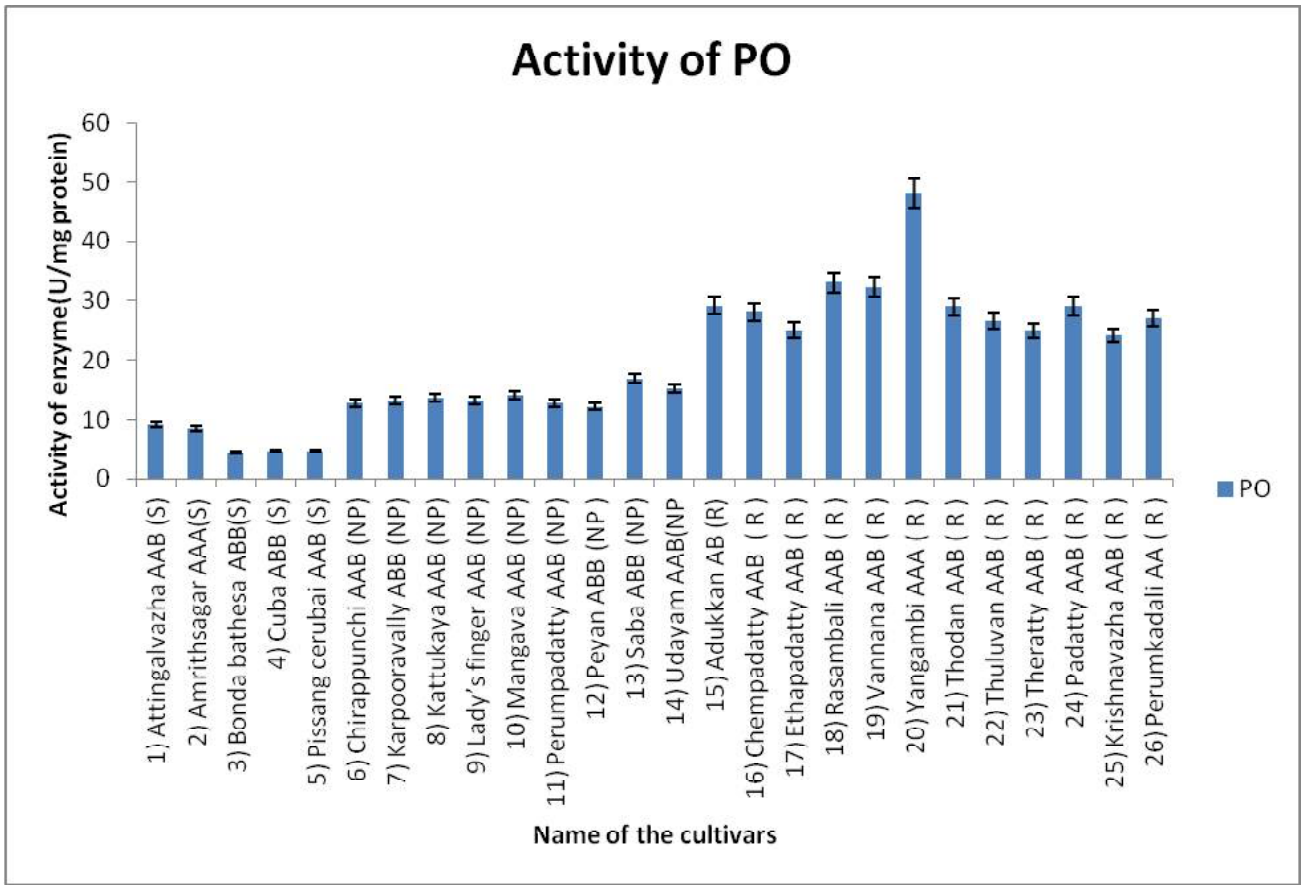

Fig. 5. Activity of Peroxidase

longicollis larvae in the pseudostem of these cultivars did not result in any problem and they complete life cycle as usual.

Musa cultivars described as numbers 6-14 possessed preferably higher content of TP and TF and in the agro ecosystem they did not show any infestation by this pest (Ajitha.*et al.,* 2017). The activity of enzymes and the secondary metabolites which are designated as NP are equal to the *Musa* cultivars which were categorized as NP cultivars by *O. longicollis* as described in the previous paper (Kavitha *et al.,* 2017b). Peculiarity of NP cultivars is that even though they were cultivated for commercial purpose in large numbers. *O. longicollis* preferentially avoid them in presence of *Nendran, Palayankodan* and *Kappa* (all three are HS) which are abundant in the agro ecosystem (Kavitha.*et al.,* 2015a) and on which the pest showed extreme preference (Padmanabhan and Sundararaju 1999; Kavitha.*et al.,* 2015b).

The cultivars described as 15-26 possessed very high content of TP, TF together with very high activity of PAL, PO and PPO. Rearing *O. longicollis* in these cultivars has resulted mortality of larvae within 7 days. Previous study by the investigation in another set of *Musa* cultivars showed that rearing of *O. longicollis* in some pest resistant cultivar has resulted wide spread cytopathological changes in the hemocytes of larvae (Kavitha.*et al.,*2016) leading to mortality of them and such cultivars designated as resistant cultivars. In the present study, cultivars numbered as between 15-26 caused immobility on fourth day and death on sixth or seventh day of keeping them in these cultivars.

Yangambi, the 20th cultivar in the present study was reported as possessing very high content of phenols and is resistant to infestation by Nematodes (Fogain 1996; Valette *et al.,* 1997). Increased activity of PO and PAL were reported in brinjal having resistance against nematodes (Rajasekhar *et al.,*1997; Sirohi and Dasgupta 1993). It has been established that PAL is a very important enzymes involved in the plant defense mechanism which is evolved in phenyl propaniod pathway which imparts resistance against various types of pests (Ramesh Kumar *et al.,* 2012). Many investigators have reported the importance of

PAL, PO, PPO in many crop plants and these enzymes showed elevated activity under the influence of pest infestation (Felip Otalvaria *et al.,* 2002, Valette *et al*.,1998).

Agricultural practices chiefly aimed only at financial gain has resulted sharp depletion of diversity of many crop plants. In case of *Musa* cultivars also farmers are extremely selective in cultivating commercially viable cultivars and eliminating those cultivars which are not commercially viable. Commercially less viable cultivars at the same time, possessed many desirable traits such as strong defense mechanism against various pests, folk lore use against various ailment and in many cultural activities. So conservation of such commercially less viable crop plants has prime importance, especially for deciphering the mechanism of pest resistance.

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Wetland Ecology and Fish Diversity of Lake Veli

FOUZIA J.* AND G. PRASAD

Department of Zoology,
Karyavattom, University of Kerala, Tvm.
email : fouzjia@gmail.com ; fouziaj09@gmail.com,

Wetland, Veli lake, Estuary, Invasive species, over exploitation, Ichthyofauna, Diversity.

Wetland is the general term applied to the land areas that are seasonally or permanently water logged including lakes, rivers, estuaries and fresh water marshes. Ramsar Convention provided the frame work for the conservation of wetlands. Monitoring of the occurrence of species has been the basis for assessing the effects of environmental impact (Spellberg, 1992). Fishes are the key stone species which determines the abundance and distribution of other organisms and good indicators of water quality and pollution.

The present hydro-ecological study was conducted in the Veli Lake. The Veli Lake is the smallest estuarine wetland in the southwest coast of Kerala (08° 31 and 08° 31 ´ NL and 76° 522 30´ to 76° 532 30´ EL) situated at Thiruvananthapuram. The study highlights the fishery potential and diversity in relation to the prevailing wetland ecological status of Veli Lake. Fish diversity was assessed by the use of standard keys and surveys. Descriptive statistics was prepared and processed by the help of PALSTAT software. Total 30 species of fishes belonging to 19 families were identified during the present study.

Both Cyprinidae and Mugilidae were the dominant families with four species each, which together constitute higher percentage (13.33%) to the total fish population in the present study. Both Cyprinidae and Mugilidae were observed as the dominant family with higher number of species. The ichthyofaunal diversity of the lake showed a declining trend in the present study. Oreochromis mossambicus and Clarias gariepinus were the exotic species collected during the study. Exotic species invasion, eutrophication, habitat destruction, climatic disturbances, overfishing, ecological degradation, industrial pollution and lack of prudent habitat

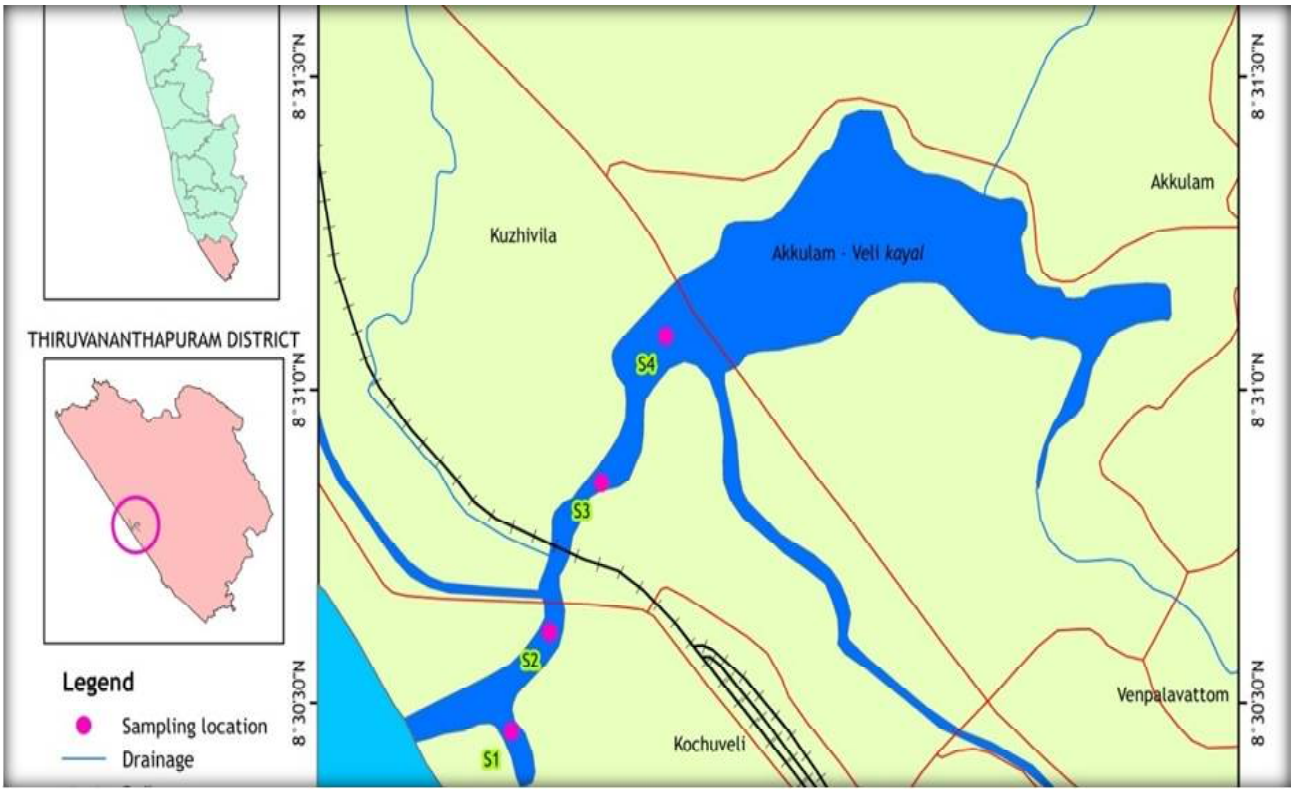

Fig.1.1 Study Site: Veli Laketab.1.1 Fihes Of Veli Lak

management were considered as the major reasons for the biodiversity loss in Veli Lake

Wetland is the general term applied to the land areas that are seasonally or permanently water logged including lakes, rivers, estuaries and fresh water marshes (Alexander and Mc Innes, 2011). Ramsar Convention provided the frame work for the conservation of wetlands (Elliot and Whitfield, 2011). According to the Ramsar Convention, wetlands are areas of marsh, fen, peat land or water, whether natural or artificial, permanent or temporary, with water that is static or flowing, fresh, brackish or salt, including areas of marine water and the depth of which at low tide does not exceed 6m (http:// www.ramsar.org).

Wetlands have been steadily and rapidly disappearing across the world. The wetlands all over the world faces severe ecological crisis (Barbier et al., 2011). Now days, the wetlands are depleting in an ever increasing rate. Wetlands are one of the most threatened habitats of the world (Erwin, 2009). Estuarine waters serve as nursery grounds for fishes (Fischer and Eckmann, 1997). Fishes play a key role in the provision of ecosystem services essential to the maintenance of wetlands. Fishes can be used as the bio indicator of pollution (Ananthan et al., 2005).

The present ecological study was conducted in the Veli Lake. The Veli Lake is the smallest estuarine wetland in the southwest coast of Kerala (08° 31 and 08° 31 ´ NL and 76° 522 30´ to 76° 532 30´ EL) situated at Thiruvananthapuram (fig.1.1.). The lake remains separated from the Arabian Sea by a sand bar. During monsoon a temporary connection, 'pozhi', is established between the sea and the lake due to the sand bar break after the cessation of the rain. The lake is a shallow one with a mean depth of 2.4 meter and has a length of 1.25 km and width 0.44 km.

Two canals, the Channankara (Parvathy puthenar) and the Kulathur join the lake at the northern side. On the South, the Veli Lake is connected to the Akkulam Lake by a narrow channel. The Kannammoola canal drains at Akkulam Lake carrying all the water from the city. In the close vicinity of the lake are two factories, the English Indian clay factory in the southern bank of the lake and the Travancore Titanium Products in the eastern side. In fact, the lake is being polluted heavily. The place is becoming the point of waste disposal from hotels, hospitals and industries. The Lake has been deteriorated because of the pollution load due to the industrial discharge from Travancore Titanium Products and English Indian clay factory.

Very little information is available on the fish diversity and richness of Lake Veli. Monitoring of the occurrence of species has been the basis for assessing the effects of environmental impact. Approximately 60% of the total world fishery population faces problems stemming from sewage discharge, over fishing and land use pattern (Taylor et al., 2005). Fishes are the key stone species which determines the abundance and distribution of other organisms and good indicators of water quality and pollution (Kupschius and Tremain, (2001).

OBJECTIVES

The present study aimed to provide baseline information on the status and distribution of fishes in wetland Lake Veli. The study highlights the fishery potential and diversity in relation to the prevailing wetland ecological status of Veli Lake. The study also assesses the present threats of diversity loss of fish community in Veli Lake.

MATERIALS AND METHODS

Fish samples were collected using cast net and gill net. Fish samples were preserved in 10% formaldehyde and identified by using standard keys of Munro (1982), Jhingran (1991), Jayaram (1999), Nelson (2006) and Fish Base (Froese and Pauly, 2013). Fish diversity was assessed by the use of standard keys and surveys for 2 years. Descriptive statistics was prepared and processed by the help of PALSTAT software.

RESULTS AND DISCUSSION

Results are given on the tables1.1 -1.2. Total 30 species of fishes belonging to 19 families were identified during the present study (tab.1.1). Both Cyprinidae and Mugilidae were the dominant families with four species each, which together constitute higher percentage (13.33%) to the total fish population in the present study. This was followed by the family Cichlidae (10%) comprised of three species. The families Anguillidae, Gobidae and Channidae comprised with two species each, which contributes 6.66% to

Table 1. Fishes of Veli Lake.

SI No	FAMILY	N	Scientific name
1	Anguillidae	1	*Anguilla bicolor bicolour*
		2	*Anguilla bengalensis*
2	Bagridae	3	*Mystus gulio*
3	Heteropneustidae	4	*Heteropneustes fossilis*
4	Gobidae	5	*Glossogobius giuris*
		6	*Oxyurichthys microlepis*
5	Anabatidae	7	*Anabas testudineus*
6	Mugilidae	8	*Mugil cephalus*
		9	*Liza tade*
		10	*Liza parzia*
		11	*Liza macrolepis*
7	Megalopidae	12	*Megalops cyprinoides*
8	Carangidae	13	*Caranx carangus*
9	Centropomidae	14	*Ambassis commersoni*
10	Channidae	15	*Channa striatus*
		16	*Channa marulius*
11	Teraponidae	17	*Terapon jarbua*
12	Lutjanidae	18	*Lutjanus argentimaculatus*
13	Cyprinidae	19	*Rasbora daniconius*
		20	*Puntius filamentosus*
		21	*Puntius sarana*
		22	*Puntius vittatus*
14	Gerridae	23	*Gerres filamentosus*
15	Sillaginidae	24	*Sillago sihama*
16	Engraulidae	25	*Stolephorus indicus*
17	Clariidae	26	*Clarias gariepinus*
18	Cichlidae	27	*Etroplus maculates*
		28	*Etroplus suratensis*
		29	*Oreochromis mossambicus*
19	Syngnathidae	30	*Ichtyocampus carce*

Table 2. Family Wise Percentage of Fishes From Veli Lake.

FAMILY	Percentage of fish species from 2009-2011
Anguillidae	6.66
Bagridae	3.33
Heteropneustidae	3.33
Gobidae	6.66
Anabatidae	3.33
Mugilidae	13.33
Megalopidae	3.33
Carangidae	3.33
Centropomidae	3.33
Channidae	6.66
Teraponidae	3.33
Lutjanidae	3.33
Cyprinidae	13.33
Gerridae	3.33
Sillaginidae	3.33
Engraulidae	3.33
Clariidae	3.33
Cichlidae	10
Syngnathidae	3.33

the total family. The remaining families were represented in 3.33% each to the total family (Table 2.)

Both Cyprinidae and Mugilidae were observed as the dominant family with higher number of species. The ichthyofaunal diversity of the lake showed a declining trend in the present study. *Oreochromis mossambicus* and *Clarias gariepinus* were the exotic species collected during the study. While *O. mossambicus* dominates all indigenous fish fauna and *C. gariepinus* just started to make its appearance. This fish can also be able to destroy the eggs of other fishes and steadily replace the native ichthyofauna (Bijukumar and Sushama, 2000). Exotic species invasion lead to the sharing of niches inhabited by indigenous fauna (Nandan, 2012).

Habitat specificity analysis of the collected species showed the specific categorization of the fish habitat as resident, marine and freshwater. *Liza tade, Liza macrolepis, Caranx carangus, Gerrus filamentosus, Stolephorus indicus* and *Ambassis commersoni* recorded during the present study were emigrants from the sea. While some species like *Anguilla bicolor bicolor, Anguilla bengalensis, Glossogobius giuris, Mugil cephalus, Megalops cyprinoides, Lutjanus argentimaculates, Oreochromis mossambicus, Etroplus suratensis, Ichthyocampus carce, Etroplus maculatus, Anabas testudineus, Oxyurychthis microlepis* and *Sillago sihama* were residents of the lake ecosystem. *Heteropneustis fossilis, Channa striata*, *Clarius gareipinus, Mystus gulio, Rasbora daniconius* and

Terapon jarbua were fresh water species.

IUCN red list analysis of data revealed the specific categorization of fish status in the lake. The fish species *Anabas testudineus, Megalops cyprinoides* and *Liza tade* were classified as the data deficient category specified by IUCN (http:// www.iucn.red list.org). *Anguilla biclolor bicolor* and *Anguilla bengalensis* categorized as near threatened category, *Puntius sarana* was included under vulnerable category, *Sillago sihama, Liza parzia, Caranx carangus, Stolephorus indicus, Rasbora daniconius, Lutjanus argentimaculatus* and *Oxyurichthys microlepis* were categorized as not evaluated category. The remaining fish species were classified as the least concerned category. Over fishing has contributed to declining species abundance of Veli Lake. as reported elsewhere (Harikrishnan et al., 2011).

Indiscriminate and illegal fishing practices such as electric fishing, light fishing and poisoning prevail in the backwaters of Kerala. The reduced fish diversity eventually decreases the production of native species and may creates extinction of several species through competition, predation and the disruption of local ecosystems as suggested by Kurup and Ravindran (2006).The stress sensitive fish species were reduced due to ecological disturbances, which lead to less diversity and greater dominance of resistant species in Veli Lake. Now the indigenous fauna of the lake replaced gradually by pollution and stress resistant species such as *O. mossambicus*. Distribution pattern of fishes may be disturbed as a result of stressful polluted environment in Veli Lake as reported by Bassi (2014).

Excessive levels of nutrients in Veli Lake may lead to eutrophication and extensive growth of aquatic weeds, similar observations were reported by Nandan (2012). Drastic reductions in population size and low diversity of fishes in Veli Lake attributed to habitat loss, exotic species invasion and pollution. Industrial pollution contributed from the nearby factories, pesticide and fertilizer pollution, domestic pollution and eutrophication may contribute the diversity reduction of fishery fauna in Veli Lake. Exotic weeds such as *Eichhornia crassipes* and *Salvinia* spp. prevailed in Veli Lake, decomposing of these weeds increases the eutrophication rate as suggested by Babu et al. (2010).

SUMMARY AND SIGNIFICANCE

Marked reduction of fish species richness and diversity in Veli Lake can also be attributed to the changing ecological conditions. Total 30 species of fishes belonging to 19 families were identified during the present study. Both Cyprinidae and Mugilidae were the dominant families with four species each, which together constitute higher percentage (13.33%) to the total fish population in the present study. Both Cyprinidae and Mugilidae were observed as the dominant family with higher number of species.

The ichthyofaunal diversity of the lake showed a declining trend in the present study. *Oreochromis mossambicus* and *Clarias gariepinus* were the exotic species collected during the study. An integrated management plan has to be formulated for the conservation of Veli Lake, which should reflect the carrying capacity of the lake for anthropogenic activities and should optimize the sustainable use of existing natural resources. Low diversity and lower species richness indicates stressed condition of the Lake. The low fish diversity indicates the non availability of stress and pollution free breeding and feeding grounds of the lake.

CONCLUSION

The rich ichthyofaunal diversity of the lake showed a declining trend in the present study. Mugilidae and Cyprinidae were the dominant family groups with higher number of species. Human interferences and unsustainable developments irrevocably damage and destroy ecology and fish diversity of Veli Lake. Thus any degradation of the wetland can have an adverse impact on the environment and fishery potential. Exotic species invasion, eutrophication, habitat destruction, climatic disturbances, overfishing, ecological degradation, industrial pollution and lack of prudent habitat management were considered as the major reasons for the biodiversity loss in Veli Lake.

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Exomorphology of Seeds of Some Halophytic Species of Arabian Gulf Coast, Saudi Arabia

M. BASAHI[1], J. THOMAS[2], R. RAJAKRISHNAN[2], A.H. ALFARHAN[2] AND S. KHALAF [2]

[1.]*Dept. of Biology, College of Science & Arts, Sajir, Shaqra University, P.O. Box-33, Shaqra, 11961, Saudi Arabia*
[2.]*Dept. of Botany & Microbiology, College of Science, King Saud University, P.O. Box 2455; Riyadh 11451, Saudi Arabia.*
[*]*email : jathomas@ksu.edu.sa*

ABSTRACT

Members of the subfamilies Salsoloideae and Salicornioideae (Amaranthaceae) are taxonomically problematic groups primarily due to the absence of indicative characters available to demarcate tribal- and generic-level frontiers; an after effect of short flowering stage prior to fruiting and the loss of succulent vegetative morphology upon drying. Seed coat morphology of 4 dominant representative halophytes (*Anabasis setifera, Halocnemumstrobilaceum, Halopeplisperfoliata* and *Seidletziarosmarinus*) found in the sabkhas of Arabian Gulf coast were investigated to ascertain whether the emerged results support current taxonomic groupings. An average 100 mature seeds were examined with the help of SEM to confirm the variations in seeds such as seed shape, dimensions,sculpture and hilum shape. The results of exo-morphological and primary embryological variations of seeds of both submiliesare potentially informative for phylogenetic analyses.

Key words *Amaranthaceae, Disseminules, Exomorphology, Salsoloideae, Salicornioideae.*

Seed micro-morphology has been effectively applied in a variety of systematic studies, and scanning electron microscopy (SEM) has enhanced character evaluation in different genera of families such as Amaranthaceae, Caryophyllaceae (Amini et al., 2011) etc. Significant number of research has focused on intrageneric variations in seed coat structure (Segarra and Mateu, 2001; Barloto and Forget, 2007; Abdel Khalik and Vander Maesen, 2002) or on variations among closely interrelated genera (Abdel Khalik, 2013; Gabr, 2014). Exomorphological studies on seeds of coastal plants or halophytes in general are seldom studied in Saudi Arabia. Halophytes constitute an important group of range plants in saline habitats of Arabian Peninsula (Khan and Qaiser, 2006) and play a vital role in providing a variety of ecosystem functions for the stabilization of biodiversity (Al-Fredan, 2008; Deil, 1998).The species selected for this study were part of the obligate halophytic vegetation belonging to the subfamilies Salsoloideae andSalicornioideae ofAmaranthaceae, an extended family including the former goosefoot family, Chenopodiaceae.

The subfamilies,Salsoloideae andSalicornioideae, contain majority of leaf-less halophytes. Members of these groups arecommonly found in all coastal and inland sabkhas such as coastal zones, estuaries, salt lakes etc. and are considered some of the most salt-tolerant terrestrial plants in the world (Short and Colmer, 1999). Four halophytes (*Anabasis setifera, Halocnemumstrobilaceum, Halopeplisperfoliata and Seidlitziarosmarinus*) growing in the Arabian Gulf coast have been selected for studying the seed exomorphic characters.As per the current circumscription, genus *Halocnemum* contains two species, *H. strobilaceum* M. Bieb and *Halocnemumyurdakulolii*Yaprak (Yaprakand Kadereit, 2008). The former is distributed from South Europe and North Africa to the saline habitats of Asia and Mongolia (Freitag, 1991) while the latter is endemic to GöksuDelta Içel-Silifke, South Anatolia. The articulated stems,glomerulate short shoots and partially fused tepels along with few scale-like leaves mark the genus unique among other members of Salicornioideae (Chaudhary, 1999). *Halocnemum* is closed related to *Halostachys*, a Eurasian species with papillose stems and perianth with partially fused segments. Genus *Anabasis* contains 28 species distributed in North Africa and other arid regions of Eurasia, of which only 4 species are reported from Saudi Arabia. Among the species reported under *Anabasis*, *A. setifera* is the most widespread species in the Kingdom, often found along the edges of salt marshes and sometimes on volcanic soils too (Boulos, 1996). Among the three recognized species under*Halopeplis*, only *H. perfoliata* is present in Saudi Arabia while genus *Seidletzia* is represented by only one species, *S. rosmarinus*. All species selected for this study are desert salt tolerant plants often used as forage for domesticated livestock, particularly camels during extreme drought (Hadi,

2009).

Several studies have been conducted on the seed coat structure for providing additional information onChenopodiaceae (Bassett & Crompton, 1982; Shepherd *et al.*,2005; Zare and Keshavarzi, 2007) Fabaceae (Duran *et al.* , 2010),Caryophyllaceae (Memon et al., 2010). Although several regional or global taxonomic treatments of Chenopodiaceae (Scott, 1977; Freitag, 2000) have been appeared in the past few decades, there has been no comprehensive survey examining morphological and anatomicalcharacters of fruits and seeds of the subfamilies except for afew studies on the structural anatomy of fruits and seeds (Shepherd et al., 2005). Dissemenules, particularly seeds tend to exhibit less phenotypic plasticity, in contrastwith other parts such

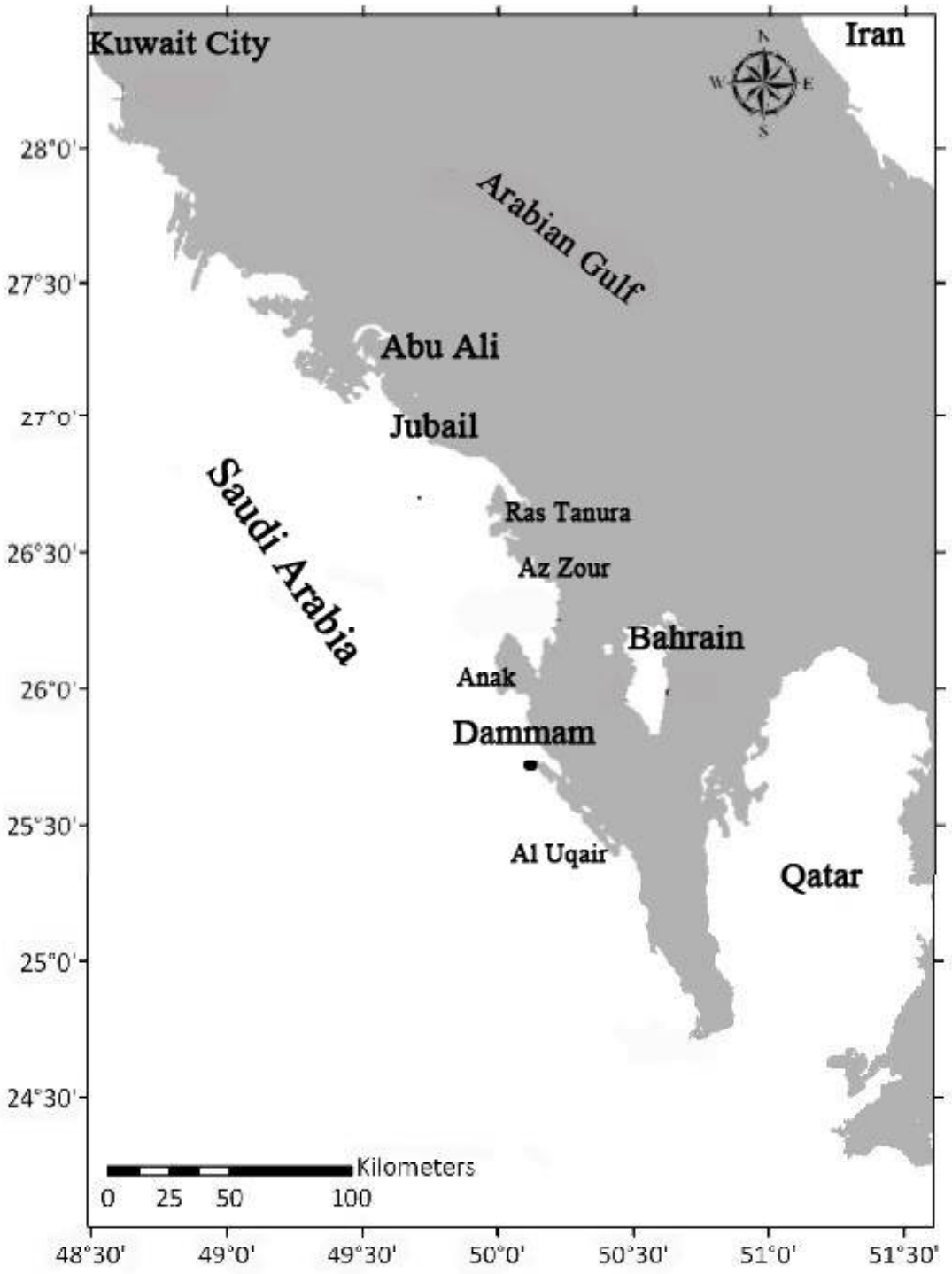

Fig. 1. Collection localities of taxa in Arabian Gulf coast in Saudi Arabia.

as overall habit, stature, leaves, etc. (Bonilla-Barbosa et al., 2000).

Exomorphological studies were applied on a number of randomly selected genera in Saudi Arabia (Hammouda and Bakr, 1971; Soliman et al., 2010) or specifically on genera, such as*Tephrosia* (Ghamdi and Al-Zahrani, 2010); *Acacia* (Hassan and Al-Farraj, 1989); *Indigofera* (Al-Ghamdi, 2011). Seed coat characters are often found useful in the circumscription of species as they are stable and not affected by environmental factors (Davis and Heywood, 1963). Corner (1976)mentions the importance of using electron microscopy (SEM)in morphological studies of seeds,which often contribute significantly to the identification of several morphological characteristics for seeds, as the surface of seed(Garnock-Jones,1991) and the external anatomy of the seed (Singh andDathan, 1972).This study aims to identify different morphological characteristics of the seeds of four salt tolerant plant species from the Arabian Gulf coast of Saudi Arabia by using scanning electron microscope (SEM) for the evaluation of their possible use for taxonomic considerations.The results help to provide additional seed morphological characters for delimiting the genera from their immediate relatives.

MATERIAL AND METHODS

The present investigation included fresh seeds of four halophytic species fromdifferent localities in the Arabian Gulf. The seeds of four species, such as *Anabasis setifera* (KSU-21501/15-10-2010), *Halocnemumstrobilaceum* (21508/12-12-2010), *Halopeplisperfoliata*(21509/12-12-2010) and *Seidletziarosmarinus* (21517/15-10-2010) were identified by comparing with similar samples housed in the Herbarium (KSU) at the Dept. of Botany and Microbiology, King Saud University, Riyadh and later confirmed by taxonomists at the Herbarium. All populations were located along the western coast of Arabian Gulf, such as in Abu Ali, Az-Zour, RasTanurah and Al-Uqair (Fig. 1).Important characteristics of external features of seeds included theseed-coat surface, size, shape, placement of the hilum andpresence of different structures, such as aril, elaiosoms, caruncle, etc.(Esau, 1977; Zoric et al., 2010).200 seeds of each species belonging to 1-3 populations were examined in order to determine the mean and standarddeviation and to guarantee the consistency of characters.At least 10 mature seeds were taken into account for recording gross morphology and size parameters. Seeds have been examined under stereomicroscope to ensure that all seeds used in the study are of normal size and growth. Seeds were mounted directly on metal stubs using double-sided adhesive tape and coated with a thin layer (ca 25 nm) of gold by Twin Coater JEC-550 device. The SEM micrographs were taken in a SEM-JSM-6380 LA at an accelerating voltage of 10-15 kV. The main parameters used in this study include, general shape and appearance of the seeds, exomorphological features such as testa cells, protuberances if any, etc. A descriptive terminology on the shape and various sculpture patterns follows Amini et al. (2011) while testa sculpturing follows Yildiz (2002) and Minuto et al. (2006).Representative specimens (Table 1) of the selected taxa were lodged with the Herbarium (KSU) at the Department of Botany & Microbiology, King Saud University, Riyadh, Saudi Arabia.

RESULTS

Though the characters of the studied species are self-explanatory, they are exemplified in Figs 2-5 for further clarification. Most of the characters and their states as defined in Table 2 are recorded for the first time for the species studied.Selected seed features of possible taxonomic importance of the studied taxa are presented in Table 2 and the SEM micrographs of seeds showing surface characters are shown in Figs. 2-5. Surface characters of examined taxa were variable, particularly the colour, shape and seed surface. Seeds are either vertically placed in *Anabasis* and *Halocnemum* or horizontal in *Halopeplis* and *Seidlitzia*.

Seed size

The length of the seed of *H.strobilaceum* ranged from 0.86 to 0.9 mm and width ranged from 0.65 to 0.72 mm in AzZour and Abu Ali Islandsrespectively. In *H.perfoliata*, the length varies from 0.78 to 0.84 and width varies from 0.56 to 0.62 mm in all the populations of Abu Ali Island and AzZour respectively. Seed dimensions of Al-Uqair and Anak populations of *Halopeplisperfoliata* and *S.rosmarinus* show slight variations in length, from 1.34 mm (Anak) to 1.5 mm (Al-Uqair) and width, from 1.07 to 1.11mm respectively. The mean seed length and width of

Table 1. Habit, habitat and distribution of investigated taxa.

Species	Subfamily	Tribe	Voucher specimen	Habit	Habitat	Dist. in Saudi Arabia
Anabasis setifera	Salsoloideae	Salsoleae	KSU-21501	Subshrub	Inland sabkha.	Northern, central and Eastern Provinces.
Halocnemumstrobilaceum	Salicornioideae	Salicornieae	KSU-21508	Subshrub	Coastal Sabkha.	Arabian Gulf coast and northwestern Red Sea coast.
Halopeplisperfoliata	Salicornioideae	Salicornieae	KSU-21509	Subshrub	Coastal and inland sabkhas.	Eastern and western coasts, North-central Province.
Seidlitziarosmarinus	Salsoloideae	Salsoleae	KSU-21517	Subshrub	Coastal and Inland sabkhas.	Central, eastern and northern Provinces.

Table 1. Morphological characteristics of seeds of five halophytes growing in the Arabian Gulf coast

Characters		Species								F-Value
		Halocnemumstrobilaceum	***Halocnemumstrobilaceum***	***Halocnemumstrobilaceum***	***Halopeplisperfoliata***	***Halopeplisperfoliata***	***Anabasis setifera***	***Anabasis setifera***	***Seidlitziarosmarinus***	
Location in Saudi Arabia		Az-Zour	Ras-Tanura	Abu-Ali	Az-Zour	Abu-Ali	Al-Uqair	Anak	Al-Uqair	
Seed	Mean ofLengthmm(L)±SE	0.86± 0.066^{a}	0.80±0.042^{a}	0.90± 0.093^{a}	0.84±0.044 a	0.78± 0.043^{a}	1.50± 0.186^{b}	1.34± 0.147^{b}	2.18±0.208 c	281.85 *
	Rangeof Length(mm)	0.76– 0.99	0.73– 0.88	0.76– 1.04	0.79– 0.95	0.72– 0.84	1.2–1.87	1.09– 1.67	1.9–2.49	
	Mean ofWidthmm(W) ±SE	0.65± 0.068^{a}	0.59± 0.048^{a}	0.72± 0.075^{a}	0.62± 0.041^{a}	0.56± 0.041^{a,f}	1.11± 0.125^{b}	1.07± 0.091^{b}	2.03± 0.178^{c}	428.71 *
	Rangeof Width(mm)	0.54-0.74	0.50-0.65	0.60-0.89	0.55-0.70	0.50-0.62	0.91-1.37	0.93-1.20	1.83-2.30	
Ratio(L/W)X100 ±SE		133.80±5.16^{a}	135.60±2.82 a	126.70±4.71^{a,b}	133.77±.06 a	139.37±4.07^{a}	136.42±4.40^{a}	125.35±3.08 a,b	107.55±1.81^{b}	6.93*
Seed-shape		Globose	Globose	Globose	Elliptical	Elliptical	Globose	Globose	Globose	
Seed-surface		Papillate	Papillate	Papillate	Papillate	Papillate to rough	Papillate	Papillate	Rough	
Colour		Brown	Brown	Brown	Brown	Brown	Black	Black	Black	
Sculpturing testa		Pentagonal	Pentagonal	Pentagonal	Pentagonal	Pentagonal				
Seed orientation		Vertical	Vertical	Vertical	Horizontal	Horizontal	Vertical	Vertical	Horizontal	
Endosperm		Absent	Absent	Absent	Present	Present	Absent	Absent	Present	
Endosperm position		-	-	-	Central	Central	-	-		
Numberof theseedsexamined forlengthandwidth		100	100	100	100	100	100	100	100	

*P<0.0001SE=Standarderror

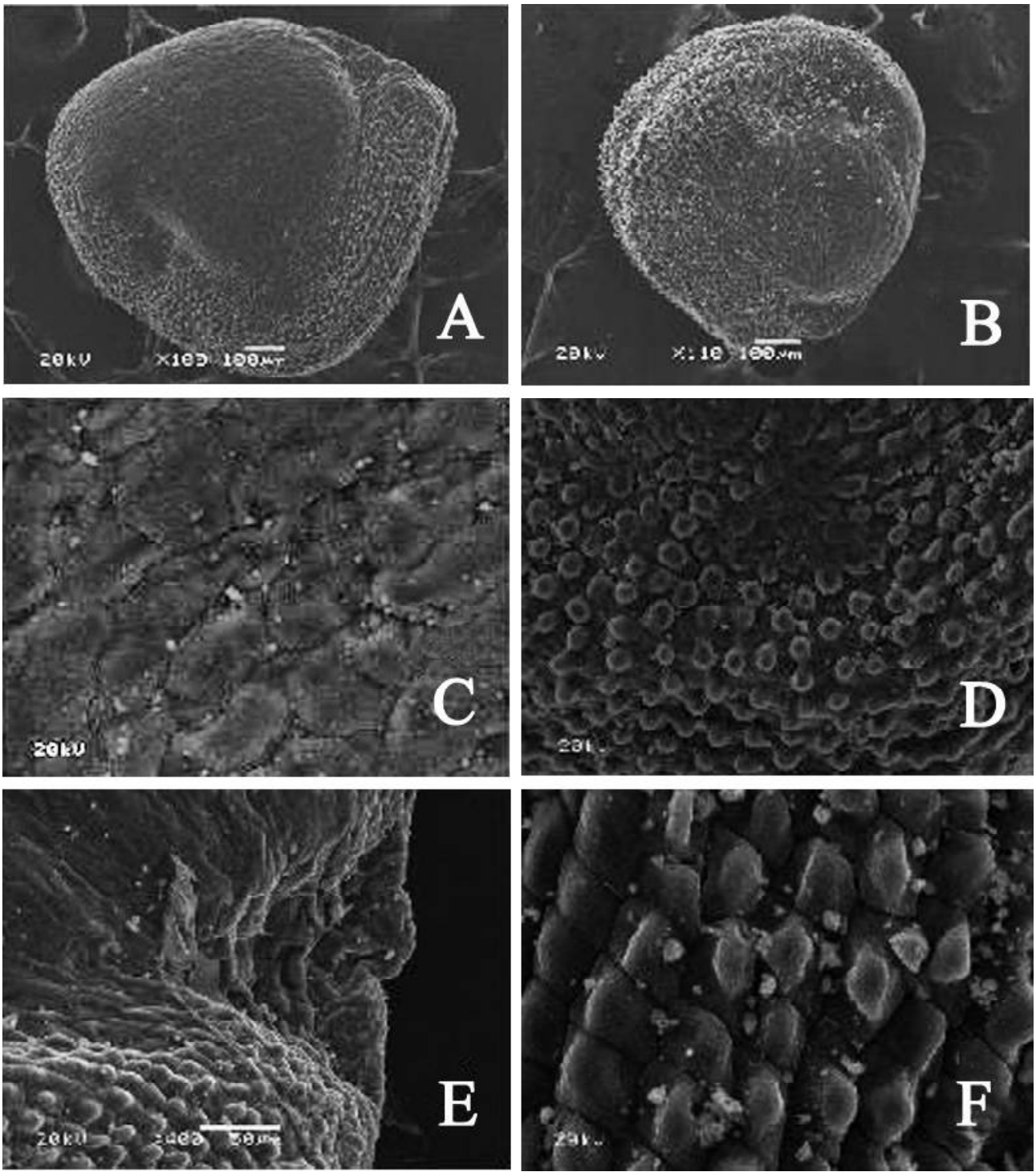

Fig. 2. *Halocnemumstrobilaceum*: A. Front view of the seed 100X, B. Dorsal view of the seed 110X; C. Superficial front part amplifier 500X; D. superficial dorsal part amplifier500X; E. hilum shape400X; F. superficial dorsal part amplifier 1000X

*Seidlitzia*populations collected from Al-Uqair is 2.18 and 2.03 respectively.Statistical studies (ANOVA TEST) have shown that there issignificant difference ($P<0.0001$) in seed dimensions (length, widthand Ratio length/width X 100) of plant species.

Seed shape

Shapes of seeds of all studied species were oval to circular (as demonstrated by ratio length / width of × 100). The seeds of *H.strobilaceum* are compressed-ovoid while it is circular to lenticular in *Anabasis setifera,* circular in *Seidlitziarosmarinus* and elliptical in *H.perfoliata* with centralendosperm. Seeds of H. perfoliata are slightly flattened on the sides. Endosperm is absent in Halocnemum and

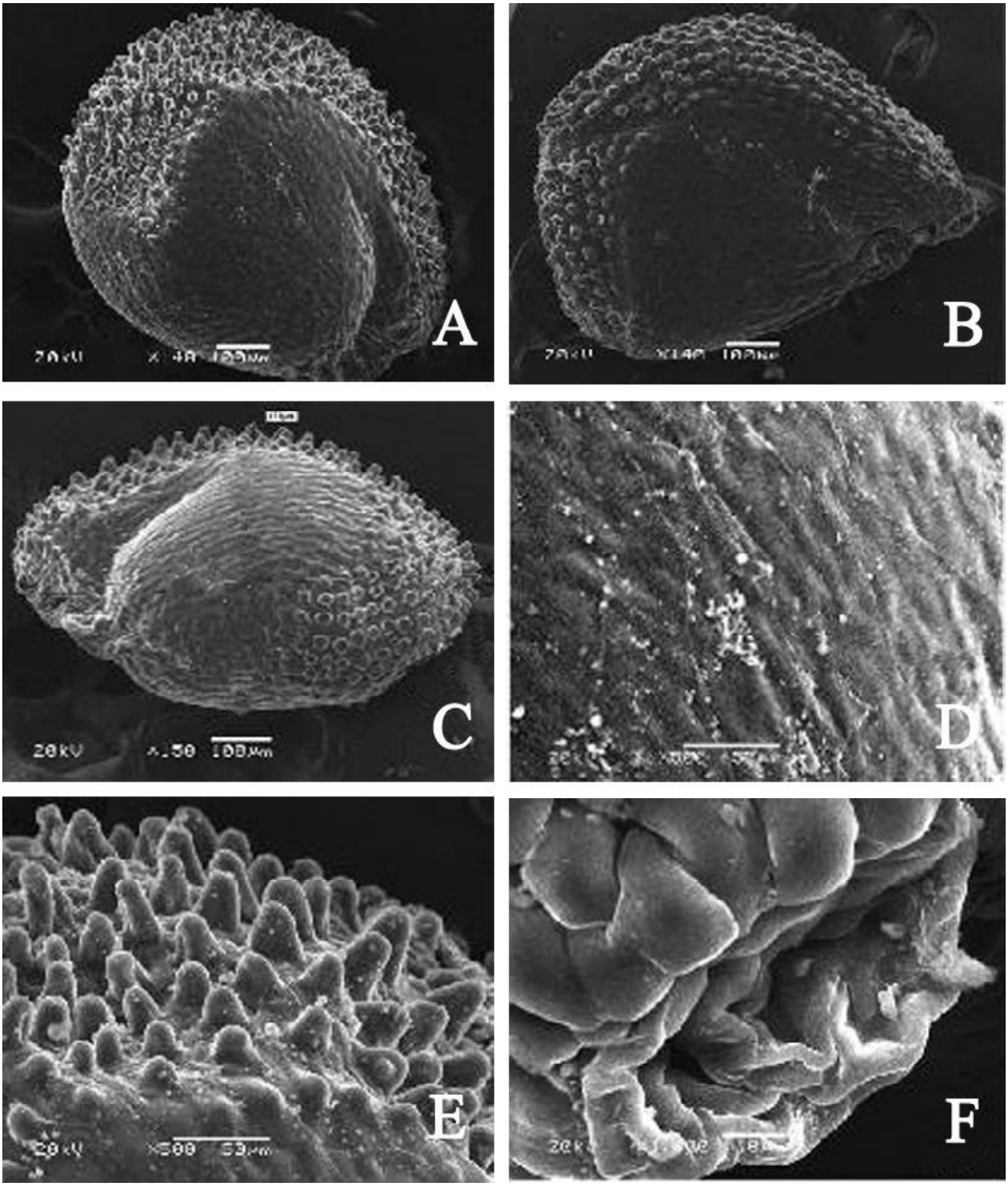

Fig. 3. *Halopeplisperfoliata.*A. Front view of the seed 140X , B. Dorsal view of the seed 140X, C. Side view of the seed 150X, D. Surface part amplifier500X, E. Superficial dorsal part amplifier500X , F. hilum shape 1000X

Anabasis. Embryo various-shaped from spiral (*Anabasis, Seidletzia*), hook-shaped (*Halopeplis*) to arcuate (*Halocnemum*).

Seed colour

The seeds in most populations of *H. strobilaceum* and *H. perfoliata* are brown in colour while that of *A. setifera* and *S.rosmarinus* are black.

Sculpturing

The exomorphology of the seeds shows that the

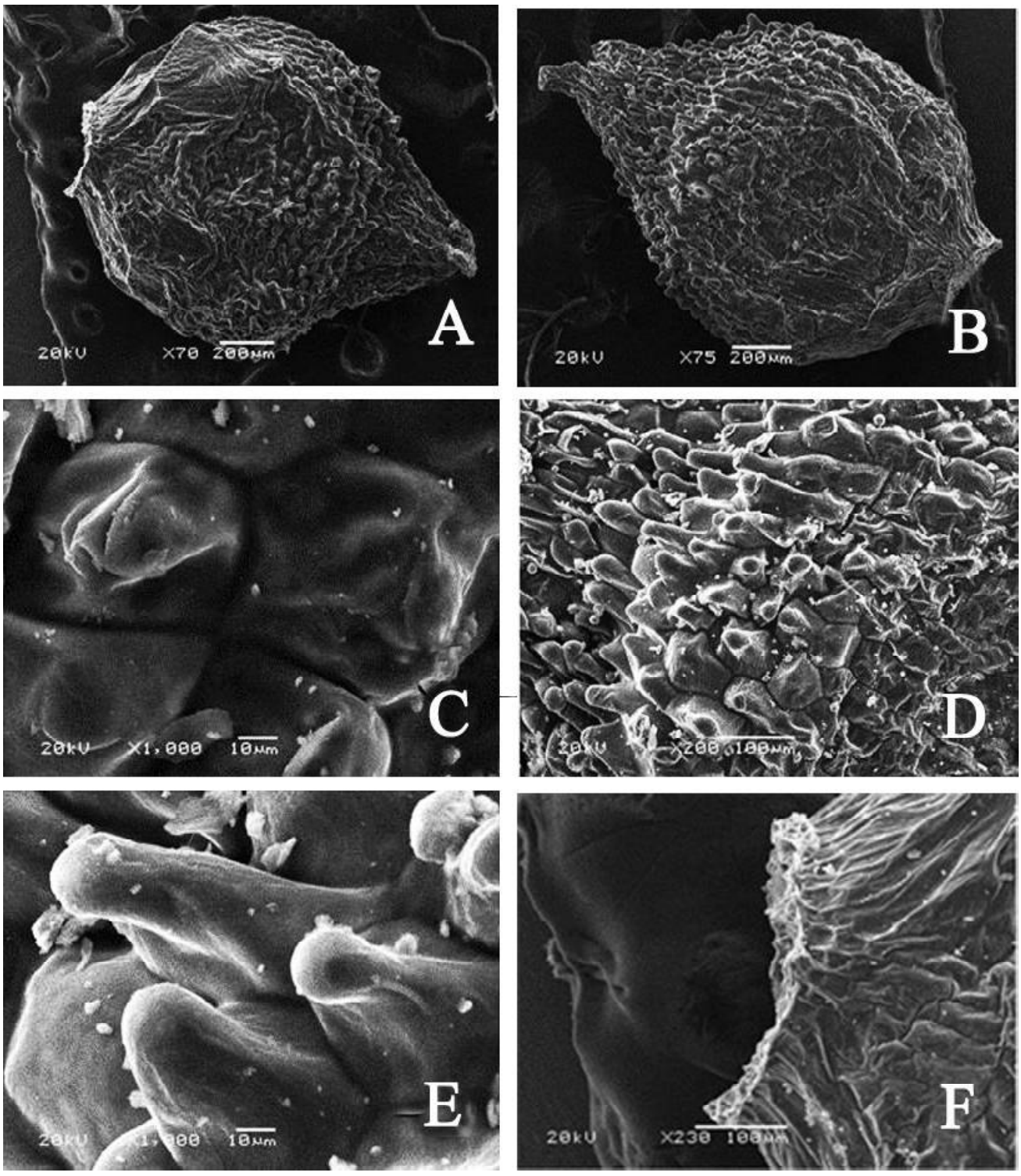

Fig. 4. *Anabasis setifera*: A. Front view of the seed 70X, B. Dorsal view of the seed 75X, C. View shows branched channels 1000X, D. Surface part amplifier200X, E. Surface part amplifier1000X, F.hilum shape 230X

surface of the seed is constructed of regularly arranged cells of various shapes and types. There is a slight increase in the cell length among cells located along the middle layers. However, majority of cells are pentagonal in most species. In *H.strobilaceum*, the surface is smooth to papillose. Protuberances (papillae) are significantly unequal, obscure, and smooth on the dorsal side and slightly papillate along the raphe (Fig. 2). In *H. perfoliata* the lower and upper ventral region appears smooth or slightly rough (Fig. 3). The seed coat of majority of members inSalsoloideae including *A. setifera* and *S.*

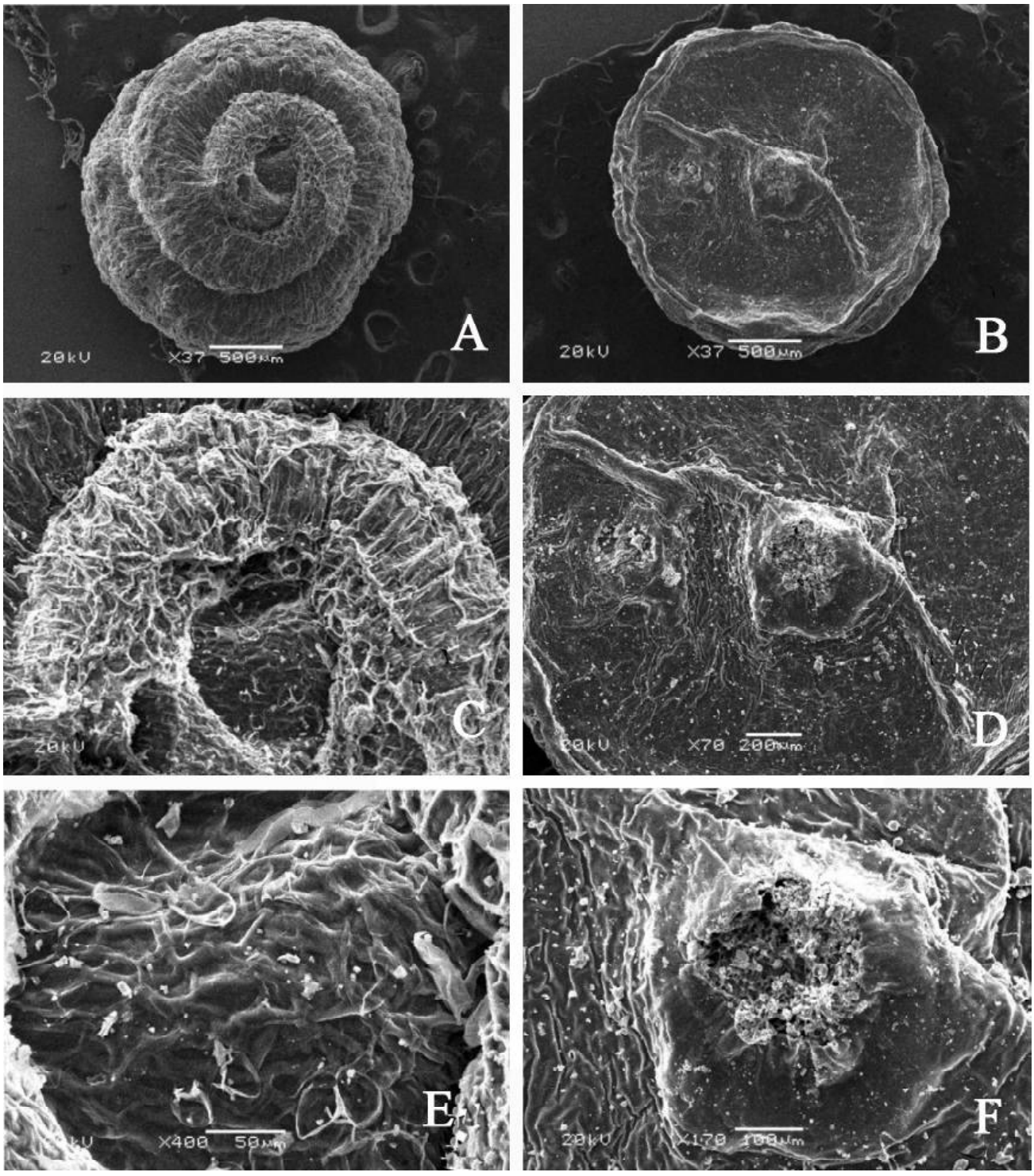

Fig. 5. *Seidletziarosmarinus:* A. Front view of the seed 30X, B. Superficial front part amplifier 200X, C. Superficial front part amplifier 400X, D. Dorsal view of the seed 35X, E. Dorsal part amplifier 70X, F. hilum shape 170X

*rosmarinus*consists of non-differentiated two-layered cell wall.However, it lacks macrosclereids and tannin in its cell wall.In *A. setifera*, the structure of seed-coat seldom varies around the perimeter (Fig. 4).

DISCUSSION

Species included in this study are important in the Kingdom because of their leadingrolein forming communities in coastal sabkhas. However, identity of these taxa, along with some of other chenopods such as Atriplex, Chenopodium and Salsola had been in a state of confusion due to the lack of clear cut characters (Chaudhary and Akram, 1986) or showing extreme vegetative plasticity in habit and leaf shapes

(Mandaville, 1990; Al-Turki and Ghafoor, 1996).Apart from the vegetative and floral characters, seed characters have been accepted as potentially useful at both the generic and species levels (Moss, 1954) because they are less inclined to show variability in the field or remain relatively unchanged upon drying (Shepherd et al., 2005). Seed position, shape, ornamentation of testa, embryo shape and perisperm presence, etc. are important in the phylogenetic point of view as these characters are highly variable in majority of members of Salicornioideae. Histological characters of the pericarp of majority of members in Salsoloideae, could be seen in the upper part of the fruit while in the lower half, the characters are either absent or insignificant (Sukhorukov, 2008).Single layered epidermis with papillae is found in several species of *Anabasis*. Papillae are to be found all over the surface or in the upper half (Sukhorukov, 2008). The seed coat of *A. setifera* is appeared to be thin, represented by 2-4-layered outer epidermis. This type of apomorphic feature ischaracterstic of the subfamily Salsoloideae and therefore not resistant to long term environmental degradations. The exomorphological characters of seed of *A. creatacea* showed that it consists of two or three cell layers of compressed cells with a thickness of 10–25(35) ìm and a thin outer cuticle. Some of the earlier studies pertaining to Salsoloideaehad reported small sized seeds in *A. setifera* (Blackwell, 1977). Overall the the subepidermal layers of *Anabasis* are well developed when compaired with that of *Salsola*(Sukhorukov (2008).

Although the characters ofcampylotropousovule with curved embryos isconstant inthe familyChenopodiaceae, variations such as shape, size or curvature have been observed in members of the subfamily Salicornioideae. Embryos of some members in Salicornioideae (e.g. *Salicornia*) are large enough to inhabit the entire seed (Shepherd et al., 2005).

Exomorphological characters are more or less constant in all species reported under *Halopeplis*. The characters reported for *H. amplexicaulis* from Iberian Peninsula indicated that the seeds are subreniform(0,5X 0,8 mm), marginally flattened on the sides,brownish epidermis, comma-shaped embryo embedded in copious starchy endosperm (Blanche and Molero, 1987). Ornamentation under SEM showed significant variations on the dorsal and ventral and dorsal sides of seeds, being smooth and papillate respectively.Seed surface characters of *Halopeplis* differ from other members of Salicornioideae like*Arthrocnemum* by its brown colour. However, the characters closely resemble with majority of seed surface characters of *Salicornia* except for the smooth episperm at the albumen region (Ball and Brown, 1970)

Members of Salicornioideae and Salsoloideae are generally devoid of endosperm in their seeds. Yet, a thin layer of endosperm was observed in a few members ofAmaranthaceaeincluding one of the species studied, such as *H.perfoliata* (Shepherd, 2005). The exomorphology of fruits and seeds of *Anabasis* is more or less similar to other members in Salsoloideae except for some exceptions like several layers of sclerenchymatous tissue beneath epidermal layers (e.g. *Halothamnusbottae*) or noticeably thickened outer periclinal cell walls of outer epidermis (*S.rosmarinus*).These differences in the lengths of seeds among the studied species and other plant species in the family indicated variations in genetic sequence.

The spiral embryo (spirally coiled)observed in all examined species is agreeing with many previous scientific studies (Mahabale andSolanky, 1953; Devi &Rao, 1976; Berggren, 1981),and also agreeing with the shape of embryo in some of the other species (*Salsola*) in the subfamily Salsoloideae(Berggren, 1981). There are significant differences in embryo shape in some other genera (*Atriplex, Beta* and *Chenopodium*) of subfamily Chenopodioidea (Berggren, 1981).The seed exomorphology of almost all species in Salsoloideae are more or less similar to that of *Anabasis*. Yet some species show some significant characters in the pericarp structure. For example, presence of mechanical parachymatous tissues which often present in several layers appear to be distinct in *Legenantha* and *Halothamnus* (Sukhorukov, 2008).The pericarp structure of *S. rosmarinus*Bunge which is noticeably thickened (25-40 ìm)is more or less similar to that of *Iljiniaregelii*(Bunge), a central Asian species.

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Genomic DNA Extraction and RAPD Analysis of Papaya (*Carica papaya* L.)

B. BINDU

Krishi Vigyan Kendra, Kollam, Kerala Agricultural University
Kerala
email : drbindusanthosh@gmail.com

ABSTRACT

Papaya (*Carica papaya* L.) is one of the most important tropical fruit crop grown in India. In Kerala, the crop has gained popularity due to its nutraceutical value. DNA isolation was done from young emerging leaves of papaya using hexa decy1 trimethyl ammonium bromide (CTAB) method with slight modification. Papaya hybrids selected for the study were Pusa Nanha x Solo, Pusa Dwarf x Coorg Honeydew, Pusa Nanha x Coorg Honeydew and Solo x Coorg Honeydew. DNA yield of different papaya hybrids ranged from 540 mg ml^{-1} (Solo x Coorg Honeydew) to 840 mg ml^{-1} (Pusa Nanha x Coorg Honeydew). The ratio of A_{260}/A_{280} ranged from 1.78 (Pusa Dwarf x Coorg Honeydew) to 1.86 (Pusa Nanha x Coorg Honeydew). All the 10 primers yielded amplification products with the DNA of Pusa Nanha x Coorg Honeydew. Primers OPA-03, OPA-04, OPA-12, OPB-04 and OPB-17 gave 40 scorable bands, with an average of 8.0 bands per primer. The highest number of scorable bands (12 bands) was given by the primer OPA-03. The number of bands resolved per amplification was primer dependent and varied from four to twelve. Results of the trial revealed that the largest cluster in dendrogram was formed by three hybrids - Pusa Nanha x Coorg Honeydew, Pusa Dwarf x Coorg Honeydew and Pusa Nanha x Solo. The second cluster contained only one hybrid - Solo x Coorg Honeydew. The pair wise similarity coefficient values varied from 0.389 to 0.714. The minimum similarity coefficient detected in the present study was 0.389, suggesting a genetic differentiation among the papaya hybrids

Key words *Papaya, DNA, RAPD, Primer, Hybrid, Genetic differentiation*

Papaya (*Carica papaya* L.) is now emerging as an important fruit crop of Kerala. Nutritive value, high yielding potential, year - round fruiting behaviour and short pre-bearing period make papaya unique among the fruit crops. Its fruits are recognized for papain, a proteolytic enzyme extensively used in cosmetics and pharmaceutical industries. Due to awareness of multifold uses of papaya for table, processing and papain extraction purposes, it is slowly emerging from the status of a homestead crop to that of commercial crop. Preparation of high-quality DNA is a prerequisite for succeeding in subsequent molecular biology research. There are many specialized DNA extraction methods. Tropical plants are often rich in cellulose, polysaccharides, polyphenols, proteins and lipids, which complicate the nucleic acid separation and purification. A wide variety of DNA extraction techniques have been developed for isolation of DNA from containing high polysaccharide and polyphenol components and applied these methods in papaya. Characterization of papaya hybrids is generally done based on morphological and a gronomic traits, isozyme analysis etc. However, these approaches are subjected to environmental influences. Recently, DNA based molecular markers like RAPD (Random Amplified Polymorphic DNA), RFLP (Restriction Fragment Length Polymorphism), AFLP (Amplified Fragment Length Polymorphism) have been widely used for genetic characterization, as they are not affected by environmental conditions. Among these RAPD marker technique is quick, reliable and widely applicable. DNA isolation was done from young emerging leaves of papaya using hexa decy1 trimethyl ammonium bromide (CTAB) method with slight modification. The present study was aimed to develop an efficient protocol for genomic DNA extraction from papaya.

MATERIALS AND METHODS

For genomic DNA extraction four papaya Hybrids were used *viz* Pusa Nanha x Solo, Pusa Dwarf x Coorg Honeydew, Pusa Nanha x Coorg Honeydew and Solo x Coorg Honeydew. DNA was extracted from young leaves using hexadecyl trimethyl ammonium bromide (CTAB) method (Dellaporta *et al*., 1983) with slight modification. 0.50 gram of papaya leaves were used for the study. The contaminating RNA was removed by digestion with 5.00 µg of RNAase for one hour at 37°C. To this 200 µl of phenol: chloroform : isoamylalcohol (25:24:1) was added and

centrifuged at 10,000 rpm for 10 minutes at 4°C. The upper phase was collected and to this 200 µl of chloroform: isoamylalcohol was added. Again centrifugation was done at 10,000 rpm for 10 minutes at 4C. The upper phase was collected. To this 200 ml of choloroform : isoamylalcohol was added again and centrifuged at 10,000 rpm for 10 minutes at 4°C. Clear upper phase was collected and to this 100 µl of 3M sodium acetate and 1000 ml of cold absolute ethanol was added. On gentle mixing, DNA strings became visible. Again centrifugation was done at 10,000 rpm for 10 minutes at 4°C and the supernatant decanted retaining the pellet. The DNA pellet was washed with 70.00 per cent ethanol, then centrifuged at 10,000 rpm for five minutes at 4°C and the supernatant decanted retaining the pellet The pellet was allowed to air dry and then dissolved in 100 ml TE buffer and stored at 4°C.

DNA quantification was done with the help of UV- Vis Spectrophotometer. The absorbance was measured at 260nm and 280nm. The spectrophotometer was calibrated at 260nm and 280nm wave length using TE buffer in which DNA was dissolved. The optical density of the DNA sample dissolved in the buffer was recorded both at 260 nm and 280 nm. The quality of DNA was assessed from the ratio of the OD values recorded at 260 nm and 280 nm. A ratio between 1.8 and 2.0 indicates good quality of DNA. Agarose gel electrophoresis was carried out in horizontal gel electrophoresis unit. Agarose concentration used was 0.8 per cent for visualizing genomic DNA and 1.4 per cent for visualizing amplified products.

The required agarose was weighed out. This was added to 1x TAE buffer. Agarose was dissolved by boiling. After cooling to 50°C, ethidium bromide was added to a final concentration of 0.50 mg ml^{-1}. This mixture was immediately poured in to a preset template with appropriate comb. After solidification, the comb was removed and the gel was mounted in an electrophoresis tank filled with 1 x TAE running buffer. The DNA sample was mixed with required volume of gel loading dye (6.0 x loading dye *viz.,* 40.00 per cent sucrose, 0.25 per cent bromo phenol blue). Each well was loaded with 20 ml of the sample. One of the wells was loaded with 1.50 ml of molecular weight marker, in required volume of gel loading dye. Electrophoresis was performed at 60 volts until the loading dye reached three fourth of the length of the gel. The gel was visualized using a ultravisible transilluminator. Random Amplified Polymorphic DNA analysis was performed using the selected primers to amplify the DNA of all the four papaya hybrids *viz*., Pusa Nanha x Solo, Pusa Dwarf x Coorg Honeydew, Pusa Nanha x Coorg Honeydew and Solo x Coorg Honeydew. Photographs of the amplification profile obtained were taken with the help of gel documentation system. The RAPD bands were represented as '+' (plus) for presence and '-' (minus) for absence and recorded PCR was repeated twice inorder to assess the reproducibility. Reproducible bands were scored for the presence (+) or absence (-) for all the four papaya hybrids. A genetic similarity matrix was constructed using the Jaccard's coefficient method. Based on the similarity coefficient a dendrogram was constructed) by following the nearest neighbour (single link) method. Association between the hybrids were found out from the dendrogram.

RESULTS AND DISCUSSIONS

As the biochemical composition of plant tissues and species varies considerably, it is difficult to use the same protocol for DNA isolation for different plant species. Even closely related species may require different DNA isolation procedures (Weising *et al*., 1995). In the present study DNA isolation was done from young emerging leaves of papaya using hexa decy1 trimethyl ammonium bromide (CTAB) method (Dellaporta *et al*., 1983) with slight modification. The quantity and quality of the isolated DNA depends on the source of tissue as well as efficient disruption of plant cell wall (Babu, 2000). Mondal *et al*. (2000) opines that tender leaves contains actively dividing cells with less intensity of extra nuclear materials like proteins, carbohydrates and other metabolites that interfere with isolation of nucleic acids, which in turn improve the quality of DNA. Tender leaves also facilitate easy cell disruption for DNA extraction.

In the present study the DNA yield of different papaya hybrids ranged from 540 mg ml^{-1} (Solo x Coorg Honeydew) to 840 mg ml^{-1} (Pusa Nanha x Coorg Honeydew). The ratio of A_{260}/A_{280} ranged from 1.78 (Pusa Dwarf x Coorg Honeydew) to 1.86 (Pusa Nanha x Coorg Honeydew). Sereena (2004) observed

Table 1. Primer associated banding patterns with the DNA of papaya hybrid using 10 primers

Sl. No.	Primers	Number of faint bands	Number of intense bands	Total number of bands
1	OPA-03	3	6	9
2	OPA-04	2	2	4
3	OPA-12	7	2	9
4	OPA-13	0	1	1
5	OPA-14	1	1	2
6	OPA-15	1	2	3
7	OPB-04	2	2	4
8	OPB-08	0	1	1
9	OPB-10	1	1	1
10	OPB-17	2	3	5

that the yield of DNA of different papaya varieties ranged from 600 mg ml^{-1} to 8035 mg ml^{-1} and purity ratio of 1.65 to 2.2.

Agarose gel electrophoresis was used for analyzing the genomic DNA isolated from different papaya hybrids as well as for the RAPD analysis. Agarose 0.8 per cent was found optimum for genomic DNA and 1.4 per cent for RAPD analysis. Lim *et al*. (1999) used 0.8 per cent agarose for visualizing genomic DNA. While Mulcaby *et al*. (1993) and Sereena (2004) used 0.9 per cent agarose for the same purpose. Prasannalatha *et al*. (1999), Mondal *et al*. (2000) and Sereena (2004) observed the separation of amplified products through 1.4 per cent agarose gel. Higher concentration of two per cent agarose has been reported by Mulcahy *et al*. (1993) and Galderisi *et al*. (1999). Voltage level of 60 V proved to be the most suitable for RAPD.

The buffer 1 x TAE performed better for band separation. Galderisi *et al*. (1999) reported that amplification of the PCR products was obtained at a voltage of 100 to 120 V. The PCR amplification was carried out using ten primers (Operon Inc., CA, USA) of Kit A and Kit B, with the DNA of papaya hybrid Pusa Nanha x Coorg Honeydew.All the ten primers yielded amplification products with the DNA of papaya hybrid Pusa Nanha x Coorg Honeydew (Table1). A total of 39 RAPDs (average of 3.9 bands per primer) were generated, of which 36 bands (92.31 per cent) were polymorphic. The individual primer has the capability to amplify the less conserved and highly repeated regions of the genomic DNA (Prasannalatha *et al*., 1999).

Five primers which produced the highest number of brands as well as the highest number of intense bands were selected for amplifying DNA from four papaya hybrids. The PCR reaction was repeated at least twice in order to check the reproducibility. Data from five primers that gave reproducible product formation on at least two runs alone were included in statistical analysis. The five primers used in this analysis were OPA-03, OPA-04, OPA-12, OPB-04 and OPB-17. This was in accordance with the findings of Somasree *et al* (1998) who observed 102 distinct bands with an average of 9.3 bands per primer, while evaluating genetic relationships among papaya varieties using primers. Muthulakshmi *et al*., (2007) obtained 40 scorable bands with an average of ten bands per primer in papaya. OPA-12, OPB-04, OPB-17) yielded 40 scorable bands with an average of 8.0 bands per primer. The number of bands resolved per amplification was primer dependent and varied from four to twelve (Table 2).

Table 2. Nucleotide sequences of primers and total number of informative RAPD markers amplified with them in the papaya hybrids

Primer	Sequence	Number of informative RAPD markers
OPA-03	AGTCAGCCAC	12
OPA-04	AATCGGGCTG	7
OPA-12	TCGGCGATAG	11
OPB-04	GGACTGGAGT	4
OPB-17	AGGGAACGAG	6

The five primers employed in this analysis yielded 40 scorable bands, with an average of 8.0 bands per primer. This was in accordance with the findings of Stiles *et al*. (1993) who observed 102 distinct bands with an average of 9.3 bands per primer, while evaluating genetic relationships among papaya varieties using primers. Sereena (2004) obtained 40 scorable bands with an average of ten bands per primer in papaya. In the present studies, the number of bands resolved per amplification was primer dependent and varied from a minimum of four to a maximum of twelve bands. Jaccard's similarity coefficient ranged from 0.389 to 0.714 among different papaya hybrids used in the present study. The highest value (0.714) for similarity index was obtained for the papaya hybrid Pusa Nanha x Coorg Honeydew with Pusa Dwarf x Coorg Honeydew. The least similarity coefficient value (0.389) was that of papaya hybrid Pusa Dwarf x Coorg Honeydew with Solo x Coorg Honeydew. The largest cluster in dendrogram was formed by three hybrids: Pusa Nanha x Coorg Honeydew, Pusa Dwarf x Coorg Honeydew and Pusa Nanha x Solo. Second cluster contained only one hybrid Solo x Coorg Honeydew. The minimum similarity coefficient detected in the present study was 0.389, suggesting a genetic differentiation among the papaya hybrids.

CONCLUSION

Genomic DNA was successfully isolated from four papaya hybrids using CTAB method. All the ten primers, six primers from OPA and four primers from OPB series yielded amplification products with isolated DNA. A total of 39 RAPDs were obtained from ten primers of which 36 bands were polymorphic. The five primers employed in this analysis yielded 40 scorable bands, with an average 8.0 bands per primer. The number of bands resolved per amplification was primer dependent and varied from 4 to 12 bands. The highest number of scorable bands of twelve was given by the primer OPA-03 and least number of bands (four) by primer OPB-04. The hybrid Solo x Coorg Honeydew and Pusa Nanha x Solo yielded three specific bands, which was absent in other hybrids with primer OPA-04. Using primer OPA-12, papaya hybrid Solo x Coorg Honeydew yielded one specific band which is absent in other hybrids. With primer OPB-17, papaya hybrid Pusa Nanha x Coorg Honeydew yielded three specific bands, which was absent in all other papaya hybrids selected for the present study. Jaccard's similarity coefficient ranged from 0.389 to 0.714 among different papaya hybrids used in the present study. The highest value (0.714) for similarity index was obtained for the papaya hybrid Pusa Nanha x Coorg Honeydew with Pusa Dwarf x Coorg Honeydew. The least similarity coefficient value (0.389) was that of papaya hybrid Pusa Dwarf x Coorg Honeydew with Solo x Coorg Honeydew. The largest cluster in dendrogram was formed by three hybrids: Pusa Nanha x Coorg Honeydew, Pusa Dwarf x Coorg Honeydew and Pusa Nanha x Solo. Second cluster contained only one hybrid Solo x Coorg Honeydew. The minimum similarity coefficient detected in the present study was 0.389, suggesting a genetic differentiation among the papaya hybrids.

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Genetic Diversity of *Orthosiphon aristatus* Accessions from Different Geographical Regions Using RAPD and ISSR Markers

CHITHRA VIJAYAN[1], K MURUGAN[2] AND G M NAIR[3]

[1]*Dept. of Botany & Biotechnology, Sree Narayana College, Kollam.*
[2]*Dept. of Botany & Biotechnology, Govt. Arts College, Thiruvananthapuram*
[3]*Biotechnology Commission, KSTEC*
email:chithraadersh@gmail.com

ABSTRACT

Medicinal herbals show considerable variation in the production of secondary components and indicate that the synthesis of these defensive metabolites can be regulated by a plant to certain extent, depending on the ability of the plant to acclimate to changes in the physical environment. The information on the genetic diversity of the five accessions of *Orthosiphon aristatus* evaluated with diverse systems were consensus with each other evolving better clarity on the genetic relationship among the studied *O. aristatus* accessions in hierarchical order of their resolving abilities. In the analysis of genetic diversity using five *O. aristatus* accessions collected from five districts of Kerala, three primers out of the twenty decamer primers failed to generate any amplification product, for RAPD analysis. The rest of the primers developed 75 polymorphic robust loci which revealed 48.07% polymorphism. An average number of 9.17 bands per selected primer were obtained for RAPD. For ISSR markers, out of 20 oligonucleotide primers screened, 17 were selected for further analysis based on clarity, scorability and reproducibility of banding patterns. A total of 66 fragments with different lengths were clearly amplified from five accessions, among which 54 bands (81.8%) were polymorphic. Of the twenty primers employed, 85% showed amplification, while only 75% produced polymorphic fingerprints. The band amplified using ISSR primers were in the range of 500 bp to 2kb. Based on the data a dendrogram was constructed to reflect the similarity and diversity among the accessions. The results obtained provide a short and snappy view of the total genetic variability for *Orthosiphon* species in Kerala and thus help in evolving pragmatic strategies for breeding improved varieties of *Orthosiphon* for pharmaceutical applications.

Key words *Orthosiphon aristatus, RAPD, ISSR markers, dendrogram, polymorphism*

The advent of molecular methods has supplemented traditional taxonomic methods in the DNA based tools to examine phylogeny and systematics (Althoff et al., 2007; Banfer et al., 2004). Information on the levels of genetic variation within and among natural populations provides fundamental insights on the genetic diversity and for improving the effective selection of the populations for pharmaceutical purposes (Domyati et al., 2011)For reasonably accurate and unbiased estimates of genetic diversity, adequate attention has to be devoted to (1) sampling strategies (ii) utilization of various data sets on the basis of the understanding of their strength and constraints (iii) choice of genetic distance measures, clustering procedures and other multivariable methods in analysis of data and objective determination of genetic relationships. Judicious combination and utilization of statistical tools and techniques, such as boot strapping, is vital for addressing complex issues related to data analysis and interpretation of results from different types of data set, particularly through clustering procedures.

MATERIALS AND METHODS

In the present study five different accessions of *Orthosiphon aristatus* were collected from five districts of Kerala, India (Table-1). The collected plants were grown initially in pots and maintained under identical growth conditions at KUBOT green house, Kariavattom, Thiruvananthapuram.

DNA based markers

The two main DNA based markers used in this study for the characterization of *Orthosiphon aristatus* accessions were Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR).

Plant DNA Extraction

Total genomic DNA was extracted from fully expanded leaves of actively growing tillers (first or second leaf from the tip) from collected from five districts of Kerala using CTAB method of Maroof et al.(1984) with some modifications. The purity of the DNA samples was determined spectrophotometrically by measuring absorbance at 260 nm and 280 nm.

Table 1. Provinces of the accessions included in the study

Accession no.	Place of collection	Geographical location
Acc 1	Malappuram	75 to 77 east longitude and 10° to 12° north latitude
Acc 2	Wayanad	11. 27' to 15. 58' north latitude and 75. 47' to 70. 27' east longitude.
Acc 3	Ernakulam	9 °57' North latitude and 76 ° 15' East longitude.
Acc 4	Thiruvananthapuram	8° 42' N latitude,77 - 35°E longitude
Acc 5	Nagercoil	77° 15' and 77° 36' of the eastern longitudes and 8° 03' and 8° 35' of the northern Latitudes.

Arbitrary primers

Twenty oligo nucleotide decamer arbitrary RAPD primers and a set of 20 anchored microsatellite ISSR primers (Genei, Banglore) were performed on DNA from the *Orthosiphon* accessions for PCR amplification. On the basis of reproducible and maximum number of distinctly scorable bands, 17 RAPD primers (Table-2) and 17 ISSR primers (Table-3) were selected for further studies.

The enzymatic PCR amplification of the genomic DNA with different primers was performed in a MJ Research Thermal–Cycler, PTC–200 (MJ Research, USA) programmed for 35 cycles for both RAPD and ISSR. To reduce the possibility of cross contamination and variation in the amplification reactions, master-mixes (all reagents except DNA were pre-mixed and aliquoted) of the reaction constituents were always used.

RAPD analysis

PCR analysis with RAPD primers

RAPD amplification of genomic DNA was conducted according to Williams et al. (1990) using 20 µl of reaction mixture containing 2µl of 10XPCR buffer, MBI Fermentas (750mM Tris – HCl pH 8.8, 200mM (NH4)2SO4, 0.1% Tween 20); 2µl dNTP; 25mM MgCl2 (MBI Fermentas); 2mM primer; 1U Taq DNA polymerase (Fermentas # EPO402) and 25ng genomic DNA. The gels were run at a constant current of 200mA for approximately 4hrs, stained with ethidium bromide (5µl/100ml) and photographed with the gel documentation system (Syngene, USA). The DNA mass ladder markers (Genei Mass Ruler TM DNA ladder), were used for determining the size of each band.

Table 2. List of primers used for RAPD analysis

No.	Primer sequence ($5^{l} - 3^{l}$)
S-150	CCCGATTCGG
S-127	CCGATATCCC
S-113	GACGCCACAC
S-142	GGTGCGGGAA
S-190	AGGGCCGTCT
S-183	GAGCCCTCCA
S-162	GGAGGAGAGG
S-150	TTATCGCCCC
S-119	CTGACCAGCC
S-196	AGCGTCCTCC
S-164	CCGCCTAGTC
S-143	CCAGATGCAC
S-166	AAGGCGGCAG
S-104	GGAAGTCGCC
S-187	GAACCTGCGG
S-191	TGCCCGTCGT

Table 3. List of primers used for ISSR analysis

Sl. No.	Primer
1	$(AG)_8G$
2	$(GA)_8C$
3	$(CT)_8G$
4	$(CA)_8A$
5	$(GT)_8C$
6	$(TC)_8C$
7	$(AC)_8C$
8	$(AC)_8G$
9	$(TG)_8C$
10	$(AG)_8YT$
11	$(AG)_8YC$
12	$(AG)_8YA$
13	$(GA)_8YT$
14	$(GA)_8YC$
15	$(CT)_8RC$
16	$(CT)_8RG$
17	$(CA)_8RG$

ISSR profiling

ISSR amplification generally followed the method of Vijayan (2004). PCR amplification profile consisted of first step at 940C for 2min followed by 35 cycles of 94^0C for 30 sec, 50^0C for 30 sec and 72^0C for 2min and final incubation of 10 min at 72^0C. The banding patterns were photographed under ultraviolet light utilizing a photo documentation system, Syngene Corporation, USA.

RAPD and ISSR, each fragment that was amplified using a primer was treated as a unit character. The presence or absence of a band was considered as a single feature to which relevant values of '1' and '0' were attributed. The data entry was done into a binary data matrix as discrete variables for all the marker systems. The total number of bands, distribution of band across accessions, number of polymorphic bands, and average number of bands per primer were calculated.

Cluster Analysis

The binary data matrix from polypeptide, RAPD and ISSR marker systems were analyzed using Unweighted Pair Group Method using Arithmetic average (UPGMA) –Phenogram and a sequential agglomerative, heirarchial and nested cluster analysis was obtained. This is a clustering program which compresses the patterns of variation into two-dimensional branch diagrams, the dendrogram (Sneath and Sokal, 1973). To evaluate the robustness of the grouping formed, the binary data matrix was subjected to boot strapping using the software program WINBOOT developed at IRRI to determine the confidence limits of the UPGMA – based dendrogram (Yap and Nelson,1996).

RESULTS AND DISCUSSION

RAPD analysis: The analysis of genetic diversity using five *Orthosiphon* accessions and twenty decamer primers showed that three primers failed to generate any amplification product. Using the described DNA purification and amplification conditions with the selected primers, good and clear patterns could be obtained for the various accessions under study. When screening all the accessions, 5-13 amplicons per selected RAPD primer were scored, originating a total of 156 fragments (Fig 1). The primers developed 75 polymorphic robust loci which revealed 48.07% polymorphism. An average number of 9.17 bands per selected primer were obtained for RAPD. A maximum number of 13 amplicons were amplified with primer S142 while the minimum number of fragments (3) was amplified with primer S105 with 100% polymorphism (Table- 4). The RAPD data of the accessions of *Orthosiphon* was extended further for statistical analysis in order to measure the genetic distances among the accessions.

Statistical analysis of the genotypic data

Even though diagnostic RAPD bands were observed, most of them were either faint or not repeatedly formed in all the representative individuals of the five accessions. Thus a large number of potentially informative bands were eliminated from consideration. Distance matrix analysis of the RAPD

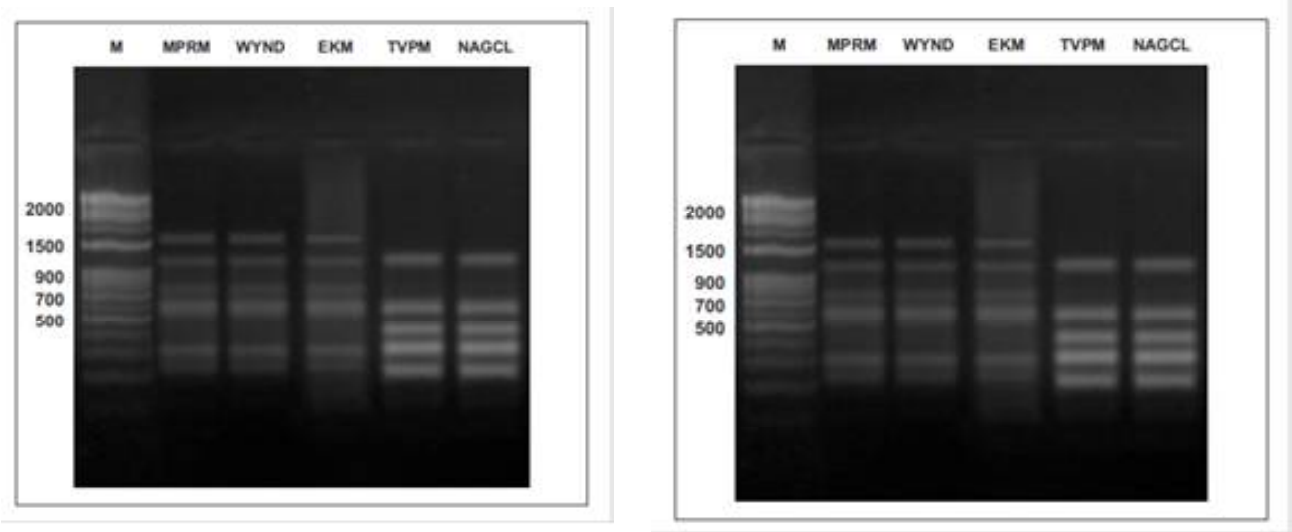

Fig. 1. RAPD fingerprints of the five accessions showing DNA polymorphism

Table 4. Distribution of RAPD bands detected for different primers across the accession of *O.aristatus*

Primer	Total no. of bands	No. of markers per accession					Total no. of polymorphic bands
		Acc1	Acc2	Acc3	Acc4	Acc5	
S-150	6	6	6	6	6	6	0
S-127	6	6	6	6	6	6	0
S-113	8	7	4	4	4	4	5
S-142	13	13	13	13	13	13	0
S-190	10	6	9	5	4	4	9
S-183	4	4	3	4	3	4	2
S-105	4	3	2	2	1	2	4
S-162	8	4	4	4	4	4	8
S-150	5	5	5	5	5	5	0
S-119	8	6	5	3	2	2	8
S-196	7	5	5	5	5	5	4
S-164	10	5	5	5	5	5	10
S-143	4	3	2	2	3	3	4
S-166	3	1	2	3	1	2	2
S-104	8	2	4	4	4	2	8
S-187	4	4	2	2	2	2	3
S-191	8	3	5	4	3	3	8

data based on Euclidean data was used for generating dendrogram using the Unweighted Pair Group Method Analysis, UPGMA (Fig 2).

Accessions from Nagercoil and Thiruvananthapuram clustered together to form the first major cluster. Second major cluster had all the remaining three accessions grouped together into two subphenons with the accession from Malappuram as an outlier. Ernakulam and Wayanad grouped together to form the second subphenone with 86.2% bootstrap value.

Clustering of accession from Nagercoil and Thiruvananthapuram into a separate group was substantiated with cent percentage boot strap value. As evident, the accessions from Malappuram, Wayanad and Ernakulam demonstrated a greater relatedness representing a major group in the phylogenetic tree through cluster analysis .

ISSR analysis: The reaction mixture composition and reaction conditions were empirically determined. This exercise involved optimization of primer concentration and annealing temperature for each primer. A total of twenty ISSR primers based on dinucleotide repeats were used to amplify the accessions of *Orthosiphon*. Out of 20 oligonucleotide primers screened 17 were selected for further analysis based on clarity, scorability and reproducibility of banding patterns. From the analysis of ISSR markers, the 17 primers used, produced bands which were clearly visualized on the gels (Fig 3). A total number of 66 fragments with different lengths were clearly amplified from five accessions, among which 54 bands (81.8%) were

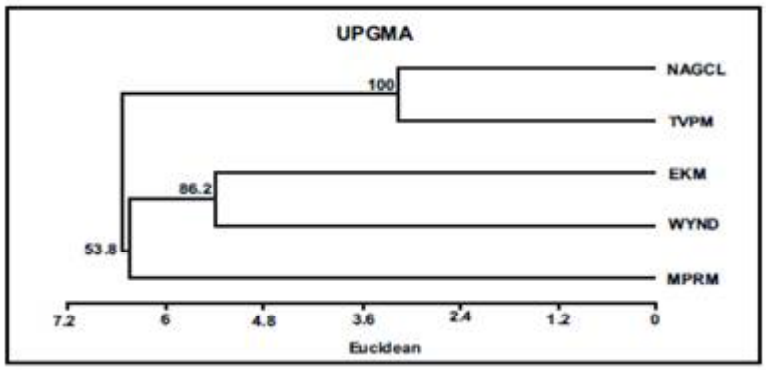

Fig. 2. Dendrogram of the 5 *Orthosiphon* accessions based on RAPD analysis data with 14 primers. Number at the branch indicate the probability supporting the branch.

polymorphic. Among the primers studied, the highest number of bands was generated with primer 14, 12 and15 (6 bands), while the lowest number of bands were generated with primer 9 and 13. Of the twenty primers employed, 85% showed amplification, while only 75% produced polymorphic fingerprints. Mostly, dark and prominent bands were scored, although bands of lower intensity but with higher reproducibility were also included in the analysis.

The band amplified using ISSR primers were in the range of 500bp to 2kb. (Table -5) gives a description of primers and the fingerprint patterns obtained by using 17 selected primers. Of all the amplified profiles, the 66 best and highly reproducible patterns were selected for further analysis.

Statistical analysis of the genotypic data

The finger print patterns of the five samples analyzed in the current study using various ISSR primers were used for the cluster analysis. This analysis in the form of a dendrogram (Fig 4) was used as the basis to infer intraspecific relationships in the species *Orthosiphon*. Each of the accession maintains its independent status in the dendrogram. The generation of this dendrogram (Fig 4). involved the computation of a similarity co efficient matrix, and the cluster analysis was based on these values by the Unweighted Pair Group Method with Arithmatic Average (UPGMA). The confidence limits for using the Winboot program to perform UPGMA based bootstrapping, tested the groupings produced by this method. Two thousand boot strap replications were carried out as suggested by Hedges, 1992 and the values were obtained in terms of the percentage of the number of times a group would be found in the bootstrap replications. The results of the consensus

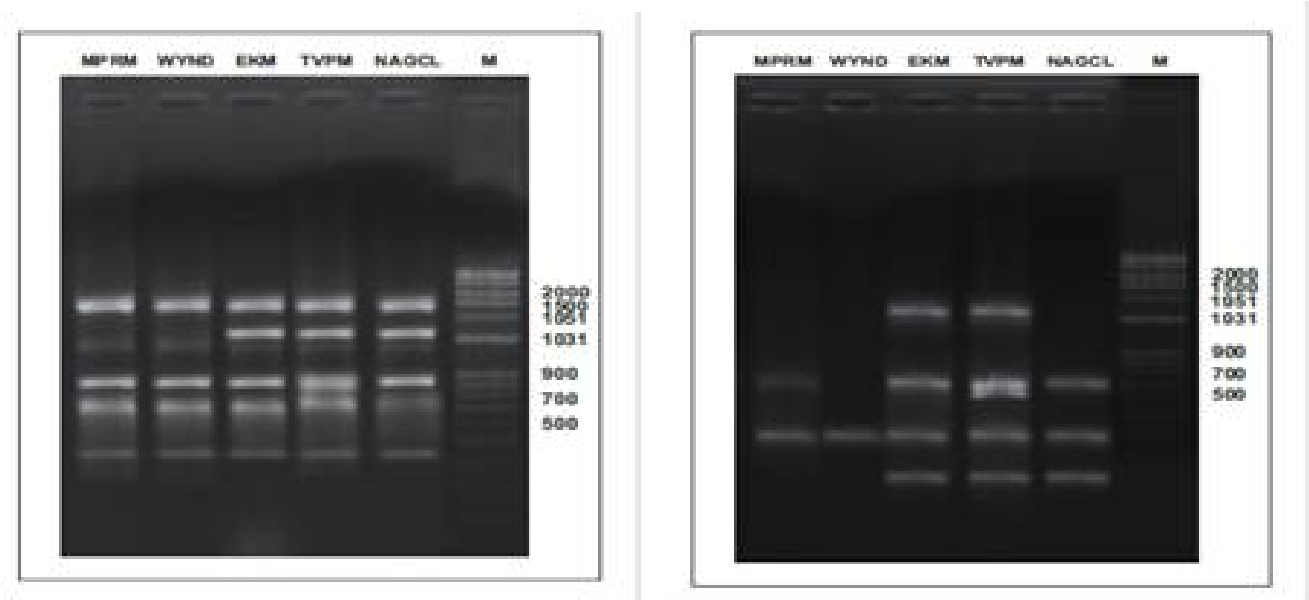

Fig. 3. Amplification product generated by the genomic DNA of the accessions of *Orthosiphon* using ISSR primers.

Table 5. ISSR banding pattern of the five accessions of *Orthosiphon aristatus*

Sl. No.	Primer	Total no. of bands	No. of markers per accession					Total no. of polymorphic bands
			Acc1	Acc2	Acc3	Acc4	Acc5	
1	$(AG)_8G$	5	4	4	3	4	3	4
2	$(GA)_8C$	5	4	4	5	4	4	3
3	$(CT)_8G$	4	1	3	3	4	1	4
4	$(CA)_8A$	3	3	3	3	3	3	0
5	$(GT)_8C$	3	2	2	2	2	1	3
6	$(TC)_8C$	4	4	3	1	1	1	3
7	$(AC)_8C$	2	2	2	2	1	1	5
8	$(AC)_8G$	5	3	3	5	5	5	2
9	$(TG)_8C$	1	1	1	1	0	1	1
10	$(AG)_8YT$	4	2	1	3	3	2	4
11	$(AG)_8YC$	3	2	2	2	2	2	2
12	$(AG)_8YA$	6	2	1	4	5	3	5
13	$(GA)_8YT$	1	1	1	1	1	1	0
14	$(GA)_8YC$	6	4	5	3	5	3	4
15	$(CT)_8RC$	6	4	5	5	4	5	4
16	$(CT)_8RG$	4	3	2	1	4	3	6
17	$(CA)_8RG$	4	2	3	2	3	1	4

tree from ISSR data indicated that the tree was divided into 2 major clusters each having two and three accessions respectively.

In general, the consensus tree indicates that the species belonging to the adjacent geographical areas group together with a high bootstrap value (61.9 – 98.9%). Apart from these observations, three major groups are seen in the dendrogram, group I *Orthosiphon aristatus* accessions comprising those from Wayanad, Ernakulam, and Malappuram and group II comprising accessions from Thiruvananthapuram and Nagercoil.

Although many plant species produce high levels of secondary metabolites, comparatively little is known about the temporal variability of the production and concentrations of these compounds, either in terms of quantitative or qualitative aspects. Earlier studies in many species complexes have indicated the inadequacy of evidence from external morphology alone for interpreting variations between accessions. To verify the limitations evidenced in the use of phenotypic and biochemical data for taxonomic purposes, which can be overcome by molecular analysis, DNA extracted from the five accessions was analyzed by RAPD and ISSR markers.

Results of RAPD analysis are independent of environmental influences and tissue type and provide greater resolution than proteins and the procedure is faster and easier than other molecular analysis (Kumar et al., 2009). Three subgroups were formed by the cluster analysis of the molecular data confirming that each accession represents a unique genetic identity and that genetic variation was associated with the geographical origin. The accession collected from Malappuram, which showed the highest percentage of free radical inhibition, stood apart as single from the rest of the accessions.

It is reported that RAPD analysis of *Allium schoenoprasum* (Friesen and Blattner, 2000) and *A.sativum* (Maab and Klaas, 1995) generated

dendrograms that mirrors the geographical distribution. Jo et al. (2012) have reported that the cluster analysis placed garlic accessions in accordance with their geographical origin, and that the data can be used for the identification of genetic groups. In the present study also most of the geographically closer accessions form tightly linked groups.

Here, molecular classification was in agreement to some extent with geographic distribution of the accessions. Although the 5 accessions studied have been clustered into 2 groups, the genetic similarity among them was high as expected for individuals belonging to the same species, however suggesting a narrow genetic pool in *Orthosiphon* accessions. This indicates that the extent of intraspecific variations in the species is relatively less. This agrees with the report of Goulao et al. (2001) in discrimination and estimation of genetic similarities among apple (*Malus domestica* Borkh), where 41 cultivars studied have been clustered into several subgroups, but the genetic similarity among them was high suggesting a narrow genetic pool in apple cultivars. RAPD-based molecular markers have been found to be useful in differentiating different accessions of *Taxus wallichiana* (Shasany et al., 2000), neem (Farooqui et al., 1998), *Juniperus communis* L. (Joshi et al., 2004), *Codonopsis pilosula*(Fu et al., 1999), *Allium schoenoprasum* L.(Friesen and Blatter 2000), *Andrographis paniculata* (Padmesh et al., 1999) collected from different geographical regions. RAPD have also been applied for characterisation of many plant species at the genetic level (Hosokawa et al., 2000). In coffee, the differences in morphology and geographical origin of the genotypes were reflected in the RAPD patterns. Maab and Klaas (1995) used RAPD to analyze intra specific differentiation of garlic (*Allium sativum* L.) and were able to group the accessions and to indicate the genetic distance between them. Wang and Goldman (1999), using this method, evaluated the genetic distance and diversity among 37 accessions of *Beta vulgaris*.

ISSR markers have been used with success to identify and determine relationships at the species population and cultivar levels in many plant species, including several aromatic and medicinal plants (Farajpour et al., 2011). In this study, we have used the ISSR markers as tools, in survey of the genomes of five *Orthosiphon* accessions obtained from different parts of Kerala and Tamilnadu. In total, 72.7% of the bands generated using ISSR were polymorphic, which provided evidence of a large genetic diversity among the tested accessions. The observations of the monomorphic band profiles among accessions reflect the maintenance of allele composition. Results summarized in the dendrogram generated through ISSR analysis define genomic relationships among the analyzed accessions within species.

ISSR markers have been preferred to RAPD markers for genotyping and assessment of genetic diversity mainly due to their high reproducibility (Huang and Sun, 2000). They are also used for determining the genetic relationships as they amplify large number of DNA fragments per reaction, representing multiple loci across the genome (Kaushik et al., 2003). Our results corroborated with this view as was evidenced by the higher number of polymorphic and discriminant fragments detected by ISSRs than that by RAPDs. This difference is mainly due to the DNA fragments

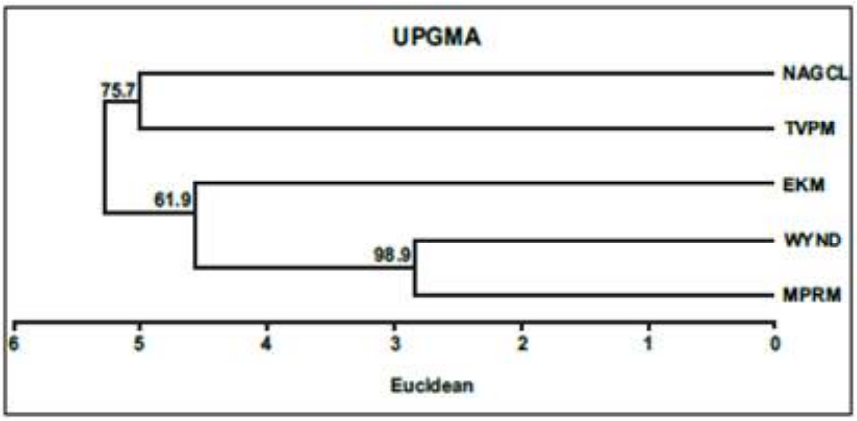

Fig 4. Cluster diagram based on genetic distance calculated from 17 ISSR primers of 5 *Orthosiphon* accessions. The values on the dendrogram give the stability of nodes estimated with the boot strap procedure.

targeted by the two methods. The observed difference between ISSR and RAPD might be explained in terms of functional constraints since some RAPD bands may be associated with functionally important loci. In fact the ISSR's have a high capacity to reveal polymorphism and offer great potential to determine intra and inter- genomic diversity as compared to other arbitrary primers like RAPD's. This explains why a higher level of polymorphism associated with ISSR based marker, as observed in the present study, was evident in many other studies related to a variety of plant species (Zhou et al., 2003). Similarly, different accessions of *Cannabis sativa* (Kojoma et al., 2002) have been discriminated using ISSR markers and those of *Arabidopsis thaliana* (Barth et al., 2002) have been differentiated using cleaved amplified polymorphic sequence and ISSR markers. Varying levels of correspondence between molecular and morphological data have been reported in scientific literature (Perrie and Brownsey 2005). In general, the use of both molecular and morphological markers is recommended because each data set provides complementary information with greater power of resolution in genetic diversity analysis. The use of both morphological and molecular markers classify genotypes better than employing only continuous phenotypic variables or only discreet phenotypic variables when assessing genetic diversity and phylogenetic relationships. Both molecular and morphological markers are also valuable for the identification of distinct populations or genotypes for conservation, optimum sites for germplasm collection, and ongoing changes in the pattern of diversity over time. Additionally, morphological and molecular markers are useful for the evaluation and utilization of genetic resources, the study of diversity of pre-breeding and breeding germplasm, and for the protection of the breeder's intellectual property rights (Franco et al., 2001).

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Phytochemical Screening of *Cnidoscolous chayamansa* Mc Vaugh, Euphorbiaceae Leaf Extract using various solvent

D. DEVIPRIYA* AND B.L. PADMASREE

Department of Botany, SN College for Women Kollam
**Email: jp.devi.jp@gmail.com*

ABSTRACT

The tree spinach (*Cnidoscolous chayamansa* Mc Vaugh, Euphorbiaceae) is cultivated in parts of its native range, especially Mexico, Panama and Guatemala, for its edible leaves and medicinal virtues.The plant contains a white latex, wild forms are usually armed with stinging hairs, but cultivated forms are unarmed. The young leaves and shoots, detoxified by cooking, are eaten as a vegetable, rich in protein, calcium, iron, carotene, riboflavin, niacin and ascorbic acid. They can be eaten alone or in combination with other vegetables in stews and soups. Present study concentrated to identify the best solvent for the extraction of phytochemicals from the plant.

Key words *Cnidoscolous chayamansa, Phytochemical analysis, Extraction*

The systematic screening of plant species with the purpose of discovering new bioactive compounds is a routine activity in many laboratories. The research on the medicinal plants should be extended with the identification of the active principles in the plants. Scientific examination of the remedies could lead to standardization and quality control of the products to ensure their safety. It is after such evaluation that they can be approved for use in the primary health care. Exploration of the chemical constituents of the plants and pharmacological screening will thus provide us the basis for developing new life-saving drugs. Phytochemistry is the branch of chemistry that deals with the enormous variety of compounds found in plants. Medicinal plants have been identified and used throughout human history. Asia represents one of the most important centers of knowledge with regard to the use of plant species for treatment of various diseases. Kunwar states, it has been estimated that the Himalayan region harbors over 10,000 species of medicinal and aromatic plants, supporting the livelihoods of about 600 million people living in the area. Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions. In 2001, researchers identified 122 compounds used in modern medicine which were derived from "ethnomedical" plant sources. People switched over to synthetic items later considering the benefits of cleanliness, purity, durability, convenience and economy factors. We therefore need to develop new set of uses of herbal products to fit the present tastes, habits and the life style. Today a number of variants of herbal products in solid, liquid and paste forms are needed for various market segments and use of the products.

Tree spinach is (*Cnidoscolus chayamansa)* otherwise known as Cabbage Star, or Chaya, a monoecious, much branched, shrub or small tree growing up to 3-5 metres tall (Breckon, 1979). The plant contains white latex, wild forms are usually armed with stinging hairs, but cultivated forms are unarmed. The plant is cultivated in parts of its native range, especially Mexico, Panama and Guatemala, for its edible leaves and medicinal virtues. The young leaves and shoots, detoxified by cooking, are eaten as a vegetable. A good flavour, they are boiled and used as spinach. Rich in protein, calcium, iron, carotene, riboflavin, niacin and ascorbic acid. The plant is said to have many medicinal benefits, ranging from the ability to strengthen fingernails and darken greying hair. It is also used to cure alcoholism, diabetes, insomnia, skin disorders, venereal diseases, gout, scorpion stings and to improve brain function and memory. Numerous flavonoid compounds have been isolated from the leaves. Most medicinal properties have never been experimentally tested. It is used as treatment for various illnesses (i.e., alcoholism, diabetes, insomnia, gout, scorpion stings, skin disorders, and venereal diseases). Diabetic rabbits, fed increasingly higher quantities of the leaves, showed a significant drop in blood sugar levels. It should be noted however that leaves should be cooked thoroughly to remove its high hydrocyanic acid content.

MATERIALS AND METHODS

Phyrochemical screening was performed for alkaloids, flavonoids, terpenoids, saponins and glycosides. The color intensity or the precipitate formation was used as analytical responses to these tests. The objective of the present study was to investigate the presence of various phytochemicals using acetone, ethanol, water and chloroform extracts of dried leaf of *Cnidoscolous chayamansa*.

Table 1

Chemical constituent		Acetone	Chloroform	Ethanol	Aqueous
Flavanoids	Ferric chloride test	+ +	+ + +	+	+++
	Alkaline reagent test	++	+ + +	+	+++
	Lead acetate solution test	+ +	+ + +	+	+++
Terpenoids		+ +	+ + +	+	-
	Bromine water Test	+ +	+ + +	+	-
Alkaloids	Hager's test	+ +	+ + +	+	-
Glycosides	Keller Killiani test	+ +	+ + +	+	+
Saponins		-	-	-	-

(-) Negative test (absence of turbidity, flocculation and precipitation)

(+) Weak positive test

(++) Positive test (Reactive product and not a turbidity flocculation)

(+++) Test strongly positive (If the reagent produce a precipitate or flocculation heavy)

Fresh leaf were collected from the garden of Department of Botany, Sree Narayana College for Women Kollam. The leaf was dried at room temperature and powdered using mortar and pestle. The powdered plant was extracted using acetone, ethanol, chloroform and in water.

Preperation of plant extract: 10gm of powdered sample were weighed separately and dipped into each of the four extraction solution and extracted using Sochlet extraction for 24 hour and is filtered which were used for preliminary phytochemical screening.

Various phytochemical tests were carried out using all the solvent extract using standard methods. Following standard procedures were used for detecting the flavonoids, terpenoids, Alkalids and Glycosides.

Test for Flavonoids:

a. Ferric chloride test- Test solution when treated with few drops of ferric chloride solution would result in the formation of blakish red colour indicate the presence of flavonoids.

b. Alkaline reagent test- Test solution when treated with sodium hydroxide solution , shows increase in the intensity of yellow colour which would become colourless on addition of few drops of dil. Hydrochloric acid, indicate the presence of flavonoids.

c. Lead acetate solution test- Test solution when treated with few drops of lead acetate (10%) solution would result in the formation of yellow precipitate.

Test for Terpenoids: To 2ml of the extract add 2ml of chloroform and 3ml of conc. sulphuric acid along the sides of the test tube , a reddish brown colouration at the interface.

Test for glycosides: Bromine water Test: Test solution was dissolved in bromine water and observed for the formation of the yellow precipitate to show a positive result for the presence of glycosides.

Test for Alkaloids: Hagers test- Test solution was treated with few drops of Hagers reagent(Saturated picric acid solution). Formation of yellow precipitate would show a positive result for the presence of Alkaloids.

Test for Glycosides: Keller Killiani test- Test solution was treated with few drops of glacial acetic acid and Ferric chloride solution and mixed. Concentrated sulphuric acid was added, and observed for the formation of two layers. Lower reddish brown layer and upper acetic acid layer which turn bluish green would indicate a positive test for glycosides.

Test for Saponins: 5ml of each extract was shaken vigorously with 5ml of distilledwater in a test tube and warmed. The formation of stable foam was taken as an indication for the presence of saponin.

RESULTS AND DISCUSSIONS

The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites such as alkaloids, flavanoids, glycosides, phenols, saponins etc. The result obtained from qualitative analysis of plant extract shows the presence of these active constituents except saponins.are

presented in table 1. The phytochemical analysis carried out on the dry leaf show better result in chloroform extract than ethanol, acetone and aqueous solution.

Presence or absence of certain important compounds in an extract is determined by colour reactions of the compounds with specific chemicals which act as dyes.This procedure is a simple pre-requisite before going for detailed phytochemical investigation. Various tests have been conducted to find out qualitatively the presence or absence of bioactive compounds. Now a day standardization of herbal drugs is a topic of great concern. They are subjected to variability as derived from heterogenous sources. Among the various extract, chloroform extract show better result when compared with ethanol and acetone extract of *Cnidoscolous chayamansa*. Water extract shows good result for only flavonoides.

Plants are healthy and natural resource of life. In particular, medicinal plants are of great importance with endless therapeutic properties useful for curing various diseases with an advantage of being natural . At present, there are un-countable products in market,with adverse side effects on once heath. Terpenoids are small molecular products synthesized by plants and are probably the most widespread group of natural products. Terpenoids show significant pharmacological activities, such as antiviral, antibacterial, antimalarial, anti-inflammatory, inhibition of cholesterol synthesis and anti-cancer activities . (Mahato and Sen, 1997). Some flavonoids have inhibitory activity against organisms that cause plant diseases, e.g. *Fusarium oxysporum*. Sandhya *et al.,* 2006. Alkaloids can also be used to treat psychiatric and palpitation It has been reported that total alkaloids extracted from extract of WS roots, caused relaxant and antispasmodic effects against various agents that produce smooth muscle contractions in intestinal, uterine, tracheal, and vascular muscles (Lakshmi *et al.,* 2000). Glycosides can suppress and soothe irritant dry coughs. They have a helpful sedative and relaxant effect on the heart and muscles when taken in small doses. They are significantly diuretic. (Veena, *et al.,* 2011)

Not all secondary metabolites or natural products such as penicillin, morphine, and paclitaxel (Taxol) can be fully synthesized due to their very complex structures that are too difficult and expensive on industrial scale. Hence, there is an urgent need to search for alternative remedies as naturally occurring biologically active secondary metabolites from plant origin. Considering the deleterious effects of synthetic antibiotics, isolation, purification and characterization of novel types of plant secondary metabolites could be a safer alternative to synthetic compounds (Bajpai and Kang, 2011).

CONCLUSION

Certain phytochemicals act in many ways on various types of disease complex, and may potentially contribute in the field of pharmacology as natural supplements to control various infectious diseases in future as the fast and reliable alternatives. Many non-natural, and synthetic drugs cause severe side effects that were not acceptable except as treatments of last resort for terminal diseases such as cancer. The metabolites discovered in medicinal plants may avoid the side effect of synthetic drugs, because they must accumulate within living cells.

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Avifauna of Kandachira Wetlands of Ashtamudi Lake, Kerala, India

B. HARI[1*], S. JISHA[2] AND B. SREEJITH[3]

[1 & 2] P.G. Department of Zoology, S.N. College, Kollam, Kerala, Pincode- 691001
[3] Kallethu Veedu, Neeravil, Perinad P.O., Kollam, Kerala, Pincode- 691601
**email : hariprashobh@gmail.com*

ABSTRACT

Kandachira wetlands are associated with the southernmost branch of the Ashtamudi backwater ecosystem, Kollam district, Kerala State. Avifauna of Kandachira wetland was studied during January to December 2016. The study described the species composition, abundance and resident status of birds. Fortnightly observations were made using binocular and zooming digital cameras. A total of 81 species of birds belonging to 15 orders and 40 families were reported from the study area during the period. Family Ardeidae (11 species) showed maximum species diversity followed by Scolopacidae (8 species). Little Egret (12%), Indian Pond Heron (10.3%), Little Cormorant (8%), Great Egret (7.8%), Indian Cormorant (6%) were the most abundant resident species present in the study area.70% of the bird species recorded belongs to resident and 30% to migrant category. Highest species numbers were recorded in the month of November (41) and lowest on July (18). Highest numbers of birds were recorded in the month of March (530) and lowest was recorded on the month of June (121). *Anhinga melanogaster* (Oriental Darter) *Threskiornis melanocephalus* (Black Headed Ibis), *Mycteria leucocephala* (Painted Stork) *Limosa limosa* and (Black-tailed Godwit), were spotted from the study area which are listed as near threatened (NT) and Ciconia episcopus (Woolly-necked Stork) as vulnerable (VR) birds in the 'IUCN Red List of Threatened Species'. The major threats identified for the birds are anthropogenic activities such as reclamation of the wetland area for construction, aquaculture activities, fishing and solid waste disposal. The importance of this wetland as one of the habitat for the migratory shorebirds along the Kerala coast is also discussed.

Key words *Birds; Avifauna; Kandachira Wetlands; Ashtamudi Lake; Conservation; Near Threatened.*

Wetlands are areas of marsh, ponds swamps with water that is static or flowing. Wetlands are complex and productive ecosystems that occupy six percent of the earth's land surface (Maltby and Turner, 1983; Maltby 1986; Unni, 2002). Wetlands are extremely important areas throughout the world for wildlife protection, recreation, sediment control, flood protection, education and scientific research. Wetland supports rich biodiversity and complicated food chain and provide unique habitat for a wide range of flora and fauna (Mitsch and Gosselink, 2000). The wetland of India is important for the presence of large number of migratory birds (Sandilyan et al., 2010). Wetland associated areas are important foraging places for many species of migratory birds during winter season mainly because these wetlands are located in the Central Asian Flyway. Wetlands are important habitat for birds which uses them for feeding, roosting, nesting and raring young ones (Weller, 1999; Stewart, 2001). Ali and Ripley (1983) reported that 273 species of birds in India can be considered as waterfowls, those depend on wetland ecosystem. These birds use wetland habitat either entire or during certain part of their life. The use of wetland by birds during the breeding cycle range widely; with some depend almost totally on wetlands for breeding, feeding or shelter during the breeding cycles. However, the life of birds in the wetland can be influenced by various factors such as food availability, total area of wetland and abiotic factors in the wetland (Paracuellous, 2006). In a wetland ecosystem, biotic factors are mostly depended upon the season and hydrography. Furthermore, anthropogenic activities resulted in serious loss or degradation of wetland habitat endangering many species of birds and their potential invertebrate pays (Wada et al., 1996; Wetland International, 1996). About 66% if the population of known water birds in Asia has been experiencing drastic decline, mainly due to human induced factors (Wetland International, 2010).

The wetlands and other water bodies of Kerala have been explored by many researchers and birdwatchers for the avifauna, (Neelakantan, 1969, 1970, 1981, 1982; Neelakantan et al., 1980; 1993; Neelakantan and Sureshkumar, 1980). Avifauna of major wetland ecosystems of Kerala was studied and the importance of wetlands in the sustenance of these avifauna and their conservational aspects were discussed in several works (Kurup, 1991; Jayson and

Table 1. Checklist of Avifauna recorded from the Kandachira wetlands of Ashtamudi Lake Kerala, India with their abundance, residential, IUCN category and breeding status

Sl. No/ Order	Common Name	Scientific Name	Family	Abundance	IUCN category	Resident Status	Breeding Status
Order	**Accipitriformes**						
1	Kite, Black	*Milvus migrans*	Accipitridae	C	LC	R	
2	Kite, Brahminy	*Haliastur indus*	Accipitridae	C	LC	R	B
Order	**Anseriformes**						
3	Duck, Lesser Whistling	*Dendrocygna javanica*	Anatidae	C	LC	R	
Order	**Caprimulgiformes**						
4	Swift, Asian Palm	*Cypsiurus balasiensis*	Apodidae	U	LC	R	
Order	**Charadriformes**						
5	Godwit, Black-tailed	*Limosa limosa*	Scolopacidae	U	**NT**	M	
6	Greenshank, Common	*Tringa nebularia*	Scolopacidae	C	LC	M	
7	Redshank, Common	*Tringa totanus*	Scolopacidae	C	LC	M	
8	Ruff	*Calidris pugnax*	Scolopacidae	R	LC	M	
9	Sandpiper, Common	*Actitis hypoleucos*	Scolopacidae	C	LC	M	
10	Sandpiper, Marsh	*Tringa stagnatilis*	Scolopacidae	U	LC	M	
11	Sandpiper, Wood	*Tringa glareola*	Scolopacidae	C	LC	M	
12	Snipe, Common	*Gallinago gallinago*	Scolopacidae	R	LC	M	
13	Lapwing, Red-wattled	*Vanellus indicus*	Charadriidae	C	LC	R	
14	Plover, Pacific Golden	*Pluvialis fulva*	Charadriidae	C	LC	M	
15	Stilt, Black-winged	*Himantopus himantopus*	Recurvirostridae	C	LC	R	
16	Jacana, Pheasant-tailed	*Hydrophasianus chirurgus*	Jacanidae	R	LC	R	
17	Snipe, Greater Painted	*Rostratula benghalensis*	Rostratulidae	R	LC	R	
18	Tern, Whiskered	*Chlidonias hybrida*	Laridae	C	LC	M	
Order	**Ciconiformes**						
19	Stork, Painted	*Mycteria leucocephala*	Ciconiidae	R	**NT**	M	
20	Openbill, Asian	*Anastomus oscitans*	Ciconiidae	C	LC	M	
21	Stork, Woolly-necked	*Ciconia episcopus*	Ciconiidae	U	VR	R	
Order	**Columbiformes**						
22	Pigeon, Rock (Feral Pigeon)	*Columba livia*	Columbidae	U	LC	R	B
23	Pigeon, Gray-fronted Green	*Treron affinis*	Columbidae	R	LC	R	
Order	**Coraciiformes**						
24	Bee-eater, Blue-tailed	*Merops philippinus*	Meropidae	U	LC	M	
25	Green bee-eater	*Merops orientalis*	Meropidae	U	LC	R	
26	Kingfisher, Common	*Alcedo atthis*	Alcedinidae	C	LC	R	
27	Kingfisher, Pied	*Ceryle rudis*	Alcedinidae	R	LC	R	
28	Kingfisher, Stork-billed	*Pelargopsis capensis*	Alcedinidae	C	LC	R	

Sl. No/ Order	Common Name	Scientific Name	Family	Abundance	IUCN category	Resident Status	Breeding Status
29	Kingfisher, White-throated	*Halcyon smyrnensis*	Alcedinidae	C	LC	R	
30	Roller, Indian	*Coracias benghalensis*	Coraciidae	R	LC	R	
Order	**Cuculiformes**						
31	Coucal, Southern	*Centropus parroti*	Cuculidae	U	LC	R	B
32	Cuckoo, Jacobian	*Clamator jacobinus*	Cuculidae	R	LC	R	
Order	**Gruiformes**						
33	Crake, Ruddy-breasted	*Zapornia fusca*	Rallidae	R	LC	R	
34	Waterhen, White-breasted	*Amaurornis phoenicurus*	Rallidae	C	LC	R	B
Order	**Passeriformes**						
35	Shrike, Brown	*Lanius cristatus*	Laniidae	R	LC	M	
36	Swallow, Barn	*Hirundo rustica*	Hirundinidae	R	LC	M	
37	Wagtail, Forest	*Dendronanthus indicus*	Motacillidae	R	LC	M	
38	Babbler, Jungle	*Turdoides striata*	Leiotrichidae	R	LC	R	
39	Bulbul, Red-whiskered	*Pycnonotus jocosus*	Pycnonotidae	U	LC	R	
40	Crow, House	*Corvus splendens*	Corvidae	C	LC	R	B
41	Crow, Large-billed	*Corvus macrorhynchos*	Corvidae	U	LC	R	
42	Treepie, Rufous	*Dendrocitta vagabunda*	Corvidae	C	LC	R	B
43	Drongo, Black	*Dicrurus macrocercus*	Dicruridae	C	LC	R	
44	Flowerpecker, Pale-billed	*Dicaeum erythrorhynchos*	Dicaeidae	R	LC	R	
45	Munia, Tricolored	*Lonchura malacca*	Estrildidae	U	LC	R	B
46	Starling, Rosy	*Pastor roseus*	Sturnidae	R	LC	M	
47	Myna, Common	*Acridotheres tristis*	Sturnidae	C	LC	R	B
48	Myna, Jungle	*Acridotheres fuscus*	Sturnidae	U	LC	R	
49	Oriole, Indian Golden	*Oriolus kundoo*	Oriolidae	U	LC	M	
50	Oriole, Black-hooded	*Oriolus xanthornus*	Oriolidae	U	LC	R	
51	Prinia, Ashy	*Prinia socialis*	Cisticolidae	U	LC	R	B
52	Prinia, Plain	*Prinia inornata*	Cisticolidae	R	LC	R	
53	Tailorbird, Common	*Orthotomus sutorius*	Cisticolidae	U	LC	R	B
54	Bushchat, Pied	*Saxicola caprata*	Muscicapidae	R	LC	R	
55	Robin, Oriental Magpie	*Copsychus saularis*	Muscicapidae	U	LC	R	
56	Sunbird, Long-billed	*Cinnyris lotenius*	Nectariniidae	U	LC	R	B
57	Sunbird, Purple	*Cinnyris asiaticus*	Nectariniidae	R	LC	R	
58	Sunbird, Purple-rumped	*Leptocoma zeylonica*	Nectariniidae	U	LC	R	
59	Warbler, Blyth's Reed	*Acrocephalus dumetorum*	Acrocephalidae	U	LC	M	
60	Warbler, Clamorous Reed	*Acrocephalus stentoreus*	Acrocephalidae	R	LC	R	

Sl. No/ Order	Common Name	Scientific Name	Family	Abundance	IUCN category	Resident Status	Breeding Status
Order	**Pelecaniformes**						
61	Egret, Little	*Egretta garzetta*	Ardeidae	C	LC	M	
62	Heron, Gray	*Ardea cinerea*	Ardeidae	C	LC	M	
63	Heron, Western Reef	*Egretta gularis*	Ardeidae	R	LC	M	
64	Bittern, Black	*Ixobrychus flavicollis*	Ardeidae	U	LC	R	
65	Bittern, Cinnamon	*Ixobrychus cinnamomeus*	Ardeidae	U	LC	R	
66	Bittern, Yellow	*Ixobrychus sinensis*	Ardeidae	R	LC	R	
67	Egret, Cattle	*Bubulcus ibis*	Ardeidae	C	LC	R	
68	Egret, Great	*Ardea alba*	Ardeidae	C	LC	R	
69	Egret, Intermediate	*Ardea intermedia*	Ardeidae	U	LC	R	
70	Heron, Indian Pond	*Ardeola grayii*	Ardeidae	C	LC	R	
71	Heron, Purple	*Ardea purpurea*	Ardeidae	C	LC	R	
72	Ibis, Glossy	*Plegadis falcinellus*	Threskiornithidae	U	LC	M	
73	Spoonbill, Eurasian	*Platalea leucorodia*	Threskiornithidae	R	LC	M	
74	Ibis, Black-headed	*Threskiornis melanocephalus*	Threskiornithidae	C	**NT**	R	
Order	**Piciformes**						
75	Barbet, White-cheeked	*Psilopogon viridis*	Megalaimidae	U	LC	R	
76	Woodpecker, black-rumped flameback	*Dinopium benghalense*	Picidae	R	LC	R	
Order	**Podicipediformes**						
77	Grebe, Little	*Tachybaptus ruficollis*	Podicipedidae	R	LC	R	
Order	**Psittaciformes**						
78	Parakeet, Rose-ringed	*Psittacula krameri*	Psittacidae	U	LC	R	B
Order	**Suliformes**						
79	Cormorant, Indian	*Phalacrocorax fuscicollis*	Phalacrocoracidae	C	LC	R	
80	Cormorant, Little	*Microcarbo niger*	Phalacrocoracidae	C	LC	R	
81	Darter, Oriental	*Anhinga melanogaster*	Anhingidae	U	**NT**	R	
C- Common; U-Uncommon;R-Rare; LC- Least Concern; VR- Vulnerable; NT- Near Threatened; R-Resident; M-Migrant;B- Breeding in the study area							

Sivaperuman, 1999; Sivaperuman and Jayson, 2000, 2012; Vijayakumar, 2005; Kumar, 2006; Narayanan et al., 2011; Aarif and Basheer, 2012; Aarif et al., 2015). However, no concerted efforts were made to study the avifauna associated with the wetlands of the Ashtamudi Lake which is the second largest Ramsar sites in Kerala, except regular Asian water fowl census. The present study describes the annual species composition, abundance and resident status of avifauna of Kandachira wetland which is the eastern part of the Southern branch of Ashtamudi Lake. It also allows a first evaluation of the importance of this natural habitat as a stopover for migratory birds and the threats faced by them.

MATERIAL AND METHODS

Ashtamudi Wetland (Ashtamudi Kayal, area - 61.4 km^2), Ramsar site No. 1204, is near Kollam City ($8^0$45'-$9^0$28'N and $76^0$28'-$77^0$17'E) in Kerala and falls in Kollam City Corporation and adjoining Grama Panchayats. This extensive estuarine system, the second largest and deepest in Kerala, is connected to sea and is of extraordinary importance for its hydrological functions and biodiversity. The study site, Kandachira wetland (08°56'1.7376"N and 76°3646.332" E) is one among the important wetlands of Ashtamudi Lake with an area of 1.5 km^2. It is located in the eastern part of the southernmost branch of Ashtamudi Lake. This wetland is approximately 10km away from the Kollam city. Many migrants are choosing this location as a wintering area during post monsoon season. This water logged area was connected to the Ashtamudi backwaters through two regulators. The major portions of the wetland are flat areas that remain submerged throughout the year and with muddy banks and bottom. Many paddy fields are also associated with this wetland ecosystem.

Biweekly inspections to the study area were made for a period of one year (January to December 2016). Binoculars (Nikon Aculon A211; 8x 42) and Cameras (Nikon Coolpix P600; Nikon P900 and Nikon DSLR D5300 with AF-S NIKKOR 70-300 mm lens) were used for bird watching and image capture. Censuses were carried out in the morning (06.30 am to 10.00 am) throughout the study period. The method of total count was employed to census the bird population. In this method, representative blocks were identified and the birds in these blocks were counted using binoculars. Birds were identified using physical feature with the help of field guides and standard referenced books (Ali and Ripley 1987; Neelakantan 1996; Grimmet et al. 2011). Birds were identified up to species level and details like number of birds in the habitat were also recorded. Abundant status of the birds are categorized in to common (C) - seen on most of the visits, uncommon (U) - seen on a few visits, and rare (R) - seen once or twice. Residential status of bird species was classified into resident (R) and Migrant (M) mainly as per Kazmierczak (2000) and Grimmet et al. (2011). Winter visitors from Central Asian countries are included in migrants. Some birds which are breeding in different habitat of Kerala but seen in Kandachira are also included in resident category.

RESULTS AND DISCUSSION

The list of avifauna spotted from the Kandachira wetland ecosystem during January to December 2016 is presented in Table 1. A total of 81 spices of birds belong to 15 orders and 40 families were reported from Kandachira wetland. Narayanan et al. (2011) listed out 225 species of birds belonging to 15 orders and 59 families from Kuttanad wetland, southern portion of Vembanad Kole Ramsar site of India. Similarly, Aarif and Bahseer (2012) reported a total of 57 species of birds belong to 16 families from Mavoor wetland of Kozhikode District, Kerala. Kumar (2006) recorded a total of 140 species of birds in 49 families from Bharathapuzha River Basin. A total of 167 species of birds belonging to 16 orders and 39 families were recorded from the Kole wetland of Thrissur District of Kerala by Sivaperuman and Jayson (2000).

Kandachira wetland is found to be a feeding and breeding area for many passerine birds also. The order Passeriformes possess the most diversified 15 families and 26 species (Fig. 1) followed by order Charadriformes with 6 families and 14 species. Order Pelecaniformes possess 2 families and 14 species. Maximum number of species was recorded from families Ardeidae (11 species) followed by Scolopacidae (8 species) (Table 1). Only 54% of the birds were belonged to the wetland category. Though a wetland dominated area, 46% of the birds belong to non-wetland category. This may be due to the edge

effect as the three boundaries are land and only south-eastern boundary is backwaters. Similar observations were made by Narayanan et al. (2011) in Kuttanad area where 52.2% birds belong to non-wetland categories and most of the birds were seen at the eastern boundaries of Kuttnadu where Kuttanad wetlands meet midland areas of Kerala.

Among the birds recorded 30% to the migrant and 70% belong to the resident category. Wetland depended birds formed major portion of these migrants. Aarif and Basheer (2012) reported 17 migrant species from Mavoor wetland. Narayanan et al. (2011) reported 86 migrant species from Kuttanad wetland area. Kannan and Pandiyan (2012) reported 24 migratory species among the shorebirds of Pulicat Lake, India. 49 migratory birds were reported by Kumar (2006) from the Bharathapuzha River basin during 1998-2004.

Altogether a total of 6,543 of birds were recorded from Kandachira during the study period. Of the total population, numerically abundant birds are Little Egret, *Egretta garzetta* (12%); Indian Pond Heron *Ardeola grayii* (10.3%); Little Cormorant *Microcarbo niger* (8%); Great Egret *Ardea alba* (7.8%) and Indian Cormorant *Phalacrocorax fuscicollis* (6%). Similar records were made by Sivaperuman and Jayson (2000) and they have reported that Little Egret, Cattle egret, Little Cormorant, Pond Heron, Intermediate Egret, and Whiskered Tern were the dominating bird species in the Kuttanadu wetland areas. Aarif and Basheer (2012) reported that Little Egret, Cattle Egret, Little Cormorant, Pond Heron, Purple Moorhen, Purple Heron, Night-Heron Little Grebe, Lesser Whistling Duck, River Tern and Whiskered Tern as abundant birds in the Mavoor wetlands.

The peak bird population in the Kandachira wetland was observed during period of November to March and the lowest in June to July. Highest number of birds were recorded during the month of March (530) and lowest in June (121). The shore bird population increased during December to March which may be due to progressive lowering water level which enables the birds to feed on the available food resources. Both number and species diversity drastically decreased in June-July months and it might be due to the effect of South-west monsoon rains and low food resources due to flooding (Kannan and Pandiyan, 2012). Highest number of species was recorded on the month of November (41) and lowest on July (18). The relative abundance of birds during the winter season might be related to the availability of food, habitat conditions of the species. The distinct seasonality of rainfall and seasonal variation in the abundance of food resources may result in seasonal changes in the species abundance of birds (Gaston et al., 2000; Karr and Roth 1971).

Breeding of 12 species were recorded in these wetlands. Among them, White Breasted Waterhen, Ashy Prinia and Black Headed Munia were identified as frequent breeders in this wetland during the study period. Breeding of 22 species of birds were reported by Aarif and Basheer (2012) in Mavoor wetlands. Narayanan et al. (2011) reported that 55 species were breeding in the Kuttanad wetlands. Relatively low numbers of breeding species in the present study site may be due to low area and high anthropogenic disturbances. No heronries were sighted in this wetland area during the study period.

Five globally threatened species such as *Anhinga melanogaster* (Oriental Darter) *Threskiornis melanocephalus* (Black Headed Ibis), *Mycteria leucocephala* (Painted Stork) *Limosa limosa* (Black-tailed Godwit), were spotted from the study area which are listed as near threaded (NT) and Ciconia episcopus (Woolly-necked Stork) as vulnerable (VR) category (IUCN, 2017). Narayanan et al. (2011) recorded 10 near threatened species from the Kuttanad wetlands and Kumar (2006) reported four near threatened (NT) bird species from the Bharathapuzha River basin.

Among the 81 bird species reported from this study area, 24 of them are migrants. Furthermore, this wetland is coming under the Central Asian – Indian Flyway, thus protection to winter visitors need high priority. During the study many anthropogenic factors which threat the Kandachira wetland ecosystems and the bird population were identified. Among them the prominent was habitat destruction due to land reclamation for building houses and roads. Felling of the trees and clearing the natural vegetation adjacent to the wetland area and conversion of wetland area for aquaculture and other activities causes habitat reduction for these birds. Alterations in the land use

pattern have created the major threat to the bird population. Menace of solid waste disposal to the waterlogged areas chocked the natural tidal flow of water and also the plastic waste materials seems to be spread over the benthic habitat and may hinder the feeding activity of many of the waders. Fishing with the small sized gill nets and dip nets has reducing even the small sized fishes and it may affect the food availability to birds. Hand picking of even under-sized bivalves may deplete their stock and reduces the food availability of birds like Asian Open Bill Storks. Thus uncontrolled and unethical fishing practices deplete the food sources of wetlands. However, there is a ray of hope for the migratory birds as the local people were aware of the presence of winter visitors and they won't allow anybody to pouch the birds. There were no reports on poaching of the birds by shooting, poisoning or using of bird traps. No signs of dead birds were located in the study area during the period.

Strict implementation of land use policies in this part of the wetland is necessitated. Awareness programmes on the consequences of solid waste disposal in these wetland area for local people need to be conducted by local government bodies in association with various non-governmental organisations. Long term monitoring of avifauna of this wetland is an important component of effective conservation-oriented research and management. Additional information on important wintering visitors from this wetland is still needed. Such data will enhance our ability to make conservation strategies and policies with regard to the Central Asia Flyway with valuable information on the wintering shorebird conservation and protection.

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An Investigation on the Epidendric Periphyton of Two Mangrove Ecosystems, Asramam and Kandachira of Ashtamudi Lake, Kollam (Dist.), Kerala, India

S. JISHA*, B. HARI AND A. NEETHU

P.G. and Research Department of Zoology,
Sree Narayana College, Kollam, Pincode - 691001, Kerala, India
**email : jishasooriya@gmail.com*

ABSTRACT

The diversity and species composition of epidendric periphyton (periphyton attached to submerged tree limbs and roots, or on other wood surfaces) of areal roots of *Bruguiera cylindrica* from two mangrove ecosystems of Ashtamudi backwaters, Kollam District, Kerala were studied through fortnight samplings for a period of three months. Qualitative as well as quantitative estimation of the abundance of epidendric periphyton community from both Kandachira (Station 1) and Asramam (Station 2) were carried out. Systematic positions of periphyton community were found out. Sampling from Kandachira revealed that Class Bacillariophyceae (62.22%) dominated among 45 species collected and identified. From Asramam, 36 species were identified, of which 58.33% comprised of Class Bacillariophyceae. Other classes collected and identified from Ashtamudi Lake were Coscinodiscophyceae, Zygnematophyceae, Euglenophyceae, Cyanophyceae and Chlorophyceae. Atmospheric temperature, water quality parameters such as temperature, pH, salinity, turbidity, dissolved oxygen (DO), biological oxygen demand (BOD), acidity, and total alkalinity were analysed on a biweekly basis during the period of study. Soil pH and soil moisture content were estimated. Soil texture at both stations were analysed and were dominated by Silt. The results of the comparative analysis showed that Asramam seemed to be more polluted with relatively high BOD levels and represented by a good number of pollution indicator species, especially the presence of Cyanophyceans. The study revealed the pollution status of the ecosystems and the chances of using periphyton as good ecological indicators.

Keywords *Periphyton, epidendric algae, Soil parameters, Hydrological parameters, Kerala, India*

The term Periphyton encompasses a wide variety of algae, bacteria, micro-invertebrates found intimately associated with submerged substrata (Stevenson, 1998). Periphytic biofilms are an integrated micro-system composed of biotic components which are seen in aquatic ecosystems. They composed of algae, fungi, bacteria, protozoa and metazoan and abiotic components like substrata, extracellular polymeric substance, and detritus (Wu, 2017) Periphyton communities are a significant contributor to primary productivity almost comparable to the contributions of phytoplankton in environments that are rich in organic load (Saikia et al., 2013). Periphyton helps in the nutrient turnover and transfers of energy to successive trophic levels and thus maintains the food web (Saikia, 2011). They also contribute the essential oxygen to the polluted water bodies and act as cleansers by removing organic contaminants. Planktonic algae enhances the productivity and they also act as good indicators of water quality (Palmer, 1980; Odum, 1997). Periphyton community range their habitats from almost all available substrata, natural and to some artificial substrata. Among them, roots of mangrove provide an ideal place of colonization and also present a unique array of both micro-floral and faunal groups. Mangroves are the most productive buffer zone that can tolerate wide range of salinity fluctuations; also contribute to nutritive sediments which offers huge primary production which indirectly promotes secondary production (Bardarudeen et al., 1996).

Periphyton communities can reflect the health status and nutrient availability in aquatic ecosystems. Their assessment may help to estimate the extent of pollution. Presence and quantity of some species of diatoms may give accurate information on the stability and degree of pollution of aquatic ecosystems (Kiran et al., 2006). Degree of variations in species composition and community structure of periphyton may be due to the constantly altering ecosystem and it can be regarded as the ecological impact on them. However, magnitude of impact may vary according to the habitat and the available substratum. Diversity studies on periphyton community from mangroves are undergoing around the world. Studies on periphyton

Table 1. Systematic positions of epidendric periphytic algae collected and identified from stations 1 & 2 during the study period

Sl.No.	Periphyton	Class	Order	Family
1	*Achnanthes xigua*	Bacillariophyceae	Achnanthales	Achnanthaceae
2	*Achnanthes* sp.	Bacillariophyceae	Achnanthales	Achnanthaceae
3	*Achnanthidium minutissimum*	Bacillariophyceae	Achnanthales	Achnanthidiaceae
4	*Amphora* sp.	Bacillariophyceae	Thalassiophysales	Catenulaceae
5	*Closterium* sp.	Zygnematophyceae	Desmidiales	Closteriaceae
6	*Cocconeis pediculus*	Bacillariophyceae	Achnanthales	Cocconeidaceae
7	*Cocconeis placentula*	Bacillariophyceae	Achnanthales	Cocconeidaceae
8	*Cocconeis* sp.	Bacillariophyceae	Achnanthales	Cocconeidaceae
9	*Coscinodiscus granii*	Coscinodiscophyceae	Coscinodiscales	Coscinodiscaeceae
10	*Coscinodiscus* sp.	Coscinodiscophyceae	Coscinodiscales	Coscinodiscaecae
11	*Cosmarium* sp.	Zygnematophyceae	Zygnematales	Desmidiaceae
12	*Cyclotella meneghiniana*	Bacillariophyceae	Pennales	Naviculoideae
13	*Cyclotella* sp.	Bacillariophyceae	Pennales	Naviculoideae
14	*Cymbella* sp.	Bacillariophyceae	Cymbellales	Cymbellaeceae
15	*Diploneis* sp.	Bacillariophyceae	Naviculales	Diploneidaceae
16	*Encyonema* sp.	Bacillariophyceae	Cymbellales	Cymbellaceae
17	*Euglena acus*	Euglenophyceae	Euglenales	Euglenaceae
18	*Fragilaria* sp.	Bacillariophyceae	Pennales	Fragilariaceae
19	*Frustulia* sp.	Bacillariophyceae	Naviculales	Amphipleuraceae
20	*Gleocapsa* sp.	Cyanophyceae	Chroococcales	Chroococcaceae
21	*Gomphonema* sp.	Bacillariophyceae	Cymbellales	Gomphonemataceae
22	*Gyrosigma* sp.	Bacillariophyceae	Naviculales	Pleurosigmataceae
23	*Lyngbya* sp.	Cyanophyceae	Nostocales	Oscillatoriaceae
24	*Melosira* sp.	Bacillariophyceae	Melosirales	Melosiraceae
25	*Merismopedia* sp.	Cyanophyceae	Chroococcales	Merismopedioideae
26	*Microspora* sp.	Chlorophyceae	Microsporales	Microsporaceae
27	*Navicula gregaria*	Bacillariophyceae	Naviculales	Naviculaceae
28	*Navicula transitans*	Bacillariophyceae	Naviculales	Naviculaceae
29	*Navicula* sp.	Bacillariophyceae	Naviculales	Naviculaceae
30	*Nitzschia palea*	Bacillariophyceae	Bacillariales	Bacillariaceae
31	*Nitzschia gracilis*	Bacillariophyceae	Bacillariales	Bacillariaceae
32	*Nitzschia obtusa*	Bacillariophyceae	Bacillariales	Bacillariaceae
33	*Nitzschia recta*	Bacillariophyceae	Bacillariales	Bacillariaceae
34	*Nitzschia reversa*	Bacillariophyceae	Bacillariales	Bacillariaceae
35	*Nitzschia sigma*	Bacillariophyceae	Bacillariales	Bacillariaceae
36	*Nitzschia* sp.	Bacillariophyceae	Bacillariales	Bacillariaceae
37	*Nostoc* sp.	Cyanophyceae	Nostocales	Nostocaceae
38	*Oscillatoria sancta*	Cyanophyceae	Oscillatoriales	Oscillatoriaceae
39	*Oscillatoria limosa*	Cyanophyceae	Oscillatoriales	Oscillatoriaceae
40	*Oscillatoria princeps*	Cyanophyceae	Oscillatoriales	Oscillatoriaceae

Sl.No.	Periphyton	Class	Order	Family
41	*Oscillatoria* sp.	Cyanophyceae	Oscillatoriales	Oscillatoriaceae
42	*Oscillatoria tenuis*	Cyanophyceae	Oscillatoriales	Oscillatoriaceae
43	*Phormidium* sp.	Cyanophyceae	Nostocales	Oscillatoriaceae
44	*Pinnularia conica*	Bacillariophyceae	Naviculales	Pinnulariaceae
45	*Pinnularia* sp.	Bacillariophyceae	Naviculales	Pinnulariaceae
46	*Pleurosigma* sp.	Bacillariophyceae	Naviculales	Pleurosigmataceae
47	*Spirogyra* sp.	Zygnematophyceae	Zygnematales	Zygnemataceae
48	*Spirulina major*	Cyanophyceae	Oscillatoriales	Oscillatoriaceae
49	*Surirella* sp.	Bacillariophyceae	Surirellales	Surirellaceae
50	*Synedra* sp.	Bacillariophyceae	Fragilariales	Fragilariales
51	*Zygnema* sp.	Zygnematophyceae	Zygnematales	Zygnemataceae

diversity in the aquatic ecosystems of Kerala especially Ashtamudi backwaters are scanty. In this context, the present study is an attempt to understand the diversity of epidendric periphyton communities in Kandachira and Asramam mangroves and also the water and soil quality parameters.

MATERIALS AND METHODS

Study Sites

An Ashtamudi backwater is known as "Gate way of backwaters" and is the second largest wetland ecosystem in Kerala. It is included in the list of wetlands of international importance. The backwater supports patches of mangrove ecosystems in sheltered regions. Ashtamudi backwaters support a good number of fishes, birds, crustaceans and molluscans. All the eight arms of the Ashtamudi are seriously threatened by various anthropogenic activities. Two stations with patches of mangroves, Kandachira and Asramam, which lie on Ashtamudi backwaters, were selected for the periphyton study. Station 1,Kandachira mangrove ecosystem is the southern part of the Ashtamudi estuary ($8^0$45`-$9^0$28`N and $76^0$28`-$77^0$17`E). Kandachira (8.5617376^{0}N and 76.3646332^0 E) is one of the eight water channels of Ashtamudi is nourished by mangroves. Station 2,Asramam mangroves is situated near the K.S.R.T.C. bus station (8.895888^{0}N and 76.5920927^{0}E). The region is blessed with the Ashtamudi Lake but its water quality is deteriorating day by day. The site provides warning signs of pollution and some regions are very much similar to solid and liquid waste dumping yards.

Methods

Qualitative and quantitative estimation of epidendric periphyton from the mangrove, *Bruguiera cylindrica* were carried out from the samples of both stations. Triplicate samples of periphyton, water and soil were collected fortnightly from both stations for a period of three months and brought to the laboratory for further analysis.

Qualitative as well as quantitative estimation of the abundance of epidendric periphyton community from both Kandachira (Station 1) and Asramam (Station 2) were carried out using standard procedures (Biggs and Kilroy, 1994). The roots of mangroves of length 10cm were sampled out and the periphytic algae were scrapped out and preserved in 4% formalin for further analysis. The samples were qualitatively and quantitatively analysed with the help of a light microscope. Systematic positions of periphyton community were found out and analysed using standard keys and identifying manuals (Biggs and Kilroy, 1994; Newell and Newell, 1988 and Santhanam et al., 1987).

Temperature was measured using a precision mercury thermometer; salinity using hand refractometer; water pH and conductivity were measured by a Multi Parameter Instrument; turbidity using Nephalo turbidity meter; acidity and alkalinity by acid-base titration and dissolved oxygen (DO) and biochemical oxygen demand (BOD) were estimated by Winkler's method. These were estimated using standard guidelines (APHA, 1989). Sediment samples were collected from two stations using a locally

Table 2. Comparison of epidendric periphyton collected and identified from stations 1 & 2 during the study period

Sl. No.	Periphyton	Station 1	Station 2
1	*Achnanthes xigua*	+	+
2	*Achnanthes* sp.	+	+
3	*Achnanthidium minutissimum*	+	-
4	*Amphora* sp.	+	+
5	*Closterium* sp.	+	+
6	*Cocconeis pediculus*	-	+
7	*Cocconeis placentula*	+	+
8	*Cocconeis* sp.	-	+
9	*Coscinodiscus granii*	+	-
10	*Coscinodiscus* sp.	+	+
11	*Cosmarium* sp.	+	-
12	*Cyclotella meneghiniana*	+	-
13	*Cyclotella* sp.	+	+
14	*Cymbella* sp.	+	+
15	*Diploneis* sp.	+	+
16	*Encyonema* sp.	-	+
17	*Euglena acus*	+	+
18	*Fragilaria* sp.	-	+
19	*Frustulia* sp.	+	-
20	*Gleocapsa* sp.	+	+
21	*Gomphonema* sp.	+	-
22	*Gyrosigma* sp.	+	+
23	*Lyngbya* sp.	+	+
24	*Melosira* sp.	+	+
25	*Merismopedia* sp.	+	-
26	*Microspora* sp.	+	+
27	*Navicula gregaria*	+	+
28	*Navicula transitans*	+	-
29	*Navicula* sp.	+	+
30	*Nitzschia palea*	+	+
31	*Nitzschia gracilis*	+	-
32	*Nitzschia obtusa*	+	-
33	*Nitzschia recta*	+	-
34	*Nitzschia reversa*	+	+
35	*Nitzschia sigma*	+	-
36	*Nitzschia* sp.	-	+
37	*Nostoc* sp.	+	-
38	*Oscillatoria sancta*	-	+
39	*Oscillatoria limosa*	-	+
40	*Oscillatoria princeps*	-	+

Sl. No.	Periphyton	Station 1	Station 2
41	*Oscillatoria* sp.	+	+
42	*Oscillatoria tenuis*	+	+
43	*Phormidium* sp.	+	+
44	*Pinnularia conica*	+	+
45	*Pinnularia* sp.	+	+
46	*Pleurosigma* sp.	+	+
47	*Spirogyra* sp.	+	-
48	*Spirulina major*	+	+
49	*Surirella* sp.	-	+
50	*Synedra* sp.	+	+
51	*Zygnema* sp.	+	+
'+' indicates presence and '–' indicates absence			

designed corer and were treated based on the parameters aimed. For soil texture studies, sieve analysis method was followed. Soil pH and soil moisture content were estimated using standard procedure (Verma and Agarwal, 1998). The number of periphytons, air temperature and water parameters collected from two sampling stations were statistically analysed using t'-test (Snedecor and Cochran, 1968).

RESULTS & DISCUSSION

A total of 51 species of epidendric periphytic algae were collected and identified from the mangroves of Ashtamudi backwaters during the study period. Of which 45 species were from Station 1, Kandachira and 36 species from Station 2, Asramam. Based on the identification, a checklist of periphytic algae was prepared. Systematic positions of periphytic algae were found out and displayed in Table 1.

Comparative study was done based on the availability of periphyton from stations 1 & 2 and the data was tabulated (Table 2). They consisted of 6 classes, of which Class Bacillariophyceae dominated in both stations (Kandachira- 62.22% and Asramam- 58.33%). Apart from it, Coscinodiscophyceae (Station 1- 4.4%, Station 2- 5.6%),), Zygnematophyceae (Station 1- 11.1%, Station 2- 5.6%), Euglenophyceae (Station 1-2.2%, Station 2-2.8%), Cyanophyceae (Station 1- 17.8%, Station 2- 25%) and Chlorophyceae (Station 1- 2.2%, Station 2- 2.8%) were recorded.

A quantitative study of epidendric periphyton from both stations showed distinct variations in the number and diversity of species (Fig. 1). 27 species were commonly occurred in both stations. Nasser and Sureshkumar (2014) studied the habitat-wise variation in periphytic-microalgal assemblages in the Vazhachal forest division of Chalakkudy river basin. They reported 97 species of micro algae and stated that the periphytic algal composition can vary according to different habitats. *Navicula* sp. was numerically most abundant species at Kandachira and *Oscillatoria* sp. was most dominant at Asramam. *Pinnularia* sp., *Achnanthes* sp., *Melosira* sp.,and *Cymbella* sp.and. were the other dominant members. *Synedra* sp., *Euglena* sp., and *Diploneis* sp. were numerically very low throughout the samplings in both stations. 2nd, 3rd and 4th samplings showed maximum number of species while 6th sampling showed comparatively less amount of periphyton. Onset of monsoon may have an impact in the reduction of periphyton. Sampling from Kandachira revealed that Class Bacillariophyceae (62.22%) dominated among 45 species collected and identified. From Asramam 36 species were identified and of which, 58.33% comprised of Class Bacillariophyceae. Other classes included were Coscinodiscophyceae (Station 1- 4.44%, Station 2- 5.55%), Zygnematophyceae (Station 1- 11.11%, Station 2- 5.55%), Euglenophyceae (Station 1-2.22%, Station 2-2.77%), Cyanophyceae (Station 1- 17.77%, Station 2- 25%) and Chlorophyceae (Station 1- 2.22%, Station 2- 2.77%).

The result of the analysis of water quality parameters is presented in Table 3. The t- test showed that there exists no significant variations (P>0.05) in air temperature, water temperature, pH, salinity, acidity,

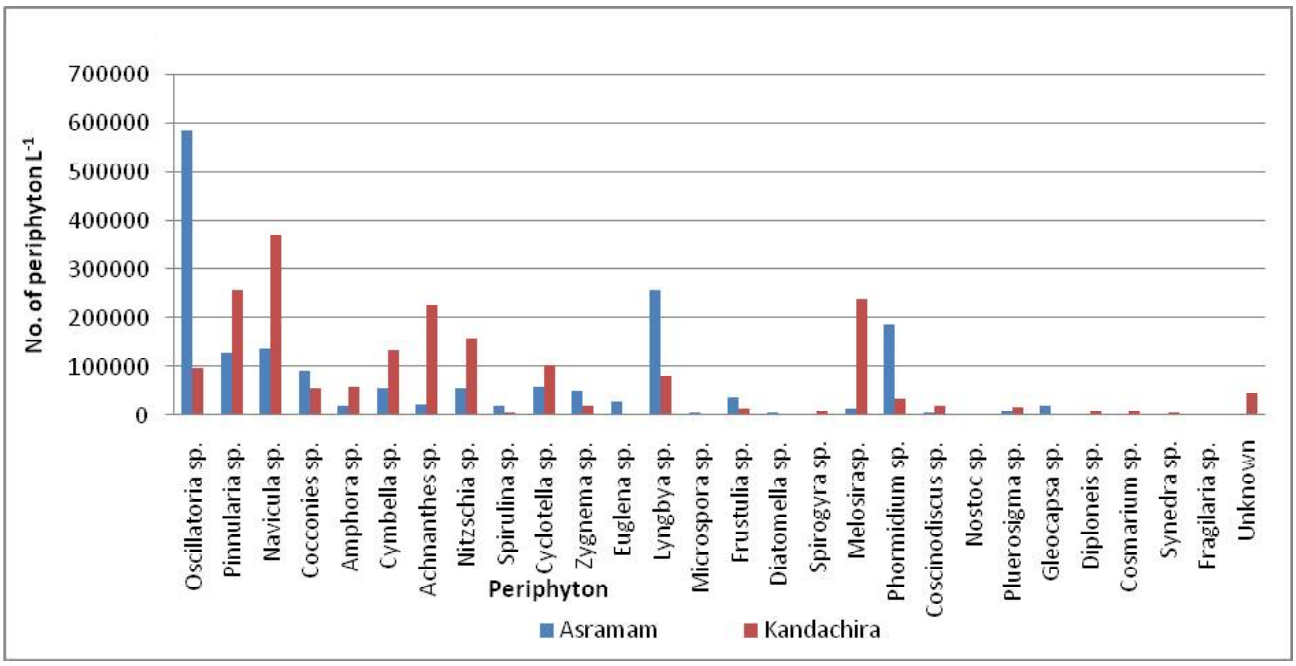

Fig. 1. Number of periphyton on mangrove roots from two sampling stations

phenolphthalein alkalinity of two sampling stations. However, turbidity, DO, BOD, methyl orange alkalinity and total alkalinity showed significant variations ($P<0.05$) between stations.

The values of BOD and DO showed polluted nature of the aquatic body in Station 2 relative to Station 1. Cyanophyceaens dominated on the roots from Station 2. In polluted Asramam mangroves, the filamentous algae showed its prominence in number which may be due to rapid nutrient enrichment, low salinity, low DO, high BOD and high Turbidity. *Oscillatotria* sp. was the most dominant periphytic algae from Asramam.*Oscillatora* sp., *Spirulina* sp., *Lyngbya* sp.and *Gleocapsa* sp. were the most dominant Cyanophyceans (Table 4). Significant variations ($P<0.05$) in the number of Cyanophyceans were found among Station 1 & 2 (Table 4). The Dhanya et al. (2015) carried out extensive investigation on the Cyanobacteria in Kuttanadu Paddy Wetlands, Kerala. They suggested that the dominance of Oscillatoriales may be due to high nutrient status of paddy fields. They also reported the presence of *Phormidium* sp. and *Leptolyngbya* sp. Similar results were obtained in the present study also. Hence, occurrence of *Osillatoria* sp., *Phormidium* sp. and *Lyngbya* species can be regarded as biological indicators of high nutrient status and reveals the pollution status of Station 2 (Asramam mangroves). According to Jafari and Gunale (2006), the presence of *Anabaena* species also denotes the organic load of a particular aquatic ecosystem.

Rafia et al. (2013) showed the dominance of Bacillariophycean members among other periphyton communities. They also reported the prevalence of some species like *Closterium* sp., *Zygnema* sp., *Amphora* sp., *Cymbella*s p., *Epithemia* sp., *Fragilaria* sp., *Navicula* sp., *Synedra* sp., *Lyngbya* sp. and *Phormidium* sp. This is in accordance with the present study also. The dominance of Class Bacillariophyceae was also reported by Ashok et al. (2014) in similar systems. Sarika et al. (2015) also reported the prevalence of Oscillatoriales (*O. sancta, O. limosa, O. subbrevis, O. vizagapatensis* and *O. nigro-viridis*)in polluted aquatic systems.In this study also, Oscillatorians dominated in Station 2 and points out heavy organic load in the system. These results strongly reflect the anthropogenic disturbances in the Ashtamudi backwaters especially Asramam region. Organic load had worsened the condition and has been indicated by the growth of filamentous algae like *Oscillatoria* sp., *Lyngbya* sp., *Phormidium* sp. etc. The low DO and high BOD values are indicators of pollution. The Turbidity values in some of the samplings have reached up to 202 NTU. Mathew and Nair (1980) points out that low salinity favours the algal growth. The onset of monsoon contributes to low salinity level and rapid enrichment of organic pollutants which may accelerate the growth of

Table 3. Water quality parameters of two sampling stations

Water Quality Parameters	Station 1#	Station 2#	t –test Significance
Water temperature (°C)	30.0±1.6	30.5±1.6	NS
pH	8.0±0.4	7.5±0.3	NS
Salinity (ppt)	26.8±8.7	27.0±13.3	NS
Turbidity (NTU)	62.0±49.9	153.7±74.5	*
Dissolved Oxygen (mg L^{-1})	6.1±1.4	2.7±2.7	*
Biological Oxygen Demand	6.7±2.7	27.2±13.8	*
Acidity (mg L^{-1})	30.2±49.7	25.2±17.7	NS
Phenolphthalein Alkalinity (mg L^{-1})	70.0±62.9	33.3±53.2	NS
Methyl orange Alkalinity (mg L^{-1})	826.7±32.7	1913.7±970.7	*
Total Alkalinity (mg L^{-1})	896.7±79.4	1946.7±949.6	*

Mean ±STD
NS - Not significant (P>0.05)
* P<0.05

(phytoplankton) algal species. In the present study salinity value decreased with the advance of monsoon season.

The present study revealed the presence of *Oscillatoria, Phormidium* and *Lyngbya* species in mangrove habitats and similar observations were made by Nedumaran et al., (2008). Sebastian and Ammini (2013) studied the diversity of filamentous algal species and points out that the presence of certain algae can indicate the pollution status in the lakes. The authors inferring from their studies concluded that *Spirogyra* sp. was the most widely distributed followed by *Oscillatoria rubescens* and was responsible for the bloom formation. In the present study, *Oscillatoria* sp. was greater which may responsible for bloom and scum formation in Station 2, Asramam. Along with microfloral communities, some faunal groups were also identified from the periphyton community of Station 1 (Kandachira mangroves). These included barnacles, nauplius, crustaceans and rotifers. Soil samples collected from the bottom of two stations were also analysed. Soil pH (Station 1- 5.5, Station 2- 6) and soil moisture content (Station 1- 5.2%, Station 2- 6.1%), were estimated for the period of study. Soil texture at both stations were analysed and both were dominated by Silt. 41.6% silt, 26.6% clay, 10.6% fine sand and 21.2% coarse sand were recorded from the soil samples of Station 1 and 51.6% silt, 13.7% clay, 6.1% fine sand and 28.6% coarse sand from Station 2.

Adverse anthropogenic effects, oil spills from fishing boats and industries are considered to be the important reasons behind the deterioration of backwaters in Kerala. The municipal waste and solid waste contamination has developed foul smell in

Table 4. Presence various species of Cynobacteria (No. of cell L^{-1}) in the areal roots of mangrove tree, *Bruguiera cylindrica* (Mean of six samplings) from two stations

Cynobacteria	Sampling Stations		t –test Significance
	Kandachira	Asramam	
Oscillatoria sp.	96111	584444	*
Spirulina sp.	5556	17778	*
Lyngbya sp.	78889	254445	*
Gleocapsa sp.	0	17778	*

NS - Not significant (P>0.05)
* P<0.05

Asramam area. The drainage system from the hospitals discharges into the backwater, which contribute a lot to the adverse condition. The water quality of Ashtamudi backwaters is deteriorating day by day especially in the areas which join with built-up area of Kollam city, TS Canal. It currently receives all untreated sewage, slaughter house wastes, direct disposal of human excreta from hanging latrines, houseboats seafood processing factories and hotels. The mangroves fringing here are in threat due to these anthropogenic activities. The rampant pollution prompted to study the extent and impact of pollution happening to the aquatic ecosystem and to monitor the system through estimation of periphytic microalgae.

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Lignification in Relation to the Influence of Desiccation Stress in the Forked Fern *Dicranopteris linearis*.

C.H. KAVITHA[1] AND K. MURUGAN [2]

[1]*Department of Botany, St. John's College, Anchal, Kollam*
[2]*Department of Biotechnology and Botany, Govt. Arts College, Trivandrum, 695 014, Kerala, India*
email :kavithach1979@gmail.com

ABSTRACT

Investigation of lignification process and its physiological significance under desiccated condition in*Dicranopteris linearis*was attempted. The response of peroxidases, polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) in relation to relative water content of the leaf tissues to water deficit treatment using PEG (poly ethylene glycol) fromday 2 to 10 was investigated. Therelative water content (RWC)of the fronds showed a marginal decrease during 2d desiccation followed bydecrease to52% during 10d desiccation when compared to control (86 to 89%) and the data were statistically significant at 5% level.The activity of guaiacol peroxidase (POX) was enhanced with highest level during 6th and 8th day of desiccation treatment.The activity of PAL and PPO was also varied in the desiccated fronds as compared to the control.The higher expression of POX activity suggests that they perform function apart from ROS scavenging to lignification by coordinating PAL and PPO to incorporate phenol and lignin into the cell walls.The anatomical characterization of the rhizome, petiole and frond revealed heavy lignification even in the control plant. The lignin level and its involvement to mitigate desiccation a positive correlation with desiccation periods i.e., increased from 2.09 (2d) to 3.00 (10d) mg/ g tissue.The histochemical localization of lignin corroborates with the lignin content in the fronds.Further studies are warranted to analyze the molecular expression of the genes involved in stress tolerance in the fern.

Key words *Dicranopteris linearis; Lignification; Peroxidases; Phenylalanine ammonia-lyase; Polyphenol oxidase; desiccation stress*

Environmental stresses have adverse effects on plant growth and productivity and are expected to become variable, severein the scenario of climate change. Plants display different mechanisms to evade drought stress. Plant's tolerance to stress includes a cascade of events or reactions leading to alleviation of potential stress-induced cellular injuries. The development of lignin biosynthesis has been considered to be one key factor that allowed plants to flourish in terrestrial ecosystems. Lignins are cell wall phenolic heteropolymers that arecovalently bound to cellulose, other polysaccharides, andproteins of the cell wall. The functional significance of lignin is associated mainly with the mechanical support allowing plants to stand, with water transport in the xylem tissuesaswell as barrier for the growth of pathogens and infection (Boudet, 2000). Moura et al., (2010) studied the effect of biotic and abiotic stresses on changes in the lignin content and composition in plants and found that stressors are responsible for regulating cell wall lignification process.Different types of abiotic stresses, such as mineral deficiency, drought, UV-B radiation and low temperatures, as well as biotic stresses, such as infection by fungi,bacteria and viruses, cause changes in the lignin content of plants.

Lignification is a complex processthat involves several different phenolic substrates andenzymes (Valentines et al., 2005).Some of the key enzymes catalysing the biosynthesisof lignin include peroxidase (POX), polyphenol oxidase(PPO), and phenylalanine ammonia-lyase (PAL). POXin either the free (cytosolic POX) or bound state (cell wall POX), hasbeen shown to be involved in monolignol polymerizationto lignin (Fry, 2004). PPO are involved in theoxidation of polyphenols into quinines and is also associatedwith lignification (Cervilla et al., 2009). PALis the entry-point enzyme in the phenylpropanoidbiosynthesis pathway and it catalyzes deamination ofphenylalanine to *trans*-cinnamate. Branch pathways lead to the synthesis of compounds that have diverse functions in plants. The resulting phenolics are often converted into more reactive species by phenol oxidases and peroxidases (Heath, 1980).

Little data is known about the effects of droughton lignin biosynthesis.Katerji et al., (1997) documented that severe drought stress due to soil salinity leads to increase in cell wall lignification of sugar beets.In this juncture the aim of the present study is to evaluate the correltaion of peroxidases, PPO and

PAL with lignin andrelative water content (RWC)duringdesiccation treatment in the forking fern *Dicranopteris linearis*.

MATERIALS AND METHODS

Plant material and desiccation treatment:*Dicranopteris linearis* (Burm.f.) Underw. was collected from the wild habitats ofPonmudi Hills, Thiruvananthapuram, Kerala. *Dicranopteris linearis* is a terrestrial fern that grows horizontally at ground level and possess characteristic forking fronds which grows on stalked compoundstem. It is commonly known as forking fern.*In vitro* experiments were conducted against five different desiccation regimes such as 2, 4, 6, 8 and 10 d usingpoly ethylene glycol(PEG) treatments under controlled conditions and also subsequent rehydration. A set of control was also maintained under optimal conditions.

Measurement of relative water content: Relative water content (RWC) was calculated using the following formula RWC (%) = [(FW – DW) / (TW – DW)] × 100, where FW is sample fresh weight, TW is sample turgid weight, and DW is sample dry weight.

Determination of activity lignification-related enzyme assays

Peroxidase (POX) activity was assayed using guaiacol as substrate (Sanchez et al., 1995).The cytosolic and cell wall bound peroxidase was extracted separately and also assayed.

The isolation and assay of PAL was according to the method of Ke and Saltveit (1986).

Isolation and assay of poly phenyl oxidase (PPO) was according to the method ofLiu et al.,(2005).

Determination of lignin

Lignin content was measured by the modified acetylbromide procedure (Hatfield et al., 1999). Lignin washistochemicallylocalized using the Wiersner test as described by RosBarcelo, (1998).

Statistical analysis: The values were expressed as the mean of six replicates ± standard error (SE). Student t-test ($p< 0.05$) was used to check statistical significance.

RESULTS AND DISCUSSION

Relative water content

The changes in water level of D. linearis leaves during different desiccation stress treatments were significant. Initially at 2 d desiccation time points, there was a marginal decrease in relative water content (RWC) i.e., 85.5%. Meanwhile, after 4th, 6th and 8th day of desiccation stress, RWC in the leaves rapidly decreased to a level lower than the RWC of the control condition i.e., 74.6, 67.2 and 60%. A further decrease in RWC to 52% was noticed in 10 day desiccated fern. The RWC value of the control ranged from 86 to 89 %. The results revealed that the potentiality of *D. linearis* fronds to maintain optimal leaf water status under stress during different treatment periods. Gradual decrease in water content during desiccation give ample time for the plant to develop mechanisms such as replacement of water with non-aqueous molecules or reduction in cellular volume by folding of cell wall thereby reducing loss of cellular water and also by altering the mechanical features like wet *vs* dry cell walls.

Changes in activities of lignification-related enzymes

The two major enzymes of phenyl propanoid metabolism (PPM), such as phenylalanine ammonia lyase (PAL) and peroxidase (POX) were isolated from the control, desiccated and rehydrated frond tissue and assayed spectrophotometrically, in order to ascertain their role in lignification.Each value was a mean of 3 replicates. The specific activity was expressed in units' mg^{-1} protein. One unit of PAL activity is equivalent to µg of cinnamic acid released by the deamination of L-phenyl alanine, under ambient condition.One unit of POX is the amount of enzyme required to oxidize one micromole H_2O_2 /min.

PAL, a key enzyme in the phenyl propanoid path-way, is involved in the defence response of plant cells.Effects of desiccation on PAL activity in frond tissues of*D.linearis*is shown in Table 1. PAL activity increased gradually and the highest activity was reported at the desiccation period fromday6 to 8 as compared to the control. A sig-nificant increase in PAL activity was observed in the fern at all tested

Table 1. Effect of desiccation stress on phenylalanineammonia lyase (PAL U/mg protein), cytosolic peroxidase activity (CY-POX U/mg protein), cell wall peroxidase activity(CW-POX U/mg protein) an poly phenol oxidase (PPO U/mg protein) in *D.linearisp*< 0.01.Abbrevations: D-Desiccated; R- Desiccated and subsequently rehydrated

	Control	2 dayD	2 dayR	4 dayD	4 dayR	6dayD	6 dayR	8 dayD	8 dayR	10 dayD	10 dayR
PAL	2.35±0.01	4.6±0.04	6.53±1.9	13.2±0.72	18.51±0.37	18.69±0.24	26.69±0.76	25.54±0.23	26.77±0.68	23.23±0.29	21.44±0.87
CY-POX	5.99± 0.88	9.89± 0.32	9.74± 0.48	11.85± 0.09	12.10± 0.26	21.6± 0.39	21.2± 0.99	25.74± 0.35	24.1± 0.12	30.56± 0.08	27.45± 0.45
CW-POX	3.186± 0.02	3.99± 0.04	3.85± 0.65	6.64± 0.92	5.85± 0.3	11.3± 0.18	8.42± 0.43	15.7± 0.056	7.18± 0.38	16.77± 0.72	7.56± 0.01
PPO	13.69±0.97	15.48±0.86	14.25±0.22	25.29±0.07	30.75±0.77	31.05±0.19	36.38±0.54	19.34±0.63	21.32±0.54	17.31±0.32	15.56±0.11

desiccation periods, and the activity increased by 10 fold during 6 day of desiccation. Thus, the present study indicates that desiccation stress resulted in an activation of PAL activity which is responsible for the increase of phenol and in turn lignin concentration in the fern.PAL is considered to be responsible for the conversion of phenylalanine to *trans*-cinnamic acid, a keyintermediary in the biosynthetic pathway of phenolicand lignin (Rivero et al., 2001). It has been reportedpreviously that PAL isgenerally stimulated in plant tissues exposed to environmental stresses. Further, studies indicated that PALenhancement in stress conditions is due to H_2O_2 generation,which occurs as a primary response against stress(Jouili and El Ferjani, 2003). Increased PAL activity canbe indirectly correlated to the lignification reaction in the fern to counteract desiccation stress.

Apart from the oxidative polymerization of cinnamyl alcohol to lignin (lignin biosynthesis), the enzyme POX was also involved in other functions like pathogen resistance, oxidation of fatty acids and phenols, phytochrome catabolism, fruit ripening, cross linking of cell wall polysaccharides and biosynthesis of extensin and auxin metabolism (Hiraga et al., 2001).To analyse the response of peroxidaseactivity to desiccation treatment, the two peroxidases such as cell wall bound and cytosolic wereexamined.The cell wall peroxidases participate in themodulation of cell wall properties during plant growth partly through catalysing the formation of covalentcrosslinks after oxidation of ester- and ether-bound phenolic acids and also through the oxidative coupling of cinnamoylalcohol moieties to generate lignin (Iiyama et al., 1994).In the present study Cytosolic POX expressed remarkable activity than cell wall bound POX (Table 1) suggesting the pertaining role of it in the scavenging H_2O_2 generated inside the cells due to the stressful environment. Meanwhile, the mean assay data of cell wall bound POX suggest its normal role in lignification (Table 1). A 5 fold increase in the activity of CWPOX during the 10th day of desiccation treatment corroborates with the lignification level of the fern.

Another enzyme involved in ligninmetabolism is poly phenol oxidase (PPO), which catalyses the oxidation ofpolyphenols into quinines (Cervilla et al., 2009).PPO is a copper-containing protein widely distributed in the plant kingdom that catalyzes theoxygen-dependent oxidation of monophenols or ï-diphenols to *o*-quinones. The *o*-quinones are highlyreactive substances that can react with amino acids,peptides and proteins, thus altering the structuraland functional properties of the cell. In the present investigation, there was anincreasedtrend of PPOactivity during the desiccation period with peak activity recorded at 6th and 8th day of desiccation treatment.Cervilla et al., (2009) demonstrated that PPO

Table 2. Lignin content (mg/g tissue) of the fern against desiccation stress

	Control	2 day D	4 day D	6 day D	8 day D	10 day D
Lignin	1.7	2.09	2.25	2.35	2.8	3.00

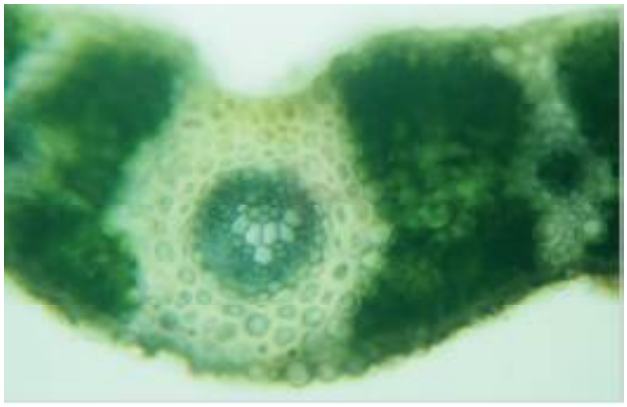

Fig.1a. Control plant without lignin staining

increasedin response to abiotic stresses such as water deficitand heavy metals. PPO activity may regulate theredox state of phenolic compounds and become involvedin the phenylpropanoid pathway (Nakayama et al., 2000).Numerous studies have indicated that phenol-oxidizing enzymes may participate in defence reactions, inducing resistance to stress (Schneider and Ullrich, 1994; Stewart et al., 2001).

Analysis of the lignin content

The lignin level and its association with resistance against desiccation in the fronds of the fernwere evaluated. Lignin content showed a positive correlation with desiccation periods i.e., increased from 2nd day to 10th day of desiccation period (Table 2). The level in control was 1.7 mg/g tissue. The analytical data was supported by the activity of enzymes related with lignin biosynthesis in the fern and also cell wall bound

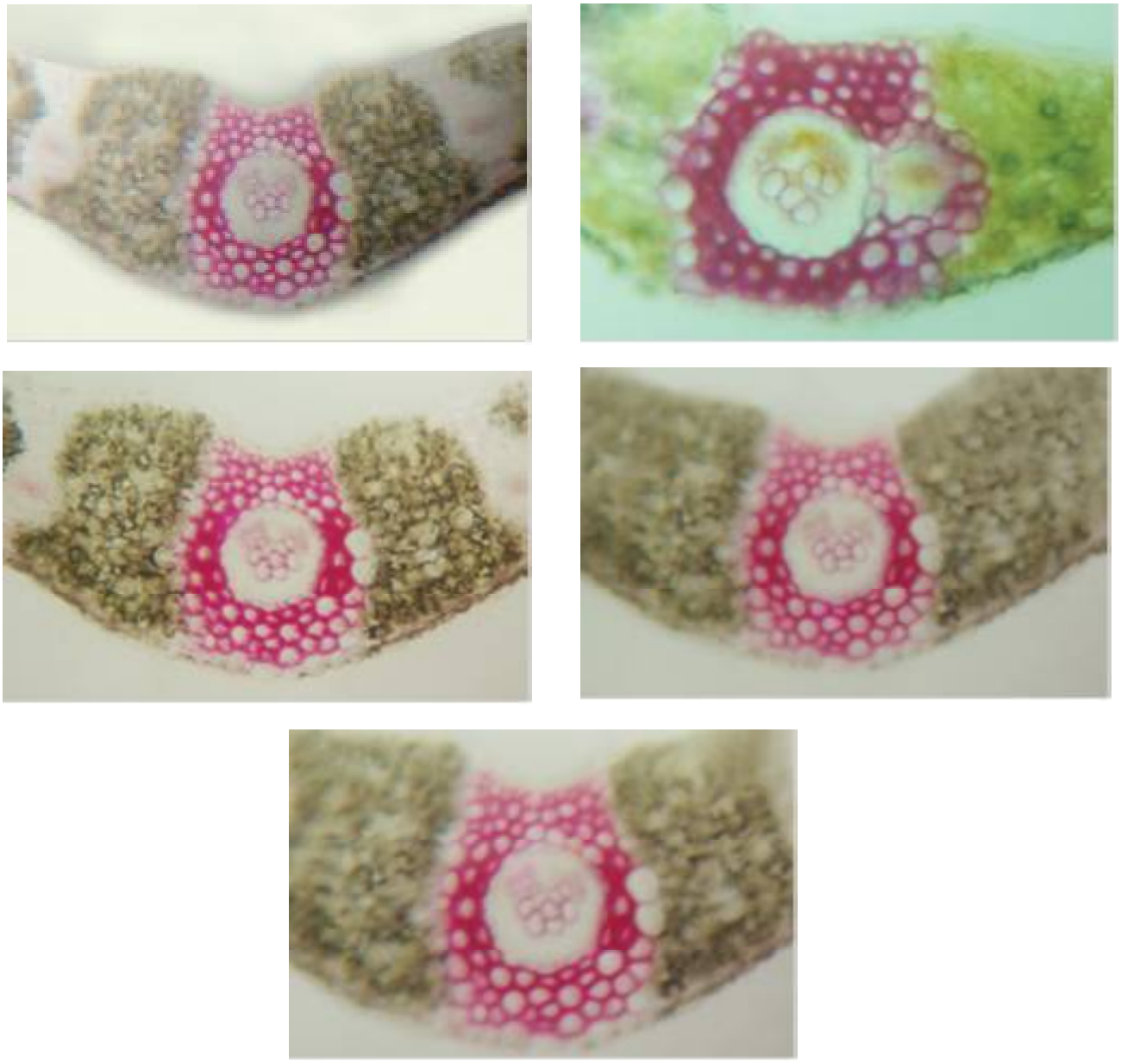

Fig. 1. Histochemical localization of lignin in the desiccated leaf tissue(b) 2 Day; (c) 4 day; (d) 6 day; (e) 8 day; (f) 10 day

POX activity. In addition, the histochemical localization of lignin in terms of reddish deposits corroborates with the lignin content in the fronds (Fig. 1a-f).

Bok-Rye et al., (2007) studied the biosynthesis of lignin and its functional significance during water stress in the leaves of *Trifoliumrepens*subjected to 28 days of drought and noticed reduced leaf growth along with the increased lignin content. The activities of enzymes involved in the biosynthesis of lignin revealed that the enzyme activities depend on duration and intensity of desiccation exposure.Lignin content has been reported in plants that were exposed to heavy metal, and wasassociated with an increased activity of lignifying POXs (Schutzendubel et al., 2001). $H_2O_2$is produced during different metabolic processes, such as during the formation of lignin in cell walls. The accumulation of H_2O_2under water deficit stress was suggested as a signallingmolecule triggering lignification (Lee et al., 2007). On the other hand, the high level of H_2O_2 in apoplastic space may accelerate strengthening of cell walls via cross bonding molecules (Zarra et al., 1999).

In our previous study we have showed an up regulation in the level of H_2O_2 in the fern following the desiccation treatment (Kavitha and Murugan, 2016). In this juncture, the present results regarding lignin content in leaves suggest there is an interaction between H_2O_2 and lignin content in *D.linearis* subjected to desiccation stress.The induction of lignification-related enzymes may also be a defence mechanism to protect the plants from long-term drought stress.

CONCLUSION

Desiccation treatment in the fern from 2 to 10 day results in a significant decrease of leaf relative water content. This wasaccompanied with changes in the activity of peroxidasesand lignifying-related enzymes. Cell wall and cytosolic peroxidases were significantlyactivated in desiccated fronds. The activation of PAL and PPOwas also observed. These results suggest that peroxidases in association with PAL and PPO participate in lignification and involve in incorporation of phenol and lignin into the cell walls.The change in the lignin content of the fronds also supports the activity of the enzymes.

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A Study on the Tensile Behaviour of High Performance of Coconut Leaves treated with Methyl methacrylate

K.R. KAVITHA

Department of Botany, Sree Narayana College Sivagiri, Varkala, Kerala, India
email: drkavithakr@gmail.com

ABSTRACT

The effects of electron beam irradiation on the mechanical properties of Coconut leaf fibre reinforced high impact Methyl methacrylate (MMA) were studied. In recent times efforts are being made to improve the quality of different plant materials. Agricultural or biodegradable materials have played a major role in human life. Kerala is the largest producers of Coconut palms. The coconut leaf has been used in different purposes. However the coconut leaves undergoes rapid deterioration and needs annual replacement. The purpose of the present investigation is to study the possibility of irradiation methods to reduce leaf deterioration of coconut palms. The collected leaves were categorized in to tender, mature and dried leaf bits. 20 samples from each of the samples were immersed in a beaker containing 150ml of unstabilized Methyl methacrylate (MMA) monomer for 180 hrs. The irradiation process was carried out using electron beams for inducing the polymerization of monomer infiltrated leaf bits. For electron beam irradiation, the samples were grouped in to three sets for giving radiation doses 5 kGy, 10 kGy and 15 kGy. 20cm long and 2cm wide specimens were used for tensile testing on an Instron testing machine. The mean value of the property measured was compared with the control samples without any monomer treatments. From these studies, assure that the monomer treated Coconut leaf samples cured by electron beam irradiation at particular doses made a change in their mechanical properties (tensile strength) thereby enhancing the durability and longevity of the samples. These converted leaf bits offers a big possibility of making high utility commercial products accessible to ordinary people.

Key words *Tensile Behaviour, High Performance Coconut Leaves, Methyl methacrylate*

The coconut palm (*Cocos nucifera* Linn.), a valuable tropical resource, supply food, energy and many economically viable products. In Kerala traditionally eco-friendly palm leaf woven houses, umbrella, baskets, separating screen etc are common. Natural fibres have some advantages over man made fibres, including low cost, light weight, renewable character, high specific strength and modulus and availability in a variety of forms throughout the world (Mohanti et al, 2000, Rajulu et al 2002)

Coconut leaf thatch is a cheap roofing material used widely in India (Kerala), Sri Lanka and in some Polynesian countries by millions of households. The leaves are available to extend of 5.6 million ton in Kerala whereas the worldwide production may exceed 42 million tons. The large availability of this renewable resource can contribute much in solving the housing problems in the developing world (Pillai, 1982). However the thatch made from coconut leaves undergoes rapid deterioration and needs annual replacement. A number of empirical studies have been reported on the prevention of deterioration (Pillai, et al 1981, Rama verma, 1957, Purushotham, et al 1953). Damage and deterioration to palm leaves are usually the result of insect damage, climatic factors (variations in humidity and temperature), staining, splitting and cleavage and mechanical damage. There is no systematic study of the properties of these palm fibers except for some brief reports regarding mechanical properties (Sathyanarayana et al, 1981) and details on the chemical characterization and structure (Venugopal et al, 1984). In view of these limited uses, a lot of this abundantly available resource is going waste. However, if diversified uses for these fibers can be thought of, it would not only increase the utilization of this abundant and renewable resource but also help in increasing employment opportunity in the rural sector since the fibre industry is a cottage industry.

Although a reduction in strength is observed during deterioration, it is interesting to note that there was an increase in strength of leaf when the green leaf was dried to a moisture content of 15 % (Pillai et al 1982).

The ability of high energy radiation like gamma and electrons to induce chemical and physical changes in materials in general and specially polymers and elastomers in particular, have been the basis for research since 1938 (Chapiro, A 1962). The effect of irradiation on mechanical, thermal and degradation

properties of biodegradable polymer bionolle and its composites was studied by Mubarak and Idriss Ali, 1999. The enhanced tensile strength of bionolle was obtained when it was exposed under 20kGy radiation.

Although brief reports regarding the mechanical properties of palm fibres (Satyanarayana et al 1986) are available studies dealing with the mechanical property of Coconut palm leaf bits after treatment with monomer and radiation is almost nil. Hence to determine the durability of leaf bits a mechanical property study, tensile strength was undertaken.

MATERIALS AND METHODS

Fresh healthy leaf bits of coconut (*Cocos nucifera*) were collected from Taliparamba of Kannur district of Kerala. The collected leaves were categorised in to tender, mature and dried leaf bits. During sample preparation, leaf bits without midrib were prepared with length X breadth 20X2 cm. Thus prepared leaf bits were grouped separately.

C1T1 – Coconut tender leaf bits

C1T2 – Coconut mature leaf bits

C1T3 – Coconut dried leaf bits

In order to minimize variations in the properties, the following procedure was followed during sample collection. Leaf bits at the bottom most part of the crown of mature palms was taken. Then specimen samples of required size were cut out from the leaflet at a desired distance of approximately 0.2m from the petiole end of the leaflet and the rest of the leaflet rejected.

20 samples each from the above mentioned samples were immersed in beaker containing 150ml of unstabilized methyl methacrylate monomer for 180 hrs. After monomer infiltration for 180 hours, they were taken out and air dried for about 2 to 4 hours in order to evaporate excess monomer. The air dried and monomer infiltrated samples were covered in zip lock polythene bags separately.

In the present study, the irradiation process was carried out using electron beams for inducing the polymerization of monomer infiltrated leaf bits. For electron beam irradiation, the samples were grouped in to three sets for giving radiation doses 5kGy, 10kGy and 15kGy. The process was carried out in 200mev accelerator machine, Electron beam centre, Kharghar, Mumbai for enhancing longevity and durability of samples.

20cm long and 2cm wide specimens were used for tensile testing on an Instron testing machine. The leaf specimens could be readily gripped and were breaking almost at the middle of the specimen. The ultimate tensile strength is the load at break per unit area of the specimen. A guage length of 5cm was employed for tensile testing. All experiments were repeated with 5 samples and the average taken. The measurements were tabulated. $UTS = \frac{\mathrm{B/L}}{\mathrm{area}}$

Here B/L is the breaking load.

RESULTS AND DISCUSSIONS

The Coconut leaf bits immersed in monomer and treated with electron beam at different doses showed a great increase in tensile strength as compared with the control samples without any treatments.

In the control leaf samples of Coconut without any treatments, the tensile strength is found as 23.72MPa, 22.19MPa and 31.92 MPa in tender, matured and dried leaf bits respectively. (Table 4)

The tensile strength of Coconut leafbits immersed in monomer and cured under 5 kGy electron beams is measured as 49.04 MPa, 42.19 MPa and 40.83 MPa respectively in tender, matured and dried leaf bits.(Table 1)

The tensile strength of Coconut leaf samples immersed in monomer and cured under 10kGy electron beams are 37.55 MPa, 42.02 MPa and 42.30 MPa in tender, matured and dried leaf bits respectively.(Table 2)

The 15kGy treated leaf bits of Coconut samples showed tensile strengths 52.21 MPa, 51.99 MPa and 47.98 MPa respectively in tender, matured and dried leaf bits.(Table 3)

When electron beam radiation passes through matter, its energy is transferred to the molecules of absorbing medium by various mechanisms. The primary reactions for the ionization or the excitation of molecule take place at certain rates in the radiated matter. The ability of high energy radiation like gamma and electrons to induce chemical and physical changes in materials in general and specially polymers and elastomers in particular, have been the basis for research since 1938 (Chapiro, A 1962). In the subsequent reaction steps, stable molecules or free

Table 1. Table showing the Tensile strength of *5KgY samples*

Samples	Thickness (mm)	Width (mm)	Maximum force (N)	Tensile strength (MPa) MEAN & SD
EC1T1	0.176	19.6	157.2	49.04±1.64
EC1T2	0.256	19.6	250	42.19±0.93
EC1T3	0.292	20.2	220.5	40.83±0.83

Table 2. Table showing the Tensile strength of *10KgY samples*

Samples	Thickness (mm)	Width (mm)	Maximum force (N)	Tensile strength (MPa) MEAN & SD
EC1T1	0.196	21.2	155.2	37.55±0.73
EC1T2	0.204	22.2	185.8	42.02±0.54
EC1T3	0.284	21	300.0	42.30±1.14

Table 3. Table showing the Tensile strength of *15KgY samples*

Samples	Thickness (mm)	Width (mm)	Maximum force (N)	Tensile strength (MPa) MEAN & SD
EC1T1	0.164	19.6	147.5	52.206±0.57
EC1T2	0.172	20.8	190.8	51.99±1.4
EC1T3	0.256	18.8	228	47.98±0.64

Table 4. Table showing the Tensile strength of *CONTROL samples*

Samples	Thickness (mm)	Width (mm)	Maximum force (N)	Tensile strength (MPa) MEAN & SD
CC1T1*	0.26	16.4	28.7	23.72±0.65
CC1T2*	0.22	18.8	98.3	22.19±0.71
CC1T3*	0.16	18.8	98.5	31.92±0.69

EC1T1 – Electron beam treated Coconut tender leafbits

EC1T2 – Electron beam treated Coconut matured leafbits

EC1T3 – Electron beam treated Coconut dried leafbits

* - Control Samples

radicals and ions formed. In further reactions with certain systems, this brings about chemical chain reaction that is capable of producing new chemical structure. The irradiated matter then exhibits changed physical and chemical properties. A good deal of work has been reported on the employment of gamma radiation as a polymerization initiator. Investigation of the alteration of the properties of wood in which graft co.polymerization had been initiated by gamma irradiation of wood impregnated with a suitable monomer styrene. There are other ionizing radiations-notably electron beams which will be equally effective in initiating graft co. Polymerization, and which are more convenient radiation source for experimental work in this field (Ramalingam, et al, 1968). Radiation induced polymerization techniques suggests a possible preservation method for the preservation of Botanical specimens (Merwin L Brown and R.W.Funsch, 1970). Polymerization of methyl methacrylate and methyl, ethyl and N-butyl acrylates was carried out in a wide range of dose rated, 10 - 10^6 rad/s by gamma ray and electron beam irradiation (Kanae Hayashi et al 1987). Biodegradable polymers constitute a family of polymers that are designed to degrade through action of living organisms or like that. They offer a possible alternative to traditional non-biodegradable polymers where recycling is unpractical or un economical. The

other method to tackle this problem is to use natural fibers as the reinforcement in polymers (Rozman HD et al 2001, Countinho FMB et al, 2000, Harikumar KR, 1999).

The electron beam (EB) irradiation technique is being increasingly utilized to modify the surfaces of various polymer materials like fibers, textiles and films. Cotton fabrics have been coated with pigment colours using EB to improve colour fastness, tensile mechanical resistance (El Naggar AM et al 2005). A novel non coloured and colourless polymer of gum Arabic can be produced by radiation induced polymerization techniques. Chemical and physical changes in gum Arabic were induced by treatment with ionizing radiations with up to 100kGy doses. Polymerized gum Arabic can be obtained by irradiations at high solute concentrations (Tsuyoshi Katayama et al, 2005). Natural fibers that have been studied as a substitute for glass and other non – biodegradable composite components are included hemp, flax, jute, banana, kenaf etc (Baiardo, M et al 2004). Other advantages of using the natural fiber are also due to its low density, high toughness, comparable specific strength properties, ease of separation and reduction in tool wear (Bessadok et al, 2009). Chemical modification and electron beam irradiation proved to be important to improve the fibre matrix adhesion so as to produce composite materials with superior strength (Zimpaloni, M et al 2007).

The increase of dose irradiation has increased the tensile strength of Pine apple leaf fibre composites. It can be seen that the tensile strength of PALF composites without irradiation is about 21.10 MPa. Meanwhile by using higher dose irradiation (100kGy), the composite has brought improvement of tensile strength value up to 40.25 MPa. The increase in dose may be a result of crosslinking reaction and interaction of free radicals produced in irradiated matrix (Ratnam,CT et al 2007).

However, no such studies have been carried out so far to enhance the utilization of Coconut palm leaves by treating them with a monomer and polymerize it through electron beam induced polymerization techniques. Tensile strength of leaf bits after treatments was compared with the control. Durability of polymerized leaf bits are tested by biological degradation assessment of the treated polymerized samples, hence the novelty and importance of the study.

From the results it is evident that there is a steady increase in the tensile strength of all the monomer immersed samples irradiated with electron beams under different doses and the increase in tensile strength is almost double the tensile strength of the control samples without any treatments. The tensile strength of 15kGy treated Coconut palm leaf bits are higher as compared to the 5kGy and 10kGy electron beam treated samples. From this studies assure that the monomer treated Coconut leaf samples cured by electron beams at particular doses made a change in their mechanical properties (tensile strength) thereby enhancing the durability and longevity of the samples.

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Phytorepellency and Mortality Effect of Five Selected Plants Against Rice Weevil, *Sitophilus oryzae*

K.P. SHARANYA AND M. KRISHNENDU

Department of Botany, NSS College Pandalam, Pathanamthitta, Kerala

ABSTRACT

The present study was conducted to evaluate the phytorepellency and mortality effects of five plants which possess aromaticity and belong to different families, against the stored food grain insect pest namely *Sitophilus oryzae* which inhabit stored rice grain. The selected plants are *Azadirachta indica, Ocimum sanctum*, Aegle *marmelos, Lantana camara* and *Eupatorium rugosum*. The study conducted in two steps. Crushed leaves, leaf extract and powdered leaf of five aromatic plant species were tested for their insecticidal activities against *Sitophilus oryzae*. Responses varied with plant material and exposure time. The first step was repellency test, insect showed highest repellency against *A. indica,* followed by other selected genera. The same plant itself showed the highest mortality percentage. Significance of the present study is traditionally it's possible to avoid the damaging effects of *S. oryzae* using plants without going for any chemical products.

Key words Phytorepellency, *S. oryzae, O. sativa, A. indica, O. sanctum, A. marmelos, L. camara, E. rugosum*

Rice, wheat and maize are the world's three leading food crops. Among these three major crops, rice is by far the most important food crop for people. Also it is the most important and staple food of the world's population. Every year cereals such as wheat, rice etc suffers heavy losses during the storage due to insect pest infection. Most of the world's harvested food is destroyed annually by insect and rodent pests. *Sitophilus oryzae* is a common rice weevil and it is one of the most important storage pests, which causes severe damage to raw cereals throughout the world. One pair of *Sitophilus oryzae* can reproduce about one million of its species within a period of three months under favorable conditions (Thomas *et.al.* 2002) and the adults are internal feeders and cause serious quantity and qualitative losses to the grains.

However, due to lack of good and efficient storage facilities, most rice grains are still being stored traditionally in granaries (Odeyemi *et al*., 2010). Control of stored-product insect populations is primarily dependent upon continued applications of liquid and gaseous insecticides. Although effective, their repeated use for several decades has disrupted biological control system by natural enemies and lead to outbreaks of insect pests. (Champ and Dyte, 1977).

Many plant secondary metabolites play an important role in plant–insect interactions and therefore are used in many parts of the world since ancient days. Plant materials that are safe for human consumption and have the ability to retain their insecticidal potency overtime should be recommended to farmers. There is a need to look for an alternative method that is easy, less poisonous and less detriment for pest control. However, very little research has been performed on the development of affordable organic pesticides which offer same control levels as synthetic, to weevil. Hence the present study aimed to examine the effect of five locally available plants for its repellency and mortality effect against *Sitophilus oryzae*.

Objectives of the study include Control *Sitophilus oryzae* in stored rice and analyse the repellency and mortality effect of the five selected plants against *Sitophilus oryzae.*

MATERIALS AND METHODS

Selected plants are used in three forms in this study, crushed leaves, leaf extract in water and dried powdered leaf. Water extract is obtained by grinding the leaves with motor and pestle. Powdered form is obtained by shade drying the selected plants for some days and finely powdered within a blender.

INSECTS TESTED

Live rice weevils were collected from the storage area of the rice trader. Cultures of *S. oryzae* were maintained in the laboratory without exposure to any insecticide on rice grain, in plastic containers. Figure 5 shows the habit as well as habitat of *Sitophilous oryzae.*

PLANTS SELECTED

Five aromatic plant species were selected and collected from Pandalam NSS College campus. The plants selected in this study were *Azardirachta indica*, *Ocimum sanctum, Eupatorium rugosum*, *Lantana camara* and *Aegle marmelos*. The leaves of plants

were washed with distilled water; air dried for several days and macerated using domestic blender. As the investigation proceed the plant leaves combination were also used. The combinations include Neem – Aegle, Neem – Lantana, Neem - Eupatorium, Neem – Ocimum, Aegle –Lantana, Aegle - Eupatorium, Aegle – Ocimum, Lantana - Eupatorium, Lantana – Ocimum and Eupatorium - Ocimum. These combinations were used in the analysis of both repellency and mortality study against *Sitophilus oryzae*.

Leaves of the above mentioned five plants and their combinations are used in the present study to know about the repellency and mortality effect on *Sitophilus oryzae*. The present study was conducted in two steps, repellency study and mortality study. Leaves of 5 aromatic medicinal plant species were tested for their insecticidal activities against *Sitophilus oryzae* using direct contact application. Responses varied with plant material and exposure time. In the test with filter paper method, both repellency and mortality activity against the species was produced by using crushed leaves, leaf extract and leaf powder. Repellency was tested for some hours while mortality was checked within two days after treatment. The untreated one in the case of mortality study and half part of petridish during repellency study was maintained as control. Each treatment was replicated three times.

FILTER PAPER METHOD

The effect of plant leaves on adult movement was assessed by filter paper as described by Tripathi *et al*. (2000). For this the plant part used was leaf only but in three forms which includes crushed leaves, leaf extract and powdered leaves.

REPELLENT ACTIVITY

The Whatman 1 filter paper of 9.0 cm diameter was divided into two equal halves. One half was considered as control by avoiding the addition of any plant parts and so, is filled with 10g of clean, undamaged and disinfested rice grains which were weighed with the aid of weighing balance into Petri dishes; while the other part was considered as treated, therefore along with 10 g of rice, plant part were also provided. Dosage for repellency study was ten grams of rice for ten *Sitophilus oryzae* along with 1g of crushed leaves, 1g of powdered leaves and 1ml of water leaf extract, which were added separately on separate petridishes in triplates, during each of the leaf form repellency study. The plant leaf extract treated filter paper was allowed to air dry until the solvent was totally evaporated at room temperature before placing into the petri dish. 10 weevils were released in the middle of each half of the filter paper side on petridish for each time. The dishes were covered immediately to prevent any escape of the insects as well as escape of the plant aroma. Each treatment was replicated three times and observations were made on the number of insects on treated and untreated half of the filter paper after three and four hours of treatment.

The repellency of different plants against *S. oryzae* was assessed for that the movement of *S. oryzae* from the side of treated filter paper to the side of untreated filter paper was noticed and recorded. More number of *S. oryzae* on the untreated or control side shows the repellency activity of each leaf extract form against the rice weevil.

MORTALITY STUDY

Mortality of five different plants against rice weevil *Sitophilus oryzae* was also assessed by filter paper method. Filter-paper circles of 9 cm diameter was taken and placed in petidishes of 9 cm diameter. Four petridishes were used at a time for mortality study. Twenty *Sitophilus oryzae* were introduced into each Petri dish containing 20 g of rice grains and plant materials. Control petridish contained only rice and rice weevil. The other three petridishes contain 2 g crushed leaves, 2ml leaf extract and 2 g of powdered leaves respectively along with rice and Sitophilous. The dishes were shaken properly for thorough mixing to ensure uniform coating of the powders or leaves on the rice grains. The dishes were covered immediately after introducing rice weevil to prevent any escape of the insects and escape of the plant aroma. For mortality study too three replicates were placed overnight and the mortality of the insect was counted. The numbers of rice weevils in the control were compared with the other three petridishes showed the effect of those five different plant leaves on rice weevil. Mortality counts were taken at 48 hours post-treatment. This procedure was carried out separately for all the five plant materials at its three leaf forms. The test insects were certified dead if they do not respond when probed slightly with office pin and at each observation dead insects were removed after counting.

RESULT

Among all the combination of plant species tested crushed leaf of *Azadirachta indica* showed the strongest repellency against *Sitophilus oryzae* with repellency of 95% followed by leaf extract and leaf powder of *Azdirachta indica* and *Ocimum sanctum* as well as crushed leaves of *Ocimum sanctum* and *Aegle marmelos*. These showed 90% of repellency. Other extract with significant repellency activity were that of *Aegle marmelos* in leaf extract and leaf powder. It exihibited 85% of repellency against rice weevil. Out of the 5 genera selected *Eupatorium rugosum* showed the least repellency of 75% in crushed leaves and 70% when given in leaf extract and powder form. The details are shown in the table 1 below. Graphical representation of repellency ativity is given in figure 1. The photographs showing repellency study are given in figure 5.

Table 1. Repellency percentage in selected genera

Genera selected	Repellency in percentage (%)		
	Crushed leaf	Leaf extract	Leaf powder
A. indica	95	90	90
O. sanctum	90	90	90
A. marmelos	90	85	85
L. camara	85	80	80
E. rugosum	75	70	70

Phytorepellency study with the combination of 5 selected plants were done and the result has been coordinated and given in table 2, which shows the repellency effect of selected plants in different combination against *S. oryzae*. The graphical representation is also given in figure 2. Combination effects on *S. oryzae* were almost similar to that of repellency study given during single plant evaluation. A significant difference observed was that when leaf, extract and powder of Neem given along with the leaf, extract and powder of *Ocimum* respectively increased the repellency upto 5% and thus showed 95% repellency. In addition to this Neem along with other four genera seemed to be raised the repellency effect against the rice weevil but at the same time Eupatorium and Lantana had very less effect on *S. oryzae* even in combination compared to other genera.

MORTALITY STUDY

Mortality study using 5 genera within 2 days generally exhibited below average mortality. The data showing mortality effect has been given in table 3 and its graphical representation is shown in figure 3 and photograph is given in figure 5. Among them over 30% of mortality at 48 hours after treatment was achieved using crushed leaves of *A.indica* and *O.sanctum* as well as leaf extract and powder of *A.indica* and extract powder of *O. sanctum* showed only 20% of mortality. In all the three forms of leaves used, *A.marmelos* and *L.camara* showed only 20% mortality. In the group of five plants least mortality that is 10% was observed in *E. rugosum*.

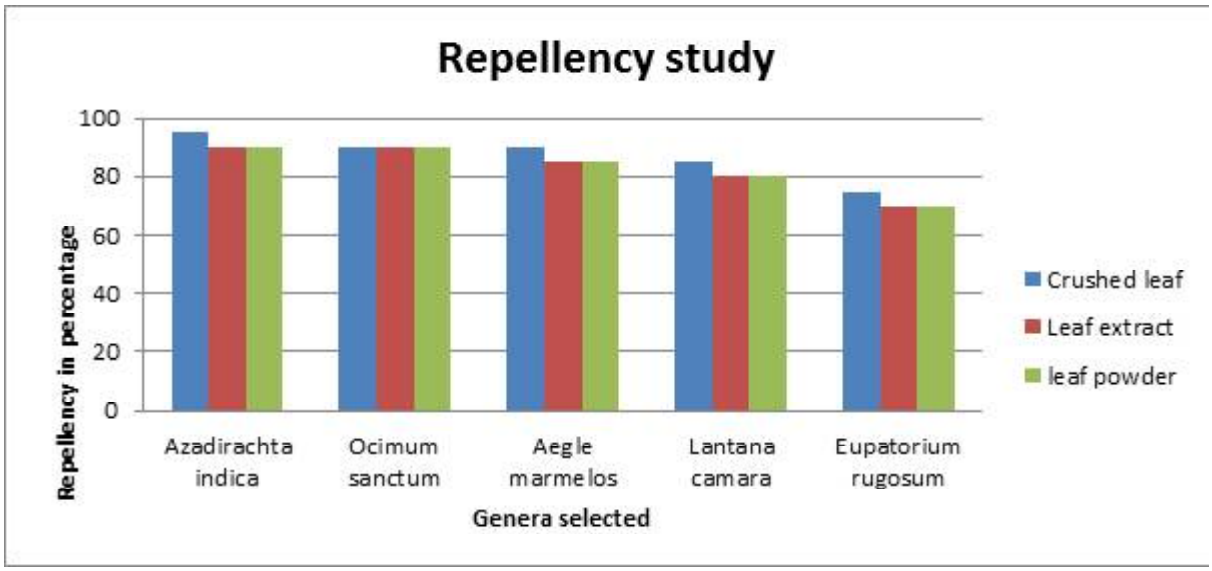

Fig. 1. Graphical representation of repellency percentage

Table 2. Repellency percentage of plant combination used

Plant combinations used	Repellency in percentage (%)		
	Crushed leaf	Leaf extract	Leaf powder
Neem-Ocimum	95	95	95
Neem-Aegle	90	90	90
Neem-Lantana	85	85	85
Neem-Eupatorium	80	80	80
Ocimum-Aegle	85	85	85
Ocimum- Lantana	80	80	80
OcimumEeupatorium	75	75	75
Aegle -Lantana	75	75	75
Aegle –Eupatorium	75	75	75
Lantana-Eupatorium	70	70	70

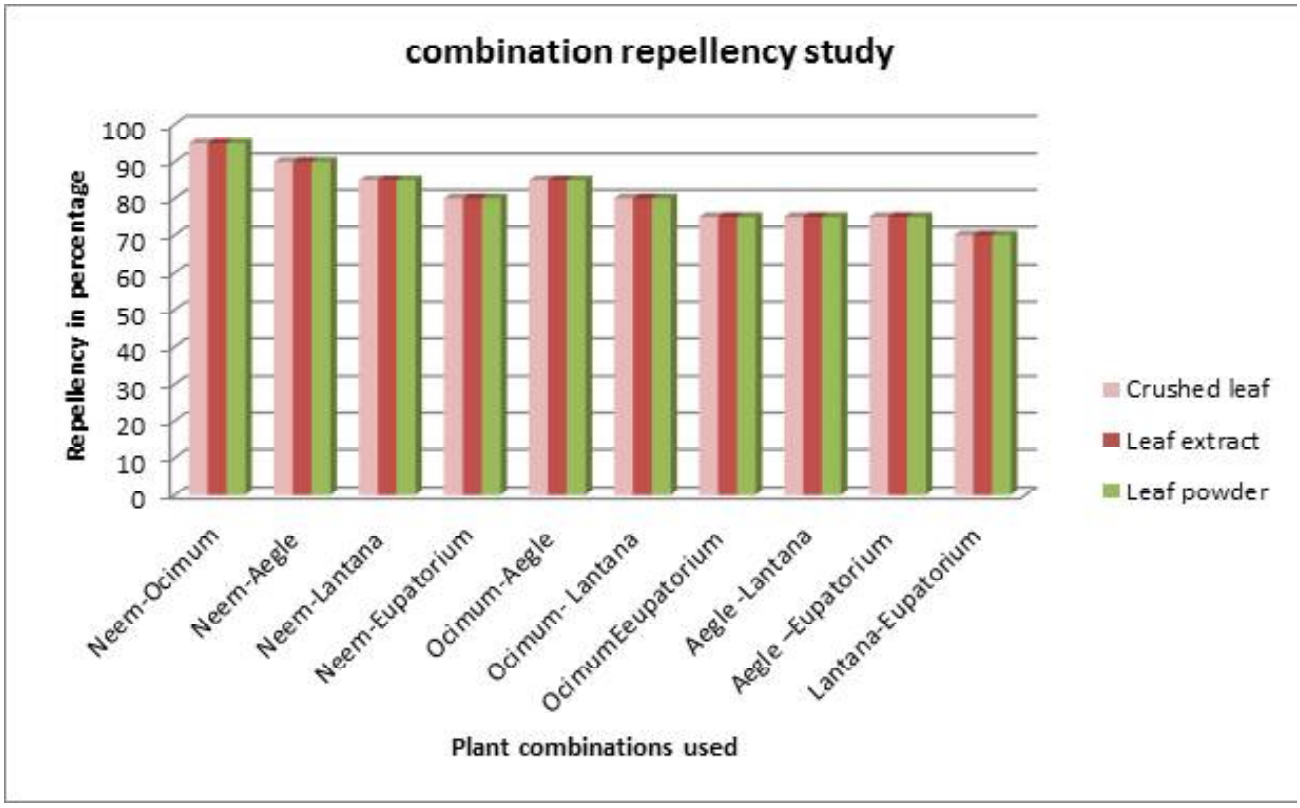

Fig. 2. Graphical representation of repellency in percentage

COMBINATION EFFECT ON MORTALITY

Similar to single plant leaf provided, combination of the selected plants also didn't show variation in mortality percentage. The combination mortality rate is given in table 4 and its graphical representation has been shown in figure 4. The highest mortality percentage observed during the combination effect was 30% in crushed leaf of *Neem- Ocimum, Ocimum-Aegle* and *Aegle -Lantana*. The same percentage could observe in leaf extract and powder form of *Neem* and *Ocimum* combination. 20% mortality activity was exhibited by the three forms used which include *Neem-Aegle, Neem-Lantana* as well as *Ocimum-Aegle* and *Ocimum-Lantana* except its crushed leaf form. All other combination showed only 10% mortality after two days of treatment given to the rice weevil-*Sitophilus oryzae*.

Table 3. Mortality percentage of selected genera Control

Genera selected	Mortality in percentage (%)			
	Control	Crushed leaf	Leaf extract	Leaf powder
A. indica	0	30	30	30
O. sanctum	0	30	20	20
A.marmelos	0	20	20	20
L. camara	0	20	20	20
E. rugosum	0	10	10	10

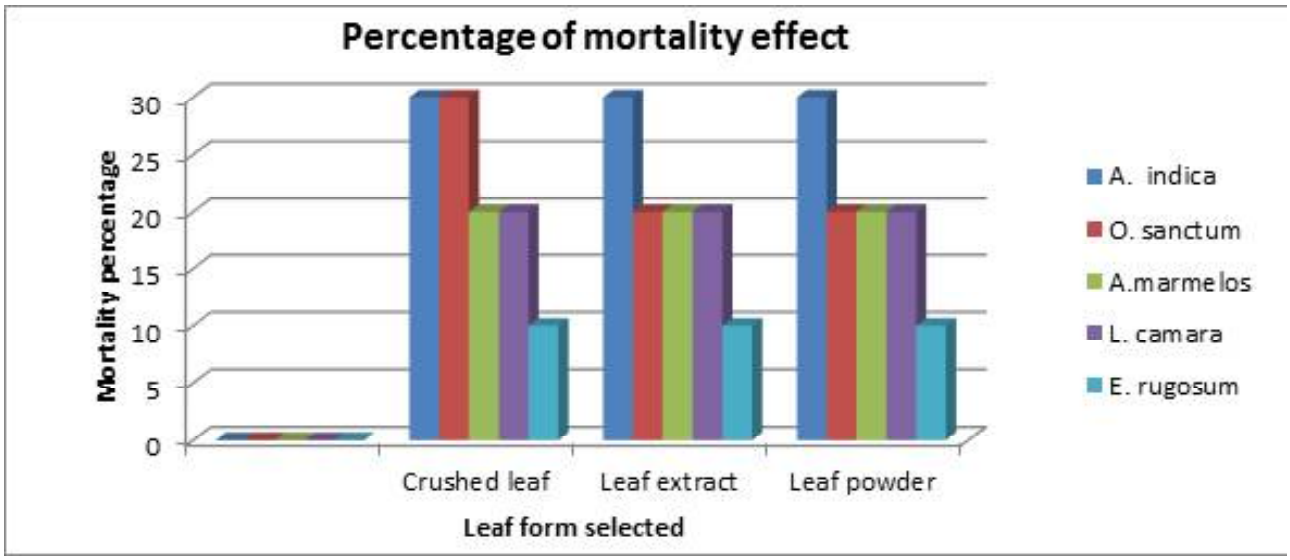

Fig. 3. Graphical representation of Mortality percentage

Table 4. Mortality percentage of plant combination used

Plant combinations	Mortality in percentage (%)			
	Control	Crushed leaf	Leaf extract	Leaf powder
Neem-Ocimum	0	30	30	30
Neem –Aegle	0	20	20	20
Neem -Lantana	0	20	20	20
Neem-Eupatorium	0	10	10	10
Ocimum-Aegle	0	30	20	20
Ocimum-Lantana	0	30	20	20
Ocimum-Eupatorium	0	10	10	10
Aegle-Lantana	0	10	10	10
Aegle- Eupatorium	0	10	10	10
Lantana-Eupatorium	0	10	10	10

DISCUSSION

Plant powders were traditionally used as grain protectants (Isman, 2000, Rajasekhar *et al*, 2012). The mode of action of plant powders may vary but with low to moderate dosages, the effect is repellant or toxic. These studies show parallelism with the present study where leaf powder showed significant insecticidal activity. According to our study leaf powder, extract as well as crushed leaf of plants act against the pest, especially *A. indica* leaves. The similar result was observed by Rahman *et.al* (1999) where they reported that the ethanol extract of *A. indica* and *C. longa* used against *S. oryzae* and *S. granaries* showed repellant effect. Saljoqi *et.al*, in

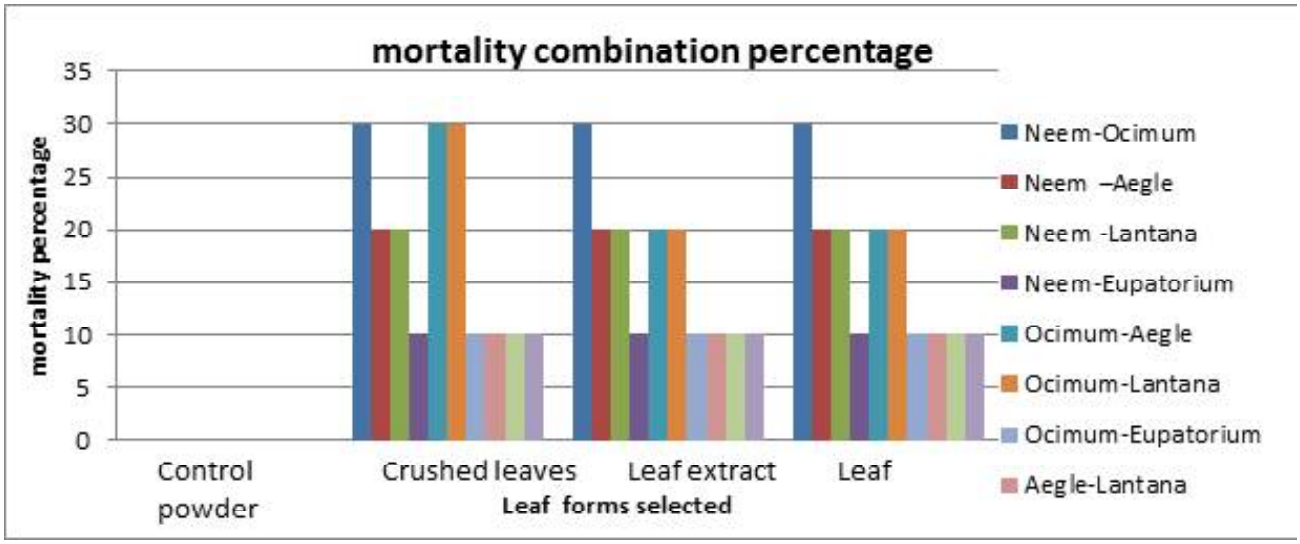

Fig. 4. Graphical representation of mortality percentage of plant combinations used

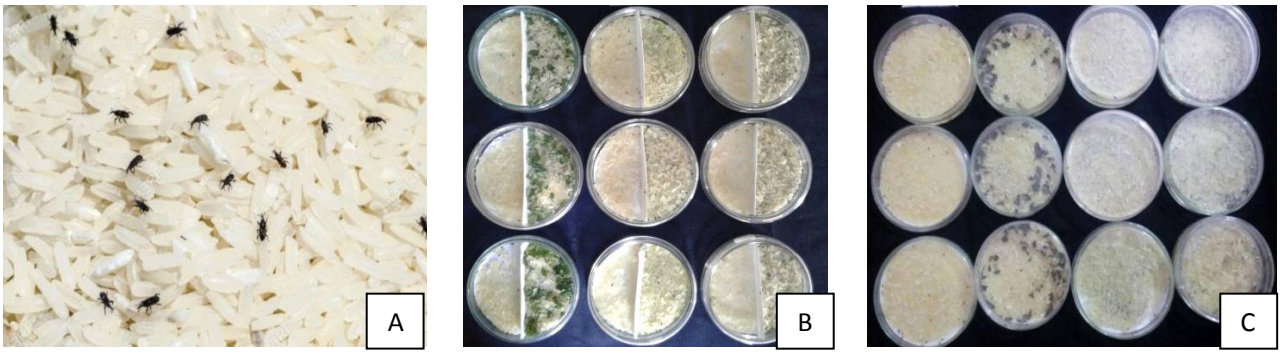

Fig. 5. habit of *Sitophilus oryzae* (A), Repellency study (B), Mortality study (C)

2006 also reported that *M. azadarach* is most effective against *Sitophilus oryzae* on wheat grain.

Many plant extracts and essential oils are known to possess repellent and insecticidal activities against various stored product insects, (Hill and Schoonhoven, 1981; Desmarchelier, 1994). El-Nahal *et al*. (1989) studied the toxic effect of the *A. calamus* essential oil on adults of five stored-product insect species. Asawalam *et al*. (2012) reported that the plant powders can lead to suffocation and death of storage insect pest. *Ocimum tenuiflorum* (Tulsi) recorded 91.7% of weevil mortality 12 weeks after the treatment. In this study also the genus Ocimum exhibited significant levels of repellency and mortality.

All these studies point to the fact that plant product possess significant repellency or mortality action against many stored food pests. The present study also reveals the same.

CONCLUSION

The present study was conducted to analyse the repellency and mortality study of five selected aromatic plants against *S.oryzae*. Many plant extract are known to possess repellent and insecticidal activities against various stored food grains. It is an accepted fact that stored product insect species are a threat to farmers as it causes severe damage to stored food grains. One such storage insect pest is *S.oryzae*, which inhabit the stored rice grain. This species leads to the destruction of millions of stored rice every year. Traditionally there are many ways to avoid pest from stored food.

In conclusion, the present study was an attempt to revive the traditional way of removing the damaging effects of *S.oryzae*, for this screening and evaluation of five plant leaves against the rice weevil *Sitophilus oryzae* has been done. Only the leaf part has been used for repellency and mortality study in three forms, crushed leaf, leaf extract and powdered leaf. All of them exhibited repellent activity against *S. oryzae* adults. The highest repellency as well as mortality percentage was exhibited by *A.indica*. Strong insect repellent activity noted in all the plants used in the study, indicate the presence of active compounds in

the plant components and hence indicate promising sources of plant based insecticides. This result suggests that these active materials have potential to provide grain protectant and may be exploited for rice weevil control in grain storage in an environment friendly way. The future perspective of this work could be the isolation and evaluation of effective compounds from these active plant materials.

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Potential of Pineapple Peel as Natural Resource of Antioxidants

A. S. LUBAINA AND G. S. RESHMA

Department of Botany, Christian College
Kattakada
email : lubainanizam@gmail.com

ABSTRACT

With the increase in production of processed food products, the amount of food wastes generated is increasing alarmingly. Fruit peels are generally considered as wastes and are normally discarded. Pineapple (*Ananas comosus* L.) is one of the most important fruits of total world production, its peel constitute 50 to 65 % of total weight of the fruits. Pineapple peel remains as the primary byproduct left over after processing thrown away as waste are potential source for various phytochemicals. Their characterization and valorization convert them into high value products with application in diverse fields such as pharmaceutics, food or cosmetics and also reduce the environmental impact created by the wastes. The antioxidant potentiality of aqueous and ethanol extracts were evaluated by various antioxidant assays. The aqueous and ethanol extracts showed positive correlation between concentrations of the extracts with DPPH (2, 2- diphenyl 1-picryl hydrazyl) radical scavenging activities. Similarly the other antioxidant potential like FRAP (Ferric reducing antioxidant power), superoxide radical scavenging activity, hydroxyl radical scavenging assay displayed remarkable potentials and were comparable with synthetic oxidants such as ascorbate and gallate. The peels of pineapple hold a tremendous potential to serve as a source of newer, effective, safer and better antioxidant agents which can reduce the hazardous effect of synthetic antioxidants.

Keywords *Pineapple peel; antioxidant potentiality; DPPH; FRAP; ascorbate; gallate.*

Fruit wastes and their by-products are formed in great amounts during industrial processing and its disposal being one of the major problems faced by all parts of our state. These wastes if not disposed properly are seen to cause serious environmental problems such as water pollution, unpleasant odors, explosions and combustion, asphyxiation, and greenhouse gas emissions due to its rapid decay and eventually becoming a source of insect multiplication. So they need to be managed or they can be utilized. Converting organic waste to useful products is a better application rather than they have been land filled.Recycling of food waste is one of the big challenges in agricultural sector. Fruit processing is done to add value to the fresh fruits products in a number of ways viz. canning, drying, freezing and new ingredient creation. In fruit processing industry and fruit shops, fruit peels are thrown or dumped as wastes, but the fact is that the peels are having several biological activities similar to other fractions (Suree, 2010).It is anticipated that discarded fruit waste material can be utilized for further industrial processes like fermentation, bioactive compound extraction etc. In this regard, several efforts have been made in order to utilize fruit wastes obtained from different sources. Fruit peels are a source of sugars, minerals and organic acid, dietary fibers and phenolics which have a wide range of actions which includes antioxidants, antimutagenic, cardio preventive, antibacterial and antiviral activities (Adams *et al.,*2006). Use of fruit waste as a source of polyphenols and antioxidants may have considerable economic benefit to food processing. The antioxidant potentialities of fruit wastes have produced the basis of several applications in pharmaceuticals, alternative medicines and natural therapy.

Since pineapple (*Ananas comosus L.*) has the second highest production volume of all tropical fruits in the world, the production of processed items results in massive waste generation, estimated about 40-50 % from fresh fruit as peels and core. The pineapple waste is either used as animal feed or disposed to the soil as a waste that can cause environmental problems. Indeed, efforts have been attempted in order to utilize pineapple wastes, which have already been used as the substrate for the production of bromelain and organic acids (Dacera etal., 2009), bio-ethanol and biogas (Nigam, 1999). The pineapple residues with rich in sugars such as sucrose, glucose and fructose and other components like minerals and vitamins (Abdullah and Hanafi, 2008). The pineapple peel contained an appreciable amount of insoluble fibre-rich fraction which primarily consisted of cellulose, pectin substances, hemicellulose, and notable proportions of lignin **(**Huang *et al.,* 2011**).** In this scenario, the present study, aims to recycling pineapple wastes for evaluating their antioxidant potentialities thereby introducing an eco-friendly process technology from pineapple peel.

MATERIALS AND METHODS

Plant material

The material used for study was the peel of queen variety of pineapple fruit collected from fruit processing industy at Vazhakulam, Muvattupuzha, Kerala which is the main cultivation region of pineapple in Kerala.

Soxhlet hot continuous extraction

250 g pineapple peel were finely chopped, air dried in shade at room temperature, powdered and successively extracted with 100 ml of ethanol and water for eight hours using soxhlet hot continuous extraction method. The extracts were lyophilized and were stored at 4^0C for further studies.

Antioxidant potentiality

Evaluation of DPPH radical scavenging activity

The free-radical scavenging activity of the different concentration of three extracts were measured with the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) in terms of hydrogen- donating or radical-scavenging activity. 3 ml of 0.2 mM DPPH in methanol was added to 100 µl of different extracts, at different concentrations (12.5-200 µg/ml). After 30 min, the absorbance was measured at 517 nm according to the procedure of Soler *et al.*, (2000) with slight modifications. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The antioxidant activity of the extract was expressed as IC_{50}, which was defined as the concentration (µg/ml) of extract that inhibits the formation of DPPH radicals by 50%. Ascorbic acid used as reference compounds. Each study corresponded to three experiments, performed in triplicate. The scavenging activity was estimated based on the percentage of DPPH radicals scavenged by the following formula:

$$\% \text{ scavenging} = [(A_0 \text{ “ } A_s) / A_0] \times 100$$

Where A_0 is absorption of control, A_S is absorption of tested extract solution.

Ferric reducing antioxidant power (FRAP)

The reducing capacity of the different concentration of three extracts was measured following the method of Benzie and Strain (1996). The method was simple and reliable measures the reducing potential of an antioxidant reacting with a ferric 2,4,6 - tri-pyridyl-S- triazine [Fe(III)-TPTZ] complex and producing a coloured ferrous 2,4,6-tripyridyl-S-triazine [Fe(II)-TPTZ] complex by a reductant at low pH, was adopted. The assay was carried out in a total volume of 1.0 ml containing a suitable aliquot of flavonoid extract (at concentration dependent (125-2000 µg/ml) in of freshly prepared FRAP reagent (prepared by mixing 10 volume of 22.78 mM sodium acetate buffer, pH 3.6, 1.0 volume of 20 mM ferric chloride and 1.0 volume of 10 mM 2, 4, 6-tripyridyl- s-triazine solution prepared in 40 mM HCl (in a ratio of 10ml : 1 ml : 1 ml). Before starting the reaction, both FRAP reagent and reagent blank were pre-incubated for 5 min at 30ºC. Subsequently different concentration of samples were added to the FRAP reagent and incubated at 37ÚC for 1 min. Absorbance was recorded at 593 nm against reagent blank. Quercetin was used as standard for calculating the total antioxidant power.

Estimation of superoxide radical scavenging activity

Superoxide scavenging was determined by the nitroblue tetrazolium (NBT) reduction method (Liu (1997). The reaction mixture consisted of 1 ml of NBT solution (l M NBT in 100 mM phosphate buffer, pH 7.4), 1 ml NADH solution (l M NADH in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of different concentrations of the flavonoid extracts and ascorbic acid (50 mM phosphate buffer, pH 7.4) was mixed. The reaction was started by adding 100 µl of PMS solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) in the mixture. The tubes were uniformly illuminated with an incandescent visible light for 15 min and the OD was read at 530 nm before and after the illumination. The % inhibition of superoxide generation was evaluated by comparing the absorbance values of the control and experimental tubes. The abilities to scavenge the superoxide radical were calculated by using the following formula:

$$\% \text{ scavenging} = (1 - A_e/A_0) \times 100$$

Where A_0 is the absorbance without sample, and A_e is absorbance with sample.

Hydroxyl radical scavenging activity

The effect of hydroxyl radicals of different concentration of three extracts was assayed by using the deoxyribose method Elizabeth and Rao (1990).2-Deoxyribose is degraded on exposure to hydroxyl radicals generated by Fenton’s reaction. Controls were

prepared in methanol. The reaction mixture contained 450 µl of 0.2 M sodium phosphate buffer (pH 7.0), 150 µl of 10 mM 2-deoxyribose,150 µl of 10 mM $FeSO_4$-EDTA, 150 µl of 10 mM H_2O_2, 525 µl of H_2O, and 75 µl of sample solution (125-2000 µg/ml). The reaction was started by the addition of H_2O_2. After incubation at 37ºC for 4 h, the reaction was stopped by adding 750 µl of 2.8% trichloro acetic acid and 750 µl of 1%TBA in 50 mM NaOH, the solution was boiled for 10 min, and then cooled in water. The absorbance of the solution was measured at 520 nm. Gallic acid used as positive controls. The ability to scavenge the hydroxyl radical was calculated using the following equation:

% Hydroxyl radical scavenging activity = (1-absorbance of sample/absorbance of control) x 100

RESULTS AND DISCUSSION

Antioxidant activity

Phenolic compounds are the most wide spread secondary metabolites in the plant kingdom. Phenolic content can be used as an important indicator of antioxidant capacity and can be used as a preliminary screen for any product when intended to be used as a natural source of antioxidants in functional foods (Viuda-Martos *et al.,* (2011). It is believed that the phenolic and/or polyphenolic compounds biosynthesized in the plant sample might be responsible for antioxidant activity. Many methods have been proposed to evaluate the antioxidant potentiality of natural sources of antioxidants. However, the antioxidant capacity of plant extracts cannot be evaluated by any one single assay because of the complex nature of phytochemicals present in them and solvent used for extraction . Therefore, it is essential that in order to assess the antioxidant capacity of any plant, more than one solvent and more than one antioxidant assays had to be performed (Chanda and Nagani, 2010). Besides, well known and traditionally used antioxidants from tea, fruits, vegetables and spices, some natural antioxidant are already exploited commercially either as antioxidant additives or as nutritional supplement (Rahman *et al*., 2012). Fruits represent one such source of dietary antioxidants thereby necessitating the call for their increased consumption in recent times. The growing demand for natural antioxidants observed in food industry, forces the search for new sources of these compounds. As fruit peels are rich in many constituents including the phenolic and flavonoid compounds, it can be served as a good natural antioxidant. Therefore various antioxidant protocols such as DPPH scavenging activity, FRAP assay, superoxide radical scavenging activity and hydroxyl radical scavenging activity were used for the determination of antioxidant potentiality of aqueous and ethanol the extracts from pine apple peel

DPPH

The free radical chain reaction is widely accepted as a common mechanism of lipid peroxidation. Radical scavengers may directly react with and quench peroxide radicals to terminate the peroxidation chain reaction and improve the quality and stability of food products (Soares *et al*., 1997). DPPH has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances. In the DPPH assay, the antioxidants were able to reduce the stable radical DPPH to the yellow coloured diphenyl-picrylhydrazine. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH–H by the reaction. DPPH is usually used as a reagent to evaluate free radical scavenging activity of antioxidants. Aqueous and ethanol extract of pineapple peel showed varying level of scavenging activities over DPPH free radicals (45.85± 0.76% and 54.74± 1.5% at 200ig/ml respectively) (Table1).There scavenging activities might be due to the presence of phenolic content. The results of the DPPH scavenging activities of pineapple extract are at par with that of ascorbic acid. Here the alcohol extract showed high scavenging activity of DPPH radicals compared to that of aqueous extract which may attributable to its significant hydrogen donating ability (Table 1). It could be inferred from the results that increasing the concentration of the extract reduce the level of the free radical in the mixture, that would ultimately raise the potential to scavenge or chelate free radicals by the pineapple extract, hence an increase in the antioxidant properties. Gnanasaraswathi *et al*., (2014) studied the DPPH radical scavenging activity of toluene and methanol extract of fruit peels from orange, papaya and mixed fruit. The results obtained by them were similar to the results obtained from ethanol extract of the present study.

Table 1. DPPH radical scavenging activity of water and ethanol extract of pineapple peel.Values are mean ± SD of three independent replications

Concentration (µg/ ml)	% of inhibition± SD		
	Ascorbate (standard)	Aqueous extract	Ethanol extract
12.5	7.29±0.18	11.41± 0.05	18.80± 0.23
25	15.83±0.32	18.53± 0.21	22.07± 0.34
50	20.28± 0.41	25.23± 0.43	28.92± 0.84
100	56.04± 0.54	36.26± 0.54	38.24± 0.79
200	62.98± 1.2	45.85± 0.76	54.74± 1.5

FRAP

Antioxidants can be explained as reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species (oxidant) is reduced at the expense of the oxidation of another antioxidant. The FRAP assay measures the antioxidant effect of any substance in the reaction medium as reducing ability. Antioxidant potential was estimated from their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) complex (Siddhuraju and Becker, 2007). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging. Ferric reducing power assay shows the ability of the extract to donate electron to ferric ion, reducing it into ferrous ion. . Ethanol extract of pineapple peel showed the highest ferric reducing power (139.53± 1.02% at 2000 µg/ml), followed by water (116.12± 1.41% at 2000 µg/ml) (Table 2). The values are comparable with antioxidant potentialities of many plant products (Muhammad *et al.,*2008).

Superoxide radical scavenging activity

The superoxide anion scavenging ability of the extracts was determined using SOD assay kit-WST. Superoxide dismutase (SOD) is an enzymatic antioxidant that can scavenge superoxide anion radical (O_2^{-}) by catalyzing the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. Superoxide (O_2^{-}) radical is known to be harmful to cellular components and act as a precursor of the more reactive oxygen species, contributing to the tissue damage and various diseases. This assay is based on the measurement of superoxide dismutase inhibition activity. In this assay, the superoxide anion reduce WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2.4-disulfophenyl)-2H- tetrazolium) to generate the water-soluble formazan dye in the testing solution, which is measured using spectrophotometer at 560 nm. In the presence of an enzymatic antioxidant, the reduction of WST-1 can be inhibited by neutralizing O_2^{-}.Thus, the SOD activity can be quantified by measuring the decrease in the color development at 560nm.The ethanol extract of pineapple peel exhibited the highest superoxide anion scavenging ability with inhibition rate of 73.12± 1.01% at 2000 ìg/ml than the aqueous extract (52.25± 0.54%at 2000ìg/ml) (Table 3). Chanda and Dave (2009) reported the antioxidant and antimicrobial potential of pomegranate peel extract. It showed strong

Table 2. FRAP potential of water and ethanol extracts of pineapple peel. Values are mean ± SD of three independent replications

Concentration (µg/ ml)	% of inhibition± SD		
	Quercetin (standard)	Aqueous extract	Ethanol extract
125	59.45± 0.98	41.76± 0.58	44.68± 0.23
250	63.57± 0.69	47.67± 0.41	50.19± 0.54
500	88.19± 1.12	56.09± 0.72	66.15± 0.89
1000	96.68± 1.3	78.57± 0.87	87.07± 0.94
2000	158.76± 0.99	116.12± 1.41	139.53± 1.02

Table 3. Super oxide anion radical quenching effects of water and ethanol extracts of pineapple peel.Values are mean ± SD of three independent replications

Concentration (µg/ ml)	% of inhibition± SD		
	Ascorbic acid(standard)	Aqueous extract	Ethanol extract
125	41.19± 0.12	23.08± 0.35	21.19± 0.65
250	47.53± 0.34	33.47± 0.87	38.68± 0.57
500	62.70± 0.59	35.50± 0.64	49.01± 0.68
1000	76.32± 0.44	39.29± 0.76	56.45± 0.47
2000	90.68± 0.35	52.25± 0.54	73.12± 1.01

Table 4. Hydroxyl radical scavenging activities of water and ethanol extracts of pineapple rind.Values are mean ± SD of three independent replications

Concentration (µg/ ml)	% of inhibition ± SD		
	Gallic acid(standard)	Aqueous extract	Ethanol extract
125	7.29± 0.09	11.41± 0.20	18.80± 0.34
250	15.83± 0.12	18.53± 0.42	22.07± 0.52
500	20.28± 0.35	25.23± 0.36	28.92± 0.49
1000	56.04± 0.64	36.26± 0.59	38.24± 0.67
2000	62.98± 0.91	45.85± 0.78	54.74± 0.59

superoxide scavenging ability as it could scavenge 90% of superoxide radical at a concentration of 50ìg/ml and the result was comparable to that of aqueous extract of pineapple peel.

Hydroxyl radical scavenging activity

The hydroxyl radical is a highly damaging species in free radical pathology, capable of damaging biomolecules of the living cells. Hydroxyl radical reacts with nucleotides in DNA and cause strand breakage leading to carcinogenesis, mutagenesis and cytotoxicity. Antioxidant activity of aqueous and ethanol extracts of pineapple peel by hydroxyl radical scavenging activity was presented in Table 4. Both extracts exhibited good hydroxyl radical scavenging activity which was dose dependent. Ethanol extract and water extract have moderate hydroxyl radical scavenging effects up to the doses in comparison to control set. The activity was found to increase at higher dose levels. Ethanol extract of pineapple was the most effective (54.74± 0.59% at 2000ìg/ml) over aqueous extract (45.85± 0.78% at 2000ìg/ml). The scavenging activity obtained for gallic acid standard was 62.98± 0.91%at 2000ìg/ml.

CONCLUSION

Pineapple peels are good sources of phenolic compounds that have very potent antioxidant activity. The aqueous and ethanol extracts of pineapple peel showed varying antioxidant properties in the entire antioxidant assay. Among them ethanol fraction showed the greatest quenching activity in terms of DPPH, FRAP, Hydroxyl radical scavenging activity and Superoxide radical scavenging activity. From the antioxidant activity of the peels, it can be concluded that the peels may have potentials to manage and prevent oxidative stress.

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In Vitro Culture Establishment and Regeneration in *Riccia billardieri* Mont. & Nees Ex Gottsche, Lindenb. & Nees.

MAHESH S., MURUGAN K, LAIJA S NAIR & MINI I

Post graduate and Research Department of Botany, University College, Thiruvananthapuram, Kerala, India- 695 034
*email:*maheshlaija@gmail.com

ABSTRACT

***Invitro* culture as a tool for conservation is very common, but its applications in Bryophytes has been rather limited. This study was aimed at standardising surface sterilisation protocols using Sodium dichloroisocyanurate. Regeneration patterns were also analysed during 4 weeks of incubation. The gametophytes of *Riccia* were grown on basal MS media with half strength macro salts lacking a sugar source. Plantlets regenerated over a 3 week culture period on a 16 hr light and 8 hr dark photoperiod. The resulting plantlets, showed dichotomous branching but lacked the typical rosette like colonies. Rhizoid development was also recorded by the end of four weeks.**

Key words *Culture Establishment, Regeneration, Riccia billardieri, Lindenb. & Nees.*

The use of *in vitro* culturing for conservation of plant species ranging from the most primitive bryophytes, to the most evolved angiosperms has been increasing steadily for the past few decades, primarily due to the recognition granted to such methods by global initiatives like, The Convention on Biological Diversity and Global Strategy for Plant Conservation.

In spite of a prominent role in biogeochemical cycling and a ubiquitous terrestrial presence, *invitro* conservation studies on Bryophytes are indeed a rarity in comparison to angiosperms. Bryophytes, non-vascular, non-flowering plants, are often over looked in conservation initiatives (Tan and Pocs, 2000). The rampant habitat destruction and interruption or termination of developmental or reproductive stages due to anthropogenic or natural causes are major reasons for the loss of these minute plants. *In situ* conservation of bryophytes are as such an untried and untested area of study at least in Indian conditions, where adequate taxonomic inventories themselves are lacking. So the need for the hour is to develop conservation protocols, including *invitro* systems for these plants, like those which exist in many European countries.

Bryophytes in spite of their insignificant size are rich storehouses of a cornucopia of chemicals many of which show novel biological action (Asakawa, 2012). Even among the bryophytes, liverworts are a discriminated group as far as biochemical prospecting or conservation studies are concerned. Only about 10% of liverwort species have been chemically analysed, although they are potentially rich sources of new secondary metabolites. In the present study, an attempt has been made to the *invitro* culture of *Riccia billardieri,* a thalloid liverwort changing the time and concentrations of sterilising agent, with an aim to standardise these conditions to aid in direct regeneration of the plant.

MATERIALS AND METHODS

Fresh green gametophytes of *Riccia billardieri* were collected from the Kallar riverine belt of Thiruvananthapuram district. The plants were replanted in pots for three weeks with riverine sand as the potting media which helped in eliminating major contaminants from the potential explants. Healthy looking plants from these were selected for *invitro* manipulation.

In vitro culture

Media and glasswares were sterilized by autoclaving at 120^0C and 15 lb/sq.in. for 25 min. Selected thalli were washed thoroughly for 1 hr under running tap water, before being transferred to conical flasks containing 0.5% Tween 20 (v/v) with constant agitation for 10 minutes. Sterile distilled water was used to rinse the material 3 to 4 times, to remove all traces of the detergent. Surface sterilisation using SDICN, at 0.05%, 0.1%, 0.5% and 1% were studied, for treatment times of 15, 30 sec, 1, 3 and 5 minutes respectively. This was followed by rinsing using sterile double distilled water thrice, after which the gametophytes were observed for any visible colour changes. Thalli which showed slight yellowing at the margins were discarded. Green thalli were transferred to covered petri plates and left aside for inoculation. Sterile scalpels were used to excise approximately 3 inch pieces containing the growing initials at the apical notch region of the thalli for use as explants.

Media composition and incubation

MS basal media (Murashige and Skoog, 1962) with 1/2 strength macro salts, lacking exogenous sugar

supplementation, and 0.4% Gelrite was used for culturing. Addition of a carbon source is still a primary requirement in conventional micropropagation systems for most plants, although it is unnecessary for most bryophytes (Duckett et al., 2004). The semi solid transparent media allowed easy visual inspection of the developing plantlets. Subsequent to inoculation the cultures were incubated at 22^0C in a photoperiod of 16 hrs light and 8 hrs of darkness. 10 replicates for each test condition, were maintained. Cultures were inspected daily for visible signs of contamination and/ or regeneration, for a period of three weeks. Cultures which showed visible regeneration were subcultured using the same media after three weeks.

RESULTS AND DISCUSSION

Standardisation of concentration and time of action of SDICN

Survival rates of explants in this study were taken to be a combination of greenish thalli which survived without visible contamination after 1 week of incubation. Algicides and fungicides were not included for sterilisation as they have been reported to induce somaclonal variations, so also exogenous hormones. Continued subculturing, treatment with PGR and other additives, and intervening callus phase can cause genetic changes in plants and lead to progenies with altered characteristics, referred to as somaclonal variation. Somaclonal variation is unpredictable in nature and can be both heritable (genetic) and non-heritable (epigenetic) (Jain, 2001). It is a problem for all *in vitro* grown plants, but is of particular concern for conservation collections, where production of clonal material is desired for re-establishment programs (Edson *et al*. 1996). Direct regeneration without any intervening stages were the final results aimed at in this study, and hence only surface sterilisation protocols were followed, to reduce the impact of chemicals on the explants.

A combination of sterilant concentration and time of action was evaluated for maximum survival percentage of explants after one week of inoculation. Although SDICN is non phytotoxic (Parkinson et al., 1996), the complete absence of secondary and protective tissues in *Riccia* resulted in significant yellowing and death of explants. A lower treatment time on the other hand resulted in higher percentage of contamination.

Exogenous and endogenous contamination of culture by micro organisms like fungi and algae are the biggest problem in the tissue culture of bryophytes. Use of regular phyto disinfectants like sodium or calcium hypochlorites and mercuric chloride, cause extreme tissue damage which are often fatal for bryophytes. This to a large extent has resulted in unearthing novel and less phytotoxic chemicals like Sodium dihydroisocyanurate (SDICN). SDICN has proved to be a reliable sterilizing agent (Parkinson et al., 1996; Niedz and Bausher, 2002) and has been used effectively to initiate plants from many taxonomic groups into axenic culture. Phytotoxicity of the compound is low, making it ideal for use where plant material is limited or rare (Parkinson et al., 1996). Survival rate of explants at more than 90% were observed in treatments of 0.05, 0.1 and 0.5% SDICN upto 1 minute (Table 1). As the time of treatment increased there was an expected drop in survival percentage, possibly due to phytotoxicity. Treatment times of above 3 minutes resulted in significant yellowing and gradual loss of vigour in the explants over a period of 3 weeks. Parkinson (1996) has reported that SDICN although non phytotoxic can penetrate and kill tissues on older portions and cut ends of explants. Survival rates had to be balanced with percentage of explants which survived without visible contamination, to arrive at an optimal combination for standardisation. Survival rates of 90% and above among the explants, were observed for all treatments by SDICN with a treatment time less than 3 minutes (Table 1). The contamination rates by fungi fell to 1% when 0.1% SDICN was allowed to act upto 3 minutes (Table 2). Significantly the above treatment allowed for survival of 98% of explants without visible fungal contaminants, hence this combination was standardised as the optimal method for sterilisation in this study.

Explant development

The use of *invitro* culture as a tool for rapid regeneration and multiplication of bryophytes dates back to the development of such techniques as reported by Lal (1984). In comparison to many vascular plants, bryophytes are relatively easy to culture and a number of initiation and propagation protocols have been published (Basile and Basile 1988; Kowalczyk *et al*.1997; Sabovljevic *et al*. 2003; Duckett *et al*. 2004; Rowntree 2006; Sabovljevic *et al*. 2009). The explants which became depigmented during sterilisation, developed a green colouration by the 3rd day and thallus initiation was noticed by the 5th day after inoculation, single as well as multiple points

Table 1. Effect of SDICN concentration & time of action on Explant survival (%).

Time of Action	Concentration of SDICN % (w/v)			
	0.05	**0.1**	**0.5**	**1**
15 sec	95	95	90	90
30 sec	95	95	90	90
1 minute	90	90	80	85
3 minutes	87	98	75	70
5 minutes	60	85	75	60

of germination were visible (Plate1. a,b). Growth was restricted to the apical notch regions of the explants. Expansion and enlargement of the growing points were visible during the 3 weeks before subculturing. By the 14th day the growing points started showing typical dichotomous branch initiation, although a definite median furrow was lacking as in normal terrestrial thalli (Plate 1. c,d). The colour of the points varied from light green to dark green. Rhizoidal initiation was also noticed on some of the explants. The rhizoids appeared as hyaline threads, significantly from all over the thalloid mass (Plate 1. e,i). Those explants which were typically dorsiventrally oriented lacked rhizoidal masses, they showed scanty growth of rhizoids that too from the ventral side adnate to the medium. This peculiar growth pattern of the rhizoids were reported in European liverworts (Rowntree, 2006). 4 weeks after inoculation, the regenerating explants were sub cultured onto the same media, to prevent media exhaustion and accumulation of chemical leachates. Lateral emergences showing typical dichotomous branching patterns were visible by this time. *Riccia* in nature tends to form compact rosettes in young colonies, before this growth pattern is disrupted. In the present study the thalloid masses although showing typical dichotomous branching, never attained the compact rosette shapes (Plate 1. g,h). Dichotomous branching occurred only in those gametophore regions, which were in contact with the media, a finding which was earlier noticed and reported in *Marchantia linearis* by Krishnan & Murugan (2014). The aerial portions of the thallus developed into an amorphous mass, and regenerated masses of hyaline rhizoids (Plate 1.i).

In comparison with plantlets produced by conventional systems, those produced by photoautotrophic systems with a sugar-free medium showed, in many cases, better growth, higher quality, lower contamination rate *in vitro*, and higher percentage survival *ex vitro* (Aitken-Christie *et al.*, 1995; Zobayed *et al.*, 2004). In the present study a start has been made in this direction, by standardisation of a sterilisation protocol and regeneration of thalli clones. The study warrants further emphasis on the anatomical and phytochemical aspects of the cultured plantlets *vis a vis,* normal field grown specimens.

Table 2. Contamination (%).

Time of action	Concentration of SDICN % (w/v)			
	0.05	**0.1**	**0.5**	**1**
15 sec	100	85	85	70
30 sec	100	60	70	62
1 minute	100	15	10	40
3 minutes	98	1	0	0
5 minutes	90	0	0	0

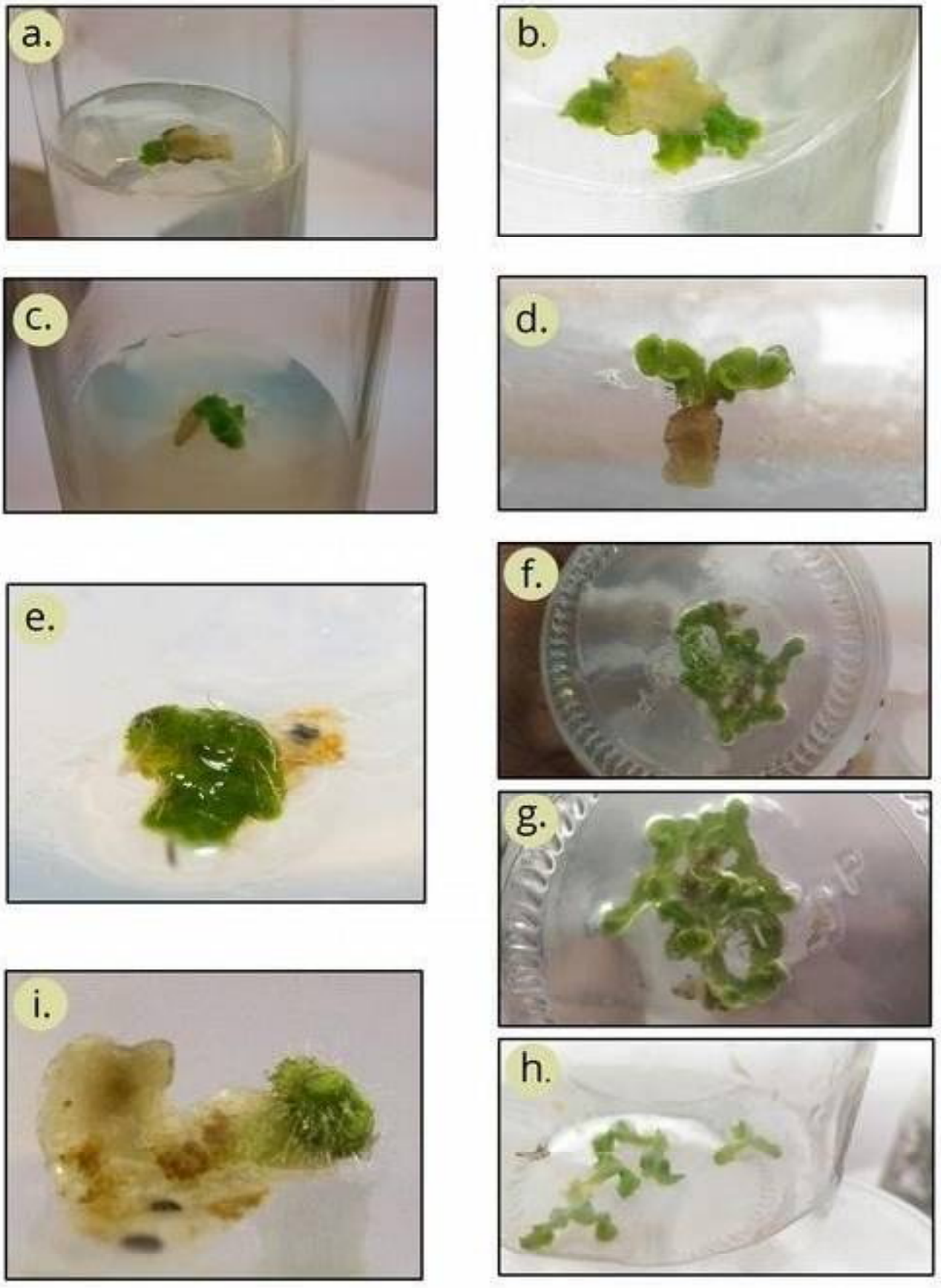

Plate a. Single initiation, b. Multiple points of initiation, c. Dichotomous growth initiation, d. Thalloid growth & enlargement; e. Hyaline rhizoid initiation; f. Scale outgrowhts from ventral thallus surface; g&h. Thalus proliferation & spread; i. Rhizoid proliferation from regenerated mass.

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A Potential Landscape for Medicinal Plants Diversity of Andavar Malai, Gobichettipalayam, Erode District, Tamil Nadu

MONICA, K.[1], P. VEKASHENI[2], K.P. ASHWIN KUMAR[1] AND K. KARTHIKA[1*]

[1]*Department of Botany, Kongunadu Arts and Science College, Coimbatore, Tamil Nadu.*
[2]*Department of Botany, Bharathiar University, Coimbatore, Tamil Nadu.*
[*]*email : karthika1431989@gmail.com*

ABSTRACT

The present investigation was aimed to explore the vast knowledge about the medico potentiality of the plants was undertaken in Andavar malai, Valliampalayam, Gobichettipalayam, Tamil Nadu during September 2016-March 2017. This investigation has resulted in providing the information on 100 plant species belongs to 46 families and 86 genera. These plants were used for curing various diseases like skin disease, diabetes, ulcer, malaria, jaundice and asthma.

Keyword *Andavar malai, Gobichettipalayam and Medicinal Plant Diversity.*

India is blessed with a variety of aromatic and medicinal plants. Owing to this, India is considered as a Botanical Garden of the world and a treasure house of the biodiversity. They considered as a local heritage with global importance. It is endowed with a rich wealth of medicinal plants. They also play pivotal role in the lives of people. The Western Ghats region is known for the wealth of the biodiversity and is known as one of the 18 hotspot of biodiversity recognized as the globe. It is estimated to harbor approximately 2000 known species, of that nearly 1800 species of higher plants are listed in the red data book in that 171 are known from Tamil Nadu.

Herbal medicine has long being recognized as one of the oldest forms of remedies used by humans (Eisenberg *et al*., 1998). There is a abundant undocumented traditional knowledge of herbal remedies used to treat diseases in most cultures (Raul *et al*., 1990). Plant represents an enormous pool of natural resource that can produce various products and chemical for the advantage of all life forms and ethno botany reveals historical and present plant use to fulfill a wide variety of human needs (Kumar *et al*., 2012).

World Health Organization (WHO) reported that 80% of the world populations depends on indigenous medicine and that the majority of traditional therapies involve the use of plants extract or of their active constituents (WHO, 2003; Rahman *et al*., 2013). There are over 2000 species of wild edible plants in the world, yet fewer than 20 species now provide 90% of our food (Ladio and Lazada, 2004). Utilization of plants for medicinal purpose in India has been documented long back in ancient literature because they are essential for human survival (Ahmad, 1999).

Documentation of indigenous and traditional knowledge is very important for the future critical studies leading to sustainable utilization of natural resource and to face the challenges of biopiracy and patenting indigenous and traditional knowledge by others. Knowledge on the use of medicinal plants is enormous but if this is not rapidly researched and recorded, indications are that it will be lost with succeeding generations. Considering the above facts, the main objectives of this study is to identify and documenting the plant species used for the treatment and prevention of various diseases and ailments in the study area of Andavar Malai, Gobichettipalayam, Erode.

MATERIALS AND METHODS

Study area

Valliampalayam of Gobichettipalayam is situated at 699 ft, 213 m above msl in Erode District of Tamil Nadu. Western Ghats forms the border of the region resulting several hillrocks and Bhavani river traverses across the region. It lies in 11.45^{o}N longitude and 77.43^{o}E latitude. Temperature is moderately warm, except during summer months when it is hot. Rainfall is moderate to high; soil mainly consists of black loam, red loam and red sand. In general, soil in and around is fertile and good for agriculture. The study was conducted in the Andavar malai located in the village of Valliampalyam, Gobichettipalayam, Tamil Nadu. It possess a good biodiversity of plants, birds, animals, herbal plants, *etc*., (Fig. 1).

Fig.1. The study area, Andavar Malai, Gobichettipalayam, Erode.

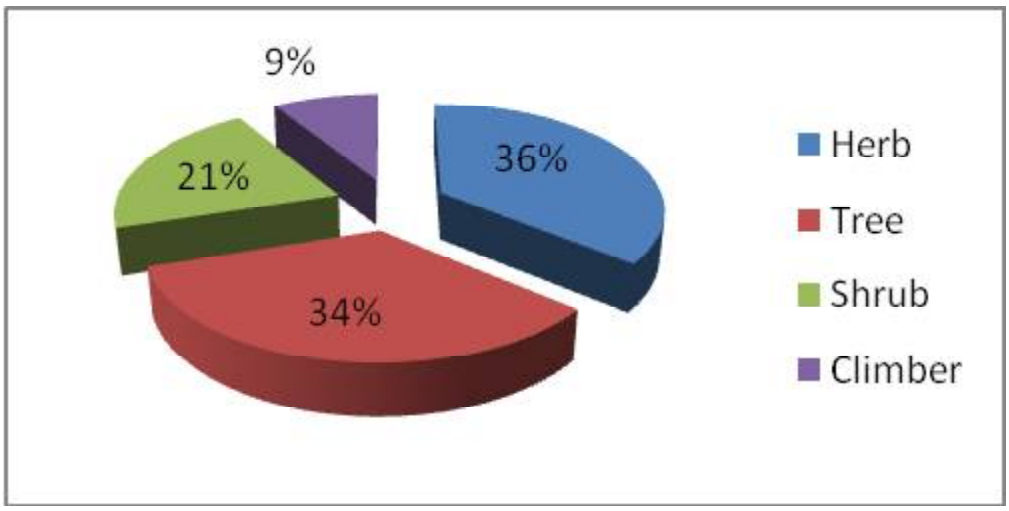

Fig. 2. Life form analysis of medicinal plants of study area.

Data collection

The wealth of medicinal plants knowledge among the people of these hills are based on 500 years of beliefs and observations. This knowledge has been transmitted orally from generation to generation. After seeking their consent, the traditional medicine practitioners were interviewed using semi-structure questionaries and open ended conversations. The informants are the custodians of indigenous knowledge on herbal medicines. Traditional healers are divided into two broad groups of herbalists who mainly use herbs while diviners also invoke ancestral sprits to guide them in their healing.

During the field survey, old and experienced persons were interviewed and cross checked in a way to explore their traditional knowledge, habitat, medicinal uses of the plants, their status, *etc*., The photographs of the plants were taken under natural habitat. Each plants presented were provided with botanical nomenclature followed by author citation. The vernacular names of the plants were given in tamil and english with their medicinal uses and ailments treated.

RESULT

The present investigation has been undertaken during September 2016 to March 2017 and it has resulted in providing information on 100 plant species (Table 1 and Plate I). These species belonging to 46 families and 89 genera were utilized by the people communities of Valliampalaym village,

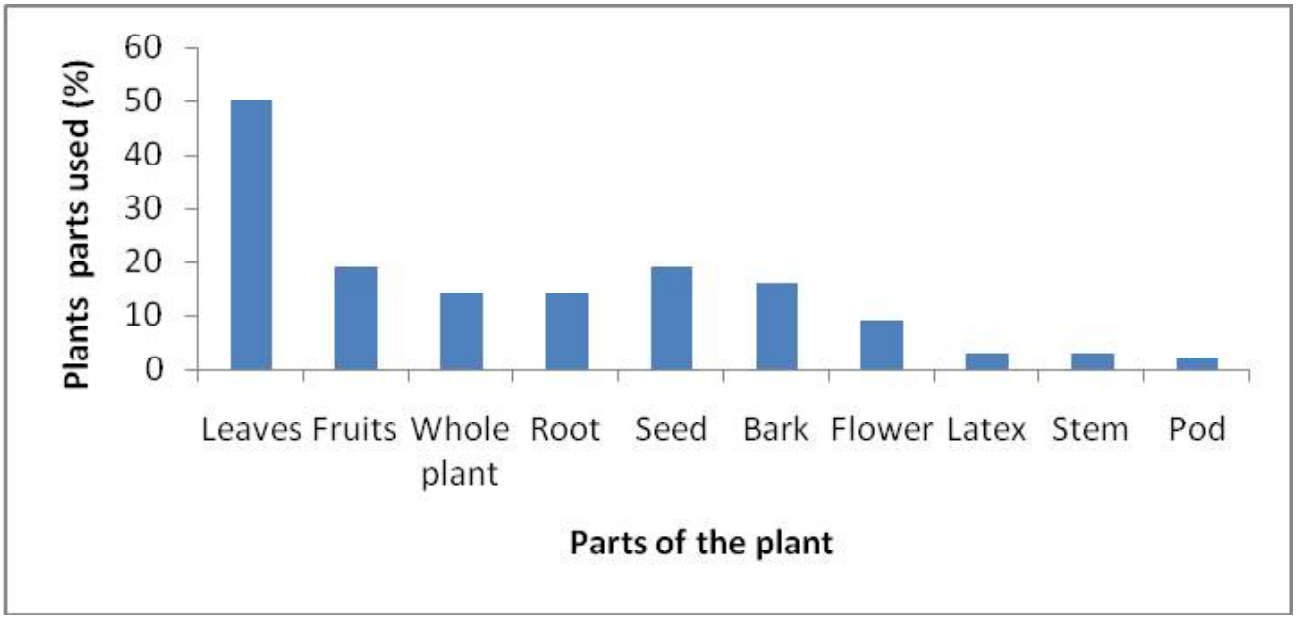

Fig. 3. Analysis of percentage of plants parts used for medicinal purpose.

Table 1. List of medicinal plants and their medico-potentiality against various ailments.

S.No	Binomial name	Family	Life form	Vernacular name	Parts used*	Mode of Usage	Ailments treated
1.	*Abutilon indicum* Linn.	Malvaceae	Shrub	Thuthi	L	Paste	Tuberculosis
2.	*Acalphya indica* Linn.	Euphorbiaceae	Herb	Kuppaimeni	W.P	Paste	Itching and scabies.
3.	*Achyranthes* aspera Linn.	Amarnthaceae	Herb	Naayuruvi	L	Paste	Body strength, skin, disease and tooth ache.
4.	*Adhatoda vasica* Nees.	Liliaceae	Shrub	Adhatoda	L	Paste	Bronchitis, asthma and throat infection
5.	*Aegle marmelos* Linn.	Rutaceae	Tree	Vilvam	L	Paste	Chronic diarrhea, dysentery, peptic ulcer, anti-oxidant, anti-viral, anti-diabetes and cardio protective.
6.	*Albizzai amara* Roxb	Mimosaceae	Tree	Arappu	Fr	Decoction	Boils, ulcers and erysipelas.
7.	*Allamanda catharatica* Linn.	Apocynaceaa	Climber	Allamanda	L &B	Paste	Diarrhea, jaundice, malaria, vomiting and cold.
8.	*Aloe vera* Linn.	Liliaceae	Herb	Kathalai	L	Gel	Stomach ache and skin disease.
9.	*Alternathera sessilis* Linn.	Amarnthaceae	Herb	Ponnanganni	W.P	Decoction	Anti-Ulcer, diuretic and memory enhancer.
10.	*Amaranthus viridis* Linn.	Amarnthaceae	Herb	Kuppai Keerai	L	Raw	Anti- ulcer and rectifies vitamin deficiency
11.	*Andrographis paniculata* Burm.f.	Acanthaceae	Herb	Nilavembu	W.P	Decoction	Diabetes and viral fever.
12.	*Annona squamosa* Linn.	Annonaceae	Tree	Sitapalam	Fr, B & Se	Decoction & Powder	Anti-cancer, Anti-oxidant, regulates RBC counts.
13.	*Areca catechu* Linn.	Arecaceae	Tree	Paaku	Se	Raw	Diuretic, digestive, anthelmintic, astringent and cardio tonic.
14.	*Areva lanata* Linn.	Amarnthaceae	Tree	Poolapo	L & R	Raw	Head ache, Demulcient, Snakebite and constipation.
15.	*Asystasia gangetica* Linn.	Acanthaceae	Shrub	Ganges primrose	R	Paste	Asthma, high blood pressure, piles, ear ache, diabetes and ulcer.
16.	*Azadirachta indica* A. Juss.	Meliaceae	Tree	Neem	L	Paste	Skin disease and small pox.
17.	*Barleria prionitis* Linn.	Acanthaceae	Shrub	Porcupine flower	W.P	Paste	Rheumatism, head ache, and skin disease.
18.	*Barleria tomemtosa* Linn.	Fabaceae	Tree	Iruvatchi	B	Paste	Skin problems, swelling of liver, intestinal worms, wounds and ulcers.
19.	*Bauhinia variegata* Linn.	Caesalpinaceae	Tree	Mandharai	B & FI	Decoction & juice.	Sore throat, coughs, haemorrhagic, bleeding piles and blood in urine.
20.	*Boerhavia diffusa* Linn.	Nyctaginaceae	Herb	Mookaratti	W.P	Decoction.	Anti-diabetic, diuretic, anti-cancer, anti-inflammatory, indigestion, scabies and jaundice.
21.	*Callistemon lanceolatus* D.C.	Myrtaceae	Tree	Bottle brush	L	Decoction	Cold and cough.
22.	*Calotropis gigantea* R.Br	Asclepiadaceae	shrub	Erukku	L	Paste	Dysentery, diarrhea, mental disorders, cough, cold, scorpion-sting, fever and jaundice.
23.	*Canavalia gladiate* Linn.	Fabaceae	Herb	Sword bean	L	Paste	Anti-nutritional and allergy causing.
24.	*Canna indica* Linn.	Cannaceae	Herb	Kalvani	Se	Decoction	Demulcient, stimulant, diuretic dropsy and diaphoretic.
25.	*Capsicum annum* Linn.	Solanaceae	Herb	chilly	Fr	Raw	Malaria

S.No	Binomial name	Family	Life form	Vernacular name	Parts used*	Mode of Usage	Ailments treated
26.	*Cardiospermum helicacabum* Linn.	Sapindaceae	Climber	Mudakatham	L	Decoction	Arthritis and cancer.
27.	*Carica papya* Linn.	Caricaeae	Tree	Papaya	Latex	Raw	Malaria
28.	*Cassia angustifolia* Linn.	Caesalpinaceae	Herb	Indian senna	R, L & Pod	Powder	Purgative, adominal distention skin diseases, rheumatoid arthritis and blood purifier.
29.	*Cassia auriculata* Linn.	Caesalpinaceae	Shrub	Aavaram	FI	Paste	Stomach Pain.
30.	*Cassia fistula* Linn.	Fabaceae	Tree	Konnai	B & R	Decoction	Cancer, epilepsy, diarrhea, glandular tumors, skin disease and constipation.
31.	*Catharanthus roseus* G, Don.	Apocynaceae	Shrub	Cayenne jasmine	L	Juice	Diabetes and malaria.
32.	*Chloris barbata* Linn.	Poaceae	climber	Swollen finger grasss	L	Paste & juice	Skin disease, diarrhea, diabetes, fever and anti-inflammatory.
33.	*Cissus quadrangularis* Linn.	Vitaceae	Climber	Pirandai	Se & L	Paste	Obesity and diabetes.
34.	*Citrus aurantium* Linn.	Rutaceae	Tree	Narthangai	Fr	Juice	Myocardial infarction
35.	*Citrus limon* Burm.f.	Rutaceae	Tree	Elumichai	L & FR	Decoction	Cold.
36.	*Cleome viscose* Linn.	Caparidaceae	Herb	Asian spider flower	L, Se &R	Paste	Fever, rheumatism, headache and skin diseases.
37.	*Clitoria ternatea* Linn.	Fabaceae	Climber	Sangupoo	R	Decoction	Swelling, paining, piles, brain weakness, small pox and increases urination.
38.	*Cocos nucifera* Linn.	Arecaceae	Tree	Coconut	Fr	Paste &oil	Headache.
39.	*Commelina benghalensis* Linn.	Commelinaceae	Climber	Bengal day flower	L	Paste	Wounds and fever.
40.	*Commiphora caudate* Engl.	Burseraceae	Herb	Hill mango	L & B	Gum	Mouth ulcers, wound healing and rheumatoid arthritis.
41.	*Croton bonplandianun* Baill.	Euphorbiaceae	Herb	Ban thulsi	W.P	Decoction	Dysentery and liver disorders.
42.	*Cyanodon dactylon* Pers.	Poaceae	Herb	Arugampul	W.P	Paste	Stops bleeding from piles, asthma, skin disease, cuts and wounds.
43.	*Cyperus rotundus* Linn.	Cyperaceae	Herb	Kora pul	L	Paste	Skin disease
44.	*Datura metal* Linn.	Solanaceae	Herb	Oomathai	L, R & Fl	Dried decoction powder	Indigestion, bronchitis, skin disease, hair loss, nausea, dizziness and anesthesia.
45.	*Eucalyptus globatus* Labill.	Myrtaceae	Tree	Eucalyptus	L	Vapour	Cough, cold, joint pain, skin disease and cardio tonic.
46.	*Euphorbia antiquorrum* Linn.	Euphorbiaceae	Shrub	Chaturakalli	Dried Latux	Raw	Cough, bronchitis and asthma.
47.	*Euphorbia hirta* Linn.	Euphorbiaceae	Herb	Amman pacharisi	Latex & L	Raw	Gastro-intestinal disorders, asthma, hypertensive, hypoglycemic, local parasiticide, bowel complaints, cough and breast pain.

S.No	Binomial name	Family	Life form	Vernacular name	Parts used*	Mode of Usage	Ailments treated
48.	*Ficus benghalensis* Linn.	Moraceae	Tree	Banyan	B ,R,L & Se	Latex	Ulcer, fever, leprosy and inflammation.
49.	*Grewia hirsute* Vahl.	Malvaceae	Shrub	Sakkarai palam	R,Fr, L & St	Powder	Bleeding disorders, dysuria and heart disease
50.	*Hibiscus rosa-sinensis* Linn.	Malvaceae	Shrub	Hibiscus	Fl	Oil	Stimulates immune system, stomach disorders, destroying abusive bacteria and fungi in intestinal tracts, laxative, pain reliever, ability to restore body tissues, wound healing, dandruff and facial edema.
51.	*Ipomea obscura* Linn.	Convolvulaceae	Climber	Morning glory	Se	Syrup	Oedema, oliguria, ascariais and various mental disorders.
52.	*Ixora coccinea* Linn.	Rubiaceae	Shrub	Idly poo	R,L &Fl	Paste	Dysentery
53.	*Jatropha gossipifolia* Linn.	Euphobiaceae	Shrub	Wild cassava	L & B	Paste	Wounds, skin disease, gum disease and insufficient breast milk.
54.	*Lantana camara* Linn.	Verbenaceae	Shrub	Unni	L	Juice	
55.	*Lawsonia inermis* Linn.	Lythraceae	Shrub	Maruthani	R,L, & Se	Paste	Skin disease, anemia and jaundice.
56.	*Leucas aspera* Spr.	Lamiaceae	Small tree	Thumbai	W.P	Paste	Epilepsy and headache
57.	*Lycopersicon esculentum* Mill.	Solanaceae	Shrub	Tomato	Fr	Raw	Skin disease and cosmetics
58.	*Mangifera indica* Linn.	Anacardisceae	Tree	Mango	Fr & Fl	Raw &Vapour	Syphilitic, uteristis, ulcer, vomiting, skin disease,malaria and diarrhea.
59.	*Manilkara zapota* Linn.	Sapotaceae	Tree	Sapota	Fr	Raw	Free radical scavenger,constipation and detoxifying agent.
60.	*Melothira madraspatana* Cogn.	Cucurbitaceae	Climber	Mosumosukai	L	Paste	Hair growth
61.	*Mimosa pudica* Linn.	Mimosaceae	Herb	Touch me not	W.P	Paste	Regulate menstruation, stimulate blood circulation, liver disorders, headache, arthritis, cough, wounds and ulcers.
62.	*Mimusops elongi* Linn.	Saptoceae	Tree	Magilam	B &Fr	Paste	Dental problems.
63.	*Momordica charantia* Linn.	Curcurbitacea e	Climber	Bitter gourd	Fr	Juice	Diabetes, malaria and cancer
64.	*Moringa oleifera* Linn.	Moringaceae	Tree	Drum stick	Fl, L & R	Soup, sauce & condiment	Anthelmentic and adjuvant.
65.	*Murraya koiengii Spreng*.	Rutaceae	Shrub	Karuvepilai	L	Juice & raw	Vermicide
66.	*Nerium oleander* Mill.	Apocynaceae	Shrub	Arali	L	Paste	Skin disease
67.	*Nyctanthus arbortristis* Linn.	Oleaceae	Tree	Pavalamalli	R	Raw	Fever, anti allergic, ulcerogenic, asthma, arthritis, chonic fever and joint pains.
68.	*Ocimum cannum* Linn.	Lamiaceae	Herb	Hoary basil	L	Oil	Diabetes, cold, fever, headache, skin disease, antibacterial, anti-fungal and anti-viral.
69.	*Ocimum tenuiflorum* Linn.	Lamiaceae	Sub-shrub	Thulsi	L	Raw	Constipation, sore throat, respiratory disorders stress and reduction of blood cholesterol level.

S.No	Binomial name	Family	Life form	Vernacular name	Parts used*	Mode of Usage	Ailments treated
70.	*Oldenlandia umbellata* Linn.	Rubiaceae	Herb	Chaaya ver	W.P	Decoction	Bronchial asthma and febrifuge.
71.	*Parthenium hysteroporous* Linn.	Asteraceae	Herbaceous weed	Carrot grass	W.P	Decoction	Skin inflammation, rheumatoid pain, urinary tract infection and malaria
72.	*Peristrophe bicalyculata* Nees.	Acanthaceae	Herb	Kali anghedi	W.P	Juice & oil	Fever, dyspepsia, swelling and bronchitis.
73.	*Phylanthus emblica* Linn.	Euphorbiaceae	Tree	Amla	Fr	Decoction & Raw	Jaundice, leucorrhea, urinary infection, dysentery, cough and bronchitis.
74.	*Phyllanthus amarus* Schum.	Euphorbiaceae	Herb	Kizhanelli	R	Decoction	Jaundice
75.	*Phyllanthus madraspatana* Linn.	Phyllanthaceae	Herb	Melanelli	W.P	Raw	Jaundice, flu, head ache, diabetes and menstruation.
76.	*Pongamia pinnata* Linn.	Fabaceae	Tree	Pungam	L	Paste	Laxative and digestion.
77.	*Portulaca oleratia* Linn.	Portulaceae	Herb	Pig weed	L	Paste	Skin rashes, ulcers, burn and insect stings.
78.	*Prosopis cinerea* Linn.	Mimosaceae	Tree	Vanni maram	L, FI & B	Tonic	Anthelmintic and leprosy.
79.	*Prosopis juliflora* DC.	Mimosaceae	Tree	Veli mul	B, L & P	Paste & Powder	Anti-bacterial and purgative
80.	*Psidium guajava*	Myrtaceae	Tree	Guava	Fr & se	Edible &oil	Anti-inflammation, anti-oxidant and cancer.
81.	*Punica granatum* Linn.	Punicaceae	Shrub	Pomegranate	Se& L	Juice	Dysentery
82.	*Quisqualis indica* Linn.	Combretaceae	Shrub	Irangun malli	Se, Fr & R	Paste & decoction	Jaundice, liver failure and hepatitis.
83.	*Rhynchosia minima* Linn.	Fabaceae	Herb	Jumby beam	R & B	Paste & Decoction	Diarrhea, heart problems and haemorrhoids.
84.	*Riccinus communis* Linn.	Euphorbiaceae	Tree	Aamanaku	R	Decoction	Menstrual problem, malaria and skin disease.
85.	*Sanesvieria roxburghiana* Schult.	Asparagaceae	Herb	Marul kalang	St	Juice	Vitality to the body gonorrhea heart disease.
86.	*Sida acuta* Brun.f.	Malvaceae	Herb	Karunthotti	L	Paste	Head ache and wound healing.
87.	*Solanum nigrum* Linn.	Solanaceae	Herb	Manathakalai	W.P	Raw	Corroding ulcer, anthrax, rheumatic joint pains, inflammation in the kidney, bladder, gonorrhea, skin disease, dysentery and bronchitis.
88.	*Solanum torvum* Sw.	Solanaceae	Shrub	Sundakai	Fr & Se	Vapour	Mouth sores, ulcer, fever and skin disorders.
89.	*Solanum trilobatum* Linn.	Solanaceae	Climber	Thuduvalai	L & Se	Paste	Good for asthma, cold, cough, flu and prevent throat infection.
90.	*Tabebuia rosea* DC.	Bignoniaceae	Tree	Rosy trumpet	Fl, R & L	Decoction	Fever, pain, tonsil inflammation and constipation.
91.	*Tamarindus indica* Linn.	Fabaceae	Tree	Tamarind	Fr, L, Fl & Se	Raw & RoaST	Stomach ache, indigestion, skin disease, fever and acts as a laxative.

S.No	Binomial name	Family	Life form	Vernacular name	Parts used*	Mode of Usage	Ailments treated
92	*Tectona grandis* Lf.	Lamiaceae	Tree	Teak	Fl, B & Se	Paste	Anemia and inflammatory disease.
93.	*Tephrosia pupurea* Linn.	Fabaceae	Shrub	Kolingli	R&L	Paste	Leprosy, ulcer, skin disease, asthma and tumors.
94.	*Terminalia arjuna* Roxb.	Combretaceae	Tree	Marudham	L, St, B & Fr	Paste	Astringent, diuretic, febrifuge, anti-dysenteric, hypertension, cirrhosis of liver, fever, contusions, cardio tonic and earache.
95.	*Thevetia peruviana* Pers.	Apocynaceae	Tree	Yellow oleamder	St, R, & L	Decoction	Skin boils and ring worms.
96.	*Tribulus terrestris* Linn.	Zygophyllaceae	Herb	Nerunji	L	Decoction	Regulates blood pressure and blood circulation in liver and kidney.
97	*Tridax procumbens* Linn.	Asteraceae	Herb	Vettukai poondu	L	Paste	Swelling, skin disease and wounds.
98.	*Vitex negundo* Linn.	Lamiaceae	Tree	Nochi	L	Juice	Head ache, tooth ache, ulcers, fever and asthma.
99.	*Wrightia tinctoria* R.BR.	Apocynaceae	Tree	Pala indigo	Fl, Fr & Se	Dye	Diarrhea, piles, skin disease like ring worm and leprosy.
100.	*Zizyphus jujube* Mill.	Rhamnaceae	Tree	Elandhai	R & L	Paste	Insomnia, palpitations and irritability.

*W.P- Whole Plant ; Fr – Fruit ; Fl – Flower; St – Stem; R –Root; L – Leaf; B - Bark.

Gobichettipalayam. They widely used many plants as wild edible plants such as *Mangifera indica*, *Cassia fistula* and *Leucas aspera*. *Mimosa pudica* and *Tridax procumbens* are used as medicine. From life form analysis, herbs were highly used as a medicine and found to be the dominant form followed by shrubs, trees and climbers. (Fig.2). Among the medicinal plant families represented, Euphorbiaceae and Fabaceae registered the dominant families with 8 species each followed by Solanaceae with 6 species, Amaranthaceae, Apocynaceae and Lamiaceae with 5 species each and Rutaceae, Mimosaceae, Malvaceae and Acanthaceae with 4 species each. The other 26 families were contributed with 1 species each. For each reported species we provided the botanical name of the plant, family, vernacular name, habit, parts used, mode of usage and ailments treated. Among the different plant parts used, leaves(50%) were the most frequently used for the preparation of medicine solely (or) mixed then root(14%), flower(9%), latex and stem(3%) and pod (2%) (Fig.3). The preparation and utilization of plant parts were grouped into 13 categories of the most commonly used method of preparation was paste (38%), decoction (26%), raw (9%), oil (5%), powder (4%), vapour, gel, gum, latex and dye(1%).The most important methods used were direct application of medicinal oil and are mainly with diseases like skin disorders, wounds, poison bites, rheumatism, body pain, headache, diabetes, jaundice, ulcer and asthma.

DISCUSSION

India is recorded as a paradise of vegetation due to rich diversity of agro climatic, socio cultural conditions prevailing in country. In India the Eastern Himalayas were recorded as one of the 26 hotspots of the world. The Western Ghats hold about 4000 flowering plant species of which 1500 species restricted only to it (Nayar, 1997). Traditional system of health care is passed down from generation to generation within a society is still the prevalent system found within the remote rural areas of the country. At present more than 50% of the secrets of the plants pertaining to health care management exist and the rest vanished. There, the present study was aimed to explore the vast knowledge about the medico potentiality of the plants.

Herbal medicines are considered the oldest forms of health care known to mankind on this earth. Prior to the development of modern medicine, traditional system of medicine that have evolved over the centuries within various communities are still

Table 2. Analysis of plant species which cures various diseases.

S.No	Skin disease	Diabetes	Ulcer	Malaria	Jaundice	Asthma
1.	*Achranthes aspera*	*Achranthes aspera*	*Aegle marmelos*	*Mangifera indica*	*Cassia fistula*	*Asytasia gangetica*
2.	*Aloe vera*	*Andrographis paniculata*	*Mangifera indica*	*Mimosa pudica*	*Tribulus terrestris*	*Nyctanthus arbortistis*
3.	*Cassia fistula*	*Asparagus racemosus*	*Mimosa pudica*	*Riccinus communis*	*Tamarindus indica*	*Cyanodon dactylon*
4.	*Acalypha indica*	*Azadiracta indica*	*Momordica charantia*	*Catharanthus roseus*	*Phyllanthus emblica*	*Euphorbia hirta*
5.	*Calotropis gigantean*	*Momordica charantia*	*Vitex nigundo*	*Carica papaya*	*Ziziphus jujuba*	
6.	*Chloris barbata*	*Ocimum sanctum*	*Amaranthus viridis*	*Capsicum annum*		
7.	*Lawsonia inermis*	*Phyllanthus emblica*	*Asytasia gangetica*			
8.	*Mangifera indica*	*Mangifera indica*	*Portulaca oleraceae*			
9.	*Ocimum sanctum*	*Mimosa pudica*	*Solanum torvum*			
10.	*Riccinus communis*	*Terminalia arjuna*	*Nyctanthus arbortistis*			
11.	*Tamarindus indica*	*Tamarindus indica*	*Alternathera sessilis*			
12.	*Theporsesia perpurea*	*Vinca rosea*	*Ficus benghalensis*			
13.	*Tribulus terrestris*	*Vitex nigundo*	*Albezzia amara*			
14.	*Tridax procumbens*	*Aegle marmelos*	*Bauhinia tomentosa*			
15.	*Cyperus rotundus*					
16.	*Eucalyptus globules*					
17.	*Lycopersicon esculentum*					
18.	*Portulaca oleraceae*					
19.	*Wrightia tinctoria*					
20.	*Barleria prionitis*					

maintained as a great traditional knowledge base in herbal medicine (Mukhejee and Wahil, 2006). The popularity of herbal in traditional medicine has been linked to their higher likelihood of containing pharmacological active compared to woody plants.

Leaves are the main photosynthetic organs containing photosynthetase which might be responsible for medicinal values (Balick *et al.,* 1996 and Ghorbani, 2005). The indigenous communities throughout the world, mostly utilized leaves for the preparation of herbal medicines. This is the reason why leaves were used mostly is that they are collected very easily than

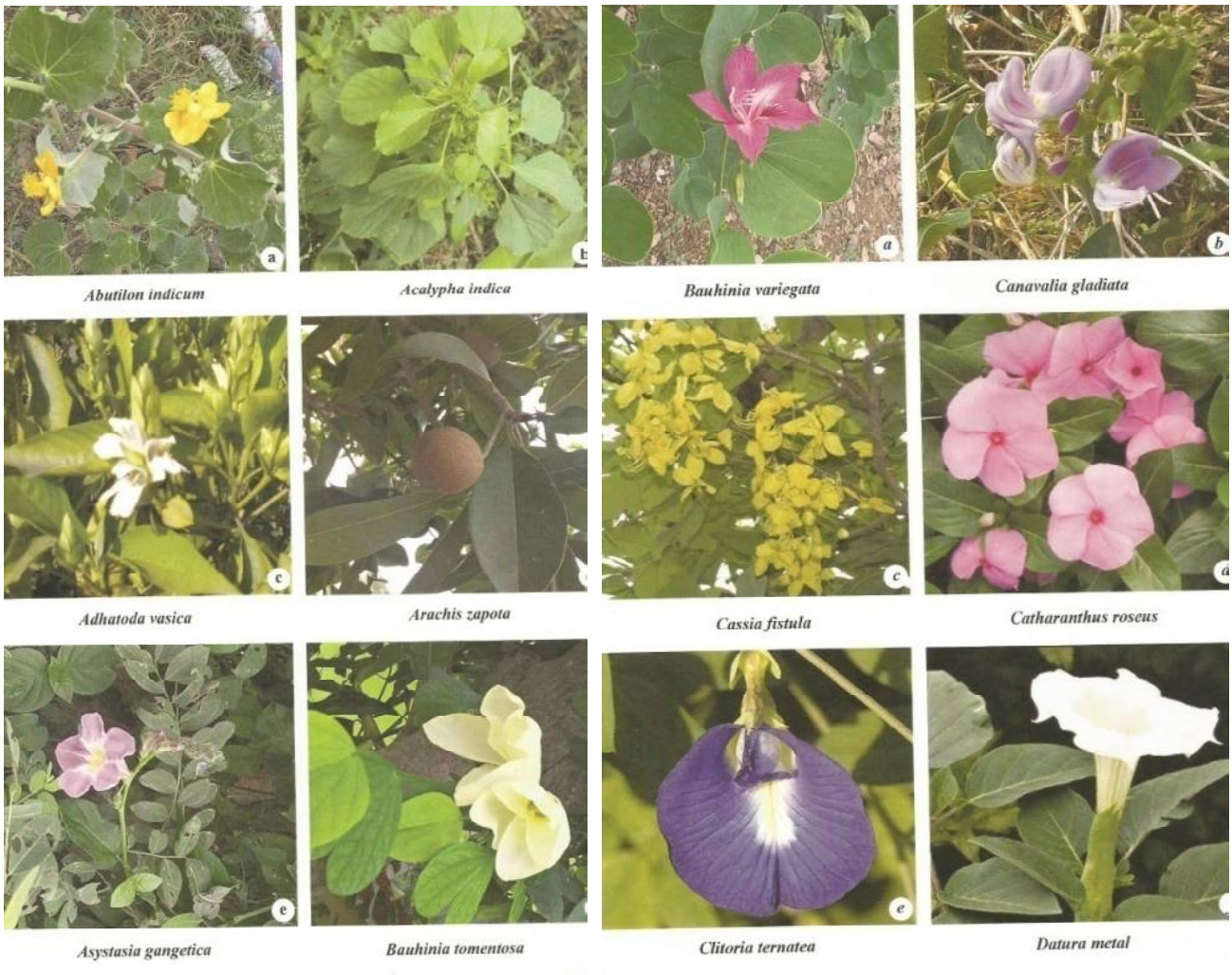

Plate I – Medicinl plant diversity of Andavar Malai, Gobichettipalayam,Erode(Dt.).

the underground parts, flowers and fruits (Giday *et al*., 2009 and Telefo *et al*., 2011)

A total of 100 species from 46 families used for the treatment of various ailments are depicted in Table 1. In the present study, collected medicinal plants and their botanical names were arranged in alphabetical order followed by the family name, vernacular name, useful parts and medicinal uses. Among the total number of ethno medicinal plants 36 species are herbs, 9 species are climbers, 21 species are shrubs and 34 species are trees (Fig.2).

The rich diversity of different plant species in the Andavar malai may be due to the presence of different microclimatic sites like open habitats, shaded habitats by broad free canopy coverage, slightly ever wet places, hedges with the habitat of more soil organic matter, etc in the common macroclimatic of semi arid conditions.

CONCLUSION

The modern generations is inclined towards the use of allopathic medicines and it seems that traditional knowledge of medicinal plants could be lost. There is a threat of losing this wealth of knowledge in the near future. The awareness and documentation about the medicinal floristic wealth or plant medicinal values will helpful in the preservation of traditional medicinal practices and the plant wealth of the concerned site. The present study exhibits a lot of information on plant species with immense value. Knowledge and use of herbal medicine for the treatment of various ailments among the local people in Andavar malai, Gobichettipalayam, Erode district, Tamil Nadu. The documented plants have potential of being used in drug development.

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In Vitro Culture of *Philonotis falcata* (Hook.) Mitt., A Moss Species from Nilgiri Hills

NISHA K.K.*, APSARA I G, REJITHA R F, ANCY ANTONY, SARANYA A S, SRUTHY A CHANDRAN AND SWATHY R U

Department of Botany, All Saints' College, Thiruvananthapuram
**email: nishabot@gmail.com*

ABSTRACT

***In vitro* culture of the moss *Philonotis falcata* (Hook.) Mitt. collected from the Nilgiri hills, Tamil Nadu was attempted. Sporophyte with mature capsule was found to be the best explant for axenic culture establishment. Effective surface sterilization was achieved with 0.1% $HgCl_2$ for 1 minute. The spores were released from the capsule under sterile conditions and were germinated *in vitro* using MS basal medium. The presence of sucrose (3%) triggered spore germination and the protonema became visible within 30 days of inoculation. Protonema failed to regenerate mature gametophyte even after 80 days of culture and this may be due to the absence of growth regulators in the medium.**

Keywords *Philonotis, protonema, in vitro culture, moss, bryophyte*

Bryophytes are the second largest group of higher plants after flowering plants, with ~15,000species worldwide (Hallingbäck and Hodgetts, 2000).Though *in vitro* culturing of bryophytes, especially mosses was successfully established very early, further studies in this area was limited due to the problem of material availability, genetic variability of the material and low level of species biology knowledge (Duckett *et al*., 2004). Various physiological, genetical, morphogenetic, ecological and evolutionary problems could be more easily studied in bryophytes rather than in vascular plants (Sabovljeviæ*et al*., 2003). Besides, *in vitro* culture of bryophytes also play a significant role in rare species conservation and the isolation of biologically active and new compounds (Batra*et al*., 2003; Cvetiæ *et al*., 2005; Gonzalez *et al*., 2006; Mallon *et al*., 2007; Rowntree *et al*., 2011).

Nilgiri hills are the part of Western Ghats biodiversity hotspot and are very rich in bryophytes especially mosses. *Philonotis,* belonging to the family Bartramiaceae, is a large genus with 169 species which are widely distributed throughout the World. In India it is represented by 26 species (Lal 2005). *Philonotis falcata* (Hook.)Mitt.is a Southeast Asiatic taxon (Koponen, 1996). The plants are medium-sized, slender, green to yellowish green and grow in dense tufts. Stem is a whorl of subfloral branches. Leaves are spirally arranged, triangular-ovate from broad base. Perichaetial leaves are more rectangular but not much differentiated. Seta apical, erect or slightly sinuose, red to brownish. Capsule is horizantal to pendulous, ovoid, brownish.

The aim of the present study was to establish stable *in vitro* culture of this species and examine its development under axenic conditions.

MATERIALS AND METHODS

Plant material

The plant selected for the study, *Philonotisfalcata* (Hook.) Mitt., was collected from near Ooty lake, part of Nilgiri hills, Tamilnadu (altitude - 2,220 m). Voucher specimens were deposited in the All Saints' College Herbarium (Fig.1A & 1B).The specimens were identified with the help of Dr. Manju C Nair, Zamorin's Guruvayurapan College, Kozhikode. For *in vitro* studies, sample specimens stored in plastic bags at -4^0C were used.

Establishment of *in vitro* cultures

Both sporophyte and gametophyte were used as explants. The plants were separated carefully from the impurity, placed in conical flask, covered with cheese cloth, and rinsed with tap water for 30 minutes. Sporophyte and gametophyte shoots were then disinfected with 0.1% $HgCl_2$ for 1 minute. Later they were rinsed three times in sterile deionized water to remove the toxic $HgCl_2$.

Murashige and Skoog (1962) (MS)

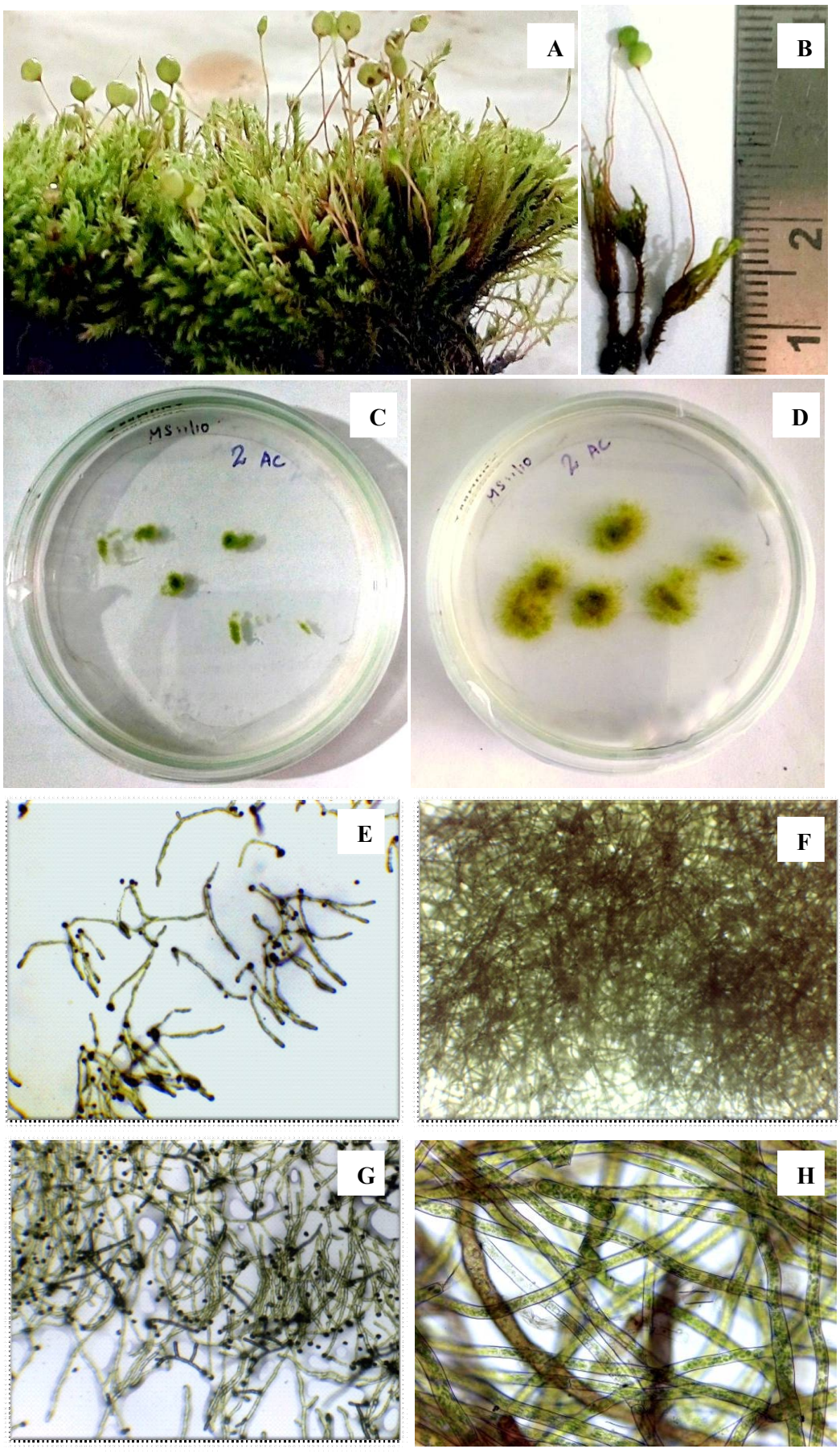

Fig. 1 A & B- *Philonotis falcata* gametophyte with sporophyte; **C-** Protonema after 5 weeks of culture; **D-** Protonema after 12 weeks of culture; **E-** Germinating spores; **F & G-** protonema under 100x magnification; **H-**Protonema under 400x magnification

medium (HIMEDIA) containing Murashige and Skoog mineral salts and vitamins, 100 mg/l inositol, 0.70% (w/v) agar was used as the basal medium. In order to observe the influence of sucrose and/or mineral salts on the morphogenesis of this species, the following medium compositions were used:

MS1: half strength of MS mineral salts, 1.5% sucrose;

MS2: half strength of MS mineral salts, 3% sucrose;

MS3: MS mineral salts, 1.5% sucrose;

MS4: MS mineral salts, 3% sucrose

The pH of the medium was adjusted to 5.7. The cultures were maintained under an illumination of 2000±200 lux for 14h/d, at 18±1ºC.

RESULTS AND DISCUSSION

The attempts to establish the axenic culture from gametophytes failed since the high concentration for surface sterilization killed the plant material or due to low concentration of surface sterilization it was overgrown quickly with fungi and bacteria. Surface sterilization of the sporophytes was more successful. Mature unopened capsules were selected and surface sterilization was done using 0.1% $HgCl_2$ solution for 1 minute. After sterilization, the capsules were opened in sterile conditions and the spores were taken out with sterile needle and inoculated to the mineral salt containing media.

The highest percentage (95%) of spores germinated on basal MS medium supplemented with 3% sucrose (MS4) (Fig. 1E). Spore germination of *P. falcata* occurred 25 days after initiating *in vitro* culture. Visible protonema can be noticed 30 days after inoculation, and it grows rapidly at 18 ±2°C (Fig. 1C). Even after 80 days of spore germination it remained in protonemal stage (Fig. 1D) and failed to regenerate mature gametophyte. This may be due to the lack of any growth regulators in the medium provided.

Schoefield (1981) stated that in most bryophytes spores germinate 7-30 days after exposure of spores to good conditions. Vujièiæ *et al* (2011) reported that when media contained sucrose the spore germination was quicker (2-4 days) than on sucrose free media.

In native conditions protonema have to achieve a certain size which then produce enough amount of kinetin-like growth regulators. This is a trigger for bud induction or passing from filamentous to meristemal growth (Vujièiæ *et al*., 2011). In our study, in all the media tested, the protonema remained small and that may be the reason for non-regeneration of mature gametophyte even after 80 days of growth. Supplementing the media with growth regulators may promote the formation of mature gametophyte.

Thus we have successfully established protonemal culture of *Philonotis falcata.* Surface sterilization is easier to achieve on sporophyte than on gametophyte. The optimum condition for axenic culture is to grow on MS medium enriched with sucrose (3%), at 18-20°C. The results obtained here can be used for developing a system for propagation and ex situ conservation of other rare and/or threatened *Philonotis* species as well.

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Extraction Isolation and Structural Elucidations of Certain Active Principles from *C. longa*

M.P. SALINI*[1] ANTONY AKHILA THOMAS[2] AND S. USHA[3]

[1&3]*Dept. of Zoology, Sree Narayana College for Women, Kollam*
[2]*Dept. of Zoology, PG and Research Centre, Fatima Mata national College, Kollam*
**email : salinimpsalini@gmail.com.*

ABSTRACT

India is one of the hotspot of medicinal plants. Medicinal plants are regarded as the special gift from god, they yields all remedies for most of the diseases.Now days the protection of these are not so easy due the habitat loss. Ayurveda regarded as the ancient group of medicinal system. Medicinal plants are the chief ingredient of Ayurveda. Most of the plants undergone destruction due to various meansof habitat loss. Majority of them are extinct.In India Ayurveda is an important discipline, comprising a long lived traditional knowledge. The regional ethnic or tribal groups in various part of the country protected these plants as their real medicines for their well-being. Some are under the verge of extinction. There are medicinal plants are there which has numerous applicability. *Curcuma longa*is one of the medicinalplants with traditional application. It has numerous medicinal properties like antimicrobial, antioxidant, antihealmtnethic etc. In the present study are deals with the extraction, isolation and structural elucidations of some active principles from *Curcuma longa*. The main active components were Curcumin, demethoxy curcumon and bis demethoxycurcumin.

Key words *Curcuma longa,extraction, isolation and structural elucidations,active principles, Curcumin, demethoxy curcumin and bis demethoxy curcumin.*

India's ancient civilization is greatly merged with the goodness of medicinal plants. Farnsworth and Soejarto(1991) define medicinal plant as"All higher plants that have been alleged to have medicinal properties *i.e.,* effects that relate to health to be useful as drugs by western standards, or which contain constituents that are used as drugs".Most of the medicinal properties of these herbs is due the presence of secondary metabolites. Secondary metabolitesare chemicals produced by plants for which no role has yet been found in growth, photosynthesis, reproduction, or other "primary" functions (Sultana *et al.,* 2010; Saline,2017).Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Sasidharan *et al.,*2011).The analysis of bioactive compounds present in the plant extracts involving the applications of common photochemical screening assays, chromatographic techniques such as HPLC and, TLC as well as non-chromatographic techniques such as immunoassay and Fourier Transform Infra Red (FTIR) etc(Handa *et al.,* 2008, Sasidharan *et.al.,*2011).Chang *et al.,* 2011 extracted curcumin from curcuma *longa* by solvent extraction method.HPLC analysis revealed that all the samples had high percentage of curcumin.Thajeswari *et al.,* 2013, have documented assimilation of curcuminoids from *Curcuma longa* by IR,UV and TLC by using different solvent systems. The main curcuminods were Curcumin, demethoxy curcumin, bisdemethoxycurcumin. The solvent system dichloromethanae; chloroform: ethyl acetate(0.5:8:1.5 v/v) with Rf value 0.690,0.687,0.682 showed better resolution for three curcuminoids respectively. Kulkarni. e*t al.*,2012 extracted and purified curcuminoids from turmeric.Isolation and purification by column chromatography. Methanol extraction showed maximum yield of each curcuminoids. Separation of curcuminoids were tested in TLC chloroform: methanol at 95:5 showed Rf value at 0.67,0.60,0.51 as Curcumin,D emethoxycurcumin, Bisdemethoxycurcumin. Kanadaswamy and Subramsanian (2013)with the help of HPTLC method has isolated and quantified curcumin in four varieties of turmeric and compared with standard. Hanisa *et al.,*014 analyzed in liquid chromatography-mass spectrometry-electrospray ionization to identify plant active principles of *Curcuma longa* and *Centella asiatica*. In the present study *Curcuma longa* was selected for the phytochemical analysis. The plants were subjected to preliminary screening. Active principles from *C.longa* (curcumin, demethoxycurcumin and bisdemethoxycurcumin), was extracted and isolated. The quantification was done using HPLC. After extraction the structural elucidation was carried out using Infrared, Mass and UV spectroscopy. Subsequently each extracts were selected for biological prediction studies using PASS software.

Table 1. Preliminary screening of *Curcuma longa*.

Class of compounds	Tests performed	*C.longa*
Carbohydrates	Molisch's test Fehling's test	++ ++
Phenols	Phosphomolybdic acid test	+++
Flavanoids	Shinoda test Lead acetate test	++ ++
Tannins	Braemer's test	+
Alkaloids	Draggendrof's test	+++
Glycosides	Legal's test	---
Saponins	Foam test	++
Anthraquinones	Borntragers test	++
Amino acid test	Ninhydrin test	---
Fixed oils and fats		++

(+++= group is present as the major class,++= moderate quantity,+=presence of only in traces,- = absence).

MATERIALS AND METHODS

Collection and processing of plant material

Tubers of *Curcuma longa* (Zingiberaceae family) was collected from the local stations of Kollam district, Kerala. The collected samples has been identified by Pankaja Kasthuri Ayurveda Research centre, Kattakkada, Trivandrum and samples deposited in herbarium.The rhizomes of *Curcuma longa* were taken for processing, all debris were washed out, cut into small pieces. Air dried samples were powdered by electronic mill.

Preliminary screening was done as per Harborne,2008. Methanolic extract of sample were prepared in order to process for obtaining major chemical constituents. The main phytochemical analysis done was Test for carbohydrates(Molisch's test, Fehling's test),Test for Phenols(Phosphomolybdic acid test),Test forflavonoids (Shinoda test,Lead acetate test),Test for tannins(Braemer's test),Test for alkaloids(Draggendorf's test),Tests for Glycosides(Legal's test),Test for Saponins(Foam test),Test for Anthraquinones(Borntrager's test),Test for Amino acids(Ninhydrin test),Test for fixed oils and fats,etc. The extract was dissolved in 200 ml methanol, and then passed anhydrous sodium sulphate for the removal of aqueous matter. The material was then subjected to column chromatography after defatting. About 500 g of silica gel (Column Chromatography Grade- Mesh size 60-120) was kept in oven for 1hr at 105ÚC for activation. It was cooled in a desiccator for 1 hour. A column of length 60cm and diameter 1.5cm was used for doing column chromatography. The column was packed up to 2/3 portions with the previously activated silica gel G (E. Merck) by wet packing procedure. The methanol extract was then mixed well and dried by continuous stirring with activated silica gel of mesh size 60-120. The upper part was then packed with the dried extract mixed with the silica gel. The top most part of the column was covered by cotton so as to avoid the disturbance while pouring the solvent.The solvent system used for the elution of the column for *Curcuma longa* is as follows(Each proportion was of 100 ml each,20 X5). Total 4 combinations was selected(5 X4 = 20);Toluene: Ethyl acetate (95:5),Toluene: Ethyl acetate (90:10),Toluene: Ethyl acetate (80:20),Toluene: Ethyl acetate (70:30).Similar fractions were clubbed together after analyzing with TLC.TLC fixed at Toluene:Ethyl acetate(9:1)for *curcuma longa* .

Structural Elucidations was done with the help of Ultraviolet-visible spectroscopy (UV-vis)(Shimadzu, Kyoto, Japan),Infrared spectroscopy (IR) (Shimadzu, Kyoto, Japan),Mass spectrometry (MS)(FSMS 1010A SHIMADZU) was employed for the present study.Biological predictions of compounds were done using a program: PASS (Prediction of Activity Spectra for Substances).

HPLC Method for Quantification of extracts.

Samples were analyzed by HPLC in a Shimadzer LC 20A0 liquid chromatography system with SPD-M20AuV detector in isocratic mode. Each sample (100 mg/ml) and standard (1mg/ml) was loaded for the detection. From the chromatogram, concentration of each active principle can be detected.

RESULTS AND OBSERVATIONS

From the preliminary screening the data obtained was listed in Table.1.Carbohydrate content found in *C.longa*. Phenolic content was high in *C.longa.* Flavanoids was moderately found in *C.longa.* Tannin was found in trace amount in *C.longa.* Alkaloid content was high in C. *longa*.Glycoside was absent in *C.longa*. Moderate amount of anthraquinones are found in *C.longa*.Fixed oils and fats are moderately present in *C.longa.*

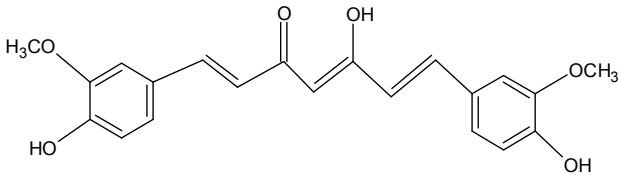

Fig. 1. Structure Of Curcumin.

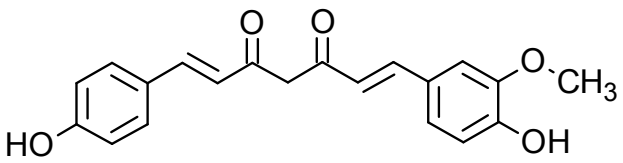

Fig. 2. Structure Of Demethoxy curcumin

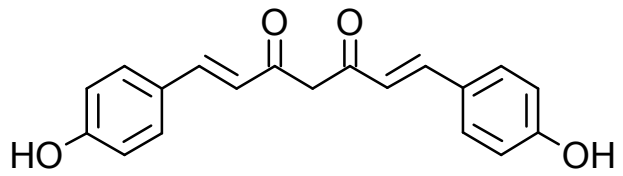

Fig. 3. Structure Of Bis Demethoxy curcumin

From the PASS studies it is clear that curcumin have Pyruvate decarboxylase inhibitor, Ubiquinol-cytochrome-c reductase inhibitor, Aspulvinone dimethylallyl transferase inhibitor, Testosterone 17beta-dehydrogenase (NADP+) inhibitor properties. Demethoxycurcumin have 1-Acylglycerol-3-phosphate O-acyltransferase inhibitor, Beta-carotene 15,15'-monooxygenase inhibitor, Aspulvinone dimethylallyltransferase inhibitor, Chlordecone reductase inhibitor, Gluconate 2-dehydrogenase (acceptor) inhibitor, Membrane permeability inhibitor properties.Some of the pharmacological properties of Bisdemethoxycurcumin includes Reductant, Aspulvinone dimethylallyltransferase inhibitor,Beta-carotene 15,15'-monooxygenase inhibitor, Ubiquinol-cytochrome-c reductase inhibitor , Dehydro-L-gulonate decarboxylase inhibitor , Glutathione thiolesterase inhibitor properties.

HPLC OF SELECTED ACTIVE PRINCIPLES.

HPLC chromatogram of *C.longa* sample was shown in figure.4. The percentage yield, retention time and all other details are tabulated in Table.2. The percentage composition of curcumin (Table.6.) was about (0.53%). The percentage composition of demethoxy curcumin in the present sample was about (0.23%). The percentage composition of demethoxy curcumin was about (0.42%).

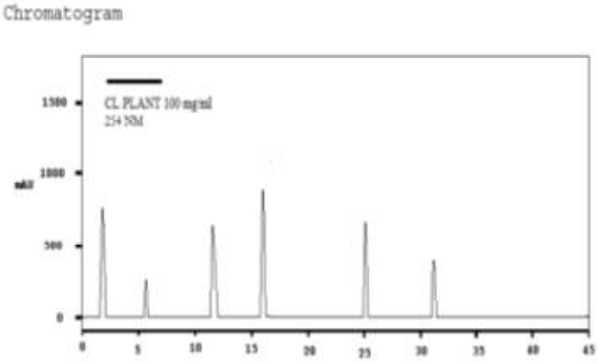

Fig.4. Chromatogram of *Curcuma longa* (sample).

Table 2. Profiling of chromatogram of *Curcuma longa* (sample).

Peak number	Retention time in Min	Peak area obtained under the curve.
1	2.02	756
2	5.45(CL-2)	244
3	11.01	599
4	15.85(CL-1)	998
5	25.02(CL-3)	610
6	31.00	485

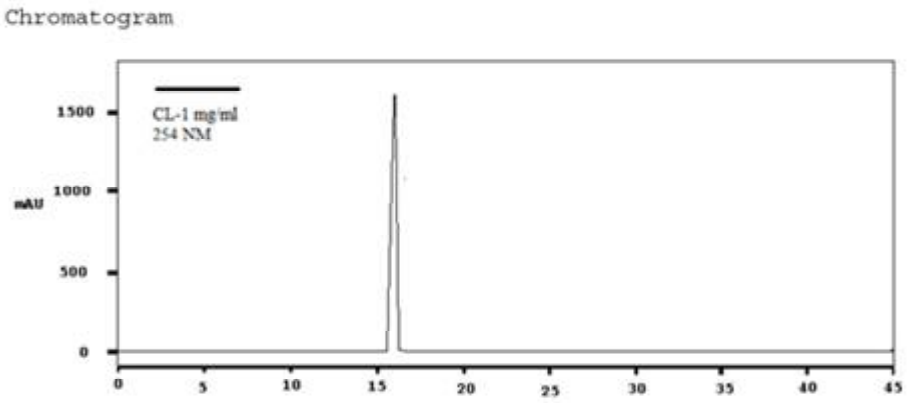

Fig. 5. Chromatogram of Curcumin (standard).

Table 3. Profiling of chromatogram of Curcumin (standard).

Peak number	Retention time in Min	Peak area obtained under the curve.
1	15.85(CL-!)	1854

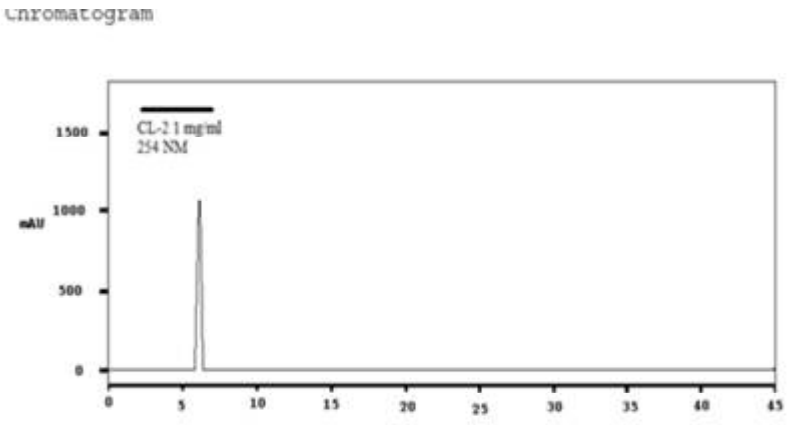

Fig. 6. Chromatogram of Demethoxy curcumin(standard)

Table 4. Profiling of chromatogram of Demethoxy curcumin (standard)

Peak number	Retention time in Min	Peak area obtained under the curve.
1	5.45(CL-2)	1024

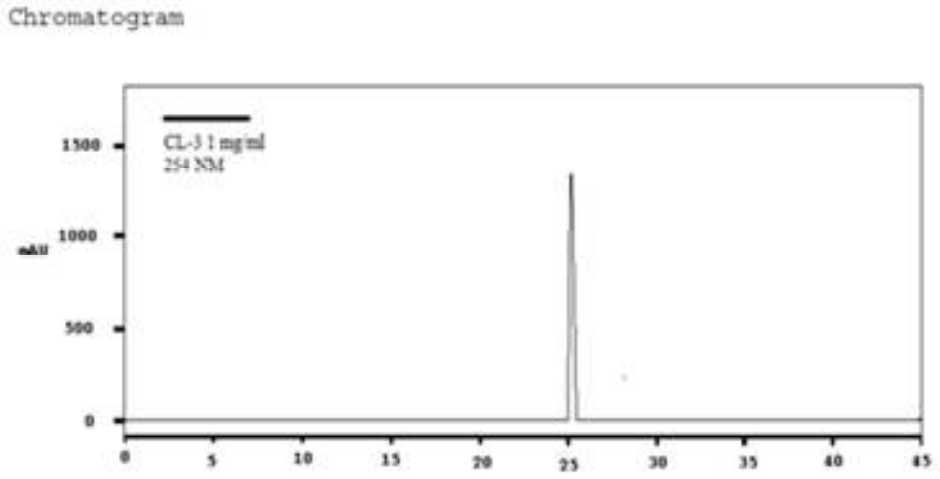

Fig. 7. Chromatogram of Bis Demethoxy curcumin (standard)

Table 5. Profiling of chromatogram of Bis Demethoxy curcumin(standard)

Peak number	Retention time in Min	Peak area obtained under the curve.
1	25.04(CL-3)	1438

Table 6. Percentage composition of each active principles under the study.

Active principles	Retention time in min	Peak area obtained under the curve	Amounts (%)
• Curcumin	15.85	998	0.53
• Demethoxy curcumin	5.45	244	0.23
• Bis demethoxycurcumin	25.02	610	0.42

DISCUSSION

As the active components of medicinal plants are important, more and more studies are going on to extract and isolate the said compounds. *Curcuma longa* is known as Indian saffron, have numerous applicability in phytochemistry.In present study curcumin,demethoxycurcumin,bisdemethoxycurcumin was the main focus of isolation and identification. Curcumin,diferuloylmethane is the primary agent also responsible for the brilliant color of *curcuma longa*(Jurenka,*et al.,*2009). Kulkarni*et al.,* (2012) extracted curcuminoids and also purified the curcuminoids from turmeric (*Curcuma longa*).In this study the three curcuminoids from *C.longa* was selected for the study. The three extracts were isolated through column chromatography and the purity was checked with TLC. After extracting the main active components the structural elucidation of curcuminoids were done using IR,Mass and UV spectra.IR spectrum of the three curcuminoids was also compared with the previous works(Nabati. *Et al.,* 2014,Sabu and Saxena,2014).Mass and UV spectral study was also got similar results as Pant.*et al., (*2013).Revathy *et al., (*2011) has reported the isolation, purification and identification of curcuminoids from turmeric by column chromatography. The individual Curcuminoids collected from the column chromatography was dissolved in methanol and heated. After complete dissolution added chloroform to get the ratio methanol: chloroform 5:2 and kept at 5°C for overnight. The crystals were precipitated with petroleum ether. The purity of Individual crystals was analyzed in HPLC.Almeida *et al.,* 2005 quantified curcuminoid pigments by HPLC.Thejeswari *et al.,* 2013, assimilated the curcuminoids from *Curcuma longa* by IR,UV,and TLC by different solvent systems. In the present study also major curcuminoids such as curcumin, demethoxycurcumin, bisdemethoxy curcumin were extracted, isolated and structural elucidation undertaken. HPLC of these three were also done and documented. In a work of Kulkarni *et al.,*2012, Curcumin, Demethoxycurcumin and

Bisdemethoxycurcumin shows single peak at the retention time of 9.213, 8.533, 7.915 min respectively in methanolic extraction. Rohman *et al.,*2012 has studied about the structural characterization of curcumin and also found a correlation with the present work. Singh *et al.,*2007 extracted the major active components and also reported their antimicrobial activities.In the present study a pilot approach was under taken to characterize the structure and composition of three curcuminoids from *Curcuma longa*.

ACKNOWLEDGMENT

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Anticancerous Potential of *In vivo* and *In vitro* Developed Roots of *Pseudarthria viscida* (L.) Wight & Arn.

SANGEETHA G.[1] AND SWAPNA T. S[2]*

[1]*Department of Botany, University College, Thiruvananthapuram-34, Kerala, India.*
[2]*Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram, Kerala, India.*
**email:Swapnats@Yahoo.Com*

ABSTRACT

The excessive collection of medicinal plants has resulted in its gradual disappearance from the natural habitat and at present their number is highly reduced in nature. To conserve the genetic stocks of many plants *in vitro* propagation can be utilized successfully. *Pseudarthria viscida* (L.) Wight & Arn. (Fabaceae) commonly called "Moovila" is such an important medicinal plant extensively collected and now disappearing from the wild. Most important part of this plant is roots which is astringent, thermogenic, digestive, anthelmintic, anticancerous, cardio and rejuvenating tonic. The main aim of proposed work is the production of *in vitro* roots and evaluation of the preliminary phytochemicals as well as anticancerous activity of *in vivo* and *in vitro* developed roots of *Pseudarthria viscida*. For *in vitro* root formation NAA, 2, 4-D, IBA and IAA at a range of 0.5 to 3mgl^{-1} were used in MS medium. The preliminary phytochemical study was done for the detection of phytoconstituents, using standard tests. For cytotoxic analysis, HCT-15 (colon cancer cell line) and L929 normal fibroblast cell lines were selected and different concentration of extracts from both *in vivo* and *in vitro* developed roots (10µg ml^{-1}, 50µg ml^{-1}and 100µg ml^{-1}) were added in the cell cultures suspension. The cytotoxic effect was determined by MTT cell viability assay and the effects were expressed as IC_{50}. Methotrexate was used as standard drug. The results indicated that maximum number of rooting was noticed on MS medium with 2.5 mgl^{-1}of NAA and 2.5 mgl^{-1}of 2, 4-D after 29 days of inoculation. In preliminary phytochemical evaluation, glycosides, flavonoids, alkaloids, terpenoids and tannins were noticed and which could be the reason for cytotoxic activity of the plant. Crude extracts of both *in vivo* and *in vitro* developed root of *Pseudarthria viscida* showed potential cytotoxic activity against HCT-15 cell line with IC_{50} values of 36±0.01µg ml^{-1}and45±0.01µg ml^{-1}respectively. They exhibited IC_{50} exceeding 100µg ml^{-1}for L929 normal cell line. The present study thus confirmed that like *in vivo* developed roots, *in vitro* regenerated roots of *Pseudarthria viscida* (L.) possess good cytotoxic activity against HCT-15 cancer cell line and conservation of this plant without disturbing its natural habitat could be done by *in vitro* root induction.

Key words *Pseudarthria viscida*, *In vivo* roots, *In vitro* rooting, Cytotoxic analysis, MTT cell viability assay, Methotrexate.

Medicinal plants are one of the important natural wealth of a country and their role is inevitable to provide health care service to common man. Hundreds of medicinal plants are at risk of extinction, threatening the discovery of future cure for disease. Interest and support for the conservation and development of medicinal plants is increasing in all parts of the world. As per World Health Organisation estimates, almost 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs. In India, medicinal plants sector has traditionally occupied an important position in the socio cultural, spiritual and medicinal arena of rural and tribal lives. The conservation of plant genetic resources has long been realized as an integral part of biodiversity conservation (Hoareau & Silva, 1999).

The habitat destruction and over harvesting have resulted in gradual disappearance of many medicinally important plants from its natural habitat. At present their number is highly reduced in the wild. Beside their vast economic and pharmaceutical potential, some of these plants cannot be cultivated for commercial purposes due to low seed viability, germination rate and high mortality of seedlings in young stages. To conserve the genetic stocks of such plants *in vitro* propagation can be utilized successfully (Meena and Thomas, 2011). One of such important medicinally important plant needed to be conserved is *Pseudarthria viscida*

Pseudarthria viscida (L.) Wight & Arn. is a perennial viscid pubescent semi erect diffuse under shrub, belonging to the family Fabaceae. It is distributed throughout India especially found in river basins and in hills up to above 900m (Krithikar & Basu, 1918). The roots are with astringent, thermogenic, digestive, anthelmintic, antiinflammatory, antifungal, antidiarrhoel, antioxidant, aphrodisiac, nervine, febrifuge, cardio and rejuvenating properties. They are useful in vitiated conditions of cough, bronchitis, asthma, tuberculosis, helminthiasis, cardiopathy, fever, hemorrhoids, gout,

Table 1. *In vitro* root induction of *Pseudarthria viscida* (L.) Wight & Arn.

Hormonal Concentration (mgl^{-1})				Explants	Response	Rooting (%)	No of days
NAA	IBA	IAA	2,4-D				
0.5	-	-	-	MS		40.00±0.03	25
1.0	-	-	-	MS	+	58.30±0.01	23
1.5	-	-	-	MS	++	72.66±0.02	25
2.0	-	-	-	MS	++	74.66±0.03	27
2.5	-	-	-	MS	++	**91.00±0.04**	29
3.0	-	-	-	MS	++	38.16±0.02	18
-	0.5	-	-	MS	+++	53.33±0.01	20
-	1.0	-	-	MS'	+	69.16±0.03	25
-	1.5	-	-	MS	++	63.50±0.29	27
-	2.0	-	-	MS	++	68.33±0.03	26
-	**2.5**	-	-	MS	++	**71.26±0.02**	28
-	3.0	-	-	MS	++	39.33±0.02	21
-	-	0.5	-	MS	+++	24.00±0.39	23
-	-	1.0	-	MS	+	31.56±0.04	20
-	-	1.5	-	MS	+	29.16±0.03	26
-	-	2.0	-	MS	+	32.50±0.02	27
-	--	2.5	-	MS	+	58.33±0.02	23
-	-	**3.0**	-	MS	+	**76.06±0.03**	26
-	-	-	0.5	MS	+	61.16±0.03	25
-	-	-	1.0	MS	+++	80.00±0.29	24
-	-	-	2.0	MS	++	68.66±0.03	20
-	-	-	**2.5**	MS	++	**89.32±0.02**	29
-	-	-	3.0	MS		69.22±0.12	25

diabetes, hyperthermia and general debility. Major chemical compounds reported to be present in the roots are 1,5 dicaffeoyl quinic acid, oleic acid, tetradecanoic acid, rutin, quercetin, gallic acid, ferulic acid and caffeic acid (Vijayabaskaran *et al*., 2010). The present investigation was undertaken to the production of *in vitro* roots and evaluation of the preliminary phytochemicals and anticancerous activity of these *in vitro* regenerated roots of *Pseudarthria viscida*.

MATERIALS AND METHODS

In vitro rooting of *Pseudarthria viscida*

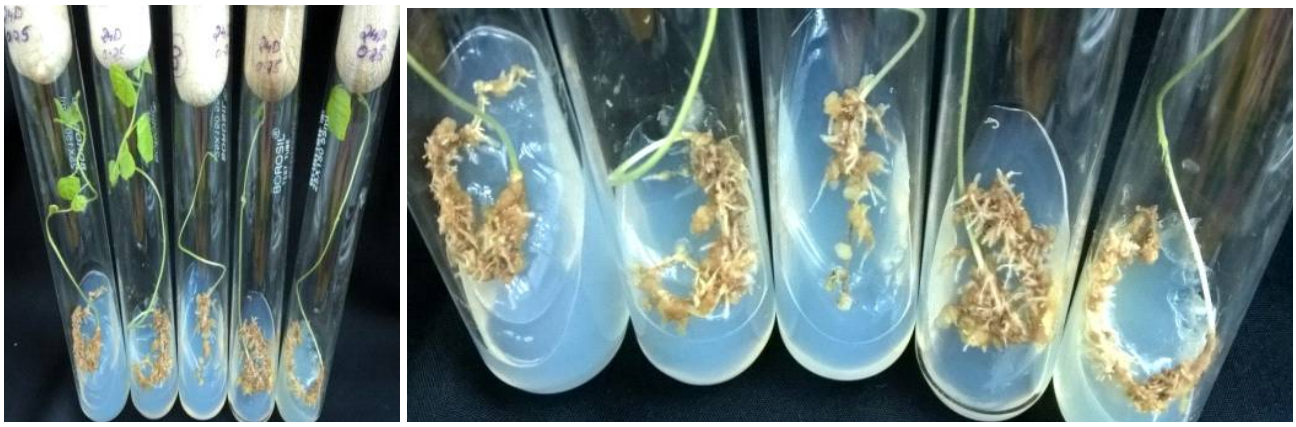
The *in vitro* multiplied microshoots after elongation were transferred for *in vitro* root formation in MS medium (Murashige & Skoog, 1962). Different auxin growth regulators like NAA, IAA, IBA and 2, 4- D were used at a range of 0.5- 3.0mgl^{-1} concentration for *in vitro* root induction in *P. viscida*. The elongated shootlets were individually cultured in culture tubes containing the rooting media. After eight weeks, percentages of root formation and root numbers of each treatment were recorded (Meena & Thomas, 2011).

In vitro regenerated root were then collected from the culture tubes and cleaned, weighed, oven dried, defatted with petroleum ether and finally extracted with pure methanol. This concentrated extract of *in vitro* regenerated roots was further used for preliminary phytochemical evaluation and anticancer analysis.

Preliminary phytochemical studies

The preliminary phytochemical studies of methanol extract of *in vivo* and *in vitro* developed root *Pseudarthria viscida* were done for the detection of phytoconstituents such as glycosides, alkaloid, terpenoids, steroids, tannin, flavonoids, phenols, saponins and coumarins using standard chemical tests (Kokate, 1986).

a. Rooting in 0.25 mg l^{-1} concentration of NAA



b.. Rooting in 0.25 mg l^{-1} concentration of 2-4D

c. Rooting in 0.3 mg l^{-1} concentration of IAA

d. Rooting in 0.25 mg l^{-1}concentration of IBA

Fig.1. *In vitro* root induction of *Pseudarthria viscida* (L.) Wight & Arn.

Glycosides (Keller - Kiliani test)

To the solution of the extract in glacial acetic acid, few drops of ferric chloride and concentrated sulphuric acid are added, and observed for a reddish brown ring at the junction of two layers.

Alkaloids (Dragendroff's test)

The plant extract was evaporated to dryness and the residue was heated on a boiling water with 10 ml of 2 % sulphuric acid for 2 minute. After cooling, the mixture was filtered and treated with a few drops of Dragendorff's reagent. Formation of orange brown precipitate showed the presence of alkaloid.

Flavonoids (Shinoda test)

Four ml of extract solution was treated with 1.5 ml of 50 % methanol solution. The solution was warmed and metal magnesium was added. To this solution, 5-6 drops of concentrated hydrochloric acid was added and red colour was observed for flavonoids.

Steroids (Liebermann Burchard Reaction):

Four ml of extract was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. Then concentrated sulphuric acid was added slowly. The formation of green bluish colour indicated the presence of steroids.

Terpenoids (Salkowski Reaction)

The extract was dissolved in 2 ml chloroform and followed by 3 ml of concentrated sulphuric acid was poured along the side walls to form a layer. Formation of reddish brown inter phase indicated the presence of terpenoids.

Saponins (Frothing test)

Five ml of extract was heated with 5 ml of distilled water and the frothing indicated the presence of saponins.

Phenols (Ferric chloride test)

The extract was added with 2 drops of 2 % ferric chloride solution and the presence of dirty green indicated the presence of phenols.

Tannins (Lead acetate test)

The extract was added with few drops of 1% lead acetate solution. The presence of yellow or red precipitate indicated the presence of tannins

Coumarins (Alchoholic sodium hydroxide and hydrochloric acid test)

The extract was added with sodium hydroxide and then dissolved in alcoholic sodium hydroxide. Then the concentrated hydrochloric acid was poured through the sides of the test tube. Appearance and the disappearance of yellow colour indicated the presence of coumarins.

***In vitro* cytotoxic analysis:**

HCT-15 colon cancer cell line and L929 normal fibroblast cell lines were purchased from NCCS Pune, were maintained in Dulbecco's modified eagle's media and grown to confluency at 37°C and 5 % CO_2 in a humidified atmosphere in a CO_2 incubator. The cells were trypsinized (500 µl of 0.025% Trypsin in PBS (Phosphate Buffered Saline)/ EDTA (Ethylene diamine tetra acetic acid) solution) for 2 minutes and passed to T flasks in complete aseptic conditions and incubated. Crude extract from *in vivo* and *in vitro* developed roots of *Pseudarthria viscida* were added in the concentration of 10µg, 50 µg and 100 µg were taken from a stock concentration of 100 mg ml^{-1} and was added, then incubated for 24 hours. The anticancer effect was determined by MTT cell viability assay (Sumitra & Krunal Nagani, 2013).

MTT cell viability assay

MTT assay is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with isopropanol and thereby released formazan. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

The cell culture suspension with crude methanol extracts from *Pseudarthria viscida* at concentration of 10µg ml^{-1}, 50µg ml^{-1} and 100µg ml^{-1} from a stock of 100mg ml^{-1}, was washed with 1x PBS and then added with 200µl MTT solution to the culture (MTT - 5mg $volume^{-1}$ dissolved in PBS), followed by incubation for 3 hours at 37°C. Media was removed, washed with 1x PBS and 200µl of DMSO was added.

Table 2. Preliminary phytochemical studies of *Pseudarthria viscida*

Sl No.	Phytoconstituents	*In vivo* developed root extract of *P. viscida*	*In vitro* regenerated root extract of *P. viscida*
1	Glycosides	+	+
2	Alkaloids	+	+
3	Flavonoids	++	+
4	Terpenoids	+	+
5	Steroids	+	+
6	Phenols	++	+
7	Tannins	+	+
8	Coumarins	+	-

Incubated at room temperature for 30 minutes until the cell got lysed and colour was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2 minutes to precipitate cell debris (Masters, 2000). OD was read at 540 nm using DMSO as blank. Percentage viability was calculated using the formula

Percentage viability = {OD of test/OD of control} x100

RESULTS AND DISCUSSION

In vitro rooting of *Pseudarthria viscida*

In *Pseudarthria viscida,* maximum *in vitro* root formation was noticed on MS medium supplemented with 2.5mgl^{-1}NAA concentration (91.00±0.04%)after 29 days of inoculation**.** Similarly 2.5mgl^{-1}2,4-D concentration were also showed higher *in vitro* root induction like 89.32±0.02%. In the case of IBA, 2.5mgl^{-1} concentration showed only 71.26±0.02 % *in vitro* root induction. But in IAA, 2.5mgl^{-1}concentration showed 58.33±0.02% root induction where as in 3.0 mgl^{-1} concentration, 76.06±0.03% of *in vitro* root formation was observed after 26 days of inoculation (Table 1 and Fig 1).NAA and IBA were generally found to be most effective for induction of roots in legumes as was observed in plants like *Pterocarpus marsupium* (Chand & Singh, 2004).

Preliminary phytochemical studies

Conventional protocols detected the presence of important secondary metabolites such as glycosides, alkaloids, flavonoids, terpenoids, steroids, phenols and tannins which could be the reason for cytotoxic activity of *in vivo* and *in vitro* developed roots of the plant (Table 2).

In vitro cytotoxic analysis:

In the present study, methanol extract of both *in vivo* and *in vitro* developed roots of *Pseudarthria viscida* showed potential cytotoxic activity against HCT-15 cancer cell lines and L929 cell lines. Methotrexate was used as standard drug. Cells which are pretreated with various concentrations of root

Table 3. Cytotoxic effect of the standard drug methotrexate and methanol extracts of *in vivo* and *in vitro* regenerated root of *Pseudarthria viscida*

Compound name	Conc. (µgml^{-1})	% of viability	
		HCT-15	L929
Standard drug (Methotrexate)	10	60.52±1.20	86.34±0.31
	50	31.33±0.74	64.56±0.82
	100	4.45±0.20	56.40±0.56
In vivo developed root extract of *P. viscida*	10	73.21±0.78	95.23±0.56
	50	39.08±0.21	89.70±0.61
	100	10.50±1.32	85.47±0.87
In vitro regenerated root extract of *P. viscida*	10	80.28±0.28	97.32±0.92
	50	52.73±0.16	91.88±0.23
	100	28.49±1.23	85.04±0.02

Table 4. IC_{50} values of Methotrexate and methanol extracts of both *in vivo* and *in vitro* developed root of *Pseudarthria viscida* by MTT assay.

Compound name	IC_{50} ($\mu g ml^{-1}$)	
	HCT-15	L929
Standard drug (Methotrexate)	22±0.08	>100
In vivo developed root extract of *P. viscida*	36±0.01	>100
In vitro regenerated root extract of *P. viscida*	45±0.01	>100

extracts (10µg ml^{-1}, 50 µg ml^{-1} and 100 µg ml^{-1}) showed decrease in percentage of HCT-15 cells viability such as crude extract of *in vivo* developed root showed 73.21±0.78%, 39.08±0.21% and 10.50±1.32% cell viability whereas *in vitro* regenerated root extract showed 80.28±0.28%, 52.73±0.16% and 28.49±1.23% cell viability for the sample concentrations10 µg ml^{-1}, 50 µg $ml^{-1}$and 100 µg ml^{-1} respectively and the results were significant(Table 3, Fig. 2-3).

The cytotoxic effects were expressed as IC_{50}. Crude extracts of both *in vivo* and *in vitro* developed root of *Pseudarthria viscida* showed potential cytotoxic activity against HCT-15 cell line with IC_{50} values of 36±0.01µg $ml^{-1}$and 45±0.01µg ml^{-1}respectively. They exhibited IC_{50} exceeding 100 µg/ml for L929 normal cell line. IC_{50} value of crude root extract showed high cytotoxicity effect compared to standard drug methotrexate (Table 4).To be a good drug, the IC_{50}values of such agent should be sufficiently low to avoid any possible unspecific effects. The American National Cancer Institute assigns a significant cytotoxic effect of promising anticancer product exerts an IC_{50} value < 40 µg/ml. Like *in vivo* developed roots, *in vitro* regenerated roots of *Pseudarthria viscida* could be proposed as a promising anticancer agent. Similar type of Cytotoxic effect against HCT-15 and HePG2 cell lines were

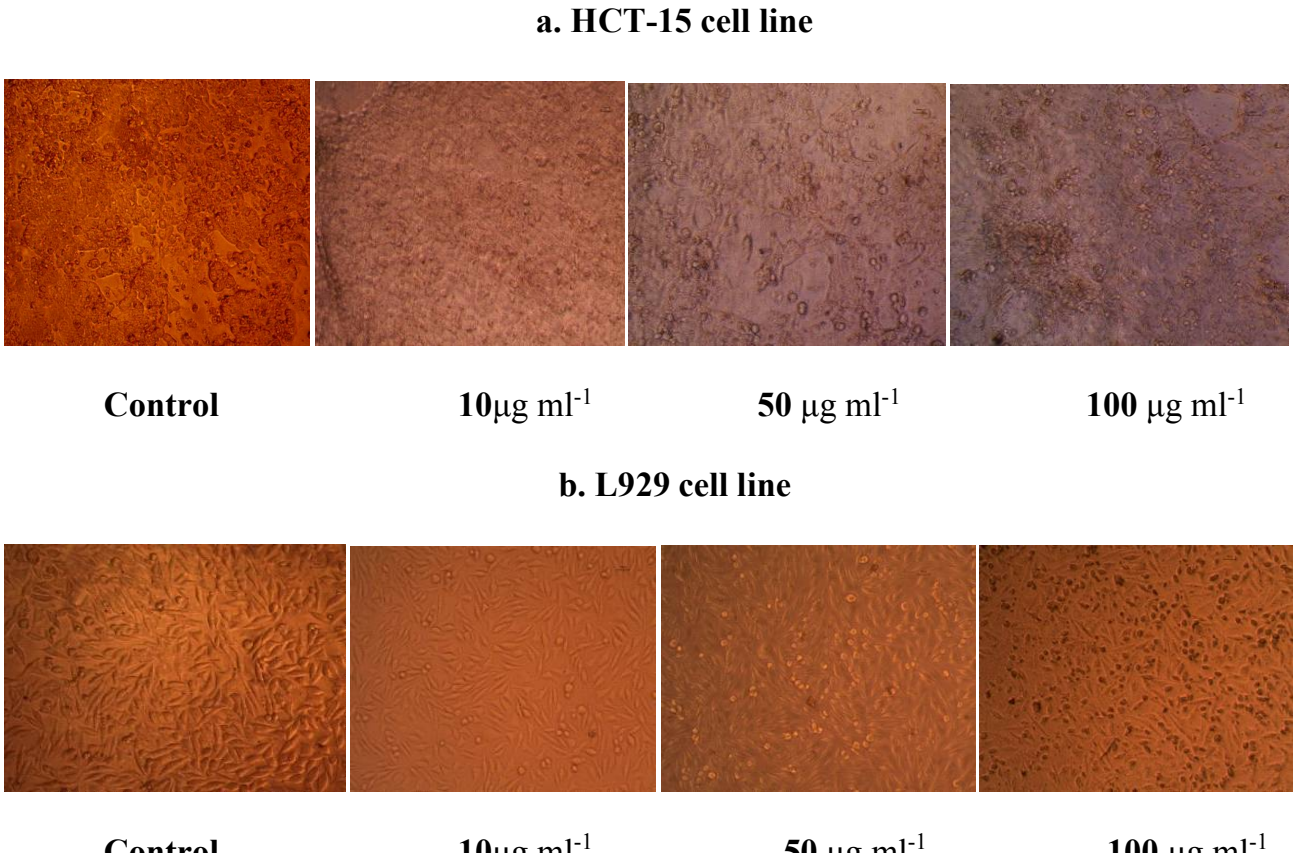
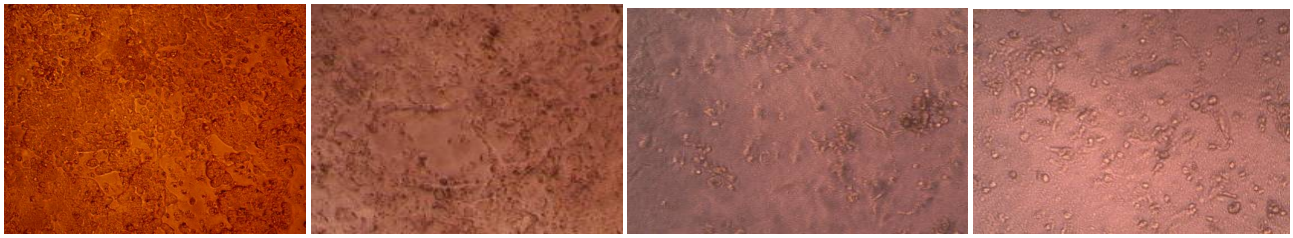


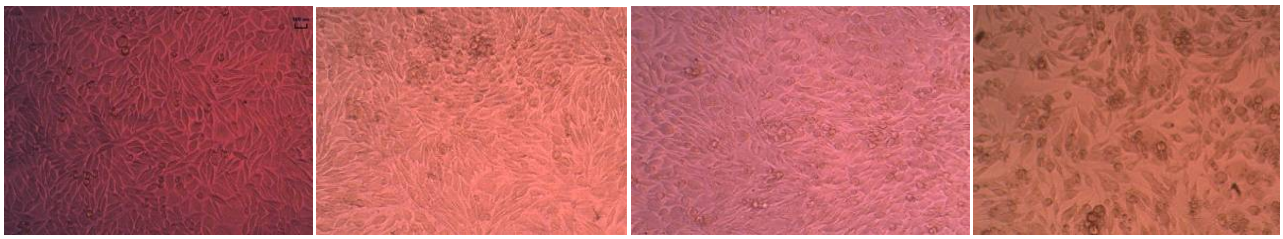
Fig.2. Cytotoxic effect of methanol extract of *In vivo* developed root of *Pseudarthria viscida*

a. HCT-15 cell line.



Control **10**μg ml^{-1} **50** μg ml^{-1} **100** μg ml^{-1}

b. L929 cell line



Control **10**μg ml^{-1} **50** μg ml^{-1} **100** μg ml^{-1}

Fig.3: Cytotoxic effect of methanol extract of *In vitro* regenerated root of *Pseudarthria viscida*

reported in *Sophora interrupta* by Vithya *et al*., 2012. Cytotoxicity effect against cancer cells were reported in the ethanolic extract of both *in vitro* and *in vivo* developed tuber of *Ceropegia pusilla* (Kalimuthu *et al*., 2014).

Like *in vivo* developed root, *in vitro* regenerated root extract of *P. viscida* contain numerous bioactive molecules with promising pharmaceutical properties like antioxidant, anticancer activities etc. The excessive collection of *P. viscida* for pharmaceutical purposes and Ayurvedic preparations has resulted in its disappearance from the natural habitat. Therefore, this study suggested the use of *in vitro* developed plants and roots of *P. viscida* instead of using wild plant for pharmaceutical purposes, which helps to conserve *P. viscida* from disturbing its wild habitat.

CONCLUSION

In the present study, maximum *in vitro* root induction were noticed on MS medium with 2.5 mgl^{-1}of NAA and 2.5 mgl^{-1}of 2, 4-D after 29 days of inoculation. In preliminary phytochemical evaluation, important phytoconstituents like glycosides, alkaloids, flavonoids, terpenoids, phenols and tannins were noticed which could be the reason for cytotoxic activity of *in vivo* and *in vitro* developed roots of the plant. Crude extracts showed potential cytotoxic activity against HCT-15 cell line with IC_{50} values of 36±0.01μg ml^{-1} and 45±0.01μg ml^{-1} in both *in vivo* and *in vitro* developed roots respectively and IC_{50} for normal cell lines were found to be very high when compared to cancer cells, which indicated that the crude extracts showed high cytotoxicity effect against cancer cells and can be considered as safer for normal cells. The present study thus confirmed that *in vivo* and *in vitro* produced roots of *Pseudarthria viscida* (L.) possess good cytotoxic activity. Conservation of *Pseudarthria viscida* could be done by *in vitro* root induction without disturbing its wild habitat. Further studies are necessary to evaluate and isolate the active principles responsible for these activities.

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A Comparitive Investigation to Distinguish Two Closely Resembling Families of Gamopetalae, In Some Selected Genera – An Anatomical Overview

SHARANYA K.P.*, RENCY BABU C P AND GEETHU P G

Department of Botany, NSS College Pandalam, Pathanamthitta, Kerala
**email : sharanyakp4@gmail.com*

ABSTRACT

The aim of present study was to distinguish two gamopetalous families namely Lamiaceae and Verbenaceae by investigating the anatomical characteristics of total ten selected members, five from each family. The anatomical parameters selected include stomatal types, stomatal intensity, trichome types and trichome intensity. The significance of this study lies in the fact that, some of the Verbenacean members based on Bentham and Hooker system of classification are now found to be shifted to Lamiaceae, according to APG system of classification. This is analogous to the present anatomical study result, which point out the fact that anatomical features of leaf, petiole, stem etc have found application in systematics, particularly for resolving controversial taxonomic problems.

Key words *Gamopetalae, Verbenaceae, Lamiaceae, Trichome, Stomata*

From ancient times, plants have been classified differently at different times. Common four major system of classification are practical, artificial, natural and phylogenetic system of classification. In 1998 a classification of the angiosperms namely Angiosperm Phylogeny Group (APG) system was introduced based on molecular phylogenetics. Now a day's molecular techniques such as molecular markers are used to classify plants. These methods are very costly. So a simple way is needed to differentiate a genus between plants. Anatomical data help to study the ranges of variability of characters in same genus or different genus. It has been successfully employed for the elucidation of phylogenetic relationship. In the present study the characteristic traits used to solve the comparison between Lamiaceae and Verbenaceae includes length and width of stomata, guard cell, subsidiary cell, trichomes and stomatal index. Also stomatal and trichome type were considered.

Major difference between Verbenaceae and Lamiaceae

- Verbenaceae has terminal style where as Lamiaceae has gynobasic style.
- Verbenaceae has racemose inflorescence where as Lamiaceae has racemose as well as cymose arrangements.
- Verbenaceae traditionally include woody, shrubby plants while Lamiaceae consist of mostly herbaceous plants.

There is only a little difference between these families. Also several genera from Verbenaceae were transferred to Lamiaceae in 1990s on the basis of phlylogenetic studies of DNA sequences. So, based on these aspects, the main objective of the present study is to evaluate the anatomical characteristics of selected plant species and identify the plant family through these anatomical characters.

Objectives of the study include the comparison of anatomical characters between Verbenaceae and Lamiaceae, such as study of trichome variability, intensity and type, stomatal type and stomatal index study, compare length and width of guard cell and subsidiary cells.

MATERIALS AND METHODS

Sample materials of ten plants comprising five genera from both Verbenaceae and Lamiaceae families respectively, based on Bentham and Hooker's system of classification have been investigated in the study. Plants were collected from the localities of Pandalam, Pathanamthitta. The genera selected for the study are listed below.

VERBENACEAE MEMBERS : 1. *Lantana camara,* **2**. *Vitex negundo,* **3**. *Clerodendrum thomsoniae,* **4**. *Duranta repens,* **5.** *Stachytarpheta indica*

LAMIACEAE MEMBERS : 6. *Ocimum sanctum,* **7.** *Leucas aspera,* **8.** *Hyptis suaveolens,* **9.** *Coleus aromaticus,* **10.** *Mentha spicata*

The stem, petiole and leaf anatomical characteristics were investigated in cross sections taken in laboratory conditions. The cross section of

stem, petiole as well as leaf and peels of leaf were taken with the help of razor blade and hand respectively. These peels and sections were placed on glass slides and dyed using a drop of 1% safranin stain, kept for 1 minute, washed with water and then mounted in a drop of glycerin to prevent the drying. A cover slip was placed above it. These slides were examined under a light microscope (10x) while the stomatal study was done with the help of high power lens (40x). During microscopic investigation, their coloured pictures were taken by a microphotography apparatus. The data were tabulated and analyzed. The parameters studied include,

1. Study of trichome morphology

Length of trichome was measured using ocular micrometer. Type, intensity and number cells were also noted.

2. Study of stomata

Stomata on the lower side of leaves only were considered. Length and width of stomata, guard cells, and subsidiary cells was measured using ocular micrometer. Also stomatal index (S.I) was calculated as defined by Salisbury (1927)

$$\text{Stomatal Index} = \frac{S \times 100}{E + S}$$

Where,

I- Stomatal Index.

S- No. of stomata per unit area.

E- No. of epidermal cells per unit area.

3. Study of stem, petiole and leaf anatomy

Epidermis characteristics, trichome intensity, cells of hypodermis, cortex were identified. Also arrangement of vascular bundle and pith were noted.

RESULT

The stem, petiole, leaf, stomata and trichome anatomy of five plants from both Verbenaceae and Lamiaceae have been investigated on the present study. There are differences and similarities between the genera, regarding the anatomical aspects of plant parts like stem, petiole and leaf but significant variation has been observed in case of stomata and trichome. The detailed anatomical observation of each genus in selected parameters has been given below.

Transverse section of stem shows similarity between the plants studied but a few differences are there. Petiole anatomy does not show much variation in selected plants. It shows similarity with stem anatomy. Leaf section shows a little similarity among the plants. All plants in Lamiaceae bears thin cuticle but in Verbenaceae all possess thick cuticle except *C. thomsoniae.*

TRICHOME MORPHOLOGY

Trichome morphology has an important role in genera delimitation within Lamiaceae and Verbenaceae family. They have also an important taxonomic role in species differentiation. In the present study trichomes was analyzed. The main types of trichomes investigated based on length, type, intensity, number of cells and their distribution among the genera are studied and summarized in table 1. The photographs are represented in figure 2. The characters of taxonomic interest were the presence of glandular and non-glandular trichomes, its number of cells, length and intensity. Types of trichomes show considerable variation among different genera in Lamiaceae and Verbenaceae therefore afford valuable characters in delimiting the families. It has been noted that number and length of trichomes was different for Verbenaceae and Lamiaceae members.

Normally Verbenaceae members have less number of trichomes except *Clerodendrum thomsoniae* and *Vitex negundo.* In Lamiaceae all five selected plants has numerous trichomes. All plants in Lamiaceae have both glandular and non-glandular trichomes. But in Verbenaceae except *Stachytarpheta indica* and *Duranta repens*, all possess glandular and non-glandular trichomes.. Among Verbenaceae members *Lantana camara* have the longest trichomes. Trichomes in all genera of Lamiaceae and Verbenaceae are more or less similar in appearance except in some critical measurements. Among the ten plants investigated *Ocimum sanctum* possesses the largest trichomes i.e. 0.549 mm and shortest trichomes are present in *Vitex negundo* and *Clerodendrum thomsoniae.* Number of cells in trichomes is highest in Ocimum sanctum, 4 to 5mm. Graphical representation of trichome length has been plotted in figure 1.

Table 1. Showing Trichome analysis of selected plants

Family	Genera	Type		Intensity	Length	No. of cells
		Glandular	Non glandular			
Verbenaceae	*C. thomsoniae*	+	+	Numerous	0.07	1
	D. repens	-	+	Less	0.084	2- 4
	L. camara	+	+	Less	0.224	1
	S. indica	-	+	Less	0.21	2- 4
	V. negundo	+	+	Numerous	0.07	1
Lamiaceae	*C. aromaticus*	+	+	Numerous	0.364	1
	H. suaveolens	+	+	Numerous	0.21	2- 6
	L. aspera	+	+	Numerous	0.224	2
	M. spicata	+	+	Numerous	0.294	2-4
	O. sanctum	+	+	Numerous	0.549	4-5

Stomatal Study

Regarding stomata, stomatal type and index represents an important feature in genera delimitation. The result of stomatal study is summarized in table 2. The photographs are presented in figure 3. Graph showing stomatal index is given in figure 4.

The total ten genera analyzed, all genera were amphistomatous i.e. bears stomata on both surfaces expect *Vitex negundo*. *Lantana camara* bears highest stomatal length, guard cell length and width. In Lamiaceae all five genera possess diacytic stomata. In Verbenaceae *S. indica, L. camara* and *V. negundo*

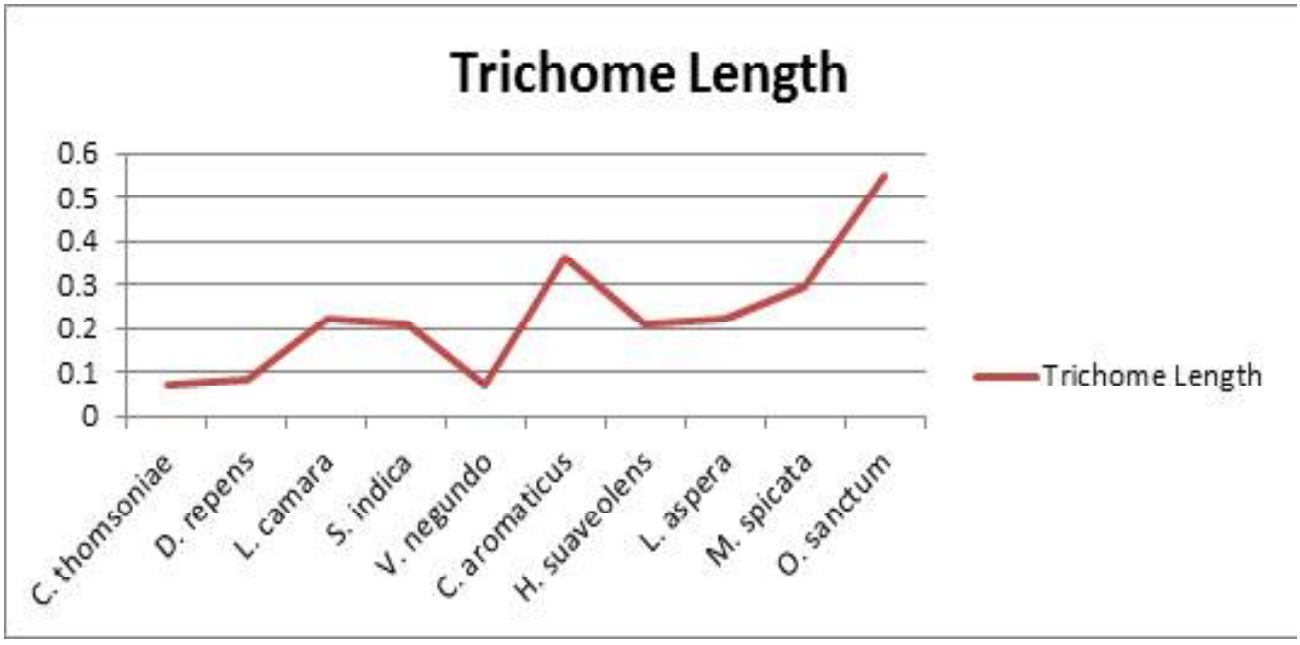

Fig. 1: Graphical representation of trichome length

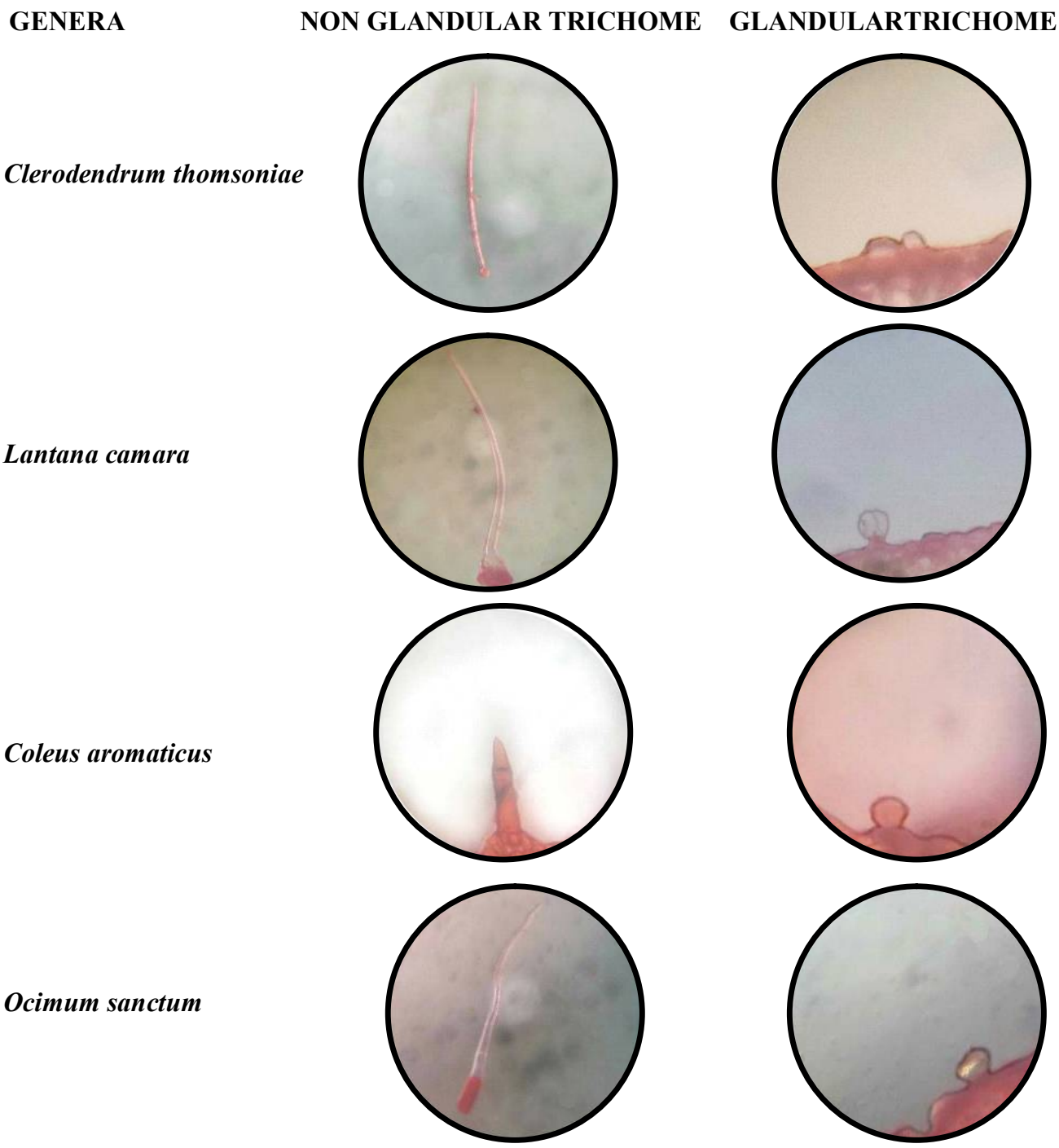

Fig. 2. Trichomes in Verbenaceae and Lamiaceae plants

consist of anomocytic stomata while *D. repens* and *Clerodendrum thomsoniae* possess anisocytic and diacytic stomata respectively. In this study it is found that most of the Lamiaceae members bear stomatal index from 20 to 24, of which *H. suaveolens* bears highest stomatal index. In Verbenaceae *S. indica* possess highest stomatal index i.e. 30, also in genera *S. indica, L. camara* and *V. negundo* subsidiary cells are absent.

DISCUSSION

The present study is quiet significant. Trichome importance of epidermal features is emphasized by many researchers (Metcalfe and Chalk, 1950; Singh and Dube, 1993; Moon *et.al.,* 2009). In most of the species trichomes were as much denser on the abaxial

Table 2. Showing stomatal study on selected plants

Family	Genera	Type	Index	Stomata		Guard cell		Subsidiary cell	
				Length	Width	Length	Width	Length	Width
Verbenaceae	*C. thomsoniae*	Diacytic	22	0.028	0.014	0.028	0.028	0.042	0.028
	D. repens	Anisocytic	28	0.028	0.014	0.042	0.028	0.056	0.028
	L. camara	Anomocytic	25	0.042	0.014	0.056	0.042	_	_
	S. indica	Anomocytic	30	0.028	0.014	0.042	0.028	_	_
	V. negundo	Anomocytic	15	0.028	0.014	0.028	0.028	_	_
Lamiaceae	*C. aromaticus*	Diacytic	23	0.028	0.014	0.042	0.028	0.056	0.028
	H. suaveolens	Diacytic	24	0.028	0.014	0.042	0.028	0.07	0.028
	L. aspera	Diacytic	21	0.028	0.014	0.042	0.028	0.056	0.042
	M. spicata	Diacytic	23	0.028	0.014	0.042	0.014	0.056	0.028
	O. sanctum	Diacytic	20	0.028	0.014	0.028	0.014	0.056	0.028

surfaces. In the present study presence or absence of trichomes as well as their types can be useful in characterizing the genera. The stomata were found only in the lower surface of the leaf, on the same level with the epidermis (Metcalfe and Chalk1950).

Trichome diversity may be taxonomically significant in Lamiaceae and closely related families at various taxonomic levels (Metcalfe and Chalk, 1950; Mathew and Shah, 1981; Xiang *et al.,* 2010). In Lamiaceae, non-glandular and glandular trichomes are distinguished with peltate and capitate trichomes as the basic types of glandular trichomes (Cantino, 1990; Fahn, 2000). Capitate glandular trichomes constitute a significant taxonomic character of the Lamiaceae (Navarro and El Oualidi, 2000).

In all members of Lamiaceae investigated in our study showed diacytic stomata. This investigated result is parallel with the study concluded by Metcalfe and Chalk (1950). The stomata of the Lamiaceae are described by Metcalfe and Chalk (1950) as mostly diacytic and intermixed with anomocytic. Cantino (1990) concluded that anomocytic and diacytic were most frequent types in both Lamiaceae and Verbenaceae. He also found five different types of stomata in Mentheae. Inamdar and Bhatt (1972), who studied 33 species in 17 genera of the Lamiaceae, found that in the majority of species, stomata occurred exclusively on the lower leaf surface.

All five members of Lamiaceae as well as *V. negundo* and *C. thomsoniae* possess quadrangular stem. This result is analogous with that of Metcalfe and Chalk (1972), where they mentioned that in many genera and species of the Lamiaceae, the stems are quadrangular. The stem, a typical characteristic of Verbenaceae, is quadrangular (Watson and Dalwitz, 1992). The petiole anatomy showed variability in different Lamiaceae species. This is supported by Gupta and Bhambie, 2008. Glandular and non-glandular trichomes are commonly found in Verbenaceae. Inamdar (1969) and Yashodhara *et al.*, 2001 also reported the same. (Munsif *et al.,* 2007) described that trichomes are mostly observed were micro hair on both surfaces of *V. negundo*.

According to Metcalfe and Chalk (1950), the stomata in the family Verbenaceae have been described as diacytic, paracytic and anomocytic. In our study among the five members, three showed anomocytic stomata while the other one showed diacytic stomata (Ramassamy and Kannabiran, 1995). In leaves the outer surface of the upper epidermis is covered by a cuticle with non-glandular hairs on its surface. These hairs are short and conical. These results show a parallelism with the record of Metcalfe

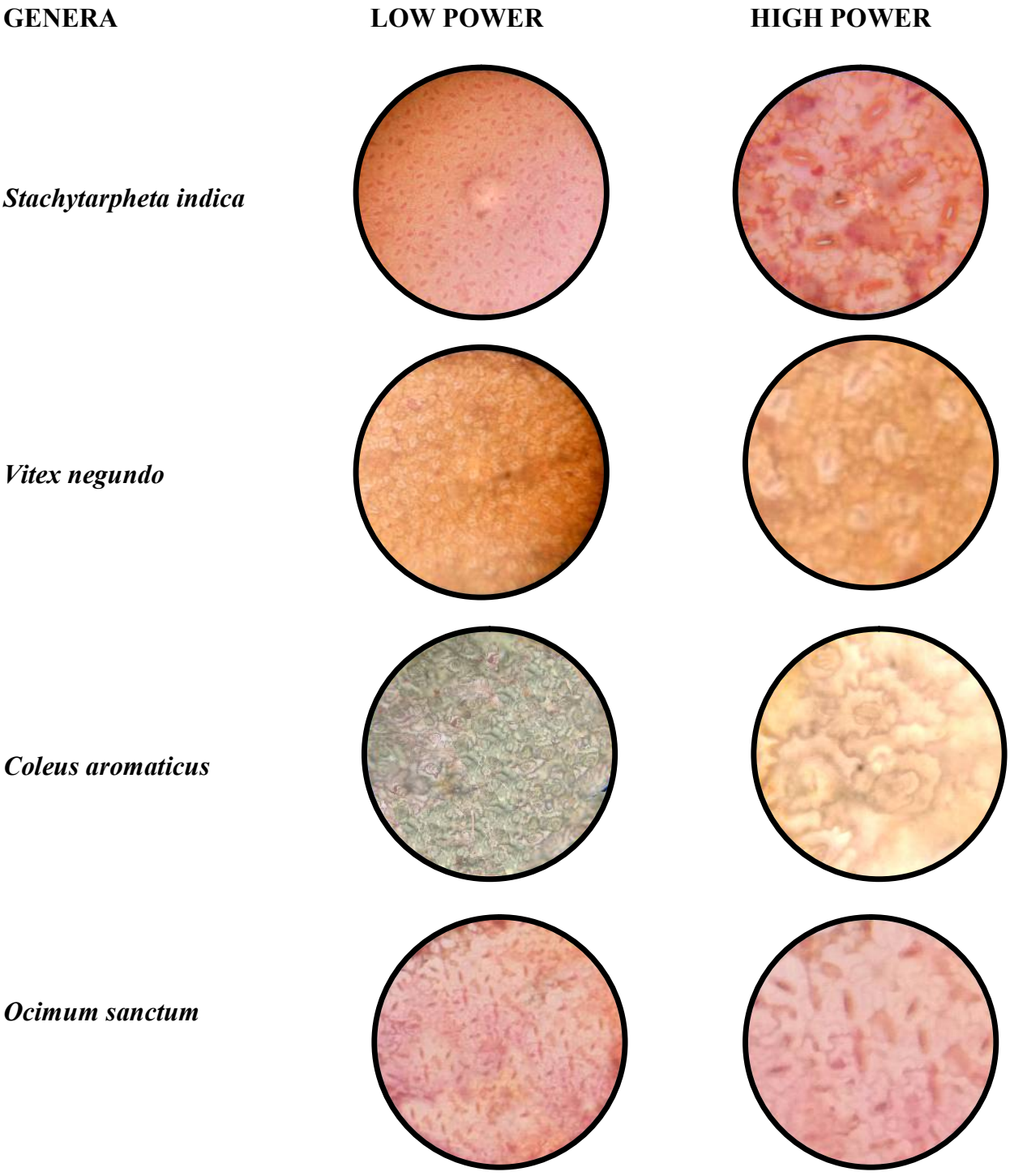

Fig. 3. Stomatal type in Verbenaceae and Lamiaceae plants

and Chalk (1957).

CONCLUSION

The present investigation is an attempt to distinguish two closely relating families Verbenaceae and Lamiaceae. There are some taxonomic uncertainties within the plants. It is extremely difficult to distinguish some plants in Verbenaceae and Lamiaceae because of their great morphological similarity. The only major morphological difference between these two families is that Verbenaceae possess tubular or oblique corolla whereas Lamiaceae possess bilabiate corolla. Sometimes, the anatomical keys are more convincing than morphological ones, but it is well known that they have low applicability in botany. But in the present study numerous anatomical

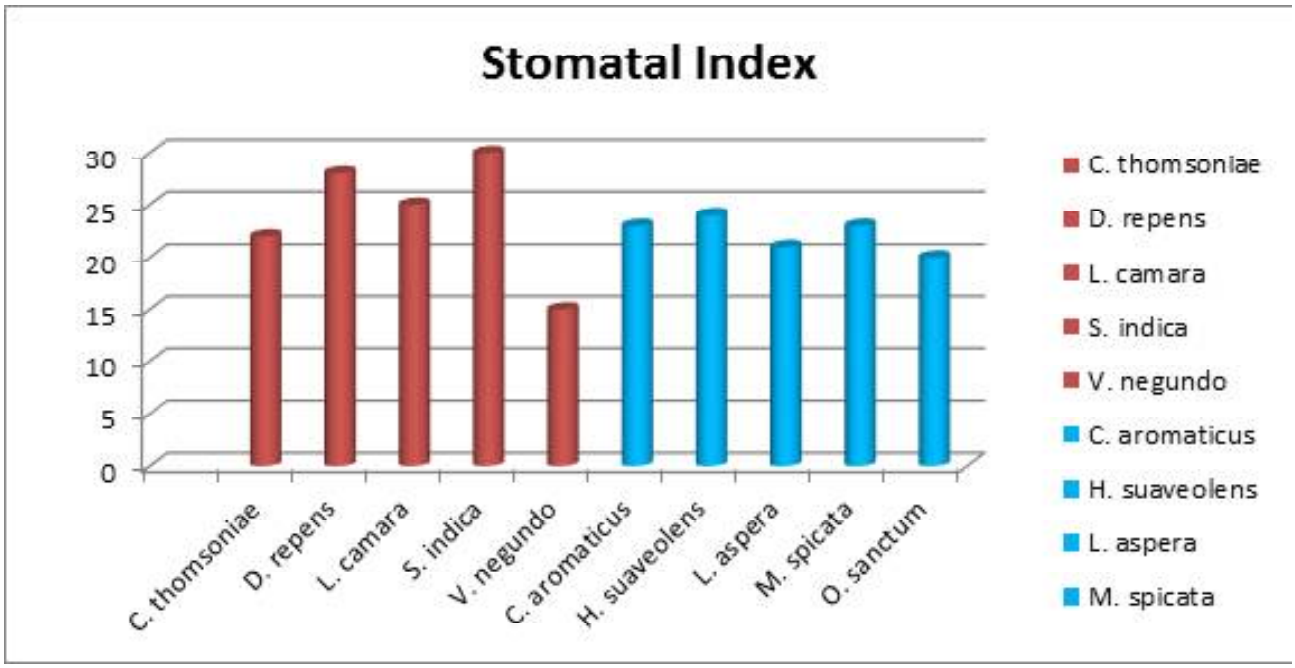

Fig. 4. Graphical representation of stomatal index

characters were found significant for the delimitation of families Verbenaceae and Lamiaceae. The anatomical parameters used to differentiate the two gamopetalous families; Verbenaceae and Lamiaceae successfully in this study were stomatal type, stomatal index, type of trichomes in epidermis, trichome intensity and trichome length. It is also important to notice similarities in leaf and petiole anatomical structures.

In conclusion, the objective of this study was to evaluate the taxonomic value of plant anatomical characteristics in two different families, to see if they provide additional perspectives on the taxonomic problems. According APG system II (2003) *Vitex negundo* and *Clerodendrum thomsoniae* has been moved from Verbenaceae to Lamiaceae family. This study also showed similar result. From this study it is concluded that taxonomic identification of family of plants through anatomy is not only widens scope of taxonomy but also provides an efficient approach to identify the plant, which otherwise would have require complete information on the plant for its correct identification.

The present investigation also question the position of *Lantana camara* in Verbenaceae family because throughout our study it could observe that certain characteristics of *Lantana* show similarity towards the other Lamiacean members including the two genera recently shifted to Lamiaceae from Verbenaceae family. Therefore this study foresees a chance of reshifting Lantana from Lamiaceae to Verbenaceae in far future.

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Anthocyanin- A Potential Natural Food Colourant From *Ipomoea batatas* (L.) Lam Genotypes

SHEEBA M. J.[1], KAMALAM P.[2] AND VIJAYAVALLI B.[3]

[1]*Department of Botany, T.K.M College of Arts and Science, Kollam.*
[2]*Division of Crop Improvement, Central Tuber Crops Research Institute, Sreekariyam - 695017*
[3]*Department of Botany, University of Kerala, Kariavattom*
email :sheebatkmbot@gmail.com

ABSTRACT

Sweet potato (Ipomoea batatas (L.) LAM.) belonging to the family Convolvulaceae is a herbaceous dicot widely grown throughout the tropics and warm temperate regions of the world. The anthocyanin content in flowers, leaves (young & mature) stems and tubers of ten sweet potato cultivars viz.S.Bhadra,S.Rethna,KV-4,A-144,91-7,95-1,94-57,96-4,94-84 and Kanjangad local were done. The colour of anthocyanin ranged from red to purple in different cultivars. The highest value of anthocyanin was recorded in the fiowers followed by young leaves, mature leaves and stems. The maximum absorbance value was noted in S. Bhadra and 94-86 for flowers and young leaves. Investigations on tubers indicated the presence of anthocyanin only in the tuber skin and rind except 91-7 and KV-4.

Key words *Anthocyanin, Absorbance value, Ipomoea batatas.*

Anthocyanins are the largest group of water-soluble pigments in most fruits, flowers and leaves and are typically glycosides usually occurring in cell sap solutions of vacuoles (Ferry and Ward, 1959; Mayer and Anderson, 1959). Their role as attractants for pollinators and seed dispersers as well as an aid in plant classification is well evident, many of them behave as indicators, tending to turn red under acid conditions and blue at alkaline pH (Arditti and Dunn, 1968). Recently they have been used as a natural colourants in food processing (Cascon *et al.,* 1984). Bassa and Francis (1987) suggested that the anthocyanins in sweet potato roots are more stable of the three anthocyanin sources tested. According to Narayan *et al.* (1999), anthocyanins have been found to be a potent antioxidant compared to classical antioxidants. Anthocyanins have been demonstrated to have a wide range of biochemical and pharmacological effects including anticarcinogenic, antiatherogenic, anti-inflammatory, antimicrobial and antioxidant activities (Mazza and Miniati, 1993; Girard and Mazza, 1998). However the anthocyanin pigments in sweet potato have received very little attention.

MATERIALS AND METHODS

Anthocyanin pigments were separated from the tubers, stems, leaves (young and mature) and flowers of all genotypes. The method adopted for the separation of anthocyanin was according to Harbone (1973). 2 gms of fresh sweet potato parts of each genotype were grinded, soaked in 10ml of 1% HCl in methanol solution for 10 minutes, certrifuged and filtered. The supernatant was evaporated in a water bath and concentrated to 5ml. To 1ml of the concentrate, 4ml of 1% HCl in methanol was added. After the required dilution, the mixture was subjected to absorption spectrophotometry and visual observation studies (FAO, 1983 – general method). Extraction was performed with three replications from each material. Colour value, a measurement for anthocyanin content, can be calculated by the formula, absorbance at 530 nm x dilution rate (JFAA, 1993). Colour retention test of anthocyanin was evaluated by keeping the sweet potato extracts in ordinary room temperature (FAO, 1983).

RESULTS AND DISCUSSION

Phenotypic analysis

The genotypes Sree Bhadra, Sree Rethna and 94–86 were purple coloured in all the five plant parts observed for colouration viz., young leaf, mature leaf, stem, flower and tuber skin. The genotype Kanjangad local showed purple colour in four parts viz., young leaf, mature leaf, flower and tuber skin. The corolla throat showed deep bluish purple colouration in all the ten genotypes. The tuber skin colour was purple in Sree - Bhadra, Sree Rethna, A – 144, 94 – 86, 94 – 57, 96 – 4, 95 – 1 and Kanjangad local. The genotypes KV – 4 and 91 – 7 showed cream white tuber skins. The young leaves of Sree Bhadra, Sree Rethna, 94 – 86 and Kanjangad local were deep purple in colour, while A–144 showed medium purple colouration. The margin of the mature leaf was light purple in colour in the genotypes Sree Bhadra, Sree Rethna, 94 – 86 and

Table 1. Colourations observed in the five main plant parts (young leaf, mature leaf, stem, flower and tuber skin) of the ten sweet potato genotypes.

Genotype	Colour of				
	Young leaf	Mature leaf	Stem	Flower	Tuber skin
94 – 57	Green	Green	Light purple at nodes	Throat is deep purple	Medium purple
Sree Rethna	Deep purple	Marginal side is light purple	Light purple	Throat is deep purple	Deep purple
91 – 7	Green	Green	Green	Throat part is deep purple	Cream white
Sree – Bhadra	Deep purple	Light purple at marginal side	Light purple at nodes	Throat part is deep purple	Deep purple
KV – 4	Green	Abaxial leaf vein is light purple	Green	Throat part is deep purple	Cream white
95 – 1	Light purple at marginal side	Green	Light purple dots	Throat part is deep purple	Deep purple
94 – 86	Deep purple	Light purple at marginal side	Light purple	Throat part is deep purple	Deep purple
96 – 4	Green	Green	Green	Throat part is deep purple	Medium purple
Kanjangad local	Deep purple	Light purple at marginal side	Green	Throat part is deep purple	Deep purple
A – 144	Medium purple in marginal side	Green	Green	Throat part is deep purple	Medium purple

Kanjangad local. The genotypes Sree Rethna and 94 – 86 showed light purple stems. In Sree Bhadra and 94-57, the nodal portion of the vines were also purple (Table 1).

Sree Rethna and 94–86 showed high intensity in the extracts from young leaves, flowers and tuber skin parts. The genotypes Sree Bhadra, Sree Rethna, 95–1, Kanjangad local and 94–86 showed high intensity in tuber skin extracts, while 94–57, 96–4 and A–144 showed medium intensity. In KV–4 and 91–7 with creamy white tuber skins, the tuber skin extracts showed no purple colouration, 91–7, showed purple

Table 2. Intensity of purple colouration in the extract from the five plant parts of the ten genotypes of sweet potato

Genotype	Intensity of purple colour of extract of:				
	Young leaf	Mature leaf	Stem	Flower	Tuber skin
94 – 57	Absent	Absent	Low	High	Medium
Sree Rethna	High	Low	Low	High	High
91 – 7	Absent	Absent	Absent	High	Absent
Sree - Bhadra	High	Low	Low	High	High
KV – 4	Absent	Low	Absent	High	Absent
95 – 1	Low	Absent	Low	High	High
94 – 86	High	Low	Low	High	High
96 – 4	Absent	Absent	Absent	High	Medium
Kanjagad local	High	Low	Absent	High	High
A – 144	Medium	Absent	Absent	High	Medium

Fig. 1. Variation in the purple colour of the extract of young & mature leaves of sweet potato genotypes

colouration only in the floral extracts. Purple colouration was absent in the extracts of young, mature leaves and stem in 96-4. On the whole, the genotypes KV–4 and 91-7 showed a poor performance in the anthocyanin content in contrast to Sree Bhadra, Sree Rethna, 94–86 and Kanjangad local, which performed well (Fig. 1 & 2, Table 2).

Spectrophotometrical analysis

Absorbance values of anthocyanin pigments contributing to the purple colouration in young leaves, mature leaves, flower and tuber skin extracts are presented under five sub headings.

Young Leaves

Only six genotypes viz., Sree Bhadra, Sree Rethna, 94 – 86, 95 – 1, A – 144 and Kanjangad local showed the maximum absorbance at 525 nm, with high values of absorbance ranging from 500 to 550 nm. The remaining four genotypes viz., KV–4, 94–57, 91–7 and 96–4 did not show any absorbance. In the six genotypes, absorbance peaks was observed to

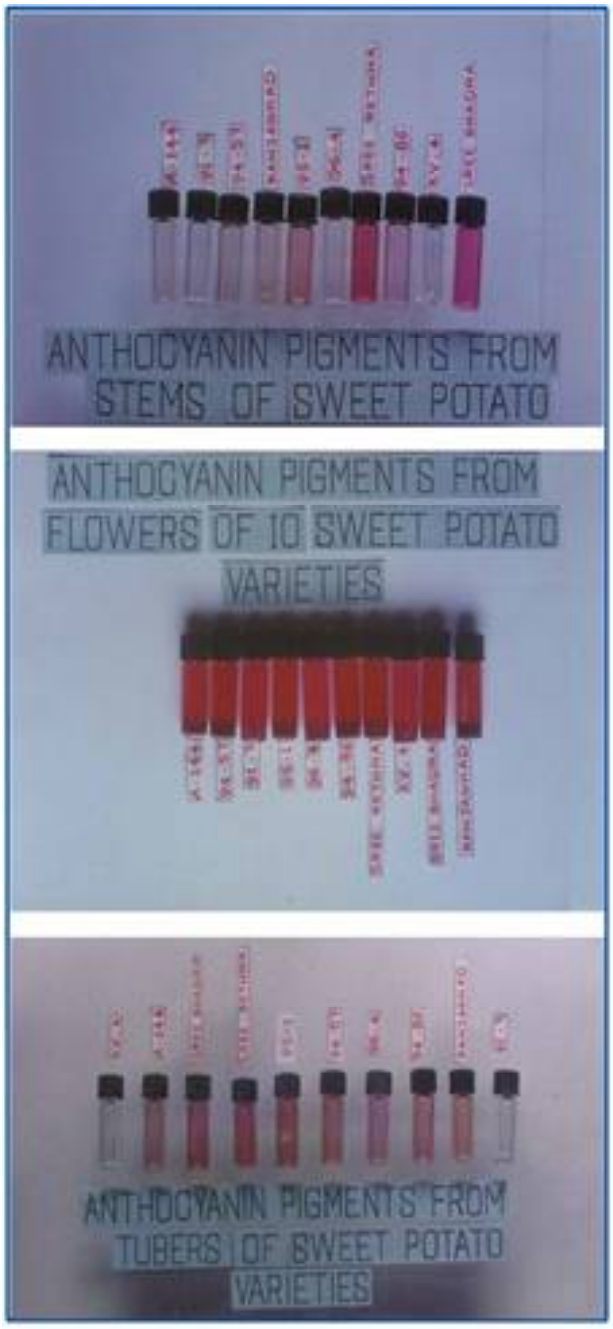

Fig. 2. Variation in the purple colour of the extract of stems,flowers & tubers of sweet potato genotypes

be absorbance was very low in the higher wave length regions (650nm to 725nm). Maximum absorbance was shown by Sree Bhadra and 94–86 followed by Sree Rethna and Kanjangad local (Table 3).

Mature Leaves

Sree Bhadra, Sree Rethna, KV–4, 94–86 and Kanjangad local showed absorbance maxima at 525 nm, with the highest values for Sree Bhadra followed by 94 – 86 and Sree Rethna. Five genotypes viz., A –

Table 3. Absorbance maxima of anthocyanin pigments in the five main plant parts of the ten sweet potato genotypes

Sl No	Genotypes	Young leaves			Mature leaves			Stem			Flower			Tuber skin		
		Wave length (nm)			Wave length (nm)			Wave length (nm)			Wave length (nm)			Wave length (nm)		
		500	525	550	500	525	550	500	525	550	500	525	550	500	525	550
1	Sree Bhadra	1.6120	1.8111	1.6130	0.3101	0.4911	0.2911	0.2767	0.3047	0.2550	1.7810	1.8112	1.7900	1.6120	1.6710	1.6230
2	Sree Rethna	1.4611	1.7471	1.4621	0.2919	0.3811	0.2711	0.2600	0.2900	0.2610	1.5788	1.7410	1.5001	1.1120	1.5120	1.2120
3	KV – 4	0	0	0	0.1004	0.1616	0.1005	0	0	0	1.5170	1.7310	1.4611	0	0	0
4	A – 144	1.1516	1.3755	1.2123	0	0	0	0	0	0	1.1234	1.7012	1.1214	0.8110	0.8910	0.8110
5	91-7	0	0	0	0	0	0	0	0	0	1.0112	1.7131	1.1316	0	0	0
6	95-1	0.6071	0.6984	0.6011	0	0	0	0.1001	0.1992	0.1002	1.0123	1.7242	1.1374	0.8221	1	0.8200
7	Kanjangad local	1.2129	1.7131	1.2910	0.1190	0.1330	0.118	0	0	0	1.1432	1.7531	1.5634	0.7139	1.1210	0.7810
8	94-57	0	0	0	0	0	0	0.1000	0.1880	0.1200	1.0931	1.7281	1.0320	0.7151	0.9127	0.7120
9	96-4	0	0	0	0	0	0	0	0	0	1.0931	1.7661	1.1000	0.6181	0.8231	0.6111
10	94-86	1.5339	1.8100	1.6478	0.3440	0.4081	0.2112	0.1790	0.3040	0.1810	1.7391	1.8171	1.7818	0.8261	1.0021	0.8001

144, 91 – 7, 95-1, 94 – 57 and 96 – 4 showed no absorbance. In the higher wave length regions (625 nm to 725 nm) absorbance was very low in all the genotypes (Table 3).

Stem

The genotypes Sree Bhadra, Sree Rethna, 95–1, 94–57 and 94–86 showed absorbance peaks at 525 nm. The highest absorbance was shown by Sree Bhadra and 94 – 86. The genotypes KV–4, A – 144, 91 – 7, Kanjangad local and 96–4 showed no absorbance. In the higher wave length regions (575 nm to 725 nm) absorbance was very low in all the genotypes (Table 3).

Flowers

All the ten genotypes exhibited maximum absorbance at 525nm, showing good absorbance within the range 500nm to 550nm. The highest absorbance was observed in the genotypes Sree Bhadra and 94 – 86. All the genotypes except Sree Bhadra and Sree Rethna showed low absorbance in the longer wave length range from 600nm to 725 nm (Table 3).

Tuber Skin

The genotypes Sree Bhadra, Sree Rethna, A–144, 95–1, Kanjangad local, 94–57, 96–4 and 94–86 showed maximum absorbance at 525nm. Better absorbance was shown by these genotypes within the range of 500nm to 550nm. The highest absorbance was noticed for Sree Bhadra, followed by Sree Rethna. In the higher wave length regions 600nm to 725nm, the performance of the genotypes was poor. The genotypes KV–4 and 91-7 did not show any absorbance (Table 3).

The extracts from young leaves, mature leaves, stem, flower and tuber skin parts of the genotypes Sree Bhadra, Sree Rethna and 94 – 86 showed maximum absorbance values of anthocyanin at 525 nm. Kanjangad local showed maximum absorbance at 525 nm for the extracts from young leaves, mature leaves, flower and tuber stem.

Colour value of anthocyanin contents

Colour values corresponding to the anthocyanin contents of the five main plant parts of sweet potato at 525nm absorbance are shown in Table 4.

Sree Bhadra, Sree Rethna, and 94–86, showed high colour values of anthocyanin content in young leaf, flower and tuber skin parts, while 95–1 showed high colour value in its flower and tuber skin parts only.In young leaf extract, the maximum colour value was observed for Sree Bhadra and 94–86 (10.8) followed by Sree Rethna and Kanjangad local (10.2). The mature leaf of Sree Bhadra showed maximum colour value (1.96) followed by those of 94–86 (1.6) and Sree Rethna (1.52). Maximum colour value of anthocyanin content in stem extracts was observed in Sree Bhadra and 94–86, followed by Sree Rethna. Flowers of all the ten genotypes showed high colour values of anthocyanin contents. In the case of tuber skin, the maximum colour value was noticed in Sree Bhadra (9.6) followed by Sree Retna (9). The genotypes 95–1, Kanjangad local and 94–86 also showed good performance. The colour value of anthocyanin content was zero in leaves, stem and tuber

Table 4. Colour value of anthocyanin contents of five main plant parts at 525 nm absorbance in the ten genotypes of sweet potato

Sl. No.	Genotype	Young leaf		Mature leaf		Stem		Flower		Tuber skin	
		1	2	1	2	1	2	1	2	1	2
1	94 – 57	0	0	0	0	0.18	0.72	1.72	10.3	0.91	5.4
2	Sree Rethna	1.7	10.2	0.38	1.52	0.29	1.10	1.74	10.4	1.50	9
3	91 – 7	0	0	0	0	0	0	1.71	10.3	0	0
4	Sree - Bhadra	1.8	10.8	0.49	1.96	0.30	1.20	1.81	10.8	1.60	9.6
5	KV – 4	0	0	0.16	0.64	0	0	1.73	10.3	0	0
6	95 – 1	0.6	3.6	0	0	0.19	0.76	1.72	10.3	1.00	6
7	94 – 86	1.8	10.8	0.40	1.60	0.30	1.20	1.81	10.6	1.00	6
8	96 – 4	0	0	0	0	0	0	1.76	10.5	0.82	4.8
9	Kanjagad local	1.7	10.2	0.13	0.52	0	0	1.75	10.5	1.12	6.6
10	A – 144	1.2	4.8	0	0	0	0	1.70	10.2	0.80	4.8

Note:

1. Absorbance value, 2. Colour value

parts of 91–7, and in the leaves and stem parts of 96–4 (Table 4).

Anthocyanins are the most important and wide spread group of colouring matter in plants. These intensely coloured water soluble pigments are responsible for the pink, scarlet, red, violet and blue colours in the petals, leaves and flowers of plants. Anthocyanin pigments in sweet potato are used as a natural colourant in food processing (Cascon *et al.,* 1984). Anthocyanins usually occur in the cellsap solutions of vacuoles (Ferry and Ward, 1959; Mayer and Anderson, 1959), and serve not only as attractants for pollinators and seed dispersers (Arditti and Dunn, 1968) but also are potent antioxidants (Narayan *et al.,* 1999). Bassa and Francis (1987) suggested that the anthocyanin in sweet potato roots are more stable than the three anthocyanin sources tested. Although their role as valuable aids in the classification of plants has been well established (Arditti and Dunn, 1968), very little efforts have been made towards the study of anthocyanin pigments in sweet potato.

In the present study, the anthocyanin pigments in different plant parts viz., leaves, stem, flowers and tuber skin were studied from ten genotypes of sweet potato.The visual observations of fresh parts of plants and their extract showed deep purple colouration in the young leaves and tuber skin of Sree Rethna, Sree Bhadra, 94-86 and Kanjangad local, and in flowers of all genotypes. Medium intensity of purple colour was noticed in young leaves and tuber skin of A-144 and in the tuber skin of 94-57, whereas the stem and mature leaves showed only a low intensity of purple colouration in all genotypes. Intensity of purple colour was high in young leaves, flower and tuber skin compared to stem and mature leaves. Visual observation indicated that genotypes differ widely in the intensity of purple colouration in the different plant parts.

Spectrophotometrical analysis of anthocyanin contents revealed maximum absorbance value (mav) of anthocyanin was at 525nm, for all parts of genotypes. According to Harbone (1973) the maximum absorbance value of anthocyanin is exhibited at a wave length of 475-550nm. The maximum absorbance of anthocyanin pigments in the extract of young leaves was high in Sree Bhadra, Sree Rethna, 94-86 and Kanjangad local (1.7 – 1.8) and medium in 95-1 and A-144 (0.6 - 1.2). Absorbance value of anthocyanin pigments in the flowers was high (1.70 – 1.81) in all the genotypes. The tuber skin of Sree Rethna and Sree Bhadra showed high (1.5 – 1.6) absorbance value of anthocyanin pigments, with that of 94-57, 95-1, 94-86, 96-4, Kanjangad local and A-144 being medium (0.8 – 1.1) absorbance value. The stem and mature leaves showed very low absorbance value (0 – 0.49) in all the genotypes.

The absorbance values obtained from spectrophotometrical analysis were used to determine the colour value which is an indicator of the amount of anthocyanin contained in different plant parts. The young leaves of Sree Rethna, Sree Bhadra, 94-86, and Kanjangad local showed high colour value (10.2 – 10.8), with low values (3.6 – 4.8) being observed in 95-1 and A-144. Mature leaves and stem of all the genotypes had low colour value (0 – 1.9), while the flowers of all the genotypes gave a high colour value (10.2 – 10.8). High colour value of anthocyanin (9 – 9.6) was observed in the skin tuber of Sree Rethna and Sree Bhadra, medium in 94-57, 95-1, 94-86 and Kanjangad local (5.4 – 6.6), and low (4.8) in 96-4 and A-144. Lila *et al.* (2003a) were reported that tuber skins and tender leaves for certain varieties of sweet potato have attractive hues of pink colour due to varying concentrations of anthocyanins. Spectral analysis showed two major absorption peaks at 525nm and 320nm. Colour value of anthocyanins ranged from 4.5 to 6.8. Anthocyanin content decreased progressively from tender leaves to mature leaves.

In the present study, anthocyanin pigments were seen to be located only in the purple tinged parts of the plant. The cultivars showed variation in the intensity of colouration ranging from red to purple in different plant parts. Imbert *et al.* (1966) had also reported red to purple range of expression of pigment colouration. The solution containing anthocyanin from sweet purple potato was bright red at P^{H}_{3} (Hong *et al.,* 2004). The highest absorbance value was observed in flowers, young leaves and tuber skin, while mature leaves and stem showed low values.Hence variations in the intensity of anthocyanin pigment may be utilized as an additional evidence in the categorization of the varieties of sweet potato.

Optimal conditions of environmental factors like temperature, nitrogen efficiency etc. influence the production of anthocyanin pigments (Bonner and Galston, 1952). However Mayer and Anderson (1959) were of opinion that the genetic capacity for anthocyanin synthesis varies from genotype to genotype, as some cultivars fail to produce anthocyanin even under favourable environmental conditions. So they concluded that the synthesis of anthocyanin is a gene controlled function and may be used for varietal identification and classification. Genotypic diversity in sweet potato regarding anthocyanin synthesis was also reported by Yoshinaga *et al.* (1999 &2000).

Anthocyanin from natural sources have problems of stability to light and heat. Bassa and Francis (1987) suggested that anthocyanin in sweet potato roots are more stable, and may relied upon as potential colourants. A variety containing anthocyanin in high quantity is considered to be a good source of natural food colourant. The performance of genotypes with regard to anthocyanin contents, revealed that Sree Rethna, Sree Bhadra, 94-86 and Kanjangad local may be considered as good sources of natural food colourants and such natural sources may help the preservation of health in human beings. Besides the uses as a natural colourant it could be used as a raw material for bread, juice and noodles. A wide range of biochemical and pharmacological effects including anticarcinogenic, antinflammatory, antimicrobial and antioxidant activities have been recognized recently for anthocyanin (Mazza and Miniati, 1993; Girard and Mazza, 1998). This calls for further improvements in the flesh colouration of the local varieties (purple fleshed) to increase their anthocyanin contents.

CONCLUSION

Anthocyanin pigments were located only in the purple tinged parts of sweet potato. The cultivars showed variation in the intensity of colour of anthocyanin content in different plant parts. Anthocyanin content was highest in flowers for all sweet potato genotypes compared to other plant parts. The anthocyanin content in the flowers remained constant in all genotypes. Anthocyanin content was the highest in flowers followed by young leaves and tuber skin, while the mature leaves and stem showed the lowest quantities. The colour of anthocyanin ranged from red to purple colour in different cultivars. So the visual observation and colour value of anthocyanin pigments in the different plant parts may be utilized in future studies for the assessment of genotypic variations. Based on the performance of genotypes in anthocyanin content, Sree Bhadara, Sree Rethna, 94-86 and Kanjangad local may be considered as good sources of natural food colourants.

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Quantitative Phytochemical Analysis of Leaf, Stem And Root in *Euphorbia rothiana* Spreng. (Euphorbiaceae), The Nilgiris Western Ghats, Tamil Nadu

R. ANUSUYA DEVI*, B. VEENA AND K. THENMOZHI

PG and Research Department of Botany, Kongunadu Arts and Science College, Coimbatore - 641 029. Tamil Nadu, India.
**email :* anusuyamsc2015@gmail.com

ABSTRACT

***Euphorbia rothiana* Spreng. is an important medicinal plant. It used in hypertensive agent in traditional medicine.The present study deals with the analysis of Phytochemical constituents by qualitative and quantitative analysis of leaves, stem and root were done using Petroleum ether, chloroform, ethyl acetate and ethanol extracts. Phytochemical screening method was used to identify qualitative analysis of bioactive component. Alkaloids, flavonoids, phenols, terpenoids, triterpinoids, steroids, cardio glycosides and carbohydrates were analysed. Alkaloids, flavonoids, and phenols were highly present various extracts of leaves stem and root. Cardio glycosides triterpinoids and carbohydrates were minimum present in the various extracts. The bioactive component ie., phenolics, tannin and flavonoids were found in all those solvent. The higher concentration of phenolics and tanins were resulted from ethyl acetate following by chloroform. While non polar solvent has lower in concentration.The present study confirm the traditional medicinal usage of the plant practiced by the traditional healers of southern district of Tamil Nadu, India.**

Key words *Euphorbia rothiana, Medicinal plant, qualitative and quantitative analy*

The evaluation of all the drugs is based on phytochemical and pharmacological approaches which leads to the drug discovery referred as natural product screening Foye *et al*., (2008). Secondary metabolites are chemically and taxonomically diverse compounds with obscure function. They are widely used in human therapy, veterinary, agriculture, scientific research and countless other areas. Any part of the plant may contain active components such as bark, leaves, flowers, roots, fruits and seeds (Gordon and David, 2001). The Euphorbiaceae, in common English sometimes called euphorbia's, which is also the name of a genus in the family, is a large family, the spurge family, of flowering plants with about 300genera and 6,500 species. *Euphorbia laeta* Heyne ex Roth. syn. *Euphorbia rothiana* Spreng. Belonging to the family Euphorbiaceae. It is an annual or perennial erect herb with copiously branched stem, Leaves alternate, linear-lanceolate or oblanceolate. The seeds are used by the tribes of Madhya Pradesh to remove warts (Mishra and Misra, 1984). They are used as an hypertensive agent in traditional medicine.The present investigation to find out the qualitative analysis from the leaves, stem and root.

MATERIALS AND METHODS

Collection of Plant material

Fresh leaves, stem and root parts of *Euphorbia rothiana* (Fig. *1* & 2) were collected from Dhottabetta hills, Niligri District. The authenticity of the selected plant material was confirmed by comparing with the reference specimen preserved at Botanical Survey of India, Southern Circle, Coimbatore. The leaf, stem and root parts were dried in shade at room temperature, chopped and ground to a fine powder in a mechanical blender. For extraction (50 g) of coarsely powdered plant samples were subjected to successive solvent extraction with petroleum ether, chloroform, ethyl acetate and ethanol using Soxhlet apparatus. The extracts were concentrated to dryness under reduced pressure using rotary vacuum evaporator, lyophilized and stored at -20oC for further phytochemical and in vitro antioxidant studies.

Fig. 1. Study Area "The Nilgiris"

Preparation of crude plant extracts

50 g of coarsely powdered plant samples were subjected to successive solvent extraction with petroleum ether, chloroform, ethyl acetate and ethanol. The extracts were concentrated to dryness under reduced pressure using rotary vacuum evaporator (Supervac R-185, India), lyophilized to remove traces of water molecules and the lyophilized powders were stored at -20°C for further studies.

Qualitative phytochemical analysis

The concentrated extracts were subjected to qualitative tests for the identification of various phytochemical constituents according to the method set forth by Trease and Evans (1989) ; and Sofowora (1993).

Quantitative phytochemical analysis

Major non-enzymic antioxidants of the plant extracts were determined using standard quantitative methods.

Total phenolics and tannins

The total phenolic content of plant extracts was determined using Folin-ciocalteu reagent according to the procedure described by Siddhuraju and Becker (2003). In this method, 20 ìg of the extract (dissolved in the respective solvent) was taken in a test tube and made up to the volume of 1.0 mL with distilled water. Then 0.5 mL of freshly prepared Folin-ciocalteu phenol reagent (1:1 with water) and 2.5 mL of 20% sodium carbonate solution were added sequentially in each tube. The mixtures were agitated and left in the dark at laboratory temperature for 40 min for the development of colour.The absorbance was recorded at 725 nm against the reagent blank using Shimadzu – UV- 160 spectrophotometer (Japan). A calibration curve of gallic acid was constructed, and linearity was obtained in the range of 10-50 ìg/ mL. Using the standard curve, the total phenol content of the extract was calculated and expressed as gallic acid equivalent (GAE) mg/ g extract. Using the same extract, tannin content was estimated after treatment with polyvinyl polypyrrolidone (PVPP) as described by Siddhuraju and Manian (2007).One hundred milligrams of PVPP was weighed in a 100 ×12 mm test tube and to this, 1.0 mL distilled water and 1.0 mL of tannin containing phenolic extract were added. The contents were vortexed and kept at 4ºC for 15 min. Then the sample was centrifuged (5000 rpm for 10 min at laboratory temperature) and the supernatant was collected. This supernatant has only simple phenolics other than tannins (the tannins would have been precipitated along with the PVPP). The phenolic content of the supernatant was measured, as monitored above and expressed as the content of free phenolics on a dry matter basis. From the above results, the tannin content of the extract was calculated as follows:

Tannin (mg GAE/ g extract) = Total phenolics (mg GAE/ g extract) – Free phenolics (mg GAE/ g extract)

Total flavonoid content

The total flavonoid content was determined spectrophotometrically using the method adopted by Zhishen *et al*. (1999). 0.5 mL of appropriately diluted extract solution was mixed with 2.0 mL of distilled water and subsequently with 0.15 mL of 5% sodium nitrite solution and maintained for 6 min. Then, 0.15 mL of 10% aluminium chloride solution was added and allowed to stand for 6 min, and finally 2.0 mL of 4% sodium hydroxide solution was added. Final volumes of the contents were made up to 5.0 mL with distilled water and were mixed thoroughly. After 15 min of incubation at laboratory temperature, the absorbance was determined against blank at 510 nm. The total flavonoid content was determined using a standard curve with rutin. The mean of the three values were expressed as milligrams of rutin equivalents (mg RE)/ g extract on a dry weight basis.

Fig. 2. *Euphorbia rothiana* Spreng.

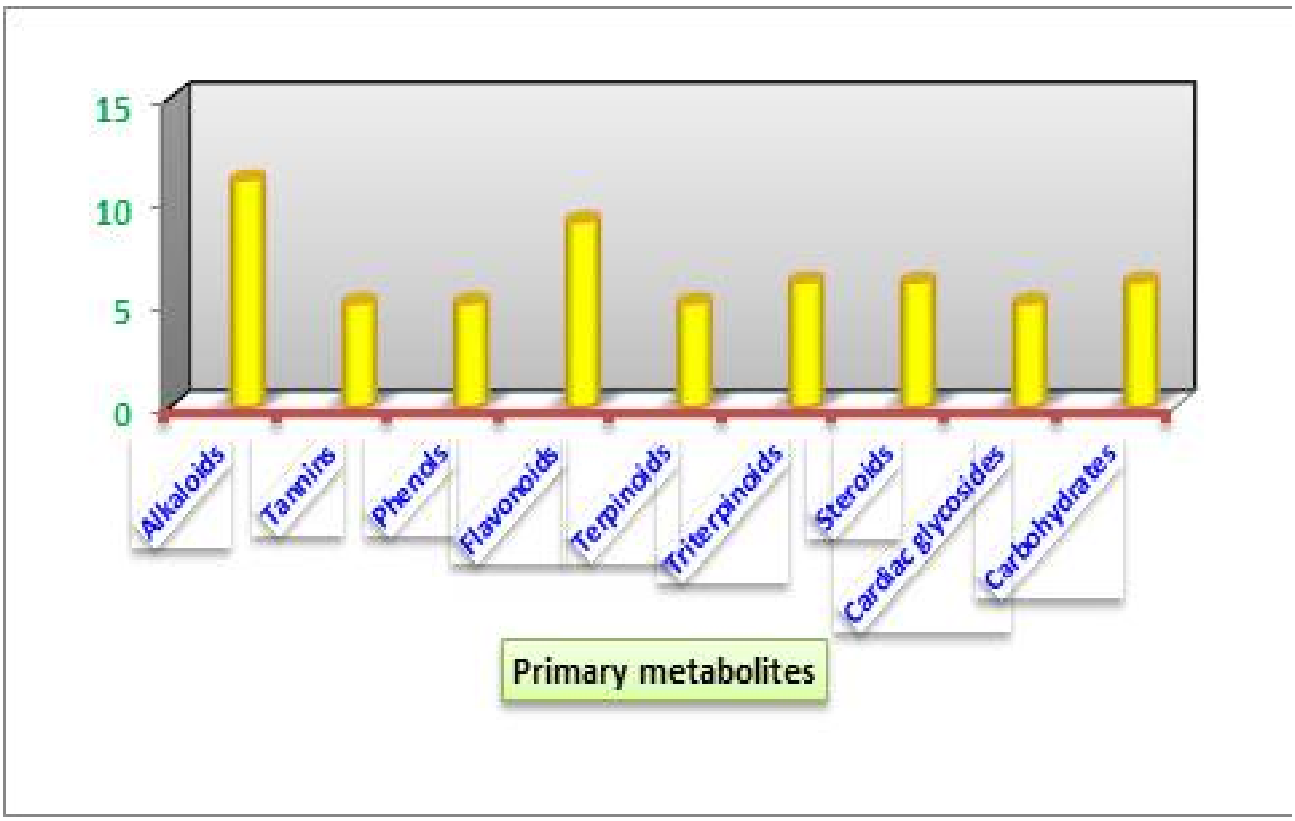

Fig. 3. Qualitative phytochemical analysis of *Euphorbia rothiana* plant parts.

Table 1. Total phenol, tannins and total flavonoid contents of *E. rothiana* plant parts.

Parts	Extracts	Total phenolics#	Tannins#	Totalflavonoid@
Leaf	Petroleum ether	46.5±0.02^{g}	3.85±0.006^{j}	8.2±3.01^{i}
	Chloroform	38.3±0.1^{h}	4.53±0.1^{i}	23.2±5.9^{e}
	Ethyl acetate	73.0±0.07^{a}	35.60±0.07^{a}	4.1±0.5^{k}
	Ethanol	59.4±0.04^{e}	25.62±0.01^{d}	17.3±0.4^{g}
Stem	Petroleum ether	15.2±0.03^{k}	3.17±0.05^{j}	12.8±1.8^{h}
	Chloroform	17.7±1.0^{j}	4.98±0.04^{h}	59.7±0.3^{a}
	Ethyl acetate	60.8±0.03^{d}	22.7±0.1^{e}	24.8±0.6^{d}
	Ethanol	15.4±0.01^{k}	10.6±0.05^{f}	22.7±3.5^{f}
Root	Petroleum ether	67.8±0.03^{b}	33.5±0.04^{b}	31.6±0.5^{c}
	Chloroform	63.9±0.4^{c}	1.1±0.2^{k}	52.7±0.2^{b}
	Ethyl acetate	47.8±0.2^{f}	31.5±0.03^{c}	5.0±0.9^{j}
	Ethanol	32.2±0.2^{i}	5.4±0.06^{g}	2.1±1.3^{l}

*Values are mean ± standard deviation (SD) of three independent experiments. Values not sharing a common letter in a column are significantly different ($P<0.05$).

Values expressed as mg GAE/g extract; @ Values are expressed as mg RE/g extract.

RESULTS AND DISCUSSION

Qualitative phytochemical analysis

In preliminary phytochemical analysis, the presence of major secondary metabolites such as alkaloids, flavonoids, phenols, saponins, terpenoids, steroids, cardiac glycosides tannins and carbohydrate were attempted and depicted. Among the various solvent types examined ethyl acetate and ethanol extracts indicated the presence of all the phytochemical constituents tested (Fig. 3). Preliminary qualitative phytochemical analysis made for the leaf, stem and root parts of E.rothiana revealed the presence of alkaloids, flavonoids, phenols, tannins, terpenoids and triterpenoids. These secondary metabolites are reported to have many biological and therapeutic properties (Narender *et al.,* 2012; Vishnu *et al*., 2013; Benedec *et al*., 2013; Charalampos *et al*.,2013).

Quantitative phytochemical analysis

These compounds are considered to be most important antioxidant of plants and they constitute one of the major groups of compound acting as primary antioxidant or free radical terminator (Sulaiman *et al*., 2011). In the present study, the Ethyl acetate extract of *E. rothiana* leaf exhibited higher content of phenolic compounds (73/mg GAE/g extract) (Table.1) and this might offer a good source of nutritional antioxidant defense against reactive oxygen species.

Tannin like polypenolic compounds is used as a sword to destroy free radicals and saves cellular macromolecules. The ethylacetate extract of *E. rothiana* leaf has been to shown to possess substantial amount of free phenolic content (35.6 mg GAE/g extract) (Table.1) and this might offer antioxidant action against reactive oxygen species involved in the initiation of deleterious free radical reactions (Sazzad *et al.,*2013).

Flavonoids are the most diverse and wide spread group of natural components and are likely to be the most important natural phenolics (Manickam *et al.,* 2012). Studies on flavonoid derivatives have shown a wide range of antibacterial, antiviral, anti inflammatory and anti-allergic activities (Di carlo *et al.,* 1999; Montoro *et al*., 2005). In the present study, the chloroform extract of *E.rothiana* stem (Table.1) has been shown to possess substantial amount of flavonoids (59.7 mg RE/g extract) (Table.1), which are thought to have positive effect of human health.

CONCLUSION

The phytochemical constituents are mainly responsible for these medicinal properties of the plants. Now a day, medicinal plant based drug industries and enterprises were increasing day by day in this juncture, scientific validation of traditional medicinal plants is required to confirm their therapeutic properties and hence the commercial production. The qualitative phytochemical profile revealed the presence of important secondary metabolites in the leaf, stem and root parts of the plant. However, the ethanolic extract presented higher amount of secondary metabolites than the other solvent studied. Similarly in quantitative phytochemical assay, the ethyl acetate and chloroform extracts of leaf and stem yielded substantial amount of total phenolics, free phenolics and flavonoid contents. Therefore the present study reveals that the study species has a reliable source of bioactive compounds which were highly correlated to their therapeutic properties.

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Microhabitat Diversity and Distribution of Angiosperms in Selected Granitic Hillocks of Palghat Gap of Southern Western Ghats

ARABHI, P*[1,2], AMRUTHA, M. A[3] AND MAYA C. NAIR[3]

[1]*Environmental Resources Research Centre, Thiruvananthapuram*
[2]*Department of Botany, Baselius College, Kottayam*
[3]*Post Graduate and Research Department of Botany, Govt. Victoria College, Palakkad, Kerala, India*
**email : arabhip1@gmail.com*

ABSTRACT

Angiosperm diversity in low altitude granitic hillocks between latitude 10° 36'38.19"N -10° 41'40.16"N and longitude 76° 32'34.90"E- 76° 37'32.73"E in Palghat gap region of southern Western Ghats were analyzed by selecting nine sample hillocks with special reference to microhabitat diversity. Granitic hillocks possess unique vegetation where different floristic components get adapted to different microhabitats. Each microhabitat is characterized with specific species associations and peculiar abiotic parameters. During the documentation, the study area was found to be supporting 189 angiosperms belonging to 59 families. Floristic diversity is characterized by the predominance of Fabaceae followed by Poaceae and Cyperaceae. These unique ecosystems possess nine microhabitats where the Soil-Rich Areas have highest elemental contribution followed by Exposed Rock Surfaces. While analyzing the life form spectrum, therophytes were found to be dominating in these rocky outcrops followed by phanerophytes and hemicryptophytes. Of the total taxa, six species were found to be endemic and six were falling under RET category. Most floristic elements have an Indo-Pacific affinity in phytogeographic comparison followed by African and South American elements. Based on Flowering Plants of Kerala (Sasidharan, 2011), 16 taxa showed extended distribution in Palakkad. Anthropogenic activities like quarrying, construction, etc. were found to flatten these unique natural ecosystems. The information generated on floral diversity and microhabitats can be used for the formulation of conservation strategies of such fragile ecosystems.

Key Words *Granitic hillock;, Palghat Gap; Microhabitat diversity.*

Inselbergs are rock outcrops rise abruptly from the surrounding landscape, have a patchy distribution, and represent centers of diversity and endemism for both animal and plant life (Hopper and Withers, 1997). They are usually of Precambrian age, with sparse vegetation cover (Seine *et al*., 1998).Granite inselbergs occur as mostly dome-shaped rock outcrops in all climatic and vegetational zones of the tropics. Consisting of Precambrian rocks, they form ancient and stable landscape elements. Due to harsh edaphic and microclimatic conditions, the vegetation of inselbergs differs markedly from those of the surroundings (Porembsky *et al.*,1997). The ecological and evolutionary processes that operate in these ancient environments differ significantly from comparatively more recent environments (Hopper, 2009). Inselbergs influence the water and nutrient supplies of surrounding landscapes (Schut *et al.,* 2014).Well defined inselberg habitats like cryptogamic crusts, rock pools, monocotyledonous mats, shallow depressions, crevices and clefts and ephemeral flush vegetation can be distinguished based on physiognomy (Porembsky,2007).

Small and medium-sized hillocks are a common sight in the midlands of Kerala and most of them are covered with all kinds of plants and provide a rich grazing area for cattle. Ecologically they are critical to the region, as they are the main repositories of water resources keeping the wells of surrounding areas well filled and providing drinking water to the people. In Kerala, there are two types of hillocks; laterite and granite hillocks. Laterite hillocks are more prevalent in Northern Kerala and granite hillocks in Southern Kerala. Palghat gap region of Southern Western Ghats is endowed with numerous low altitudinal hillock systems and each such isolated units have its own unique assemblage of floral elements due to spatial and ecological isolation from the surrounding vegetation corroborated by the influence of the Palghat gap features. Each microhabitat has its own plant composition and some plants are found in two or more microhabitats.Recent plant explorations revealed more additions to the known plant species of the area, some of which turned out to be new to science and endemic to the locality (Jose *et al*, 2013; Jose *et al*, 2015).

Low altitude hillock systems found in the neighborhood of human settlements frequently show signs of destructive human influences like fire, grazing, quarrying,dumping of wastes etc. and that may cause considerable damage to the very sensitive inselberg plant communities. Particularly dangerous are invasive

weeds that are capable of replacing indigenous species on such ecosystems. Most of the hillocks in the district of Palghat gap region fall under granitic category where there is severe exploitation of these ecosystems for rocks. With this background, the present paper summarizes the angiosperm diversity with associated diverse microhabitats of selected granitic hillocks in the Palghat gap region of southern Western Ghats.

MATERIALS AND METHODS

Study Area

Documentation of angiosperm diversity in nine selected granitic hillocks between 10° 36' 38.19" N - 10° 41' 40.16" N and longitude 76° 32' 34.90" E- 76° 37' 32.73" E under Nemmara Forest Division in the Palghat gap region of southern Western Ghats was carried out between October 2016 and September 2017. The study area included nine different hillocks of varying altitudes and includes Vizhumala (10° 37'13.58" N,76° 32'34.90" E), Kokkarnikunnu (10° 40' 73.70" N, 76° 33' 59.97" E), Vellappara (10° 41' 40.16" N,76° 35' 20.61" E) Paruthikunnu (10° 41' 21.91" N,76° 34' 53.86" E), Perinkunnam Kottamala (10° 40' 53.25" N,76° 34' 42.38" E) Edamalakunnu (10° 36' 38.19" N,76° 35' 36.09" E), Vamala (10° 38' 14.35" N,76° 36' 54.50" E), Muringamala (10° 40' 24.67"N,76° 37' 32.73" E) and Mallampara (10° 40' 58.62" N,76° 34' 37.63"E). Of these Vizhumala is the highest and largest hillock in the study area.The plants were collected and identified using standard flora along with reference to local herbaria MH and CALI and enumerated based on APG IV (Chase *et al.,* 2016). The nomenclature validation is followed with IPNI (www.ipni.org) and the plant list (www.theplantlist.org). The plant diversity was documented and different microhabitats were identified. The phytogeographical affinities of the vegetation was worked out (Cox, 2001), the RET taxa assessment was based on IUCN (2017) and the elements were photographed using digital camera Sony Cyber shot DSC HX7V.

RESULTS AND DISCUSSION

During the study 189 species of flowering plants belonging to 59 families were documented and 6 of them were found to be endemic to peninsular India and southern Western Ghats and the species were found to be uniformly distributed in 9 microhabitats.

Microhabitat diversity in granitic hillocks

Plants are generally adapted to various microhabitats and these microhabitats were characterized by peculiar abiotic factors such as soil, water, light etc., and explicit species composition. The most common microhabitat types of the study area have been described along with their characteristic species composition (Figure-1, Table-1)

a) Ephemeral flush vegetation (EFV)

The common species of such open rocky area with poor soil deposition are *Desmodium triflorum*, *Drosera indica*, *Murdannia semeteres*, etc. About 22 species were recorded from this microhabitat, in which the above 3 species were exclusively adapted and confined to this microhabitat.

b) Exposed rock surfaces (ERS)

These rocky plateaus, flat or uneven, are directly exposed to sunlight. This microhabitat recorded 51 species, of which 8 species were specifically confined to this microhabitat. They include *Sesamum prostratum*, *Chamaecrista mimusoides, Tephrosia maxima, Cleome aspera, Tridax procumbens, Allmania nodiflora, Boerhavia erecta and Portulaca grandiflora*,

c) Rock crevices or fissures (RCF)

Granitic hillocks possess several rock crevices and fissures with very thin soil deposition which provides a unique ecological niche for some species. The common species of such habitats are *Cyanotis arachnoidea* and *Polygala persicarifolia* and about 12 species were recorded from this particular microhabitat and these two species were found exclusively in this habitat.

d) Small Ephemeral Pools (SEP)

These shallow depressions which remain filled with water during rainy season possess *Rotala malampuzhensis, Fimbristylis aestivalis*, *Fimbristylis argentia*, *Cyperus iria*, *Dopatrium nudicaule*, *Lindernia sessiliflora,* etc. This micro ecosystem recorded 17 species of which the above 6 species were strictly confined to this microhabitat.

e) Soil-Filled Depressions (SFD)

These depressions in the rocky area where water and soil accumulates were commonly inhabited by

Bulbostylis puberula, Mitrasacme indica, Mollugo pentaphylla, Oldenlandia dineshii, Setaria pumila, etc. About 34 species were recorded from this microhabitat and the above-mentioned 5 species were found with specific distribution in this habitat.

f) Soil-Rich Areas (SRA)

These soil-rich microhabitats with more than 20cm soil-thickness are mainly occupied by species like *Boerhavia diffusa*, *Breynia retusa, Cardiospermum halicacabum, Chrysopogon aciculatus, Commelina benghalensis, etc*. The survey recorded about 91 species from this microhabitat and 26 of them were specifically confined to this area.

g) Tree Cover and Tree Associated (TCTA)

The soil rich area where trees are dominating makes a suitable habitat by providing their shades for some shade loving plants. *Holarrhena pubescens*, *Getonia floribunda*, *Wrightia tinctoria*, *Cleistanthus collinus*, *Streblus asper*, etc. are the common species of such habitat. About 85 species were recorded from this microhabitat and 43 of them were specifically confined to this microhabitat.

h) Boulders (B)

These microhabitats consist of isolated rocks or large rocks in groups. They are usually covered by lichens and bryophytes. *Cyanotis papilionacea*, *Kyllinga bulbosa* and *Bulbostylis barbata* are the common species found in such microhabitat.

i) Crust Edges or Cliffs (CEC)

The edges of the rocky hillocks act as a microhabitat for some plants like *Eriocaulon xeranthemum*, *Utricularia lazulina*, *Cyanotis papilionacea*, *Kyllinga bulbosa,* etc.

Flowering plant diversity in granitic hillocks

From the study area, 189 species belonging to 59 families were recorded. Among them, 3 species were Magnolids belonging to 2 families, 47 species were monocots belonging to 9 families. The remaining 139 species recorded were eudicots belonging to 48 families. The relative dominance follows the order with highest elemental contribution by Fabaceae followed by Poaceae and Cyperaceae. According to Porembski (2007) Poaceae, Cyperaceae and Fabaceae form the most speciose families in most of the rocky outcrop systems. The surveyed hillocks also showed similar condition and these three families together contribute 20% of total floristic composition.

Plant associations and adaptive traits

Adaptive traits help many plant species to overcome harsh environmental stresses such as drought, high temperature and nutrient deficiency. Plant species showing such traits observed in hillock systems are insectivorous plant species such as *Drosera indica* and *Utricuaria lazulina,* succulent plant species such as *Cyanotis papilionacea and Cyanotis arachnoidea* and semi-parasitic plant species such as *Parasopubia delphinifolia* and *Striga augustifolia.*

Phytogeographical affinities

The plants recorded from the study area showed different phytogeographical affinities. The number of plants showing different phytogeographical affinities is listed in Table-1.The critical evaluation of the affinities showed that most elements have an Indo-Pacific range in distribution followed by African and South American affinities.

Life form spectrum analysis

Biological spectrum of the flora based on the life form was prepared by following Raunkiaer (1934) life form classes. Therophytes are the dominant element with about 50 taxa which include plants like *Commelina benghalensis, Abelmoschus angulosus, Abrus precatorius,* etc. Geophytes include 4 species and they are *Curculigo orchioides, Curcuma decipiens, Cyperus rotundus* and *Fimbristylis falcata*. About 18 species were recorded as hydrophytes and they include plants such as *Rotala malampuzhensis, Dopatrium nudicaule* etc. About 41 species were recorded as Hemicryptophytes and they include plants like *Commelina diffusa, Boerhaavia erecta, Anisochilus carnosus* etc. The present study identified 35 species as Chamaeophytes and it includes plants like *Heliotropium marifolium, Alternanthera sessilis, Blepharis integrifolia* etc. The vegetation includes 41 species as Phanerophytes and it includes species like *Acacia auriculaeformis, Butea monosperma, Cleistanthus collinus* etc.

Table 1. Species distribution pattern in different microhabitats of granitic hillocks.

Sl. No.	Name of Plant Species	Microhabitat	Phytogeographic affinity
1	*Abelmoschus angulosus* Wall. ex Wight &Arn.	TCTA	Indo-Pacific and Australia
2	*Abrus precatorius* L.	TCTA	Indo-Pacific, Africa and South-America
3	*Acacia auriculiformis* Benth.	TCTA	Australia
4	*Allmania nodiflora (L.) R.Br. ex Wight*	ERS	Indo-Pacific
5	*Alternanthera sessilis* (L.) R.Br. ex DC.	TCTA	Indo-Pacific, South America and Africa
6	*Alysicarpus vaginalis* (L.) DC	TCTA	Indo-Pacific and Africa
7	*Ampelocissus tomentosa* (B. Heyne& Roth) Planch..	TCTA	Indo-Pacific
8	*Anacardium occidentale* L.	TCTA	South America
9	*Andrographis alata* (Vahl) Nees	TCTA	Indo-Pacific
10	*Andrographis echioides* (L.) Nees	ERS, TCTA	Indo-Pacific
11	*Anisochilus carnosus* (L.f.) Wall	ERS, TCTA	Indo-Pacific
12	*Annona squamosa* L.	ERS, TCTA	South America
13	*Argyreia bracteata* Choisy	ERS, TCTA	Indo-Pacific
14	*Asparagus racemosus* Willd.	TCTA	Indo- Pacific and Africa
15	*Azadirachta indica* A.Juss.	TCTA	Indo-Pacific
16	*Bambusa bambos* (L.) Voss	TCTA	Indo-Pacific
17	*Biophytum reinwardtii* (Zucc.) Klotzsch	EFV, SRA	Indo-Pacific
18	*Blepharis integrifolia* (L. fil.) E. Mey. &Drege	SRA, TCTA	Indo-Pacific
19	*Boerhavia diffusa* L.	SRA	Indo-Pacific, South America and Africa
20	*Boerhavia erecta* L.	ERS	Indo-Pacific, South America and Africa
21	*Bombax ceiba* L.	ERS, TCTA	Indo-Pacific, Africa and Australia
22	*Borassus flabellifer* L.	TCTA	Indo- Pacific and Africa
23	*Breynia retusa* (Dennst.) Alston	SRA	Indo-Pacific
24	*Bridelia retusa* (L.) A.Juss.	TCTA	Indo-pacific and Africa
25	*Bulbostylis barbata* (Rottb.) C.B. Clarke	B, ERS, EFV	Holarctic, Indo-Pacific and Africa
26	*Bulbostylis puberula* Kunth	SFD	Indo-Pacific and Africa
27	*Butea monosperma* (Lam.) Taub.	TCTA	Indo-Pacific
28	*Cajanus rugosus* (Wight &Arn.) Maesen	TCTA	Indo-Pacific
29	*Calotropis gigantea* (L.) Dryand.	TCTA	Indo-Pacific
30	*Canthium rheedei DC*	ERS, TCTA	Indo-Pacific
31	*Cardiospermum halicacabum* L.	SRA	Indo-Pacific, South America and Africa
32	*Casearia esculenta* Roxb.	TCTA	Indo-Pacific
33	*Cassia fistula* L.	TCTA	Indo-Pacific

Sl. No.	Name of Plant Species	Microhabitat	Phytogeographic affinity
34	*Cassytha filiformis* L.	TCTA	Indo-Pacific, Africa and Australia
35	*Catharanthus pusillus (Murray) G.Don*	ERS, SRA	Indo-Pacific
36	*Catunaregam spinosa* (Thunb.) Tirveng	TCTA	Indo-Pacific
37	*Cayratia pedata* (Lam.) Gagnep	SRA, TCTA	Indo-Pacific
38	*Centranthera tranquebarica* (Spreng.) Merr.	EFV, SEP	Indo-Pacific and Australia
39	*Chamaecrista mimosoides* (L.) Greene	ERS	Indo-Pacific, Africa and South-America
40	*Chromolaena odorata* (L.) R.M.King&H.Rob.	TCTA	South America
41	*Chrysopogon aciculatus* (Retz.) Trin.	SRA	Indo-Pacific and Australia
42	*Cissus vitiginea* L.	ERS, TCTA	Indo-Pacific
43	*Cleistanthus collinus* (Roxb.) Benth. exHook.f.	TCTA	Indo-Pacific
44	*Cleome aspera* J.Koenig ex DC.	ERS	Indo-Pacific
45	*Cleome viscosa* L.	SRA, ERS, TCTA	Indo-Pacific, South America and Africa
46	*Commelina benghalensis* L.	SRA	Africa, Indo-Pacificand Holarctic
47	*Commelina diffusa* Burm.f.	EFV, ERS, SFD, SRA	Holarctic, Indo-Pacific, Africa and South America
48	*Commelina paludosa* Blume	SRA	Indo-Pacific
49	*Commelina wightii* Raizada	SRA, ERS	Indo-Pacific
50	*Cordia dichotoma* G.Forst.	TCTA	Indo-Pacific and Australia
51	*Croton hirtus* L'Hér.	ERS, TCTA	Indo- Pacific and South America
52	*Curculigo orchioides* Gaertn.	SFD, SRA, TCTA	Indo-Pacific
53	*Curcuma decipiens* Dalzell	SRA, TCTA	Indo-Pacific
54	*Cyanotis arachnoidea* C.B.Clarke	RCF	Indo-Pacific
55	*Cyanotis axillaris* (L.) D.Don ex Sweet	SRA, SFD	Indo-Pacific
56	*Cyanotis cristata* (L.) D.Don	SRA	Indo-Pacific and Africa
57	*Cyanotis papilionacea* (Burm.f.) Schult. &Schult.f.	EFV, SRA, RCF, SFD, B, CEC	Indo-Pacific
58	*Cyanthillium cinereum (*L.) H.Rob	SRA, TCTA	Indo-Pacific, Africa and South America
59	*Cymbopogon nardus* (L.) Rendle	SRA, RCF	Indo-pacific and Africa
60	*Cyperu sdubius* Rottb.	EFV, SFD	Indo-Pacific
61	*Cyperus clarkei* T.Cooke	SFD, SRA	Indo-Pacific
62	*Cyperus compressus* L.	EFV, SRA, ERS	Indo-Pacific, Africa and South-America
63	*Cyperus cyperinus* (Retz.) Suringar	SFD, SRA	Indo-Pacific, Australia, Africa and South America
64	*Cyperus iria* L.	SEP, SFD	Indo-Pacific and Africa
65	*Cyperus rotundus L.*	ERS, SRA	Indo-Pacific, Australia and Africa

Sl. No.	Name of Plant Species	Microhabitat	Phytogeographic affinity
66	*Dactyloctenium aegyptium* (L.) Willd.	SRA	South-America
67	*Desmodium triflorum (L.) DC.*	EFV	Indo-Pacific and Australia
68	*Digitaria ciliaris*(Retz.) Koeler	SRA	Indo-pacific and Africa
69	*Dioscorea wallichii* Hook.f.	TCTA	Indo-Pacific
70	*Dipteracanthus prostratus (*Poir.) Nees	SRA	Indo-Pacific
71	*Dopatrium nudicaule* (Willd.) Benth.	SEP	Indo-Pacific
72	*Dregea volubilis*(L.f.) Benth. exHook.f.	SRA, TCTA	Indo - Pacific
73	*Drosera indica* L.	EFV	Indo-Pacific, South America and Africa
74	*Duranta erecta* L.	TCTA	South America
75	*Eclipta prostrata* (L.) L.	SRA	Indo-Pacific, South America and Africa
76	*Ehretia microphylla* Lam.	SRA	Indo-Pacific and Australia
77	*Eragrostis unioloides* (Retz.) Nees ex Steud.	EFV, SRA	Indo-Pacific and Africa
78	*Eranthemum capense* L.	SRA	Indo-Pacific
79	*Eriocaulon xeranthemum* Mart.	EFV, ERS, SFD, CEC	Indo-Pacific and Africa
80	*Euphorbia hirta* L.	SRA	South America, Africa and Indo-Pacific
81	*Euphorbia thymifolia* L.	SRA, ERS	Indo-Pacific
82	*Evolvulus alsinoides* (L.) L.	ERS, SRS	Indo-Pacific
83	*Ficus religiosa* L.	TCTA	Indo-Pacific
84	*Fimbristyli saestivalis* Vahl.	SEP	Indo-Pacific and Australia
85	*Fimbristylis argentea* (Rottb.) Vahl	SEP	Indo-Pacific
86	*Fimbristylis falcate* (Vahl) Kunth	ERS, SRA	Indo-Pacific and Holarctic
87	*Fimbristylis littoralis* Gaudich.	SEP	Indo-Pacific
88	*Firmiana simplex* (L.) W.Wight(Sterculiaurens)	TCTA	Indo-Pacific
89	*Flacourtia indica* (Burm.f.) Merr.	SRA	Indo-pacific, Africa and South-America
90	*Garnotia courtallensis* (Arn. &Nees) Thwaites	ERS, TCTA	Indo-Pacific
91	*Getonia floribunda* Roxb	TCTA	Indo-Pacific
92	*Glycosmis pentaphylla* (Retz.) DC.	SRA	Indo-Pacific
93	*Helicteres isora* L	SRA	Indo-Pacific and Australia
94	*Heliotropium marifolium* J.Koenig ex Retz.	EFV, RCF, SEP, SRA	Indo-Pacific
95	*Heliotropium rottleri* Lehm.	EFV, RCF, SEP	Indo-Pacific
96	*Hemidesmus indicus* (L.) R. Br. ex Schult.	SRA, TCTA	Indo-Pacific
97	*Holarrhena pubescens* Wall. ex G.Don	SRA, TCTA	Indo-Pacific
98	*Holoptelea integrifolia* Planch.	TCTA	Indo-Pacific
99	*Hybanthus enneaspermus* (L.) F.Muell.	SRA, ERS	Indo-pacific, Australia and Africa
100	*Hyptis suaveolens*(L.) Poit.	SRA, TCTA	South-America

Sl. No.	Name of Plant Species	Microhabitat	Phytogeographic affinity
101	*Impatiens balsamina* L.	ERS, SRA	Indo-pacific
102	*Indigofera uniflora* Roxb.	ERS, SFD, RCF	Indo-Pacific
103	*Ipomoea pes-tigridis* L.	ERS, SFD, TCTA	Indo-Pacific, South-America and Africa
104	*Ischaemum barbatum Retz.*	SRA	Indo-Pacific
105	*Jasminum coarctatum* Roxb.	SRA, TCTA	Indo-Pacific
106	*Jatropha curcas* L.	TCTA	South-America
107	*Jatropha gossypiifolia* L.	ERS, SRA, TCTA	Indo-Pacific and Africa
108	*Justicia tranquebariensis* L.f.	ERS, SRA	Indo-Pacific
109	*Kyllinga bulbosa* P.Beauv.	B, CEC, SFD	Indo-Pacific, Africa and South-America
110	*Kyllinga nemoralis* (J.R.Forst. &G.Forst.) Dandy ex Hutch. &Dalziel	RCF, SFD, SRA	Indo-Pacific, Africa and South-American
111	*Lagerstroemia speciosa* (L.) Pers.	TCTA	Indo-Pacific
112	*Lantana camara* L.	SRA, TCTA	South America
113	*Leucas aspera* (Willd.) Link	SRA, TCTA	Indo-Pacific and Africa
114	*Lindernia ciliata* (Colsm.) Pennell	EFV, ERS, SFD, SRA, SEP,	Indo-Pacific and Australia
115	*Lindernia crustacea* (L.) F.Muell.	SFD, ERS	Indo-Pacific, South America and Africa
116	*Lindernia rotundifolia* (L.) Alston	EFV, SFD, SEP	Indo-Pacific, South America and Africa
117	*Lindernia sessiliflora* (Benth.) Wettst.	SEP	Indo-Pacific and Africa
118	*Ludwigia hyssopifolia* (G.Don) Exell	SEP, SRA	Indo-pacific, Africa and South-America
119	*Macaranga peltata (*Roxb.) Müll.Arg.	TCTA	Indo-Pacific
120	*Mallotus philippensis* (Lam.) Müll.Arg.	TCTA	Indo-Pacific and Australia
121	*Martynia annua* L .	ERS, SRA	Indo-pacific and South America
122	*Melochia corchorifolia* L.	ERS, SRA	Indo-Pacific, Africa and South-American
123	*Merremia tridentata* (L.) Hallier f.	ERS, TCTA	Indo-Pacific and Africa
124	*Microstachys chamaelea* (L.) Müll. Arg.	ERS, RCF	Indo-Pacific and Australia
125	*Mimosa pudica* L.	SRA	South America, Africa and Indo-Pacific
126	*Mitracarpus hirtus* (L.) DC	ERS, SFD, SRA, TCTA	Africa and South America
127	*Mitrasacme indica* Wight	SFD	Indo-Pacific and Australia
128	*Mollugo pentaphylla* L.	SFD	Indo-Pacific, South America and Africa
129	*Morinda pubescens* Sm.	TCTA	Indo-Pacific
130	*Murdannia dimorpha* (Dalzell) G.Brückn.	SFD, SRA	Indo-Pacific

Sl. No.	Name of Plant Species	Microhabitat	Phytogeographic affinity
131	*Murdannia semitere*s (Dalzell) Santapau	EFV	Indo-Pacific
132	*Mussaenda frondosa* L.	SRA, TCTA	Indo-Pacific
133	*Naravelia zeylanica* (L.) DC.	SRA, TCTA	Indo-Pacific and Africa
134	*Naregamia alata* Wight &Arn.	SFD, SRA	Indo-Pacific
135	*Oldenlandia corymbosa* L.	ERS, RCF, SRA, SEP	Indo-Pacific and Africa
136	*Oldenlandia diffusa* (Willd.) Roxb.	SRA,SFD	Indo-Pacific, Africa and Holarctic
137	*Oldenlandia dineshii* Sojan& V. Suresh	SFD	Indo-Pacific
138	*Orthosiphon thymiflorus* (Roth) Sleesen	SRA	Indo-Pacific
139	*Panicum sumatrense Roth*	SRA	Indo-Pacific
140	*Parasopubia delphiniifolia (L.) H.-P. Hofm. &Eb. Fisch.*	EFV, SEP, SFD, SRA	Indo-Pacific
141	*Passiflora foetida* L.	SRA, TCTA	South-America and Indo-Pacific
142	*Pavetta indica* L.	TCTA	Indo-Pacific
143	*Pennisetum pedicellatum* Trin.	ERS, SRA	Indo-Pacific and Africa
144	*Pennisetum polystachyon* Schult.	SRA, TCTA	Indo-Pacific and Africa
145	*Perotis indica* (L.) Kuntze	SRA, SFD	Indo-Pacific
146	*Phyllanthus tenellus* Roxb.	SFD, ERS	Indo-Pacific
147	*Physalis minima* L.	SRA	Indo-Pacific, South-America and Africa
148	*Polyalthia cerasoides* (Roxb.) Bedd.	SRA, TCTA	Indo-Pacific
149	*Polygala chinensis* L.	EFV, SRA	Indo-Pacific
150	*Polygala persicariifolia* DC	RCF	Indo-Pacific
151	*Pongamia pinnata (L.) Pierre*	TCTA	Indo-Pacific
152	*Portulaca grandiflora* Hook.	ERS	Indo-Pacific, South-America and Holarctic
153	*Pouzolzia zeylanica*(L.) Benn.	SRA	Indo-Pacific
154	*Pterocarpus marsupium* Roxb.	TCTA	Indo-Pacific
155	*Pycreus polystachyos*(Rottb.) P.Beauv.	EFV, SRA	Indo-Pacific, Africa and South America
156	*Rhamphicarpa fistulosa* (Hochst.) Benth.	EFV, RCF	Indo-Pacific
157	*Rotala malampuzhensis* R.V.Nair	SEP	Indo-Pacific
158	*Saccharum spontaneum* L.	SEP, SFD	Indo-Pacific and Africa
159	*Santalum album* L.	TCTA	Indo-Pacific
160	*Scoparia dulcis* L.	SRA, TCTA	South America
161	*Senna tora* (L.) Roxb.	SRA	South-America
162	*Sesamum indicum* L.	ERS, SRA, TCTA	Indo-Pacific and Africa
163	*Sesamum prostratum* Retz.	ERS	Indo-Pacific
164	*Setaria pumila* (Poir.) Roem. &Schult.	SFD	Indo-Pacific and Africa
165	*Sida acuta* Burm.f.	SRA, TCTA	Indo-Pacific, South America and Africa

Sl. No.	Name of Plant Species	Microhabitat	Phytogeographic affinity
166	*Sida cordata* (Burm.f.) Borss.Waalk.	SRA, TCTA	Indo-Pacific, South America and Africa
167	*Sida cordifolia* L.	SRA, TCTA	Indo-Pacific, South America and Africa
168	*Spermacoce alata* Aubl.	ERS, SFD	South -America
169	*Spermacoce pusilla* Wall.	ERS, SFD	Indo-Pacific
170	*Sporobolus indicus* var. *diandrus*(Retz.) Jovet & Guédès*	SRA, RCF	Indo-Pacific and Australia
171	*Sporobolus tenuissimus* (Schrank.) Kuntze	SRA	Indo-Pacific and Africa
172	*Stachytarpheta jamaicensis* (L.) Vahl	ERS, SRA, TCTA	South America
173	*Streblus asper* Lour.	SRA, TCTA	Indo-Pacific
174	*Striga angustifolia* (D. Don) C.J. Saldanha	EFV, SFD	Indo-Pacific
175	*Strychnos nux-vomica* L	TCTA	Indo-Pacific
176	*Synedrella nodiflora*(L.) Gaertn.	SRA, TCTA	South America and Indo-Pacific
177	*Tephrosia maxima* (L.) Pers.	ERS	Indo-Pacific and Africa
178	*Tephrosia purpurea* (L.) Pers.	ERS, SRA	Indo-Pacific and Africa
179	*Terminalia paniculata* Roth	TCTA	Indo-Pacific
180	*Trianthema portulacastrum* L.	ERS, SRA	Indo-Pacific, South America and Africa
181	*Tridaxprocumbens* (L.) L.	ERS	Indo- Pacific and South America
182	*Urena lobata* L	SRA	Indo-Pacific, South America and Africa
183	*Utricularia lazulina* P. Taylor	EFV, SFD, CEC	Indo-Pacific
184	*Vitex altissima* L.f.	TCTA	Indo-Pacific
185	*Wrightia tinctoria* R.Br.	TCTA	Indo-Pacific
186	*Xylia xylocarpa* (Roxb.) Taub.	TCTA	Indo-Pacific
187	*Ziziphus jujuba* Mill	SRA, TCTA	Indo-Pacific, Africa and South-America
188	*Ziziphus oenoplia* (L.) Mill.	SRA, TCTA	Indo-Pacific and Australia
189	*Zornia gibbosa* Span	ERS, SFD	Indo-Pacific and Australia

Endemic plants of low altitude hillocks

During the study 189 species of plants belonging to 59 families were collected and 6 of them were found to be endemic to peninsular India and southern Western Ghats viz. *Commelina wightii* Raizada, *Murdannia dimorpha* (Dalzell) G.Brückn, *Curcuma decipiens* Dalzell, *Naregamia alata* Wight & Arn., *Heliotropium rottleri* Lehm., *Rhamphicarpa fistulosa* (Hochst.) Benth and *Utricularia lazulina* Taylor.

Rare, Endangered and Threatened taxa in low altitude hillocks

Of the 189 species reported, *Commelina wightii* Raizada found to be near threatened, *Oldenlandia*

Fig. 1. The common Microhabitat types on the granitie hillocks

dineshii Sojan & Suresh critically endangered and *Cayratia pedata* (Lam.) Gagnep, *Cleistanthus collinus* (Roxb.) Benth. ex Hook. f., *Santalum album* L. and *Pterocarpus marsupium* Roxb. were found to be vulnerable (IUCN,2017)

Extended distribution of taxon to Palakkad district based on Flowering Plants of Kerala (Sasidharan, 2011)

Based on Flowering Plants of Kerala (Sasidharan, 2011), 16 taxa showed extended distribution in Palakkad district and they were *Cassytha filiformis* L., *Murdannia dimorpha* (Dalzell) G. Bruckn., *Bulbostylis puberula* Kunth. ,*Cyperus clarkei* T. Cooke , *Fimbristylis falcata* (Vahl) Kunth., *Sporobolus tenuissimus* (Schrank.) Kuntze, *Cymbopogon nardus* (L.) Rendle, *Eragrostis uniloides* (Retz.) Nees ex Steud., *Cajanus rugosus* (Wight &Arn.) Maesen, *Chamaecrista mimosoides* (L.) Greene. *Tephrosia maxima* (L.) Pers., *Polygala chinensis* L., *Cleome aspera* J. Koenig ex DC. *Pavetta indica* L., *Blepharis integrifolia* (L. fil.) E. Mey. &Drege and *Centranthera tranquebarica* (Spreng.) Merr.

Threats and consequences in low altitude hillocks

These low altitude granitic hillocks were facing several threats mainly due to anthropogenic pressures created as a part of rapid urbanization. For the expansion of National Highway many of such hillocks were destroyed. Quarrying was another major threat faced by rocky hillocks and many crusher units were working in this area which have demolished many of the rocky outcrops which caused serious threats to the flora and fauna. The encouragement for monoculture plantations like Teak, Acacia, etc. also destroyed the microhabitats in this ecosystem. By considering these open rocky areas as 'wastelands', nearby villagers dumped wastes which deteriorated the edaphic quality of the ecosystem. Growth of invasive plants like *Chromolaena odorata, Mimosa diplotricha* etc. was also a major threat to the existence of the natural and unique vegetation of such low altitude hillocks. These hillocks were more susceptible to forest fire due to the presence of dry vegetation during the hot summer. Most of the hillocks had fire indicating taxa like *Hyptis suaveolens and* grasses which got dried up during the hottest months of Palakkad district. Hence, concerted efforts must be taken by the policy makers in order to protect these unique ecosystems which in turn can maintain the watersheds in and around each of the hillocks.

CONCLUSION

The microhabitat diversity of granitic low altitude hillocks presented unique ecosystems and the study showed nine different microhabitats with associated plant combinations. The Soil-Rich Areas had highest elemental contribution with 90 taxa followed by Exposed rock surfaces (ERS) with 51 taxa. Almost all the hillocks had tree cover with about 85 taxa. From the study area, 189 species belonging to 59 families were recorded. The most represented family was Fabaceae followed by Poaceae and Cyperaceae. The overall vegetation of the study area was dominated by therophytes followed by phaneropytes and hemi cryptophytes. The predominance of therophytes indicated a disturbed environmental condition where phanerophytes could not establish themselves. Of the 189 taxa recorded, 6 taxa were found to be endemic and six of the taxa were found to be RET taxa. Anthropogenic activities including overgrazing, overharvesting and developmental activities reduced the macro elements of the vegetation. Conservation strategies have to be formulated for the sustenance of floristic diversity and ecological services of these ecosystems.

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Antidiabetic Effect of Purified Anthocyanin from *In Vitro* Culture of *Begonia malabarica* Lam.

ASWATHY JM* AND MURUGAN K

Plant Biochemistry and Molecular Biology Laboratory,
Department of Botany, University College,
Thiruvananthapuram, 695 034
email: aswathyjm@gmail.com

ABSTRACT

Many herbals were validated for diabetes mellitus therapy by pharmacological people. The present study aims to evaluate the glycemic attributes in normal, sub and mild diabetic rats treated with purified anthocyanin from *in vitro* culture of *Begonia malabarica* Lam. Friable calli from leaf segments of *B. malabarica* were cultured to initiate cell suspension cultures on liquid MS media. Liquid MS medium fortified with 2, 4-D (0.1 mg L^{-1}) + BAP (0.5 mg L^{-1}) showed significant production of suspension cultures without aggregated or clumped cells on day 14 i.e., 8.090 g. Subsequently, the physiochemical parameters were standardized to enhance the biomass production and the synthesis of anthocyanin. The chemical elicitors trailed were phenylalanine, zinc sulfate, salicylic acid (SA), GA_3, methyl jasmonate, abscisic acid (ABA). Salicylic acid and abscisic acid induced anthocyanin to remarkable level. The extracted anthocyanin was purified initially by liquid-liquid partition followed by Amberlite XAD-7. Acidified ethanol 50% and 75% could elute around 70.4 and 72.5 % of anthocyanins from the column. Purified anthocyanin was fractionated using LC-MS/MS analysis at 4.7-5.4 min. Tandem MS of the *m/z* 655.3 peak was identified as anthocyanidin Malvidin-3 –diglucoside, the major compound. Diabetes was induced in rats by a single dose administration of streptozotocin (120 mg/ kg, i.p.) or by injecting dexamethasone (10 mg/kg, i.p.) for 10 days. In normal rats, purified anthocyanin had significantly decreased the blood glucose level (BGL) in a dose dependent manner after repeated administration for 7 days. In streptozotocin -induced diabetic rats, both the extracts decreased blood sugar levels with significant improvement in glucose tolerance and body weight at the end of 1st, 2nd and 3rd week after test extract treatment. In case of dexamethasone induced insulin resis-tant diabetic rats, repeated administration of purified anthocyanin inhibited the increase in blood glucose level and improved glucose tolerance induced by dexamethasone as compared to dexamethasone induced diabetic rats. Glyburide was used as standard drug. Further, anthocyanin revealed remarkable inhibition á- amylase and á- glucosidase inhibitory activity at the concentration of 400 µg/ml (71.46 ± 1.21 %, 76.85 ± 0.75 %) with IC_{50} value of 260 µg/ml and 244 µg/ml respectively. Overall, the present results suggest that purified anthocyanin from *B.malabarica* possesses ideal hypoglycemic power coupled with antidiabetic efficacy.

Keywords *Antidiabetic, glucose, glyburide, hypoglycemic, purified anthocyanin, Begonia malabarica*

Begonia is a perennial flowering plant belonging to the family Begoniaceae with upright rhizomatous or tuberous stem. The leaves are often large, variously stotched or variegated and are usually asymmetric. Begonias are important medicinal plants due to their pool of polyphenols such as luteolin, quercetin and â-sitosterol. The leaves are commonly used by the locals for the treatment of respiratory infections, diarrhea, blood cancer and skin borne diseases. Limited reports on cultivation, breeding and phytochemical studies of *B. malabarica* are available despite its commercial importance.

Anthocyanin constitutes the largest and unique group of water-soluble natural pigments. 635 anthocyanin molecules are identified in plants that imparts vivid blue, purple, and red colour of fruits, vegetables and flowers. The colour comes from charged anthocyanin pigments related with pH of the intracellular medium. Anthocyanins usually accumulate in the floral parts but *Begonia* is an exception in which the leaves are brightly coloured with this unique pigment. Anthocyanins are mostly accumulated in the vacuoles. Anthocyanins in plants attract animals, leading to seed dispersal and pollination. Owing to strong absorption of light, they may also be important in protecting plants from UV-induced damage. Anthocyanins belong to polyphenolics synthesized via phenylpropanoid pathway. The present investigation is aimed to isolate and fractionate the bioactive anthocyanin components from the cell suspension culture of *Begonia malabarica* using LC – MS analysis followed by analysis of antidiabetic activity .

MATERIALS AND METHODS

Healthy *Begonia malabarica* Lam. plants were collected, and the identity was authenticated with the herbaria of JNTBGRI, Palode, Kerala. The voucher plant materials were deposited at the Herbarium of

Department of Botany, University College, Trivandrum (UCB 1207).

Establishment and elicitation of cell suspension culture

The cell suspension was established from 1 g calli tissue derived from the leaf explants of *B. malabarica* and was subcultured in liquid MS culture medium fortified with 2,4-D (0.1 mg L^{-1}), NAA (0.5 mg L^{-1}) and BAP (0.5 mg L^{-1}) for continuous and stable accumulation of biomass. Various physico-chemical parameters at different concentrations were employed for elicitation of anthocyanin in cell suspension such as, peptone water (0.01-0.025%), yeast extracts (0.1 – 2.5 %) phenylalanine (5-25 ìM), abscisic acid (0.05-2.0 mg/L), salicylic acid (1-100 ìM), zinc sulphate (50-150 ìM)) and methyl jasmonate (50 ìM).

After the first cycle, the cells were harvested via a Buchner funnel, washed with distilled water to remove residual medium, and filtered again. Then the weighed fresh cells (FW) were dried at 50 °C to constant dry weight (DW). Cell growth was measured based on FW and DW. The combined aqueous concentrates of anthocyanin from cell suspension culture after evaporation were purified by liquid-liquid partition against ethyl acetate followed by Amberlite XAD-7 adsorption column chromatography. Then the adsorbed anthocyanins in the column were eluted using 75% ethanol containing 7 % acetic acid (v/v) as mobile phase. Fractionation and identification was carried using LC-MS analysis.

Determination of acute toxicity (*OECD, 2001*)

The acute toxicity of purified anthocyanin from *B.malabarica* was determined by using albino Wistar rats (150--200 g) which were maintained under the standard conditions at a dose of 2000 mg/kg and observed for their mortality during 2 and 7 days study period (short term) toxic-ity and the dose increased up to 5000 mg/kg and was observed up to 7 days for their behavioral, economical and neurologi-cal profiles except slight depression in their activity.

Assessment of hypoglycaemic activity in normal rats

Fasted rats were divided in to five groups consisting of six ani-mals in each group. Groups 1 and 2 received distilled water and vehicle (3% (v/v) Tween 80 in distilled water), orally in a volume of 10 ml/kg, which served as normal control and vehicle control. Group 3 received Glyburide (5 mg/kg, p.o.) as a standard drug suspended in vehicle. Purified anthocyanin suspended in vehicle were administered at the doses of 150, 300 and 400 m/kg, p.o. in a volume of 10 ml/kg to the rats of groups 4, 5, 6 and 7, respectively. Blood samples were collected from the tail vein or by retro-orbital puncture method just prior to and at 1, 2 and 4 h after dosing for acute studies and glucose were estimated. For sub-acute studies blood sample was removed on the 4th and 7th days after 16 h of overnight fasting for glu-cose level estimation (*Kesari et al., 2006*)

Oral glucose tolerance test (OGTT) was carried by admin-istering glucose (2 g/kg, p.o.), 30 min after the extract or stan-dard drug administration after 7 days of pre-treatment period. Blood samples were collected from tail vein for glucose analy-sis prior to glucose administration (0 h) and at 1, 2 and 3 h after glucose loading.

Assessments of anti-diabetic activity in streptozotocin induced diabetic rats

Streptozotocin (Stz) was given in 50 mg kg^{e1}bw (prepared in pH 4.5, 0.1 M citrate buffer). The solution should be fresh and prepared just prior to the administration. The rats were given 5% (w/v) glucose solution in feeding bottles for next 24 h in their cages to prevent hypo- glycaemia after streptozotocin injection. After 72 h rats with BGL greater than 200 mg/dl and less than 400 mg/dl were selected and observed for consistent hyperglycaemia (fasting blood glu-cose level greater than 200 mg/dl and lesser then 400 mg/dl) up to 7 days. Such animals were divided into seven groups as follows (*Kesari et al., 2006*):

Group 1: Control (distilled water 10 ml/kg, p.o.). *Group 2:* Vehicle control (3% (v/v) Tween 80 in water 10 ml/kg, p.o.). *Group 3:* positive control (Glyburide 10 mg/kg, p.o.). *Group 4:* anthocyanin 150 group (anthocyanin extract 150 mg/kg, p.o.). *Group 5:* anthocyanin 300 group (anthocyanin extract 300 mg/kg, p.o.). *Group 5:* anthocyanin 400 group (anthocyanin extract 400 mg/kg, p.o.).

Blood samples were collected by retro-orbital puncture at 0, 1, 2, 4 and 8 h after the administration. The treatment was con-tinued for the next 21 days and blood samples were collected on 4th, 7th, 14th and 21st days after 1 h administration. Blood glucose level (BGL) was estimated at various time intervals GOD/P.O.D. kit. Oral glucose tolerance test (OGTT) on day 21 was carried out. Body weight of all animals

was measured on the 0, 4th, 7th, 14th and 21st days after 1 h of treatment with the anthocyanin/ Glyburide /vehicle. The percentage change of body weight was calculated from its initial weight.

Urine strip for glucose estimation contains 1.7% (w/w) glu-cose oxidase, 0.2% (w/w) peroxidase, 0.1% (w/w) potassium iodide, 71.8% buffer, 42.1% (w/w) non-reactive ingredients. Urine was collected on day 4, 7, 15 and 21 after administration of anthocyanin/ Glyburide /vehicle. The sugar level in urine was graded. The colour intensity of strip depends on the concentration of glucose which was compared against the standard grade given on the packet.

Assessment of mortality rate in streptozotocin -induced diabetic rats

Streptozotocin may cause ketoacidosis and may lead to death of animal. In view of this the mortality rate was monitored throughout the study. The % of mortality was calculated at the end of each week of treatment on 7th, 14th, 21st day.

Effect of anthocyanin on dexamethasone induced insulin resistant diabetic rats (*Shalam et al., 2006*). Wistar rats of either sex weighing 170-220 g were divided into seven groups consisting of six animals per group. Groups 1 and 2 received only distilled water and vehicle (3% (v/v) Tween 80 in water). Group 3 received Glyburide (10 mg/kg, p.o.) which served as reference standard group. Anthocyanin at the doses, 300 and 400 mg/kg were administered orally to the rats of groups 4 and 5, respectively. All the treatments were made for a period of 10 days. 1 h after the test drug adminis-tration all the rats received dexamethasone (10 mg/kg, p.o.) daily for 10 days except group 1 which served as the normal control group. Blood samples were withdrawn from tail vein, to determine FBG and insulin levels, prior to and on 4th, 7th and 10th days after 1 h after dexamethasone treatment. Oral glucose tolerance test was also performed with a glucose loading in these rats on the 10th day

In vitro á-glucosidase inhibition assay

The á-amylase and á-glucosidase inhibitory effect of plant extracts was determined according to the standard method.

Statistical analysis

The entire data were statistically validated using two-way ANOVA, followed by a post hoc percentage considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Cell suspension culture of *B. malabarica*

Cell suspension culture was established by culturing fresh 1 g friable calli from the leaf explants of *B. malabarica* in liquid MS media supplemented with different concentrations of BAP, 2, 4-D and NAA. Medium supplemented with 2, 4-D (0.1 mg L^{-1}) and BAP (0.5 mg L^{-1}) combinations showed well established suspension cultures i.e., suspensions without any aggregation or clumps of cells. The *in vitro* suspension of cells revealed optimal and steady biomass accumulation on day 14. The same medium and growth hormone combinations were used for further analysis of growth kinetics. The maximum fresh weight (8.0 g) and dry weight (0.85 g) was noticed at 14th day of culture in liquid MS medium complimented with 2, 4-D (0.1 mg L^{-1}) + BAP (0.5 mg L^{-1}). The time course of biomass accumulation was the typical sigmoid growth curve.

MS liquid medium supplemented with other combinations of hormones NAA + BAP showed only minimum outputs with aggregated or clumped cells. Generally, the cell growth was slow during the initial 5 days of culturing. Thereafter, biomass accumulated rapidly, and reached the highest value on the 14th day. Then the culture entered the stationary phase and declined marginally. Some cultures continued to grow even up to 30th day.

Quantification of anthocyanin

Cell suspension cultures showed insignificant accumulation of anthocyanin under elicitation by peptone water and yeast treatments. In fact, abscisic acid (ABA) (0.25 mg/L) and salicylic acid (SA) (60 µM) elicited anthocyanin remarkably i.e., 18.6 and 30.8 g/100 mL respectively. Similarly, the addition of phenylalanine, zinc sulphate, methyl jasmonate enhanced the anthocyanin synthesis in the cells but the content was lower than that of SA or ABA treatments.

Purification of anthocyanin

25 g of fresh, homogenized cell suspension samples were extracted out from the cell suspension cultures. The non-aromatic compounds were removed with the use of Amberlite XAD-7 column chromatography. Acidified ethanol with the concentration of 75% was used for the desorption of

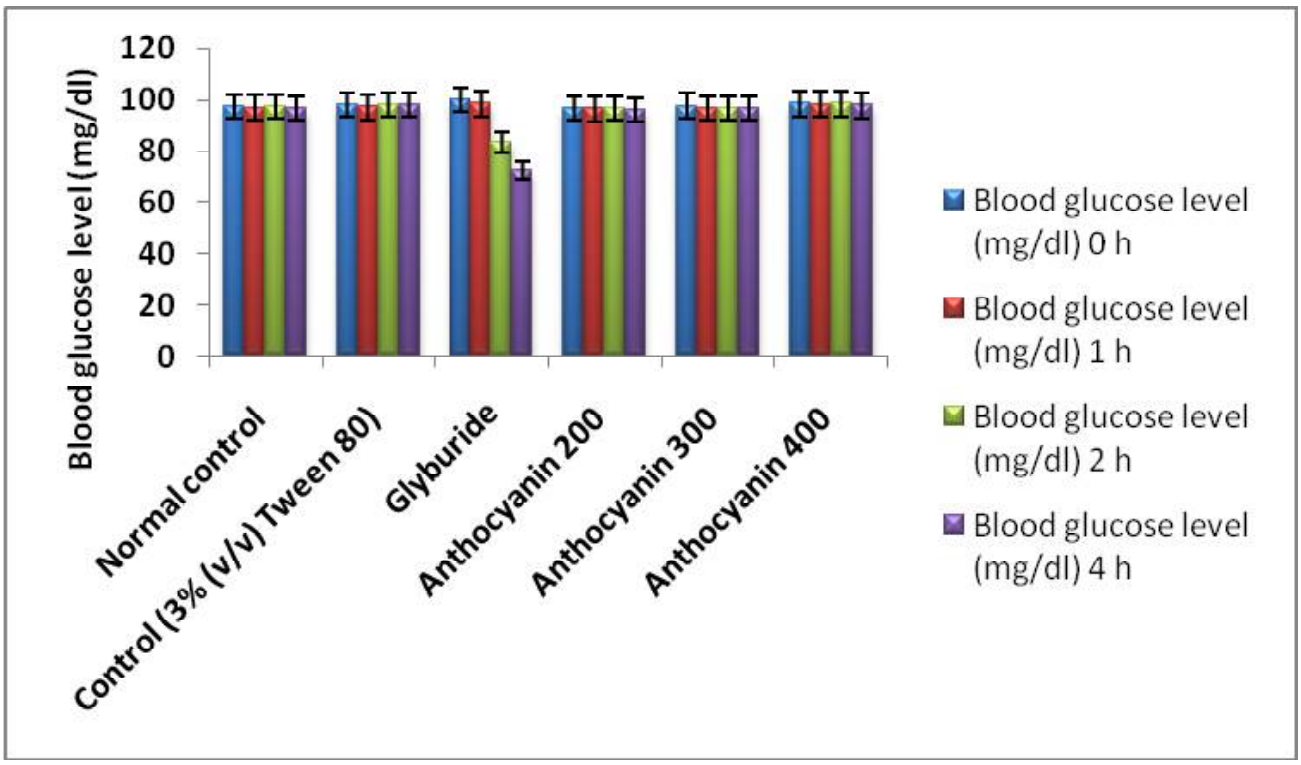

Fig. 1. Effect of anthocyanin extract by single dose administration on fasting blood glucose level in normal rats.

anthocyanins from the Amberlite XAD-7 HP column. Finally, the purified amberlite column eluted fraction was used for LC-MS analysis revealed malvidin-3 – diglucoside (*m/z* 655.3) as the major compound. The other peaks identified were malvidin or peonidin (584.3), delphinidin + glucose (459.2), cyanidin (403.2) and cyanidin aglycone (287.1). The others were sugar derivatives or minor fragments (m/z 242.3, 195.1 & 144.1).

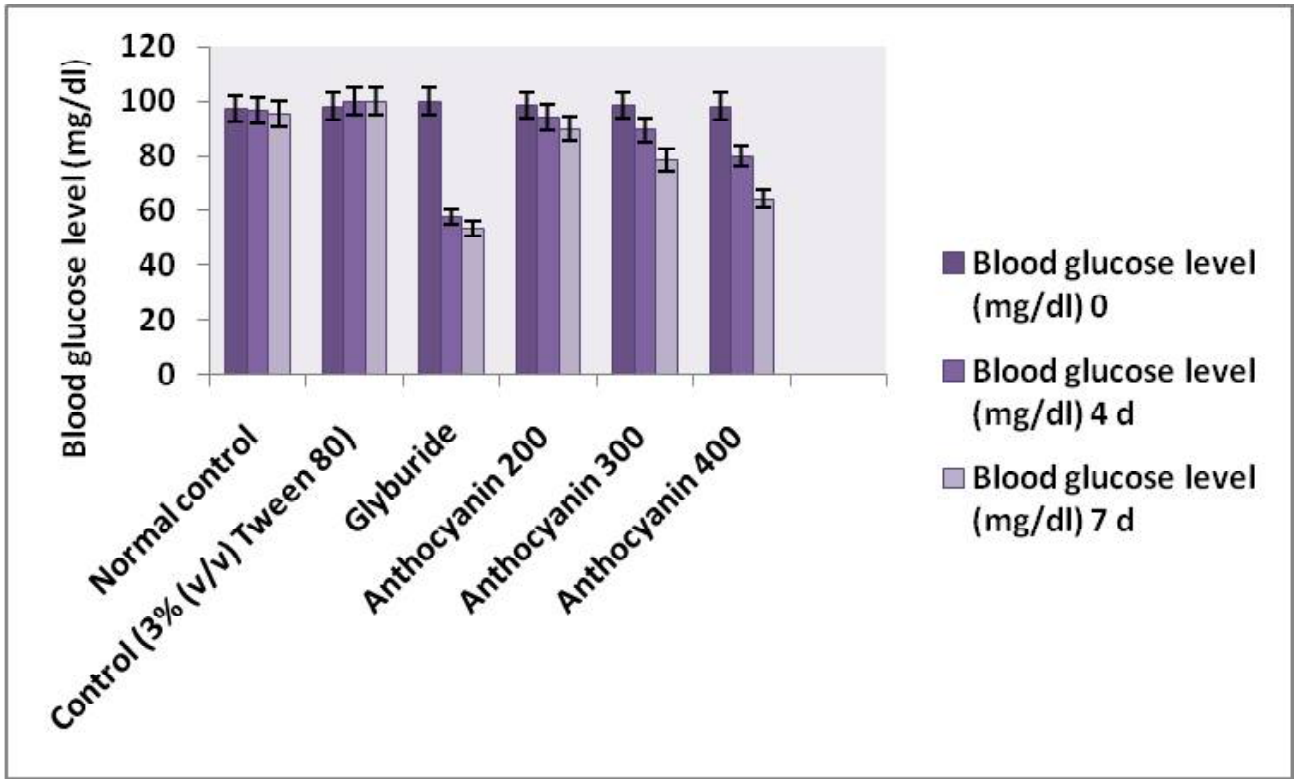

Fig. 2. Hypoglycaemic activity of anthocyanin in normal rats after repeated dose administration for 7 days.

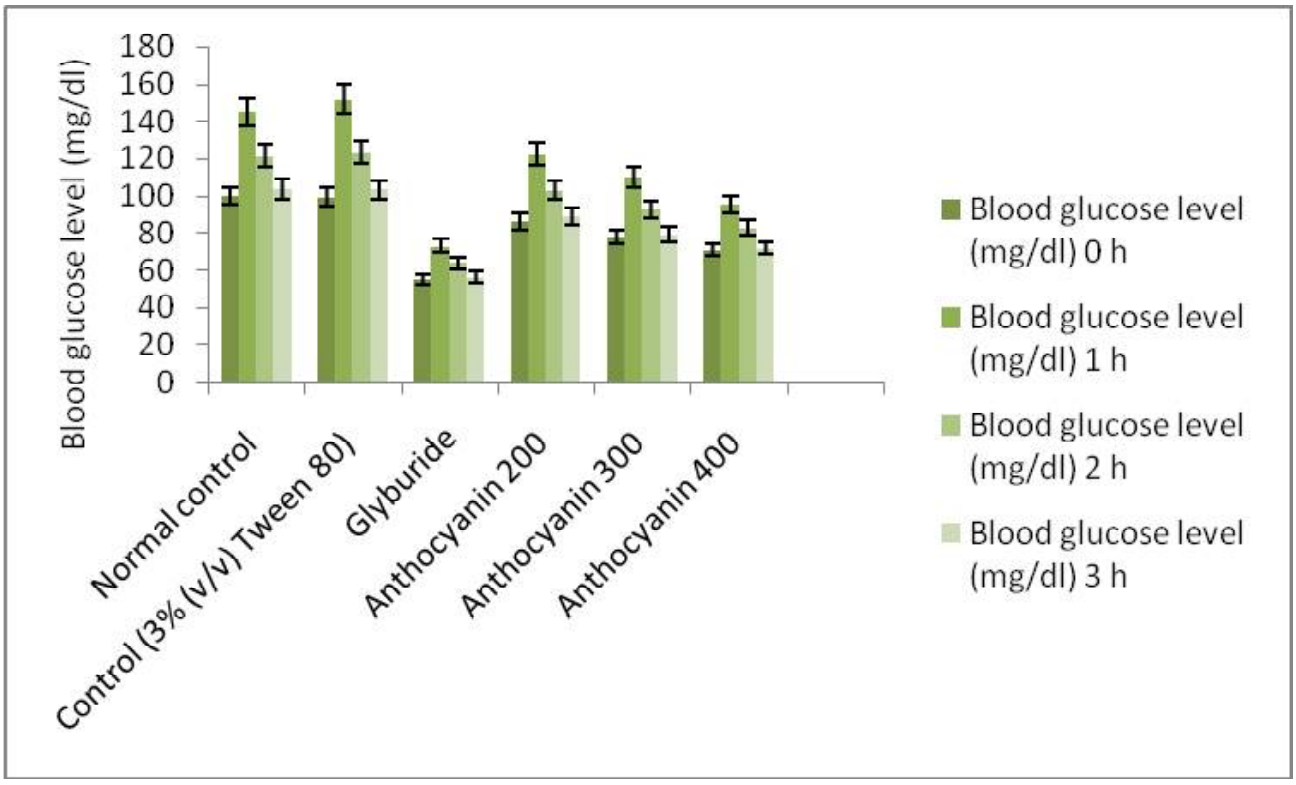

Fig. 3. Effect of anthocyanin on blood glucose level of glucose loaded hyperglycaemic rats (OGTT) after 7 days treatment.

Hypoglycaemic potential in normal healthy rats

Fasting blood glucose (FBG) levels were within the range of 96-104 mg/dl in all the groups at 0 h. Single dose administra-tion of purified anthocyanin (150, 300 and 400 mg/kg) did not remarkably reduced the blood glucose level at 1, 2 and 4 h after treatment, suggesting that the anthocyanin was not significant in terms of hypoglycaemic activity after acute treatment (Fig.1).

Repeated anthocyanin dosage had remarkably decreased the FBG from 4th day with 400 mg/kg and on 7th day with 300 and 400 mg /kg, indicating that the anthocyanin can produce hypoglycaemia on repeated administration (Fig.2). Meanwhile, single dose and repeated administration of Glyburide (5 mg/kg) significantly reduced blood glucose level when compared to the vehicle groups.

Administration of glucose (2 g/kg) to 7 days pre-treated rats significantly suppresses the rise in BGL with anthocyanin at 1 and 2 h with 300 mg/kg and at 1 h with 400 mg/kg as com-pared with vehicle control. Glyburide (5 mg/kg) showed significant suppression in BGL rise at 1 and 2 h (Fig.3).

Anti-diabetic power in streptozotocin -induced diabetic rats

Fasting blood glucose (FBG) levels in normal healthy rats were ranged from 96 to102 mg/dl. Administration with streptozotocin (120 mg/kg, i.v.) had enhanced the blood glucose level (BGL) to a range of 253-275 mg/dl after 5 days. Single dose administration of anthocyanin (150, 300 and 400 mg/kg) did not remarkably re-duced the BGL in streptozotocin induced diabetic rats, meanwhile Glyburide (5 mg/kg) reduced the BGL at 1st, 2nd and 4th h after single dose administration in streptozotocin induced diabetic rats (Fig.4).

Repeated dosage with anthocyanin extract (150, 300 and 400 mg/kg) had steadily decreased the BGL in a concentration dependent manner over a period of 3 weeks (Fig.5) i.e., a significant decrease ($P < 0.05$) in BGL on 4th, 7th, 14th and 21st days as com-pared to other animal groups. These results indicate that anthocyanin of *B. malabarica* displays hypoglycaemic activity on repeated dosage in streptozotocin - induced diabetic rats. Further, the repeated dosage of treatment of anthocyanin extracts (150, 300 and 400 mg/kg, p.o.) for 3 weeks had improved glu-cose tolerance when compared to diabetic control rats (Fig.6).

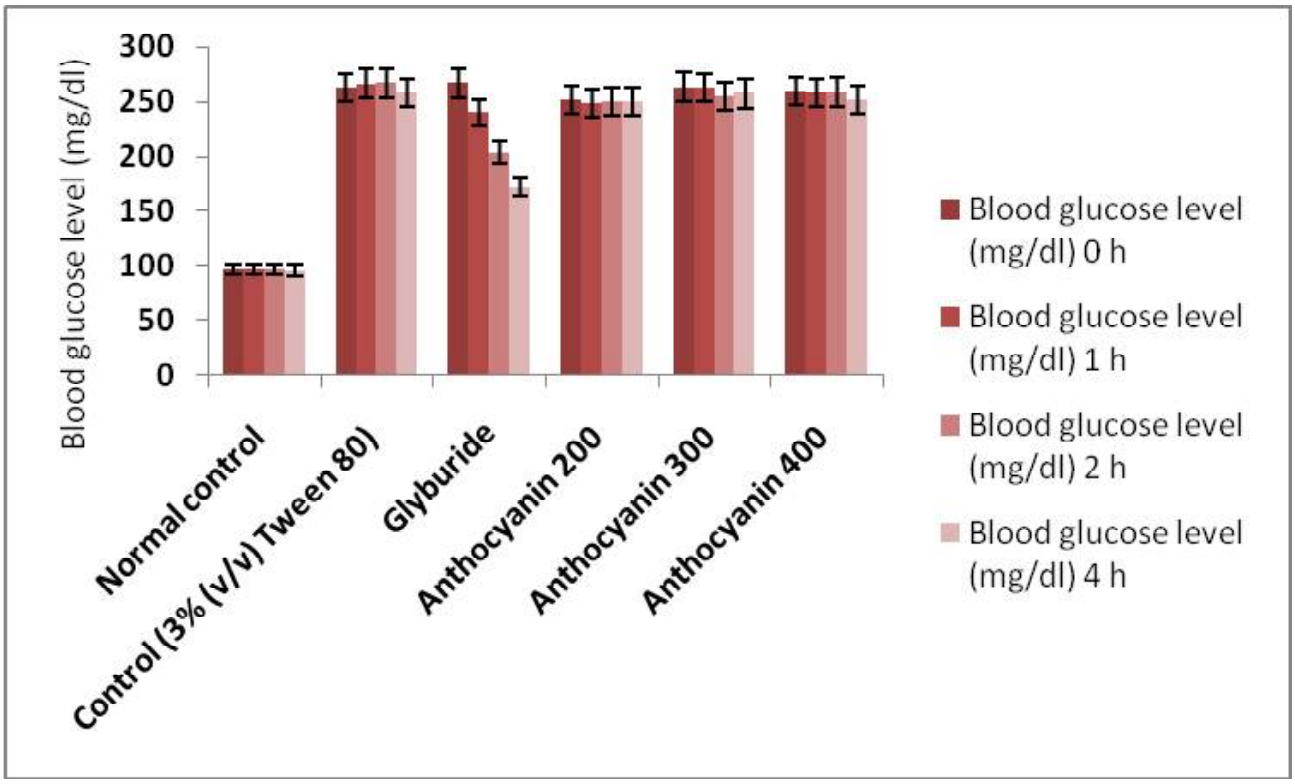

Fig. 4. Effect of single dose treatment of anthocyanin of blood glucose level on Streptozotocin -induced diabetic rats.

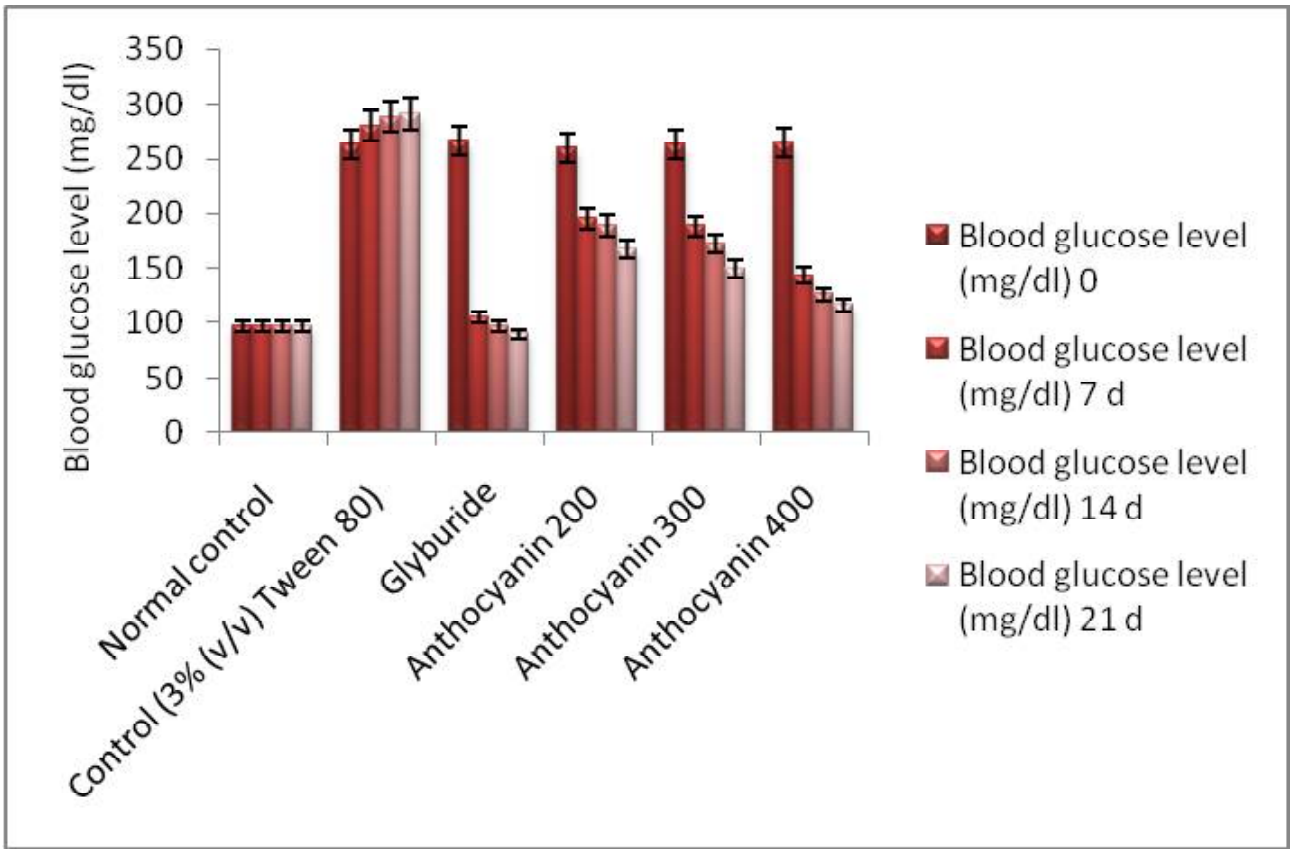

Fig. 5. Effect of repeated dose treatment of anthocyanin of blood glucose level in Streptozotocin -induced diabetic rats.

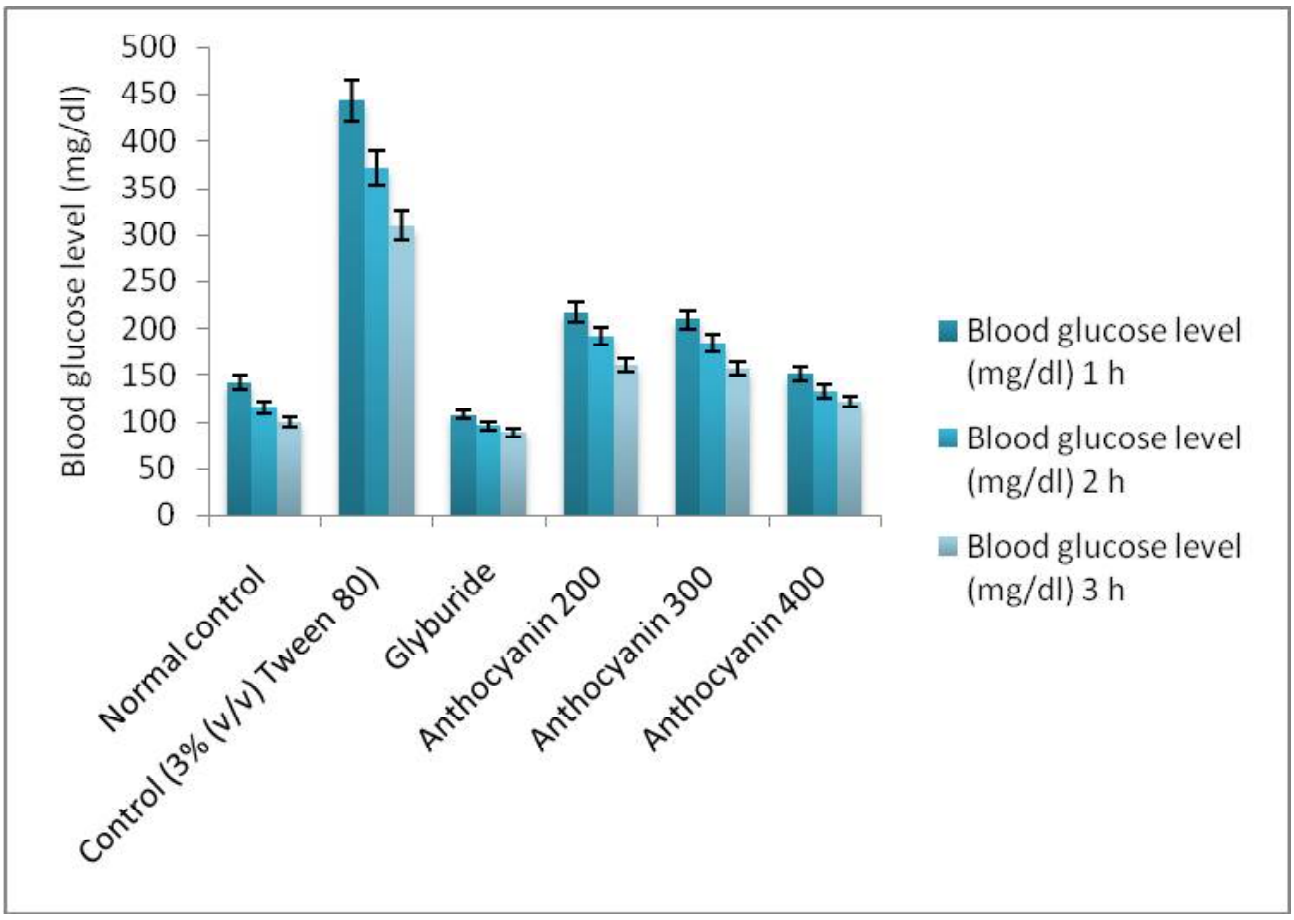

Fig. 6. Effect of repeated dose administration of anthocyanin on oral glucose tolerance test in Streptozotocin induced diabetic rats on 21st day.

Streptozotocin given rats (120 mg/kg, i.v.) showed a significant de-crease in the body weight at the end of day 7, 14 and 21 as compared to normal animals. Administration of Glyburide (5 mg/kg) had inhibited the reduction in body weight on day 4, 7, 14 and 21 in diabetic rats. These results suggest that anthocyanin extracts (300 and 400 mg/kg) were able to inhibit the body weight from day 14 onwards (Table 1).

Single administration of streptozotocin (120 mg/kg, i.v.) had resulted mortality of 45% over a period of 21 days. Continuous administration of Glyburide (5 mg/kg) had blocked the mortality in streptozotocin-induced diabetic rats throughout the study periods. Moreover, anthocyanin (150, 300 and 400 mg) administration also showed no mortality at the end of 3 weeks. These results indicated that Glyburide and

Table 1. Effect of repeated dose treatment of *B. malabarica* of body weight in streptozotocin induced diabetic rats. Values are expressed as mean } S.E.M. n= 6. P values were analyzed using One-way ANOVA followed by post hoc Tukey's test.

	Body weight (percentage change from the initial weight)			
	4 d	**7 d**	**14 d**	**21 d**
Normal control	1.46	2.59	4.53	-5.88
Control (3% (v/v) Tween 80)	-5.48	-8.66	-10.1	-10.75
Glyburide	-1.61	-2.5	-2.4	-2.3
Anthocyanin 200 mg/kg bw	-5.2	-6.6	-5.9	-5.2
Anthocyanin 300 mg/kg bw	-4.2	-3.5	-3.4	-3
Anthocyanin 400 mg/kg bw	-3.65	-3.1	-2.6	-2.3

* P< 0.05 versus the vehicle treated control group

Table 2. Urine sugar grade in alloxan-induced diabetic rats after repeated administration.

	Urine sugar (grade)			
	4 d	**7 d**	**14 d**	**21 d**
Normal control	0	0	0	0
Control (3% (v/v) Tween 80)	+++	+++	+++	+++
Glyburide	+	+/2	0	0
Anthocyanin 200	+++	+++	++	++
Anthocyanin 300	+++	+++	++	++
Anthocyanin 400	++	+	+/2	0

Values are expressed as mean grade } S.E.M. n=6.
++++=2. +++= 1. ++=0.5. + =0.25. +/2= 0.1.

anthocyanin could protect the animals against streptozotocin induced mortality.

Urine sugar grade in streptozotocin - *induced diabetic rats*

Streptozotocin administration at a single dose of (120 mg/kg, i.v.) induced the urine sugar level to grade 1 (+ + +) after day 7 s and it was further raised to grade 2 (+ + + +) within a week's period. The standard Glyburide had reduced the urine sugar level from grade 0.25 (+) to grade 0.1 (+ /2) on the day 7 and no sugar on the day14 and 21. Similarly, anthocyanin (400 mg/kg) also reduced the urine sugar level from grade 0.25 (+) to grade 0.1 (+ /2) on the day 14 and on the 21 no urine sugar level was noted (Table 2).

Impact of anthocyanin on BGL on repeated dose administration in dexamethasone induced insulin resistance diabetes in rats

Administration of dexamethasone for 10 days results a signif-icant increase of serum glucose level from 140 to 200 mg/dl compared to normal control. The anthocyanin extract 300 and 400 mg/kg along with dexamethasone for 10 days had remarkably ($P < 0.05$) reduced BGL on day 4, 7 and 10 compared to dexamethasone given group (diabetic control) (Fig.7).

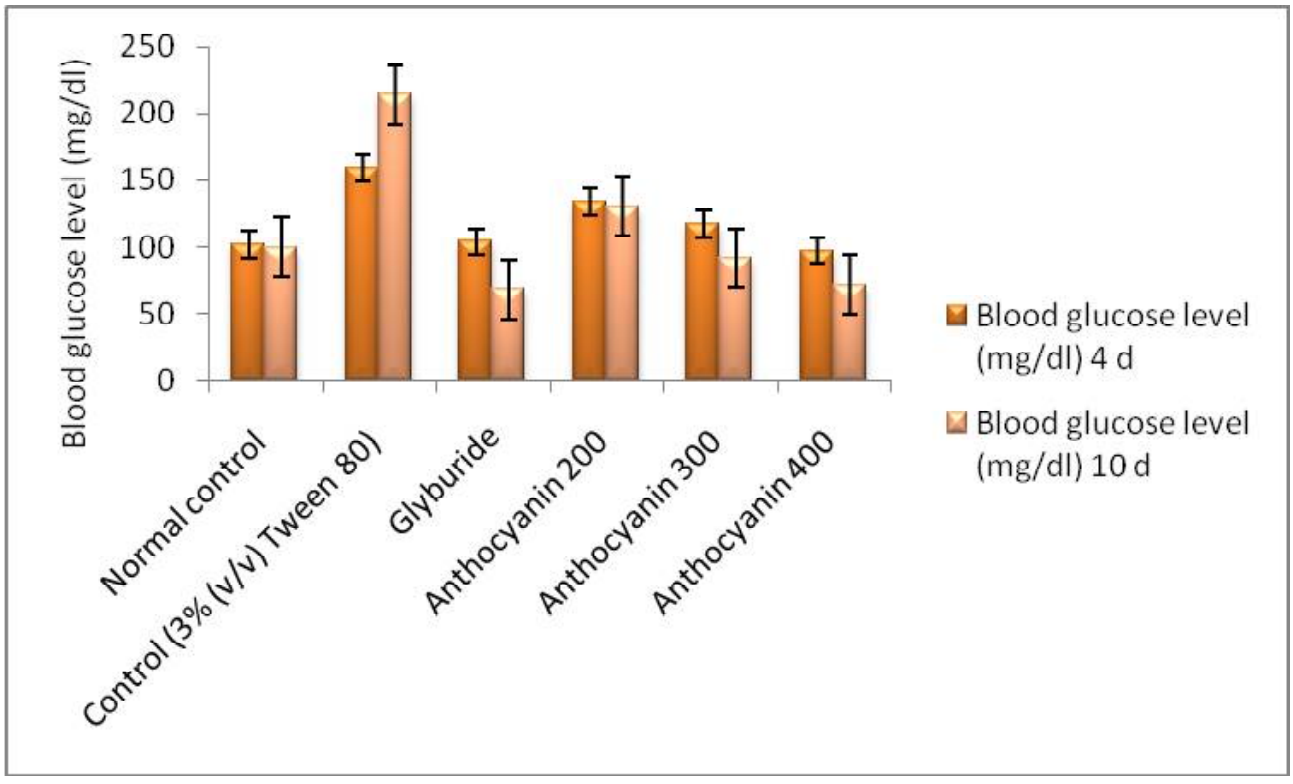

Fig. 7. Effect of anthocyanin of blood glucose level in dexamethasone induced diabetic rats.

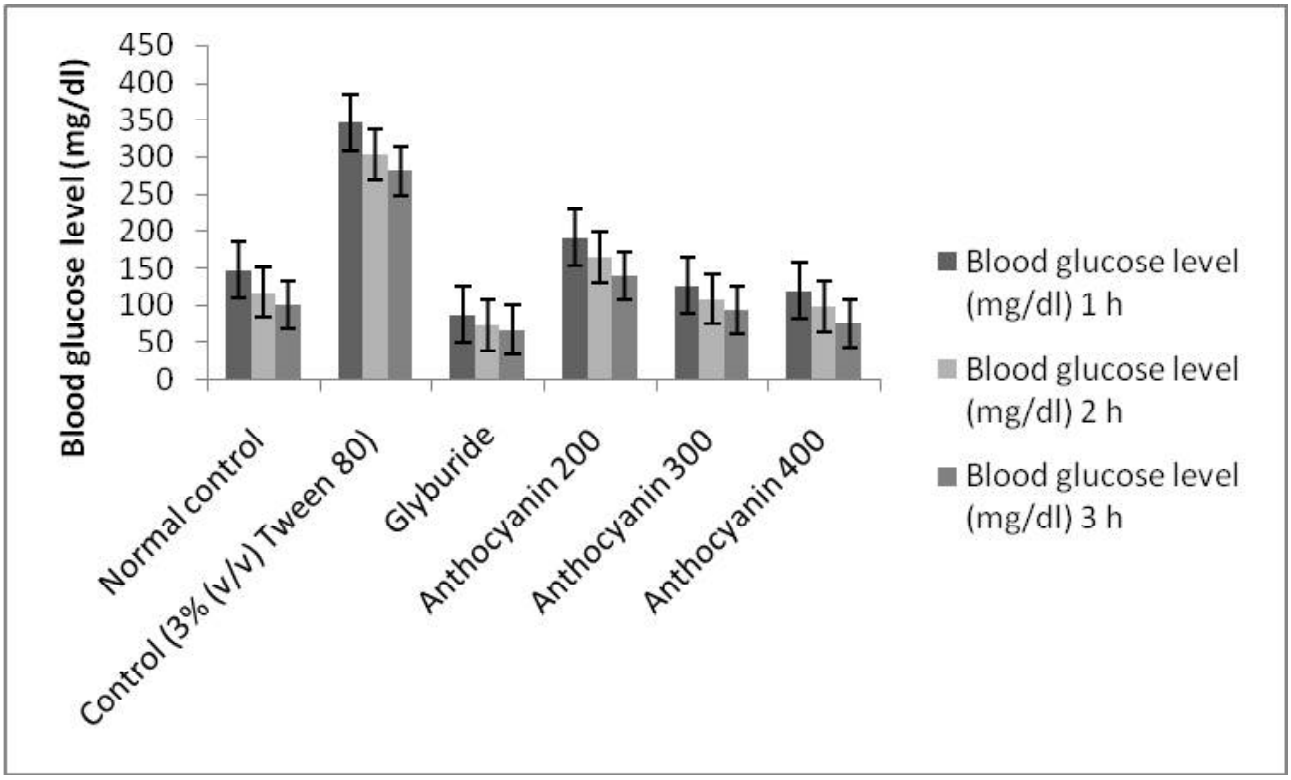

Fig. 8. Effect of repeated dose administration of anthocyanin on oral glucose tolerance test in dexamethasone induced diabetic rats on 10th day.

Change in body weight in dexamethasone induced diabetic rats

Dexamethasone caused weight reduction which was prevented significantly ($P < 0.05$) by the 300 and 400 mg/kg bw of anthocyanin extract after 10 days of treatment compared with diabetic control and standard Glyburide treated rats.

Enzymic analysis

As the last phase, the enzymic analysis of application of purified anthocyanin extract from *B. malabarica* was screened. A concentration dependent inhibitory activity of anthocyanin was noticed with both the enzymes. Purified anthocyanin showed significantly inhibited the activities of á- amylase and alpha glucosidase at the concentration of 400 µg/ml i.e., 77 ± 0.98 %, 72 ± 0.21 % with IC_{50} value 273 µl/ml and 282 µl/ml respectively. Acarbose was used as standard drug with different concentrations (Table 3).

The present study validates the anti-diabetic potentialities of anthocyanin in streptozotocin induced diabetes and dexamethasone triggered insulin resistance. Traditionally, natural food colourant was used or in combination with other plant products against diabetes mellitus. Streptozotocin administration reduces insulin release via the destruction of the â-cells of the islets of langerhans, resulting in to hyperglycaemia (Deeds *et al.,* 2011). Streptozotocin induced diabetic rats displayed a steady rise in BGL after 7 days a typical feature of diabetes mellitus. Ethnic people use herbal medicines throughout the world for mitigating diabetic issues. *B. malabarica* was used by the locals for curing diabetic disorders and therefore, an attempt was made to validate the

Table 3. Enzymic analysis of application of purified anthocyanin extract from *B. malabarica*

Quantity (µl)	Anthocyanin		Acarbose	
	α-glucosidase	α- amylase	α-glucosidase	α- amylase
100	22.8± 0.21 %	23.4± 0.21 %	33± 0.21 %	36± 0.21 %
200	43.9± 0.21 %	42.5± 0.21 %	53± 0.21 %	56± 0.21 %
300	60.8± 0.21 %	56.9± 0.21 %	72± 0.21 %	79± 0.21 %
400	77± 0.21 %	72± 0.21 %	85± 0.21 %	91± 0.21 %

effect of anthocyanin from *B. malabarica* in normoglycaemic and hyperglycaemic rats. Single dose administration of purified anthocyanin extract of *B. malabarica* was ineffective to de-crease the BGL in streptozotocin induced diabetic rats. However, repeated administra-tion of anthocyanin extract of *B. malabarica* from day 7 regulated remarkably the ele-vated BGL compared with other group.

Administration of anthocyanin extract repeatedly improved the glucose tolerance among diabetic rats on day 8, 15 and 22 when com-pared to diabetic control. Derailment of glucose tolerance is due to the lack of insulin in streptozotocin -induced diabetic rats by damging the â-cells which leads to diabetes type I. Data of the present analysis suggests that anthocyanin ex-tracts can improve the glucose tolerance, through insulin mimetic activity or improved glu-cose utilization process. Glucosuria is the common syndrome associated with diabetes. It arises due to the alteration in BGL above 250 mg/dl in diabetic rats. In the present study, the administration of anthocyanin to diabetic rats reversed their blood glucose which was also substantiated in their sugar levels of urine. The plausible mode of action of anthocyanin towards anti-hyperglycaemic activity may be via inducing either pancreatic secretion of insulin from â- cells or its release from the bounded form (Zhuo *et al.,* 2013). It was proved that sulfonylureas induce hypoglycaemia through enhancing the insulin secretion from pancreas and these molecules are active in mild streptozotocin induced diabetes, whereas they are inactive in intense streptozotocin diabetes (i.e., all the beta cells have been damaged) (Aquilante et al., 2010). Similarly, Glyburide reduced BGL in mild hyperglycaemic rats. The diabetic rats administrated with anthocyanin showed normalization of BGL compared to diabetic control. This may be due to the possibility of survival of beta cells to exert their insulin releasing effect by *anthocyanin extract of B. malabarica*. Moreover, *anthocyanin* produced hypoglycaemia in normal rats also. This tempts to suggest that *anthocyanin* is probably med-iated by enhanced secretion of insulin like compounds.

In the present study, the possibility of enhanced tissue uptake of anthocyanin cannot be ruled out. In addition, the glucose lowering ef-fect of anthocyanin was more effective as compared to normal rats revealing that it may be due to an enhancement in periph-eral glucose consumption, this reinforces the cocept that the hypoglycaemic action involves insulin like effect via peripheral glucose consumption, delay in insulin catabolism or inhibition of glucose reabsorption by the kidney. Commonly, polyphenols enhances the â-cell regeneration and peripheral glucose consumption in streptozotocin and allied compounds induced diabetic rats supports the above assumption (Chakravarthy *et al.,* 1982; Mitra *et al.,* 1996). It is well established that ROSs/ free radicals are involved in diabetogenic action of streptozotocin and plants containing isoflavonoids, phenols, t-erpenoids have been proved to be effective in diabetes due their antioxidants potentiality (González *et al.,* 2000). This suggest that the anti-hyperglycaemic activity of anthocyanin extract of *B. malabarica* may be due to ROSs scavenging potentiality which induce the â-cell regeneration against streptozotocin induced free radicals. However, the exact mechanism of anti-diabetic activity of anthocyanin extract of *B. malabarica* is still not known. One of the issue of diabetes type I is loss of weight. This may be due to the impairment in insulin action (destruction of beta cells) like the conversion of glucose into glycogen, catabolism of fats and lipolysis inhibition. All these may results in to decrease in the body weight of the animals and eventually in death. Administration of anthocyanin extract of *B. malabarica* has substantially improved the body weight loss and mortality produced by streptozotocin. The overall results of BGL, glucose tolerance, urine sugar, body weight and mortality are well correlated with each other and suggest that the anthocyanin extract of *B. malabarica* can be beneficial in reducing the effects of streptozotocin induced diabetes.

Dexamethasone induces hyperglycaemia through the derailment of lipid metabolism results into hyperlipidemia. This inturn in-crease glucose level leading to hyperglycaemia. Further, dexamethasone may cause metabolic changes resulting in reduction of food consumption and body weight decrease. Barbera *et al.,* (2001) correlated obesity due to alteration of *ob* gene expression with insulin resistance. This in turn en-hanced the BGL via impairing GLUT4 translocation. In the present analysis, dexamethasone treatment for 10 days enhaced BGL and body weight decreases. Pre-treatment with anthocyanin extract of *B. malabarica* (150, 300 and 400 mg/kg) 1 h be-fore dexamethasone for 10 days had remarkably blocked the increase in BGL caused by dexamethasone and also normalized glucose tolerance after glucose loading.

CONCLUSION

Thus, the present study proved the hypoglycemic and antidiabetic efficacy of purified anthocyanin from *B. malabarica*. The antidiabetic effect of anthocyanin was significant than the synthetic Glyburide. Enzymatic results also substantiated the potentiality of anthocyanin in regulating á- amylase and á-glucosidase activities. Further studies are warranted at molecular mechanism level to analyze the mechanism responsible for lowering of BGL is warranted.

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Brachiaria Subquadripara (Trin.) Hitch. (Poaceae) : A New Record of Kerala.

M. LIJA[1] AND M.S. BINOJ KUMAR[2]

[1]*Department of Botany, University of Kerala, Karyavattam.*
[2]*Sanathana Dharma College Alappuzha.*
email:lijaomana@gmail.com

ABSTRACT

During the systematic study on the family Poaceae, the authors came collected a species of Brachiaria from S. D. College campus. Critical studies on the species with the help of flora and herbarium, it is identified as *Brachiaria subquaripara.* This species has not reported so far from the state of Kerala. Gamble (1921) included the species in the Flora of the Presidency of Madras based on his collection from Medinpur district of West Bengal. This species is now widely distributed in Kerala.

Key words *Systematic; Poaceae; flora; herbarium.*

Biodiversity is the wide spectrum of living organisms including plants, animals, and microorganisms inhabiting in the terrestrial aquatic and other habitats. It has been estimated that our earth support about 50 million species of organisms through three billion years of evolution involving mutation, recombination and natural selection. But human history has tended to erode this diversity (Ashish Kothari 1997). The state of Kerala lies along the southern West coast of India; approximately between the latitudes 8° 18' and 12° 48' N and it has warm humid, tropical climate with an average rainfall of 3,020mm per year. These conditions support our state for diversified vegetation. Alappuzha is one of the unique district in Kerala without forest and highlands. Our present floristic study has been carried out in Santana Dharma College of this district. Even though there are several rare and endemic species due to the various biotic and abiotic impacts, the floristic diversity of the campus is declining day by day. Hence the documentation of the rich diversity is inevitable. Poaceae the fourth largest family of flowering plants, include about 700 genera and probably 10,000 species with cosmopolitan distribution. But the study of grasses has not an attracted work, the desired attention as the group is considered difficult for identification. Their spikelets being minute, the grasses require care dissection for correct determination. Being botanically neglected group, grasses need a separate detailed taxonomic investigation.

Ranga Acharya and Tadulinga Mudhaliyar (1921) in their *Handbook of Some Common South Indian* grasses described and illustrate about 103 species of Poaceae but unfortunately most of them are from the plain and only a few grasses from Kerala state . Fischer (1934-1936) described about 380 species of grasses under 126 genera from the then Presidency of Madras.It is estimated that about 200 out of the 380 species are distributed with the present political boundaries of Kerala ,Of these 10 are strictly endemic to Kerala. Sri Kumar and Nair 1991 published " *Flora of Kerala Grasses*" and reported 296 species of grasses from the state including , 2 new genus and 26 new species. Ravi (1995) reported *Dimeria chelariensis* and *Dimeria eradii* from Malappuram and Kozhikode of Kerala respectively .Mohanan and Ravi 1996 reported *Dimeria Shivarajini* from Kochu Pamba hills of Patnamthitta district. Sunil.C.N& M. Sivarajan (2000)reported *Cenchrus echinatus* from peninsular India.

MATERIALS AND METHODS

Materials collected were preserved by the procedure given by Jain and Rao (1977)& Van Baloogy (1987) . Photograhgs of the species were taken by using digital camera. Herbarium specimens are deposited in the Herabarium of S. D College .Simple microscopes were used for the observation of microscopic characters. The identification of difficult taxa was made by matching with authenticated specimens at the herbarium of Botanical Survey of India, Coimbatore and Tropical botanic Garden and Research institute ,TVPM.

RESULT

Brachiaria subquadripara (Trin.) Witch.

Griseb. In Lead.Fl.Ross.4:469.1853; Fischer in Gamble, Fl. Pres. Madras. 1767.1934; Bor, Grass Bur. Cey. Ind. Pak, 278.1960;Hook .f.Fl. Brit.Ind.7:33:1896.

Local Name : Cori Grass.

A genus of 90 species, in which about 6 occur in kerala and 1 in Alappuzha.

Annuals or perennials. Culms erect; sometimes creeping or ascending .Nodes and internodes are distinct ;nodes beared. Leaf linear- lanceolate; 10-20 ×0.5-0.7 cm long; sheaths compressed; ligule membraneos; fimbriate. Raceme terminal, 15-20cm

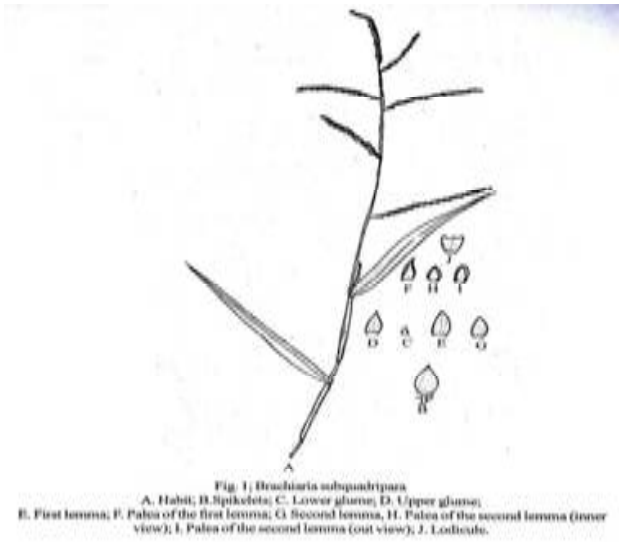

Fig. 1; Brachiaria subquadripara
A. Habit; B.Spikelets; C. Lower glume; D. Upper glume; E. First lemma; F. Palea of the first lemma; G Second lemma, H. Palea of the second lemma (inner view); I. Palea of the second lemma (out view); J. Lodicule.

height; rachis triquetrous or flat; narrowly or broadly winged. Spikelets ovoid –ellipsoid paired or single. Glumes uneaqual; membraneous ;distant; sometimes scaly; lower glume adaxial; shorter than the spikelets. Upper glume as long as the spikelets ; lower floret male or sterile; lower lemma similar to the upper glume; upper lemma crustaceous; upper palea obtuse or sub-acute. Stamens 3.Caryopsis ovoid or ellipsoid.

Distr. : West of the campus

Fl. & Fr. : Aug-Sep.

Notes : This species is not reported from any of the south Indian flora, hence the present report become a new record to south India.The Identity of the species confirmed by Dr. V.J. Nair. Botanical Survey of India,Coimbatore.

Specimen studied : West to the campus,23-09-2009 Lija 5[SDCH]

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Partial Genomic Sequencing of Phytochelatins Involved in Heavy Metal Tolerance of *Amaranthus Spinosus* L.

MARY SHEEBA A [1], MAHESH B[1], REMAKANTHAN A[1], SWAPNA T S[2] AND MINI I[1*]

[1] *Post Graduate Research Department of Botany, University College, Thiruvananthapuram, Kerala, India 695 034*
[2] *Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram, Kerala, India -695 581*
**email : dr.mini08@gmail.com*

ABSTRACT

Rapid industrialization, increased urbanization and improved agricultural practices have enhanced the level of heavy metal contaminants in the environment. Weedy species of *Amaranthus* such as *A.spinosus* was screened for heavy metal tolerance in the present study. Bioassay was conducted under laboratory conditions to assess the heavy metal tolerance of the test plant under Pb^{2+} and Cr^{6+} (100 to 200 µM) and Cd^{2+} (5 to 15 µM). The phytoaccumulation of heavy metal recorded values well above the normal level of accumulation in plants. An attempt was also made to sequence heavy metal binding phytochelatins (PCs) after extracting DNA from *A. spinosus*. Two sets of degenerate primers were designed by the screening of phytochelatin reported plants with the help of software BioEdit v.7.2.5. and Primer Premier 2.00. PCR was carried out to amplify the genomic DNA followed by its sequencing. A partial phytochelatin like sequence was obtained in *A. spinosus* and deposited in NCBI Genbank with Accession number MF174049. The BLAST results revealed relation with certain reported sequences of defence related proteins in some flowering plants.

Key words *Heavy metal tolerance; Amaranthus spinosus; Phytochelatins; Genomic sequence*

Industrialization, mining, release of waste water and sewage have resulted in the destruction of environmental quality around the globe. Heavy metal pollution is of considerable importance and relevant in the present scenario due to the increasing levels of pollution and its obvious impact on human health through the food chain (Hadjiliadis, 1997). Heavy metal toxicity in soil can be tackled through physical, chemical or biological remedial measures. Majority of ruderal weed species growing in abandoned areas and waste lands tolerate high levels of heavy metal contamination. Weedy species exhibit metal tolerance because it possesses huge biomass, efficient seed dispersal mechanism, rapid growth and capability to evade heavy metal toxicity with various protective mechanisms (Wang *et al*., 2005; Abe *et al.*, 2006).

The main mechanism for metal tolerance is chelation accomplished by multiple coordination bonds between organic molecules and metals. Organic compounds present in the cell function as ligands/ chelants, chelators, chelating agents or sequestering agents which may function as metal chelators inside the cell rendering the heavy metal into almost inactive or non toxic form. Phytochelatins (PC's) are metal chelators ubiquitous in the plant kingdom. They are small peptides rich in sulfur derived from glutathione enabling detoxification of heavy metals in plant cells. They are composed of L-cysteine, L-glutamic acid and glycine. Glutamic acid is linked to each cysteine by a y-peptide linkage and the general structure is (Glu-Cys)n-Gly (n = 2-11). The synthesis is catalysed by the enzyme phytochelatin synthases, which can bind to various metals including Cd, As, Cu or Zn. One protective strategy against excess metal is the expression of high-affinity binding sites to suppress uncontrolled binding of metal ions to physiologically important functional groups.

Some of the weed species of *Amaranthus* exhibit the potential of accumulating heavy metals from the soil and they exhibit high metal tolerant capacity. Hence the present investigation focusses on the metal tolerant capacity of *Amaranthus spinosus* common in the roadsides, abandoned fields and wastelands of Kerala. The study encompasses sequencing of the metal chelating ligand - Phytochelatins effective under Pb^{2+}, Cd^{2+} and Cr^{6+} stress.

MATERIALS AND METHODS

Bioassay was conducted under laboratory conditions to assess the heavy metal tolerance of the test plant under Pb^{2+} and Cr^{6+} (100 to 200 µM) and Cd^{2+} (5 to 15 µM) and phytoaccumulation was estimated through wet digestion method (APHA, 1993). After the isolation of genomic DNA (Rogers *et al*., 1985), PCR amplification was performed.

Two sets of degenerate primers were designed by screening the plants reported with phytochelatin sequence using the software BioEdit v.7.2.5. and Primer Premier 2.00. The designed primers were given in the table.

Sl. No.	Primer Name	Direction	Sequence (5' → 3')
1	Am-Pc-2F	Forward	TGAGVTCMGAATWRCAGAGG
	Am-Pc-2R	Reverse	AGCAGCGAGATCHTCCTCTT
2	Am-Pc-3F	Forward	GGCTATGGCGAGTTTVTAYCG
	Am-Pc-3R	Reverse	GGAACCCARTGRGGAGGATAC

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PhirePCR buffer, 1 µl DNA, 0.2 µl PhireHotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5 pM of forward and reverse primers. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) at an initial denaturation at 98°C for 30 seconds, followed by 40 cycles of denaturation at 98 °C for 5 seconds, Annealing at 60°C for 10 sec, elongation at 72 °C for 15 seconds, with a final extension at 72°C for 60 seconds.

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. The molecular standard used was 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad). After the removal of unwanted primers and dNTPs from a PCR mixture, Sequencing was performed in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). BLAST analysis was carried out to find out the similarity between the query sequences with the sequence reported in the NCBI database (Altschul *et al.*, 1990). The sequence obtained in *A.spinosus* was deposited in the NCBI GenBank through BankIt submission tool.

RESULTS AND DISCUSSION

The plant was able to withstand a concentration of 200µM of Pb^{2+} and Cr^{6+} and 15 µM of Cd^{2+} (Table 1) without much change in the morphology and functions. Higher accumulation of $Pb^{2+}$was noticed in the root (213.87 to 484.98 µg g^{-1}) whereas Cd^{2+} accumulation exhibited a trend of root> flower> stem> leaf in *A. spinosus*. Chromium accumulation was also high in *A. spinosus*. The normal range of concentration of Pb^{2+} and Cd^{2+}in plants is 0.1 to 10µg g^{-1} and were 0.2 to 1.8µg g^{-1} with a toxic limit of 100µg g^{-1} (Leeper, 1978). The values reported herein was well above the normal range of accumulation in plants indicative

Table 1. Phytoaccumulation capacity of *A. spinosus* under heavy metal stress

Metal	Treatments	Bioparts (µg g^{-1})			
		Root	Stem	Leaf	Flower
Pb	T0	3.418± 2.418	1.175± 2.151	0.885± 2.449	0.3563± 0.2760
	T1	213.87±12.236**	121.575± 1.192**	69.498± 0.9383**	25.0055± 0.4792**
	T2	233.69±10.403**	107.32± 3.415**	53.688± 1.176**	44.78± 2.106**
	T3	484.98± 103.63**	175.3± 0.67**	86.30± 0.3350**	45.261± 3.125**
Cd	T0	0.3873± 1.117	0.153± 0.4462	0.318± 0.6273	0.208± 0.4943
	T1	9.97± 2.947**	7.325± 0.2754**	8.128± 0.2973**	4.903± 1.167**
	T2	43.458± 4.262**	28.31± 0.7649**	9.355± 0.4382**	8.105± 0.3503**
	T3	37.725± 4.420**	26.38± 0.3594**	28.014± 0.9606**	30.833± 0.4100**
Cr	T0	3.895± 0.3631	1.198± 0.4260	1.8± 0.3916	0.915± 2.220
	T1	173.1± 18.634**	153.358± 2.540**	18.403± 2.010**	11.645± 1.276**
	T2	141.16± 3.564 **	162.168± 3.087**	40.338± 1.766**	21.925± 4.488**
	T3	262.98± 7.772**	127.745± 0.6740**	120.940± 2.428**	48.010± 0.5381**

T3(200µM), Cd-T1(5µM), Cd-T2(10µM), Cd-T3(15µM), Cr-T1(100µM), Cr-T2(150µM), Cr-T3(200µM). Significance at $p < 0.05$, according to the Dunnett test. ns- Non significant, *Significant; **Highly significant.

of its metal tolerance capability.

The results represented as Mean ±SE of three replicates. T0- control, Pb-T1(100µM), Pb-T2(150µM), Pb-T3(200µM).

The present study revealed that the metal tolerance may be enhanced by the production of chelants in *A.spinosus* and the plant displayed the presence of phytochelatin like gene sequence. The isolation, amplification and sequencing of genomic DNA from *A. spinosus* was performed with a view to explore the metal chelating sequence of phytochelatin in the species. The major mechanism to relieve the heavy metal stress is the synthesis of metal binding polypeptides phytochelatins. PCs can act as biomarkers for the early detection of heavy metal stress (Saba *et al*., 2013). Zagorchev *et al*. (2013) reported that in plants, phytochelatins played a defensive role not only against heavy metal stress but also in response to several abiotic stressors such as heat, saline, herbicides and UV stress. The role of phytochelatins in plant adaptive mechanism under heavy metal stress was reported (Chen *et al*., 2008; Szalai *et al*., 2013; Saba *et al*., 2013; Batista *et al.* 2014).

It was noticed that the DNA fragments obtained from *A.spinosus* (Fig. 1) amplified with the second set of primer (Am- Pc- 3F & 3R). The expected size of the phytochelatin gene fragment was 600 bp in length on the basis of Am- Pc- 3F and 3R. But the ethidium bromide stained gel showed amplified DNA band with 857 bp in length in *A. spinosus* and the size of the DNA fragment was found higher than expected size with 485 bp in length genome sequence of *Amaranthus tricolor* (Wen *et al*., 2012). The amplified DNA fragments were subjected to sequencing analysis and a nucleotide sequence was obtained.

The chromatogram of *A.spinosus* (Fig. 2) revealed revealed single peaks at each base call. The nucleotide sequence of *A.spinosus* was subjected to BLAST analysis to find out similarity

between query sequence and sequence reported in NCBI gene data bank. The results showed (Fig. 3) that the query sequence of *A.spinosus* showed greater similarity (91%) with *Beta vulgaris* subsp. vulgaris probable inactive leucine-rich repeat receptor-like protein kinase At3g03770, transcript variant X3, mRNA, 85% similarity with *Amaranthus palmeri* clone NJ_Ap_20, and 80% similarity with *Amaranthus*

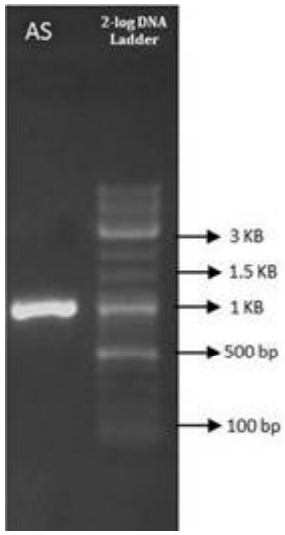

Fig. 1. Amplification of genomic DNA from *A.spinosus* with Am-Pc-3F

>SEQ2 [organism=*Amaranthus spinosus*] Phytochelatin related
CATCATTATCGTACTGTAATATATTCTGCTTATACGA
ACTATGATCAGGGTCTGGGAGGGATGGACAACCATA
CCCTAAAGAAGCTACCAAAAAATTGAATCGAA
TTGGCAATTTAAGTTTGCCTAAATTCTCAA
TAAGTAAC TGA GTGAAGAAGTTTTATCATAGAATG
ATCTCCTTTGACTAACTAATATATTGCAT
AATCATGCAATAAAATTAGGGCCGCTTAGTTACATAAC
GGGC GCTTTAAAACAG ATTTTGAGCTTATC GTTC
TGCAA AACCGTGA TACGTAACA ATTGATT TAAAA
TGAT GGAGTAATAAGCAT TATAAGC TATTATGAAGC
ATATGAGCCATTATGACTTTTTCTTTTAATATGCAT
TGGATTCTAAAAGGGCTATTGG GGCGTT TGGCAA
AGAGTTGATAGCCTGTAGCTAATAGAGTAATAGT
TGATTACATTG GCTGTTTTGACCAGATGATTTTATTG
ACTGGTTTGACTAGTTGATTTTGAATCCGCTG
CTATGAGCAACTTGTTCAAAAACAGCTTATTCG
TAACAATAAGTTGTTTCAACCCTTTATTTGTAT
CAAAATAAGCTAAAACTGCTTACACACGATTTTTTT
TCTATACAGAGCA AAAGCCTACCTGGTCGAAAAGGA
TCGCAGTTGCAGTGGAAGTTGCAAAAGGAATCC
AGTTTCTTCAAACAGGGATGATACCT GGTTTATATTC
AAACAATATAAAGACAACCAATGTTTTATTGG
ATCATAGCCTTCATGTGAAACTAAACAGCTATAA
TCTCCCTTTGTTAGCTGAAAACAAGGATAAGGTAG
CACATCAATACACCATCTTTTTTATATGTTTCAC

palmeri isolate MS-R 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene, complete cds; and transposon putative Ac, complete sequence. The sequence obtained in *A.spinosus* failed to show strict similarity with the reported phytochelatins genes. This may be probably due to the presence of introns in the amplified region. The nucleotide sequence obtained in *A.spinosus* was deposited in NCBI Genbank with Accession number MF174049.

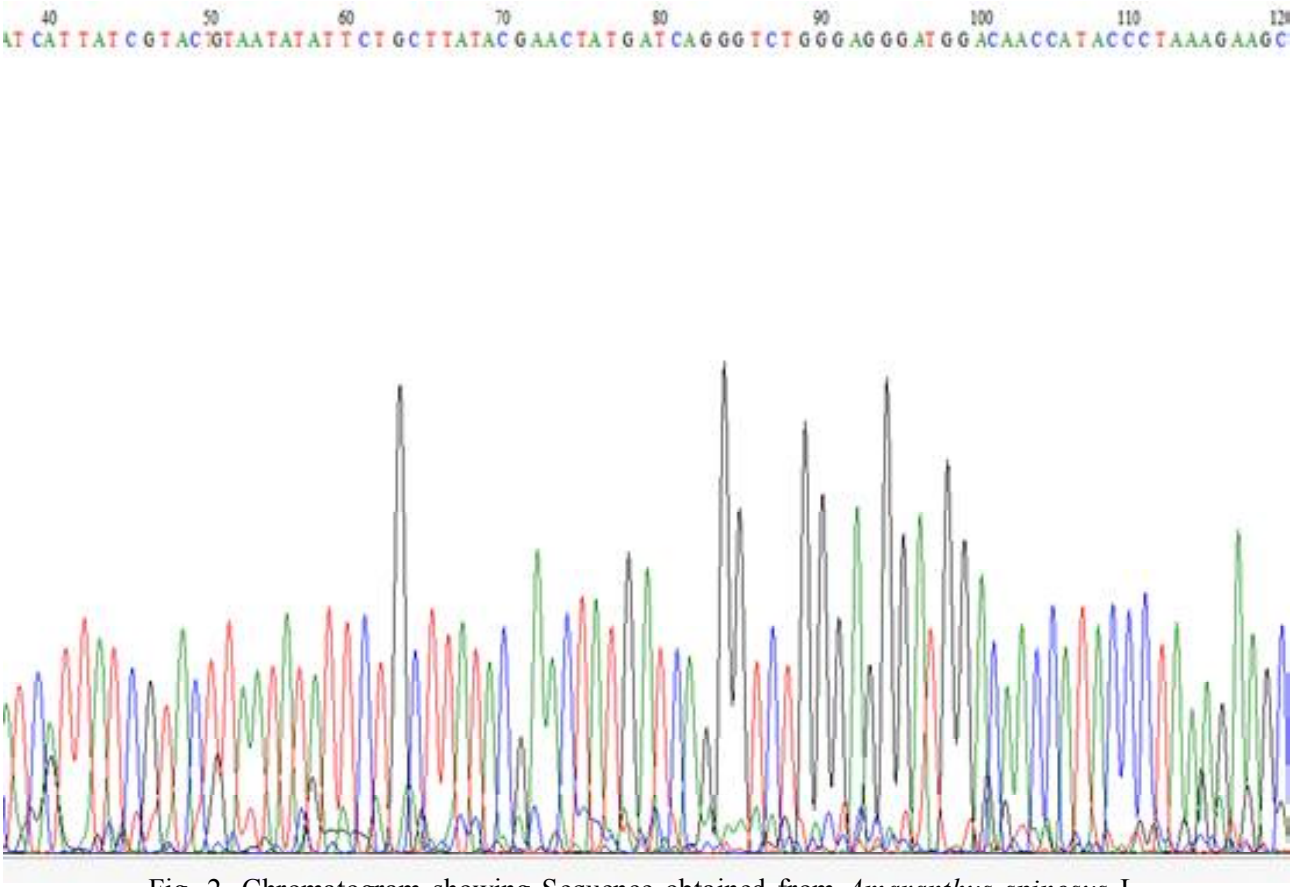

Fig. 2. Chromatogram showing Sequence obtained from *Amaranthus spinosus* L.

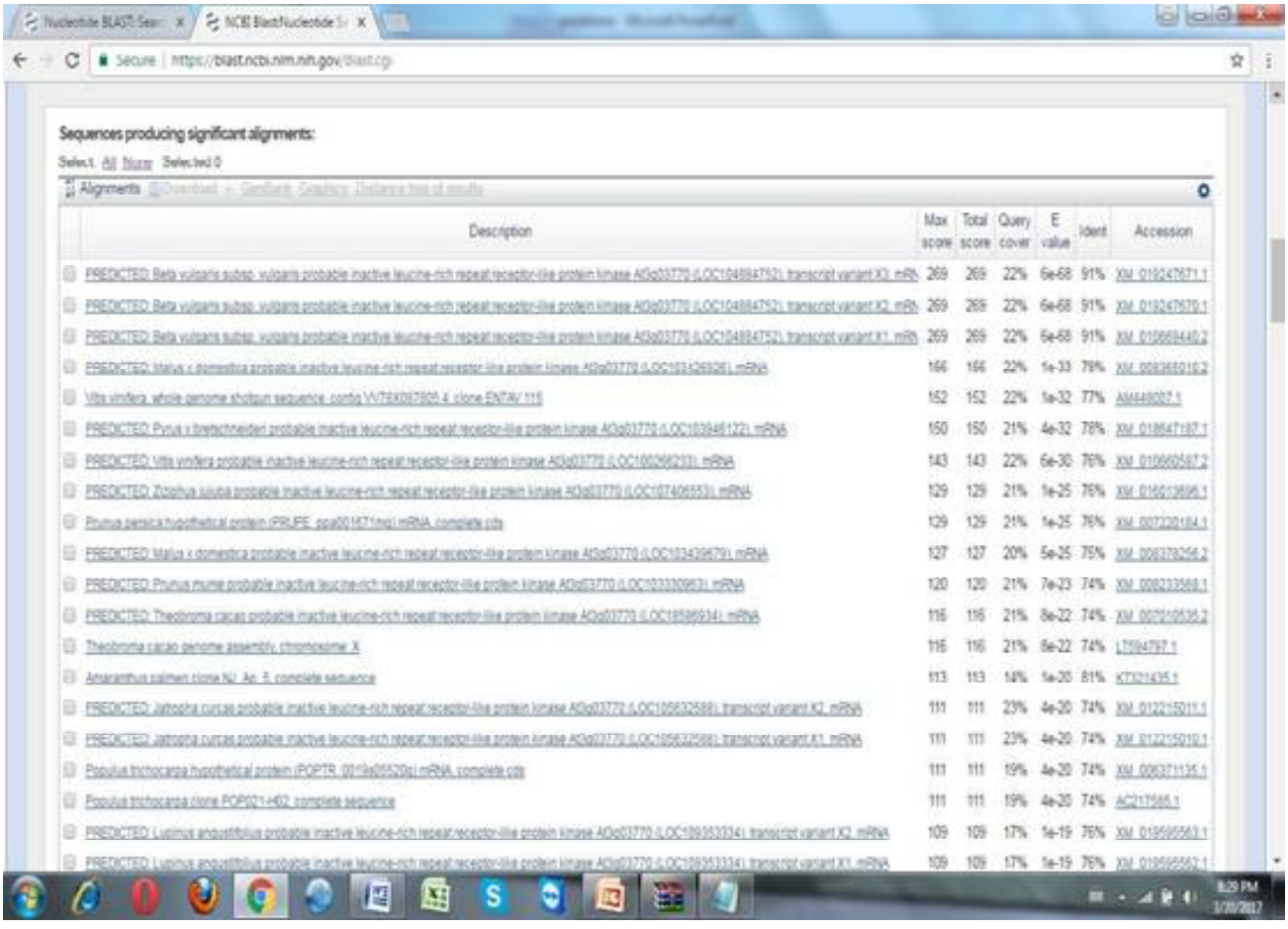

Fig. 3. Blast Results

SUMMARY AND CONCLUSION

Prolonged exposure of heavy metal toxicity pose severe threat to all living beings especially to plants through the generation of reactive oxygen species (ROS) and free radicals in plants which may interference with electron transport system. ROS create oxidative stress leading to cell damage, deterioration of macromolecules, membrane disruption, DNA damage, lipid peroxidation followed by activation of defense responses. Phytochelatins play a crucial role in heavy metal detoxification and the present study on heavy metal tolerance of *A. spinosus* may provide a baseline data on the sequencing for metal chelant, phytochelatins. The genomic sequencing of *A. spinosus* warrants full length gene isolation and sequencing for the validation of the isolated gene as phytochelatins.

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Biochemical and Molecular Mechanism of Purified Protease Inhibitor From Fruits of *Solanum Aculeatissimum* Jacq. (SAPI) as Anticancer Agent against Human Hela Cell Lines

MEENU KRISHNAN VG* AND MURUGAN K

Plant Biochemistry & Molecular Biology Laboratory, Department Of Botany, University College, Thiruvananthapuram 695 034, India.
email. meenukrishnanvg@gmail.com

ABSTRACT

Protease inhibitors (PIs) are low molecular weight proteins and are ubiquitous in all life forms. They are natural antagonists of protease which fascinated the attention of pharmaceutical and biotechnological researchers. PIs display myriad roles ranging from defense against pathogens and pests to medicine against cancer and virus replication. In this juncture, PI was isolated from the fruits of *Solanum aculeatissimum* Jacq. (SAPI) and purified via salt precipitation to sepharose affinity chromatography. The purity was confirmed by reverse phase HPLC chromatography. The molecular mass was detected using size elution chromatography (22.2 kDa). Antimetastatic analysis includes evaluation of viability of Ht29 colon cells, MG63 bone cells, HeLa cervical cells against 5 to 100 µg/ml concentrations of SAPI. Among them, HeLa cervical cells showed remarkable concentration dependent decrease in viability (39%). Further, at 50 or 100 µg/ml it caused an increase in the percentage of cells in the G0/G1 phase with a corresponding decrease in S phase of the cell cycle. Additionally, these doses caused an increase in the percentage of apoptotic cells as revealed by annexin V binding assay. Molecular analysis reveals a down regulation of cyclin D1, E, cyclin-dependent kinase expressions and retinoblastoma protein phosphorylation. Meanwhile, SAPI treatment enhanced the expression of p53 and its target gene product, p21 significantly. Further studies are warranted to confirm the efficacy of SAPI using animal models.

Keywords *Solanum*; Protease inhibitors; antimetastatic potentialities; apoptosis; cell cycle arrest.

Cervical cancer is the 4th most common cancer associated deaths across the countries globally. Population based surveys reveals that death toll of cervical cancer in developing countries was 19% in comparison to developed nations (63%) i.e., ranging from 1 (Bangladesh) to 73% (Brazil) (Gakidou et al., 2009). However, women who are with the highest risk of developing the cancer. Opportunistic screening of cervical cancer in different parts of India revealed a range from 6.9 to 10%. Human papilloma viruses have been recognized as a lead factor for inducing cervical cancer (Aswathy et al., 2015). The increased duration between initial infection and disease suggests that multiple factors like sexual- reproductive characters, sexually transmitted diseases, coinfection with HIV, smoking, malnutrition, genetical susceptibility, use of hormonal contraceptives, high parity and specific religious traditions in connection with HPV infection leads into cervical cancer (Shahina et al., 2014). 70% of cervical cancer among women was induced by HPV-16 and 18. Recent works reveal that PI3K/AKT/STAT3 signaling pathways play vital roles in regulating cervical cancer (Lee et al., 2015). Many carcinogenic signaling entities, micro and small noncoding RNAs that regulate the expression of oncogenic and tumor suppressive genes are the targets for designing drugs against cervical cancer.

Currently, synthetic drugs are the common practice of treatment against acute cervical cancer. However, clinical trials often show serious challenges including development of resistance and adverse side effects. Thus, it is utmost important to design plant based novel drugs for treating cervical cancers. Castro-Guillen et al., (2010) and Puxbaum and Mach (2009) reviewed the role of protease inhibitors (PI) as a mean to combat cancer. Cathepsin induced degradation of the extracellular matrix at the invasive front of ongo cells is one such example. Turk et al., (2004) reported that proteolytic process regulates cell proliferation and angiogenesis in many tumour cell lines. Many serine PIs have been implicated as unique regulators of development of cancer.

Dutch eggplant (*Solanum aculeatissimum* Jacq.), a common tropical weed intruded all over the hill stations of Kerala. The origin of the species is controversial i.e., South-Asia, or from Africa / South America. Consumption of ripe or unripe fruits leads to death in cattles but, the fruit decoction or fruit sap of roasted fruit has been used in ethnic medicine (Welman, 2003). In this scenario, the present study aims to evaluate the anticancer potentiality of purified PIs from *S. aculeatissimum* against cervical cancer cell lines.

MATERIALS AND METHODS

Plant material and purification of SAPI

Fresh fruits *Solanum aculeatissimum* Jacq. were collected from Munnar hills of Western Ghats, Kerala. 100 g fruits were homogenized with 250 ml of saline Tris buffer (20 mM Tris, pH 8.0; 0.15 M NaCl) containing 1 % polyvinylpyrrolidone (1:6 w/v) and filtered through chilled 4-fold muslin cloth and further, centrifuged for 15 min at 10,000 x *g*. The entire protocol was carried at 4°C (Asif-Ullah et al., 2006). The crude PI extract was fractionated by 20-90 % $(NH_4)_2SO_4$ precipitation. The $(NH_4)_2SO_4$ was removed by the process of dialysis using the same extraction buffer. The dialyzed protein showing high protease inhibition activity was subjected to different chromatography protocols such as DEAE cellulose ion exchange, Sephadex G-50 and sepharose affinity chromatography. Apparent molecular weight was obtained by Sephadex G-50 gel filtration column (0.1 M phosphate buffer, pH 7.6) calibrated with known molecular weight proteins (14.3 to 43 kDa).

Protease inhibitor activity assay

SAPI activity was determined by estimating the residual hydrolytic activity of trypsin and chymotrypsin towards the substrates BAPNA (N-benzoyl-L-arginine-p-nitroanilide) and BTPNA (N-benzoyl-L-tyrosyl-p-nitroanilide), respectively, at pH 8.0 after pre-incubation with inhibitor. Protein content was measured as per the method of Bradford (1976) using BSA by Coomassie blue staining.

SDS Page

Molecular mass and purity of PI was evaluated by SDS–PAGE (Laemmli, 1970). The molecular mass was further confirmed by size elution chromatography.

Cell Lines

Ht29 (Colon cancer cells), MG63 (Osteosarcoma cells) and HeLa (Cervical cancer cells) were procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecco's modified Eagles medium (Gibco, Invitrogen). The cells were cultured in RPMI 1640 medium (Invitrogen, developed at Roswell Park Memorial Institute), supplemented with 10% heat inactivated Fetal bovine serum, penicillin (100 U/ml), streptomycin (100 ìg/ml) and gentamycin (100 ìg/ml) and maintained in a humidified incubator containing 5% CO_2 and 95% humidity at 37°C.

MTT Assay

The viability of cells was evaluated by MTT assay and observation of cells was carried using inverted phase contrast microscope.

Colony formation assay

In this experiment, HeLa cells were seeded in a 12-well plate and allowed to stand for the next 3 days. Cells were treated with SAPI (25 and 50 µM) for 7 days. Control cells were incubated with 0.1% DMSO. The cells were maintained under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment. Colonies were fixed in methanol, stained with haematoxylin, and counted using UVP 810 software.

Wound healing assay

HeLa cervical cancer cell migration was analyzed by an *in vitro* wound-healing assay as described by Sikander et al., (2016). Briefly, HeLa cells were seeded in a 12-well plate and after 80–90% confluency, a standardized wound was made using a 200 ìL micropipette tip. Cells were treated with 25 and 50 ìM SAPI and photographed after 24 and 48 h by phase contrast microscopy. Motility of the cells was evaluated by their ability to wound closure.

Apoptosis

The FITC Annexin V/Dead Cell Apoptosis Kit with FITC annexin V and PI (propidium iodide) for flow cytometry provides a rapid and convenient assay for apoptosis. HeLa cells (Cervical cancer cells) in complete RPMI medium were added to a 24-well sterile tissue culture plate, and cultured in the presence and absence of purified SAPI in a CO_2 incubator for 24 h. The cells were washed with 50 mM cold PBS (phosphate buffered saline) at pH 7.4, collected by centrifugation at 1000 rpm for 10 min at 4°C, rinsed and re-suspended in binding buffer. HeLa cells were stained with Annexin V-FITC (0.1 mg) and propidium iodide (0.5 mg) in binding buffer. After an incubation period of 15 min in the dark at room temperature, the relative percentage of live/apoptotic/necrotic cells was determined by flow cytometry.

DNA fragmentation assay

HeLa cells density of about 0.75×10^6 cells were incubated for 24 h in the culture medium. Subsequently, the cells were treated with 50 ìg /ml SAPI for 12, 24 and 48 h. After incubation, treated

cells were trypsinized and resuspened in 1 ml of Hank's balanced salt medium. The cell suspensions were transferred into 10 ml of ice-cold 70% ethanol and stored in "20°C for 36 h. Fixed cells were centrifuged at 600 × *g* for 6 min and the resulted cell pellets were resuspended into 40 ìl phosphate-citrate buffer (0.2 M Na_2HPO_4 + 0.1 M citric acid, pH 7.8) and incubated at room temperature for 45 min. Cell suspension was centrifuged at 900 × *g* for 6 min and supernatant was gently decanted without disturbing the residue. To lyse the cells, 3 ìl of 0.25% Nonidet NP-40, 3 ìl of RNase (1 mg/ml in water) was added to the suspension and incubated for 20 min at 37°C. Further 3 ìl of proteinase K (1 mg/ml and incubated at 60°C for 20 min. After incubation, 12 ìl of gel loading buffer (0.25% bromophenol blue, 30% glycerol) was added. Finally, Samples were loaded in to 1.5% agarose gel and electrophoresis was done out at 4 V/cm for 4 h and DNA was analyzed under UV transilluminator.

Cell Cycle Arrest

HeLa cells were cultured as per standard procedures and treated with SAPI at IC_{50} concentration for 24 h. The cell samples were transferred to a 12 × 75 mm polystyrene tube or 50 ml conical flask. The samples were then centrifuged at 3000 rpm for 5 min. After centrifugation, the cell pellet forms either a visible pellet or a white film on the bottom of the tube. Appropriate volume of PBS was added to each tube (i.e., 1 ml of PBS per 1 × 10^6 cells) and the contents were mixed by pipetting several times or gently vortexing. The cells were centrifuged at 3000 rpm for 5 min. The supernatant was discarded without disturbing the cell pellet, leaving approximately 50 µl of PBS per 1×10^6 cells. Resuspend the pellet in the residual PBS by repeated pipetting several times or gently vortexing. The resuspended cells were added drop wise into the tube containing 1ml of ice cold 70% ethanol while vortexing at medium speed. Cap and freeze the tube at -20ºC. After the overnight incubation, the samples were centrifuged at 3000 rpm for 5 min. at room temperature. The supernatant was removed and 250 µl PBS was added to the pellet. Then the centrifugation was done again at the same rpm and time. The pellet was taken after discarding the supernatant; 250 µl of cell cycle reagent was added. This was incubated at dark for 30 min and analyzed using flow cytometer. Gating was performed with reference to untreated control cells and samples were analyzed.

Detection and quantification of apoptotic by Hoechst staining

To evaluate and quantify the apoptotic cells, the Hoechst staining method was performed. The cells were seeded on the chamber slides in serum-free medium overnight and then treated with 50 µg/ml SAPI. After overnight incubation, the cells were then fixed with ice-cold 75% methanol/25% acetic acid solution at room temperature. The Hoechst 33258/ PBS solution (0.5 ìg/ml) was then added. The cells were kept in the dark at room temperature for 10 min. Cells with typical apoptotic nuclear morphology such as nuclear shrinkage, fragmentation and condensation were defined as apoptotic. The apoptotic scores were counted from five random fields (for a total of 500 cells counted) for each sample, and the percentage of apoptotic cells was calculated as the number of apoptotic cells/number of total cells counted.

Western blot analysis

The entire experiment was carried at 4º C. 5 × 10^5 cells/ml healthy HeLa cells were treated with 50 µg/ml of SAPI and the control cells without SAPI vs different periods (12, 24, and 48 h) were cultured. After different periods of culture, the cells were centrifuged, the resulted pellets were washed thrice with PBS, and resuspended in 100 ìL of radio-immuno precipitation assay buffer (RIPA) (contain pH 7.5, Tris–HCl-20 mM; NaCl -150 mM, EDTA- 1 mM, Nonidet P40-1%, 0.5% deoxycholate, SDS- 0.1%, NaF- 5 mM, and cocktail -0.5%), centrifuged at 13000 rpm for 15 min to collect the supernatant. The supernatant protein concentration was measured using a bicinchoninic acid protein assay kit (Thermo Scientific Co). 50 ìg of protein from each experimental group were electrophoresed for 2 h on 10% SDS gels and then transferred to a PVDF membrane using an electroblotter for 90 min. Membranes were blocked in PBS with 0.1% Tween 20 (PBST) containing 5% non-fat dried milk power for 60 min. An antibody raised against á-tubulin (1:20000), an antibody raised against pRB (1:2000), an antibody raised against p21 an antibody (cell signaling) raised against phosphorylated pRB (1:3000), and antibodies raised against p53 (1:3000), cyclin D1 (1:200), CDK4 (1:200), and CDK2 (1:500) were diluted in PBST containing 5% non-fat milk and membranes were incubated overnight. After washing thrice with PBST for 10 min each, the blot was incubated with anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase (1:5000 dilution

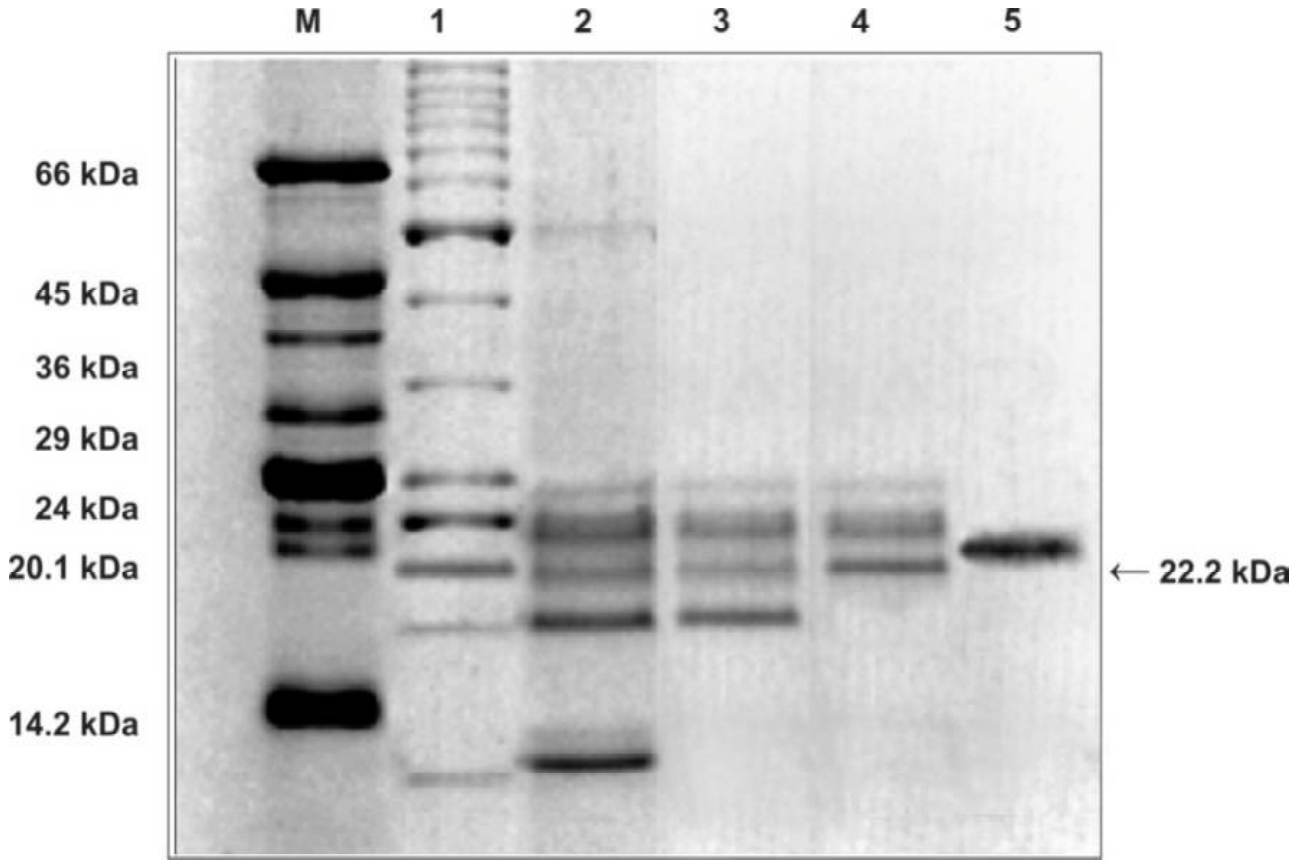

Fig. 1. *SDS PAGE of* purified SAPI

in PBST containing 5% non-fat milk) for 60 min at room temperature. After washing thrice with PBST for 10 min each, the blots were developed with a chemiluninescene detection kit, and the OD of each band was calculated by densitometric scanning instrument.

STATISTICAL ANALYSIS

In vitro and other assays were carried in triplicate and results were shown as mean ± SD. Statis-tical significance was calculated among various concentrations with one way ANOVA test. $p < 0.05$ was considered to be significant**.**

RESULTS AND DISCUSSION

Purification of the S. aculeatissimum protease inhibitor (SAPI*)*

Fruits of *S. aculeatissimum* revealed significant trypsin and chymotrypsin protease inhibitory activities i.e., 54 % and 48 % respectively. Subsequently, SAPI was purified by 4 step protocol. Initially, $(NH_4)_2SO_4$ precipitation (80-90 %) followed by dialysis against 20 mM Tris-HCl, pH 8.2 yielded 367 mg/g protein with 1.49 (trypsin) and 1.51 (chymotrypsin) fold of purification compared to the crude extract. Further, DEAE ion exchange chromatography showed one major and a minor peak. Pooled active fractions from 0.18 to 0.24 M NaCl (fractions: 9-12) were dialyzed and showed 93.2 TIU and 90.2 ClU in terms of trypsin and chymotrypsin inhibitory activities respectively. The fold of purification for Sephadex G-50 column was 52.7 and 51.8. Affinity column chromatography displayed a single prominent elution peak and was further confirmed by RP-HPLC with retention time of 10 min coinciding with the protein peak. Thus, purified SAPI yielded specific activity of 502 TIU and 433.7 CIU U/mg, with low protein content of 0.95 mg. Overall, the specific activity increased about 92.6 and 82.9 folds with 9.8 and 8.77 % yield with respect to trypsin and chymotrypsin respectively. The purity was confirmed by RP-HPLC yielded a single peak.

Molecular mass

The electrophoretic separation of SAPI by SDS-PAGE from crude to affinity chromatography yielded a single prominent band of 22.2 kDa mass (Fig. 1) and was in agreement with gel chromatography.

Cytotoxicity analysis

Generally, plant based drugs suppress cancer

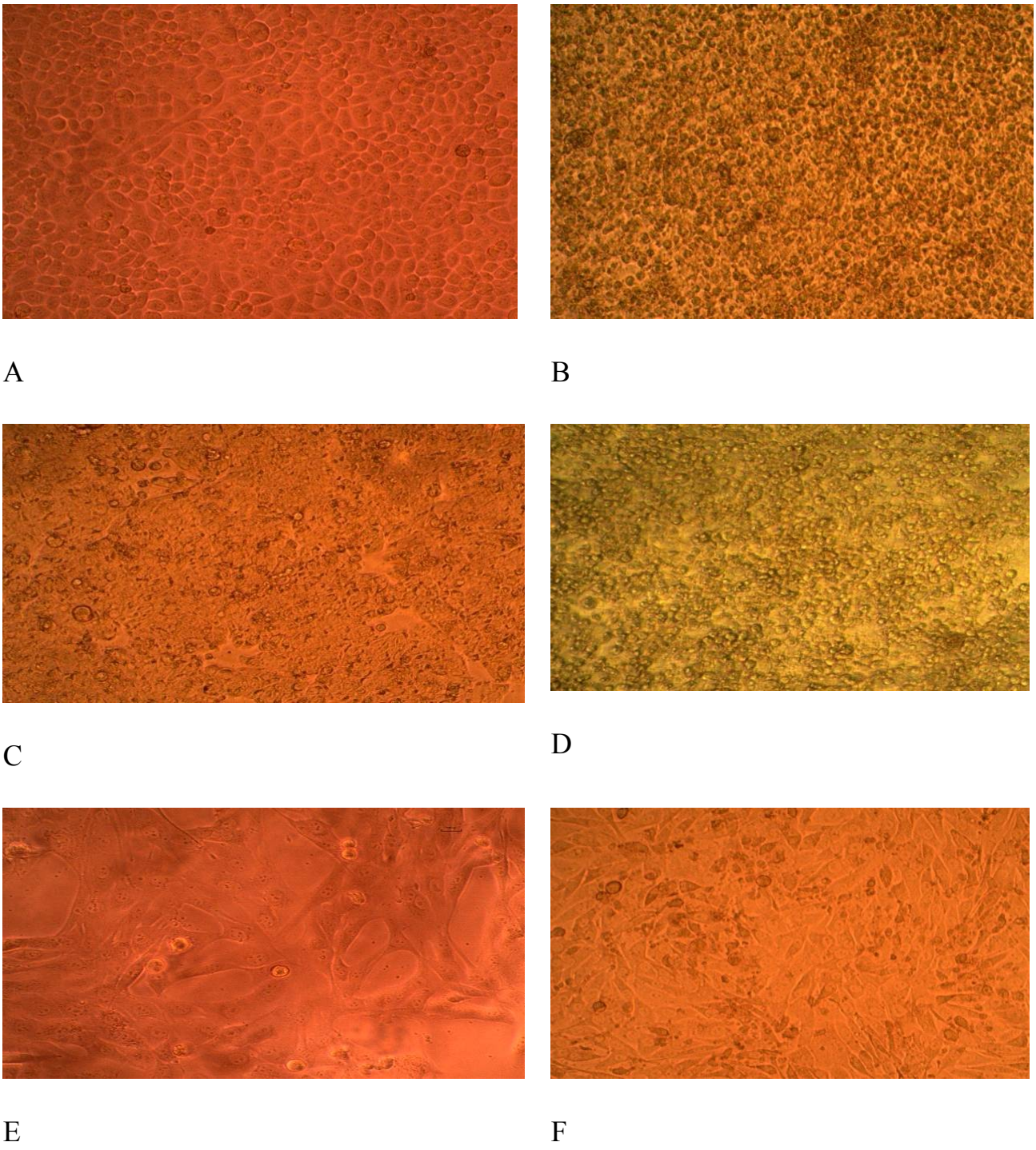

Fig. 2. Tumour cell lines culture after 96 h. A) HeLa control cells, B) 100 µg/ml SAPI treated HeLa cells, C) HT29 control cells, D) 100 µg/ml SAPI treated HT29 cells E) Mg63 control cells, F) 100 µg/ml SAPI treated Mg63 cells. All images are magnified at 40×. Images shown are representative of at least five such fields of view per sample and three independent trials.

through different phases like initiation, promotion and progression. PIs mostly affect cancer cell lines during initiation and promotion (Rakashanda et al., 2013). MTT assay is a valid accurate method and was widely used to determine cell growth and cytotoxicity, particularly in the development of drugs. Viability of MG63, Ht29 and HeLa cell lines against SAPI showed a mixed response at different concentrations (Table 1). MG63 and Ht29 colon cells were less affected as revealed by the MTT assay. Meanwhile, HeLa cervical cells displayed a concentration dependent decrease in viability. At higher doses i.e., at 100 µg/ml, the viability of the cells was decreased by 39%. The IC_{50} values for MG63, Ht29 and HeLa cell lines

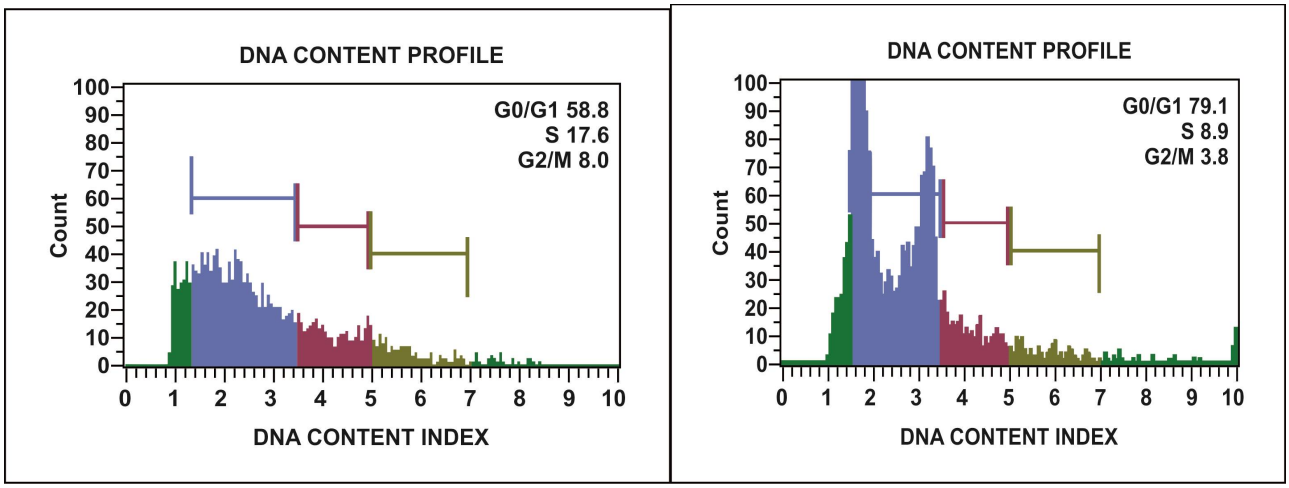

Fig. 3. Cell cycle distribution of HeLa cells in control and SAPI treated (50 µg/ml)

were 106.4, 121.7 and 43.6 respectively.

Morphology of cells and cell number were observed along different duration and concentrations. Initially, at 0 h, the cells remained normal (Fig.2). No visible morphological change was noticed in all the cell lines in control. Meanwhile, at 24 h after SAPI treatment at lower concentration, the cells still appearing normal (5 to 25µg/ml). However, after 72 h of cell culture, apparent DNA condensation within the nucleus was visible in treated cells (Fig.2). In addition, morphological changes, cell debris and reduction in cell density were noticed when compared to the control. At 96 h of cell culture, higher DNA condensation within their nucleus along with cell debris, a loss of cell adhesion and a significantly reduced cell number were clearly seen (Fig.2). The intensity of morphological damage increased significantly with concentrations i.e., from 50 to 100 µg/ml (Fig.2).

Colonogenic assay

It was employed to analyze the long term treatment effect of SAPI on proliferation of HeLa cervical cancer cells for 7 days. Results demonstrated that SAPI treatment remarkably ($p < 0.05$) reduced the number of colonies formed compared with control. The results suggest that SAPI treatment inhibits the proliferation and colonogenic potential of HeLa cervical cancer cell lines. Cysteine protease from the leaves of *Billbergia pyramidalis* was evaluated for antiproliferative action against human malignant cell lines such as human skin carcinoma cell line, human breast adenocarcinoma, HeLa, Glioma cell line Hs 683,

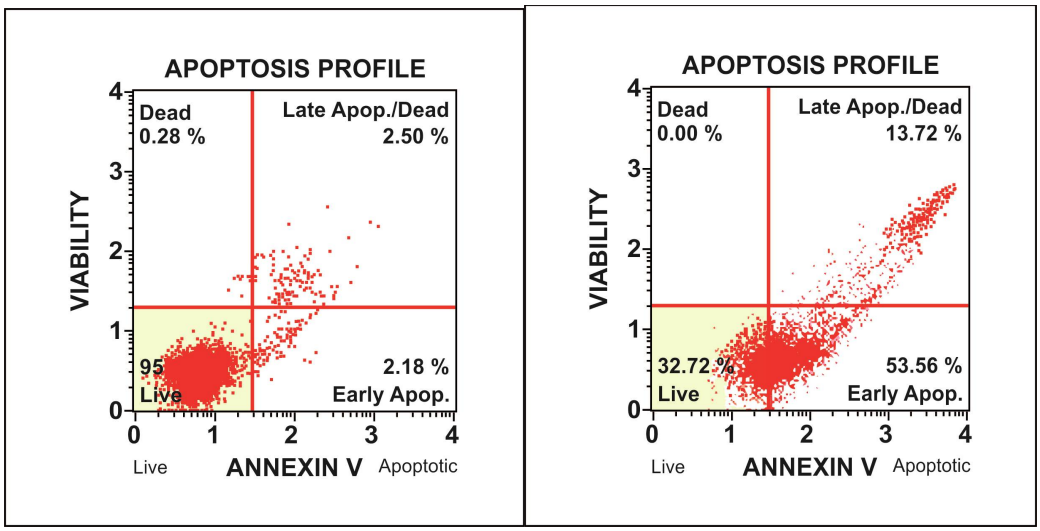

Fig. 4. Dot plot of HeLa cells – control and SAPI treated. The divisions of the plots distinguish necrotic cells (Annexin V-/PI+, left upper quadrant) from early apoptotic cells (Annexin V+/PI-, right lower quadrant) and late apoptotic cells (Annexin V+/PI+, right upper quadrant).

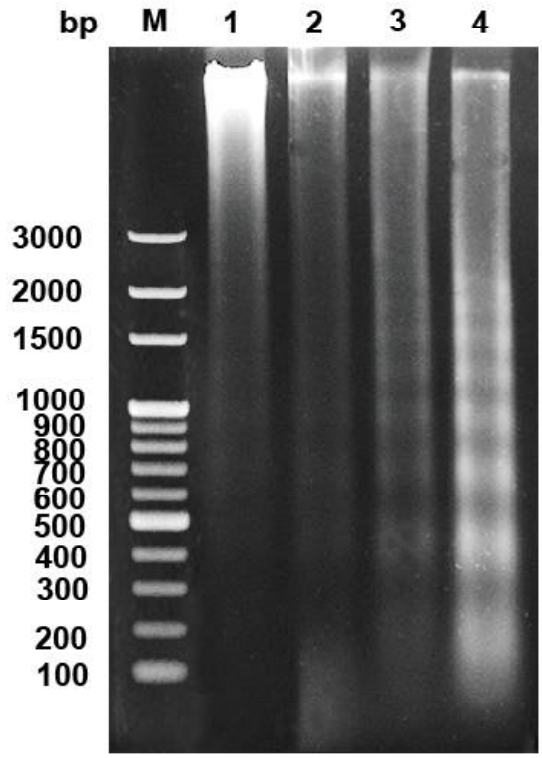

Fig.3. Agarose gel electrophoresis of 1 ig DNA (per lane) extracted from HeLa cells after 0, 24, 48 and 72 h *in vitro* culture in complete medium Lane 1 marker DNA (100-3000 bp), lane 2 DMSO solvent only (control), Lanes 3, 4 and 5 SAPI treated DNA after 24, 48 and 72 h.

and a non-cancerous 3T3- L1 Preadipocyte cell line from mouse, using MTT assay *in vitro*. It was noticed that PI was remarkable against A431 cell line only (IC_{50} value of 80.72 ìg/ml) than others. The present results are more superior to the PIs of *B. pyramidalis* (Kadiri and Anand, 2016)**.** Barla et al., (2016) proved the *in vitro* antiproliferative potential of trypsin inhibitor from testa of *Citrullus lanatus*.

Analysis of DNA content and cell cycle arrest

Cell cycle arrest has been used as a target for the cancer management. PIs positively regulate the cell cycle regulatory proteins. The cell cycle is coordinated by cyclins and usually they are up regulated in tumor cell lines. Cyclins and CDK inhibitors are degraded by the ubiquitin-proteasome pathway and this sensitize cells to apoptosis (Gillessen et al., 2003). The most common method for assessing the cell cycle is by flow cytometry which reveal the cellular DNA content at G1, S, G2 and M phases. Treatment with SAPI (50 µg/ml) on HeLa cells induced an increase in the population of cells in the G0/G1 phase and a decrease in the G2/M phase (Fig.3)**.** The untreated, control cells (Fig.3) showed a cell distribution of 58.8%, 17.6% and 8% in G0/G1 , S and G2M phase respectively when compared to SAPI treated cells i.e., nearly 79% of cells were accumulated at Go/G1 phase. During the 72 h treatment periods, SAPI enhanced the G0/G1 cells % to nearly 90 (i.e., enhanced G0/G1 peak) and reduced the G2/M percentage of cells. The control group showed a normal DNA pattern that was reflected in terms of G0/G1, S and G2/M phases of the cell cycle (Fig.3). Thus, SAPI treated cells revealed a typical apoptosis pattern of DNA content that was reflected in G0/G1, S and G2/M phases of the cell cycle, together with a sub-G0/G1-phase (referring to apoptotic cells). A pre-G0/G1 apoptotic peak was conspicuous after the cells were treated for 72 h. Similarly, an increase of SAPI dose led to an increase of apoptosis in HeLa cells i.e., 50 µM SAPI induced significant G0/G1 arrest in HeLa cells after 72 h time period. It is likely that high doses of SAPI may inhibit the cell cycle enzymes which lead to cell deaths. In addition, the cell morphology was changed and the viable cell % was decreased as revealed by MTT and flow cytometry analysis.

Apoptosis determination

The critical stage of apoptosis involves the acquisition of surface changes in the dead cells. The stain AnnexinV reveals high affinity for phospholipid phosphatidylserine (PS) and therefore bind to cells contain exposed PS molecules. On the other hand, propidium iodide (PI) penetrates the permeable cell membrane of the dead or damaged cells. Staining cells simultaneously with AnnexinV-FITC (green fluorescence) and propidium iodide (red fluorescence) effectively discriminate intact cells (FITC-PI-) from early apoptotic (FITC+PI-) and late apoptotic or necrotic cells (FITC+PI+) cells. To substantiate the increased G0/G1 population of human cervical cancer cells was due to apoptosis, the cells were labelled with FITC conjugated Annexin V and PI and subjected to flow cytometry analysis. SAPI treated cells showed 13.72% late and 53.56% early apoptosis cells when compared with the control (Fig.4). This observation may be due to the ability of PIs to regulate proteases, involved in various biological processes like cancer metastasis, tumor invasion and programmed cell death.

DNA fragmentation assay

Generally, apoptosis in cells was due to derailment of tubulin polymerization process. In order to

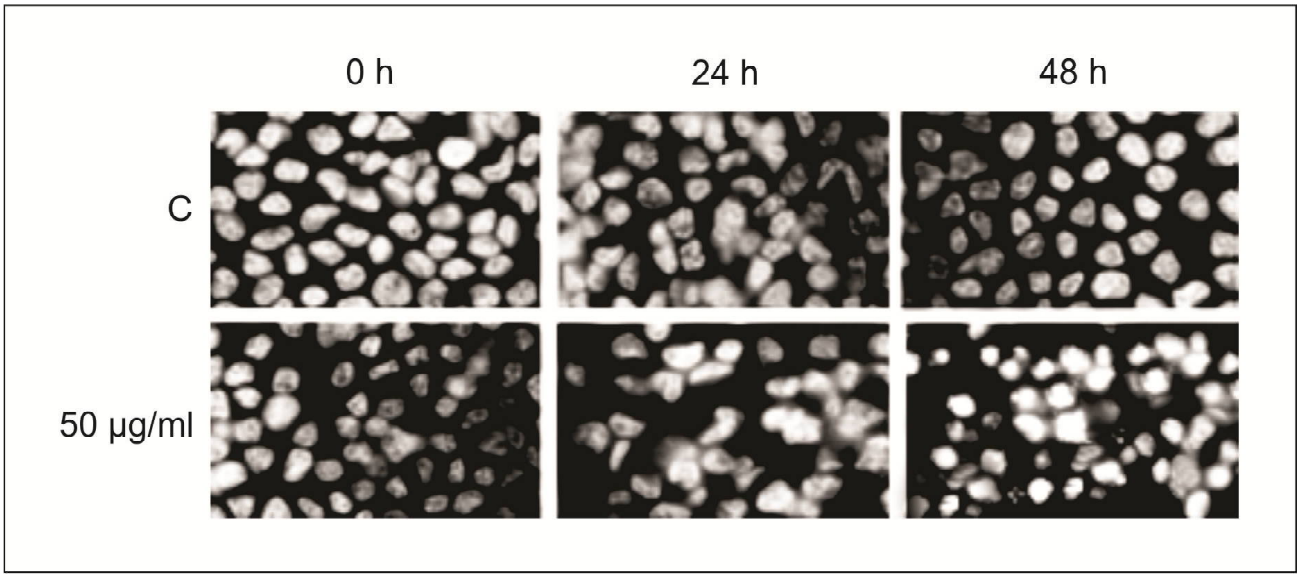

Fig. 6. Apoptosis assay using Hoechst 33342 staining of HeLa cell lines (50 µg/ml) C- control against durations.

substantiate the apoptosis, DNA ladder a hallmark assay has been carried. DNA fragmentation is the final reaction of apoptosis, and this explains the fragmentation event between the nucleosomes and forms 100-3000 bp fragments. The SAPI treated HeLa cell's nucleus has showed laddering pattern compared to control. This fragmentation was significant from 24 h onwards in treated cells, but the

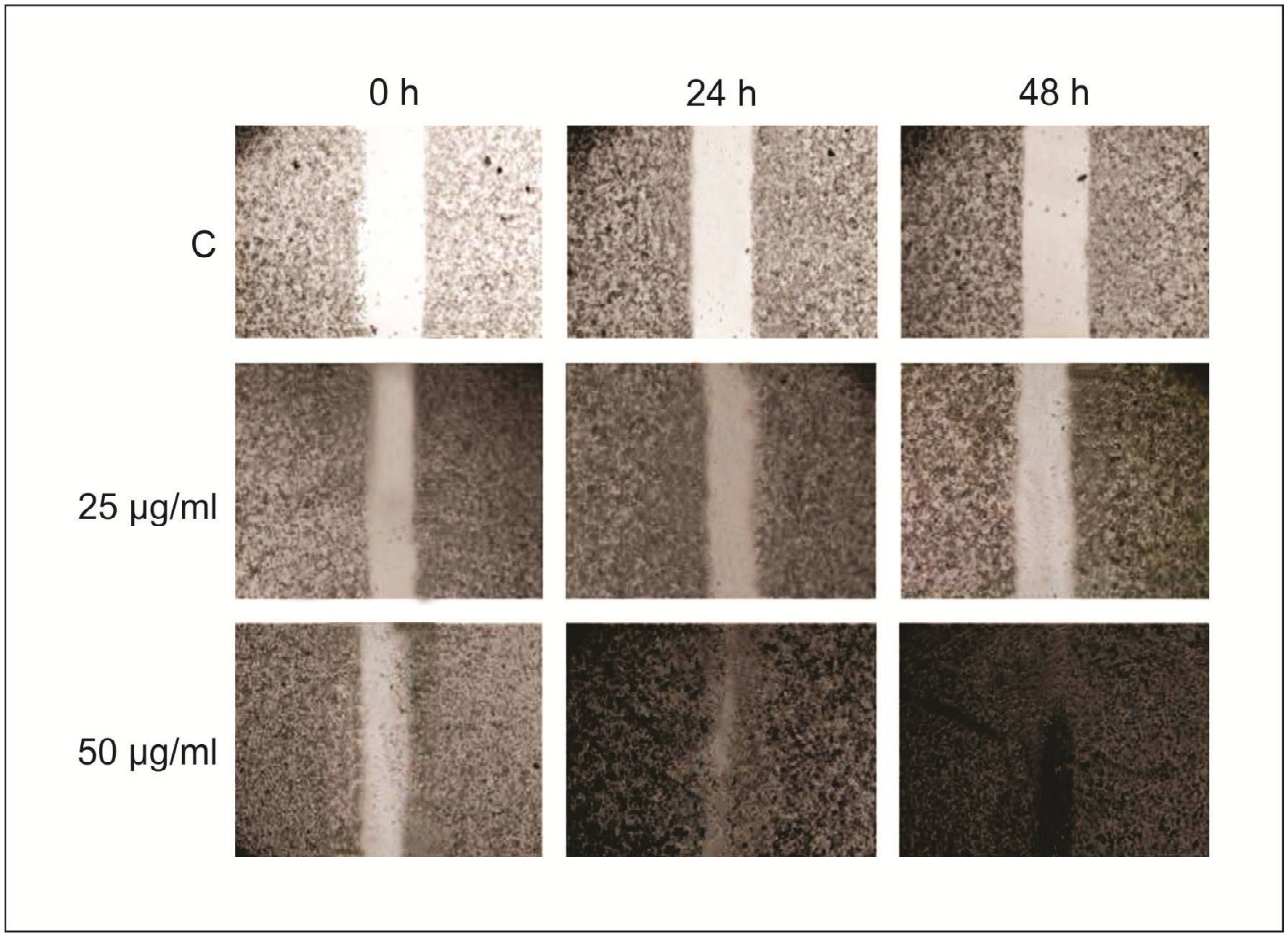

Fig.7. Effect of different concentrations of SAPI on migration of HeLa cells in control and treated groups at 0, 24, and 48 h

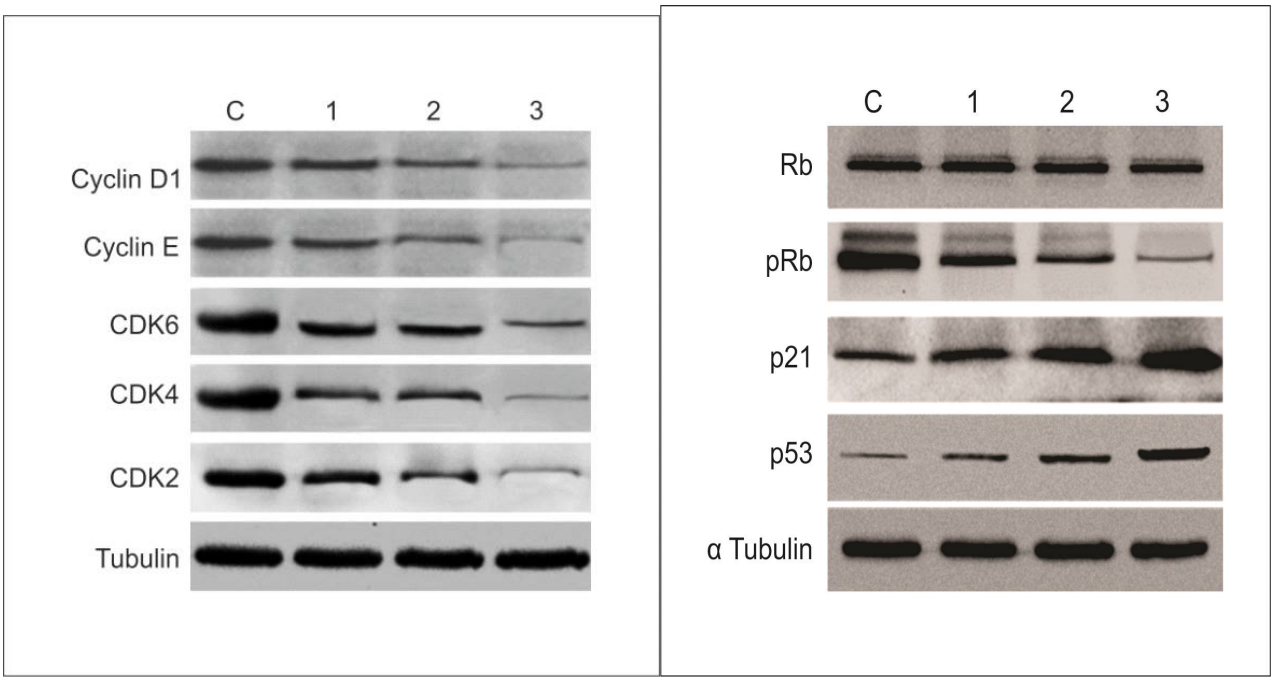

Fig.8. Effects of SAPI on expression of cyclin D1 E, CDK6, 4, 2, p21, p53 and RB, and phosphorylation of pRB in HeLa cells. Tubulin was used for normalization and verification of protein loading in Western blot analysis. Lanes 1, 2, 3, and 4 represent HeLa cells treated with 50 ìg/ml SAPI with durations 12, 24 and 48 h respectively.

control cells did not showed any symptom of DNA fragmentation (Fig.5). Hence, the present results confirm the apoptosis in HeLa cells by SAPI. Generally, DNA fragmentation was mostly as a consequence of microtubule targeted drugs and therefore, SAPI may be involved in disorganizing the microtubules in the DNA.

Generally, cell signaling pathways are regulated by various proteins while, deregulation of their activities can lead to various abnormalities, including cancer. Designing a drug which shows potentiality in regulating tumor progression and metastasis proteins is a positive approach. Many plant PIs have been reported as anti-proliferative, apoptotic inducing and chemopreventive agents against human cancer cell lines. The mechanism involved in the cytotoxic activity is found to be via up regulation of caspase-3, 9, mitochondrial membrane potential leads to increased DNA fragmentation (Li et al., 2009). This was further supported by anti-cancer activity of plant PIs, viz., Bowman-Birk TI from Hokkaido large soybean against breast cancer (MCF-7) and Hepatoma (HepG2) cell lines (Ho and Ng, 2008; Ye and Ng, 2009). and TI from *Fagopyrum esculentum* (buckwheat) against human T-acute lymphoblastic leukemia, HeLa cells and chronic myeloid leukemia cells (Park and Obha, 2004; Wang et al., 2007). Similarly, Bowman-Birk trypsin and chymotrypsin inhibitor (BBI) from soybean inhibit M5067 ovarian sarcoma via increasing expression of tumor-suppressor molecule Cx43 (Suzuki et al., 2005). BBI also suppressed the proteasomal chymotrypsin like activity in MCF-7 breast cancer cells (Chen et al., 2005). A Kunitz type trypsin inhibitor (TI) from soybean could suppress the invasion and metastasis of HRA ovarian cancer cells through blocking urokinase upregulation (Kobayashi et al., 2004). Islamov and Fursov (2007) studied the cytotoxicity of trypsin inhibitor from wheat seeds by counting the cells at the time of harvest. Relative increase in cell counts in terms of cytotoxicity i.e., 91% (50 µg/ml) was reported with trypsin inhibitors. Sen and Dutta (2012) evaluated the anti-proliferative activity of Ragi bifunctional inhibitor (RBI) on HeLa cells human chronic myeloid leukemia cells. Reduction of cellular proliferation and induction of apoptosis of HeLa cells by purified RBI was determined by MTT assay and flow cytometry analysis. The polyspecific proteinase inhibitor from *Enterolobium contortisiliquum* (EcTI) prevents proMMP activation leads to cytotoxicity against tumor cells without affecting normal tissue remodeling fibroblasts or regenerative hMSCs cells (Nakahata et al., 2011). Further, TIs from *Peltophorum dubium* and *Ipomoea batatas* also showed their potential in

Table 1. Percentage of viability of MG63, Ht29 and HeLa cell lines against SAPI treatment

Concentration (µg/ml)	Viability (%)		
	MG63	HT29	HeLa
5	87.45	97.96	58.47
10	85.97	85.27	56.02
25	64.29	81.02	47.05
50	57.83	65.20	40.54
100	51.27	59.32	39.22

cancer prevention and therapy on rat lymphoma cells (Troncoso et al., 2007) and NB4 promyelocytic leukemia cells (Zhou et al., 2007). A novel Kunitz-type serine proteinase inhibitor, termed PIVL, purified from the venom of the Tunisian snake *Macrovipera lebetina transmediterranea* specifically inhibiting trypsin activity and also integrin inhibitory activity without being cytotoxic on human glioblastoma U87 cells (Morjen et al., 2013).

SAPI induces cell cycle arrest in the G0/G1 phase. After HeLa cells were treated with 50 ìg SAPI, the percentage of cells in the G0/G1 phase increased significantly meanwhile, S-phase cells showed a marginal increase. The % of G2/M-phase cells decreased remarkably (Fig.4). This finding suggests that cell cycle distribution was blocked significantly in the G0/G1 phase when HeLa cells are treated with SAPI.

Apoptotic analysis by Hoechst 33258

To investigate the cell death, the HeLa cells were pre-treated with the SAPI. SAPI induced cell death in a duration dependent manner (Fig.6). The apoptotic percentages of cells treated with 50 ìg/ml SAPI was 80.3±1.4% at 48 h compared to 24 h (46.8±3.4). Staining of nuclei with the DNA fluorochrome Hoechst 33258 showed nuclear condensation of DNA and fragmentation of nuclei into apoptotic bodies (Fig.6).

Wound scratch assay

SAPI treatment inhibited migration of HeLa cells (Fig.7) at 24 and 48 h as determined by scratch wound assay. The results strongly suggest that SAPI has the potential to inhibit migration of cervical cancer cells. Migration is one of a hallmark of the metastatic characteristics of cancer cell lines. Thus, agents which inhibit migration of cancer cells could be used for the prevention and treatment of metastatic cancer. The present dose of SAPI significantly ($p < 0.05$) inhibits migration of cervical cancer cells, which indicate that SAPI could be an effective agent to inhibit HeLa cervical cancer cell metastasis.

SAPI regulates the expression of cell cycle-related proteins in HeLa cells

To unravel the molecular mode of cell cycle arrest in the G0/G1 phase, effects induced by SAPI associated with the level of G1-S transition-related protein expression were analyzed. Duration dependent decrease in cyclin D1 and E expression was seen in HeLa cells treated with 50 µg/ml of SAPI and was accompanied by a reduction in the amount of CDK6, 4 and 2 expressions (Fig.8).

To analyze the effect of SAPI on the phosphorylation of pRB, HeLa cells were treated with 50 µg/ml of SAPI, subsequently, proteins were analyzed using antibodies specific to the total pRB and phosphorylated pRB from 12 to 48 h duration of treatment. Expression of total RB remains almost constant while, the level of phosphorylated pRB was down regulated in a time dependent manner (Fig.8). p21, the CDK inhibitor, can block cell cycle and thereby cell proliferation (Li et al., 2012). p21 binds to cyclin E-CDK2 complexes, which leads to pRB hypophosphorylation and cell cycle arrest at the G1-S transition stage. Further analysis revealed that SAPI could induce a duration dependent increase in p21 in HeLa cells (Fig.8). Consistent with the change in p21, the p53 protein was upregulated, which suggests that SAPI induces the expression of p21 in a p53-dependent manner in the cells (Fig.8).

In the present study, it is noticed that cyclin D1, E CDK6, 4 and 2 are significantly down regulated in HeLa cells after SAPI treatment. This finding suggests that inhibition of cyclin D1, E and CDK6, 4 and 2 expressions are involved in SAPI-induced G0/G1 arrest in HeLa cells. During G1-phase progression, pRB is phosphorylated by cyclin D-CDK4, CDK6, and cyclin E-CDK2 complexes. Hyperphosphorylation of pRB inactivates its function and dissociates the E2F transcription factor from pRB, which is critical to progression to the S phase.

In addition, the expression level of RB remains constant in SAPI-treated HeLa cells, whereas the level of phosphorylated pRB decreases significantly, indicating that SAPI can suppress pRB phosphorylation. Thus, hypophosphorylated pRB combines E2Fs more tightly, induces cell cycle arrest,

and prevents proliferation. CDK activity is regulated negatively by a group of proteins called CDK inhibitors, including the protein p21 WAF1/CIP1 (p21). p21 protein binds to and inhibits the activity of cyclin E-CDK2 complexes, which causes pRB hypophosphorylation and cell cycle arrest in the G1–S transition. Expression of the p21 gene is tightly controlled by the tumor suppressor p53. The results of the present study show that SAPI treatment significantly upregulates the expression of p21 in HeLa cells. Consistent with the change in p21, the expression of p53 protein is also elevated, which suggests that SAPI may induce the expression of p21 in a p53-dependent manner in HeLa cells.

Migration and invasion of cancer cells into the extracellular matrix is mediated by cell surface related proteolytic enzymes like matrix metalloproteinases, cysteine proteases - cathepsins B and L, aspartic protease cathepsin D, and serine proteases - plasmin and urokinase plasminogen activator i.e., inhibition of protease activity by low molecular mass inhibitors are promising tool for anticancer and antimetastatic therapy (Jedinak and Maliar, 2005).

CONCLUSION

The results obtained from the *in-vitro* studies performed using the HT29, Mg63 and HeLa malignant cell lines reveals that the SAPI has variable levels of antiproliferative potentials. Moreover SAPI shows potent growth inhibitory effects against HeLa cells by inducing apoptosis through altering the cell cycle phase in the tumour cells. HDAC activity is a leading component induce cell cycle arrest in the G0/G1 phase and provide a mechanistic framework for further exploring the use of SAPI as a novel antitumor agent. Cyclin and CDK proteins expression were substantiating the antitumour potential of SAPI. Thus, it is evident that PIs can be used as drugs to treat tumours. Many new protease inhibitors are currently under clinical investigation leading to new generation therapy based on protease inhibition.

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Soil Nutrient Analysis from the Germplasm Conservation Sites of Ochlandra Species at TBGRI, Palode, Thiruvananthapuram.

S.A.NISHA* AND R. SANTHOSHKUMAR

Dept. of Botany, Mahatma Gandhi College, Thiruvananthapuram, Kerala.
**email : nishasa2014@gmail.com*

ABSTRACT

In this study ten elements essential for the plant growth were analysed in the soil samples of the germplasm conservation sites of *Ochlandra travancorica* (BAM/758) and *Ochlandra wightii* (BAM/727) from TBGRI Palode, Thiruvananthapuram District, Kerala. Soil consists of Primary and Secondary elements. P and K are the primary nutrients that plants require in large amount. Ca, Mg, and S are the three secondary elements required in smaller amounts. The micronutrients B, Cu, Fe, Mn and Zn were present in very small amount in the soil but they are as important as the primary nutrients. The experimental results in the soil samples revealed the following:, K (123.2 and 190.4(Kg/ha)), (187 and123 (Kg/ha)), S (9.12 and 5.61 ppm), Ca (231.5and 224.5 ppm), Mg (16 and 22.5 ppm), Zn (1.42 and 0.47 ppm), Fe (47 and 55 ppm), Mn (10 and 10 ppm),Cu(0.01 and 0 ppm) and B(0.43 and 0.92 ppm) from the sites of *O.wightii* and *O.travancorica* respectively. Further analysis of the soil physical parameters revealed a pH of 5 and 4.86 and electrical conductivity of 0.092 and 0.072 respectively for *O.wightii* and *O.travancorica*. Organic carbon in the samples was 1.62% and 1.91% respectively. Soil analysis is a valuable tool in nutrient management. It enables prediction and determination of proper amounts of nutrients in a given soil based on its inherent fertility and crop needs.

Keywords *Soil, Macro nutrients, Micro nutrients, Ochlandra*

Plants require sixteen elements for normal growth and out of it Potassium, Phosphorous and Nitrogen are considered as primary nutrients. Calcium, Magnesium and Sulphur are secondary elements needed in smaller amounts. Boron, Copper, Chlorine, Iron, Manganese, Molybdenum and Zinc are the seven essential micronutrients present in small quantities both in plants and soil. Soil contain large amount of the nutrients but plants use only a limited amount of the nutrients for their growth. . The macronutrients limit or co- limit the plant growth and alters the nutrient limitation on land [(2)]. *Ochlandra,* the reed bamboos are thin, tall shrubby grass that provide non-timber resources which support the economically weaker sections of the society. *Ochlandra* spp. is excellent soil binders in hilly zones of Kerala [(1)]. The reed bamboos play an important role in increasing the fertility and conservation of soil [(3)].

METHODOLOGY

The study was conducted in TBGRI, Palode, Thiruvananthapuram district in Kerala (8.7098° N, 77.1291° E). In TBGRI, the germplasm conservation of different bamboo types is done. This study focussed on the germplasm conservation of *Ochlandra travancorica* and *Ochlandra wightii*. Different spots of soil sampling were done near to the species *O.tavancorica* and *O.wightii*. The smeared soil from the surface was scraped and removed. It was done to minimise the effect of contamination from other areas and sources. The soil sample was collected by making boreholes from the shallow subsurface intervals of 30 cm. Two- five samples were randomly selected. The soil from these spots was thoroughly mixed. About 500gms of the soil samples were collected by removing roots and other materials manually and were stored in a polythene container. These samples were air-dried and were sealed.

Soil samples were collected in the month of October 2017 and the physical and chemical properties were assessed. Analysis of the soil sample includes pH (pH meter), Organic carbon (Walkley Black wet digestion method, 1947), Electrical conductivity (Conductivity meter), Available macronutrients like Phosphorous (Bray and Kurtz, 1945), Pottasium (Ammonium acetate extraction method, Flame Photometer), Calcium and Magnesium(Ammonium acetate extraction method, Atomic Absorbtion Spectroscopy) Sulphur(Barium chloride turbidimetric method, Spectrophotometer) and Available micronutrients like Iron, Manganese, Zinc and Copper(Atomic Absorbtion Spectroscopy) and Boron (Hot water extract) [(3).]This data is used to analyse and compare the nutrient status of soil and plant.

RESULTS AND DISCUSSION

In this study ten elements essential for the plant growth were analysed from the soil samples collected from germplasm conservation, TBGRI, Palode, Thiruvananthapuram District, Kerala. The analysis

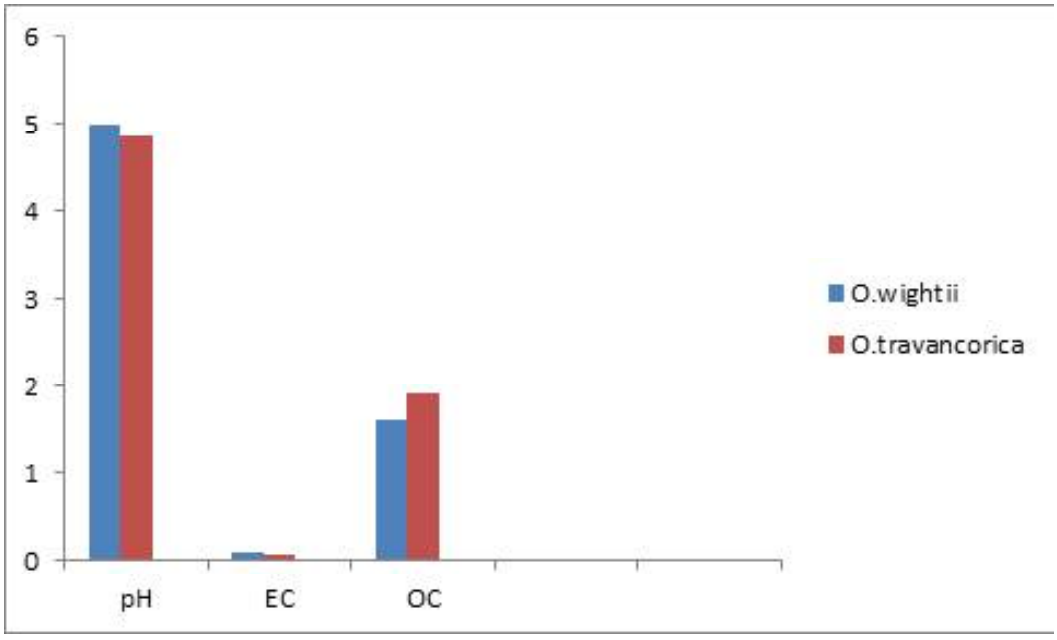
primarily interprets the pH, Electrical conductivity and Organic carbon of the sample and also the control. It focuses on the Primary plant nutrients like Phosphorous and Potassium; Secondary plant nutrients Calcium, Magnesium and Sulphur; Plant micronutrients like Iron, Manganese, Zinc, Copper and Boron.

The result of the soil sample collected during the survey studies at a depth of 30cm shows that the sample was brown in colour. Soil sample collected from the sites of Ochlandra, The basic physical characteristics at each site were recorded based on standard procedures. The values of soil pH in the germ plasm conservation sites for *O.wightii* was 5 and for *O. travancorica* was 4.86 indicating an acidic nature of soil. The EC values were 0.092 and 0.072 respectively. From the pH values, it shows that the plant nutrients within these sites are optimum for proper growth. The normal EC ranges from 0.02 to 2.0 mS/cm. Hence the soil in germplasm conservation sites is non- saline in nature. The low values in EC might be mainly due to the washing of the cations from the soil by rain.

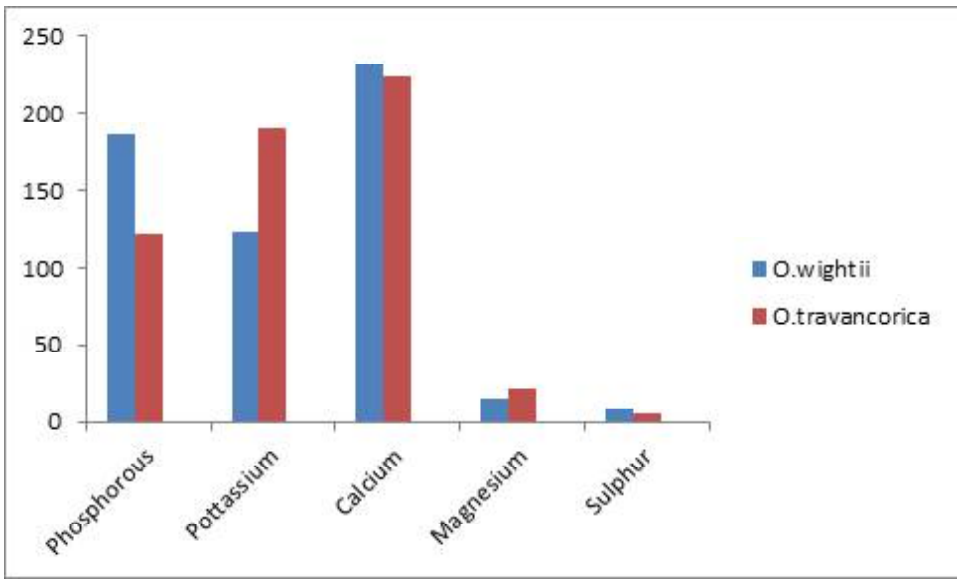
pH is more acidic where weathering and rainfall is more advanced. Soil pH when less than 5.5, it influences the nutrient availability and may result in Calcium (Ca), Magnesium (Mg) and Phosphorus (P) deficiency or excess of manganese (Mn) and iron (Fe). Micronutrients are most available in acid soils and often unavailable at high pH. All physical, chemical and biological soil properties are affected by pH.[1]. Electrical conductivity in soil water system of *O.wightii* was 0.092 and *O.travancorica* was 0.072, is a measure of concentration of soluble salts and extent of salinity in the soil. It was found to be below 1 which is harmless. EC for non- saline soil is 0 - 1.4 mS/cm while for strongly saline it ranges between 4.5 - 11.4 mS/cm. The low values for EC may be due to high rainfall in this area which washes out soluble cations from the soils.

Soil organic matter has been defined as the organic fraction of soil including plant, animal and microbial residues, fresh and at all stages of decomposition and the relatively resistant soil humus. However, soil organic matter estimate includes only those organic materials that accompany soil particles through a 2mm sieve. Carbon is the chief element present in the soil organic matter and forms 48-58% of the total weight. The values for Organic carbon in the sample of *O.wightii* are 1.62 and that of *O.travancorica* is 1.91, which are below 2%. Hence the soil analysed are deficient in organic matter and in Nitrogen also. Organic carbon determinations are often used as a basis for estimation of organic matter. The above result clearly indicates that the soil is highly acidic in nature and the range of electrical conductivity explains about the limited salinity in the soil sample. The presence of organic carbon is supposed to be the indicator of available Nitrogen status of the soil.

pH and organic carbon content are highly responsible for the degree of availability of micronutrients to plants. Calcium is essential, not only to correct soil acidity but also as a nutrient element



Graph 1. Results of pH, Electrical conductivity and Organic Carbon

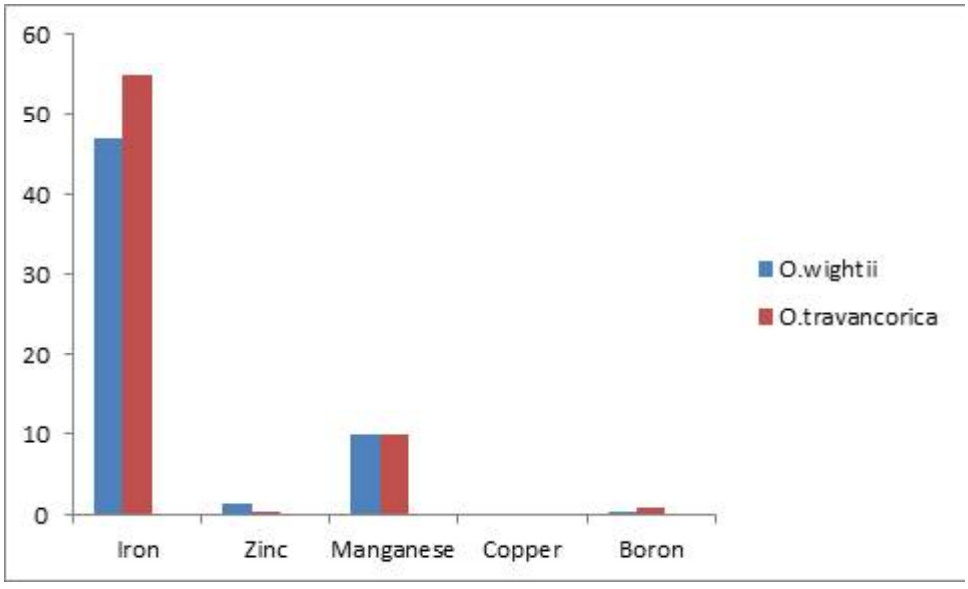


Graph 2. Results of Macronutrient analysis of soil

necessary for normal plant growth. Hence, soil Ca plays an essential role in regulating soil pH.

The Phosphorous level in both the soil samples is in excess when compared with the standard of 20-40ppm.Potassium undergoes reaction with Calcium and Magnesium and it affects the plant available potassium. The availability of potassium in the soil near to *O.wightii* was 123.2 and that of *O.travancorica* was 190.4, which shows a decline in fertility level. Calcium, Magnesium and Sulphur are considered secondary because they are essential for crop development but the uptake is usually lower than for the primary nutrients, Phosphorous and Potassium, but considerably higher than the micronutrients. Calcium and Magnesium have similar chemical properties in soil. The fertility level of both these nutrients in *O.wightii* was 231.5 and 224.5 ppm whereas in *O.travacorica*, it was 16 and 22.5 respectively. If Ca:Mg ratios are greater than 8:1, there is a chance for Magnesium deficiency. When the concentration of Mg is greater than twice those of Ca, an unfavorable plant growth and poor soil structure may occur. Sulphur containing soil generally lowers the pH. The presence of Sulphur in the soil sample of *O.wightii* was 9.12 and that of *O.travancorica* was 5.61.

Micronutrients are required in smaller amounts



Graph 3. Results of micronutrient analysis of soil

than macronutrients or secondary nutrients. Copper, Iron, Manganese and Zinc exit as minerals adsorbed to the soil particles. The presence of organic matter increases the availability of micronutrients to the plants but high carbon ratio may temporarily immobilize these nutrients until carbon decomposes. Zinc and Boron deficiencies may occur in acidic soils where leaching with low salt irrigation water occurs. Copper was almost absent in both the soil samples. Hence, indicating that the samples were deficient in copper. The optimum range for Copper is 0.2 to 2.5 ppm. The presence of Iron in the soil sample was 47ppm for *O.wightii* and 55 ppm for *O.travancorica* which is very high as the normal range for Fe varies between 2.5 to 4.5 ppm. The soil samples were highly enriched in Manganese. The soil samples from the study area showed manganese content as 10ppm in both the cases. The normal Manganese content ranges from 1.02to 2.5 ppm. The soil samples were enriched in Zinc. It was analysed to be 1.42 ppm in *Ochlandra wightii* and 0.47 ppm in *O.travancorica* respectively. The normal range was between0.51 to 1.21ppm. The presence of Boron was comparatively higher. It was 0.43 ppm in *O.wightii* and 0.92 ppm in *O.travancorica* respectively.

Among the grass family, bamboo is the heavy nutrient feeder. The nutrient content of the soil is directly related to the growth and yield of the plant. The present study has analyzed different nutrients of bamboo growing soil from the germplasm conservation sites at TBGRI, Palode.. It is concluded that the pH of the study site was acidic and the soil was saline in nature. The soil lacked in organic carbon and Nitrogen. But were enriched in micronutrients like Zn, Mn, and Fe but deficient in Copper. It helps us to determine the need of various nutrients for the proper development of the bamboos.

The results of Soil Nutrient Analysis from the Germplasm Conservation sites of Ochlandra (BAM/758) and (BAM/727) species at TBGRI, Palode, Thiruvananthapuram.

CONCLUSION

Soil parameters help to access the physical and chemical properties of soil. The chemical properties help to analyse the plant nutrient status. Hence, the analysis of the soil helps to analyse the fertility of the soil and the productivity of the ecosystem concerned. Soil nutrient help to find out the concentration of plant nutrients and is the main indicator of site fertility. Increased soil acidity reduces the nutrient availability, decreases the rate of decomposition and alters the rate of microbial function. Hence, Soil analysis provides sufficient information about the nutrient availability related to plant growth.

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Anticancer potential of *Bidens biternata* (Lour.) Merr. & Sheriff – An Ethno Medicinal Plant of Wayanadu District of Kerala

PRADEESH S[1] & SWAPNA T. S[1*]

Department of Botany, NSS College, Pandalam, Kerala - 689 501.
[*]Department of Botany, University of Kerala, Thiruvananthapuram- 695 581, Kerala, India.
**email- swapnats@yahoo.com/pradeeshnair10@gmail.com*

ABSTRACT

***Bidens biternata* (Lour.) Merr. & Sheriff, belongs to the family Asteraceae, is an erect annual herb, up to 1 m height, a wide spread weed of cultivated areas. This plant is common, particularly in Western Ghats regions of Kerala state. It is used as a leafy vegetable by Paniya, Chetti, Kani and Kattunaayika tribes of Waynadu Districts in Kerala and also to cure hepatitis, cold, cough, dysentery, asthma, inflammation etc. The aim of this work is to evaluate anticancer potential of leaves of *B. Biternata* by using cell lines like Dalton's lymphoma Ascites cells (DLA) and Ehrlich Ascites Carcinoma cells (EAC). The result of the present analysis revealed that the ethno medicinal plant *B. biternata*, possess high anticancer effect against the cancer cell lines.**

Key words *Bidens biternata, DLA, EAC and anticancer potential.*

Cancer is a general term for uncontrolled growth of abnormal cells medically known as neoplasm characterized by autonomous growth of tissues and loss of differentiation. Tumor mass may metastasis and spread to other tissues and organs (Khanum and Khan, 2007). Cancer is one of the leading causes for mortality worldwide and the inadequacy of conventional chemotherapy to reduce mortality indicated that new approaches are critically needed. It is estimated that 7.6 million people died of cancer in 2007 worldwide and it is projected to increase to 11.5 million deaths by 2030 (www.inmu.utoyama.ac.jp/en). The low efficacy of current chemotherapy accompanied with severe adverse reactions has been driving an increasing number of patients towards alternative medicines. In the United States, half of all patients with cancer have tried complementary and alternative medicine (McCann, 1997).

Therefore, there is an urgent need to develop new anticancer agents with minimum side effects. From the earliest times, herbs have been prized for their pain-relieving and healing abilities and today we still rely largely on the curative properties of plants. According to World Health Organization (WHO), 80% of the people living in rural areas depend on medicinal herbs as primary healthcare system (Sakarkar and Deshmukh, 2011). The synthetic anticancer remedies are beyond the reach of common people because of cost factor.

Herbal medicines have a vital role in the prevention of cancer and medicinal herbs are commonly available and comparatively economical (Sakarkar and Deshmukh, 2011). Out of the 92 anticancer drugs approved between 1983 and 1994 for commercial use approximately 62% are directly related to natural origin. Plant derived natural products such as flavonoids, terpenoids, alkaloids, saponins, tannins, glycosides (Arasan *et al*., 2010) etc. has received considerable attention in recent years due to their diverse pharmacological properties including antioxidant and cancer chemo-preventive effects (Roja and Heble, 1994). Free-radical damage may lead to cancer and some natural products rich in antioxidants interact with these radicals and may prevent the damage caused by them (Muneerudeen *et al*., 2013).

B. biternata is an annual herb used as green leafy vegetable and eaten by tribes. *Bidens* grows in moist, shady and cool environment with an annual rainfall of 2,786 mm in laterite soil. The chemical constituent in *B. biternata* includes sodium, potassium, carbon, phosphorous, magnesium, manganese, copper, iron, aluminium, different proteolytic enzymes, cyanogenic glycosides and alkaloids (Pradeesh *et al*., 2012). The leaves of *B. Biternata* have been used in traditional medicine to treat hepatitis, inflammation, diarrhea, asthma etc. by tribes like Paniya, Chetti and

Kattunaayika of Waynadu district of Kerala. Though the plant and its extracts have been extensively used in the folklore medicine, information from organized search of published literature does not provide evidence for its antitumor activities. So the present study has been undertaken to investigate the anticancer potential of extract of *B. biternata*.

MATERIALS AND METHOD

Collection and Preparation of Sample

Leaves of *B. biternata* were collected fresh from Western Ghats of Kerala. These were shade dried, ground well using mechanical blender in to fine powder and transferred to airtight containers for future studies.

Extraction from plant parts

The fine powder was used for extraction by using methanol. Fifty gram of sample powder was covered with cotton cloth and kept into the soxhlet apparatus for distillation. Three hundred ml of methanol was taken into the round bottom flask and heated in a mantle for 8 hours at 70°C. After completing the process, extract was collected in beaker and was kept in oven at 37°C-40°C for evaporation. The crude concentrated extract was again weighed and used for further anticancer studies. The extract was used for the anticancer analysis by using DLA and EAC cells.

In vitro anticancer studies in methanol extract and isolated compound of *B. biternata* leaves

Anticancer effect of crude methanolic extract of *B. biternata* was studied by using Dalton's lymphoma Ascites cells (DLA) or Ehrlich Ascites Carcinoma (EAC) cells. The crude methanol extracts from *B. biternata* at high concentration damage the cells and make pores on the membrane through which Trypan blue enters. The damaged cells are stained with Trypan blue stain and can be distinguished from viable cells. Since live cells are excluded from staining, this method is also known as dye exclusion method (Prasanth *et al*., 2010).

Dalton's lymphoma Ascites cells (DLA) and Ehrlich Ascites Carcinoma (EAC)

Varying concentrations (100, 500 and 1000 µg/ml) of crude methanolic extract of *B. biternata* were prepared. The cancer cells were aspirated from peritoneal cavity of cancer bearing mice and were washed thrice with normal saline. The cell suspensions (1×10^6 DLA cells in 0.1 ml) were added to tubes containing various concentrations of test extracts (10, 20, 50, 100 and 200 µg/ml) and volume was made up to 1 ml using phosphate buffer saline (PBS). Control tube contained only cell suspension. The mixtures were incubated for 3 hours at 37ºC and were added with two drops of Trypan blue dye. Dead cells take up the blue colour of Trypan blue while live cells do not take up the dye. Further percentages of dead cells were evaluated by Trypan Blue Exclusion method. The numbers of stained and unstained cells were counted separately.

% Dead cells = [Number of Dead cells/Number of viable cells + Number of Dead cells] × 100

RESULTS AND DISCUSSION

The Ehrlich tumor cells are one of the rapidly growing carcinoma with very aggressive behaviour and are able to grow in almost all strains of mice. Ascetic tumor implantation promotes local inflammatory reactions leading to increase in vascular permeability and results in intense edema formation, cellular migration and progressive ascetic fluid formation. Ascetic fluid is essential for tumor growth, since it constitutes the direct nutritional source for tumor cells (Segura *et al*., 2000). The *in vitro* anticancer activity of *B. biternata* was assessed by the Trypan blue exclusion method (Sheeja *et al*., 1997). Reduction in the viable cell count and increased non-viable cancer cell count towards normal in tumor host suggest antitumor effect against EAC (Ehrlich Ascites Carcinoma) and DLA (Dalton's lymphoma Ascites cells) cells in mice (Bala *et al*., 2010). Cyclophosphamide is used as standard anticancer compound. The results obtained from anticancer study revealed that methanol extract of *B. biternata* leaves showed remarkable (dose dependent cytotoxicity) anticancer activity against both the test cell lines (DLA and EAC). Methanolic extract of *B. biternata* showed 50.51, 80.46, 92.26% cytotoxicity in EAC compared to DLA which showed 38.16, 75.32, 87.19% cytotoxicity at the concentration of 100, 500 and 1000 µg/ml. The result of *in vitro* anticancer study in DLA

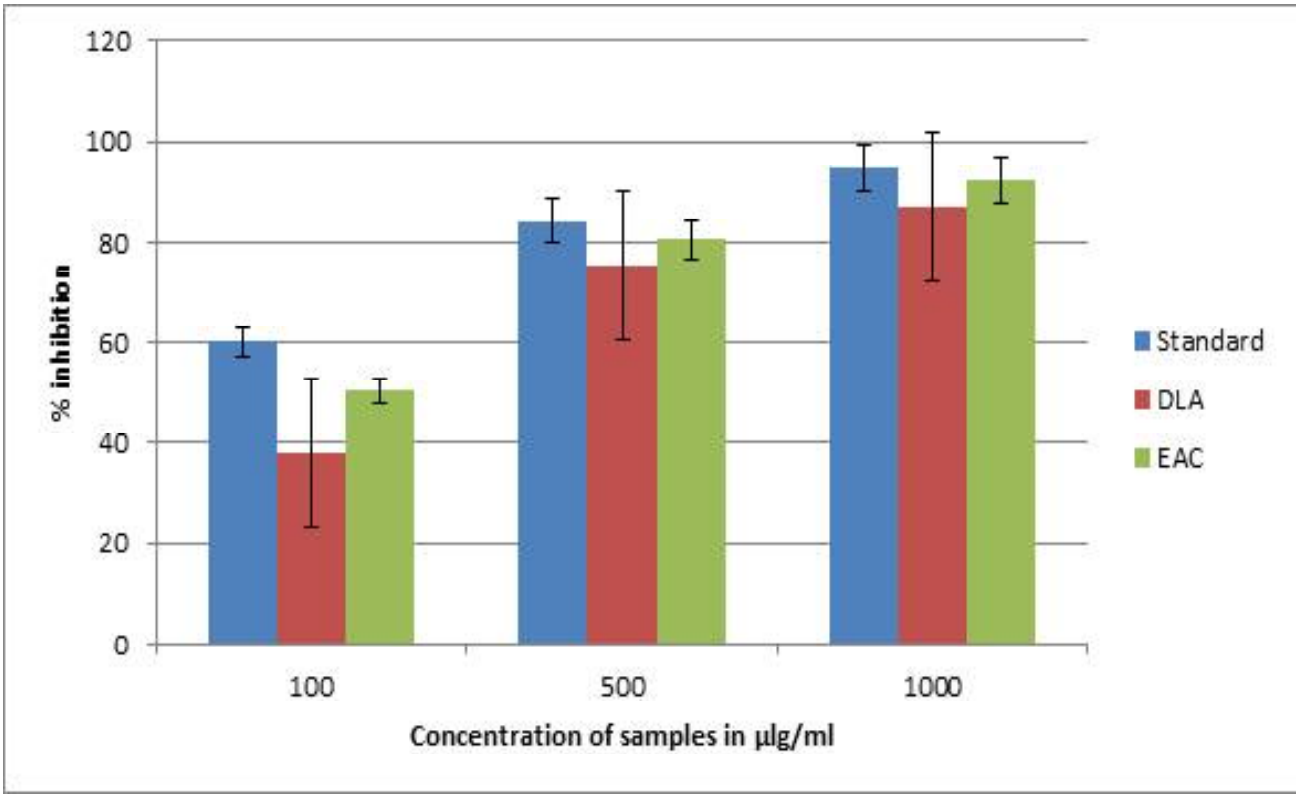

Fig. 1. *In vitro*anticancer potential of *B. biternata*

and EAC cells lines showed high activity with increasing the concentration of the extract such as 100, 500 and 1000 µg/ml of *B. biternata* as shown in Fig 1. This *in vitro* anticancer activity of methanol extract in both DLA and EAC cell lines is higher compared to standard (94.73%) and anticancer plants reported like *Tecomaria capensis* (DLA-62.58% and EAC-83.95%) belongs to Bignoniaceae (Tamil *et al*., 2013), *Bambusa bambos* (DLA-48% and EAC-60%) of Poaceae (Muneerudeen *et al*., 2013), *Desmodium triangulare* (DLA-61% and EAC-70%) of Papilionaceae (Jayaseelan *et al*., 2012) and *Euphorbia hirta* (DLA-38.5% and EAC-28.81%) of Euphorbiaceae (Anitha *et al*., 2014).

CONCLUSION

The present study provides strong evidence suggesting that *B. biternata* leaf methanol extract have *in vitro* anticancer activity against DLA and EAC cell lines. The plant extract was found to be effective against DLA induced solid tumor and EAC induced ascites tumor. This may be used to development of effective therapeutic approaches towards the prevention or treatments of various types of cancer in human beings.

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Structural Analysis of Mangrove Vegetation in Asramam, Kollam District, Kerala

N. RATHEESH[1], S. LEKSHMI[2] AND K.B. MANOJ[3]

[1]*Postgraduate Research Department of Botany and Biotechnology, Sree Narayana College, Kollam,*
[2] *Postgraduate Department of Botany and Biotechnology, Sree Narayana College Kollam*
[3]*Department Chemistry, Sree Narayana College, Cherthala*
email: ratianchal@gmail.com

ABSTRACT

A study was conducted on the structural analysis of the Mangroves in Asramam, Kollam District. A total of 6 mangrove species belonging to 4 families were enumerated. The study resulted dominance of *Sonneratia caseolaris* L, which distributed in all quadrates studied, followed by *Rhizophora apiculata.* The mangrove species *Avicennia officinalis* recorded lowest density. Maximum relative basal area was represented by *Sonneratia caseolaris* followed by *Rhizophora apiculata.* Hence these species registered the highest Importance Value Index. The true mangrove species are confined to salty-marshy environment along back waters, where as the mangrove associate species were also found in other areas apart from the mangrove environment. At present the mangrove continuity in this area are lost and are faced destruction. Urbanization, tourism development and chemical discharge are some of the major common threats that dwindle mangrove ecosystems in the study area. Conservation strategies are to be made to conserve the existing pristine ecosystem in the area.

Key words *diversity, abundance , Important Value Index,*

Mangrove forest is otherwise called mangrove community, mangrove ecosystem, tidal forest, etc. However they grow in conditions where no other plant species can survive (Hutchings and Saenger 1987). The mangrove environment is primarily saline and the muddy or sandy sediments home of variety of epibenthic, in faunal and mesofunal invertibrates. They are are highly productive ecosystems with an average production of 2,500 mg/ cm^2 per day (Bunt 1992). Due to their high primary productivity, turnover rates of organic matter and the permanent exchange with the terrestrial and marine ecosystems mangroves are of particular interest for the biogeochemical cycling of carbon and associated elements along tropical continental margins (Jennerjahn and Ittekkot 2002). Also these biological barrier reduces damaged caused by storms by limiting wave energy and preventing the land from being flooded. This has become even more apparent after 2004 Asian tsunami (Radhika 2006).

Kerala was once blessed with this amazing ecosystem (Basha 1991) but now going in a declined state. Various studies show that mangrove vegetation cover only 1905 ha in Kerala (Kurien, 1994). The distribution of mangrove in Kerala is discontinuous and patchy. Only Kannur district has good natural patches. There are approximately 755 hectare of mangrove forest. However it was reduced to 17km^2 (Kaladharan and Asokan 2012), followed by Kozhikode (293ha.) and Ernakulam (260 ha) (Mohan Kumar 1996).

The threats to the mangrove ecosystems could be broadly grouped into both natural as well as anthropogenic. The mangroves in the State are threatened with unprecedented destruction, which includes commercial exploitation of raw materials, land reclamation for agriculture, aquaculture and housing (Muraleedharan et al 2009).

In Kollam district Kerala, mangrove present in three places, namely Adventure Park Asramam, Munrothuruth islands and Kumbalam area. Asramam is the one of the most famous mangrove site in Kollam District and the mangrove spread was habitat to the highly endangered species of mangroves. The mangrove species present in the area are *Sonneratia caeseolaris, Rhizophora mucronata* and *Rhizophora apiculata.* In addition to being a major spawning ground for several edible marine species, the Asramam mangroves in the past was also home to otters and migratory birds and years ago this area contain thick and continuous mangrove without patches. Species like *Brugiera gymnorhiza* and species of *Rhizophora* are common in regions of Kollam district. But in the present scenario the disappearance of all this species along this coast. Now a days the continuity was lost and severe disruption and degradation due to developmental activities of tourism, real estate and pollutant discharges from various sources. In this ground the present study was undertaken to understand the distribution, diversity and structural analysis of mangrove ecosystem at Asramum, Kollam District and also aim to create an awareness on the declining mangrove species and its future conservation strategies.

MATERIALS AND METHODS

Study area

The present study conducted in the mangrove forest at Asramam area of Kollam District. Asramam located in the core of Kollam city in the State. The thick mangrove forest in the area is very popular all over the state. The Asramam banks of Astamudi Lake bordering the government guest house complex were a major mangrove forest belt of the state. But towards the mid 1980's a systematic destruction of mangrove was launched through a tourist development programme of State government. It paved for the creation of Adventure Park. At present major part of the mangroves are situated in Asramam Adventure Park. It was opened to public on 1980 with an area of 48 acers. So many endangering species like *Syzygium tranvencorium* and other trees are surviving there. Some of the old flora still survived there it include 250 year old *Hopea*. Years ago this area contain thick and continuous mangrove without patches but now a days the continuity was lost and due to the over exploitation and human interference.

METHODS

An area of 48 acres in the Asramam adventure park and near the link road portion was selected for detailed studies. Structural analytical studies of mangroves vegetation in Asramam was carried out during the months of March - June, 2016. The study was based on species area estimation and quadrant analysis (Michael, 1998). The quadrant size of 5x5m was fixed by species area curve method and altogether seven quadrates were selected randomly. The plant species and their individuals occurring in each quadrant were recorded. Basal area of mangrove species was measured 1.37m above the ground or highest prop root using tailoring tape. Approximate tree height should also measured. From the observations, the quantitative characters such as frequency, density, abundance, relative frequency, relative density, relative dominance, Importance Value Index (IVI) were calculated. Importance value index of each species was calculated as the sum of relative density, relative frequency and relative dominance (Ellison and Farnsworth 2001) so as to reveal relative contribution of each species to the overall stand composition. The vegetation data were analyzed to calculate the diversity indices and species richness, Shannon–Weiner diversity (H'), Simpson index and equitability were measured (Legendre and Legendre 2012). Species richness were measured (Margalef 1958). Frequency, density and abundance were calculated using following formulae:

$$\text{Frequency (F)} = \frac{\text{Number of quadrants in which the species present}}{\text{Total number of quadrates studied}} \times 100$$

$$\text{Abundance (Ab)} = \frac{\text{Number of individuals of the species in all quadrants}}{\text{Number of quadrants of occurrence of the species}}$$

$$\text{Density (D)} = \frac{\text{Number of individuals of the species in all quadrants}}{\text{Total number of quadrants studied}}$$

In addition to this relative frequency, relative density and relative dominance were calculated using following formulae:

$$\text{Relative density (RF)} = \frac{\text{Number of individuals of a species}}{\text{Total number of individuals}} \times 100$$

$$\text{Relative dominance (RD)} = \frac{\text{Total basal area of a species}}{\text{Basal area of all species}} \times 100$$

$$\text{Relative frequency} = \frac{\text{Frequency of a species}}{\text{Sum frequency of all species}} \times 100$$

Importance value Index (IVI) = Relative Density + Relative Dominance + Relative Frequency

RESULTS AND DISCUSSION

The present study results the occurrence of six true mangroves species (Table.1) belonging to 4 plant families (Avicenniaceae, Rhizophoraceae, Sonneratiaceae and Acanthaceae) and several mangrove associates in the study area (Table 2). The plant family Rhizophoraceae has three species- *Kandelia candal, Rhizophora apiculata* and *Bruguiera gymnorrhiza* (Table .3). Among this, *Rhizophora apiculata* showed highest abundance.

While species like *Sonneratia caseolaris, a* rare

Table 1. True mangrove flora of the study area at Asramam, Kollam

Sl.No	Species	Family	IUCN Status	Habit
1	*Acanthus ilicifolius* L.	Acanthaceae	Endangered	Sub shrub
2	*Avicennia officinalis* L.	Avicenniaceae	Endangered	Small tree
3	*Bruguiera sexangula*	Rhizophoraceae	Endangered	Shrub
4	*Kandelia kandal*	Rhizophoraceae	Least Concern	Small tree
5	*Rhizophora apiculata* Blume.	Rhizophoraceae	Vulnerable	Small tree
6	*Sonneratia caseolaris* L.	Sonneratiaceae	Threatened	Tree

and threatened species, present large quantity in Link Road area. It is listed critically endangered in IUCN Red list. During the course of survey, there were various types of habits like trees, shrubs and herbs were observed. One Pteridophyte species, *Acrostichum aureum* was noticed in the study area. This indicates fairly rich species diversity even present in a highly degraded condition. Years ago this area contain thick and continuous mangroves without patches and rich species diversity but the present study show that continuity was lost and patches are generated. Now mangrove growing area is restricted in link road portion and Asramam Adventure Park only.

In the present study structural features of the mangroves were studied. Structural analysis encompasses not only the study of vegetation and its internal social relationships, but also provides information on classifications of plant communities and their structure, composition, and succession relations. Importance Value Index of each species was calculated as the sum of relative density, relative frequency, relative dominance and relative basal area, so as to reveal relative contribution of each species to the overall stand composition. It was found that the highest IVI were recorded for *Rhizophora apiculata* (93.62). The lowest IVI were recorded for *Avicennia officinalis* (31.01). *Kandelia kandal* also a rare species its presence in Asramam Adventure Park, which is now included in IUCN Red list. *Avicennia officnalis* is one of the common species in Kerala but its occurrence in the present study areas are very few in number. *Bruguiera gymnorrhiza* is the another pure mangrove present in Asramam region.

Table 2. Mangrove associates from the study area

Sl no	Species	Family	Habit
1	*Acalypha indica*	Euphorbiaceae	Herb
2	*Acrostichum aureum*	Pteridaceae	Herb
3	*Acacia auriculiformis*	Fabaceae	Tree
4	*Aerva lanata*	Amaranthaceae	Herb
5	*Borhavia diffusa* L	Nyctaginaceae	Herb
6	*Clerodendrum inerme*	Verbenaceae	Shrub
7	*Crotalaria striata*	Fabaceae	Shrub
8	*Cyperus distans*	Cyperaceae	Herb
9	*Euphorbia geniculata*	Euphorbiaceae	Herb
10	*Euphorbia hirta*	Euphorbaceae	Herb
11	*Ficus religiosa*	Moraceae	Tree
12	*Hyptis suaveolens*	Lamiaceae	Shrub
13	*Ipomoes biloba.* Forssk.	Convolvulaceae	Creeping herb
14	*Mimosa pudica*	Mimosaceae	Herb
15	*Passiflora foetida*	Passifloraceae	Climbing shrub
16	*Tridax procumbens*	Asteraceae	Herb

Table 3. Frequency, Density, Abundance, Relative frequency, Important Value Index of true mangrove species in the study area

Species	Frequency (%)	Density (Individuals /m^2)	Abundance	Relative frequency (%)	Relative density (%)	Relative dominance (%)	Importance Value Index (IVI)
Acanthus ilicifolius L.	44.3	1.30	3.5	14.60	15.20	11.86	41.02
Avicennia officinalis	33.3	1.28	3	12.06	9.18	9.77	31.01
Brugaria gymnorrhiza	57.1	1.7	3	20.6	12.24	13.63	46.55
Kandelia kandal	57.1	1.28	2.24	20.68	9.18	9.18	37.95
Rhizophora apiculata	57.1	5.7	10	20.68	40.81	32.13	93.62
Sonneratia caseolaris	71	4	5.6	25.8	28.57	36.36	90.01

Density of various mangrove species present in the study area was much varied between them. Highest density was observed in *Rhizophora apiculata* (5.7 individuals/m^2) followed by *Sonneratia caseolaris* (4 individuals/m^2). On the other hand the *Kandelia candal* and *Avicennia officinalis* show same density value (1.28 individuals/m^2). The species *Bruguiera gymnorrhizae* showed with lowest density (1.7 individuals/m^2). While relative frequency of *Sonneratia caseolaris* (25.8%) is high in all regions. *Rhizophora apiculata Bruguiera gymnorrhizae* and *Kandelia candal* were showed same relative frequency (20.68%). Relative density of *Sonneratia caseolaris* is high in link road area. While in other areas *Rhizophora apiculata* (40.8%) showed highest.

Detailed investigation on the structural features of mangroves in Kerala and elsewhere studied and reported by various workers. Structural analysis of the mangrove communities at different estuarine formations revealed that there was a site specific domination of species which intern supported by adaptability of species to specific site conditions (Ewel and Bourgeois,1998). The present investigation also support this, of the several species present the *Rhizohora apiculata* shows the highest abundance. Ecosystem evaluation with respect to adaptation of species to the specific site may be attributed by physical, biological and edaphic factors (Ewel and Bourgeois,1998).

In a study at west coast in Ayiramthengu mangroves, Kollam. District, Kerala, twenty seven species belonging to 17 families has been reported (Jisha. *et al.,* 2004). Another study the mangrove flora of Ayiramthengu comprise of totally 9 mangrove species (Vishal, *et al.* 2015). In agreement with these in the present study at a small portion of the mangrove at Asramam it was observed six species.

In most of the estuaries of Kerala, the mangroves had been to large extent, converted to mixed silvi-agri-aqua cultural system and used as water ways, but were later destroyed for uses such as firewood, building materials and as a source of tannin. Quantitative structure of true mangroves was studied in terms of frequencies, density and abundance. Compared to Sundarbens of west Bengal, Kerala has very few area of mangrove forest. It was estimated that out of the 17km2 of mangroves of Kerala, a major share is present in Kannur district (Khaleel, 2005). Kollam District encompasses with highest extent of mangroves among Southern Districts. However, degradation and conversion of mangroves is profoundly experienced in many parts of Kollam especially Kayamkulam, Ashtamudi, Paravoor areas. Asramam is one of the most famous mangrove site in Kollam District had undergone severe disruption due to conversion and real estate activities. The most critically endangered species *Syzygium travancoricum* is found in very few numbers here. Similarly, *Lumnitzera racemosa*, which is one of the rare mangrove species in Kerala, has shown its restricted distribution in Asramam area of this District.

Now Asramam area consist of six species of mangrove coming under four families, of this *Sonneratia caseolaris* occur throughout the area but the number is limited compared to *Rhizophora apiculata*. The first three quadrant study shows the

Fig. 1. One of the polluted areas in the study site at Asramam, Kollam

complete absence of *Rhizophora apiculata.* The rare species *Kandelia candel* is occurring in this area. Usually this species is occurring in Malabar region.

At present the species diversity in Asramam is drastically decreasing due to human activities such as cutting of mangroves, dumping of hospitals and house hold wastes, tourism development, improper planning of developmental activities such as aquaculture, agriculture, construction of human inhabitants, mining and industrialization. Another major problem is by the difficulties of protection because of scattered geographic distribution of mangroves. Years ago this area has rich diversity of mangrove and mangrove associates, and also it was a major place for migratory birds.

Compared to other mangrove forest this area has only limited species diversity. The study shows that this area has six species it included in three family and continuity of mangrove forest was lost and now it appears as patches. Asramam is one of the famous tourist center of Kollam District because of the presence of adventure park, house boating, etc. Its beneficial to our economy but harmfully to our environment because the area is modified for the tourism development. Another important fact that pollution is one of the major threats to mangrove ecosystem (Basha, 1991). Polluted water affects the growth and multiplication of micro flora and fauna of Ashtamudi lake and also affect the mangroves. Due to the dumping of hospital waste, house hold waste, plastics, etc. will destroy the physical and chemical characteristics of Ashtamudi lake, due to all these human activities now this area look like a waste land (Fig 1).

We need to adopt certain strategies for conservation and management of mangrove ecosystems in the Asramam. Some of the conservation strategies proposed are; identification of potential mangrove areas for declaration as national park or sanctuaries, restoration of degraded and critical mangrove areas by planting suitable species, identification of endangered mangrove species and full protection for their rehabilitation, checking encroachment destruction and reclamation of mangrove areas and raising awareness among the public on the importance of mangrove and the need of their preservation.

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Effect of Nodal Position and Seasonal Variation on *In Vitro* Regeneration of *Gynochthodes ridsdalei* Razafim. & B. Bremer an Endangered Medicinal Plant Species of Southern Western Ghats

RENJI R. NAIR AND A. GANGAPRASAD*

Plant Tissue Culture and Molecular Biology Lab,
Department of Botany, University of Kerala,
Thiruvananthapuram, Kerala, India, 695581
**email: agangaprasad@yahoo.com*

ABSTRACT

An efficient *in vitro* propagation protocol for the influence of various seasons on different nodal positions of *Gynochthodes ridsdalei* (Rubiaceae) an endemic, endangered medicinal plant species of southern Western Ghats was carried out. To investigate the influence of nodal positions, nodal segments (0.5 – 1.0 cm) of the first visible node from the shoot tip to 5th nodes of the young shoot cutting were collected during the four different seasons were used for culture initiation and shoot multiplication.Murashige and Skoog (MS) medium fortified with varying concentrations of benzyl amino purine (BA)was used for shoot multiplication and differentconcentrations of indole 3 acetic acid (IAA) was used for *in vitro* rooting were used. Among the different concentrations of BA used 1.5mg/l gave maximum number of multiple shoots of 6.90 at summer season from the 3rdnode followed by 5.20 shoots in presence of 2 mg/l BA from 3rd nodal position. The explant collected during the summer season gave maximum response (86%) and low percentage of contamination (14%) followed by the winter season (78%) and least response was during the monsoon season (51%). Micro shoots were rooted in half strength MS medium supplemented with IAA (1.5mg/l) and produced an average of 15.30 roots/micro shoot within six weeks of culture.

Keywords *Micropropagation, Gynochthodes ridsdalei, endemic, endangered*

Gynochthodes ridsdalei Razafim & B. Bremer (Syn: *Morinda reticulata*) is a medicinal woody climbing species belonging to the family Rubiaceae (Fig:1). *G.ridsdalei* is an endemic, endangered species of southern Western Ghats (Mohanan & Sivadasan, 2002). It forms an important component in herbal formulation of the traditional medicine (Zhang *et al*., 2013). Due to habitat destruction, poor seed germination and seed dormancy lead to the declaration of *G. ridsdalei* as an endangered species by International Union for Conservation of Nature (Gopalan & Henry, 2000). The genus *Gynochthodes* (Syn: *Morinda*) are known to have medicinal properties due to the presence of substantial amount of anthraquinone especially in roots (Han *et al*., 2001). Propagation of medicinal plants through seeds has many limitations such as poor germination rate, seed dormancy etc. (Venkataramiah *etal*., 1980, Chand & Singh, 2004). Thus conventional propagation through seeds and vegetative cuttings is not an adequate solution to meet the demand for this endangered plant.These difficulties could be overcome by using the *in vitro* propagation system. The developed protocol could be used for the conservation and further studies in this medicinally important plant.

MATERIALS AND METHODS

Wild grown plants of *G. ridsdalei* were collected from the forest areas of Ponmudi region in the southern Western Ghats of Kerala, India. The collected plants were maintained in clay pots in the green house of Department of Botany, University of Kerala which were used as the source of explant for the present study. The taxonomical identification of the plant was done using authentic literature (Ijinu *et al*., 2011, Razafimandimbison & Bremer,2011, Sasidharan, 2004). A Voucher specimen was deposited in the Herbarium of Department of Botany, University of Kerala, Kariavattom (KUBH No. 8095).

Explant selection and surface sterilization

Young top cuttings consists of 1-5 visible nodes from the shoot tip were excised from mother plant during four different seasons. To study the influence of seasonal variation on percentage response of nodal explants, the explants were collected during different seasons viz. summer (April- May), monsoon (June-July), post monsoon (September- October) and winter season (December- January). The top cuttings with 5 visible nodes were defoliated and washed in 10 % (v/v) labolene (Qualigens, India) for 15 minutes and then in running tap water for 30 minutes. Surface sterilization of the cuttings involved immersion of 0.1% $HgCl_2$ for 5 minutes followed by 3-4 rinses in sterile distilled water to remove the trace of the sterilants. To investigate the influence of nodal position, nodal

Fig. 1. Habit of *G.ridsdalei*

segments (0.5- 1 cm) of the first visible node from the shoot tip to 5th nodes of the surface sterilized shoot cultures were dissected out and transferred separately into MS agar medium containing different concentration of benzyl amino purine for culture iniyiation and multiplication. All the operations were carried out inside the laminar air flow hood (Klenzaids, India).

Culture Media and conditions

Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) containing 3 % (w/v) sucrose (SRL, Mumbai) which served as the carbon source. Full strength MS medium was used for shoot multiplication and half strength MS medium was used for *in vitro* rooting. Plant growth regulators used were Benzyl adenine (BA- 0.5-3mg/l)for shooting and Indole 3-acetic acid (IAA- 0.5-3 mg/l)) for rooting at different concentrations were augmented with basal media. The pH of the medium was adjusted to 5.8 with 0.1N NaOH or0.1N HCl and gelled with 0.8% agar (w/v). Required volumes of the media were dispensed (15 ml) into culture tubes (25mm x 150 mm) and autoclaved for 18 min at 121^{0}C and 1.1 kgmc^{2}pressure. MS medium without any plant growth regulator served as the control.Cultures were incubated in the culture room under 8/16 h photoperiod at a light intensityof 3000lux providedby cool, white, fluorescent tubes (Philips, India

Fig. 2. Initiation of axillary bud from 3rd node

Ltd., Mumbai). The temperature and relative humidity of the culture room were maintained at 25 ± 2 ˚C and 50- 60% RH respectively.

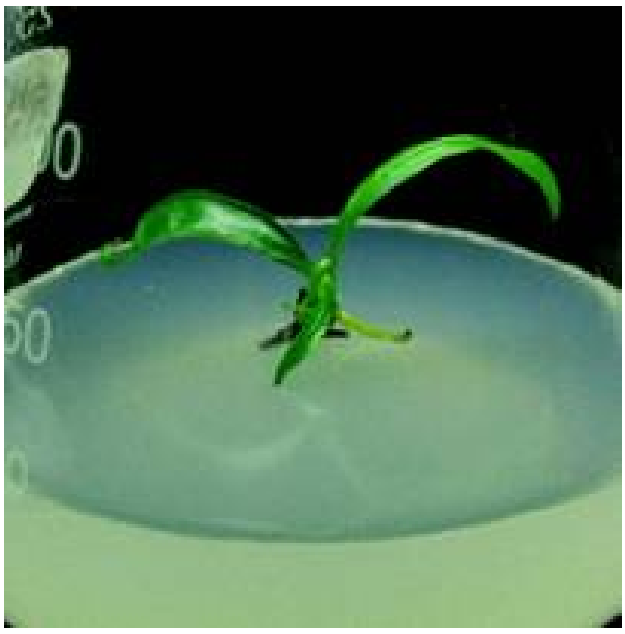

Fig. 3. Shoot formation from 1.5mg/l BA

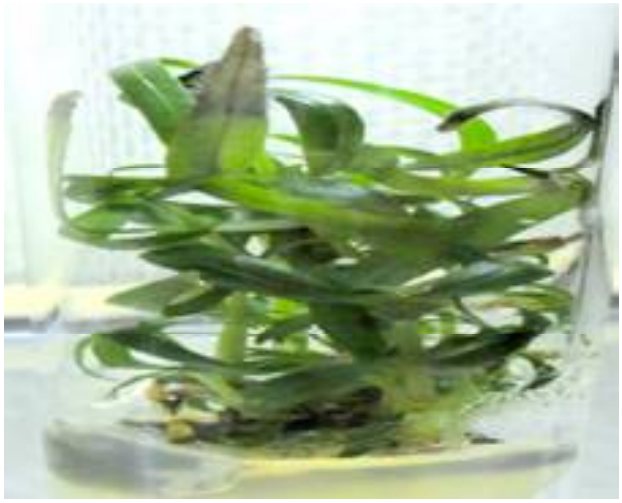

Fig. 4. Multiple shoot formation from nodal explant collected during summer season

Effect of nodal position and seasonal variation on shoot multiplication

Surface sterilized nodes (0.5- 1cm) were inoculated into MS medium supplemented with varying concentrations (0.5-3 mg/l) BA at different seasons.Seasonal effect of nodal explants on *in vitro* response were assessed. The responses of explants were recorded after 30 days of culture.

In vitro rooting and acclimatization

Shoots multiplied *in vitro* (3-4cm) having 3-4 nodes wereharvested and used for rooting. For*in vitro* rooting, individual shoots were transferred to full strength and half strength MS medium containing 3% (w/v) and 0.8% agar and supplemented with different concentrations (0.5- 3 mg/l) of root inducing growth regulator Indole 3 acetic acid (IAA),with induced

Fig. 5. Multiple shoot formation from nodal explant collected during winter season

Fig. 6. Multiple shoot formation from nodal explant collected during post monsoon season

concentrations. The cultures were incubated under 12 hr photoperiod at 25± 2°C for 45 days. After 45 days, plants with developed roots were harvested from the culture tubes and washed in tap water and planted onto plastic pots (6.7 x 7.1 cm) containing garden soil and sand (1:1) for acclimatization. The pots were covered with transparent polythene and frequent watering was carried out to maintain soil moisture. After one month, the polythene covers were removed and plants were transferred to plastic pots (16 x 16.5 cm) filled with garden soil, sand and cowdung (1:1:1) and kept in green house conditions. The pots were watered daily.

Fig. 7. Multiple shoot formation from nodal explant collected during monsoonseason

Fig. 8. Rooting in IAA

Fig. 9. Roots from 1.5mg/l IAA

Statistical Analysis

In all experiments, each treatment consisted of 10 replicates and each experiment was repeated 3 times.Data on various parameters were subjected to analysis of variance (one way ANOVA) to determine the levels of significance and mean separation was done using Duncan's Multiple Range Test ($p<0.05$).

RESULTS

Effect of nodal position and seasonal variation on shoot multiplication

Nodal explant excised during the four seasonal period of the year viz. summer (April - May), monsoon (June - July), post monsoon (September - October) and winter (December - January) showed that explant and season had significant influence on soot initiation, multiplication and percentage contamination of the nodal explants. Seasonal variation is an important factor that influences theaxillary bud proliferation as well as shoot multiplication and hold the major aspect of contamination free cultures. High percentage (86%)of morphogenesis and low percentage (14%) of contamination was observed when the nodal explants were collected during the month of April-May that is summer season over the other seasons (Table:1). Of the various position of the nodal explant excised from young shoot cuttings, the 3rd and 4th nodes responded better compared to 1st , 2nd and 5th nodes in all the seasons. The nodal explants collected during monsoon period (June- July) showed high rate of contamination. Nodal explants (3rd node) established in the summer season showed maximum number of multiple shoots (6.90) in presence of 1.5 mg/l BA followed by 5.20 shoots in presence of 2 mg/l BA from 3rd nodal position with maximum response of 86% (Table: 1) (Fig:2,3,4). Next to summer season winter season gave maximum multiple shoots that is 1.5 mg/l gave an average multiple shoots of 4.40 from the

Fig. 10. Established plants in pots

Table 1. Multiple shoot formation in *G. ridsdalei* from 1st to 5th nodal explants cultured in MS medium supplemented with different concentrations of BA collected during summer season

Conc (mg/l)	I Node	II Node	III Node	IV Node	V Node
0.5	2.40 ± 0.163^{ab}	2.70 ± 0.153^{c}	3.70 ± 0.153^{d}	3.40 ± 0.163^{c}	2.60 ± 0.258^{cd}
1	2.30 ± 0.153^{b}	3.60 ± 0.163^{ab}	4.70 ± 0.211^{c}	3.60 ± 0.306^{b}	3.60 ± 0.163^{ab}
1.5	2.90 ± 0.180^{a}	3.70 ± 0.153^{a}	**6.90 ± 0.180^{a}**	5.00 ± 0.211^{a}	4.20 ± 0.200^{a}
2	2.50 ± 0.167^{ab}	3.20 ± 0.200^{b}	5.20 ± 0.200^{b}	4.10 ± 0.233^{b}	3.30 ± 0.153^{b}
2.5	2.10 ± 0.169^{b}	2.70 ± 0.153^{c}	4.60 ± 0.211^{c}	3.50 ± 0.167^{bc}	2.50 ± 0.167^{cd}
3	2.45 ± 0.180^{ab}	2.30 ± 0.156^{c}	3.90 ± 0.200^{d}	3.30 ± 0.156^{c}	2.30 ± 0.146^{d}
(%) of response	71	75	86	79	73

Observation were made after 30days of culture

3rd node followed by 3.90 shoots from 2mg/l BA with maximum response of 78 % (Table: 4)(Fig:5). First, second, fourth and fifth nodes also developed multiple shoots but lesser when compared to 3rd node. Higher contamination rate (49%) was seen in monsoon season which is a factor for lesser response (51%) (Table:2)(Fig:7) followed by post monsoon season (68%) (Table:3) (Fig:6). Irrespective of the season maximum response and maximum number of multiple shoots were obtained from the 3rd node.

Rooting and acclimatization

Individual shoots dissected from the multiple shoot culture were transferred in full strength MS medium resulted in callusing only.Half strength MS medium supplemented with IAA (0.5- 3mg/l) produced multiple roots.Among different concentrations of IAA used, the plants rooted in presence of 1.5 mg/l IAA gave average multiple roots (15.30) with maximum length of 3.02 cm followed by 1 mg/l IAA gave 14.40 roots with maximum length of 2.26 cm (Table: 5) (Fig: 8,9).Six week old rooted plantlets in culture were kept at room temperature and they were deflasked. The deflasked rooted plantlets were transferred to plastic pots (6.7 x 7.1cm) containing soil and sand (1:1),covered with transparent polythene bags for a week. After one month, they were transferred to plastic pots (16 x 16.5cm diameter) filled with soil, sand and cow dung (1:1:1). Plants hardened for 4 weeks showed 86% survival rate (Fig:10). Three months after establishment, the established plants were free from morphological and growth defects and were suitable for field planting.

DISCUSSION

Successful initiation of tissue culture leading to

Table 2: Multiple shoot formation in *G. ridsdalei* from 1st to 5th nodal explants cultured in MS medium supplemented with different concentrations of BA collected during monsoon season

Conc (mg/l)	I Node	II Node	III Node	IV Node	V Node
0.5	1.80 ± 0.249^{b}	2.10 ± 0.133	2.30 ± 0.153	1.80 ± 0.100^{c}	1.60 ± 0.163
1	1.90 ± 0.100^{ab}	2.40 ± 0.163	2.50 ± 0.167	2.10 ± 0.180^{abc}	1.80 ± 0.133
1.5	2.20 ± 0.163^{b}	2.50 ± 0.267	2.70 ± 0.213	2.50 ± 0.221^{bc}	2.00 ± 0.167
2	2.00 ± 0.000^{ab}	2.40 ± 0.163	2.60 ± 0.163	2.20 ± 0.133^{ab}	1.90 ± 0.180
2.5	1.80 ± 0.200^{b}	2.20 ± 0.422	2.30 ± 0.300	1.90 ± 0.180^{bc}	1.60 ± 0.163
3	1.70 ± 0.213^{b}	1.90 ± 0.163	2.10 ± 0.277	1.60 ± 0.221^{c}	1.50 ± 0.167
(%) of response	39	42	51	49	45

Observation were made after 30days of culture

Table 3. Multiple shoot formation in *G. ridsdalei* from 1st to 5th nodal explants cultured in MS medium supplemented with different concentrations of BA collected during post monsoon season

Conc (mg/l)	I Node	II Node	III Node	IV Node	V Node
0.5	1.90 ± 0.800^{ab}	2.20 ± 0.200	2.40 ± 0.221^{bc}	2.00 ± 0.223^{a}	1.80 ± 0.200^{ab}
1	2.40 ± 0.163^{a}	2.50 ± 0.167	2.80 ± 0.200^{b}	2.20 ± 0.133^{a}	2.20 ± 0.153^{a}
1.5	2.60 ± 0.173^{a}	2.70 ± 0.213	3.40 ± 0.221^{ab}	2.70 ± 0.213^{a}	2.30 ± 0.156^{a}
2	2.30 ± 0.213^{b}	2.60 ± 0.613	3.10 ± 0.314^{c}	2.50 ± 0.224^{a}	2.10 ± 0.233^{a}
2.5	2.00 ± 0.000^{ab}	2.40 ± 0.163	2.30 ± 0.300^{c}	2.20 ± 0.133^{a}	2.00 ± 0.133^{a}
3	1.90 ± 0.180^{ab}	2.10 ± 0.133	2.20 ± 0.163^{a}	1.60 ± 0.221^{b}	1.50 ± 0.167^{b}
(%) of response	54	56	64	61	57

Observation were made after 30days of culture

morphogenesis depends on the season of collection of explant type and nature of the explant, age of the explant (George & Sherrington, 1984, Gamborg &Phillips,1996).The selection of correct explant and the season of its collection showed crucial influence in bacterial contamination in culture and morphogenic response (Cassels, 1991). In the present study highest percentage response and minimal contamination percentage was noticed in explants collected during the summer season. The highest percentage response of the explants collected during summer season were reported by various authors in various plants like, *Schleichera oleosa* (Saha, 2013), *Gmelina arborea* (Thakar &Bhargava, 1999), *Corylus* species (Yu & Reed, 1995).Contamination rate was high during the rainy season (June – July) in the present study due to high humidity rate in the environment which provide the most favourable condition for the growth of microorganisms (Cardoza *et al*., 1999). Similar results were also reported in *Arundinaria* (Devi &Sharma, 2009), *Corylus* species (Yu & Reed, 1995), *Boerhaavia diffusa* (Patil &Bhalsing, 2015) and *Anacardium occidentale* (Cardoza *et al*., 1999).Effect of season on bud sprouting was also noted in many tree species like *Tectona grandis* (Gupta *et al*., 1980), guava (Jaiswal & Amir, 1987), *Tecomella undulata* (Rathore *et al*., 1991), The influence of various season on the shoot regeneration ability of explants was reported inmany other species, such as *Mamillaria elongata*(Papafotiou *et al*., 2001), *Ceratonia siliqua (*Romano *et al*., 2002). In the present study, BA alone with different concentration were used for the morphogenic response of the nodal explant of *G.ridsdalei*. Benzyl adenine (BA) is reported to be one of the most effective and affordable cytokinins used in micropropagation techniques (Bairu

Table 4. Multiple shoot formation in *G. ridsdalei* from 1st to 5th nodal explants cultured in MS medium supplemented with different concentrations of BA collected during winter season

Concentration BA (mg/l)	I Node	II Node	III Node	IV Node	V Node
0.5	1.90 ± 0.180^{ab}	2.40 ± 0.167^{a}	2.80± 0.200^{abc}	2.30 ± 0.153^{b}	2.20 ± 0.133^{a}
1	2.40 ± 0.000^{a}	2.60 ± 0.163^{bc}	3.40 ± 0.200^{bc}	3.00 ± 0.122^{a}	2.60± 0.100^{b}
1.5	2.80 ± 0.249^{b}	3.20 ± 0.200^{b}	4.40 ± 0.221^{bc}	3.30 ± 0.133^{a}	3.00 ± 0.111^{b}
2	2.60 ± 0.163^{a}	2.70 ± 0.213^{a}	3.90 ± 0.163^{c}	2.90 ± 0.121^{a}	2.30 ± 0.153^{a}
2.5	2.40 ± 0.180^{a}	2.50 ± 0.167^{a}	3.10 ± 0.314^{ab}	2.80 ± 0.200^{a}	2.10 ± 0.000^{a}
3	1.90 ± 0.170^{ab}	2.30 ± 0.133^{a}	2.40 ± 0.165^{bc}	2.20 ± 0.133^{b}	2.00 ± 0.075^{b}
(%) of response	69	72	78	75	73

Observation were made after 30days of culture

Table 5. Effect of different concentration of IAA on *in vitro* rooting of *G. ridsdalei* in half strength MS medium

Concentration IAA (mg/l)	Mean no of roots/ shoot ± SE	Mean root length ±SE
0.5	11.40 ± 1.09[a]	2.86 ± 0.23[b]
1	14.40± 1.44[a]	2.26 ± 0.12[c]
1.5	15.30 ± 2.16[a]	3.02 ± 0.16[ab]
2	12.20 ± 1.34[a]	2.80 ± 0.14[ab]
2.5	12.76 ± 1.01[a]	3.14 ± 0.23[a]
3	7.50 ± 0.52[b]	2.49 ± 0.11[bc]

Observation were made after 45 days of culture

et al., 2007; Amoo *et al*., 2011). In *G. ridsdalei* 3rd and 4th nodes produced higher number of shoots than the other nodes. Anjusha & Gangaprasad (2016) reported similar observation in a related species of *Gynocthodes umbellata.* Similar observation were also recorded in *Holostemma annulare* (Sudha *et al*, 1998). Hevea and *Theobroma cacao* (Lardet *et al*,1998). The differential responsiveness of multiple shoot formation among the nodes dissected from different position of plants have been interpreted (Okubo *et al*, 1991; Bonga,1982) Suitability of BA for *in vitro* shoot proliferation was reported in other perennials of Rubiaceae family like *Mitragyna parvifolia* (Roy *et al.*, 1988), *Ochreinauclea missions* (Dalal & Rai, 2001).The effectiveness of IAA on rooting of micro shoots were earlier reported in species like *Artemisia vulgaris* L.(Sujatha & Kumari, 2007), *Enicostema axillare* (Loganathan & Bai, 2014). IAA was reported as a potential auxin for rooting of *Arachis stenosperma* and *Arachis villosa* (Vijayalakshmi & Giri, 2003).

CONCLUSION

In the present micropropagation experiment, the effect of seasonal variation and nodal positionhave an intense effect in shooting. Due to the present status of this plant and its medicinal properties and considering the poor germination of seeds micropropagation is an alternative method of conservation of this endangered plant.

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Phytochemical Screening and Evaluation of Antioxidant Potential of *Impatiens Balsamina* L. Flowers *In Vitro*

V. SREELAKSHMI*, PRABHU RAMYA, R., ARYA, V. KUMAR, ATHIRA, V. MOHAN, M. AYESHA, S. SHAHINAS, C. SUBHADRA VISHNU

Department of Biochemistry , Government Arts College, Thycaud Thiruvananthapuram, Kerala, India, 695014
**email: sreelakshmi.v.88@gmail.com*

ABSTRACT

***Impatiens balsamina* is a herbaceous plant native to southern Asia that comes under the family *Balsaminaceae*. Leaves, stem, flowers and seeds of the plant are traditionally used for various disorders in traditional systems of medicine. This study was undertaken to investigate the qualitative and quantitative phytochemical screening and antioxidant potential of methanolic extract of *Impatiens balsamina* flowers. The level of phenolics and flavonoids were assayed and expressed in terms of gallic acid and quercetin equivalents respectively. The antioxidant activity was checked in terms of total antioxidant capacity and reducing power. The extract has been found to have good phytochemical content and good antioxidant capacity. Our results indicate that the phenolics in the plant may be responsible for the antioxidant activity of the extract and the observations are in line with the traditional application of the plant. From the results, *Impatiens balsamina* flowers might be a valuable bioactive resource and would seem to be applicable as a natural antioxidant.**

Key words *Antioxidant; Impatiens balsamina;Reducing power; Total phenolic; Total flavonoids*

Free radicals are molecular species that have an unpaired electron in an atomic orbital and are capable of independent existence. Free radicals are unstable and highly reactive that they can donate or accept an electron from other molecules and behave as oxidant or reductant (Lobo, *et al*., 2010). The most important free radicals are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, peroxynitrite radical etc. Free radicals form the essential part of aerobic metabolism and are indispensable for phagocytosis, detoxification processes and signaling cascades. Usually the level of oxidants is balanced by various antioxidants in the body. Overproduction of these oxidant species in manifested in many pathological conditions like atherosclerosis, diabetes, cancer, cataract etc. Free radicals induce peroxidation of lipids in the membrane, modification of proteins and DNA mutations. Many pharmacological studies recommend the consumption of antioxidant plants and vegetables to alleviate oxidative damage by free radicals (Sreelakshmi and Abraham, 2016).

Impatiens balsamina is an annual plant belongs to the family *Balsaminaceae*. It is commonly called garden balsam or garden jewelweed. It is usually cultivated as an ornamental plant. It is an erect, sparsely-branched, soft-stemmed, up right plant growing to 20-90 cm tall. The leaves are spirally-arranged and toothed. Cup-shaped, single to mostly double flowers with incurved spurs come in different colors such as pink, rose, red, purple, white and bicolor. Flowers bloom singly or in small clusters from the leaf axils throughout the growing season.

Traditionally different parts of the plant are used for different ailments throughout the world.The leaf juice is used against warts and snake bites.The juice of flowers is applied over burns, scalds and joints to relieve pain. The decoction of the flower is an effective emetic and laxative and it promotes the flow of urine. Washing the hair with the extract of the whole plant is employed to stimulate hair growth. The powdered seeds are given to women during in order to provide strength and promote blood flow (Meenu, *et al*., 2015). Modern pharmacological studies have reported the antifungal, antibacterial, antitumor, antipruritic and antianaphylactic activities of *Impatiens balsamina*. It is also an inhibitor of 5α-reductases, enzymes that reduce testosterone levels and applicable in the fileld of androgen-related skin disorders (Ishiguro,*et al*., 2000; Wang, *et al*., 2009).The objective of the present investigation is to screen qualitatively and quantitatively the secondary metabolites in *Impatiens balsamina* flowers and to determine its antioxidant potential.

MATERIALS AND METHODS

Chemicals

All the reagents used were of analytical grade

and were purchased from SRL, Ranbaxy and Spectrochem, India.

Plant material and sample preparation

Impatiens balsamina flowers were collected from Thiruvananthapuram, Kerala. The collected material was dried under shade, powdered using a mechanical grinder and stored in air tight containers. The dried powder was extracted by random shaking method with 80% methanol in a conical flask and plugged with cotton. The extract was filtered through Whatman filter paper No. 1. The supernatant was collected and the solvent was evaporated and the dry extract was stored at 4°C in air tight bottles.

Qualitative phytochemical analysis

Evaluation of phytochemical chemical constituents of extracts was carried by qualitative chemical methods of Tiwari, *et al*., 2011. A small portion of the dry extract was subjected to tests for secondary metabolites by characteristic colour reactions.

In order to find out the presence of carbohydrates, Benedict's test was done. The extract is dissolved in water and filter. Equal volume of Benedict's solution and extracts were mixed in a test tube and heated in boiling water bath for 10 min the changes in colour to yellow, green and red indicates the presence of reducing sugars.

Biuret test was done to detect the presence of proteins. To 3 ml of extract 1 ml of 4% sodium hydroxide and 1ml of 1% copper sulphate were added. The change in colour of the solution to violet or pink indicates the presence of proteins.

The extract is dissolved in dilute hydrochloric acid and filter. The filtrate was treated with saturated aqueous solution of picric acid (Hager's reagent). Presence of alkaloids was confirmed by the formation of yellow coloured precipitate.

A small quantity of extract is dissolved in chloroform, to 2 ml of extract 5 drops of concentrated sulphuric acid was added, shaken and allowed to stand. Formation of brown ring indicates the presence of phytosterols (Salkowaski test).

The presence of tannins/phenolics was tested by ferric chloride test. Treated the extract with 3-4 drops of ferric chloride (5%) solution, formation of bluish black colour indicated the presence of phenols/ tannins.

Presence of flavonoids was done by lead acetate test. The extract was treated with few drops of lead acetate solution, formation of yellow precipitate indicated the presence of flavonoids**.** Yellow, orange, blue and violet colours indicate presence of anthocyanins, flavones and flavonones.

Keller-Killani test was done to evaluate the presence of glycosides. To 2 ml of extract, glacial acetic acid, one drop 5 % ferric chloride and concentrated sulphuric acid were added. Appearance of reddish brown colour at the junction of the two liquid layers indicates the presence of cardiac glycosides.

Presence of saponins was detected by foam test. A small quantity of the extract was shaken with 2 ml of water. Persistence of foam produced for ten minutes indicated the presence of saponins.

Determination of total phenolic content (TPC)

Phenolics level of extract was determined using Folin-Ciocalteu method as described by Singleton, *et al*., 1999. 0.5 ml sample was mixed with 3 ml distilled water and 0.25 ml Folin-Ciocalteu reagent (1N). The mixture was allowed to stand at room temperature for 2 minutes and 0.75 ml sodium carbonate (20%) was added to the mixture. The volume was made upto 5 ml with distilled water. Absorbance was measured at 765 nm after standing for 2 hours. The content of phenolics was expressed as mg gallic acid equivalents.

Determination of total flavonoid content (TFC)

Aluminium chloride colorimetric technique was used for flavonoids estimation (Chang, *et al*., 2002). 1 ml sample was mixed 0.5 ml aluminium chloride (1.2%) and 0.5 ml potassium acetate (120 mM). Absorbance was measured at 415 nm after incubating 30 minutes at room temperature. The content of flavonoid was expressed as mg quercetin equivalents.

Reducing power assay

The reducing power of extract was determined as per the method of Oyaizu, 1986. Different concentrations of sample (1 ml) was mixed with 2.5 ml phosphate buffer (0.2 M; pH 6.6) and 2.5 ml

potassium ferricyanide (0.1%), followed by incubation at 50^{0}C on a water bath for 20 min. After incubation, 2.5 ml TCA (10%) was added to terminate the reaction. The upper portion of the solution (2.5 ml) was mixed with 2.5 ml distilled water and 0.5 ml ferric chloride solution (0.01%) was added. The reaction mixture was left for 10 minutes at room temperature and the absorbance was measured at 700 nm against blank. A higher absorbance of the reaction mixture indicates greater reducing power.

Total antioxidant capacity assay (TAC)

Total antioxidant capacity of the extract was evaluated by the method of Prieto, *et al*., 1999. 0.1 ml sample solution was mixed with 1 ml phospho-molybdenum reagent and incubated at 95^{0}C for 90 minutes. The tubes were cooled to room temperature and the absorbance was read at 695nm against a blank. TAC was expressed as mg gallic acid equivalents.

Statistical analysis

All the experiments were carried out in duplicates and data reported are mean ± standard error.

RESULTS AND DISCUSSION

Impatiens balsamina was chosen for this study due to their medicinal values as well as being indigenous plant. The growing interest in the antioxidant studies of these kind of plants derived from their and low toxicity compared with those of synthetic antioxidants and its availability.

Preliminary phytochemical analysis

The phytochemical screening of crude methanolic extract of flower sample of *Impatiens balsamina*revealed the presence of some secondary metabolites such as phenolics, tannins, flavonoids, steroids and cardiac glycosides as shown in Table 1. All the phytochemical compounds detected are known to have medicinal importance (Iqbal, *et al*., 2015) and that reinforces the traditional medicinal uses of the plant.

Total phenolic content and total flavonoid content

Phenolic compounds comprise a group of secondary metabolites which have favorable effects on the plant's host. Apart from this, phenolics

Table 1. Qualitative phytochemical analysis of *Impatiens balsamina* flower

Secondary metobolite	Result
Carbohydrates	Present
Proteins	Present
Alkaloids	Absent
Phytosterol	Present
Phenolics	Present
Tannins	Present
Flavonoids	Present
Falvonones	Present
Anthocyanins	Present
Flavones	Present
Glycosides	Present
Saponins	Absent

demonstrate various biological properties that positively influence human health. Phenolic compounds exhibit redox properties due to its multiple hydroxyl groups and it allow them to act as antioxidants (Soobrattee, *et al*., 2005). Generally, the antioxidant mechanisms of phenolic compounds are by neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals (Li, *et al*., 2009). Thus the free radical scavenging ability of plant extracts can be screened from their phenolic concentration. Total phenolic content in extract was determined by Folin-Ciocalteu method using gallic acid as the standard. Flavonoids are the most common and widely distributed group of plant phenolic compounds present in plants. It is characterized by a benzo-ã-pyrone structure. Total flavonoid content in different extracts was determined by aluminium chloride method using quercetin as the standard. The extract show significant level of phenolics and flavonoids (Table 2) and this may effectively eliminate free radicals and prevent oxidative stress.

Table 2. TPC, TFC and TAC of *Impatiens balsamina* flower

TPC	15.5± 0.77 mg gallic acid equivalents
TFC	14.75 ± 0.73 mg quercetin equivalents

Reducing power and total antioxidant capacity

An antioxidant molecule can contribute an

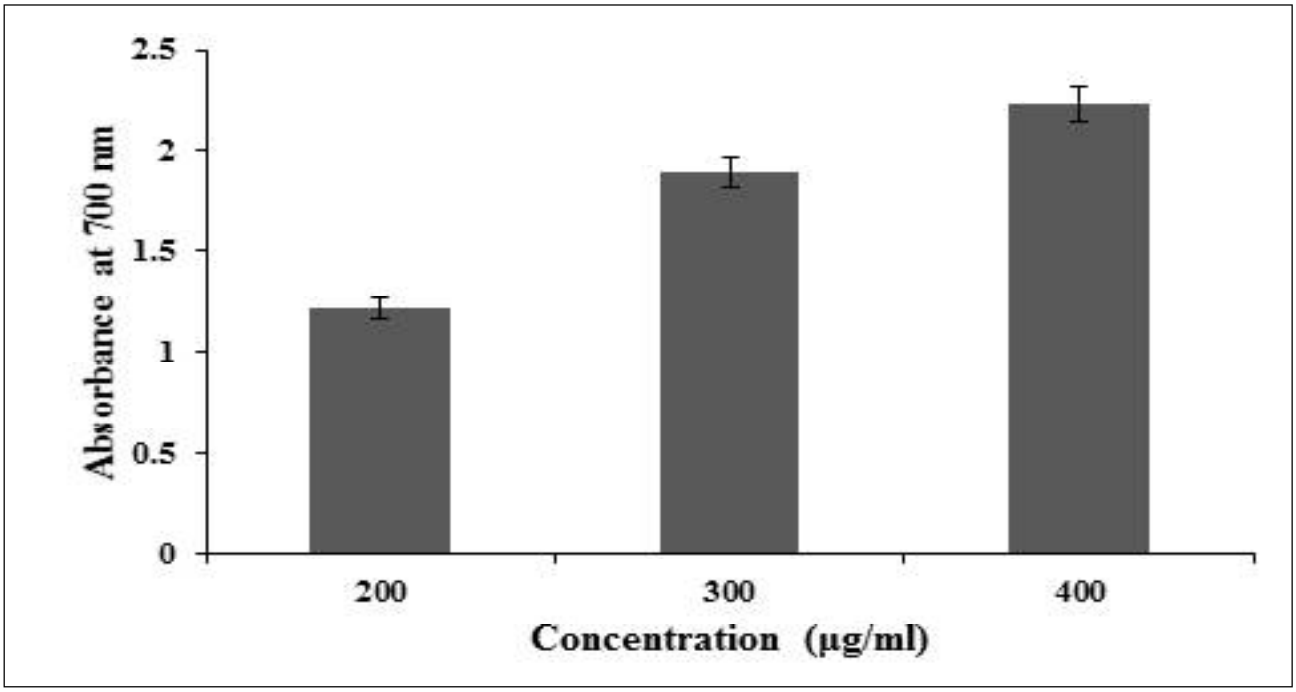

Fig 1. Reducing power of *Impatiens balsamina* flower

electron to free radicals, which leads to the neutralization of the radical. Reducing power was measured by direct electron donation in the reduction of ferrocyanide to ferricyanide in the presence of iron containing salts. The intense Prussian blue color of the complex was measured at 700 nm colorimetrically (Yen, *et al*., 1995). A higher absorbance value signifies a stronger reducing power of the extract. As shown in the figure 1, the extract shows a concentration dependent increase of absorbance indicates the potential of *Impatiens balsamina* flower extract to scavenge free radicals.

Total antioxidant capacity is the assay that evaluates both water-soluble and fat-soluble antioxidants. It is based on the reduction of Mo (VI) to Mo (V) by the antioxidants and subsequent formation of blue phosphate/Mo (V) complex at acidic pH (Aliyu, *et al*., 2012). TAC of *Impatiens balsamina* flower is found to be 90.90 ± 4.54 mg gallic acid equivalents. The result is in accordance with total phenolis, flavonoids and reducing power of the extract.

Considering the phytochemical screening, total phenolics and flavonoids, reducing capacity and total antioxidant capacity, as the indices of antioxidant activity of the extract, the findings revealed the potential of *Impatiens balsamina* as a source for natural antioxidants. It can be used as a natural antioxidant source to prevent diseases associated with free radicals. Studies are in progress for the bioassay guided fractionation and identification of the factors in charge for the activity.

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In vitro Cytotoxic Effects of Terpenoid Extract from *Hypnea musciformis* (Wulfen) J.V. Lamouroux. against Selected Cancer Cell Lines

S.S. SUMAYYA* AND K MURUGAN

Plant Biochemistry & Molecular Biology Laboratory, Department of Botany, University College, Thiruvananthapuram 695 034, India.
email: sumayyasumi01@gmail.com

ABSTRACT

Plant based research have shown that a variety of chemopreventive phytochemicals affect tumor initiation, promotion, and progression and the main difference, between botanical medicines and synthetic drugs, resides in the presence of complex metabolite mixtures shown by botanical sample which in turn exert their action on different levels and via various mechanisms. The genus *Hypnea*, red algae were traditionally used by the local people for many medicinal purposes. In particular, *Hypnea musciformi*s showed many therapeutic properties. Local fisher men communities have used the crude extract an herbal remedy for various diseases, including cancer. Considering the lack of available data for supporting this antimetastatic effect, the target of this work was to study the *in vitro* cytotoxic potential of the terpenoids from *Hypnea musciformi*s on Hep G2 (Liver Hepatocellular Carcinoma), HeLa (Cervical carcinoma), A549 (Lung cancer) cells. The following terpenoids Hexadecane, 2,6,10,14 tetramethyl, Pentadecanal, Phytolisomer, 4,8,12,16- tetramethylheptadecan-4-olide were noticed in the purified fraction of *Hypnea musciformi*s as revealed by GC-MS analysis. Regarding the cytotoxic activity based on MTT assay, the terpenoid extract showed varied levels of potential against the selected malignant cell lines. 50% cytotoxic effect was noticed against HeLa cells similar to that observed for the positive control. The results of Hep G2 and A549 cells also showed 34.7 and 39.5 % cytotoxic activity. Morphological deformities noticed in the present study substantiate the assay data. In general, the terpenoid extract from *H. musciformi*s presented cytotoxicity against the investigated tumor cell lines which confirms their antiproliferative potential.

Key words *Hypnea musciformis, terpenoid extract, cytotoxic, GC-MS, cancer cell lines*

Nowadays, the use of natural compounds and its derivatives as anticancer agents represent a highly-investigated research area. Triterpenic acids exhibit various biological and pharmacological activities, including anti-inflammatory, antimicrobial, antiviral, cytotoxic, and cardiovascular effects (Shaban et al., 2012). For *in vivo* biological screening and evaluated assays the total synthesis of this class of natural products is still not an easy strategy to obtain in required quantities. For example, commercial betulinic acid is prepared by chemical selective oxidation of the alcohol derivative (betulin) and it is also isolated in good yields together with the isomers ursolic and oleanolic acids from restricted group of plants.

The main classes of natural compounds and its derivatives were esters (Naganna et al., 2017). This class of compounds exhibit a variety of structural types including aliphatic and aromatic moieties leading to broad range of biological activities. Although the biological role of such compounds in plants has not been fully studied, various biological activities have been reported such as antibacterial, antifungal, estrogenic, antiestrogenic, inhibition of testosterone secretion, immunological, anti-inflammatory, cytotoxic, and ionophoretic properties, relaxant effect, vasodilatory effect, antagonist of calcium, etc. Natural products esters and its derivatives can be obtained from various natural sources (Alzoreky,2003), but its use as lead compounds for medicinal purposes gives an idea about the potentiality of this approach. An example of lead compound derived from betulinic acid spawned the drug Bevirimat®, which has been showed to be antiviral activity against HIV-1 entry (David. Kingston, 2011). In this juncture the present study was undertaken to analyze antiproliferative potential of terpenoid extract from *Hypnea musciformi*s (Wulfen) J.V.Lamouroux against the selected cancer cell lines.

MATERIAL AND METHODS

The marine algae *Hypnea musciformis* was collected during April 2015, from the Mandapam coast (latitude 9Ú 17' N, longitude79Ú 22' E), Gulf of Mannar. The ground samples were then kept and stored for further analysis.

Fractionation of the sample was done by silica gel Column Chromatography (CC) using petroleum

ether:ethyl acetate solvent combinations. The purified fraction was quantified for the presence of terpenoids (Ferugson,1956) and further analyzed by GC-MS.

GC-MS analysis

For GC-MS analysis, the sample was injected into a HP-5 column (30 m X 0.25 mm i.d with 0.25 ìM film thickness), Agilent technologies 6890 N JEOL GC Mate II GC-MS model. Helium was used as carrier gas with a flow rate of 1 mL/min; the injector was operated at 200 °C and column oven temperature was programmed as 50 - 250 °C at a rate of 10 °C / min injection mode. The following MS conditions were used: ionization voltage of 70 eV; ion source temperature of 250 °C; interface temperature of 250 °C; mass range of 50 - 600 mass units. A chromatogram was obtained and the mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

MTT assay

The invitro viability of cell lines were determined by MTT assay on three cell lines Hep G2 (Liver Hepatocellular Carcinoma), HeLa (Cervical carcinoma), A549 (Lung cancer) cells. MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. The cells are then solubilised with an organic solvent Dimethyl sulfoxide (Merck Inc) and the released, solubilised formazan product was measured at 540nm. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells (Arung *et al.,* 2006).

15mg of MTT was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hrs. of incubation period, the sample content in wells were removed and 3.0µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm (Laura B. Talarico et al., 2004).

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples} \times 100}{\text{Mean OD of control group}}$$

STATISTICAL ANALYSIS

All the data were mean SD of six independent experiments. Significant level was noted as $p < 0.05$.

RESULTS AND DISCUSSION

The crude extract was purified by Column chromatograpy. The fraction eluted from the column was further quantified for the presence of terpenoid i.e 4.52 mg/g. Subsequently, fraction was subjected for GC -MS analysis. Figure 1 shows the terpenes composition of *Hypnea musciformis* as detected by using GC-MS spectra technique. Retention time and the relative abundance of each compound were recognized. The GC-MS analysis of the purified fraction revealed the presence of 4 major peaks of terpenoids such as Hexadecane,2,6,10,14 tetramethyl, Pentadecanal, Phytol Isomer, 4,8,12,16 tetramethylheptadecan-4-olide. Out of which 4,8,12,16 tetramethylheptadecan-4-olide showed the highest peak followed by the diterpene, Phytolisomer compatible with their fragmentation pattern. Eman et al (2015) also reported the presence of terpenoids 4,8,12,16 tetramethyl heptadecan-4-olide; 2,6,10,14 Tetramethyl hexadecane in the filamentous green algae *Spirogyra*. Marine algae of the genus *Laurencia* were first noted for producing terpenoids containing bro-mine. Ghazala and Shameel (2005) identified diterpene phytol from brown algae while Gupta and Abu Ghannam (2011) confirmed that several types of diterpenoids and sesquiterpenoids have been found to be the main secondary metabolites of the species belonging to Dictyotales.

From the time immemorial, plants have been used for their therapeutic abilities and currently still the underdeveloped countries rely largely on the curative properties of herbals. According to WHO, 80 % of

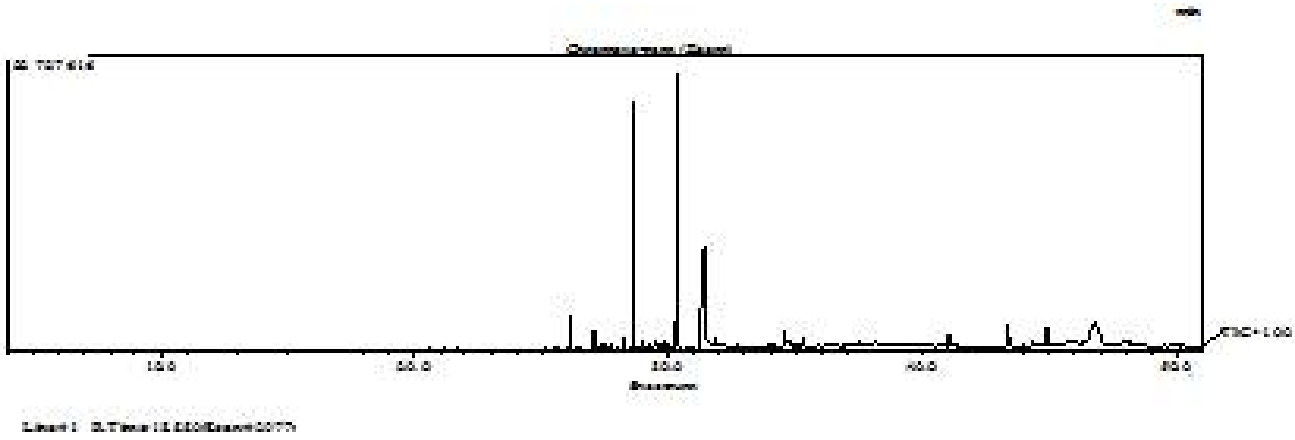

Fig. 1. GC-MS analysis showing the terpene composition in *Hypnea musciformis*.

the common people living in remote areas depend on medicinal plants for their primary healthcare system. The synthetic anticancer drugs are either costly or possess side effects. Plant based novel compounds have a vital role in the prevention and treatment of cancer and medicinal herbs are available everywhere and comparatively cheap. Remarkable pharmacological documentation carried in technologically advanced countries like USA, Germany, France, Japan and China has considerably improved quality of the herbal based medicines used in the treatment of many life style disorders. Medicinal plants safe guard the human body from cancer by enhancing detoxification functions. Certain phytochemicals derived from herbs were known to inhibit growth of cancer by regulating the activity of a particular hormones or key enzymes. Most of the herbal products reduce toxic side effects of chemotherapy and radiotherapy. Pharmacologists all over the globe are authenticating on the herbal drugs to enhance immune cells of the body against cancer. By elucidating the complex synergistic action of different components of antimetastatic herbs, the herbal crude formulations can be designed to block the malignant cells without harming normal healthy cells of the body. Herbal drugs are also significant source of synthetic drug designing. So far, pharmaceutical industries have screened more than 28,000 plants for anti-metastatic molecules. Most of the plant based outputs reveal that single ingredient to attack cancer might be not effective at the point because cancers are disturbances or stress in the body, so phytochemicals of the herbals can regulate or modify the disturbances as well as control many malignant. Herbal system of medicine has been practiced for thousands of years. Red algae preparations can be used as a cheaper alternative to the conventional disinfectants (Sticher,1977). Plants are storehouse of a pool of phytochemicals. Many studies have confirmed the usefulness of plant based drugs as good candidate of anticancer activity agents.

The terpenoid extract of *H. musciformis* showing varied growth inhibition in terms of more than 40% cytotoxicity at 100 µg/ml and may be considered to be active. The *in vitro* cytotoxicity was performed against three human cancer cell lines namely lung (A-549), liver (Hep-2) and HeLa cell lines. Against lung (A-549) cell line terpenoid extract showed 39.5% growth of inhibition. In case of liver (Hep-2) the extract showed 34.7% inhibitory activity. Where as in case of HeLa cell line terpenoid extract showed maximum activity i.e., 50%. The terpenoid extract provide a cheap and sustainable method toward disease reduction and can eventually improve the quality of life of the rural and urban poor in developing countries.

Morphological alteration of malignant cell lines upon exposure using terpenoid extract was observed under phase contrast microscope. The cells indicated the most prominent effects after exposure to the terpenoid extract. The microscopic visuals revealed the terpenoid extract to be having outstanding effect on treated HeLa cells compared to other treated cells (Fig 2.a,b,c;Table 1). The number of dead cells increased correspondingly with concentration increment of the extract treatment in regard to observation. At high extract concentration, enlargement of the cells was conspicuosly observed. 39%-50% of the cells showed membrane blebbing (demonstrated with small protrusions of the membrane)

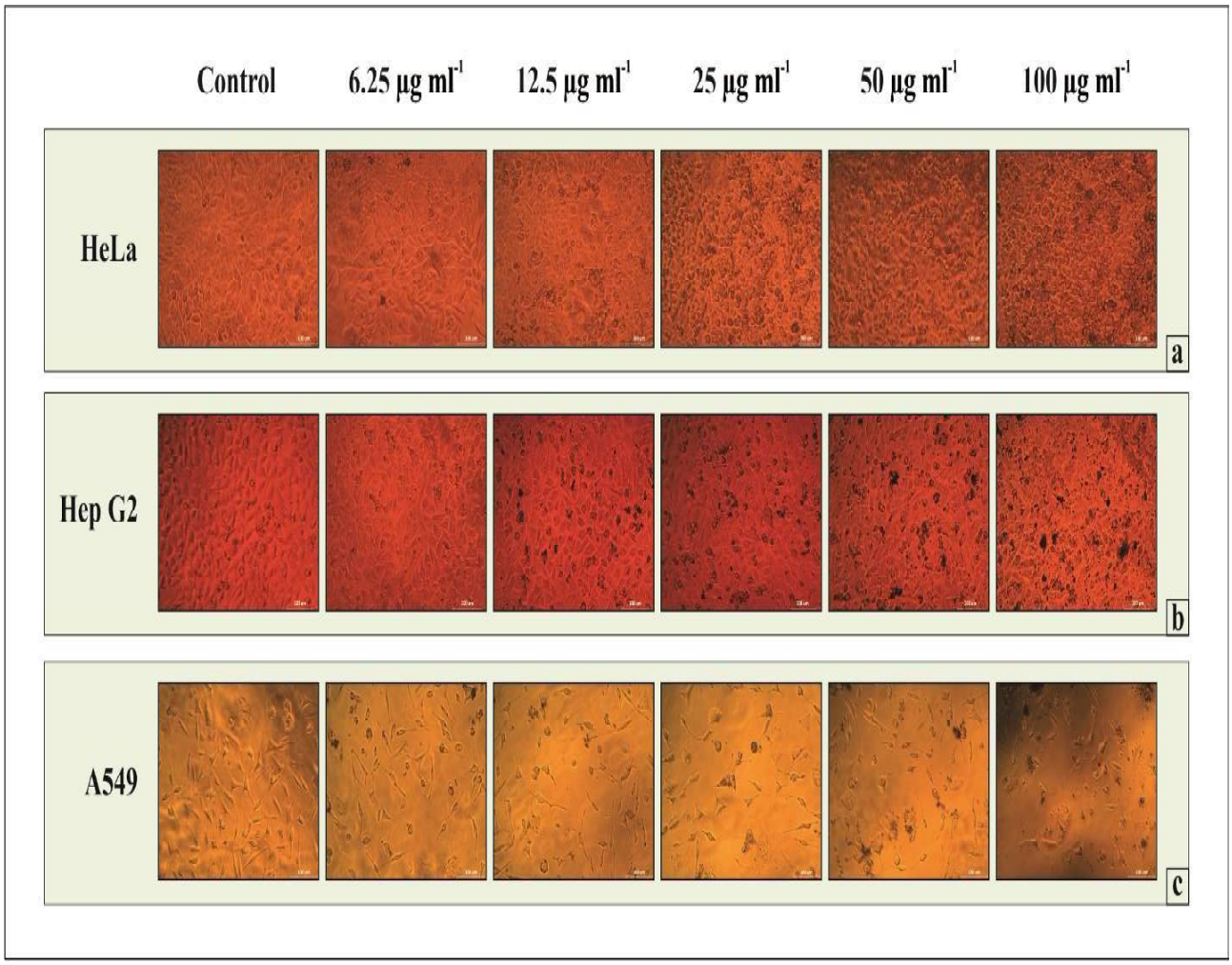

Fig. 2. a,b,c showing the anticancerous effect of terpenoids against various cell lines

and ballooning were apparent in the cells. The presence of apoptotic bodies could also be seen in the extract treated cells (Fig 2). Cells also showed extensive vacuolation in the cell cytoplasm, indicating autophagy like mechanism of cell death. Autophagosome like structures were clearly seen in the cells treated with terpenoid extract (Fig 2). At highest concentration (100 µg/mL) the cells became rounder, shrunken and showed signs of detachment from the surface of the wells denoting cell death. In fact, no inhibition was noticed with human blood lymphocytes (as non-malignant control cells).

MTT reduction is usually carrieed to study mitochondrial/non-mitochondrial dehydrogenase activity as a cytotoxic test for a variety of secondary metabolites. Therefore, the mode of action of terpenoid extract from *H. musciformis* may be via changing the enzymatic activity of mitochondria and initiate a preliminary injury that leads to cell death. Furthermore, it was also documented that polyphenolic derivatives can cause damage in the mitochondrial membrane since they provoke depolarization of the mitochondrial membranes by decreasing the membrane potential (Sezik et al.,2005) and also alter the fluidity of membranes which become abnormally permeable. Shaban et al., (2012) proved *in vitro* cytotoxicity of *Moringa oleifera* against selected human cancer cell lines. Döll-Boscardin et al., (2012) also showed *in vitro* cytotoxic potential of essential oils of *Eucalyptus benthamii* and its related terpenes on tumor cell lines Vijayarathna and Sasidharan (2012) alnalyzed cytotoxicity of methanol extracts of *Elaeis guineensis* on MCF-7 and Vero cell lines. Thus, the differential inhibition showed by the terpenoid extract of *H. musciformis* is justifiable. Shiezadeh et al., (2013) proved anti-proliferative potential of root extract from *Rheum turkestanicum* in terms of cytotoxic and apoptotic potential against human cancer and normal cells.

Table 1. Cytotoxicity analysis of *H. musciformis* against selected cancer cell lines

Sample Concentration (µg/mL)	Average OD	Percentage Viability (%)
HeLa		
Control	0.7038	100.00
6.25	0.5404	76.78
12.5	0.5095	72.39
25	0.4571	64.95
50	0.4265	60.60
100	0.3536	50.24
Hep G2		
Control	0.7562	100.00
6.25	0.6550	86.62
12.5	0.5824	77.02
25	0.5526	73.07
50	0.5133	67.88
100	0.4938	65.30
A549		
Control	0.7216	100.00
6.25	0.6694	92.78
12.5	0.6137	85.06
25	0.5845	81.02
50	0.5547	76.89
100	0.4368	60.54

CONCLUSION

In the present study, the work summarizes that the purified terpenoid extract from *Hypnea musciformis* showed selective *in vitro* cytotoxity against human cancer cell lines. Activity depends upon the mechanism of action of terpenoid extract. Many plant extracts inhibit the cancer cell lines through activating apoptosis and some through regulating growth regulators. Future studies were warranted to analyze the mechanism of terpenoid extract in inhibiting the malignant cell proliferation using *in vivo* model.

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Ethnomedicinal Survey among the Irula Tribals of Palamalai Hills, the Western Ghats of Coimbatore, Tamil Nadu

A. VENKATACHALAPATHI, AND S. PAULSAMY*

Department of Botany, Kongunadu Arts and Science College, Coimbatore - 641 029.
email: paulsami@yahoo.com

ABSTRACT

The present study is aimed to document the ethnomedicinal plants used for various ailments by the Irula tribes of Palamalai hills, Southern Western Ghats of Coimbatore, Tamil Nadu, India. The ethnobotanical survey was carried out during the period of October, 2009 to March, 2010 among Irula population residing in this area. A total number of 82 plants species belonging to 71 genera and 37 families used by them as herbal medicines to treat several common diseases such as skin diseases, dysentery, cough and cold, cuts and wounds, etc. were documented. Among the plant species Herbs were the primary source of medicine (34%) followed by trees (28%), climbers (22%) and shrubs (16%) and leaves were mostly used (53%) for various illness followed by whole plant (12%), fruit (8%), bark (6%), root (5%), stem, flower and seed (4% each), latex (2%), gum and bulb (1% each). Most of the medicine prescription by healers is decoction. Therefore, it is suggested to take-up pharmacological and phytochemical studies to evaluate the species to confirm the traditional knowledge of Irulas on medicinal plants.

Keywords *Ethnobotany, Irulas, Palamalai hills, Western Ghats.*

Ethnobotany word is made from two words ethno and botany and the term was coined by John William Harshberger in the 1896. Ethnobotany is the study of people and study of plants; this is represented good relationship between wild plants (Herbs, Shrubs and Trees) and tribal's. Ethnobotany is the branch of Ethnobiology and complete information about plants and their medicinal uses is given by ethnobotanical studies (Jitin, 2013).India is rich in ethnic diversity and indigenous knowledge that has resulted in exhaustive ethnobotanical studies. There are over 537 different aboriginal groups in India with extensive knowledge of plants (Jain, 1991). Herbal medicine is widely practiced throughout the world from time immemorable. These medicines are safe and environment friendly. The World Health Organization (WHO) defines traditional medicine as practices, knowledge and belief systems which uses minerals, plants and animal based remedies, spiritual therapies and exercises to prevent, treat and maintain well being (WHO, 2003). According to the WHO, about 80% of the population of the world depends on traditional medicine, mostly herbal remedies, for their primary health care needs (Muthu*et al.,* 2006; Newman and Cragg, 2007; Li, 2010). In the developed countries people are seeking for herbal medicine because of their scarcity side effects compared to the synthetic drugs. According to WHO 70 to 90 percent of world population especially from developing countries, use plant remedies for their health care (Belachew, 1984; Nair and Nathan, 1998). Developing countries like India, Pakistan and China have identified potential usage of medicinal plants, and integrated them in to their overall health care system (Andrew, 1982).

Irulars are small tribal community in the part of Dravidian language group which is spoken in south eastern India. They belong to the Negrito (or Negroid) race which is one of the six main ethnic groups that add to the racial mosaic of India (Deepa*et al.,* 2002). The origin of the word "Irula" is not clear. Some surmise that, it is derived from the Tamil word "Irual" implying the dark complexion of the Irula, often being spotted by villagers as distinct silhouettes in the forests and supporting their local name, the Forest People (Fuchs, 1973). They do not practice agriculture and therefore, fully depend on forest produces and wild animals. Other occupations of the Irulars include intermittent farm labour and the legendary profession of snake charming (Venkatachalapathi *et al.,* 2015; 2016).In recent years some researchershave reported various medicinal plants used by Irulatribals in Anaikatty hills, Siruvani hills and Maruthamalai hills of Coimbatore district (Palanisamy, 1993; Balasubramanian*et al.,* 1997; Nikkitha, 1999; Hamasavalli, 2001; Karthikeyani, 2003; Senthilkumar, 2004; Senthilkumar*et al.,* 2006; Geetha*et al.,* 2007;Paulsamy, 2011;Tamilselvi*et al.,* 2016). Therefore, the aim of the study was, to documentation of traditional knowledge of utilization of medicinal

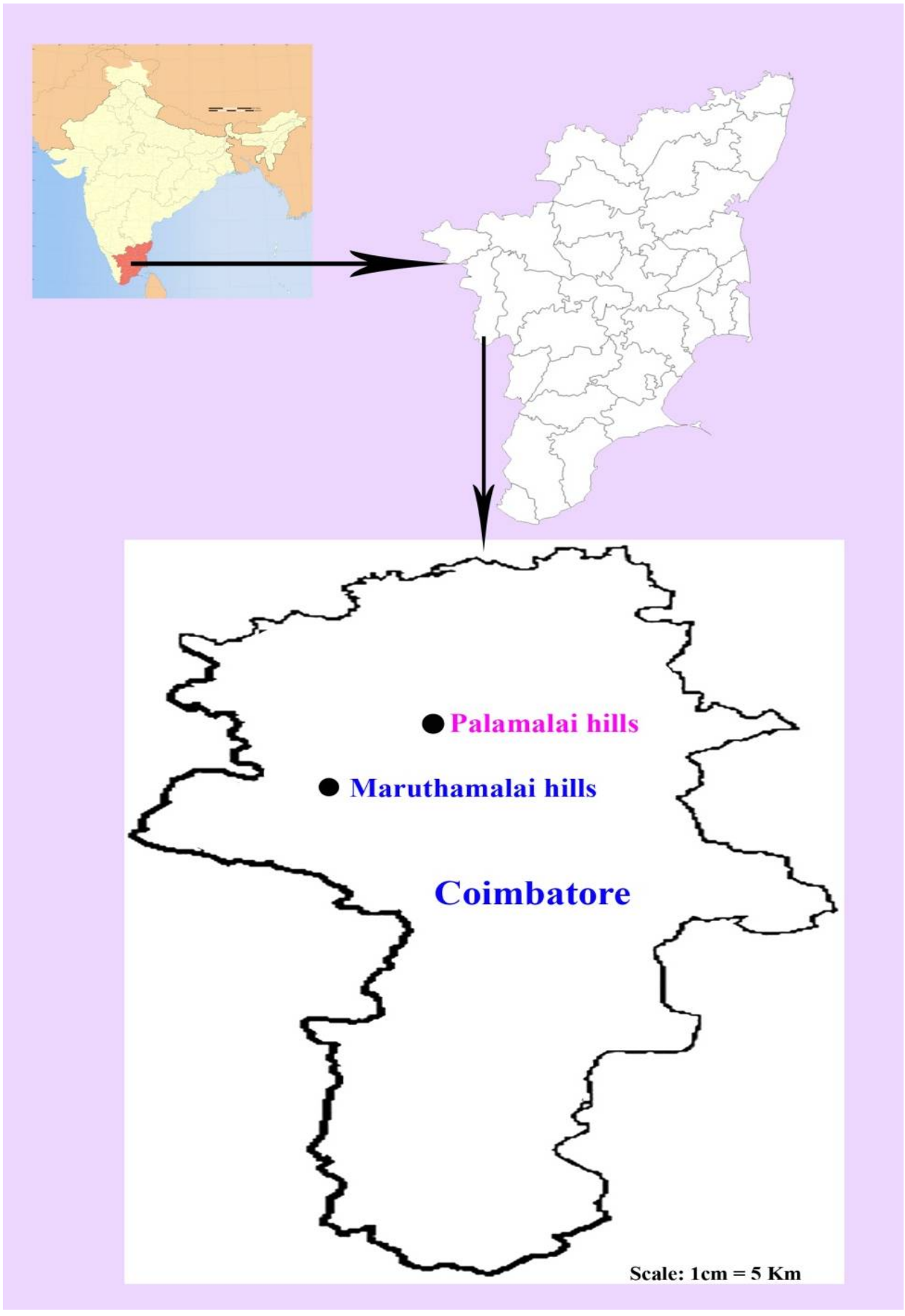

Fig. 1. Location of the study area of Palamalai hills.

plants used by Irula tribes for various ailments in Palamalai hills of Coimbatore district, Tamil Nadu.

MATERIALS AND METHODS

Study area

The present work was undertaken in the Palamalai hills located in the Coimbatore district of Tamil Nadu, South India. Palamalai is an offshoot of the Eastern Ghats geographically contiguous with the Billigrirangaa hills range as they reach out to merge with the Western Ghats at Nilgiris. It lies at an altitude of 1839 m above mean sea level and an attitude of 1400 m on the Western Ghats (Fig. 1).

Data collection

The ethnobotanical survey was carried out during October 2009 to March 2010 among Irula population residing in this area. The data on medicinal plants was recorded through interview, discussion and field observation with knowledgeable elder people using standard methods adopted by Jain (1991) and Jain and Goel (1995). Out of 19, 10 were male and 9 female respondents under the age group of 35 to 70 years. The information about plants and their local names, parts of plant used for preparation of drug and mode of administration were documented in the field survey and it was confirmed by cross-checking with respondents and also with the already existing literature.

The collected plant species were identified with help of The Flora of Presidency of Madras (Gamble and Fischer, 1915-1936) and confirmed by comparing authentic specimens in Madras Herbarium (MH) at Botanical Survey of India, Southern circle, Coimbatore and through recent floras and taxonomic revisions. The voucher specimens were deposited at the Department of Botany, Kongunadu Arts and Science College, Coimbatore, Tamil Nadu.

RESULTS AND DISCUSSION

The present study revealed the use of 82 species of plants distributed in 71 genera belonging to 37 families which were commonly used by Irulatribal healers of Palamalai hills, southern Western Ghats of Coimbatore district, Tamil Nadu for the treatment of 47types of ailments. The prominent family was Fabaceae with 9 species, followed byEuphorbiaceae with 7 species, Asclepiadaceae 5 species,Acanthaceae, Caesalpiniaceae, Mimosaceae, Rutaceae, Cucurbitaceae, Apocynaceae, Verbenaceaecontributed with 3 species each, Amaranthaceae, Asteraceae, Capparidaceae, Lamiaceae, Malvaceae, Myrtaceae,Poaceae, Sapindaceae, Solanaceae contributed with 2 species each and the remaining families are contributed with 1 species each. All the reported species were arranged

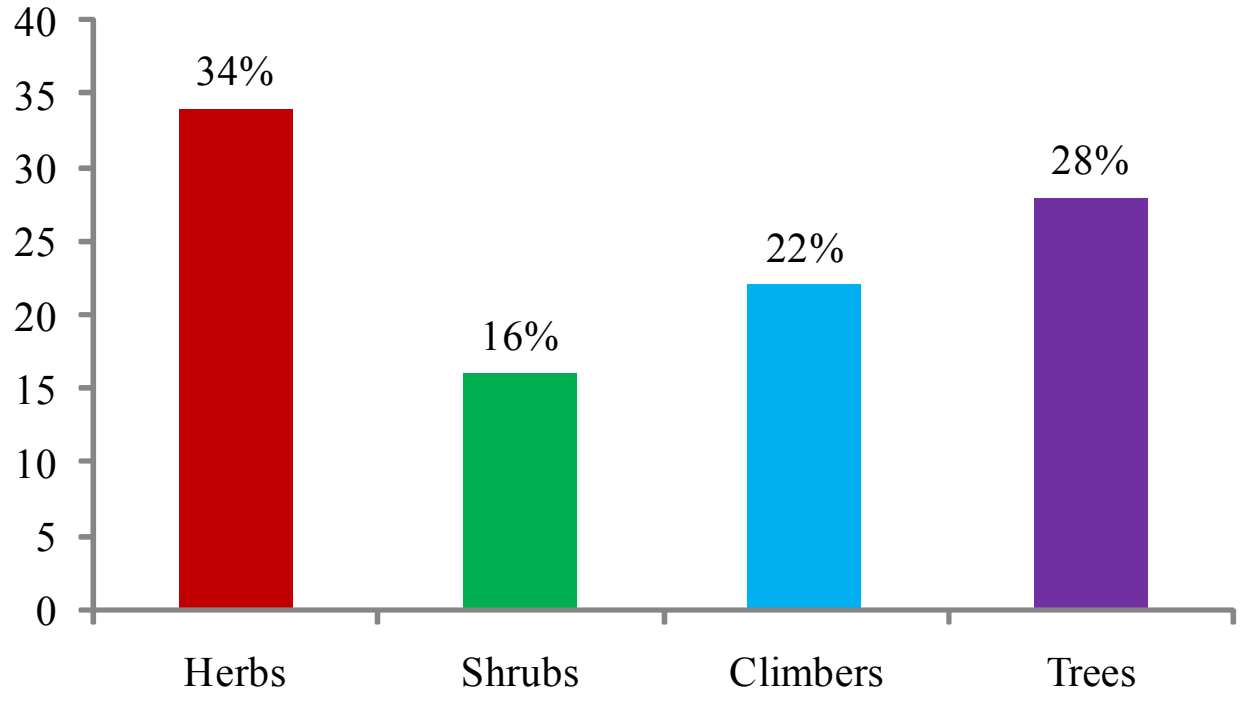

Fig. 2 Per-cent life-forms of medicinal plants used by Irulas in Palamalai hills.

Table 1. List of medicinal plant species used for their health care by Irulatribals of Palamalai hills, the Western Ghats of Coimbatore, Tamil Nadu.

S. No.	Binomial Name	Local name	Family	Parts used	Medicinal uses	Mode of administration
1	**Trees** *Acacia leucophloea*Willd.	Velvelam	Mimosaceae	Bark	Toothache	Powder
2	*A. nilotica*(L.) Del	Karuvelam	Mimosaceae	Gum	Liver problem	Decoction
3	*Aeglemarmelos*(Linn.) Corr.	Vilvam	Rutaceae	Leaf	Dyspepsia	Decoction
4	*Albiziaamara*(Roxb.) B. Boivin.	Arapu	Mimosaceae	Flower and seeds	Piles Diarrhea	Paste Decoction
5	*A. lebbeck*(L.) Benth.	Siridam	Mimosaceae	Whole plant	Anticancer	Paste
6	*Atalantiamonophylla*Corr.	KattuElumeachi	Rutaceae	Leaf	Paralysis	Paste
7	*Azadirachtaindica*A. Juss.	Vembu	Meliaceae	Bark	Stomach worms	Decoction
8	*Carica papaya* L.	Pappali	Caricaceae	Latex	Scorpion sting Snake bites	Paste Paste
9	*Cassia fistula* L.	Konrai	Caesalpiniaceae	Flower	Diabetes	Decoction
10	*Chloroxylonswieteni*aDC.	Porasu	Rutaceae	Leaf	Joint pain	Paste
11	*Emblicaofficinalis*Gaertn.	Nellikkai	Euphorbiaceae	Fruit	Cold and cough	Decoction
12	*Mangiferaindica*L.	Maamaram	Anacardiaceae	Leaf	Cracks	Paste
13	*Pongamiapinnata*(Linn.) Pierre.	Pungamaram	Fabaceae	Leaf	Ulcers Diabetes	Decoction Decoction
14	*Psidiumguajava*L.	Koiya	Myrtaceae	Leaf	Dysentery	Juice
15	*Pterocarpusmarsupium*Roxb.	Vengai	Fabaceae	Bark	Dysentery	Decoction
16	*Santalum album* L.	Chandanam	Santalaceae	Heart wood	Heart problem	Decoction
17	*Syzygiumcumini*(L.) Skeels.	Naval	Myrtaceae	Fruit	Diabetes	Raw
18	*Tamarindusindica*L.	Puliyamaram	Caesalpiniaceae	Fruit	Digestive	Raw
19	*Tectonagrandis*Linn. f.	Tekkumaram	Verbenaceae	Leaf	Skin diseases Ulcers	Paste Decoction
20	*Vitexnegundo*L.	Notchi	Verbenaceae	Flowers	Diarrhea Cardiac disorders	Decoction Decoction
21	*Wrightiaarborea*Mabberley.	Karupaalai	Apocynaceae	Bark	Kidney stones	Powder
22	*Wrightiatinctoria*(Roxb.) R. Br.	Veppalai	Apocynaceae	Leaf	Headache	Paste
23	*Ziziphusmauritiana* L.	Ilanthai	Rhamnaceae	Leaf	Wound healing	Paste
24	**Shrub** *Argemonemexicana*L.	Pirammathandu	Papaveraceae	Seed	Cracks at foot	Powder
25	*Breyniarhamnoides*(Retz.) Muell.	Sithuruvum	Euphorbiaceae	Leaf	Skin diseases	Paste
26	*Cadabaindica*Lam.	Vizhuthi	Capparidaceae	Leaf	Female sterility	Decoction
27	*Calotropisgigantea*(Linn.) R. Br.	Erukku	Asclepiadaceae	Latex	Wound healing	Paste

S. No.	Binomial Name	Local name	Family	Parts used	Medicinal uses	Mode of administration
28	*Capparisbrevispina*DC.	Sirakkali	Capparidaceae	Fruit	Body cooling	Decoction
29	*Carissa spinarum*L.	Chirukila	Apocynaceae	Fruit	Heart diseases	Decoction
30	*Cassia auriculata*L.	Avarai	Caesalpiniaceae	Leaf	Scabies Bone fractures	Paste Paste
31	*Cassia tora*L.	Tagarai	Caesalpiniaceae	Leaf	Leprosy Ulcers	Decoction Decoction
32	*Indigoferatinctoria*L.	Averi	Fabaceae	Root	Snake bites	Decoction
33	*Jasminumauriculatum*Vahl.	Uccimalligai	Oleaceae	Leaf	Burning sensation	Paste
34	*Jatrophacurcas*L.	Kattuamanaku	Euphorbiaceae	Stem	Digestion	Juice
35	Lablab purpureus (Linn.) Sweet.	Avarai	Fabaceae	Leaf	Ring worm	Paste
36	*Lantana camara*L.	Unnichedi	Verbenaceae	Leaf	Cuts and wounds	Paste
37	**Herbs** *Abutilon indicum*(Linn.) Sweet.	Thuthi	Malvaceae	Root	Fever	Decoction
38	*Acalyphaindica* L.	Kuppaimeni	Euphorbiaceae	Whole plant	Diabetes	Decoction
39	*Achyranthes aspera* L.	Naayuruvi	Amaranthaceae	Leaf	Dog bite	Paste
40	*Aervalanata*Juss.	Poola poo	Amaranthaceae	Leaf	Kidney stone	Decoction
41	*Allium cepa*L.	Vengayam	Liliaceae	Bulb	Boils	Paste
42	*Andrographisechioides*(Linn.) Nees.	Gopuramthangi	Acanthaceae	Leaf	Fever	Juice
43	*Andrographispaniculata*(Burm. f.) Wall.*ex*Nees.	Nilavembu	Acanthaceae	Bark	Fever Skin diseases Snake bite	Decoction Paste Paste
44	*Bambusaarundinacea*(Retz.) Roxb.	Mungil	Poaceae	Leaf	Wound healing	Paste
45	*Barleriabuxifolia*L.	Karaculli	Acanthaceae	Leaf	Cough	Decoction
46	*Blepharismaderaspatensis*(L.) B.	Kozhimookkan	Acanthaceae	Whole plant	Gas trouble	Decoction
47	*Cleome gynandra*L.	VeliKeerai	Cleomaceae	Leaf	Ear ache	Juice
48	*Crotalaria verrucosa*L.	Gilugiluppai	Fabaceae	Leaf	Blood purification	Decoction
49	*Cynodondactylon*Dress.	Arugampull	Poaceae	Whole plant	Eye disorder	Juice
50	*Daturametel*L.	Oomethai	Solanaceae	Leaf	Respiratory troubles	Juice
51	*Ecliptaprostrata* L.	Karasilaganni	Asteraceae	Leaf	Black hair Skin diseases Wound healing	Paste Paste Paste
52	*Euphorbia hirta*L.	Amman pacharisi	Euphorbiaceae	Leaf	Dysentery	Decoction
53	*Evolvulusalsinoides*L.	Vishnukranthi	Convolvulaceae	Leaf	Wound healing	Paste

S. No.	Binomial Name	Local name	Family	Parts used	Medicinal uses	Mode of administration
54	*Mollugonudicaulis*L.	Parpadakam	Aizoaceae	Leaf	Boils	Paste
55	*Leucas aspera* L.	Tumbai	Lamiaceae	Leaf	Bronchitis	Decoction
56	*Ocimumamericanum*L.	Naithulasi	Lamiacaeae	Leaf	Cold Cough Bronchitis	Decoction Decoction Decoction
57	*Phyllanthusamarus*Schum. &Thonn.	Kilanelli	Euphorbiaceae	Leaf	Jaundice	Juice
58	*Phyllanthusmaderaspatensis*L.	Nila-nelli	Euphorbiaceae	Leaf	Diarrhea	Decoction
59	*Sidacordifolia*L.	Nilathuthi	Malvaceae	Leaf	Ear ache	Juice
60	*Solanumnigrum*L.	Manathakkali	Solanaceae	Leaf	Mouth sores	Raw
61	*Tribulusterrestris*L.	Nerunjimul	Zygophyllaceae	Fruit	Cough	Juice
62	*Trichodesmaindicum*L.	Kavilthumbai	Boraginaceae	Leaf	Diarrhea Dysentery	Decoction Decoction
63	*Tridaxprocumbens*L.	Kinathupoondu	Asteraceae	Leaf	Wound healing	Paste
64	*Waltheriaindica*L.	Shengalipoondu	Sterculiaceae	Whole plant	Skin diseases	Paste
65	**Climbers** *Abrusprecatorius*L.	Kundumani	Fabaceae	Root	Cough Cold	Decoction Decoction
66	*Aristolochiabracteolata*Lam.	Aaduthinnappalai	Aristolochiaceae	Whole plant	Skin diseases Snake bites	Decoction Decoction
67	*Canavaliagladiata*DC.	Segapputampattai	Fabaceae	Fruit	Itching	Paste
68	*Canavaliamollis*W. & A.	Segapu	Fabaceae	Leaf	Wound healing	Paste
69	*Cardiospermumcanescens*Wall.	Mudakkathan	Sapindaceae	Whole plant	Rheumatism	Paste
70	*Cardiospermumhalicacabum*Linn.	Mudakkathan	Sapindaceae	Whole plant	Swellings Joints pains	Paste Paste
71	*Cissampelospareira*L.	Ponmusutai	Menispermaceae	Leaf	Malarial fever	Decoction
72	*Cissusquadrangularis*L.	Perandai	Vitaceae	Stem	Stomachache Swelling	Decoction Paste
73	*Clitoriaternatea*L.	Thuthi	Fabaceae	Whole plant	Fever	Decoction
74	*Cocciniaindica*Wight &Arn.	Kovaikai	Cucurbitaceae	Leaf	Diabetes	Powder
75	*Gymnemasylvestre*R. Br.	Sirukurinjan	Asclepiadaceae	Leaf	Diabetes	Powder
76	*Hemidesmusindicus*R. Br.	Nannari	Asclepiadaceae	Root	Fever	Decoction
77	*Kedrostisfoetidissima*(Jacq.) Cogn.	Nurakvel	Cucurbitaceae	Leaf	Asthma	Decoction
78	*Leptadenia reticulata* Eetv.	Palaikkodi	Asclepiadaceae	Whole plant	Fever	Decoction
79	*Pergulariadaemia*(Forsk.) Chiov.	Veliparuthi	Asclepiadaceae	Leaf	Cold Fever	Decoction Decoction
80	*Piper nigrum*L.	Kurumilagu	Piperaceae	Seed	Cough Throat infection	Powder Powder
81	*Solenaamplexicaulis*(Lam.) Gandhi.	Pulivanchi	Cucurbitaceae	Leaf	Diabetes	Decoction
82	*Toddaliaasiatica*L.	Kindumullu	Rutaceae	Leaf	Stomachache	Decoction

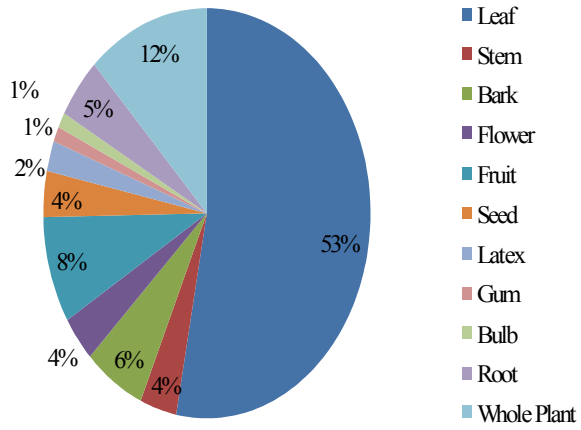

Fig. 3. Per-cent plant parts used for medicine preparation.

alphabetically and provided the botanical name of the plant, family, specimen number, local (Tamil) name, life form, part (s) used, ailments treated and mode of administration (Table-1).

Table 1.List of medicinal plant species used for their health care by Irulatribals of Palamalai hills, the Western Ghats of Coimbatore, Tamil Nadu.

Herbs were the primary source of medicine (34%) followed by trees (28%), climbers (22%) and shrubs (16%) (Fig. 2). The frequent use of among the indigenous communities is a result of wealth of herbaceous plants in their environs (Tabuti*et al.,* 2003; Uniyal*et al.,* 2006; Giday*et al.,* 2010) and a Yercaud hills harbours more number herbs as compared to trees, shrubs and climbers (Parthipan*et al.,* 2011). Among the different parts used, the leaves (53%) were most frequently used for the preparation of medicine solely or in combination with other parts. It was followed by whole plant (12%), fruit (8%), bark (6%), root (5%), stem, flower and seed (4% each), latex (2%), gum and bulb (1% each)(Fig. 3). Many indigenous communities throughout the world also utilized mostly leaves for the preparation of herbal medicines (Teklehaymanot*et al.,* 2007; Cakilcioglu and Turkoglu, 2010; Gonzalez *et al.,* 2010). The reason why leaves were used mostly is that they are collected very easily than underground parts, flowers, fruits, etc. (Ganesan*et al.,* 2004; Giday*et al.,* 2009; Rajaduraiet *al.,* 2009).

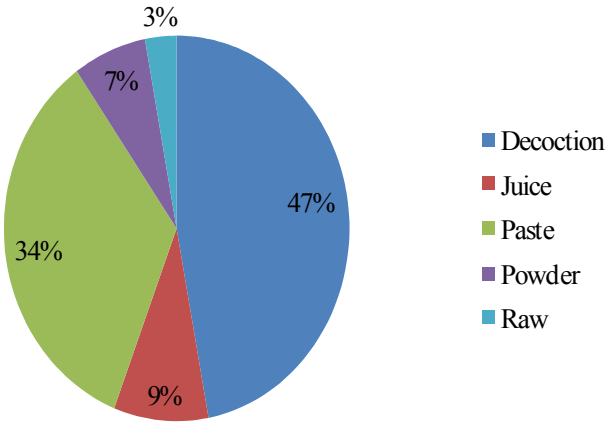

Fig. 4. Per-cent form of medicine preparation by Irula tribe.

The preparation and utilization of plant parts were grouped in to five categories (Fig. 4). Of these, most commonly used method preparation was decoction (47%) followed by paste (34%) juice (9%), powder (7%) and raw (3%). Preparation of paste for the treatment of ailments is a common practice among the tribal communities in the world (Giday*et al.,* 2007; Roosita*et al.,* 2008; Giday*et al.,* 2010). The paste was prepared by grinding the fresh or dried plant parts with oil or water. The powder was prepared by grinding of shade dried plant parts. The decoction was obtained by boiling the plant parts in water until the volume of the water reduced to minimum or required amount. The inhalation was done by the burning of plant parts and inhaled the smoke through nose or mouth (Robert and John, 1983; Shanley and Luz, 2003; Roosita*et al.,* 2008; Kaur *et al.,* 2011).

The ethnomedicinal studies evidently pointed out that, instead of trying to identify the active compounds and pharmacological actions of plants through massive collection of plants from natural sources, it is better to start investigating the efficacy of the plant based on their use in folk medicine, since most of the commercially proven drugs used in modern medicine were initially tried in crude form in traditional of folk healing practices (Fabricant and Fransworth, 2001).

CONCLUSION

All the enumerated plant species are very commonly used for various ailments by the Irula tribes of Palaalai hills, southern Western Ghats of Coimbatore district, Tamil Nadu. A few interesting observations made in the present study are: the use of *Coccinia indica* and *Pongamia pinnata* for diabetes, *Vitex negundo* for cardiac disorders, *Carica*

papaya and *Indigoferatinctoria* for poisonous snake bites, *Cardiospermumhalicacabum*for joints pains, *Cassia auriculata*for scabies and bone fractures.Although traditional medication is still practiced in this area, it is now fast disappearing due to modern life style. Hence, proper documentation and preservation of traditional skills and technology of medicinal plants is a vital necessity. Further investigations on pharmacological importance of these plants and their diversity may add new knowledge to the traditional medical and cultural systems.

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Hydroxyapatite Loaded-PEG Reinforced-Chitosan–co-Alginate Hydrogels as Templates for Bone Tissue Engineering

ANITHA RADHAKRISHNAN, SREEKANTH SREEKUMARAN AND MURALEEDHARAKURUP G

Department of Biochemistry, University of Kerala, Karyavattom, Thiruvananthapuram, Kerala, India.
email:gmkbio@gmail.com

ABSTRACT

ECM mimicking biomaterials scaffolds for bone tissue regeneration has gained potential applications in the management of end stage complications associated with various bone disorders. The bone tissue engineering strategies with various natural, synthetic and hybrid hydrogels have been well appreciated. Exploitation of the combinatorial effects of these materials for designing multi-task hydrogels is the major focus of the study. A novel hydroxyapatite (HA) loaded poly ethylene glycol (PEG) reinforced cross linked hydrogel scaffolds (HA-PCA and HA-PCAG) based on the co-polysaccharide of alginate and chitosan were designed. HA-PCA was synthesized by cross linking with Ca^{2+} at the alginate fraction and gluteraldehyde cross linking on HA-PCA hydrogel scaffold leads to the synthesis of HA-PCAG. Both the hydrogels exhibited promising physiochemical properties as evident from water content and water holding capacity, porosity, surface contact angle values and hydrophilicity. The scaffolds were hemocompatible and able to adsorb appreciable plasma proteins on to the surface. MTT assay on hydrogel extracts and direct contact assay revealed the cytocompatibility whereas live/dead assay using ethidium bromide/ acridine orange cocktail confirmed the healthy cells growing on the hydrogel networks even after 5 days of initial seeding. The proliferation of human osteoblasts on the scaffolds and increased collagen deposition substantiates their role as potential scaffolds for cell growth and as HA delivery vehicle for bone regeneration. Both the hydrogels can be promising candidates for bone tissue engineering and regeneration owing to their biocompatibility and physiochemical features.

Key words *Scaffolds; chitosan; alginate; hydroxyapatite; ECM mimic.*

Bone disorders are one of the widespread global health issues affecting the population of all ages, especially for the elders. An estimated two million bone sufferers were reported annually in United States and the health care costs around $20 billion per year (Wang *et al.,* 2014). The report describes the situation in US alone and the ratio will increase drastically when world population is considered. In fact, this statistics demands the need for novel bone reconstruction therapeutic strategies for the management of bone disorders. Even though autografting has been considered as a standard procedure, the unavailability and donor site morbidity are challenging (Sen and Miclau, 2007). On the other hand, allografting is also limited due to the risk of inflammation and infection (Flierl *et al*., 2013). Engineering of bone tissue is being explored to be a promising alternative to overcome these difficulties. Bone tissue engineering (BTE) is a combination of biomaterial scaffolds with micro-architecture mimicking the bone tissue, specific cells and signaling molecules (Steinert *et al*., 2012).

The inspiration for the microarchitecture scaffolding is attributed to the microstructure of native bone tissue. The native bone is composed of complex organic and inorganic entities bound together in a co-ordinated fashion (Kadler *et al.,* 1996). The organic part is mainly composed of biomolecules and a lion's share constituted by type I collagen (Kadler *et al*., 1996). The inorganic part mainly consists of hydroxyapatite (HA) crystals in the nano scale dimensions (Landis and Silver, 2009, Su *et al*., 2003). HA, a form of calcium phosphate is biologically active and can form mechanical interlocking with bone tissue (Dorozhkin, 2010). HA is well known for its excellent biocompatibility, mechanical compatibility and injectability for minimal invasive applications. It has been used for bone tissue engineering and several related applications in the form of bone cements, bone composites and scaffolds in combination with polymeric materials and cross linkers (Grover *et al*., 2013). Being a native bone constituent HA has become an inevitable component for bone tissue engineering scaffolds.

An ideal bone substitute should be a porous matrix to promote bone ingrowth and at the same time it should have sufficient mechanical strength to bear physiological loads during the course of healing process. It should provide sufficient cues to bond with the cells in the host tissue and pave way for cell migration and penetration (Deville *et al.,* 2006). HA

is biocompatible, non-toxic, non-inflammatory, non-immunogenic as well as osteoconductive. Still, the hardness, fragility, difficulty in remodeling and lack of flexibility makes its molding to specific implantable form strenuous, which limits the long term load bearing applications of HA (Sun *et al.,* 2011). These difficulties can be overcome by combining HA with biocompatible polymers so that the osteophilicity of the system can be retained (Zhang *et al*., 2014).

Hydrogels form ideal scaffolds for various tissue engineering applications due to their excellent compatibility imparted by the abundant water content in the bulk. Apart from their soft tissue engineering applications, hydrogels have been widely employed for hard tissue like bone owing to their capability to recreate complex interactions between the cells and their micro niche. Such a scaffold material can enhance the angiogenesis and healing process (Pandit *et al*., 2013). Injectable *in situ* cross linking hydrogels were commonly used for bone tissue engineering as these can act as a delivery vehicle for several osteoconductive cues. Hydrogels prepared from natural sources like collagen, gelatin, alginate, hyaluronic acid, etc. were extensively used for bone regeneration and engineering (Alves *et al*., 2012). But their mechanical incompatibility is challenging.

Chitosan, a natural cationic polysaccharide derived from the exoskeleton of crustaceans, is biodegradable and biocompatible and also has been hailed for its *in vitro* and *in vivo* osteoconductive effects (Li *et al*., 2005). Chitosan is a linear semi-crystalline polysaccharide composed of *N*-acetyl D-glucosamine and D-glucosamine units. The biological performance of chitosan based scaffolds is imparted by the presence of amino groups in the structure. The amino groups help to complex with other functional groups and metal ions. Moreover, the presence of –OH groups in the chitosan chains enable to form stable bonds with other species (Croisier and Jerome, 2013). Chitosan based scaffolds have been shown to possess excellent osteogenic properties applicable for designing bone substitutes. The brown sea weed derived poly anionic saccharide alginate also gained significance in tissue engineering and drug delivery applications owing to its biomimetic character. It is a linear block copolymer comprising repeated units of (1-4)-linked-D-mannuronic acid (M units) and (1-4)-linked- L-guluronic acid (G units) residues. Alginate can form instant hydrogels by chelating divalent ions like Ca^{2+} with its G residues (Lin and Yeh, 2004). Co-polymers of alginate with various natural and synthetic polymers has been employed for the fabrication of scaffolds for bone regeneration (Venkatesan *et al*., 2014). Still the hydrogel scaffolds made from these two polysaccharides fail in mechanical properties which limit their long term applications for load bearing tissues. Improved mechanical properties and porosity can be achieved by reinforcing these polysaccharides with biocompatible and slow degrading synthetic polymers.

In the present work, we have introduced a novel approach in creating a hybrid biofunctional matrix. The natural polymers alginate and chitosan were reinforced with Poly ethylene glycol (PEG) to provide sufficient mechanical integrity and HA was uniformly dispersed. Poly ethylene glycol (PEG) is an FDA-approved highly compatible synthetic linear polymer which has been widely used for several biomedical applications (Tan and Marra, 2010). Such a hybrid system incorporating alginate, chitosan and PEG has the advantage of biocompatibility along with sufficient mechanical stability. Based on this hypothesis, the present article aimed to synthesize, characterize and evaluate dual functional hydrogel scaffolds loaded with HA and interpenetrating PEG with a co-polymer of alginate and chitosan for bone tissue engineering applications.

MATERIALS AND METHODS

Materials

Sodium alginate (medium viscosity) from brown algae, chitosan (low molecular weight, degree of deacetylation (75–85 %) and hydroxypatite powder were obtained from Sigma-Aldrich (SpruceStreet, St. Louis, USA). Polyethylene glycol 4000, disodium hydrogen phosphate, sodium chloride and calcium chloride were supplied by Merck specialities (Pvt. Ltd,Mumbai), India.

Synthesis of the hydrogel scaffolds

The PEG reinforced Chitosan-co-Alginate co-polymer (PCA) hydrogel was synthesized by condensing the polysaccharides chitosan and alginate in presence of acid catalyst and PEG was uniformly interpenetrated. A hard mass so formed was then dissolved in minimum distilled water (1 ml for 1 g PCA) to form a viscous solution of PCA co-polymer.To 5g PCA solution 0.1g HA, 5ml 1% Na_2HPO_4 and 3ml

2% $CaCl_2$ were added, stirred to mix uniformly and casted overnight at 60°C. The sheet so formed was then subjected to additional cross linking with 10% $CaCl_2$ to form PEG reinforced Chitosan-co-Alginate co-polymer hydrogel scaffold (HA-PCA). Gluteraldehyde cross linked HA-PCAG was synthesized by adding 1% gluteraldehyde to the same reaction mixture for HA-PCA and subsequent steps were repeated. All the hydrogels were washed in distilled water, freeze dried, steam sterilized and stored aseptically for further evaluations.

Physiochemical characterizations

ATR-IR spectral analysis: ATR spectrum of HA-PCA and HA-PCAG were recorded by using Nicolet 5700 FTIR Spectrometer on freeze-dried samples.

SEM Analysis: The Scanning electron micrograph of the hydrogels were obtained by Field emission scanning electron microscope (Nova Nano SEM 450).Freeze dried hydrogel disks of 1cm diameter were mounted on aluminium studs and gold coated by plasma drying.

Water contact angle measurements: Surface hydrophilicity of both the hydrogels was determined by contact angle measurements. The water swollen hydrogels of rectangular dimensions were used for the evaluations. The advancing and receding contact angle measurements of these water swollen hydrogels were determined using KSV sigma 701 tensiometer employing Wilhelmy method (Thankam and Muthu, 2014b).

Mechanical Testing: Water swollen hydrogels were tested for their tensile properties using the universal automated mechanical test analyzer (Instron, model 3345, M/S Instron India Pvt Ltd, and Chennai, India). The samples were tested with a load cell of 100 N at 25°C with a crosshead speed of 5 mm/min. The data were calculated and recorded using Bluehill software (Thankam and Muthu 2014a).

Water content and holding capacity: For the determination of water parameters freeze dried samples (1cm x 1cm, n=6) of HA-PCA and HA-PCAG of known weight were swelled in distilled water. The wet weight of the swollen samples was measured at 30 minutes interval until equilibrium was reached. Then equilibrium water content and weight % swelling of the hydrogels were determined (Gnanaprakasam Thankam *et al*., 2013b).

Porosity of hydrogels: Ethanol replacement method was employed for the calculation of porosity. For the measurement, freeze dried hydrogels discs were soaked in absolute ethanol overnight (Gemeinhart *et al*., 2000).

Biostability of hydrogels: The stability of hydrogels was analyzed qualitatively in the cell culture medium DMEM containing 10% FBS at 37°C and pH 7.4 for 7 days. The long term degradation of the hydrogels was determined in the simulated biological fluid, PBS at physiological pH and temperature. To determine the extent of degradation, the weight loss and changes in pH were determined at an interval of 7days (Finosh and Jayabalan, 2015).

HEMOCOMPATIBILITY EVALUATIONS

Hemolysis assay: RBC suspension was collected from healthy human volunteers and washed twice with physiological saline. Both the hydrogel samples were extracted in sterile PBS for 48hrs. 100il of this PBS was mixed with 100il dilute RBC suspension and incubated at 37°C for 3hrs. A +ve control was set up with 100il sterile distilled water and a –ve with 100il 0.9% saline and both were mixed with equal volume of RBC suspension. After incubation it was subjected to centrifugation at 2000rpm for 10min and the OD of the supernatant was determined at 541nm. From the OD values the % hemolysis was calculated (Finosh *et al.,* 2015).

Red blood cell (RBC) aggregation assay: 1ml RBC suspension was diluted to 10ml with saline. Then 100µl hydrogel extract was added to 100ul diluted RBC and incubated at 37°C for 30min and examined under bright field microscope to evaluate RBC aggregation (Gnanaprakasam Thankam *et al*., 2013b).

Adsorption of plasma protein: 1ml diluted blood plasma (1 in 9 ml PBS) was added to PBS-swollen hydrogels and incubated for 1h at 37°C in a shaking incubator. The proteins, removed from the scaffolds, were quantified by Folin's method. The type of plasma proteins adsorbed on to the hydrogels was resolved by SDS-PAGE analysis. The adsorbed proteins were removed by vortexing, denatured and electrophoresis was done in 10% acrylamide gel with 65V for stacking gel and 130V for resolving gel. After the electrophoresis the gel was stained with coomassie brilliant blue dye for overnight, then destained and the image of the resolved gel was acquired by a scanning

system. A well was loaded with bovine serum albumin as control marker for plasma albumin adsorption (Gnanaprakasam Thankam and Muthu, 2013).

Biological evaluation of hydrogels

Cell culture: L929 fibroblast cells were used for biological evaluations. The cells were procured from NCCS, Pune, India and maintained in DMEM containing 10% fetal bovine serum and antibiotics (penicillin, streptomycin and amphotericin) in a humidified incubator with 5% CO_2. The cells were allowed to attain around 80% confluence, which were trypsinized and passaged. Media was changed once in 3 days.

Cytotoxicity assay: The cytotoxicity of hydrogels were determined by standard MTT assay on hydrogel extracts and direct contact assay by following previously published protocols (Thankam and Muthu, 2014a).

Live/dead assay: The fate of L929 fibroblasts in all the hydrogels were determined by live-dead assay using ethidium bromide (EtBr) and acridine orange (AO) as per the previously published protocols (Thankam and Muthu, 2014b.). Around 2 x 10^5 cells were seeded onto the hydrogels and allowed to grow for 5days in DMEM supplemented with 10% FBS. Then the hydrogels were washed twice in PBS and added 2 mL EtBr/AO mixture (1:1) to make a final concentration of 50µg/ml for each dye. After addition the excess dye was washed with PBS and immediately observed under a fluorescent microscope connected to a CMOS camera attached to a computer.

Responses of hydrogels for BTE

Osteoblast infiltration assay: The scaffold samples (1cm diameter) were used for studies. Initially, all the hydrogels were incubated in DMEM at 37°C in a CO_2 incubator for 24h. Then the medium was removed and 100µl HOS cell suspensions containing 3x10^5 cells was added on the scaffolds and kept undisturbed in CO_2 incubator at 37°C for 45 minutes for cell attachment to the scaffold. After the attachment 1ml cell culture medium was added carefully through the wall of the wells. The cell infiltration was quantified by modified MTT assay after one week. The cell grown scaffolds were taken out from the wells, washed twice with PBS and incubated for three hours with 1mL MTT solution (1mg/ml in PBS) under standard cell culture conditions. After incubation the scaffolds were extracted with isopropanol containing 0.01N HCL, vortexed for 10 minutes, kept for 30 minutes, centrifuged at 10000 rpm for 5 minutes to settle the cell debris and scaffold. The OD of the supernatant was read at 570 nm and used for viability calculation. A control containing cells without scaffolds and blank containing scaffolds without cells were also maintained in the same manner (Gnanaprakasam Thankam and Muthu, 2013).

Determination of collagen deposition on hydrogels

The collagen deposited on the hydrogel scaffolds by L929 cells grown for 10 days was determined by Sirius red method. The cell grown scaffolds were incubated at room temperature with a solution containing 0.1% of Sirius red in saturated picric acid for 1 hour. The unreacted stain was removed by washing extensively with 0.01 M HCL until the HCL become colourless. The stained collagen along with the hydrogel scaffold was then dissolved in 0.1 M NaOH, spun at 7000 rpm for 5 min and the OD of the supernatant was read at 530 nm. A control without scaffolds and blank containing scaffolds without cells were also treated in the similar manner. From the OD values, the percentage variation in collagen was calculated (Thankam and Muthu, 2015).

Statistical analysis

All experiments consisted of 3 to 6 samples from each group. The values are presented as means ± standard deviations. Statistical analysis was done with one way ANOVA using online calculator, Statistics Calculator version-3 beta and the level of significance was set at P value < 0.05.

RESULTS AND DISCUSSIONS

Synthesis of HA loaded PEG reinforced Chitosan-co-Alginate cross linked hydrogel scaffolds

In vitro fabrication of biological tissues often requires functional cues for the desired application. Co-polymer based hybrid scaffolds from natural polymers like alginate and chitosan with incorporation of HA simulates the composite structure of native bone tissue. In addition, the mineralized tissues like bones requires HA as a natural component. The natural bone

is a complex of organic molecules like proteins and inorganic moieties like HA. HA present in bone is chemically $Ca_{10}(PO_4)_6(OH)_2$ which forms a layer by layer hierarchical architecture (Kikuchi *et al.,* 2001). The presence of Ca and P in HA contribute its capability for inducing both intra and extra cellular responses (Marcacci *et al*., 2007). These qualities make HA a crucial component in bone tissue engineering scaffolds. So, the incorporation of HA with compatible biopolymers can mimic and sustain a natural environment for the formation of bone tissue (Mi *et al*., 2002).

Hybrid scaffolds prepared from Alginate and Chitosan has been shown to provide physiological cues for bone tissue regeneration (Venkatesan *et al*., 2015). Furthermore, HA incorporation to these hydrogels enhanced the biocompatibility and osteoconductivity (Jin *et al*., 2012). Addition of nano-HA enhanced biomineralistaion and osteo-inductive properties of alginate –chitosan copolymer scaffolds (Hye-Lee Kim *et al*., 2015). We have synthesized two HA loaded hybrid polymeric systems for bone tissue engineering applications. The polymers that we employed were, of natural (alginate and chitosan) and synthetic (PEG) origin, hailed for their excellent biocompatibility (Fig. 1A, B and C).

Chitosan is ideal for bone related applications due to its osteoconductivity and intrinsic antibacterial potential. The lysosome mediated chitosan degradation, the tunability of degradation rate and non-toxic and non-immunogenic nature of the degradation products had raised the utility of this polysaccharide for various biomedical applications (Muzzarelli, 1993). Similarly, alginate has also been hailed for its easy gelation, biocompatibility and low toxicity. But, the mechanical instabilities of these two polysaccharides hinder their performance in tissue engineering applications (Shoichet, 2010; Finosh and Jayabalan, 2012). In order to address these issues, we co-polymerized alginate and chitosan under acid catalyst to form a co-polysaccharide. The condensation reactions were mediated by –OH and –COOH groups of alginate and –COOH and $–NH_2$ groups of chitosan. PEG was then physically interpenetrated along the co-polysaccharide network and the G residues of the co-polysaccharide were then cross linked with Ca^{2+}. The calcium cross linking physically reinforced the PEG to form a semi-interpenetrating network of PCA. HA is uniformly dispersed into the polymer network to form HA-PCA. The available end –OH groups of PEG and $–NH_2$ groups of chitosan were utilized for the cross linking with gluteraldehyde to form the interpenetrating network of HA-PCAG. In short, we have synthesized two hydrogel systems loaded with HA for bone tissue engineering applications which can act as a HA delivery vehicle and also as a template for bone tissue formation. Due to extensive cross linking and PEG interpenetration these hydrogels can acquire sufficient mechanical properties to be compatible with native bone tissue.

Physiochemical characterizations

ATR spectral analysis showed the surface functionalities of HA-PCA and HA-PCAG (Fig. 2).

The peak observed around $1600cm^{-1}$ for carboxylate groups and the broad peak at around $3400cm^{-1}$ revealed ample amount of hydrogen bonded hydroxyl groups. These peaks revealed the presence of calcium alginate units on the surface. The peak at $1720cm^{-1}$ for the C=O group stretching indicated the ester bond formation and the peaks around $1100cm^{-1}$ can be attributed to the C-O-C stretching of the alginate and chitosan. The peak around $630cm^{-1}$indicates OH stretching of HA. The peaks around $1400cm^{-1}$ correspond to carbonate groups of HA. The peak around $870cm^{-1}$ indicates phosphate groups. Therefore it was concluded that alginate fraction, chitosan fraction and PEG segments are present on the surface of the hydrogel scaffolds.

The mechanical stiffness of matrices for tissue engineering has profound impact on cell adhesion and differentiation. And, the establishment of tissue specific ECM demands mechanical compatibility of the implanted scaffold.Evaluation of the tensile properties of the hydrogel scaffolds showed an increase in Young's modulus for the gluteraldehyde crosslinked variant, HA-PCAG. The tensile strength of HA-PCA increased from 0.80 ± 0.16 MPa to 1.30 ± 0.029 MPa on crosslinking with gluteraldehyde indicating increased mechanical rigidity for HA-PCAG (Table 1).From the results it was very clear that, the interpenetration with PEG has significantly contributed to the rigidity of the hybrid hydrogels (Pramanik *et al*., 2015). Both *in vitro* and *in vivo* bone regeneration has been augmented by porous hydrogels with an optimum elasticity of 60kPa (Huebsch *et al.*, 2015).

Table 1. Physiochemical characterizations and biological evaluations of the hydrogels

Parameters (n=6)	HA-PCA	HA-PCAG
Contact angle (advancing)	29.5 ± 0.39	47.65 ± 1.6
Contact angle (receding)	38.96 ± 0.19	51.12 ± 1.1
Swelling (%) (P<0.001)	256.79± 40.39	222.57±29.84
EWC (P<0.05)	59.85±2.70	69.86±3.5
Tensile Strength (MPa)	0.80 ± 0.16	1.30 ± 0.029
Porosity (%)	65.10±2.13	64.84±0.99
Pore length (μm)	15.63±2.44	18.19±2.3
Hemolysis (%)	1.92±0.03	1.3±0.14
Protein adsorption (%)	32.36±2.37	16.90±2.08
Viability (%) L929	98.44±2.96	96.03±4.89
Viability (%) HOS	102.9±0.89	99.33±1.55
Collagen deposition(%OD)	53.69±6.16	23.82±3.4

Also, PVA/Collagen nanofibrous scaffolds containing nano-hydroxypatite with tensile strength of 1.03 MPa has been proposed for non-load bearing applications as a bone ECM mimic (Asran, Henning and Michler, 2010). Inspite of the limited mechanical rigidity of hydrogels they form ideal mimics of the ECM of bone (Chicatun *et al.*, 2011). Both HA-PCA and HA-PCAG hydrogels possess appreciable tensile strength for the establishment of bone specific ECM. Such mechanically rigid hydrogels are proposed as suitable templates for osteoblast attachment and differentiation.

The water content and water holding capacity of hydrogel scaffolds are given in Table 1. Both HA-PCA and HA-PCAG attained equilibrium at 90min (Fig. 3). The water content and swelling was appreciable for both the hydrogels. The swelling of hydrogels is influenced by the balance between the concentration of ionic groups and the counter ions present in the gel structure. The entropy imparted by the counter ions contributes to the generation of additional osmotic pressure which results in a swollen hydrogel (Gerlach and Arndt, 2009). In the case of a hydrogel with free –COOH group, the H^+ is released to the medium and combine with –OH to form water. Here, the electrical neutrality is maintained by this neutralization process. But, the dissociation of the ionizable groups, especially under physiological conditions, results in an increased cation concentration and subsequent generation of osmotic pressure. The solvation of polymer chains and mixing with water also results in swelling. If the contribution of osmotic forces is much higher, the swelling continues until the equilibrium is attained. In physiological buffers, the H^+ released from the regions of hydrogels containing highly concentrated –COOH groups is consumed by the buffer. These H^+ can be supplied to the regions with lower –COOH concentration. In short, the buffers provide more opportunities for the diffusion of H^+ ions which pave way to the hydrogels to swell.

Porosity and pore interconnectivity are essential characteristics of cellular scaffolds for directed cell growth and tissue remodeling. Being non-homogenous, the bone tissue exhibits varying degrees of porosity with cortical or compact bone showing 5–10% porosity and trabecular bone possessing 50–95% porosity. Adequate porosity in tissue engineering scaffolds aids cellular infiltration and promotes vascular penetration into the scaffold interior. Porous architecture of the scaffolds is one of the determinants of scaffold biofunctionalisation. The HA-PCA hydrogels yielded a porosity percentage of 65.10 and gluteraldehyde crosslinked hydrogels exhibited 64.84% porosity (Table 1). The optimal porosity for bone tissue regeneration depends on the type of biomaterial, their surface morphologies, and growth factors incorporated for obtaining the specific scaffold architecture.

SEM analysis revealed an average pore length of 15.63 ± 2.44ìm for HA-PCA and 18.19 ± 2.3ìm for HA-PCAG (Table 1). A well interconnected porous network is visible in the SEM micrographs of both the hydrogel scaffolds (Fig. 4). A pore size ranging from 20 to 1500ìm has been reported in various bone regenerative scaffolds (Loh and Choong, 2013). For instance, silk fibroin scaffolds with pore diameters ranging from 10 to 250 ìm have been shown to induce osteogenesis and neovascularisation in engineered human stem cells *in vivo* (Riccio *et al.*, 2012). Moreover, the controlled stretching in water swollen hydrogels has introduced unidirectional pore orientation, which enhanced the cellular responses and biomimeticity in Alginate based copolymer hydrogels (Thankam and Muthu, 2014b.). During cell seeding and attachment in suitable growth medium, the swelling of hydrogels causes the stretching of pores resulting in increased volume which facilitate cellular infiltration

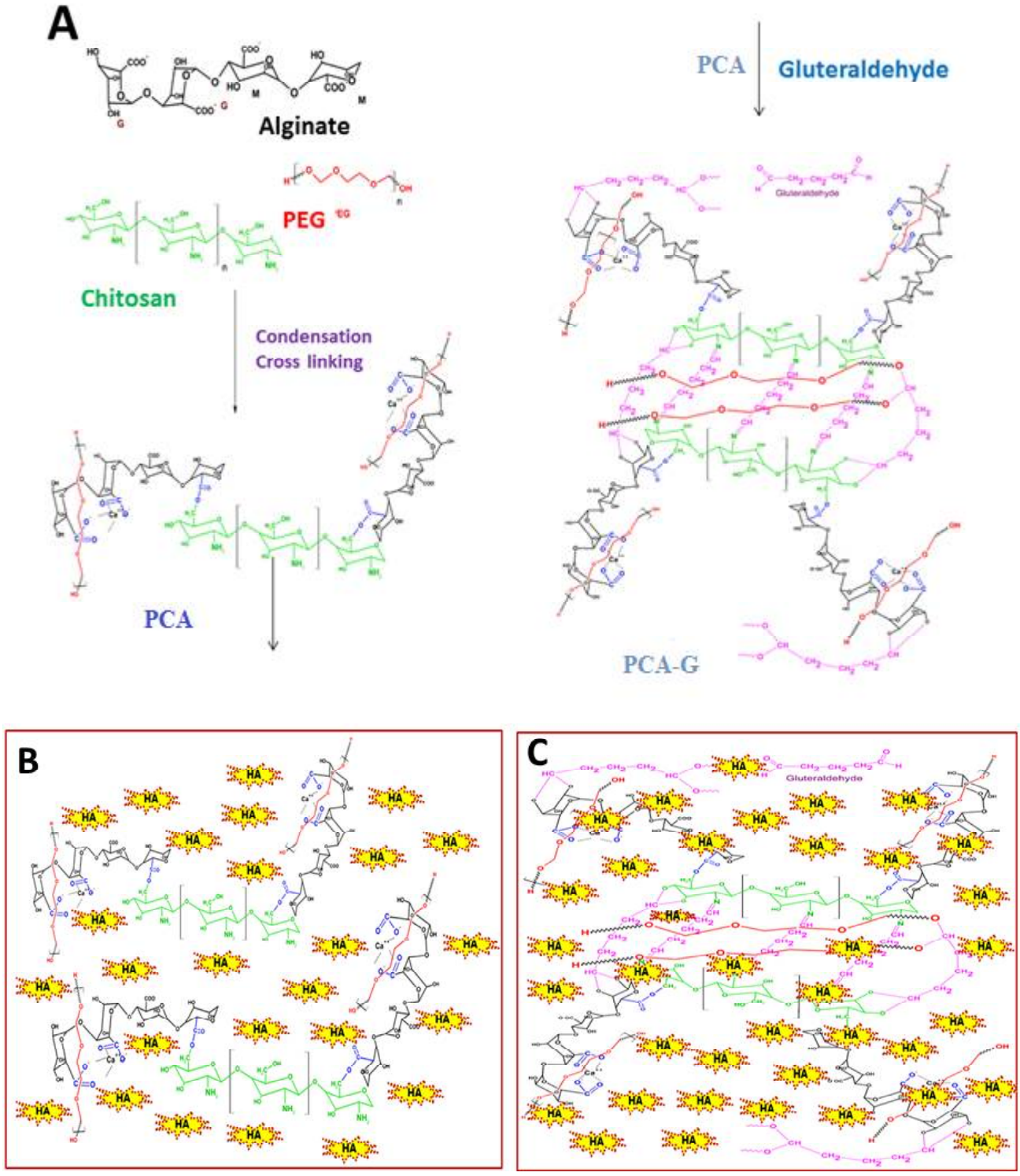

Fig. 1. Diagrammatic representation of Synthesis of (A) PCA hydrogels (B) HA-PCA (C)HA-PCAG

and growth.

The qualitative analysis of the biostability of the hydrogels was done in cell culture medium for a period of 7 days. Both the hydrogels were found to be stable and retained their shape and texture. Then the biodegradation was quantified by aging in PBS solution. The changes in dry weight and pH were monitored at a regular time interval of 7 days (Figs 5 & 6). The results revealed a progressive decrease in the dry weight of both the scaffolds during the course of time. And this decrease was an obvious indication of degradation or fragmentation of the scaffolds. The mechanism of degradation may be hydrolysis of ester bonds leading to the formation of acidic degradation

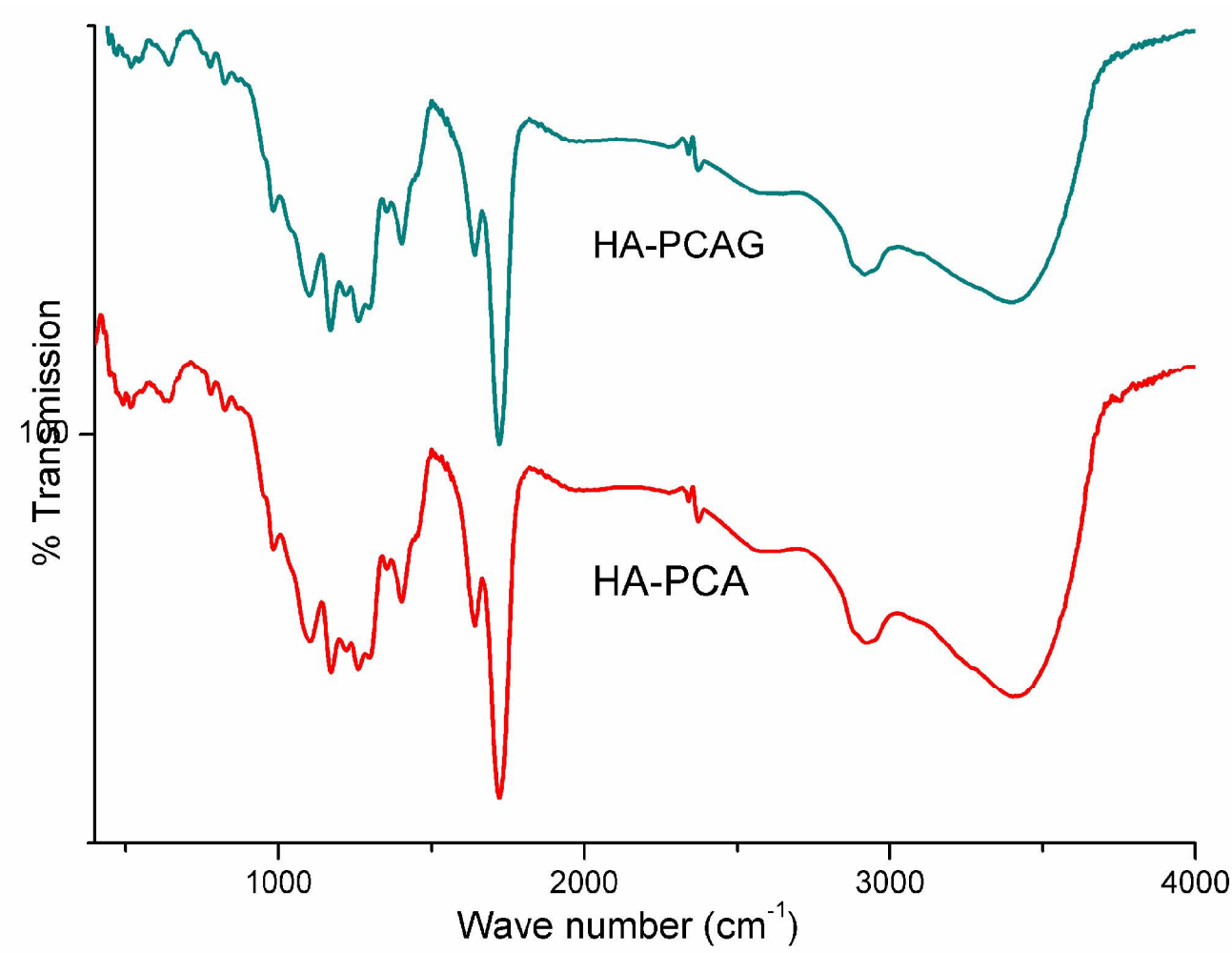

Fig. 2. IR spectral analysis of HA-PCA and HA-PCAG hydrogel scaffolds.

products which in turn accelerate further hydrolysis. This was evident from the slight drop in pH in the case of both scaffolds. This pH drop can be effectively buffered by the physiological buffers under *in vivo* conditions and the degradation products can be utilized by the body or eliminated through general circulation. Since the hydrogels were made from biological molecules, the chances of toxicity of degradation products are believed to be minimal (Gnanaprakasam Thankam *et al*., 2013a).

Hemocompatibility evaluations

Assessment of RBC integrity: The evaluation of hemocompatibility is one of the key tests for the blood contacting biomaterial implants. The biomaterials should not evoke any adverse reactions upon contact with blood. The possibility of RBC destruction can be evaluated by hemolysis and RBC aggregation assays. The percentage hemolysis of both our hydrogels were negligible and within the acceptable range of 5% (Table 1). This indicates that the chances of RBC lysis upon contact of our hydrogels with general circulation are minimal. And, the probability of hemolytic anemia, jaundice, thromboembolism and destruction of blood components associated with biomaterial implants is minimal. Similarly, the absence of RBC aggregation on contact with our hydrogels indicates the absence of rouleaux formation (Fig. 7), suggesting the minimal chances of obstruction to the normal flow of blood especially through micro capillaries. The studies on RBC clearly delineated the maintenance of normal blood rheology and RBC membrane integrity on contact of blood with both our hydrogels (Thankam and Muthu, 2013b).

Plasma protein adsorption studies: The amount of total plasma protein adsorbed on the hydrogel surface was quantified by Folin's method. The % of proteins adsorbed on the surface of HA-PCA was greater when compared with that of HA-PCAG. This may be due to the limited surface area and lower functional groups of the latter hydrogel due to extensive cross linking with gluteraldehyde. In order

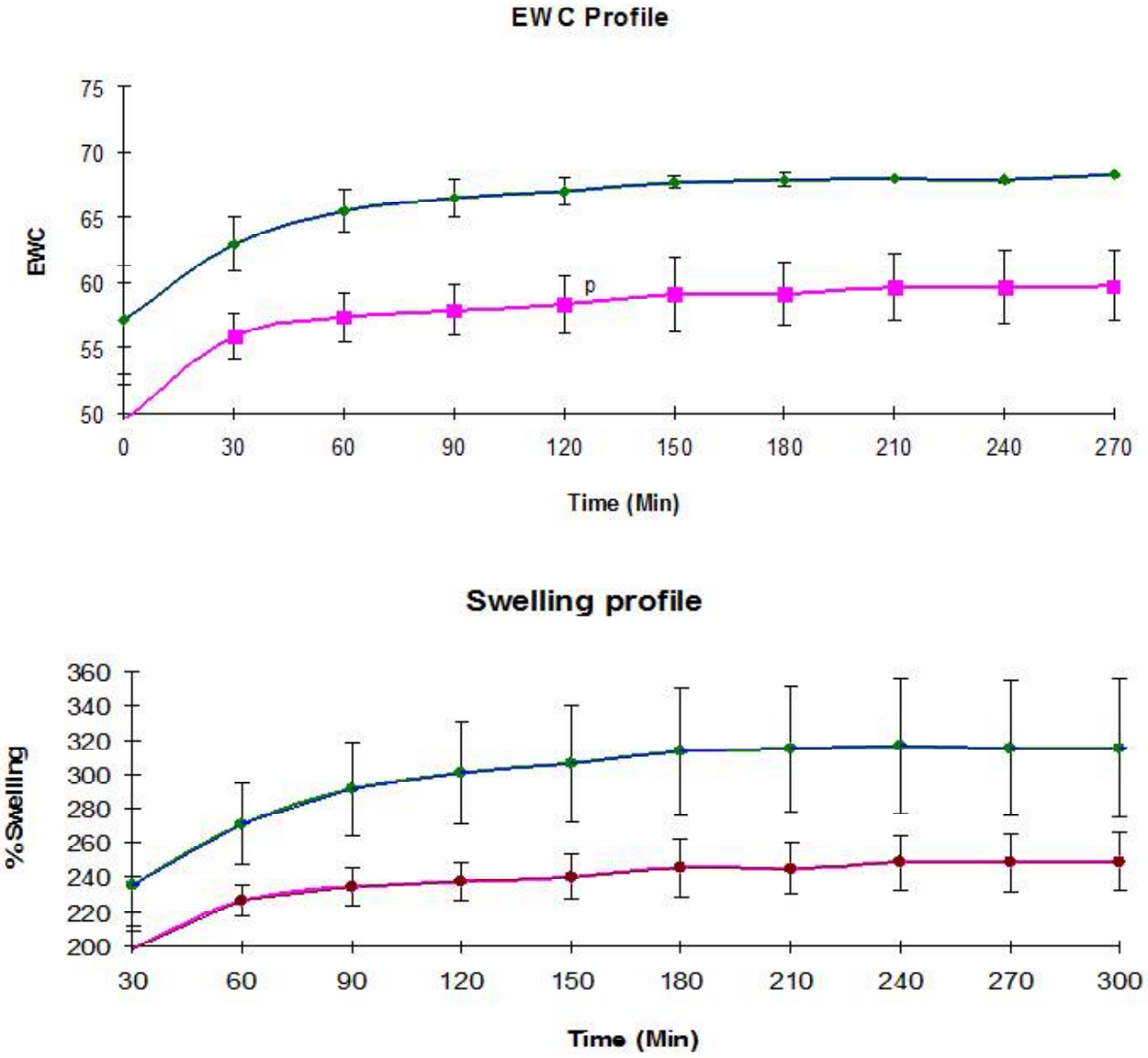

Fig. 3. Water holding capacity and swelling profile of HA-PCA and HA-PCAG hydrogel scaffolds

to evaluate the type of protein adsorbed on the hydrogels SDS-PAGE analysis was done. The band corresponding to that of albumin was prominent on both the hydrogel samples indicating albumin adsorption and passivation (Fig. 7). Upon implantation the hydrogels are subjected to interactions with water and protein from the blood plasma by a phenomenon called "Vroman effect". This is a rapid process which results in the formation of protein layer of nanometer thickness on hydrogel surface. In general, the protein adsorption on to the hydrogel depends on the surface chemistry.

HA-PCAG (B) hydrogels and SDS-PAGE analysis of plasma protein adsorption on both hydrogels showing thick bands corresponding to that of albumin.

The proteins at the interface of the hydrogel initially interact with the water and electrolytes followed by binding to the surface functional groups. The interactions of proteins with hydrogels are strictly non-covalent and mainly involve hydrogen bonding, van der Waals and electrostatic interactions. But, the extent of binding and the associated biological responses will depends on the type, charge, and conformation of the protein (Thankam and Muthu, 2013a : de Mel *et al.,* 2012). Among the major blood proteins, the adsorption of albumin on to the hydrogel surface was reported to be beneficial for tissue engineering applications as the adsorbed albumin layer contribute biocompatibility to the hydrogel (Guha Thakurta and Subramanian, 2011). Since both the

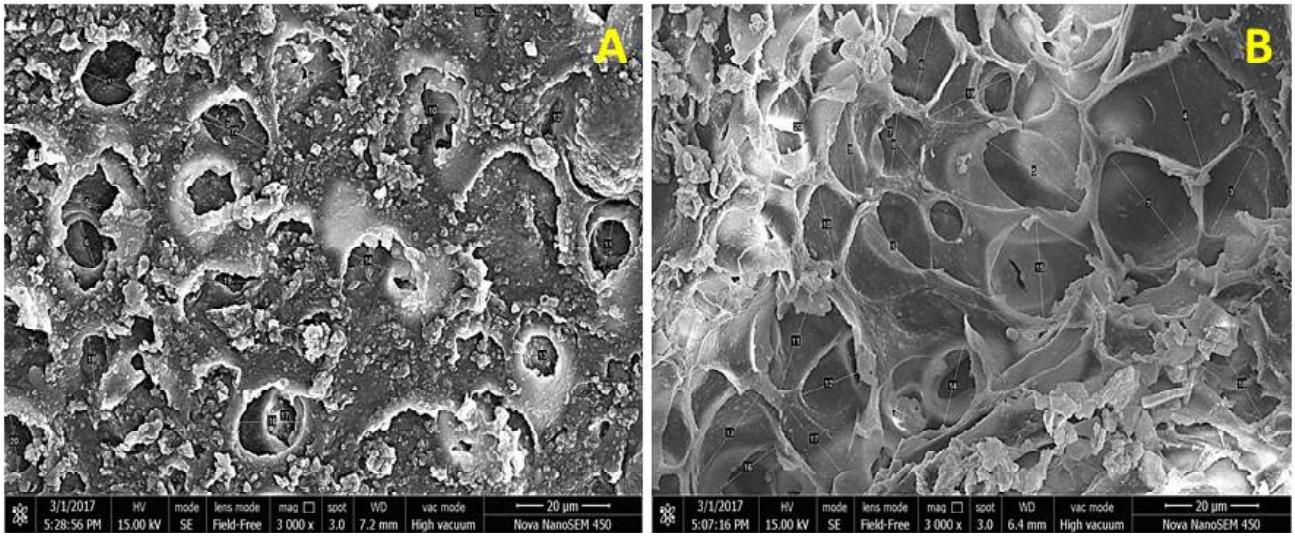

Fig. 4. FESEM micrographs of (A) HA-PCAG (B) HA-PCA.

hydrogels were hydrophilic and bear appreciable water content, the adsorption of albumin was favored on them.

Cytocompatibility Evaluations: The cytocompatibility of HA-PCA and HA-PCAG were evaluated by determining the metabolic activity of L929 fibroblasts grown on hydrogel extracts and also by the proliferation of cells on contact with the hydrogels. L929 cells grown in the extracts of both the hydrogels exhibited excellent metabolically active mitochondria and the viability was found to be greater than 96% (Table 1). From the MTT assay it was clear that the particles leaching out from the hydrogels were nontoxic to the cells. This was confirmed by the direct contact assay, where the cells retained their normal characteristic spindle morphology upon direct contact with both our hydrogels (Fig. 8). From this it was very vivid that both our hydrogels are able to support a long term cell growth which can be extrapolated *in vivo* for bone tissue engineering and regeneration (Thankam and Muthu, 2014a).

Live/dead staining of fibroblast cells grown on both the hydrogels displayed the green nucleus given by acridine orange (Fig. 8). The pore architecture was also evident in mild background fluorescence. Since AO is a permeable dye, it can easily cross the membrane barrier and intercalate the nucleic acid to display a green fluorescence when excited by blue

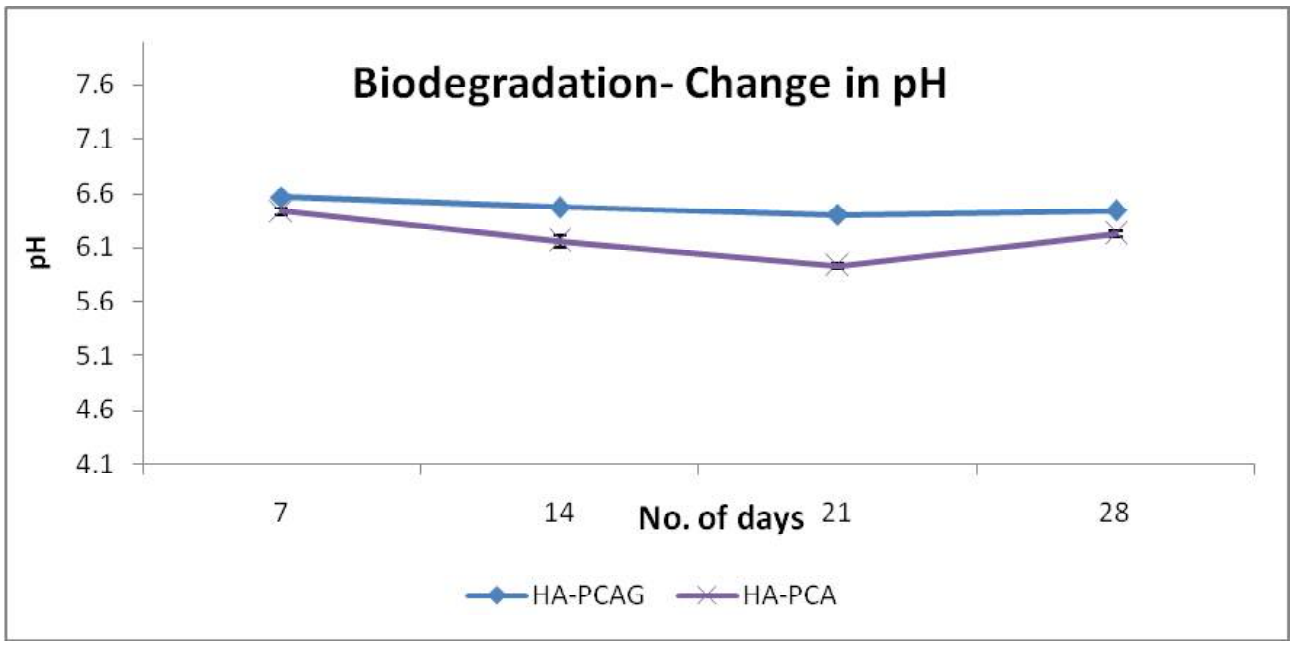

Fig. 5. Biodegradation profile of HA-PCA and HA-PCAG hydrogel scaffolds displaying changes in pH

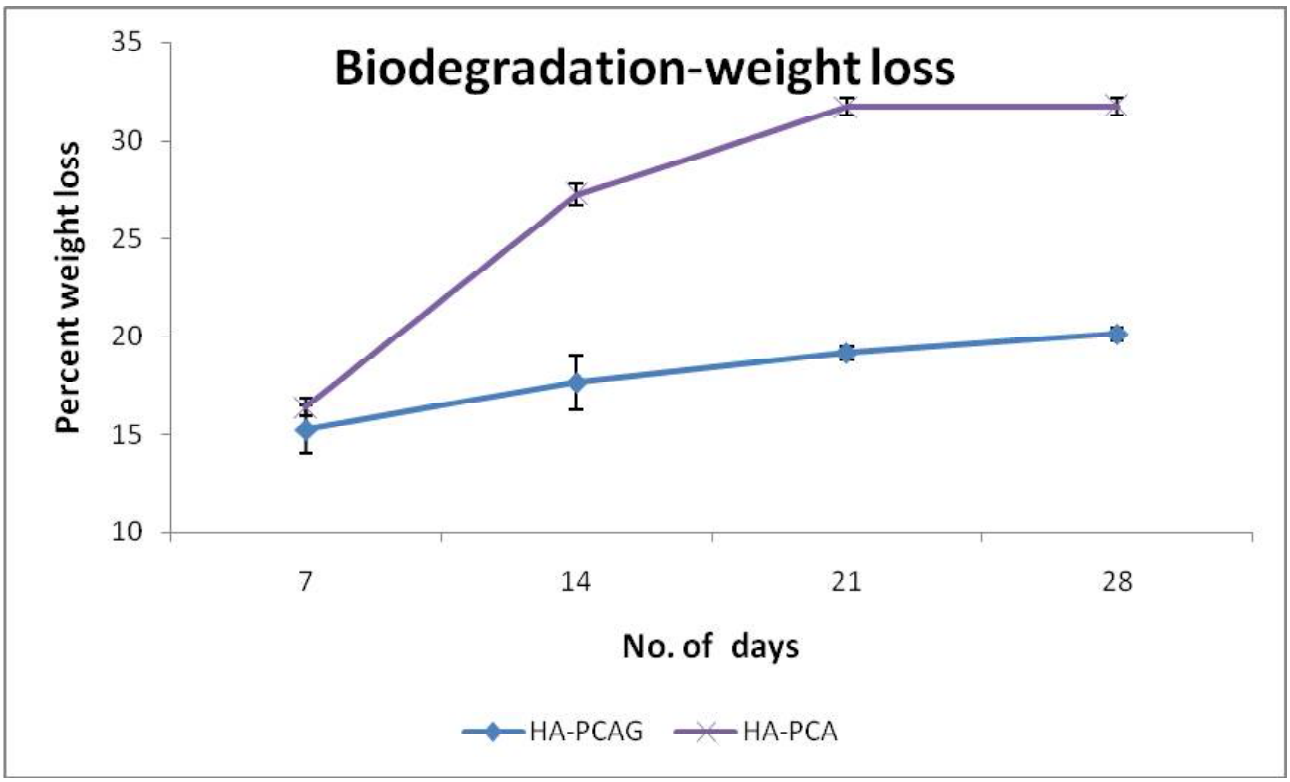

Fig. 6. Biodegradation profile of HA-PCA and HA-PCAG hydrogel scaffolds displaying progressive weight loss in physiological saline

light. On the other hand, being impermeable ethidium bromide (EB) can only enter the cells whose plasma membrane integrity is lost where it gives a red fluorescence. So, the presence of red nucleus is a sign of apoptotic cells and depending on the color intensity the health status of the cells can be evaluated. Most of the cells grown on our hydrogels displayed green nucleus indicating the cell friendly micro niche provided by the hydrogel scaffolds (Gnanaprakasam Thankam and Muthu, 2013).

Biological responses of HA-PCA and HA-PCAG to Support BTE

The viability of human osteoblast cells on the hydrogel scaffolds was also evaluated by MTT assay. An ideal template for bone regeneration should promote the proliferation of osteoblast cells. The viability percentage was found to be more than 90% indicating healthy proliferation of HOS cells on both the hydrogels (Table 1). The adherence and proliferation of bone forming cells is one of the basic requirements for bone regenerative scaffolds (Chen *et al*., 2016).

Establishment of a tissue specific ECM on biomaterial scaffolds provides biochemical cues for tissue regeneration. Collagen forms one of the major components of native extracellular matrix of the bone and plays significant role in tissue repair.L929 fibroblast cells grown on HA-PCA hydrogel showed significant increase in collagen deposition when compared to control cells as indicated by the increase in OD values.A 53% increase in collagen concentration was exhibited by HA-PCA whereas only 23% was shown by the gluteraldehyde crosslinked HA based CAP hydrogel (Table 1).The ability of the hydrogel scaffolds to direct ECM synthesis is confirmed by the above observation. Bone regeneration involves a cascade of co-ordinated events contributed by specific biological interactions. Collagen has been indicated in augmenting bone mineralization through apatite formation (Wang *et al*., 2012). Initial stages of bone repair involves increased rate of collagen synthesis and osteoblasts initiates the process of mineralization on collagen fibrils which acts as the template to further propagate bone matrix formation (Ferreira *et al*., 2012). Following tissue damage, deposition of collagen by fibroblasts initiates ECM remodeling but excess accumulation of collagen can lead to fibrosis leading to impaired matrix formation (Abraham *et al*., 2007). The process of bone remodeling establishes equilibrium

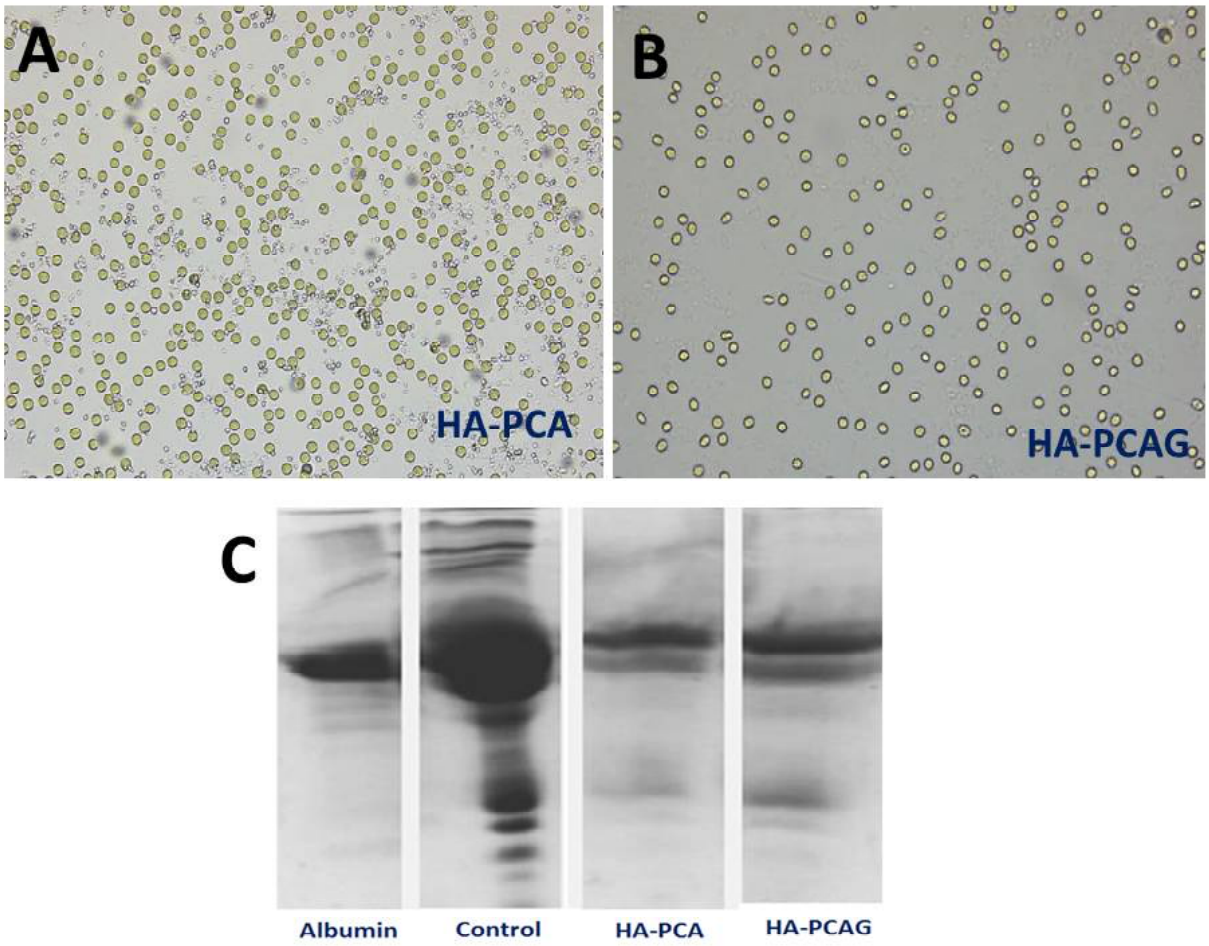

Fig. 7. RBC aggregation studies showing the absence of aggregation in HA-PCA (A), HA-PCAG (B) hydrogels and SDS-PAGE analysis of plasma protein adsorption on both hydrogels showing thick bands corresponding to that of albumin

between the rate of synthesis and degradation of collagen. Both our scaffolds have been shown to augment collagen production by fibroblasts cells, strongly indicating their role in modulating cellular interactions at the scaffold tissue interface for the establishment and maintenance of tissue specific ECM.

The current article deals with the preliminary evaluations on the compatibility of HA loaded PEG reinforced hydrogels as scaffolds for the engineering of mineralized tissues like bones. Reinforcement with PEG imparted the retention of biocompatibility of natural polysaccharides like alginate and chitosan and at the same time enhanced the mechanical compatibility too. The availability of Ca^{2+} in the hydrogels (in the form of HA) maintain the cross linked form of alginate by shielding it from Na^{+}-Ca^{2+} exchange. The gluteraldehyde cross linking on HA-PCAG impart additional strength and retention capacity to the loaded HA. So this hydrogels will release Ca^{2+} and HA in a comparatively slower than HA-PCA. These properties make HA-PCAG to be excellent for long term BTE and the reverse will be apt for HA-PCA (for short term applications due faster rate of HA release). In short, both the hydrogels can perform dual functions, as HA carrier and as scaffold for cells and signaling molecules, for bone tissue engineering. Still, more optimizations and evaluations are needed to validate this concept.

CONCLUSIONS

Copolymerized multicomponent hydrogel scaffolds have been fabricated as three dimensional microenvironments for cell adhesion and proliferation.HA-PCA and HA-PCAG hydrogels possessed appreciable physiochemical properties in terms of water content and hydrophilicity. Interpenetration of PEG conferred mechanical durability to the hydrogels. Additional crosslinking with gluteraldehyde has enhanced the tensile properties of

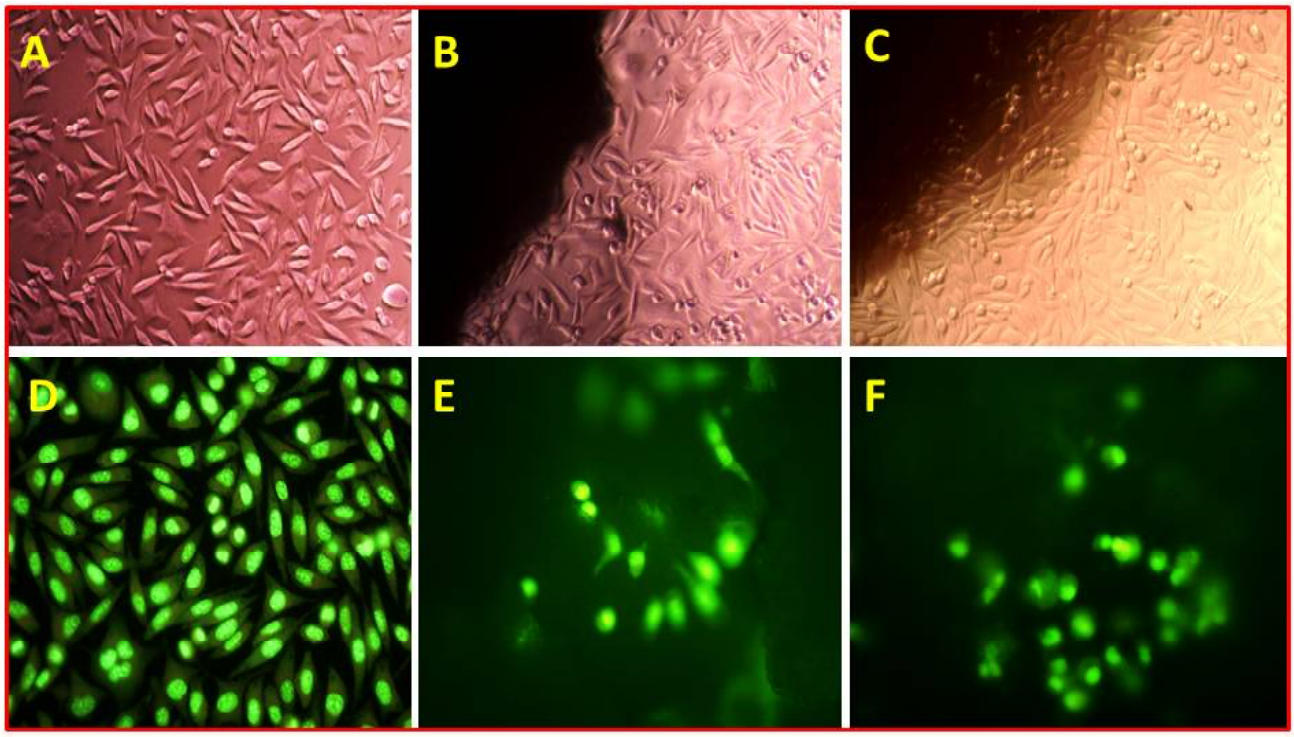

Fig. 8. Cytocompatibility evaluations of hydrogels. Direct contact assay on L929 cells—control (A), HA-PCA (B) and HA-PCAG (C) hydrogel scaffolds. Live/dead assay on L929 cells—control (D) HA-PCA (E) and HA-PCAG (F) hydrogel scaffolds

HA-PCAG, but resulted in less porosity. Both the scaffolds were found to be hemocompatible and cytocompatible. The hydrogels facilitated the growth of osteoblast cells as well as enhanced the synthesis of collagen by fibroblast cells. HA-PCA and HA-PCAG can simultaneously act as a template for tissue formation and vehicle for HA delivery and can be applied for engineering mineralized tissues like bone.

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Bioconversion of Water Hyacinth as an Alternate Substrate for Mushroom (*Pleurotus eous*) Cultivation

SHIRMILA JOSE G.[1*], ATHIRA S.V[2]., DEVIKA A.V[3]., AISHWARYA NAIR A.M[4], PARVATHY A.M[5]AND MERRY DON M[6]

[1], *Department of Botany, All Saints College, Thiruvananthapuram -695007,*
[2, 3, 4, 5, 6]*Students, Department of Botany, All Saints College, Thiruvananthapuram -695007Kerala, India.*
email : shirmijo@gmail.com,Kerala, India

ABSTRACT

This study investigate the utilization of water hyacinth as an alternative substrate for mushroom cultivation. The water hyacinth is pretty in riversand lakes due to its ability to double biomass rapidly and is considered as a menace on wateractivities in the lakes. Many strategies have been introduced such as physical removal, chemicaland biological for eradication with little focus on its economic value.Objective of this study is to cultivate oyster mushroom *(Pleurotus eous*), using novel combinations of water hyacinth compost, water hyacinth and paddy straw as a substrate. The yield data showed that the substrate of water hyacinth compost gave a significantly higher oyster mushroom production of 810g/1kg/dry. wt. followed by paddy straw bed. The lowest yield was obtained in waterhyacinth alone substrate beds.This upshot showed that the growth of edible mushroom shall help in its removal on one hand and food production on the other.

Keywords *Water hyacinth; Pleurotuseous; mushroom cultivation.*

Water hyacinth(*Eichhornia crassipes*) is an aquatic member of the pickerelweed family - Pontederiaceae. It was introduced into India as an ornamental plant nearly half a century ago (Oommachan,1971)**.** It spreads rapidly and is found practically all over the country. Itisa serious pest, covering by its dense growth vast surface of rivers, lakes and ponds. Water hyacinth has invaded paddy fields in some areas and rendered them unproductive (Zadrazil, 1980). It has resisted all attempt of eradication.

The city Thiruvananthapuram (Kerala) faces severe water logging due to drainage blocking by aquatic weeds, which was discussed in the "Operation Anantha report" (Shaji, 2017) . Although water hyacinth is seen in many canals of Trivandrum as a weed and is responsible for many problems. It prevents sunlight and oxygen from the water and dramatically reduce biological diversity in aquatic ecosystem. Proliferation of invasive water weeds and exotic species of fish had played havoc with the ecosystem of the canals which affecting back water tourism (Shaji, 2017). Many individual groups and institutes have been able to turn the problem around and find useful application for the plant. The plant though containing more than 95% water, has a fibrous tissue and high energy and protein content, and can be used for the variety of useful applications such as leaf protein and for the production of biogas (Mishra and Mishra, 2001).Oyakavaet al., (1968) utilized Water hyacinth as a substrate for the production of yeast, feeds and forages. Zadrazil (1977) reported the conversion of straw into feed by the members of *Basidiomycetes*(mushroom).

Mushroom is fleshy, spore- bearing fruiting body of a fungus with rich in protein constituting a valuable source of supplementary food. Use of mushrooms can contribute positively in facing the challenge of world-wide food shortage. The great value in promoting the cultivation of mushroom lies in their ability to grow on cheap carbohydrate materials in to protein rich edible material (mushroom fruiting body). Oyster mushroom can be cultivated in any type of lignocelluloses material like straw, sawdust, rice hull etc. Hami (1990) studied the oyster mushroom cultivation on sawdust of different woods; found that *P. ostreatus*gave the maximum yield in them.

In the present investigation, our objective is to cultivate oyster mushroom (*Pleurotus eous*), using novel combinations of water hyacinth and paddy straw as a substrate. Eradication of water hyacinth is a global problem and by using it as a substrate for the growth of edible mushroom shall help in its removal on one hand and food production on the other and that too at affordable cost.

MATERIALS AND METHOD

Experimental Design

Theexperiment was laid out in three replicates/ substrate (Paddystraw - control), water hyacinth and hyacinth compost). Water hyacinth (*Eichhornia crassipes*) collected from Veli and Vellayani lakes (Trivandrum) are used for this work

Preparation of mushroom culture tube

The fresh fruiting body of the *Pleurotuseous* was procured from Koonpura (mushroom society, Thiruvananthapuram).The sporophoretissue was placed on Potato Dextrose Agar (PDA) medium, and incubated.After seven days of incubation, a fully grown white cottony mycelium was obtained, Which was used for mother spawn preparation.

Preparation of spawn

The paddy grains were washed and soaked for 6-8 h in cold water and then boiled for 40 min. The boiled grains were drained and supplemented with 2% of $CaCO_3$. Then the excess moisture was air dried up to 60%. The treated grains (250g) were packed in polypropylene bags (size 200x300mm) and autoclaved at 121ºC for about 1 hr. The bags were taken out from the autoclave and allowed to cool for a day. After which, the bags were inoculated with pure culture of *P. eous*from the PDA slants in laminar air flow chamber. The culture and grains were mixed by shaking to distribute the mycelium uniformly. Inoculated bags were incubated at 27±2 °C for mycelial growth without light for 10-12 days until the mycelium fully covered the grains.

Water hyacinth cultivation method

Preparation of substrate

Water hyacinth (*Eichhornia crassipes*) was collected from velli and vellayani fresh water lake of Thiruvananthapuram (Kerala) city. They were brought to work area . After removal of roots, they were cut into pieces having size of around 2 cm. Pieces were soaked into clean, clear water for approximately 6-8 hrs. The excess water was drained by spreading the weed on clear floor. The chopped pieces of Water hyacinth were then given heat treatment for destruction of their waxy layer by immersing into boiling water for 30 min. After which they were dried in shade until the required 65-70% moisture content was achieved. Sterilized hyacinth substrate was filled in fresh polythene bags of size about 5cm height. A layer of spawn is added above the layer. The procedure is repeated until 3/4th height of polythene bag is filled up. Then the holes are made through out the bag to allow aeration. The filled bags are incubate 21^0 C - 23^0 C with sufficient light and humidity for 12-15 days and water sprayed the bags twice a day through out spawn running period.

Hyacinth compost cultivation method

The above treated water hyacinths was composted by adopting Rajapakse(2007) method. After the process the compost was then packed in small poly propylene bag (1000g), tied with rubber bands, and sterilized. After sterilization, the bags were allowed to cool in the laboratory and each bag was inoculated with spawn (1% total weight). The inoculated substrate bags were incubated. After full ramification, the bags were exposed in the growth room by removing the rubber bands and opening the top of bags. Watering was adequately done to increase the relative humidity of the environment to enhance sporophore emergence.

Paddy straw cultivation method

For control, commercial paddy straw substrate was taken,fresh, good quality paddy straw bits (4.5cm) were soaked inwater for upto 12 – 24 hrs and dried and packed into 2 meters height of polypropylene bags and autoclaved at150 pressures.Sterilized paddy straw is filled in fresh polythene bags ofsize about 5cm height. A layer of spawn is added abovepaddy straw layer. The procedure is repeated until 3/4thheight of polythene bag is filled up. The filled bags are Incubate.Frequent spraying of water is required in the cropping room depending upon the atmospheric humidity.

RESULT AND DISCUSSION

The water hyacinth (Fig 1), hyacinth compost and paddy straw (Fig 2) were inoculated with *P. eous*.In order to find out the effective bag, morphologicalobservations and yield of the mushroom from each bags to were recorded.

Fig. 2. Schematic representation of cultivation of *P. eous* on water hyacinth substrate.

Fig. 1. Water hyacinth(*Eichhorniacrassipes*) in vellayani (Trivandrum) fresh water lake.

The results (Fig 2) indicated that the spawn running was completed in the paddy straw bags 7to 8 days and pinheads appeared on the 11th – 15th. Pinheads turned into leaf like on 17th day and the first harvest was made at about 15 – 18 days. The second harvest will be another 2 or 3 days.The results were good in irrespective period.

For the paddy straw alone, oyster mushrooms were harvested after 12 days. The combination of waterhyacinth*(E.crassipes*) compost resulted in harvests times between the 15 and 17 days times. The substrate paddy straw was suitable substrates for early harvests compared to other substrates tested. Oyster mushrooms were harvested eight times from waterhyacinth compost.Whereas only five to six harvests were made in the remaining substrates, but no statistical differences were found in version of treatments variation. The yield data showed that the substrate of waterhyacinthcompost gave a significantly higher oyster mushroom production of 1030 g/1kg/dry. wt. followed by paddy straw alone as compared to the other treatments (Fig 3). The lowest yield was obtained in waterhyacinth alone substrate beds(563g/ dry wt).

This result was supported by Nageswaran et al.,(2003) .Biological efficiency was determined as the percentage conversion of dry substrate to oyster mushroom fresh weight, but there were no differences among the treatments.Choi (2004)reported that cotton waste is chosen as the main substrate material for oyster mushroom cultivation. . Fruit body produced under humid conditions (85-90%) is bigger with less dry matter while those developed at 65-70% relative humidity are small with high dry matter. The above results of mushroom cultivation usingwater hyacinth is reduced the aquatic weed problems tosome extent. Local villagers need to be motivated to makeuse of waterhyacinth compost as a substrate for oyster mushroom cultivation. Also, theoyster mushrooms provide an inexpensive source of proteinfor human

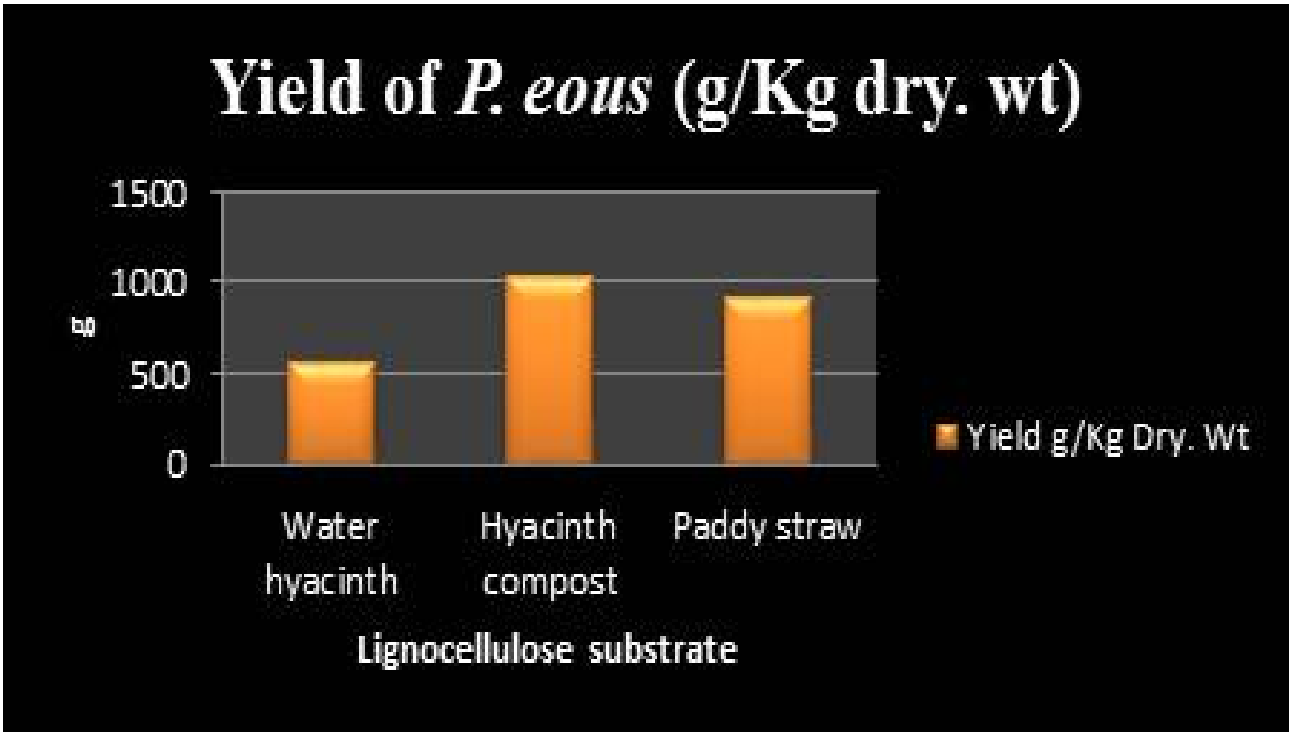

Fig. 3. Yield data of *P.eous* mushroom on three lignocelluloses waste.

consumption. Further studies are require for the nutritional composition of *Pleurotuseous* grown on water hyacinth.

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Short-Term Insulin Challenge Modifies Intracellular Ion Transport During Stress Response in Air-Breathing Fish, *Anabas testudineus* Bloch

D.S BINDULEKHA[1] AND M.C SUBHASH PETER[1,2]

[1]*Department of Zoology, University of Kerala, Kariavattom Thiruvananthapuram*
[2]*Centre for Evolutionary and Integrative Biology, University of Kerala, Kariavattom, Thiruvananthapuram*
email: binduhaiya@gmail.com

ABSTRACT

Insulin, an anabolic polypeptide hormone, has a multitude of actions on a wide range of cellular processes in vertebrates including fishes. But its role in intracellular ion transport during stress response is, however, not certain. We therefore examined whether insulin exerts any role in intracellular ion transport and how this short-term insulin modified this vital physiologic process during stress response in the metabolic and osmoregulatory tissues of air-breathing fish *Anabas testudineus*. To achieve this goal, indices of intracellular ion transporters were quantified after *in vivo* insulin (INS, 1µg g^{-1} for 30 min) challenge in fish exposed to net confinement stress for 60 min. Short-term insulin challenge produced hypoglycemia and hyperlactemia without affecting plasma urea in intact fish where a significant decrease in plasma glucose and urea was observed in stressed fish. Increased Na^+, K^+-ATPase activity was found after insulin challenge in all the tested tissues of stressed fish. H^+-ATPase activity, an index of H^+ secretion showed differential response after insulin treatment in both stressed and non-stressed fish. Likewise, *in vivo* insulin modified the gill, liver, kidney and intestinal *m*Ca^{2+}-ATPase and *m*Mg^{2+}-ATPase activities in stressed fish. Collectively, modified metabolite and osmotic responses to insulin in both stressed and non-stressed fish as evident in the glucose, urea and lactate levels and ATPase activities in the varied osmotic and non-osmotic epithelia clearly indicate a rapid and direct action of insulin in the intracellular ion signaling system in fish tissues particularly during stress response.

Key words *Fish, Insulin, ATPase, Stress, Osmoregulation*

Fishes are sensitive to numerous environmental insults as it is intimately associated with their environment. As a result, any change in the environment can alter their physiology, which would reflect in their organismal, physiological and cellular processes. Stress induces biological, physiological and behavioral changes (Wendelaar Bonga, 1997). In most fishes, for example, a general stress response (e.g., to handling) can induce elevations in metabolic rate (Baker and Brauner, 2012). Several hypophysial and extrahypophysial hormones control the activities of the osmoregulatory organs and maintain hydromineral balance in fish under varying environmental conditions (Peter *et al*., 2014). Unlike other osmoregulatory hormones, insulin as a widely recognized metabolic hormone is known for its metabolic action in fish (Polakof *et al*., 2010). It is of interest to study the role of this hormone in the intracellular ion transport in fish tissues especially during stress as every hormone acts basically by modifying some aspects of cellular metabolism.

Insulin, an anabolic polypeptide hormone, has a multitude of actions on a wide range of cellular processes in vertebrates including fishes. Insulin acts on a variety of tissues *via* insulin receptors which are ubiquitously distributed in the plasma membrane of cells (Borge and Wolf, 2003**)**. Insulin signaling in target tissue, results in large array of biological outcomes (Kahn and Flier, 2000; Le Roith and Zick, 2001; Kim *et al*., 2008). Sweeny and Klip (1998) suggested that the control of ionic homeostasis is a prominent yet often underestimated feature of insulin action. Similarly, the activation of insulin on osmotic and ion regulation has been poorly studied in teleosts (John *et al*., 1997; Kazumi *et al*., 1998). The analysis of the transporter responses to insulin thus would offer mechanistic understanding of the role of insulin in the regulation of ion transport in fish and how these cellular processes respond to insulin signaling in the intracellular milieu.

Teleost fishes possess a wide variety of ion transport proteins that mediate the active and passive movement of ions (Evans *et al*., 2005; Lorin-Nebel *et al*., 2006). Adenosine triphosphatase (ATPase), a complex set of enzyme systems found in invertebrates and vertebrates (Carfagna *et al.*, 1996), play a central role in the physiological functions of a cell as main energy transducers (Kodama, 1985). The activity response of ATPases, which are coupled with pumping of cations (Na^+, K^+, Ca^{2+} and Mg^{2+}) across the membranes, reflects the rate of transport of these cations and contributes to the understanding of the functional status of cell (Peter *et al*., 2014).

Mitochondria are mobile intracellular integrators

of metabolic and ionic signaling. Any response or adaptation to stress requires the expenditure of energy that would otherwise be utilized for maintaining normal body functions such as growth, digestion, osmoregulation, disease resistance, and reproduction (Carl, 1982; Bruce and George, 1991). Mitochondria are, therefore, among the first responders to various stressors that challenge homeostasis of the cell and organism. It is expected that extensive alterations in the energy transducing functions of these organelles will take place at the onset of stress response. Most of the anabolic and catabolic hormones are involved in the regulation of stress-induced alterations during growth phase (Pickering, 1993). Similarly, it has been well documented that the activation of the neuroendocrine systems that directs biochemical and physiologic adaptations in fish may favor them to acclimate to the hostile environment especially in stressed conditions (Wendelaar Bonga, 1997; Peter *et al.*, 2007, 2010; Lock and Wendelaar Bonga, 2008). Net-confinement was practiced in this fish since this handling stressor is known for its effect to induce stress without any toxic manipulation (Nolan *et al.*, 1999). Most changes in ion transport during stress response could activate neuroendocrine signals (Peter, 2011). Therefore, this study was undertaken to examine how insulin integrates the mitochondrial energy metabolism and intracellular ion transport during stress in freshwater climbing perch.

MATERIALS AND METHODS

Fish holding conditions

Tropical freshwater (FW) air-breathing fish, commonly known as climbing perch (*A. testudineus* Bloch) belonging to order Perciformes and family Anabantidae, was used as the test species. This native teleost fish inhabiting the backwaters of Kerala in Southern India is an obligate air-breathing fish equipped to live in demanding environmental conditions with their well defined physiological and biochemical mechanisms (Peter *et al.*, 2007, 2011). Laboratory-acclimated fish, comprising both sexes of approximate 50 g body mass and in their post-spawning phase were collected from the wild and maintained under laboratory conditions for three weeks under natural photoperiod (12 h L:12 h D) and at freshwater temperature ranging from 28^0C to 29^0C with a mean water pH of 6.2. Fish fed with dry commercial fish feed at 1.5% of body mass were used in the experiment. The regulations of Institutional Animal Ethics Committee of the University were followed.

EXPERIMENTAL PROTOCOL

Effect of *in vivo* insulin on stress

In vivo effects of a selected dose of insulin (1µg g^{-1}) (Human Mixtard from Novo Nordisk) on intracellular ion transport were studied in both non-stressed and stressed fish. Thirty-two laboratory-acclimated fish were kept as two batches of non-stressed (Batch I) and stressed fish (Batch II) that comprised two groups of eight fish each. Each fish of group 1 in batch 1 was given saline (0.65%) injection at 0 times and kept for 30 min and served as control. The second fish group in this batch was given insulin injection (1µg g^{-1}) at 0 times and kept for 30 min. Batch II fish that comprised two stressed fish groups were first kept at net confinement for 60 min. The first group of stressed fish were given 0.65% saline injection at 30 min and kept for another 30 min period. The second group of stressed fish was given 1µg g^{-1} insulin at 30 min time and held for another 30 min period. All the fish were sampled after a total period of 60 min. Insulin was dissolved in saline (0.65% NaCl) and all injections were made intraperitoneally at 9.00 am and the volume of hormone vehicle (saline) was kept as 0.1 ml.

Sampling and analysis

Feeding was stopped 24 h prior to sampling to ensure optimum experimental conditions. Both control and hormone-treated groups were handled in the same manner. After the treatment, all fish groups were anesthetized in 0.1% 2-phenoxyethanol (Sigma, St. Louis MO) solution and the blood was taken from the caudal vessels using a heparinised 23 guage syringe. The blood was centrifuged at 5000g for 5 min at 4°C to separate plasma and stored at $^{-}$80°C until analyzed. The fish were then sacrificed by spinal transsection and pieces of second gill arch, 1 cm of anterior intestine, posterior kidney and a posterior lobe of liver were excised immediately and kept in ice cold 0.25 M SEI buffer (pH 7.1) and stored at $^{-}$80°C.

Plasma metabolites

Plasma glucose (GOD/POD test kit, Span Diagnostics Ltd., New Delhi), urea (DAM kit, Span Diagnostics Ltd., New Delhi) and lactate (PAP Fluid test; Radiant Diagnostics, New Delhi) concentrations were measured colorimetrically using commercial test kits in a Systronics Spectrophotometer 2202 (Systronics, New Delhi).

Isolation of mitochondria

The tissues were washed in ice cold 0.25M SEI

buffer (pH 7.1) containing 0.3M sucrose, 20mM Na_2EDTA and 0.1M Imidazole and minced into small pieces using scissors. Tissue homogenate was prepared in SEI buffer (pH7.1) and centrifuged at 700 g for 10 min. This fraction was used for Na^+, K^+-ATPase assay. This supernatant was further centrifuged at 10,000g for 10 min. The supernatant collected was taken as post-mitochondrial suspension (PMS) and it served as the sample for cytosolic transporter assays. The mitochondrial pellets obtained were washed twice by repeating centrifugation and suspended in fresh ice cold SEI buffer. The protein concentrations in mitochondria as well as in cytosol were determined using a modified Biuret Assay (Alexander and Ingram, 1980) with bovine serum albumin as standard.

Quantification of ion-specific ATPases

Na^+, K^+-ATPase specific activity

A portion of the tissue homogenized in SEI buffer (pH 7.1) and centrifuged at 700 g for 10 minutes was used for analyzing the Na^+, K^+-ATPase activity. The ouabain-sensitive Na^+, K^+-ATPase-specific activity in tissue homogenates was quantified adopting the method of Peter *et al*., (2014). Saponin (0.2 mg protein-1) was routinely added to optimize substrate accessibility. The samples in duplicate were added to a 96-well microplate containing 100 mM L-1 NaCl, 30 mM L-1 immidazole (pH 7.4), 0.1 mM L-1 EDTA and 5 mM L- 1 MgCl2 with or without ouabain and incubated at 37^0C. The reaction was initiated by the addition of ATP and was terminated with addition of 8.6% TCA. The liberated inorganic phosphate was determined in Autoreader 4011 (Spam Diagnostics Ltd., Surat, India) at 700 nm and expressed in ìM Pi h-1 mg protein-1.

H^+-ATPase specific activity

The baffilomycin-sensitive H^+-ATPase activity in the cytosolic and mitochondrial fractions was measured as described for Na^+, K^+-ATPase but baffilomycin A was used as inhibitor (Peter *et al*., 2014). The samples in duplicate were added to a 96-well microplate containing baffilomycin A. The reaction was initiated by the addition of ATP and terminated by adding 8.6% TCA and the inorganic phosphate content was determined as above and expressed in ìM Pi h-1 mg protein-1.

Ca^{2+}-dependent ATPase specific activity

The vanadate-dependent Ca^{2+}-ATPase activity in the cytosolic and mitochondrial fractions was determined as described for Na^+, K^+-ATPase but

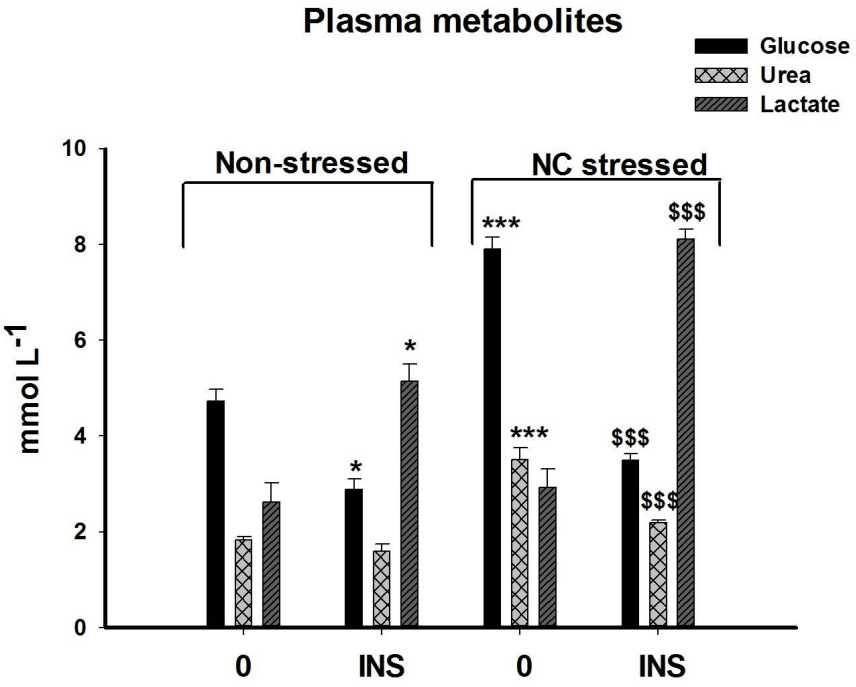

Fig. 1. Effects of *in vivo* insulin (INS 1µg g^{-1} for 30 min) on plasma metabolites (mmol L^{-1}) in fish kept in non-stressed or stressed (net confined (NC) for 60 min) condition. Each column represents mean ± SEM for eight fish. Statistical differences between fish were quoted after SNK test. . *P < 0.05, **P < 0.01 and ***P < 0.001 denote significant difference from non-stressed control fish and $P < 0.05, $$P < 0.01 and $$$P < 0.001 denote significant difference from stressed control fish.

vanadate was used as inhibitor (Peter *et al*., 2014). Samples in duplicate were added to a 96-well microplate containing either $CaCl_2$ or vanadate. The inorganic phosphate content released was determined as above and expressed in ìM Pi h-1 mg protein-1.

Mg^{2+}-dependent ATPase specific activity

The specific activity of oligomycin-sensitive Mg^{2+}- ATPase in the mitochondrial fraction was determined as described for Na^+, K^+-ATPase but oligomycin was used as inhibitor (Peter *et al*., 2014). Mitochondrial samples in duplicate were added to a 96-well microplate with or without oligomycin. The inorganic phosphate content released was measured and expressed in ìM Pi h-1 mg protein-1.

Statistical analysis

Data were collected from eight animals in each group. Statistical difference among groups was tested by means of one-way analysis of variance (ANOVA) followed by SNK comparison test. Significance between the groups was analyzed with the help of Graphpad Software (Graphpad Instat-3, San Diego) and the level of significance was accepted if $P < 0.05$.

RESULT

Effect of *in vivo* insulin in non-stressed fish

Short-term administration of insulin in intact fish produced hypoglycemia ($P<0.05$) and hyperlactemia ($P<0.05$) without affecting plasma urea (Fig.1). *In vivo* insulin for 30 min in non-stressed fish significantly increased ($P<0.001$) the gill and intestine Na^+, K^+-ATPase activity, whereas it showed significant decrease ($P<0.001$) in liver and kidney tissues (Fig.2). Significant increase ($P<0.01$) in *c*H^+-ATPase activity was found in the gills ($P<0.01$) and kidney ($P<0.05$) after insulin treatment in non-stressed fish, but a

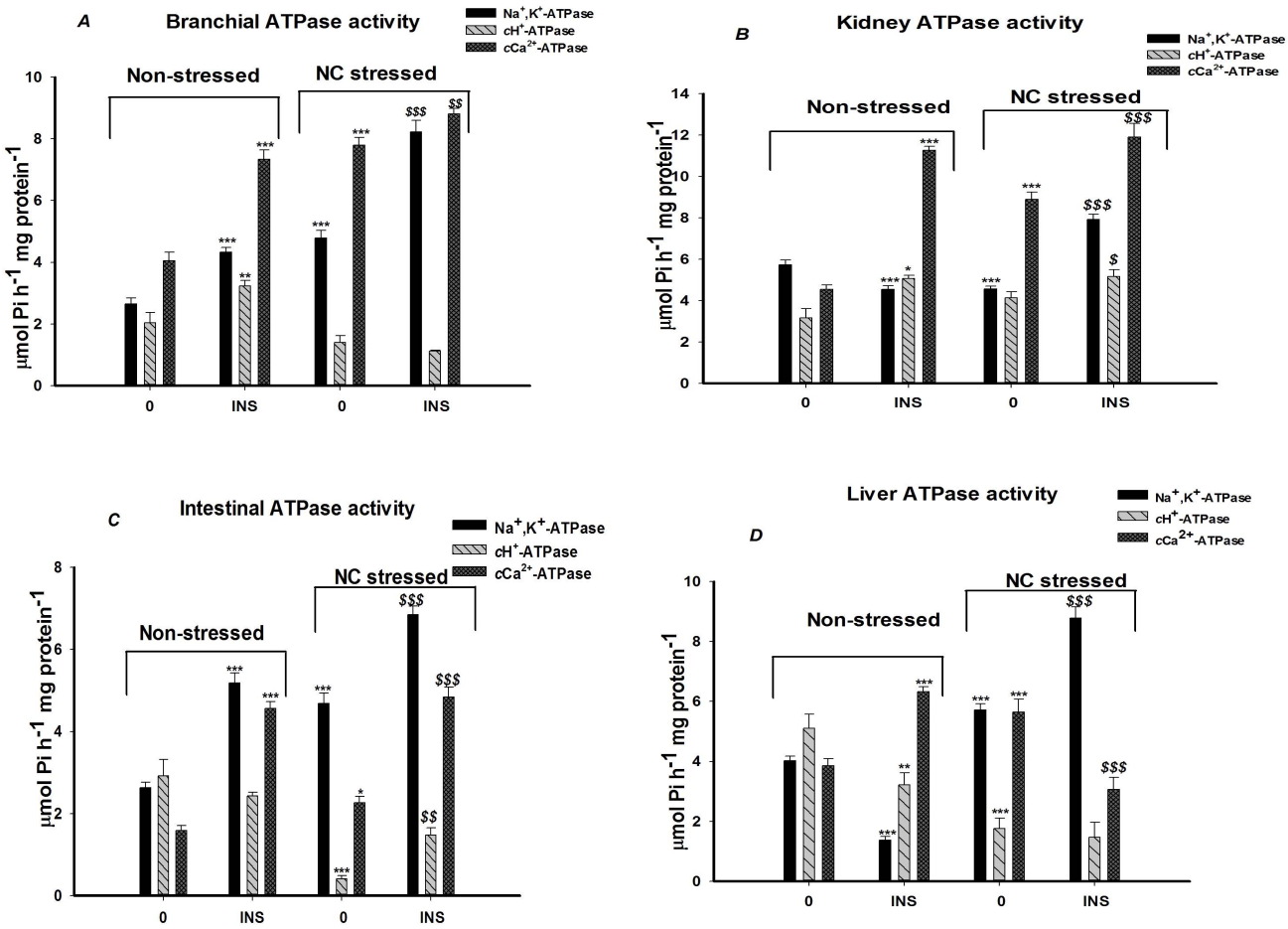

Fig. 2. Na^+, K^+-ATPase ,H^+ - ATPase and Ca^{2+}-ATPase activities in the gills (A), kidney (B), intestine (C) and liver (D) of non-stressed and stressed (net-confined for 60 min) air-breathing fish treated with either saline (control) or insulin (INS, 1µg g^{-1}). Each column represents mean ± SEM for eight fish. Statistical differences between fish were quoted after SNK test. *P < 0.05, **P < 0.01 and ***P < 0.001 denote significant difference from non-stressed control fish and $P < 0.05, $$P < 0.01 and $$$P < 0.001 denote significant difference from stressed control.

reversal (P<0.01) was found in liver (Fig.2). Insulin challenge in non-stressed fish produced a significant rise (P<0.001) in the cCa^{2+}-ATPase activity in all the tested tissues (Fig.2). Similar to cytosolic cCa^{2+}-ATPase activity, short-term insulin treatment produced significant increase (P<0.001) in the gill, kidney, intestine and liver mCa^{2+}-ATPase and mMg^{2+}-ATPase activity in non-stressed fish (Fig 3). However, gill (P<0.05) and intestinal (P<0.01) mH^{+}-ATPase activity decreased significantly after short-term insulin treatment in non-stressed fish, though it showed a reversal in kidney (P<0.05) and liver (P<0.001) mH^{+}-ATPase activity (Fig.3).

Effect of insulin in stressed fish

Short-term administration of insulin produced a substantial rise in plasma lactate whereas a significant decrease (P<0.001) in plasma glucose and urea was observed in stressed fish (Fig.1). Insulin administration increased (P<0.001) Na^{+}, K^{+}-ATPase activity in all the tested tissues of stressed fish (Fig.2). Though cH^{+}-ATPase activity in the gills and liver remain unresponsive to insulin treatment, pronounced increase in its activity was found in intestine (P<0.01) and kidney (P<0.05) of stressed fish (Fig.2). *In vivo* insulin for 30 min in stressed fish significantly increased (P<0.001) the gill, kidney and intestine cCa^{2+}-ATPase activity, whereas it showed significant decrease (P<0.001) in liver tissue (Fig.2). Similarly, mCa^{2+}-ATPase activity in the kidney and intestine remain unresponsive to insulin treatment, whereas pronounced increase (P<0.001) in its activity was found in gills and liver of stressed fish (Fig.3). However, short-term administration of insulin in stressed fish produced a significant decline (P<0.01) in the gill and intestinal mH^{+}-ATPase activity though it had no effect on liver ATPase activity (Fig.3). Similar to cH^{+}-ATPase activity, insulin challenge significantly increased (P<0.05) the kidney mH^{+}-ATPase activity in stressed fish (Fig.3). Likewise, substantial downregulation (P<0.001) was found in the gills and liver mMg^{2+}-ATPase activity to insulin challenge in stressed fish though it showed reversal in kidney and intestine tissues (Fig.3).

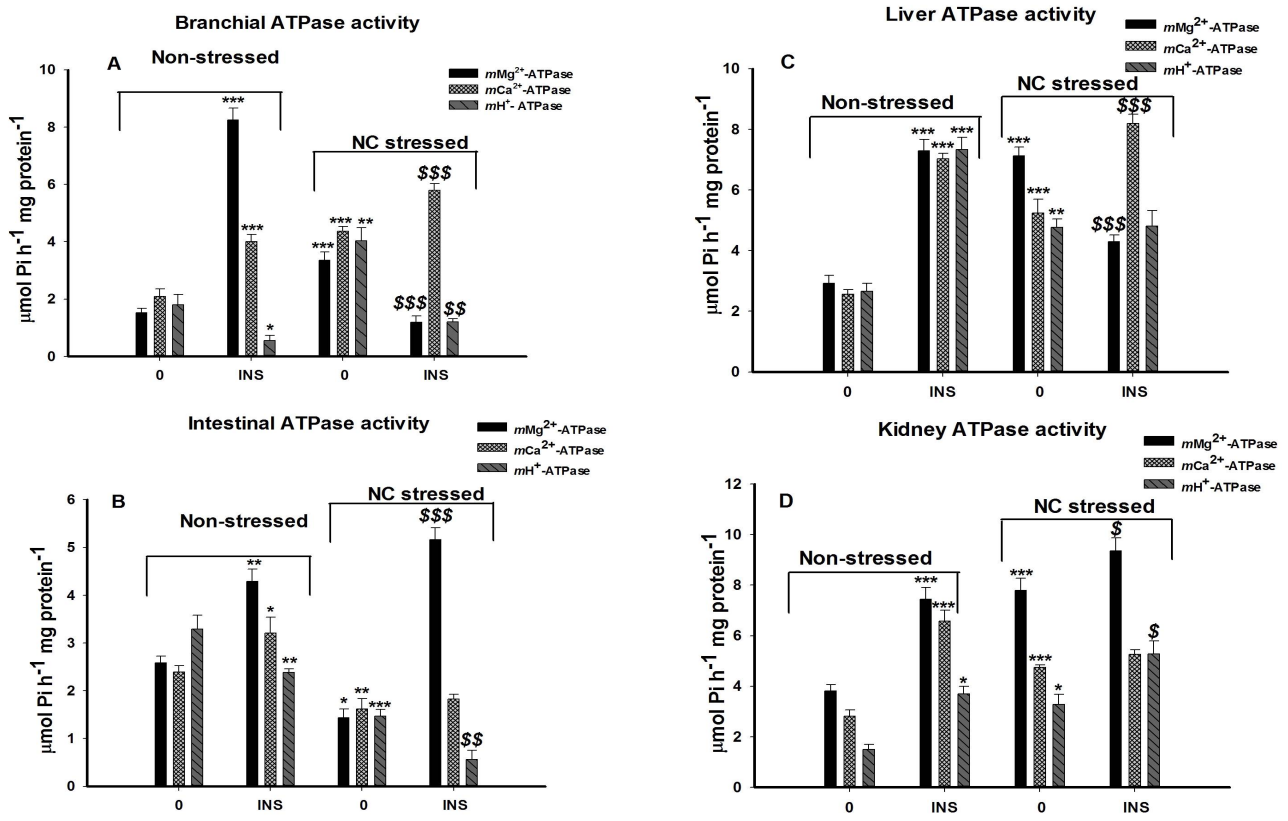

Fig. 3. Mitochondrial Mg^{2+}-ATPase, Ca^{2+}-ATPase and H^{+}-ATPase activities in the gills (A), intestine (B), Liver (C) and Kidney (D) of non-stressed and stressed (net-confined for 60 min) air-breathing fish treated with either saline (control) or insulin (INS, 1µg g^{-1}). Each column represents mean ± SEM for eight fish. Statistical differences between fish were quoted after SNK test. *P < 0.05, **P < 0.01 and ***P < 0.001 denote significant difference from non-stressed control fish and $P < 0.05, $$P < 0.01 and $$$P < 0.001 denote significant difference from stressed control.

DISCUSSION

Understanding the integrated stress response in fish offers a mechanistic view on the adaption in fish. The short-term *in vivo* administration of insulin markedly regulated the ATPase activities in different osmoregulatory and metabolic organs of perch. The major function of insulin is to control whole body glucose homeostasis. Similar to mammals, in fishes also, the most prominent response to exogenous insulin injection results in hypoglycemia (Ince, 1983), though the magnitude and duration of this effect are dependent on insulin type and dose, the route of injection, the season, nutritional status (Mommsen and Plisetskaya, 1991). The declined plasma urea status, an indication of diminished ureogenesis, after insulin treatment, supports the view that insulin alone has little effect on the mRNA levels of urea cycle enzymes (Li *et al*., 2004). The hyperlactemia after insulin injection in perch and a similar rise in plasma lactate in stressed fish after insulin treatment clearly indicate a key role of insulin in energy metabolism and its sensitivity to oxygen utilization.

Cell membrane is required to maintain a fluid state for the proper activity of a number of membrane proteins, such as Na^+-K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase (Vajreswari *et al*., 2002). Na^+-K^+-ATPase located in the cell membrane has been found to be involved in the active transport of Na^+ and K^+ across the cell membrane and it act as an index of osmoregulation in fishes (Peter *et al*., 2000). This transporter in the gill filament is a measure of the functional state of the chloride cells in branchial epithelium (Peter *et al*., 2000). Similarly, the activity pattern of Na^+-K^+-ATPase has been used extensively as a measure of hydromineral capacity (Dang *et al*., 2000; McCormick, 2001).

In fishes, gills, intestine and kidney as the major osmoregulatory organs, show integration of osmotic functions and thus maintain an optimal hydromineral balance (Evans *et al*., 2002; Marshall and Grosell, 2005). This homeostasis is maintained by a delicate balance of transport activities across the plasma and organelle membranes (Supek *et al*., 1997). It has been suggested that the mechanism of insulin-stimulation of Na^+, K^+-ATPase activity varies among different cells and tissues (Longo *et al*., 2001). In a previous study, a similar tissue specific Na^+, K^+ATPase response has also been reported in tilapia where its activity in the kidney declined, but during confinement stress its activity increased in the kidney while decreasing in the gill (Nolan *et al*., 1999). In our results, the Na^+, K^+-ATPase, that marks the osmotic competence of organs showed down regulation in the liver and kidney but not in the gills and intestine where its activity shows upregulation in non-stressed fish indicates that insulin exerts osmotic and iono-regulatory action in fish tissues. Similarly, the increased Na^+-K^+ATPase activity after insulin challenge in stressed perch emphasize an integrative and compensatory role for insulin in these target cells, where insulin demands modifications of Na^+ pump activity in tune with the stress status for a homeostatic intracellular ion transport.

Experimental findings advance an emerging paradigm that establishes insulin as a predominant postprandial anabolic hormone and also as an important regulator of mitochondrial oxidative phosphorylation (Stump *et al*., 2003; Boirie, 2003). Furthermore, by increasing mitochondrial protein synthesis and possibly decreasing protein breakdown, insulin may increase mitochondrial protein content and thus enhance mitochondrial respiration (Stump *et al*., 2003). Similarly, many studies have shown the changes of gene expression of the plasma membrane H^+-ATPase in response to a variety of environmental factors (Braun *et al*., 1986). *In vivo* insulin challenge in stressed and non-stressed perch modified the pattern of H^+-ATPase activity in all the tested tissues. A direct action of insulin on proton regulation can be seen in these tissues. The response of gill, intestine, kidney and liver mitochondrial H^+-ATPase activity after *in vivo* insulin challenge in stressed perch indicates a possible involvement of specific pattern of response during insulin challenge in stressful condition. Similarly, a potential action of insulin on proton regulation further reflect the possible involvement in intracellular ion signaling where proton transport occurs between the mitochondria and cytosol as evident in the mitochondrial and cytosolic H^+-ATPase activity.

Cells utilize two primary mechanisms for removing excess calcium from the cytoplasm, both of which are energetically expensive: active export across the plasma membrane, and import into the ER and mitochondria (Steven and Miechel, 2008). Mitochondria could alter Ca^{2+} channel activity itself or they could alter indirectly net Ca^{2+} fluxes by regulating Ca^{2+}-ATPases or by regulating the K^+ or Cl^- channels that set the driving force for Ca^{2+} entry (Markus *et al*., 2000). In our study, the results showed a modulatory effect of Ca^{2+}-ATPase in the gill, liver, intestine and kidney mitochondria and cytosol of non-

stressed fish after *in vivo* insulin treatment and may account for a specific role of insulin that could enhance the intracellular calcium transport for biochemical signaling. Since stress is an energy demanding process, irrespective of stress status of fish, insulin administration produced an increased mitochondrial Ca^{2+}-ATPase activity in the gill, liver and kidney tissues clearly indicate a role of insulin in the movement of Ca^{2+} ions. The same modulatory effect was noticed in the cytosolic Ca^{2+}-ATPase activities in the tested tissues of fish, indicating a rise in cytosolic Ca^{2+} concentration that may induce the binding of Ca^{2+} ions to calmodulin, for triggering allosteric activation of the Ca^{2+}-ATPase. The involvement of Ca^{2+}-ATPase in calcium absorption has been proposed by many researchers, since the activities of these enzymes correlate with the degree of calcium absorption in different parts of the intestinal tract under different circumstances (Schiffle and Binswanger, 1980).

Cellular Mg^{2+} content is finely regulated by precise control mechanisms at the level of Mg^{2+} entry, Mg^{2+} efflux, and intracellular buffering and compartmentation (Cole and Quamme, 2000). Mg^{2+}-ATPase is responsible for the transepithelial regulation of Mg^{2+} ions, which are essential for the integrity of the cellular membrane, intracellular elements and the stabilization of branchial permeability (Parvez *et al.*, 2006). It is well documented that intracellular organelles are the primary pool for cellular magnesium which respond to hormonal stimulation (Murphy, 2000). Mg^{2+} frequently modulates ion transport by pumps, carriers and channels, and thereby, may modulate signal transduction and the cytosolic concentration of electrolytes, such as Ca^{2+} and K^{+} (Smith and Maguire, 1993). There are also several reports suggesting that altered magnesium buffering, often in intracellular organelles, is important for regulating magnesium homeostasis (Murphy, 2000).

It has been reported that if Mg^{2+} compartmentation fluctuates in response to cellular metabolic events, then there must be an active exchange of Mg^{2+} among the different compartments (Romani, 2006). Significant elevation of Mg^{2+}-ATPase activity was observed after insulin challenge in the osmotic and metabolic organs of perch. The varied responses of Mg^{2+}-ATPase activity may indicate vulnerable energy equilibrium in the stressed fish which appears to be corrected by *in vivo* insulin. It is also reasonable to suggest that the declined Mg pump activity could be a loss of Mg availability as loss in ion-specific ATPase could also be attributed to the loss of ions due to the cellular leakage into the body fluids (Afsar *et al.*, 2012). Similarly, altered mitochondrial membrane permeability affecting enzyme activity, and thus, disruption in vital energy yielding phosphorylation process, point to an alteration in ATPase system (Afsar *et al.*, 2012). In a previous study by Romani *et al.*, (2000) reports that insulin stimulates Mg^{2+} accumulation in myocytes, but the intriguing new twist is that insulin-stimulated Mg^{2+} uptake is blocked, if insulin stimulated-glucose transport is inhibited. This response of Mg^{2+}-ATPase activity to insulin further support the earlier view that ions may serve as intracellular messengers for insulin and that the hormone regulates their translocation through ATPase (Jarret and Smith, 1974). Therefore, the specific pattern of responses of mitochondria occurred in both osmoregulatory and metabolic organs of stressed and non-stressed perch after insulin challenge clearly indicate a role for insulin in monovalent and divalent cation transport and thus provide evidence for its involvement in intracellular ion signaling in fish tissues.

CONCLUSION

The analysis of data thus provides evidence that insulin evokes specific iono-responsive actions in target cells of fish. Further the data point to modified iono-osmotic and metabolic responses of various organs to insulin treatment depends on stress status and/or internal ionic conditions in fish. Overall, our results support the hypothesis that insulin has a vital role in integrating intracellular ion transport in fish particularly during stress response in fish.

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FTIR Spectroscopic Analysis of Biochemicals Synthesised in the Mature, Germinated and Desiccated Seeds of *Vateria indica* L.

GAYATRI G.P, AJITH KUMAR K.G, PARVATHY S. NAIR, SUNIL KESAVA DETH G, DINESH BABU K.V.

Post Graduate and Research Department of Botany, Government College for Women, Trivandrum.
email :gayatribchandran@gmail.com

ABSTRACT

***Vateria indica* L. is an endemic species in Western-Ghats of India which belongs to the family Dipterocarpaceae. The species has been cited as a critically endangered plant, endemic to the Western Ghats South India, due to problems associated with its natural regeneration and seed recalcitrance (Ashton, 1998). The present study was carried out to understand the physiology and biochemistry of mature seeds during germination as well as on desiccation. Seeds germinated immediately after shedding and desiccated quickly. On desiccation, the rate of germination of seeds decreased. The critical threshold water level was found to be 47% and the seeds remain viable for seven to nine days. FTIR spectrum profile showed variations in biochemical composition in fully mature freshly fallen seeds, germinated seeds and seeds at the time of critical threshold water level. FTIR also showed the presence of so many compounds, in *V. indica*, which can be used pharmaceutically.**

Key words *Vateria indica L., Seed recalcitrance, Fourier Transform Infrared Spectrometry, Critical Threshold Water Level.*

Vateria indica L., (Indian Copal Tree) is an endemic species in Western-Ghats of India and it is commercially exploited as a source of white dammar which is a main ingredient in many Ayurvedic preparations. It belongs to the family Dipterocarpaceae which has been reported to have 98% recalcitrant species (Farnsworth, 2000). The species has been cited as a critically endangered species, endemic to the Western Ghats South India, due to problems associated with its natural regeneration (Ashton, 1998). The major problem in the regeneration of this species is seed recalcitrance in which the embryo cannot tolerate desiccation (Berjak *et al.*, 2007). The mechanism of recalcitrance has not received much attention in India in spite of the fact that our country has a vast reservoir of tropical recalcitrant species. The exact molecular mechanism involved in seed recalcitrance has not been studied completely and there are reports showing the change in physiology and biochemistry of recalcitrant seeds on desiccation (Xia *et al.*, 2012; Delahaie *et al.*, 2013; Kermode *et al.*, 2001; Pammenter *et al.*, 1999).

Fourier Transform Infrared Spectrometry (FTIR) is a powerful tool used to identify the types of chemical bonds (functional groups) present in compounds. The spectrum displays specific light wavelengths absorbed by chemical bonds which can characterise them. The objective of the present study was to understand the change in the molecular expression of *V. indica* seeds at fully matured freshly fallen stage, on germination and at the time of critical threshold water level, along with an attempt to understand its recalcitrant behaviour. It also focused on the determination of critical moisture content and the loss of viability after seed dispersal.

MATERIALS AND METHODS

Seed Collection and Germination studies

Fully matured, just fallen seeds were collected from the healthy trees of *V. indica*, during the month of May. Ten healthy seeds were randomly taken to determine the moisture content and another ten for viability test using tetrazolium salt. Twenty five seeds, arranged in five rows in a plastic tray (three replicates) containing sterile coir pith, were kept in a growth chamber with a temperature maintained between 27^0C to 30^0C and relative humidity between 80 to 90%, for germination studies. Ten seeds were dissected to remove embryos and cotyledons for FTIR analysis. Remaining seeds were allowed to desiccate under natural conditions for a period of twelve days. Ten seeds were randomly selected from the seed lot for every two days for the determination of moisture content.

FTIR spectroscopic analysis

FTIR studies were carried out for the fully matured just fallen seeds, plumule and radicle of

germinated seeds and the seeds at critical threshold water level. For FTIR analysis, the embryo, cotyledons, plumule and radicle were separated, thoroughly washed in distilled water, chopped and ground, in three different solvents viz. 100% methanol, 50% methanol and chloroform, using mortar and pestle. The homogenised sample was centrifuged and supernatant was collected. The solvent was evaporated in a rotary evaporator, the dried sample was dispersed in dry Potassium bromide and was mixed in a mortar and pressed at a pressure of 6 bars to form a Potassium bromide disc. The disc was placed in a sample cup of a diffuse reflectance accessory. The IR spectrum was obtained using Perkin Elmer 2000 Infrared Spectrophotometer. The samples were scanned from 400 to 4000 cm^{-1} for sixteen times to increase the signal to noise ratio. Samples were run in triplicate and the data was collected within one day (Manigandan *et al.*, 2015; Marimuthu and Gurumoorthi, 2013).

Statistical analysis

All statistical analysis was done using SPSS 16.0 (SPSS Inc. Chicago, USA).

RESULTS AND DISCUSSION

Change in the moisture content during embryo desiccation

The mature embryos of *Vateria indica* showed a typical recalcitrant nature in which the embryo occupied more than half of the volume of seed with large cotyledonary tissue and scanty endosperm. The embryonal axis of the mature embryo showed moisture content of 57% and 37% in the cotyledonary tissues. This is invariably a trait showed by many recalcitrant species in which the embryo retains more moisture at the time of dispersal. Germination test was conducted to understand the seed viability and result showed cent percent germination (Fig. 1) indicating that the seeds of *Vateria indica* did not show any kind of quiescence just after seed dispersal, an indication of the recalcitrance behaviour of these seeds. Tetrazolium test of just fallen mature seeds showed cent percent viability of seeds.

Fig. 1. Cent percent germination of freshly collected seeds of *Vateria indica* (moisture content in the embryonal axis, 57%) without any metabolic quiescence.

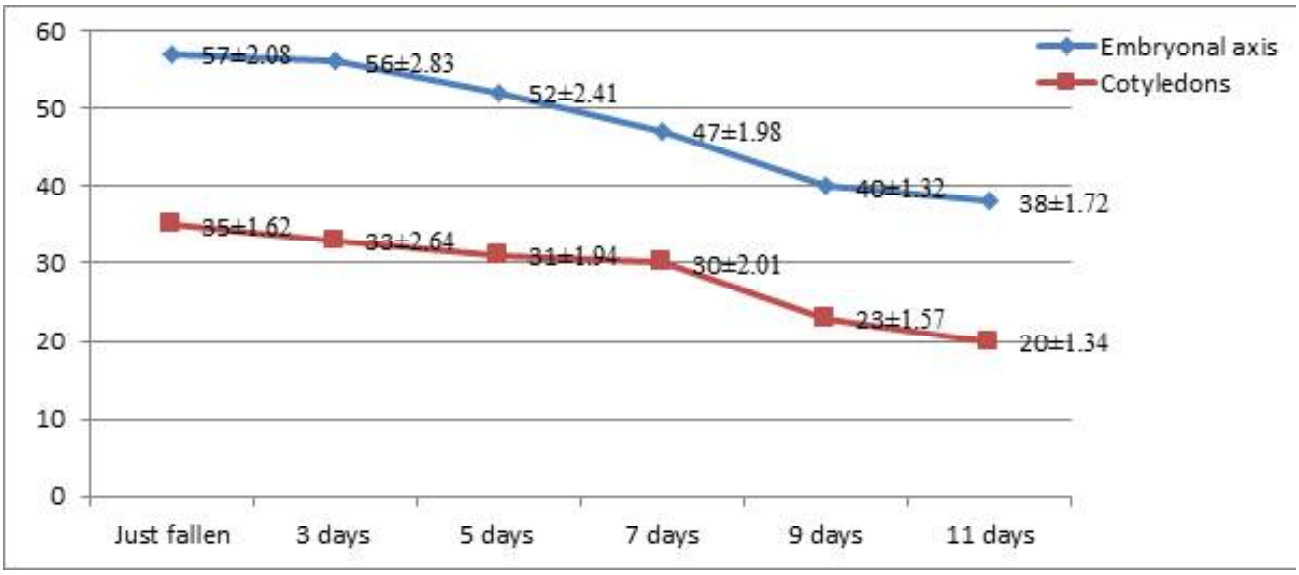

Fig. 2. Change in moisture content in the embryonal axis and cotyledons of *Vateria indica* seeds, upon desiccation. Standard deviation is also represented in the graph.

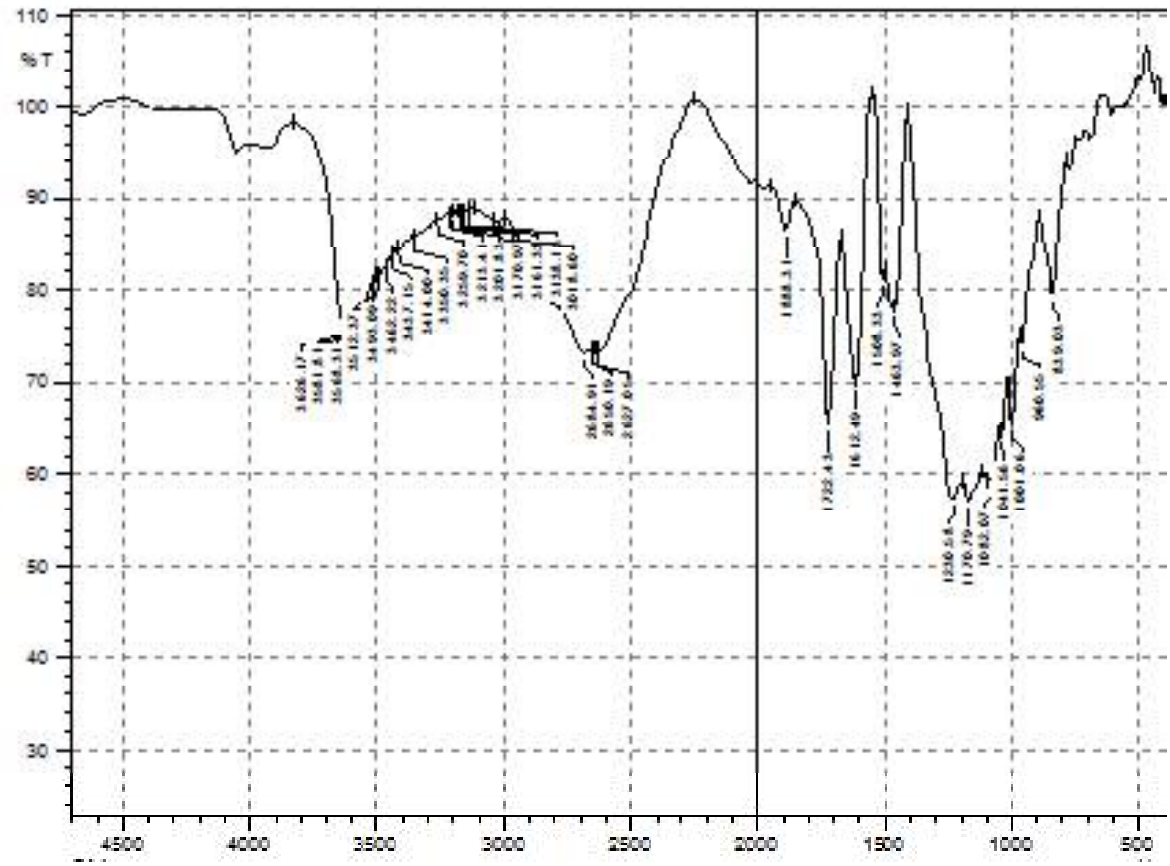

Fig. 3. (a) FTIR spectrum, of embryonal axis, of Just fallen mature seeds in 100% methanol

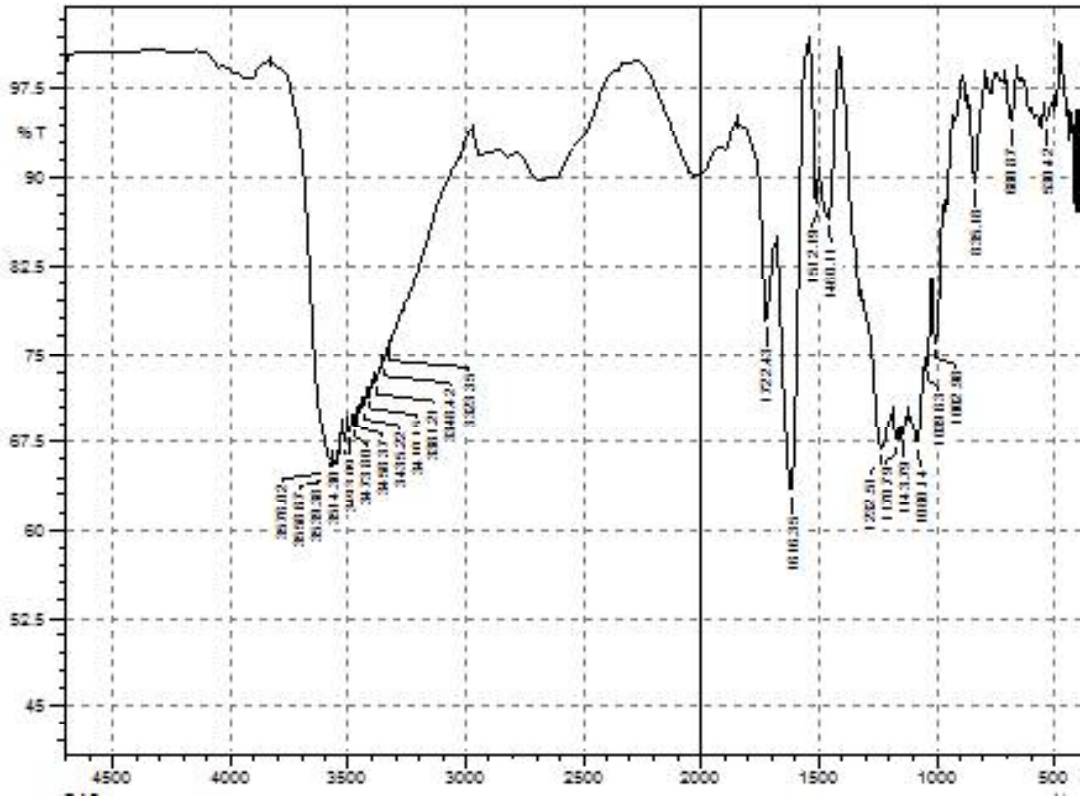

Fig. 3. (b). FTIR spectrum, of embryonal axis, of Just fallen mature seeds in 50% methanol

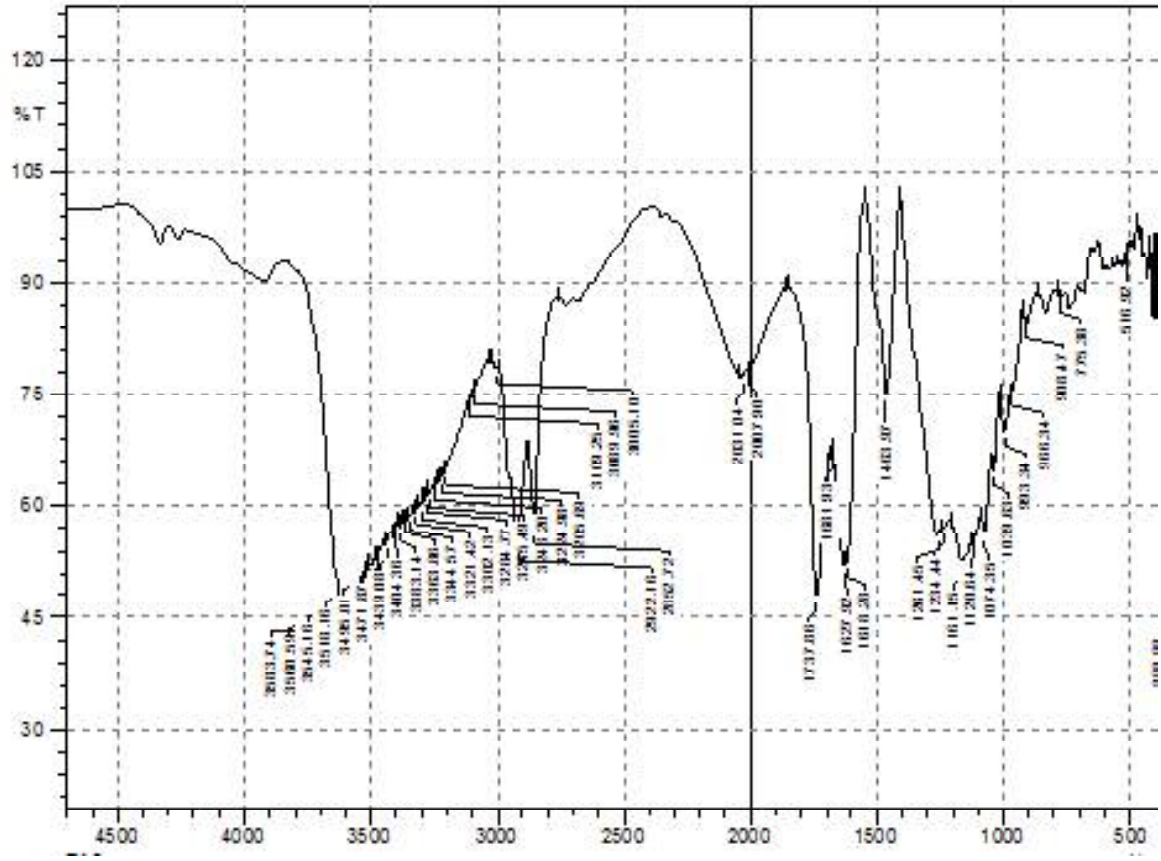

Fig. 3 (c). FTIR spectrum, of embryonal axis, of Just fallen mature seeds in chloroform

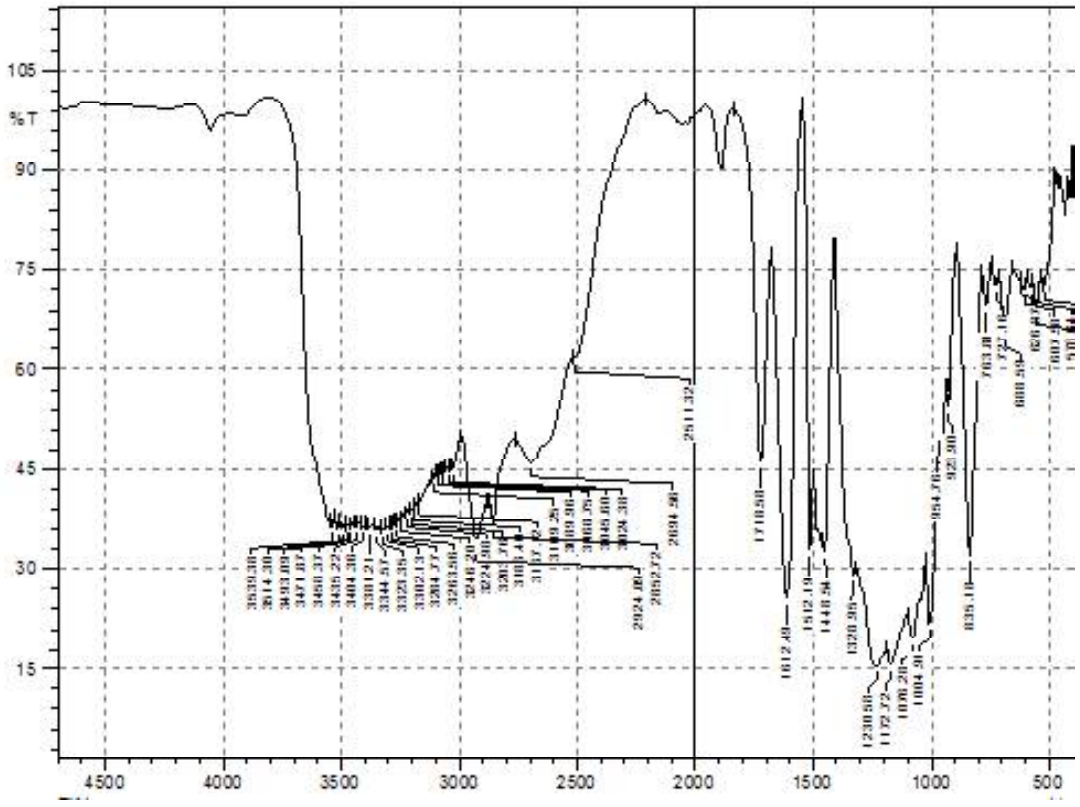

Fig. 3. (d). FTIR spectrum, of cotyledon, of Just fallen mature seeds in 100% methanol

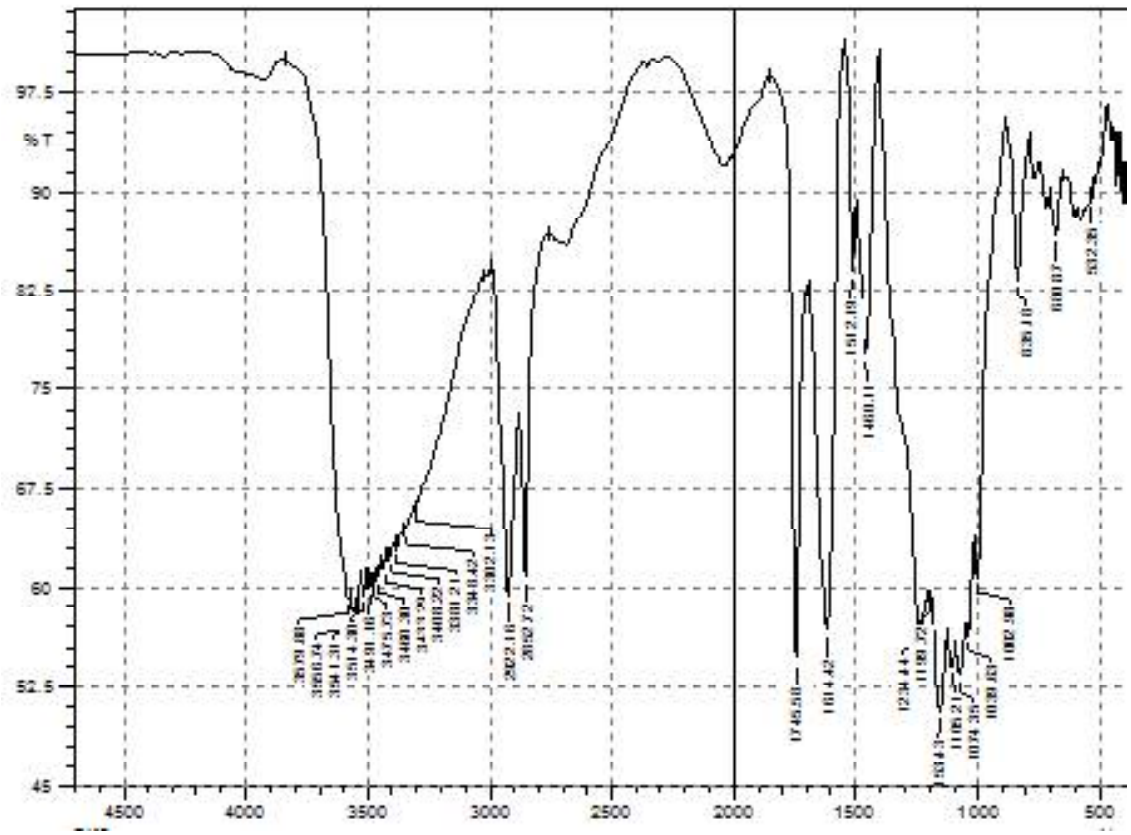

Fig. 3(e). FTIR spectrum, of cotyledon, of Just fallen mature seeds in 50% methanol

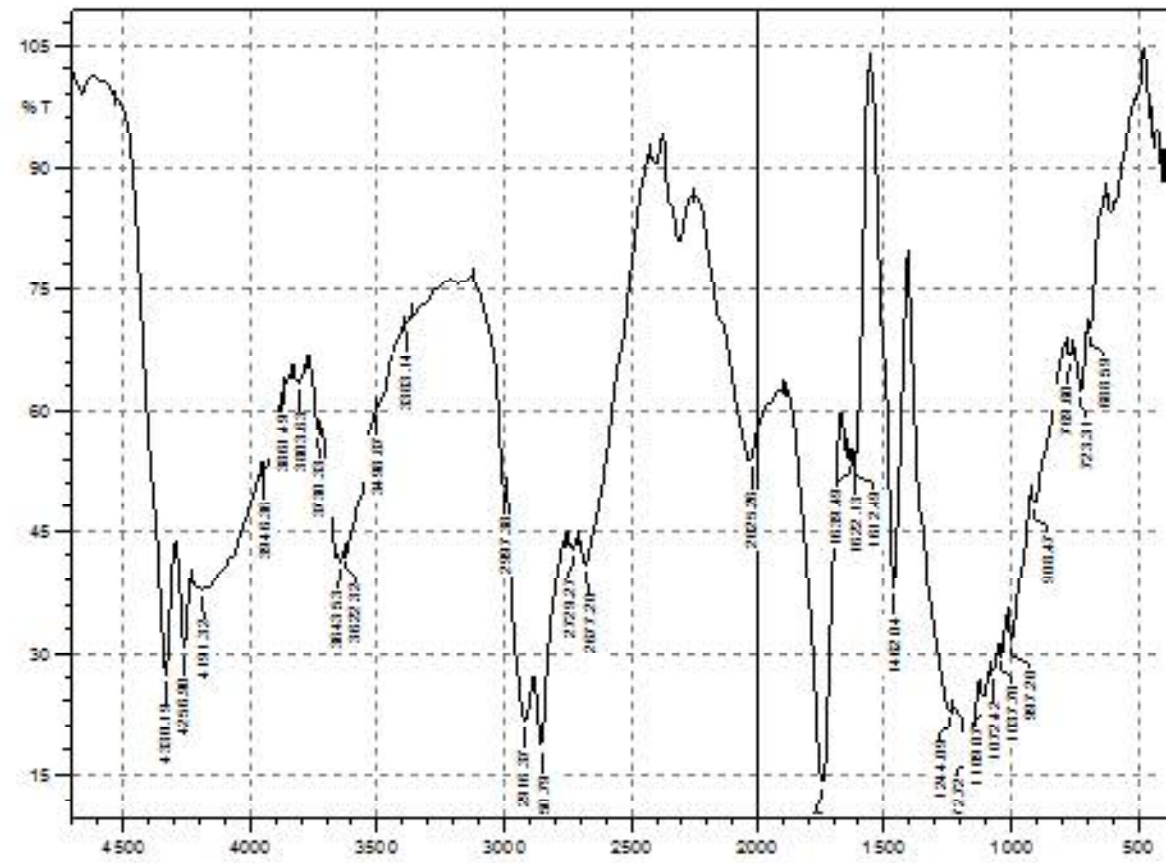

Fig. 3(f). FTIR spectrum, of cotyledon, of Just fallen mature seeds in chloroform

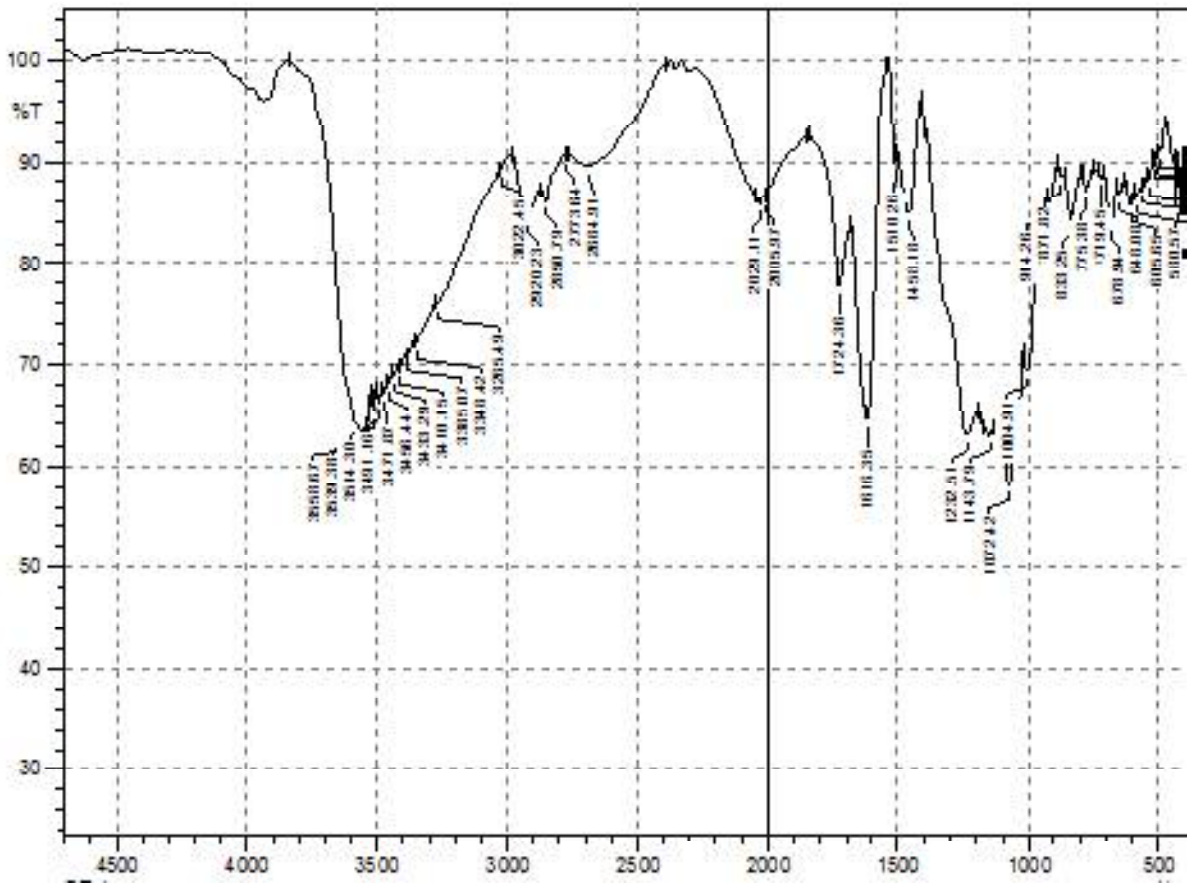

Fig 3(g). FTIR spectrum, of radicle in 100% methanol

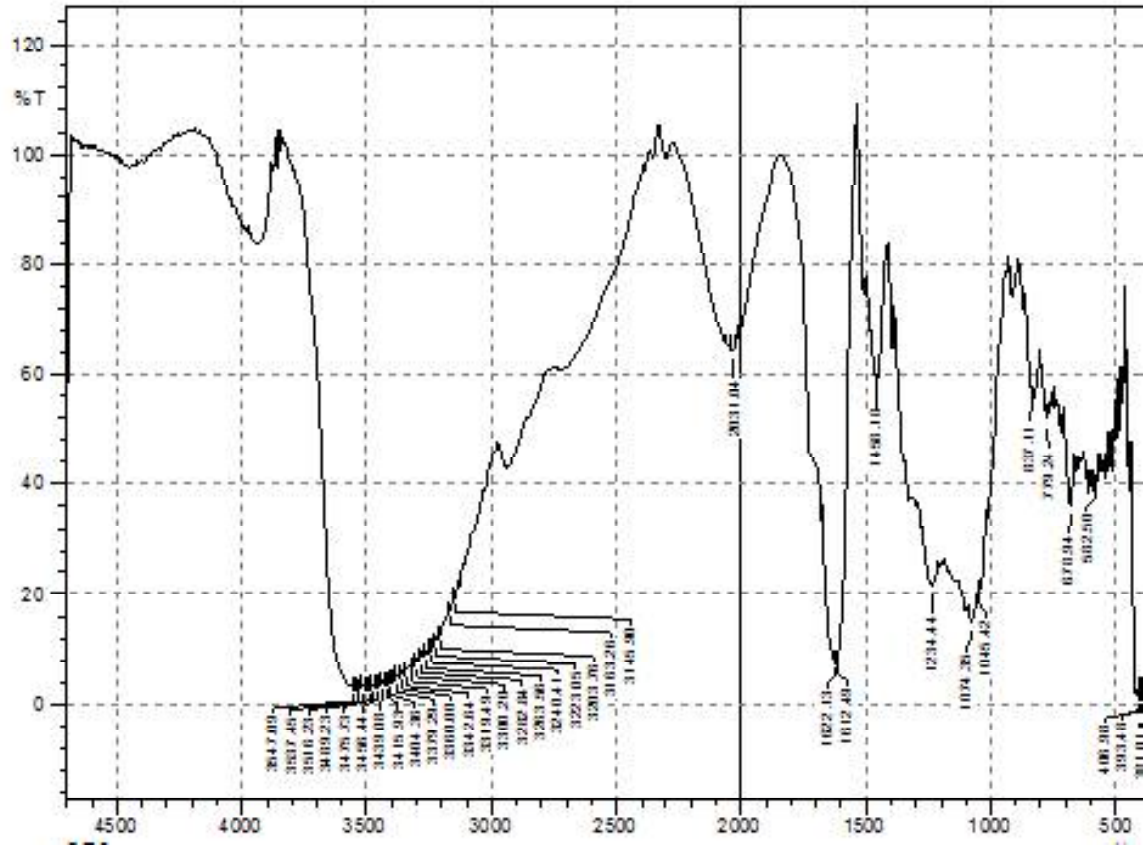

Fig. 3(h). FTIR spectrum, of radicle in 50% methanol

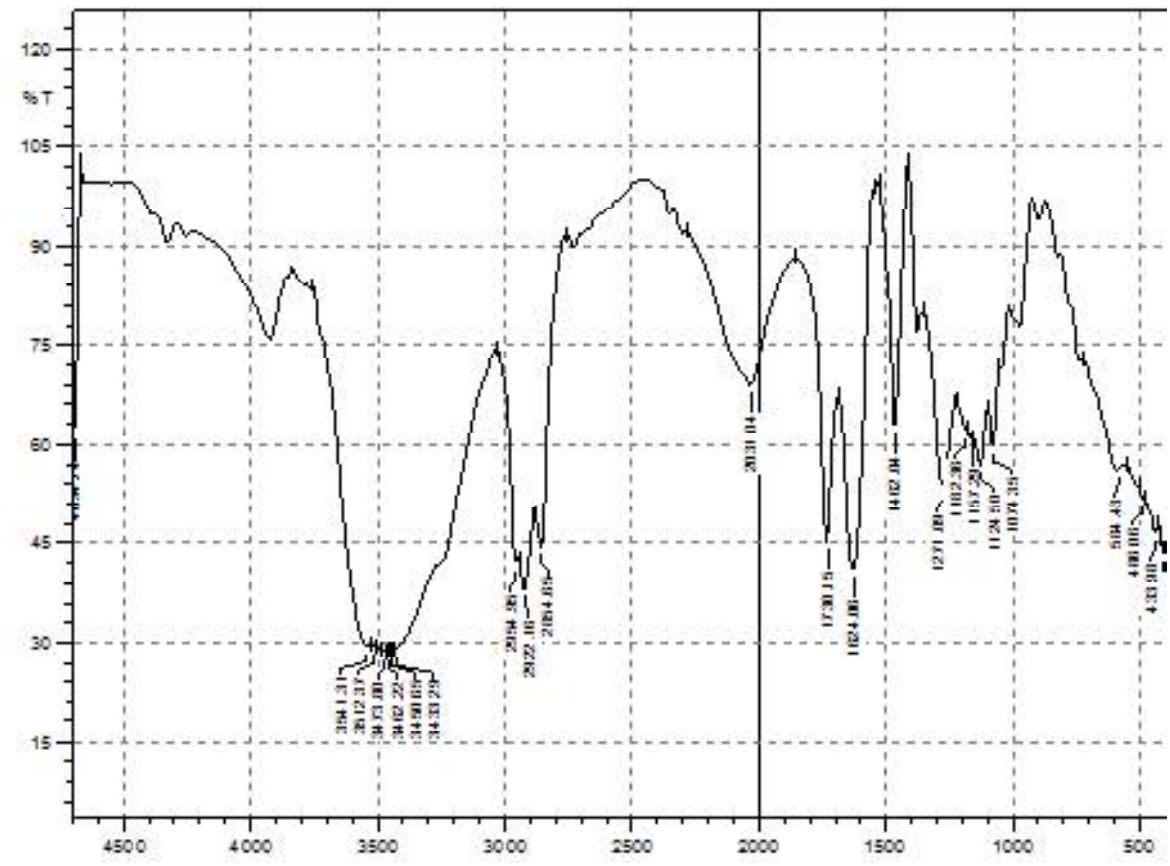

Fig. 3(i). FTIR spectrum, of radicle in chloroform

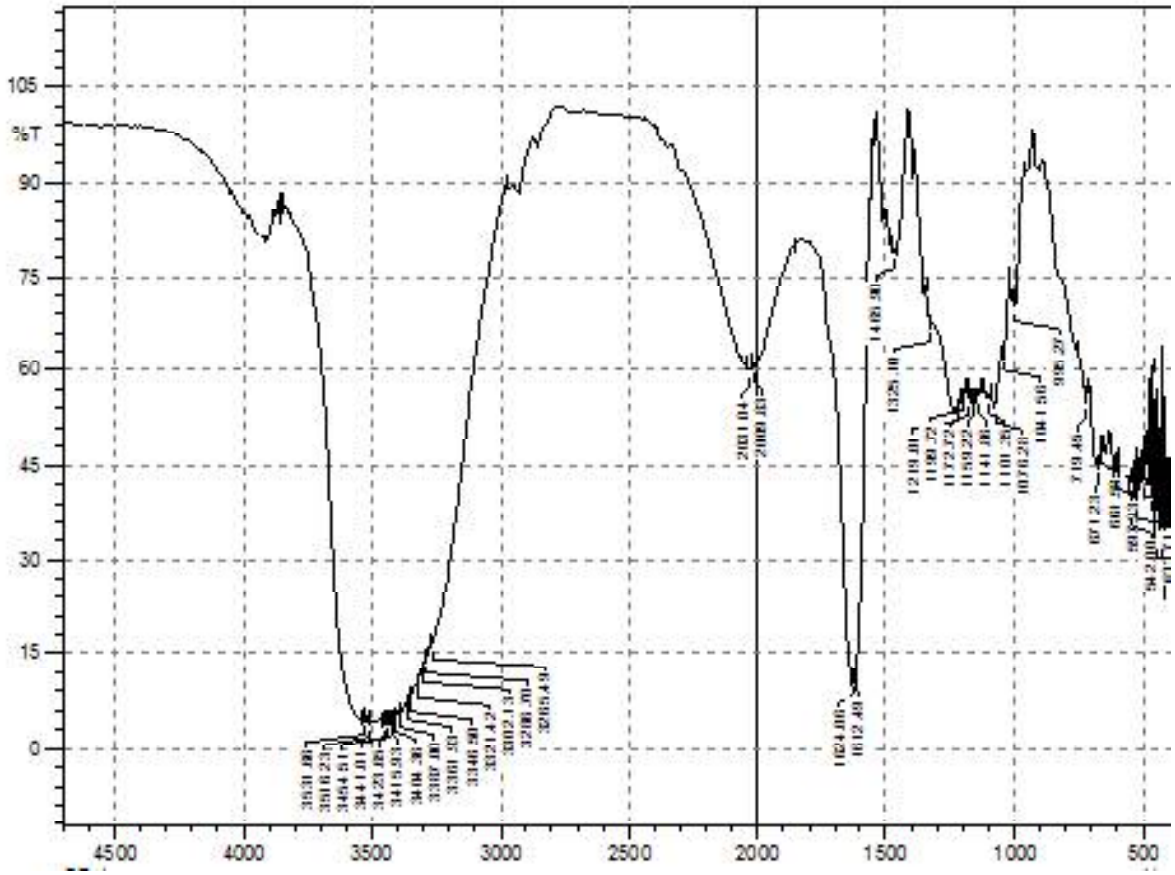

Fig. 3(j). FTIR spectrum, of plumule in 100% methanol

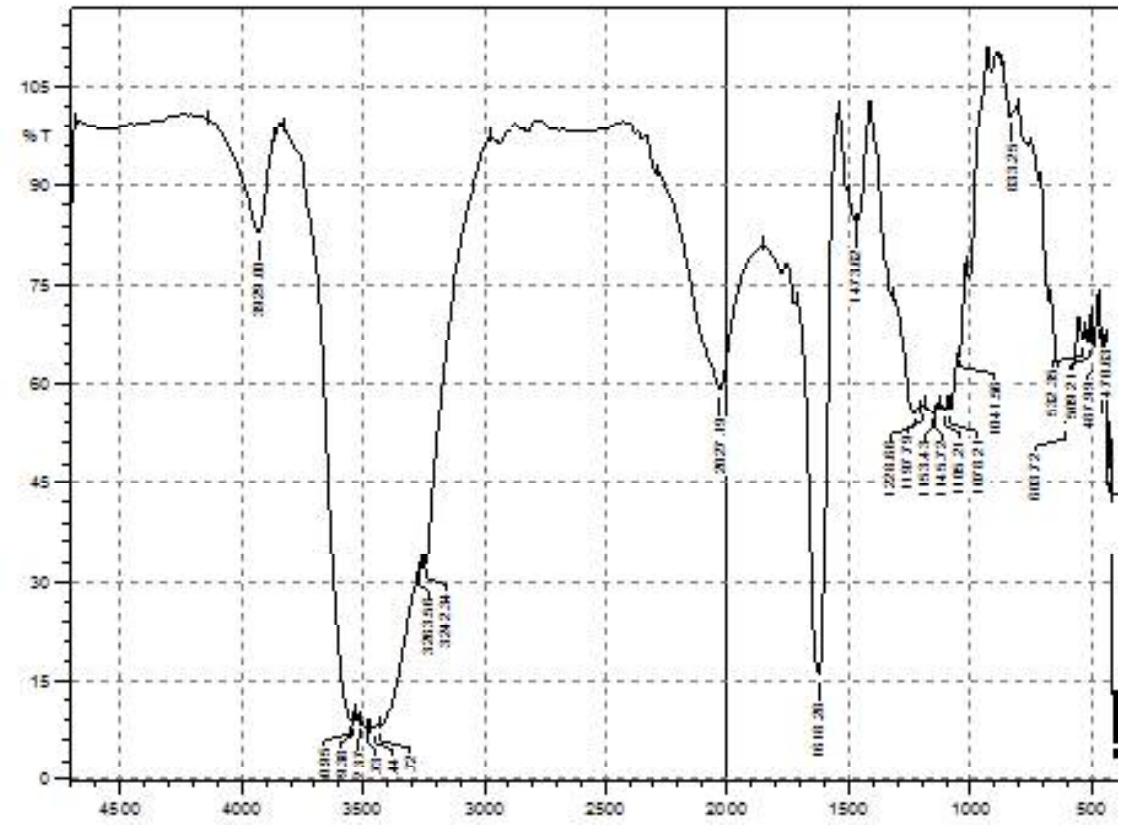

Fig. 3(k). FTIR spectrum, of plumule in 50% methanol

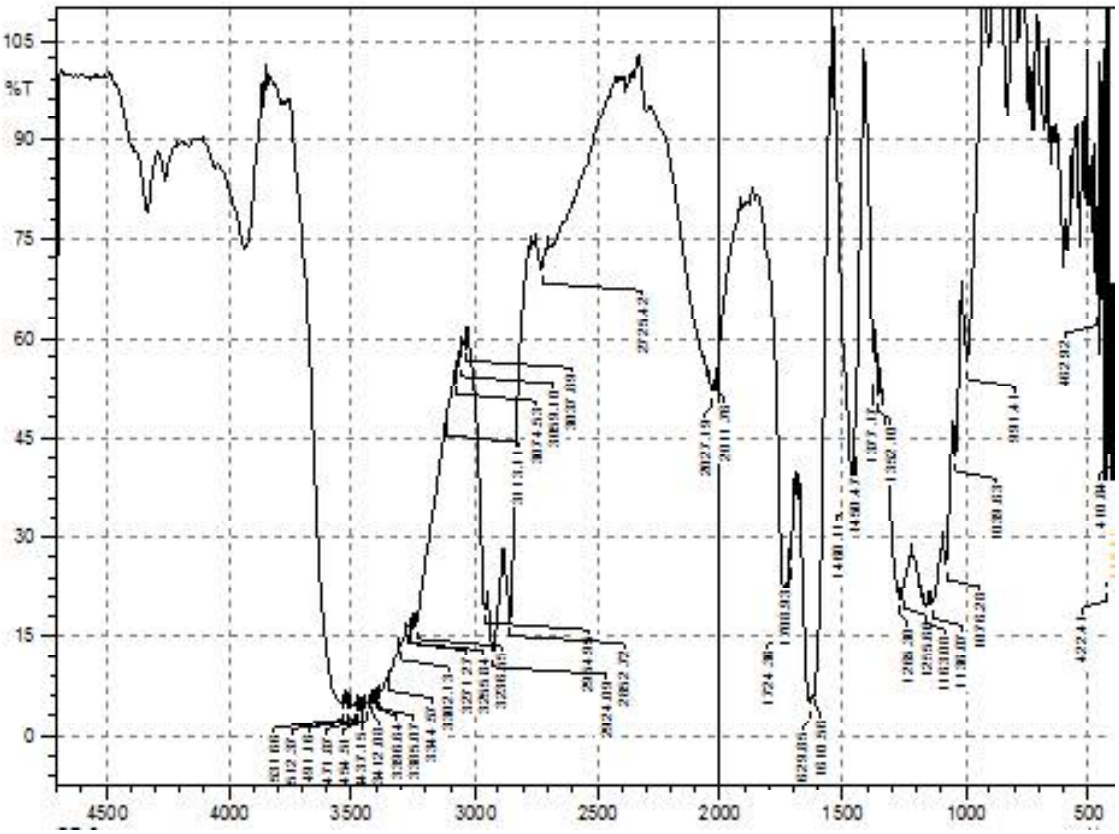

Fig. 3(l) FTIR spectrum, of plumule in chloroform

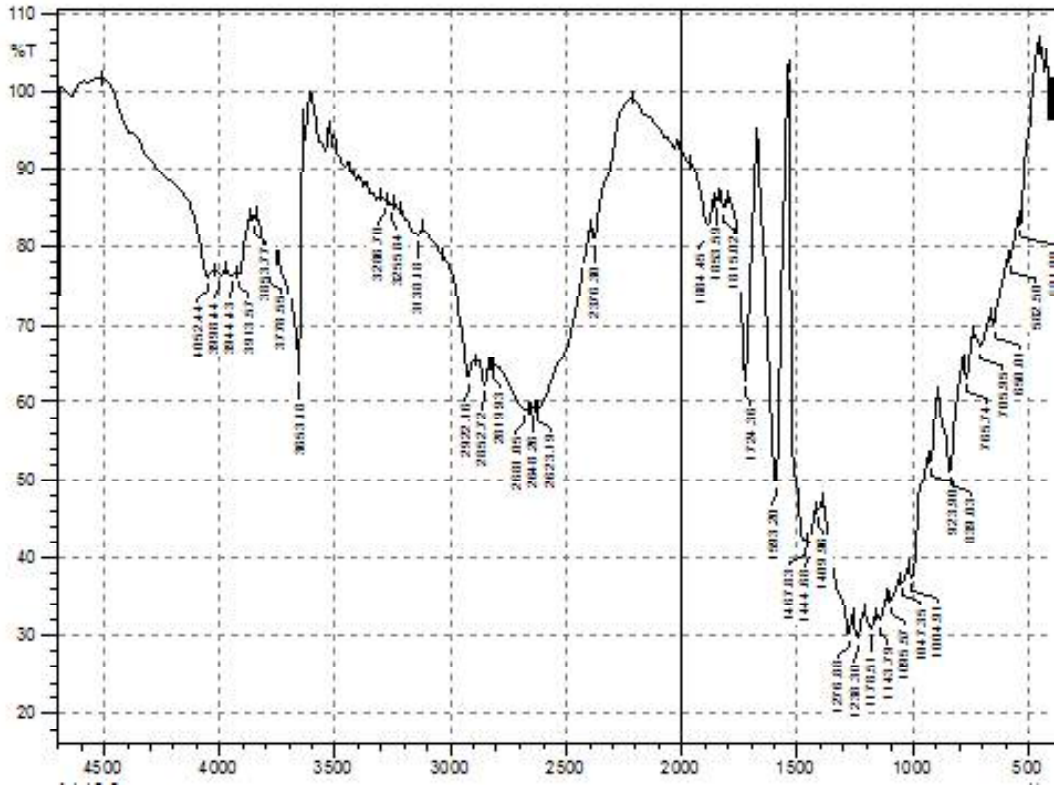

Fig. 3(m). FTIR spectrum, of embryonal axis, of threshold water level stage seeds in 100% methanol

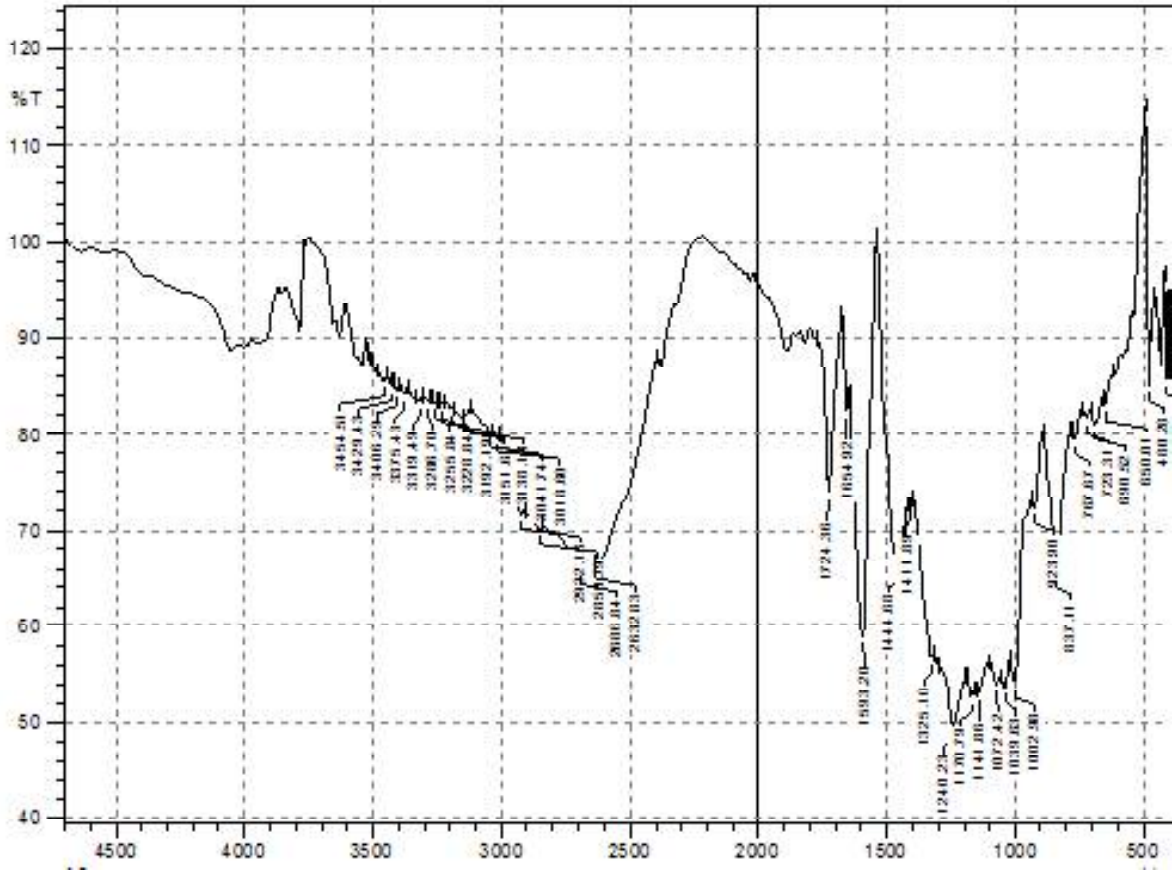

Fig. 3(n). FTIR spectrum of embryonal axis, of threshold water level stage seeds, in 50% methanol

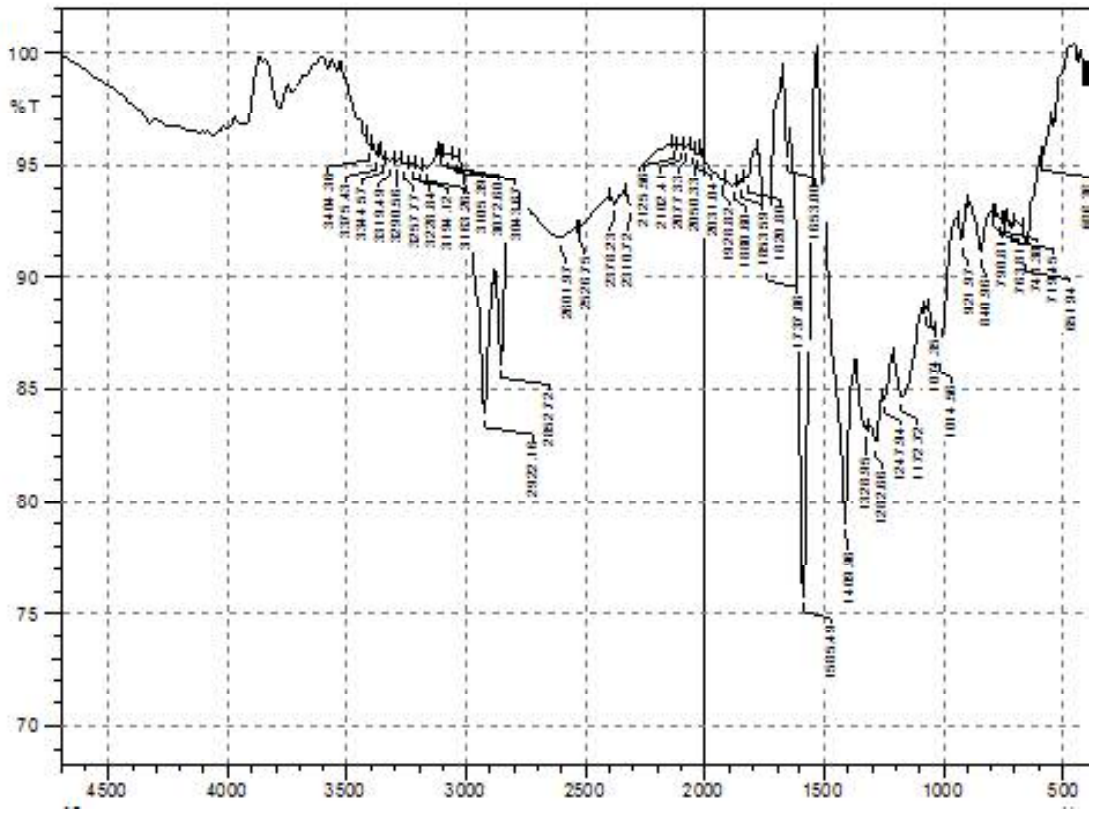

Fig. 3(o). FTIR spectrum of embryonal axis, of threshold water level stage seeds, in chloroform

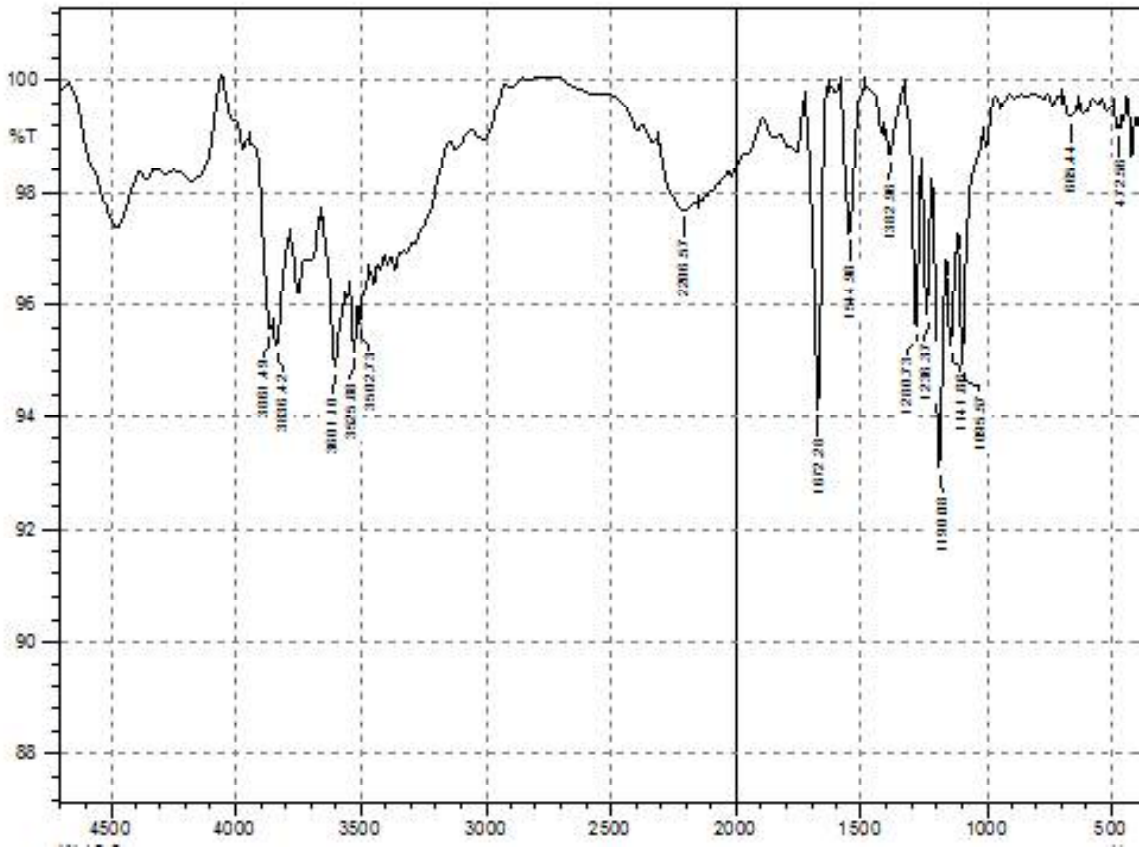

Fig. 3 (p). FTIR spectrum, of cotyledon, of threshold water level stage seeds, in 100% methanol

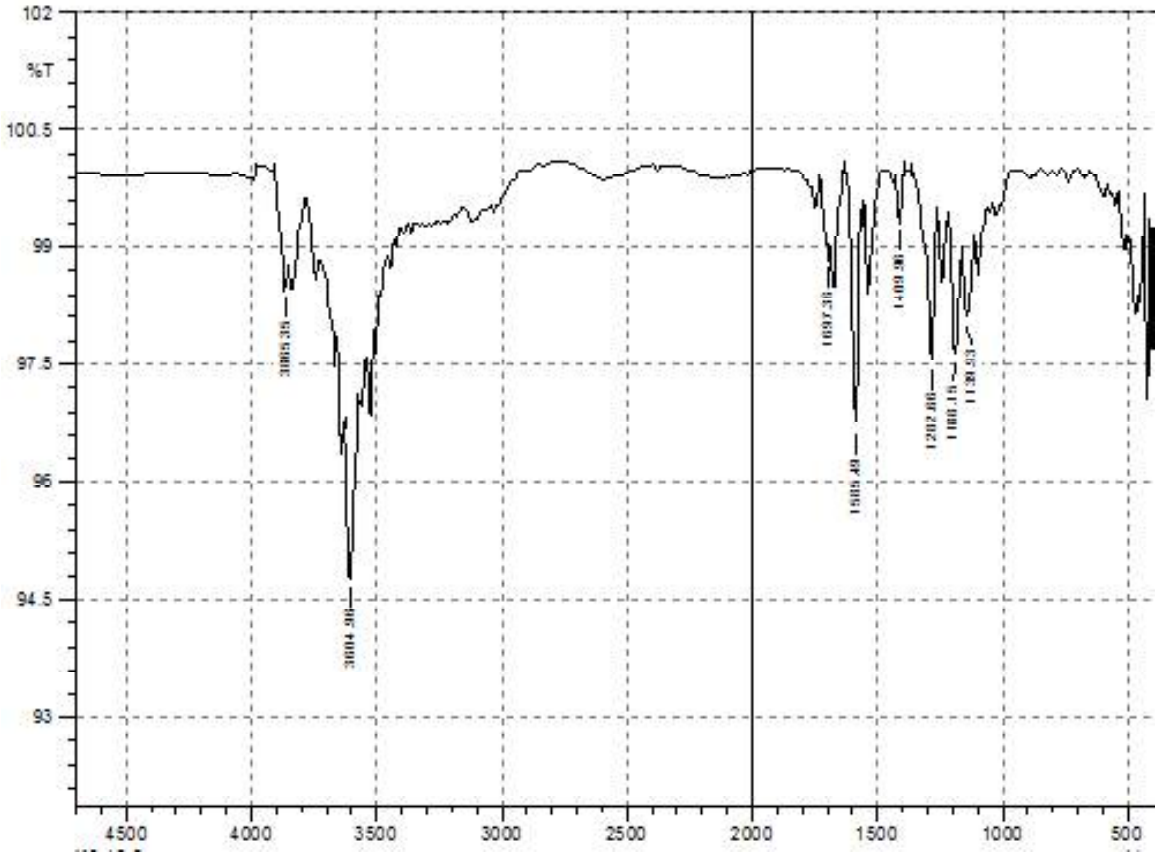

Fig. 3 (q). FTIR spectrum, of cotyledon, of threshold water level stage seeds, in 50% methanol.

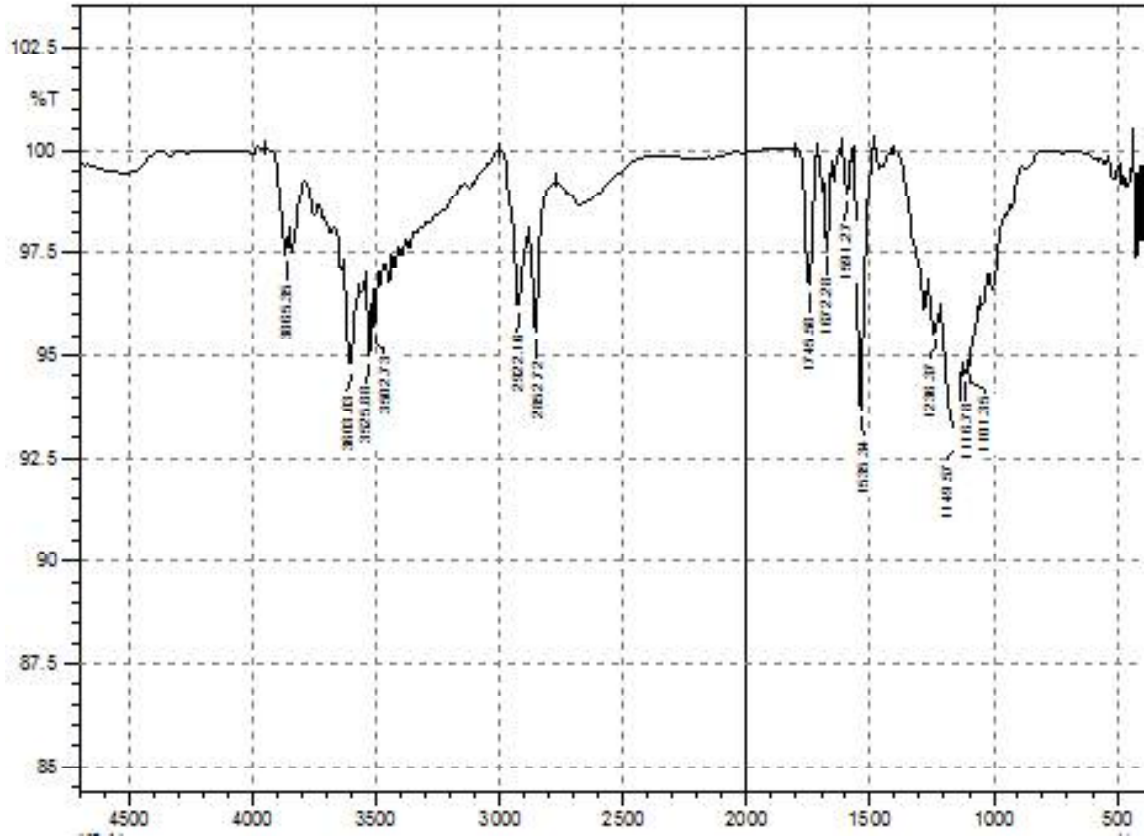

Fig. 3 (r). FTIR spectrum, of cotyledon, of threshold water level stage seeds, in chloroform

Table 1a. FTIR DATA of AXIS – Fully Mature- Freshly Fallen Seeds

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM FA3	100% METHANOL FA1	50% METHANOL FA2
1	ALKANES	C-H STRETCH	2852.72	---	---
		CH_2 BEND	1463.97	1463.97	1460.11
		CH_3 BEND	1463.97	1463.97	1460.11
2	ALKENES	C-H STRETCH	3089.96	---	---
		C=C STRETCH	1627.92	---	---
		=C-H BEND	---	---	---
3	AROMATIC RING	=C-H BEND	966.34 to 1161.15	1170.79 1082.07 1041.56	1002.98 to 1170.79
		C-H STRETCH	3089.96 3109.25	---	---
		C=C STRETCH	1463.97	1463.97	1460.11
4	ALDEHYDE	C-H STRETCH	---	---	---
		C=O STRETCH	1737.86	---	---
5	KETONES	C= O STRETCH		1722.43	1722.43
6	QUINONES	C=O STRETCH	1681.93/ 1618.28, 1627.92	1612.49	1616.35
7	ESTERS AND LACTONES	C=O STRETCH	1737.86	---	---
		C-O STRETCH	1039.63 to 1201.45	1230.58 1170.79	1232.51 1170.79
8	CARBOXYLIC ACIDS AND THEIR SALTS	O-H STRETCH	2852.72 to 3383.14	2627.05 to 3350.35	3323.35 to 3381.21
		C=O STRETCH	1234.44 1261.45	1230.58	1232.51
		C-O STRETCH			---
9	AMMONIUM SALTS	NH_4 SRETCH	3089.96 3284.77 / ---	---	---
		C=O STRETCH	1463.97 to 1627.92	---	1616.35 1512.19
10	AMIDES	N-H STRETCH		---	---
		C=O STRETCH	3109.25 to 3495.01	3138.18 to 3493.09	3323.35 o 3493.09
		N-H BEND	1618.28 to 1627.92	1612.49	1616.35
11	ALCOHOLS	O-H STRETCH	3205.69 to 3560.59	3201.83 to 3568.31	3323.35 to 3558.67
12	$1^0,2^0,3^0$ ALCOHOLS	C-O STRETCH	1074.35 to 1120.64	1082.07	1080.14 1143.79
		O-H STRETCH	---	3626.17	---
13	PHENOLS	C-O STRETCH	1039.63 to 1234.44	1001.06 to 1230.58	1002.98 to 1232.51
		O-H STRETCH	3545.16 to 3583.74	3568.31 to 3626.17	3558.67 3576.02
		O-H BEND	1234.44 to 1463.97	1230.58 to 1508.33	1232.51 1460.11

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM FA3	100% METHANOL FA1	50% METHANOL FA2
14	CARBOHYDRATES	O-H STRETCH	3089.96 to 3583.74	3018.60 to 3581.81	3323.35 to 3576.02
		C-O –C STRETCH	2852.72 to 3089.96	3018.60 3138.18	---
		C-H Stretch	1234.44 to 1463.97	1230.58 1463.97	1232.51 1460.11
15	ETHER	C-O STRETCH	1039.63 to 1261.45	1001.06 to 1230.58	1002.98 to 1232.51
16	ALIPHATIC PRI.AMINES	N-H STRETCH	3344.57	---	---
		C-N STRETCH	1039.63 to 1074.35	1082.07 1041.56	1080.14 1039.63
		N-H BEND	1618.28 to 1627.92	1612.49	1616.35
17	NITRILES	C = N STRETCH	---	---	---
18	SULPHIDES	C-S STRETCH	---	---	680.87
19	DISULPHIDES	S-S STRETCH C-S STRETCH	---	---	--- 680.87
20	POLYSULPHIDES	S-S STRETCH	---	---	---
21	SULPHUR –OXY COMPOUND	SO_2 STRETCH	---	---	---
22	PEROXIDES	C-O STRETCH	908.47 966.34 993.34	960.55	
23	ORGANIC PHOSPHATE	P=O STRETCH	1261.45	---	---
24	AROMATIC PHOSPHATES	P-O-C STRETCH	1234.44/ 908.47	1230.58/ 960.55	1232.51
25	ALIPHATIC PHOSPHATES	P-O-C STRETCH	993.34 1039.63	1041.56 1001.06	1039.63
26	HALIDES	C-F	1039.63 to 1120.64	1082.07 1041.56	1002.98 to 1143.79
		C-Cl	775.38	---	---
		C-Br	---	---	680.87
		C-I	516.92	---	532.42
27	SILICON OXY COMPOUNDS	Si –O-C	---	---	1105.21
		Si –O-Si		1082.07	---
28	AROMATIC NITROCOMPOUND	N=O	1463.97	1463.97	1512.19 1460.11
29	ALIPHATIC NITROCOMPOUND	N=O	1463.97	1463.97	1512.19 1460.11
30	ALI. SEC. AMINE	N-H STRETCH	3321.42 3322.57	3350.35	3323.35 to 3348.42
31	HETEROCYCLIC AMINE	N-H STRETCH	3439.08 TO 3471.87	3462.22	3435.22 to 3473.80
32	ALKYNE	C-H STRETCH		---	---
		C=C	---	---	---

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM FA3	100% METHANOL FA1	50% METHANOL FA2
33	ANTHEROQUINONE	C-O STRETCH	1039.63 to 1261.45	1001.06 to 1230.58	1002.98 to 1232.51
34	TERPENES	C-H STRETCH	2852.72	---	---
		C=C STRETCH	1627.92	---	---
35	CYANOGENIC GLYCOSIDES	C=N STRETCH	1627.92	1612.49	1616.35
		N=C STRETCH	---	---	---
36	ISOTHIOCYANIDE	N=C STRETCH	---	---	---
37	CARDIAC GLYCOSIDES	C-O STRETCH	1039.63 to 1261.45	1001.06 to 1230.58	1002.98 to 1232.51
		C=O STRETCH	1463.97 1618.28 1627.92	1508.33 1612.49	1616.35 1512.19
38	SAPONINS	C=O STRETCH	1463.97 1618.28 1627.92	1463.97 1508.33 1612.49	1616.35 1512.19 1460.11
		C-O STRETCH	1039.63 to 1261.45	1001.06 to 1230.58	1002.98 to 1232.51
39	SULPHONAMIDES	N-H STRETCH	3321.42 3344.57	3350.35	3323.35 3348.42
		S-O STRETCH	775.38 to 993.34	839.03 960.55	835.18
40	ALKALOIDES	N-H STRETCH	3321.42 TO 3344.57	3350.35	3323.35 3348.42
		C-N STRETCH	1039.63 to 1261.45	1041.56 to 1230.58	1232.51 1170.79 1143.79
		N-H BEND	1618.28 1627.92	1612.49	1616.35
41	TANNINS	O-H STRETCH	3545.16 TO 3583.74	3568.31 to 3626.17	3539.38 3576.02
		C-O STRETCH	1039.63 to 1261.45	1001.06 to 1230.58	1002.98 to 1232.51
42	STEROIDES	C-O STRETCH	1039.63 to 1261.45	1001.06 to 1230.58	1002.98 to 1232.51
		C=O STRETCH	1463.97 TO 1627.92	1463.97 1508.33 1612.49	1616.35 1512.19 1460.11
43	FLAVANOIDES	O-H STRETCH	3545.16 TO 3583.74	3568.31 to 3626.17	3539.38 3576.02
		C=O STRETCH	1463.97 TO 1627.92	1463.97 1508.33 1612.49	1616.35 1512.19 1460.11

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM FA3	100% METHANOL FA1	50% METHANOL FA2
44	ANHYDRIDE	C-O STRETCH	1039.63 to 1261.45	1001.06 to 1230.58	1002.98 to 1232.51
		C=O STRETCH	1463.97 TO 1627.92	1463.97 1508.33 1612.49	1616.35 1512.19 1460.11
45	MERCAPTANS	S-H STRETCH	---	---	---
46	THIOPHINES	S-H STRETCH	---	---	---
47	THIOLACIDS	S-H STRETCH	---	---	---
48	SULPHONIC ACID	O-H STRETCH(SO_3H)	3545.16 to 3583.74	3568.31 to 3626.17	3539.38 3576.02
49	THIOAMIDE&THIOUREAS	N-H STRETCH	3321.42 3344.57	3350.35	3323.35 to 3348.42
		C=S STRETCH	1039.63 to 1161.15	1041.56 1082.07 1170.79	1039.63 to 1170.79
50	CYANATE	OCN & C-OCN STRETCH	---/ 1120.64 1161.15	---/ 1170.79 1082.07	---/ 1080.14 to 1170.79
51	ISOCYANATE	N=C=O (ASYM.STRETCH)	---	---	---
52	THIOCYNATE	-SCN	---	---	---
53	THIOLS	S-H STRETCH	---	---	---
54	PHOSPHINE OXIDE	P=O STRETCH	1161.15	1170.79	1143.79 1170.79
55	PHOSPHINE	P-H STRETCH	---	---	---
		P-H BEND	908.47 to 1074.35	1082.07 1041.56	835.18 to 1080.14
56	IMINE	R_2C=N-R STRETCH	1681.93	---	---
57	THIOCARBONYL	C=S	1074.35 to 1161.15	1170.79 1082.07	1080.14 1170.79
58	ARENES	C-H	775.38	839.18	835.18
		C=S	---	1508.33 1612.49	1512.19
59	PHOSPHORAMIDE	P=O	1234.44 to 1261.45	1230.58	1232.51
60	SILANE	Si-H	---	---	---
61	=NOH OXIME	O-H STRETCH	3560.59 3583.74	3568.31 3581.81	---
		C=NSTRETCH		---	---
		N-OSTRETCH	---	---	---
62	BENZALDEHYDE	C=O STRETCH	1618.93 to 1737.86	1612.49 1722.43	1616.35 1722.43
63	ALLENE	C=C=C STRETCH	---	---	---
64	1-HEXENE	C-H STRETCH	3005.10 3089.96	3018.60	---

Table 1b.FTIR DATA of COTYLEDON – Fully Mature- Freshly Fallen Seeds

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM	100% METHANOL	50% METHANOL
1	ALKANES	C-H STRETCH	---	---	2852.72
		CH_2 BEND	1462.97	1448.54	---
		CH_3 BEND	1462.97	1448.54	---
2	ALKENES	C-H STRETCH	---	3089.96	---
		C=C STRETCH	1622.13 1639.49	---	---
		=C-H BEND	1244.09	---	---
3	AROMATIC RING	=C-H BEND	997.20 TO 1172.72	954.76	1002.98 to 1234.44
		C-H STRETCH	---	---	---
		C=C STRETCH	1462.04	---	---
4	ALDEHYDE	C-H STRETCH	2729.27	---	---
		C=O STRETCH	---	---	---
5	KETONES	C= O STRETCH	---	1718.58	---
6	QUINONES	C=O STRETCH	---/ 1612.49 TO 1639.49	1612.49	1614.42
7	ESTERS AND LACTONES	C=O STRETCH	---	---	1745.58
		C-O STRETCH	1037.70 to 1244.09	1230.58 1172.72	1234.44 to 1002.98
8	CARBOXYLIC ACIDS AND THEIR SALTS	O-H STRETCH	2677.20 to 3383.14	2511.32 to 3381.21	2852.72 to 3381.21
		C=O STRETCH	1244.09	1230.58	1234.44
		C-O STRETCH	---	---	---
9	AMMONIUM SALTS	NH_4 SRETCH	---	3045.60 to 3284.11	---
		C=O STRETCH	1462.04 to 1639.49	1612.49	1614.42
10	AMIDES	N-H STRETCH	1639.49	---	---
		C=O STRETCH	3383.14 to 3498.87	3109.25 to3493.09	3302.13 to 3491.16
		N-H BEND	1612.49 to 1639.49	1612.49	1614.42
11	ALCOHOLS	O-H STRETCH	3383.14 3498.87	3203.76 to 3539.38	3302.13 to 3556.74
12	$1^0,2^0,3^0$ ALCOHOLS	C-O STRETCH	1072.42 to 1109.07	1076.28	1105.21 1074.35
		O-H STRETCH	3622.32 to 3643.53	---	
13	PHENOLS	C-O STRETCH	1037.70 to 1244.09	1004.91 to 1230.58	1002.98 to 1234.44
		O-H STRETCH	3622.32	3539.38	3541.31 to 3579.88
		O-H BEND	1244.09 1462.04	1230.58 to1512.19	1234.44 1460.11 1512.19

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM	100% METHANOL	50% METHANOL
14	CARBOHYDRATES	O-H STRETCH	3383.14 3498.87	3068.75 to 3539.38	3302.13 to 3579.88
		C-O –C STRETCH	2850.79 2916.37 2997.38	2852.72 to 3045.60	2852.72 2922.16
		C-H Stretch	1244.04 1462.04	1230.58 to 1448.54	1234.44 1460.11
15	ETHER	C-O STRETCH	1037.70 to 1244.09	1004.91 to 1230.58	1002.98 to 1234.44
16	ALIPHATIC PRI.AMINES	N-H STRETCH	---	3344.57	---
		C-N STRETCH	1037.70 to 1072.42	1076.28	1039.63 to 1074.35
		N-H BEND	1612.49 to to 1639.49	1612.49	1614.42
17	NITRILES	C = N STRETCH	---	---	---
18	SULPHIDES	C-S STRETCH	688.59	578.64 to 688.59	680.87
19	DISULPHIDES	S-S STRETCH C-S STRETCH	688.59	607.58 578.64 to 688.59	---
20	POLYSULPHIDES	S-S STRETCH	---	---	---
21	SULPHUR –OXY COMPOUND	SO_2 STRETCH	---	---	---
22	PEROXIDES	C-O STRETCH	908.47 997.20	923.90 954.76	---
23	ORGANIC PHOSPHATE	P=O STRETCH	---	1328.95	---
24	AROMATIC PHOSPHATES	P-O-C STRETCH	908.47	1230.58 954.76 923.90	---
25	ALIPHATIC PHOSPHATES	P-O-C STRETCH	997.20 1037.70	---	1002.44 to 1039.63
26	HALIDES	C-F	1037.70 to 1109.07	1076.28 1004.91	1002.98 to 1105.43
		C-Cl	723.31 to 769.60	763.81 727.16	---
		C-Br	688.59	607.58 TO 688.59	680.87
		C-I	---	578.64	532.35
27	SILICON OXY COMPOUNDS	Si –O-C	1109.07	---	1105.21
		Si –O-Si	---	1076.28	---
28	AROMATIC NITROCOMPOUND	N=O	1462.04	1512.19 1448.54 1328.95	1512.19
29	ALIPHATIC NITROCOMPOUND	N=O	1462.04	1512.19 1448.54	1512.19
30	ALI. SEC. AMINE	N-H STRETCH	---	3323.35 to 3344.57	3348.42

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM	100% METHANOL	50% METHANOL
31	HETEROCYCLIC AMINE	N-H STRETCH	---	3435.22 to 3471.87	3433.29 to 3495.73
32	ALKYNE	C-H STRETCH	---	---	---
		C=C	---	---	---
33	ANTHEROQUINONE	C-O STRETCH	1037.70 to 1244.09	1004.91 to 1230.58	1002.98 to 1234.44
34	TERPENES	C-H STRETCH	2850.79	2852.72	2852.72
		C=C STRETCH	---	---	---
35	CYANOGENIC GLYCOSIDES	C=N STRETCH	1612.13 1639.49	1612.49	1614.42
		N=C STRETCH	---	---	---
36	ISOTHIOCYANIDE	N=C STRETCH	---	---	---
37	CARDIAC GLYCOSIDES	C-O STRETCH	1037.70 to 1244.09	1004.91 to 1230.58	1002.98 to 1234.44
		C=O STRETCH	1462.04 to 1639.49	1612.49 1512.19 1448.54	1512.19 1614.42
38	SAPONINS	C=O STRETCH	1462.04 to 1639.49	1612.49 1512.19 1448.54	1512.19 1614.42 1448.54
		C-O STRETCH	1037.70 to 1244.09	1004.91 to 1230.58	1002.98 to 1234.44
39	SULPHONAMIDES	N-H STRETCH	---	3323.35 3344.57	3348.42
		S-O STRETCH	---	763.81 to 954.76	---
40	ALKALOIDES	N-H STRETCH	---	3323.35 to 3344.57	3348.42 to 1234.44
		C-N STRETCH	1037.70 to 1244.09	1328.95 1230.58	1039.63 to 1234.44
		N-H BEND	1612.49 to 1639.49	1612.49	1614.42
41	TANNINS	O-H STRETCH	3622.32	3539.38	3541.31 to 3579.88
		C-O STRETCH	1037.70 to 1244.09	1004.91 to 1230.58	1002.98 to 1234.44
42	STEROIDES	C-O STRETCH	1037.70 to 1244.09	1004.91 to 1230.58	1002.98 to 1234.44
		C=O STRETCH	1462.04 to 1639.49	1612.49 1512.19 1448.54	1512.19 1614.42 1448.54
43	FLAVANOIDES	O-H STRETCH	3622.32	3539.38	3541.31 to 3579.88
		C=O STRETCH	1462.04 to 1639.49	1612.49 1512.19 1448.54	1512.19 1614.42 1448.54

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM	100% METHANOL	50% METHANOL
44	ANHYDRIDE	C-O STRETCH	1037.70 to 1244.09	1004.91 to 1230.58	1002.98 to 1234.44
		C=O STRETCH	1462.04 to 1639.49	1612.49 1512.19 1448.54	1512.19 1614.42 1448.54
45	MERCAPTANS	S-H STRETCH	---	---	---
46	THIOPHINES	S-H STRETCH	---	---	---
47	THIOLACIDS	S-H STRETCH	---	---	---
48	SULPHONIC ACID	O-H STRETCH(SO_3H)	3622.32	3539.38	3541.31 to 3579.88
49	THIOAMIDE&THIOUREAS	N-H STRETCH	---	3325.35 to 3344.57	3348.42
		C=S STRETCH	1037.70 to 1172.72	1172.72 1076.28	1039.63 to 1199.72
50	CYANATE	OCN&C-OCN STRETCH	---/ 1170.79 1082.07	---/ ---	---/ 1105.21 to 1153.43
51	ISOCYANATE	N=C=O (ASYM.STRETCH)	---	---	---
52	THIOCYNATE	-SCN	---	---	---
53	THIOLS	S-H STRETCH	---	---	---
54	PHOSPHINE OXIDE	P=O STRETCH	1172.72	---	1153.43 1199.72
55	PHOSPHINE	P-H STRETCH	---	---	---
		P-H BEND	908.47 to 1042.42	1076.28 954.76 923.90	835.18 to 1074.35
56	IMINE	R_2C=N-R STRETCH	1639.49	---	---
57	THIOCARBONYL	C=S	1072.42 1172.72	1172.72 1076.28	1074.35 1199.72
58	ARENES	C-H	723.31 to 798.60	835.18 763.81 727.16	835.18
		C=S	---	1512.19	1512.19
59	PHOSPHORAMIDE	P=O	1244.09	1230.58	---
60	SILANE	Si-H	---	---	---
61	=NOH OXIME	O-H STRETCH	---	---	3556.74 to 3579.88
		C=NSTRETCH	---	---	---
		N-OSTRETCH	---	954.76	---
62	BENZALDEHYDE	C=O STRETCH	1612.49 1622.13 1639.49	1612.49 1718.58	1614.42 1745.58
63	ALLENE	C=C=C STRETCH	---	---	---
64	1-HEXENE	C-H STRETCH	---	3024.38 to 3089.96	---

Table 1c. FTIR DATA of PLUMULE

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM GP3	100% METHANOL GP1	50% METHANOL GP2
1	ALKANES	C-H STRETCH		---	---
		CH_2 BEND	1450.47 1460.11	1465.90	1473.62
		CH_3 BEND	1450.47 / ---	1465.90 / ---	--- / ---
2	ALKENES	C-H STRETCH	3074.53	---	---
		C=C STRETCH	1629.85	1624.06	---
		=C-H BEND	---	---	---
3	AROMATIC RING	=C-H BEND	991.41 TO 1163.08	995.27 to 1219.01	1041.56 to 1197.79
		C-H STRETCH	3074.53 3113.11	--	---
		C=C STRETCH	1450.47 1460.11	1465.90	1473.62
4	ALDEHYDE	C-H STRETCH	2725.42	---	---
		C=O STRETCH		---	---
5	KETONES	C= O STRETCH	1708.93 1724.36	---	---
6	QUINONES	C=O STRETCH	--- / 1610.56 1629.85	--- / 1624.06	---/ 1618.28
7	ESTERS AND LACTONES	C=O STRETCH		---	---
		C-O STRETCH	1039.63 to 1265.30	1041.56 to 1219.01	1041.56 to 1234.44
8	CARBOXYLIC ACIDS AND THEIR SALTS	O-H STRETCH	2725.42 to 3396.64	3265.49 to 3387.00	3242.34 3263.56
		C=O STRETCH	1255.66 to 1265.30	1219.01	1228.66
		C-O STRETCH	---	---	---
9	AMMONIUM SALTS	NH_4 SRETCH	3037.89 to 3271.27 / ---	3265.49 3286.70 / ---	3242.34 3263.56/ ---
		C=O STRETCH	1352.10 to 1629.85	1465.90 to 1624.06	1473.62 1618.13
10	AMIDES	N-H STRETCH		---	---
		C=O STRETCH	3113.11 to 3491.16	3265.49 to 3454.51	3242.34 to 3475.73
		N-H BEND	1610.56 1629.85	1612.49 1624.06	1618.28
11	ALCOHOLS	O-H STRETCH	3236.55 to 3531.66	3265.49 to 3531.66	3242.34 to 3550.95

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM GP3	100% METHANOL GP1	50% METHANOL GP2
12	$1^0,2^0,3^0$ ALCOHOLS	C-O STRETCH	1076.28 1136.07	1076.28 1101.35 1141.86	1078.21 1105.21 1145.72
		O-H STRETCH	---	---	---
13	PHENOLS	C-O STRETCH	1039.63 to 1255.66	1041.56 to 1219.01	1041.56 to1228.66
		O-H STRETCH	3531.66	3531.66	
		O-H BEND	1255.66 to 1460.11	1325.10 1465.90	1228.66 1473.62
14	CARBOHYDRATES	O-H STRETCH	3059.10 to 3531.66	3265.49 to 3531.66	
		C-O –C STRETCH	2852.72 to3037.89	---	---
		C-H Stretch	1255.66 to 1460.11	1219.01 1325.10 1465.90	1228.66 1473.62
15	ETHER	C-O STRETCH	1039.63 to 1265.30	1041.56 to 1219.01	1041.56 to 1228.66
16	ALIPHATIC PRI.AMINES	N-H STRETCH	3344.57		---
		C-N STRETCH	1039.63 1076.28	1041.56 1076.28	1041.56 1078.21
		N-H BEND	1610.65 1629.85	1612.49 1624.06	1618.28
17	NITRILES	C = N STRETCH	---	---	---
18	SULPHIDES	C-S STRETCH	---	597.93 to 671.23	603.72
19	DISULPHIDES	S-S STRETCH C-S STRETCH	---	--- 597.93 to 671.23	603.72 603.72
20	POLYSULPHIDES	S-S STRETCH	---	---	470.63 487.99
21	SULPHUR –OXY COMPOUND	SO_2 STRETCH	1352.10 1377.17	---	---
22	PEROXIDES	C-O STRETCH	991.41---	995.27	---
23	ORGANIC PHOSPHATE	P=O STRETCH	1255.66 1265.30	1325.10	---
24	AROMATIC PHOSPHATES	P-O-C STRETCH	---	1199.72 1219.01	1197.79 1228.66/ ----
25	ALIPHATIC PHOSPHATES	P-O-C STRETCH	991.41 1039.63	995.27 1041.56	1041.56

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM GP3	100% METHANOL GP1	50% METHANOL GP2
26	HALIDES	C-F	1039.63 to 1136.07	1041.56 to 1141.86	1041.56 to 1145.72
		C-Cl	---	719.45	---
		C-Br	---	661.58 671.23	603.72
		C-I	---	522.71 to 597.93	509.21 532.35
27	SILICON OXY COMPOUNDS	Si –O-C	---	1101.35	1105.35
		Si –O-Si	1076.28	1076.28	1078.21
28	AROMATIC NITROCOMPOUND	N=O	1377.17 to 1460.11	1465.90	1473.62
29	ALIPHATIC NITROCOMPOUND	N=O	1450.47 1460.11	1465.90	1473.62
30	ALI. SEC. AMINE	N-H STRETCH	3344.57	3321.42 3346.50	---
31	HETEROCYCLIC AMINE	N-H STRETCH	3437.15 to 3471.87	3441.01 3454.51	3456.44 3475.73
32	ALKYNE	C-H STRETCH	---		---
		C=C	---	---	---
33	ANTHEROQUINONE	C-O STRETCH	1039.63 to 1265.30	1041.56 to 1219.01	1041.56 to 1228.66
34	TERPENES	C-H STRETCH	2852.72	---	---
		C=C STRETCH	1629.85	1624.06	---
35	CYANOGENIC GLYCOSIDES	C=N STRETCH	1610.56 1629.85	1624.06	1618.28
		N=C STRETCH	---	---	---
36	ISOTHIOCYANIDE	N=C STRETCH	---	---	---
37	CARDIAC GLYCOSIDES	C-O STRETCH	1039.63 to 1265.30	1041.56 to 1219.01	1041.56 to 1228.66
		C=O STRETCH	1352.10 to 1629.85	1465.90 1612.49 1624.06	1473.62 1618.28
38	SAPONINS	C=O STRETCH	1352.10 to 1629.85	1465.90 1612.49 1624.06	1473.62 1618.28
		C-O STRETCH	1039.63 to 1265.30	1041.56 to 1219.01	1041.56 to 1228.66
39	SULPHONAMIDES	N-H STRETCH	3344.57	3321.42 3346.50	
		S-O STRETCH	991.41	995.27	833.25

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM GP3	100% METHANOL GP1	50% METHANOL GP2
40	ALKALOIDES	N-H STRETCH	3344.57	3321.42 3346.50	---
		C-N STRETCH	1039.63 to 1352.10	1041.56 to 1325.10	1041.56 to 1228.66
		N-H BEND	1610.56 1629.85	1612.49 1624.06	1618.28
41	TANNINS	O-H STRETCH	3531.66	3531.66	3539.38 3550.95
		C-O STRETCH	1039.63 to 1265.30	1041.56 to 1219.01	1041.56 to 1228.66
42	STEROIDES	C-O STRETCH	1039.63 to 1265.30	1041.56 to 1219.01	1041.56 to 1228.66
		C=O STRETCH	1352.10 to 1629.85	1465.90 1612.49 1624.06	1473.62 1618.28
43	FLAVANOIDES	O-H STRETCH	3531.66	3531.66	3539.38 3550.95
		C=O STRETCH	1352.10 to 1629.85	1465.90 1612.49 1624.06	1473.62 1618.28
44	ANHYDRIDE	C-O STRETCH	1039.63 to 1265.30	1041.56 to 1219.01	1041.56 to 1228.66
		C=O STRETCH	1352.10 to 1629.85	1465.90 1612.49 1624.06	1473.62 1618.28
45	MERCAPTANS	S-H STRETCH	---	---	---
46	THIOPHINES	S-H STRETCH	---	---	---
47	THIOLACIDS	S-H STRETCH	---	---	---
48	SULPHONIC ACID	O-H STRETCH(SO_3H)	3531.66	3531.66	3539.38 3550.95
49	THIOAMIDE&THIOUREAS	N-H STRETCH	3344.57	3321.42 3346.50	---
		C=S STRETCH	1039.63 to 1163.08	1041.56 t01219.01	1041.56 to 1197.79
50	CYANATE	OCN&C-OCN STRETCH	--- / 1136.07 1163.08	--- / 1101.35 to 1172.72	--- / 1051.21 t01153.43
51	ISOCYANATE	N=C=O (ASYM.STRETCH)	---	---	---
52	THIOCYNATE	-SCN	---	---	---

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM GP3	100% METHANOL GP1	50% METHANOL GP2
53	THIOLS	S-H STRETCH	---	---	---
54	PHOSPHINE OXIDE	P=O STRETCH	1163.08	1141.86 to 1199.72	1145.72 to 1197.79
55	PHOSPHINE	P-H STRETCH	---	---	---
		P-H BEND	991.41 to 1076.28	995.27 to 1076.28	833.25 to 1078.21
56	IMINE	R_2C=N-R STRETCH	1629.85	---	---
57	THIOCARBONYL	C=S	1076.28 to 1163.08	1076.28 to 1199.72	1078.21 to 1197.79
58	ARENES	C-H	---	719.45	833.25
		C=S	---	---	---
59	PHOSPHORAMIDE	P=O	1255.66 1265.30	1219.01	1228.66
60	SILANE	Si-H	---	---	---
61	=NOH OXIME	O-H STRETCH	3551.66	---	3550.95
		C=NSTRETCH	---	---	---
		N-OSTRETCH	---	---	---
62	BENZALDEHYDE	C=O STRETCH	1610.56 to 1724.36	1612.49 1624.06	1618.28
63	ALLENE	C=C=C STRETCH	---	---	---
64	1-HEXENE	C-H STRETCH	3037.89 3059.10 3074.53	---	---

The moisture content of the axis and cotyledon dropped in response to natural desiccation. The moisture content in the axis dropped from 57% to 56% during three days of natural drying indicating innate mechanism in the seed tissues to prevent dehydration, a characteristic of recalcitrant seeds (Fig.2). However, the cotyledonary tissue had lower water content (37%) at the time of seed dispersal and it dropped to 33% after three days of desiccation. The rate of water loss in the embryonal and cotyledonary tissues was lower during the initial period of desiccation but rapid in the later stage, probably due to the loss of membrane permeability. Cent percent germination could be observed up to seventh days of desiccation. However, the rate of germination decreased drastically (60%) after seventh days of desiccation which could be attributed to the rapid loss of water due to the loss of membrane permeability. Hence, the critical moisture content in *Vateria indica* seeds is found to be 47% for the embryonal axis and 30% for cotyledonary tissues. It has been reported that seed germination, growth, DNA integrity, protein synthesis, membrane structure, organelle formation and normal embryo development are inhibited when internal hydration level drops below critical thresholds (Artlip *et al.*, 1995; Kermode,1990; MacIntyre, 1987; Osborne *et al.*, 1994).

FTIR Spectral Analysis of Phytochemicals

Extracts were prepared from the above mentioned stages, using three different solvents viz. 100% and 50% methanol and chloroform to obtain polar as well as non-polar functional groups present in the compounds. The FTIR spectra are presented in the Fig.3 (a-r).

Compounds necessary for faster germination like

Table 1 d. FTIR DATA of RADICLE

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM GR3	100% METHANOL GR1	50% METHANOL GR2
1	ALKANES	C-H STRETCH	2854.95	---	---
		CH_2 BEND	1462.04	1458.18	1458.18
		CH_3 BEND	1462.04 / ---	1458.18 / ---	1458.18 / ---
2	ALKENES	C-H STRETCH	---	---	---
		C=C STRETCH	1624.06	---	1622.13
		=C-H BEND	---	---	---
3	AROMATIC RING	=C-H BEND	1074.35 to 1182.36	1004.91 1072.42 1143.79	1045.42 to 1234.44
		C-H STRETCH	---	---	---
		C=C STRETCH	1462.04	1458.18 1510.26	1458.18
4	ALDEHYDE	C-H STRETCH	---	2773.64	---
		C=O STRETCH	1730.15		---
5	KETONES	C= O STRETCH	---	1724.36	---
6	QUINONES	C=O STRETCH	--- / 1624.06	---/ 1616.35	---/ 1612.49 1622.33
7	ESTERS AND LACTONES	C=O STRETCH	1730.15		---
		C-O STRETCH	1074.35 TO 1271.09	1004.91 to 1232.51	1045 to 1234.44
8	CARBOXYLIC ACIDS AND THEIR SALTS	O-H STRETCH	2854.65 2922.95 2954.95	2684.91 to 3385.07	3145.90 to 3379.29
		C=O STRETCH	1271.09	1232.51	1234.44
		C-O STRETCH			---
9	AMMONIUM SALTS	NH_4 SRETCH	--- / ---	3265.49 / ---	3145.90 to 3300.20 / ---
		C=O STRETCH	1462.04 1624.06	1458.18 to 1616.35	1458.18 to 1622.13
10	AMIDES	N-H STRETCH	---	---	---
		C=O STRETCH	3433.29 to 3473.80	3625.49 to 3491.16	3145.90 to 3489.23
		N-H BEND	1624.06	1616.35	1612.49 to1622.13
11	ALCOHOLS	O-H STRETCH	3433.29 to 3541.31	3265.49 to 3558.67	3203.76 to 3547.09
12	$1^0,2^0,3^0$ ALCOHOLS	C-O STRETCH	1074.35 1124.50	1072.42 1143.79	1074.35
		O-H STRETCH	---	---	---
13	PHENOLS	C-O STRETCH	1074.35 to 1182.36	1004.91 to1232.51	1045.42 1074.35 1234.44
		O-H STRETCH	3541.31	3539.38 3558.67	3537.45 3547.09
		O-H BEND	1271.09 1462.04	1232.51 to 1510.26	1234.44 1458.18

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM GR3	100% METHANOL GR1	50% METHANOL GR2
14	CARBOHYDRATES	O-H STRETCH	3433.29 to 3541.31	3265.49 to 3558.67	3145.90to 3547.09
		C-O –C STRETCH	2854.65 2922.16 2954.95	2850.79 2920.23 3022.45	---
		C-H Stretch	1271.09 1462.04	1232.51 1458.18	1234.44 1458.18
15	ETHER	C-O STRETCH	1074.35 to 1271.09	1004.91 to 1232.51	1045.42 1074.35 1234.44
16	ALIPHATIC PRI.AMINES	N-H STRETCH	---	---	3342.64
		C-N STRETCH	1074.35	1072.42	1045.42 1074.35
		N-H BEND	1624.06	1616.35	1612.49 1622.13
17	NITRILES	C = N STRETCH	---	---	---
18	SULPHIDES	C-S STRETCH	584.43	580.57 to 678.94	582.50 to 678.94
19	DISULPHIDES	S-S STRETCH C-S STRETCH	--- 584.43	605.65 580.57 to 678.94	--- / 582.50 to 678.94
20	POLYSULPHIDES	S-S STRETCH	486.06	---	---
21	SULPHUR –OXY COMPOUND	SO_2 STRETCH	---	---	---
22	PEROXIDES	C-O STRETCH	---	914.26	---
23	ORGANIC PHOSPHATE	P=O STRETCH	1271.09	---	---
24	AROMATIC PHOSPHATES	P-O-C STRETCH	---	1232.51	1234.44 / ---
25	ALIPHATIC PHOSPHATES	P-O-C STRETCH	---	1004.91	1045.42
26	HALIDES	C-F	1074.35 1124.50	1004.91 to 1143.79	1045.42 1074.35
		C-Cl	---	719.45 775.38	779.24
		C-Br	---	605.65 678.94	678.94
		C-I	584.43	580.57	582.50
27	SILICON OXY COMPOUNDS	Si –O-C	---	---	---
		Si –O-Si		---	
28	AROMATIC NITROCOMPOUND	N=O	1462.04	1458.18 1510.26	1458.18
29	ALIPHATIC NITROCOMPOUND	N=O	1462.04	1458.18 1510.26	1458.18
30	ALI. SEC. AMINE	N-H STRETCH	---	3348.42	3319.49 3342.64 3360.00

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM GR3	100% METHANOL GR1	50% METHANOL GR2
31	HETEROCYCLIC AMINE	N-H STRETCH	3433.29 to 3473.80	3433.29 to 3471.87	3439.08 to 3489.23
32	ALKYNE	C-H STRETCH	---	---	331949
		C=C	---	---	---
33	ANTHEROQUINONE	C-O STRETCH	1074.35 to 1271.09	1004.91 to 1232.51	1045.42 1074.35 1234.44
34	TERPENES	C-H STRETCH	2854.65	2850.79	---
		C=C STRETCH	1624.06	---	---
35	CYANOGENIC GLYCOSIDES	C=N STRETCH	1624.06	1616.35	1612.49 1622.13
		N=C STRETCH	---	---	---
36	ISOTHIOCYANIDE	N=C STRETCH	---	---	----
37	CARDIAC GLYCOSIDES	C-O STRETCH	1074.35 to 1271.09	1004.91 to 1232.51	1045.42 1074.35 1234.44
		C=O STRETCH	1462.04 1624.06	1458.18 1510.26 1616.35	1458.18 1612.49 1622.13
38	SAPONINS	C=O STRETCH	1462.04 1624.06	1458.18 1510.26 1616.35	1458.18 1612.49 1622.13
		C-O STRETCH	1074.35 to 1271.09	1004.91 to 1232.51	1045.42 1074.35 1234.44
39	SULPHONAMIDES	N-H STRETCH	---	3348.42	3319.49 to 3360
		S-O STRETCH	---	775.38 to 914.26	779.24 837.11
40	ALKALOIDES	N-H STRETCH	---	3348.42	3319.49 3342.64 3360.00
		C-N STRETCH	1074.35 to 1271.09	1072.42 to 1232.51	1045.42 1234.44
		N-H BEND	1624.06	1616.35	1458.18 1612.44 1622.13
41	TANNINS	O-H STRETCH	3541.31	3539.38	3537.45 3547.09
		C-O STRETCH	1074.35 to 1271.09	1004.91 to 1232.51	1045.42 1074.35 1234.44
42	STEROIDES	C-O STRETCH	1074.35 to 1271.09	1004.91 to 1232.51	1045.42 1074.35 1234.44
		C=O STRETCH	1462.04 1624.06	1458.18 1510.26 1616.35	1458.18 1612.49 1622.13
43	FLAVANOIDES	O-H STRETCH	3541.31	3539.38 3558.67	3537.45 3547.09
		C=O STRETCH	1462.04 1624.06	1458.18 1510.26 1616.35	1458.18 1612.49 1622.13

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM GR3	100% METHANOL GR1	50% METHANOL GR2
44	ANHYDRIDE	C-O STRETCH	1074.35 to 1271.09	1004.91 to 1232.51	1045.42 1074.35 1234.44
		C=O STRETCH	1462.04 1624.06	1458.18 1510.26 1616.35	1458.18 1612.49 1622.13
45	MERCAPTANS	S-H STRETCH	---	---	---
46	THIOPHINES	S-H STRETCH	---	---	---
47	THIOLACIDS	S-H STRETCH	---	---	---
48	SULPHONIC ACID	O-H STRETCH(SO_3H)	3541.31	3539.38 3558.67	3537.45 3547.09
49	THIOAMIDE&THIOUREAS	N-H STRETCH	---	3348.42	3319.49 3342.64 3360.00
		C=S STRETCH	1074.56 to 1182.36	1072.42 1143.79	1045.42 to 1074.35
50	CYANATE	OCN&C-OCN STRETCH	--- / 1124.50 to 1182.36	--- / 1143.79	--- / ---
51	ISOCYANATE	N=C=O (ASYM.STRETCH)	---	---	---
52	THIOCYNATE	-SCN	---	---	---
53	THIOLS	S-H STRETCH	---	---	---
54	PHOSPHINE OXIDE	P=O STRETCH	1157.29 1182.36	1143.79	---
55	PHOSPHINE	P-H STRETCH	---	---	---
		P-H BEND	1074.35	833.25 to 1072.42	837.11 to 1074.35
56	IMINE	R_2C=N-R STRETCH	---	---	---
57	THIOCARBONYL	C=S	1074.35 to 1182.36	1072.42 1143.79	1074.35
58	ARENES	C-H	---	719.45 to 871.82	779.24 837.11
		C=S	1462.04 1624.06	1510.26	---
59	PHOSPHORAMIDE	P=O	1271.09	1232.51	1234.44
60	SILANE	Si-H	---	---	---
61	=NOH OXIME	O-H STRETCH	---	3539.38 3558.67	---
		C=NSTRETCH	---	---	---
		N-OSTRETCH	---	---	---
62	BENZALDEHYDE	C=O STRETCH	1624.06 1730.15	1616.35 1724.36	1612.49 1622.13
63	ALLENE	C=C=C STRETCH	---	---	---
64	1-HEXENE	C-H STRETCH	---	3022.45	---

Table 1e. FTIR DATA of AXIS (Critical Threshold Water Level Stage)

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM FA3	100% METHANOL FA1	50% METHANOL FA2
1	ALKANES	C-H STRETCH	---	---	2922.16
		CH_2 BEND	---	1467.83	
		CH_3 BEND	---	1444.68	1444.68/ ---
2	ALKENES	C-H STRETCH	---	---	---
		C=C STRETCH	1653.00	---	1654.92
		=C-H BEND	---	---	---
3	AROMATIC RING	=C-H BEND	1014.56 1074.35 1172.72	1004.91 to 1178.51	1002.98 to 1170.79
		C-H STRETCH	3072.60 3105.39	---	---
		C=C STRETCH	---	1467.83	---
4	ALDEHYDE	C-H STRETCH	---	---	---
		C=O STRETCH	1737.86	---	
5	KETONES	C= O STRETCH	---	1724.36	1724.36
6	QUINONES	C=O STRETCH		---	---
7	ESTERS AND LACTONES	C=O STRETCH	1737.86	---	---
		C-O STRETCH	1014.56 to 1282.66	1004.91 to 1276.88	1002.98 to 1240.23
8	CARBOXYLIC ACIDS AND THEIR SALTS	O-H STRETCH	2526.75 to 3375.43	2623.19 to 3286.70	2632.83 to 3375.43
		C=O STRETCH	1247.94 1282.66	1238.30 to 1276.88	1240.23
		C-O STRETCH		--	
9	AMMONIUM SALTS	NH_4 SRETCH	3043.67 3290.56/ 1409.96	3138.18 3286.70/ 1409.96	3041.74 to 3286.70/ 1411.89
		C=O STRETCH	1409.96 1585.49	1409.96 to 1593.20	1411.89 to 1593.20
10	AMIDES	N-H STRETCH	1653.00	---	1654.92
		C=O STRETCH	3105.39 to 3404.36	3138.18 to 3286.70	3138.18 to 3454.51
		N-H BEND	1585.49	1593.20	1593.20
11	ALCOHOLS	O-H STRETCH	3228.84 to 3404.36	3255.84 to 3286.70	3228.84 to 3454.51
12	$1^0,2^0,3^0$ ALCOHOLS	C-O STRETCH	1074.35	1095.57 1143.79	1072.42 to 1141.86
		O-H STRETCH	---	---	---
13	PHENOLS	C-O STRETCH	1014.56 to 1247.94	1004.91 to1238.30	1002.98 to 1240.23
		O-H STRETCH	---	---	---
		O-H BEND	1247.94 to 1409.96	1238.30 to 1467.83	1240.23 to 1444.68

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM FA3	100% METHANOL FA1	50% METHANOL FA2
14	CARBOHYDRATES	O-H STRETCH	3070.60 to 3404.36	3138.18 3255.84 3286.70	3138.18 to 3454.51
		C-O –C STRETCH	2852.72 2922.16 3043.67	2819.93 2852.72 2819.93	2850.79 to 3041.74
		C-H Stretch	1247.94 to 1409.96	1238.30 to 1467.83	1240.23 to 1444.68
15	ETHER	C-O STRETCH	1014.56 to 1282.66	1004.91 to 1276.88	1002.98 to 1240.23
16	ALIPHATIC PRI.AMINES	N-H STRETCH	3344.57	---	---
		C-N STRETCH	1074.35	1047.35	1039.63 1072.42
		N-H BEND		1593.20	1593.20
17	NITRILES	C = N STRETCH	2050.33 to 2310.72	---	---
18	SULPHIDES	C-S STRETCH	586.36 651.94	582.50 to 650.01	650.01 690.52
19	DISULPHIDES	S-S STRETCH C-S STRETCH	---	---	---/ 650.01 690.52
20	POLYSULPHIDES	S-S STRETCH	586.36 651.94	582.50 to 705.95	480.28
21	SULPHUR –OXY COMPOUND	SO_2 STRETCH	---	---	1411.89
22	PEROXIDES	C-O STRETCH	921.97	923.90	923.90
23	ORGANIC PHOSPHATE	P=O STRETCH	1282.66 1328.95	1276.88	1325.10
24	AROMATIC PHOSPHATES	P-O-C STRETCH	---/ 921.97	1238.30/ 923.90	923.90
25	ALIPHATIC PHOSPHATES	P-O-C STRETCH	1014.56	1004.91,1047.35	1002.98 1039.63
26	HALIDES	C-F	1014.56 1074.35	1004.91 to 1143.79	1002.98 to 1141.86
		C-Cl	719.45 to 790.81	705.95 to 765.74	723.31 767.67
		C-Br	651.94	650.01	650.01 690.52
		C-I	586.36	524.08 to 582.50	---
27	SILICON OXY COMPOUNDS	Si –O-C	---	1095.57	---
		Si –O-Si			---
28	AROMATIC NITROCOMPOUND	N=O	1409.96	1409.96 to 1467.83	1411.89 1444.68
29	ALIPHATIC NITROCOMPOUND	N=O	1409.96	1409.96 to 1467.83	1411.89 1444.68
30	ALI. SEC. AMINE	N-H STRETCH	3319.49 3344.57	---	3319.49

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM FA3	100% METHANOL FA1	50% METHANOL FA2
31	HETEROCYCLIC AMINE	N-H STRETCH	---	---	3454.51
32	ALKYNE	C-H STRETCH	3319.49	---	3319.49
		C=C	---	---	---
33	ANTHEROQUINONE	C-O STRETCH	1014.56 to 1282.66	1004.91 to 1276.88	1002.98 to 1240.23
34	TERPENES	C-H STRETCH	2852.72	2819.93 to 2852.72	2850.79
		C=C STRETCH	1653.00	---	1654.92
35	CYANOGENIC GLYCOSIDES	C=N STRETCH		---	---
		N=C STRETCH	2102.41 2125.56	---	---
36	ISOTHIOCYANIDE	N=C STRETCH	2102.41 2125.56	---	---
37	CARDIAC GLYCOSIDES	C-O STRETCH	1014.56 to 1282.66	1004.91 to 1276.88	1002.98 to 1240.23
		C=O STRETCH	1409.96 1585.49	1409.96 to 1593.20	1411.89 to 1593.20
38	SAPONINS	C=O STRETCH	1409.96 1585.49	1409.96 to 1593.20	1411.89 to 1593.20
		C-O STRETCH	1014.56 to 1282.66	1004.91 to 1276.88	1002.98 to 1240.23
39	SULPHONAMIDES	N-H STRETCH	3319.49 3344.57	---	3319.49
		S-O STRETCH	763.81 to 921.97	---	767.67 837.11 923.90
40	ALKALOIDES	N-H STRETCH	3319.49 3344.57	---	3319.49
		C-N STRETCH	1074.35 to 1328.95	1047.35 to 1276.88	1039.63 to 1325.10
		N-H BEND	---	1593.20	1593.20
41	TANNINS	O-H STRETCH	---	---	---
		C-O STRETCH	1014.56 to 1282.66	1004.91 to 1276.88	1002.98 to 1240.23
42	STEROIDES	C-O STRETCH	1014.56 to 1282.66	1004.91 to 1276.88	1002.98 to 1240.23
		C=O STRETCH	1409.96 1585.49	1409.96 to 1593.20	1411.89 to 1593.20
43	FLAVANOIDES	O-H STRETCH	---	---	---
		C=O STRETCH	1409.96 1585.49	1409.96 to 1593.20	1411.89 to 1593.20
44	ANHYDRIDE	C-O STRETCH	1014.56 to 1282.66	1004.91 to 1276.88	1002.98 to 1240.23
		C=O STRETCH	1409.96 1585.49	1409.96 to 1593.20	1411.89 to 1593.20

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM FA3	100% METHANOL FA1	50% METHANOL FA2
45	MERCAPTANS	S-H STRETCH	2601.97???	---	---
46	THIOPHINES	S-H STRETCH	---	---	---
47	THIOLACIDS	S-H STRETCH	---	---	---
48	SULPHONIC ACID	O-H STRETCH(SO_3H)	---	---	---
49	THIOAMIDE&THIOUREAS	N-H STRETCH	3319.49 3344.57	---	---
		C=S STRETCH	1074.35 1172.72	1047.35 to 1178.51	1039.63 to 1170.79
50	CYANATE	OCN&C-OCN STRETCH	---/ 1172.72	---/ 1095.57 to 1178.51	---/ 1141.86 1170.79
51	ISOCYANATE	N=C=O (ASYM.STRETCH)	---	---	---
52	THIOCYNATE	-SCN	---	---	---
53	THIOLS	S-H STRETCH		---	---
54	PHOSPHINE OXIDE	P=O STRETCH	1172.72	1143.79 1178.51	1141.86 1170.79
55	PHOSPHINE	P-H STRETCH	2310.72	---	---
		P-H BEND	840.96 to 1074.35	839.03 to 1047.35	837.11 to 1072.42
56	IMINE	R_2C=N-R STRETCH	1653.00	---	1654.92
57	THIOCARBONYL	C=S	1074.35 1172.72	1095.57 1178.51	1072.42 1170.79
58	ARENES	C-H	719.45 to 840.95	705.95 to 839.03	690.52 to837.11
		C=S	1585.49	1593.20	1593.20
59	PHOSPHORAMIDE	P=O	1247.94	1238.30	1240.23
60	SILANE	Si-H	2102.41 to 2310.72	---	---
61	=NOH OXIME	O-H STRETCH	---	---	---
		C=NSTRETCH	1653.00	---	1654.92
		N-OSTRETCH	---	---	---
62	BENZALDEHYDE	C=O STRETCH	1653.00 1737.86	1724.36	1654.92 1724.36
63	ALLENE	C=C=C STRETCH	1928.82	---	---
64	1-HEXENE	C-H STRETCH	3043.67 3072.60	---	3018.60 3041.74

primary, secondary and tertiary alcohols, phenols, tannins, flavanoids, and sulphonic acid were altogether absent in the embryonal axis of threshold water level stage, though these compounds were detected in the cotyledons of the same stage(Fig.3m-r). This shows that these compounds were not transported from the cotyledons to the axis and it may be due to the loss of membrane structure and permeability.

Another interesting thing noticed was that, allene made its appearance only on prolonged desiccation of seeds indicating that it is having some role in delaying seed germination (Fig.3-o)

More than sixty phytochemicals were identified with the aid of FTIR. The phytochemicals were

Table 1f. FTIR DATA of Cotyledon (Critical Threshold Water Level Stage)

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM 13.6 FK3	100% METHANOL 13.6 FK1	50% METHANOL 13.6 FK2
1	ALKANES	C-H STRETCH	---		---
		CH_2 BEND	---		---
		CH_3 BEND	---		---
2	ALKENES	C-H STRETCH	---		---
		C=C STRETCH	1672.28	1672.28	---
		=C-H BEND	---	---	---
3	AROMATIC RING	=C-H BEND	1101.35 1116.78 1149.57	1095.57 to 1190.08	1139.93 1188.15
		C-H STRETCH	---	---	---
		C=C STRETCH	---	---	---
4	ALDEHYDE	C-H STRETCH	---	---	---
		C=O STRETCH	---	---	---
5	KETONES	C= O STRETCH	---	---	---
6	QUINONES	C=O STRETCH	---	---	---
7	ESTERS AND LACTONES	C=O STRETCH	1745.58	---	---
		C-O STRETCH	1101.35 to 1236.37	1095.57 to 1280.73	1139.93 to 1282.66
8	CARBOXYLIC ACIDS AND THEIR SALTS	O-H STRETCH	2852.72 2922.16	---	---
		C=O STRETCH	1236.37	1236.37 1280.73	1282.66
		C-O STRETCH	---	---	---
9	AMMONIUM SALTS	NH_4 SRETCH	---	---	---/ 1409.96
		C=O STRETCH	1535.34 1591.27	1382.96 to 1544.98	1409.96 158549
10	AMIDES	N-H STRETCH	1672.28	1672.28	---
		C=O STRETCH		---	---
		N-H BEND	1535.34 1591.27	---	1585.49
11	ALCOHOLS	O-H STRETCH	3502.73 3525.88	3502.73 3525.88	---
12	$1^0,2^0,3^0$ ALCOHOLS	C-O STRETCH	1101.35 1116.75 1149.57	---	1139.93
		O-H STRETCH	3603.03	3601.10	3604.96
13	PHENOLS	C-O STRETCH	1101.35 to 1236.37	1095 to 1236.37	1139.93 1188.15
		O-H STRETCH	3603.03	3601.10	3604.96
		O-H BEND	1236.37 1535.34	1236.37 to 1544.98	1282.66 1409.96

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM 13.6 FK3	100% METHANOL 13.6 FK1	50% METHANOL 13.6 FK2
14	CARBOHYDRATES	O-H STRETCH	3502.73 3525.88	3502.73 3525.88	3604.96
		C-O –C STRETCH	2852.72 2922.16	---	---
		C-H Stretch	1236.37	1236.37 1382.96 1544.98	1282.66 1409.96
15	ETHER	C-O STRETCH	1101.35 to 1236.37	1095.57 to1289.73	1139.93 1188.`5 1282.66
16	ALIPHATIC PRI.AMINES	N-H STRETCH	---	---	---
		C-N STRETCH	---	---	---
		N-H BEND	1519.27	---	---
17	NITRILES	C = N STRETCH	---	2206.57	---
18	SULPHIDES	C-S STRETCH	---	665.44	---
19	DISULPHIDES	S-S STRETCH C-S STRETCH	--- ---	--- 665.44	--- ---
20	POLYSULPHIDES	S-S STRETCH	---	---	---
21	SULPHUR –OXY COMPOUND	SO_2 STRETCH	---	1382.96	1409.96
22	PEROXIDES	C-O STRETCH	---	---	---
23	ORGANIC PHOSPHATE	P=O STRETCH	---	1280.73	1282.66
24	AROMATIC PHOSPHATES	P-O-C STRETCH	---/ 921.97 1236.37	1190.08 1236.37/ ---	---
25	ALIPHATIC PHOSPHATES	P-O-C STRETCH	---	---	---
26	HALIDES	C-F	---	1095.57 1141.86	1139.93
		C-Cl	---	---	---
		C-Br	---	665.44	---
		C-I	---	---	---
27	SILICON OXY COMPOUNDS	Si –O-C	1101.35	1095.57	---
		Si –O-Si	---		---
28	AROMATIC NITROCOMPOUND	N=O	1535.34	1382.96 1544.98	1409.96
29	ALIPHATIC NITROCOMPOUND	N=O	1535.34	1382.96 1544.98	1409.96
30	ALI. SEC. AMINE	N-H STRETCH	---	---	---
31	HETEROCYCLIC AMINE	N-H STRETCH	---	---	---
32	ALKYNE	C-H STRETCH	---	---	---
		C=C	---	2206.57	---
33	ANTHEROQUINONE	C-O STRETCH	1101.35 to 1236.37	1095.57 to 1280.73	1139.93 1188.15 1282.66

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM 13.6 FK3	100% METHANOL 13.6 FK1	50% METHANOL 13.6 FK2
34	TERPENES	C-H STRETCH	2852.72	---	---
		C=C STRETCH	1672.28	1672.28	---
35	CYANOGENIC GLYCOSIDES	C=N STRETCH	---	---	---
		N=C STRETCH	---	2206.57	---
36	ISOTHIOCYANIDE	N=C STRETCH	---	2206.57	---
37	CARDIAC GLYCOSIDES	C-O STRETCH	1101.35 to 1236.37	1095.57 to 1280.73	1139.93 1188.15 1282.66
		C=O STRETCH	1535.34 1591.27	1382.96 1544.98	1409.96 1585.49
38	SAPONINS	C=O STRETCH	1535.34 1591.27	1382.96 1544.98	1409.96 1585.49
		C-O STRETCH	1101.35 to 1236.37	1095.57 to 1280.73	1139.93 1188.15 1282.66
39	SULPHONAMIDES	N-H STRETCH	---	---	---
		S-O STRETCH	---	---	---
40	ALKALOIDES	N-H STRETCH	---	---	---
		C-N STRETCH	1101.35 to 1236.37	1095.57 to 1280.73	1139.93 1188.15 1282.66
		N-H BEND	1591.27	---	---
41	TANNINS	O-H STRETCH	3603.03	3601.10	3604.96
		C-O STRETCH	1101.35 to 1236.37	1095.57 to 1280.73	1139.93 1188.15 1282.66
42	STEROIDES	C-O STRETCH	1101.35 to 1236.37	1095.57 to 1280.73	1139.93 1188.15 1282.66
		C=O STRETCH	1535.34 1591.27	---	1409.96 1585.49
43	FLAVANOIDES	O-H STRETCH	3603.03	3601.10	3604.96
		C=O STRETCH	1535.34 1591.27	1382.96 1544.98	1409.96 1585.49
44	ANHYDRIDE	C-O STRETCH	1101.35 to 1236.37	1095.57 to 1280.73	1139.93 1188.`5 1282.66
		C=O STRETCH	1535.34 1591.27	1382.96 1544.98	1409.96 1585.49
45	MERCAPTANS	S-H STRETCH	---	---	---
46	THIOPHINES	S-H STRETCH	---	---	---
47	THIOLACIDS	S-H STRETCH	---	---	---
48	SULPHONIC ACID	O-H STRETCH(SO_3H)	3603.03	3601.10	3604.96
49	THIOAMIDE&THIOUREAS	N-H STRETCH	---	---	---
		C=S STRETCH	1101.35 1116.78 1149.57	1095.57 to 1190.08	1139.93 1188.15

SL NO	FUNCTIONAL COMPOUND	STRETCH/BEND	CHLOROFORM 13.6 FK3	100% METHANOL 13.6 FK1	50% METHANOL 13.6 FK2
50	CYANATE	OCN&C-OCN STRETCH	---/ 1101.35 1116.78 1149.57	---/ 1095.57 to 1190.08	---/ 1188.15 1139.93
51	ISOCYANATE	N=C=O (ASYM.STRETCH)	---	---	---
52	THIOCYNATE	-SCN	---	---	---
53	THIOLS	S-H STRETCH	---	---	---
54	PHOSPHINE OXIDE	P=O STRETCH	1149.57	1141.86 1190.08	1188.15
55	PHOSPHINE	P-H STRETCH	---	---	---
		P-H BEND	---	---	---
56	IMINE	R_2C=N-R STRETCH	1672.28	1672.28	---
57	THIOCARBONYL	C=S	1101.35 1116.78 1149.57	1095.57 1190.08	1139.93 1188.15
58	ARENES	C-H	---	---	---
		C=S	1535.34 1591.27	1544.98	1585.49
59	PHOSPHORAMIDE	P=O	1236.37	1236.37	---
60	SILANE	Si-H	---	2206.57	---
61	=NOH OXIME	O-H STRETCH			
		C=NSTRETCH	1672.28	1672.28	---
		N-OSTRETCH	---	---	---
62	BENZALDEHYDE	C=O STRETCH	1672.28 1745.58	1672.28	1697.36
63	ALLENE	C=C=C STRETCH	---	---	---
64	1-HEXENE	C-H STRETCH	---	---	---

identified based on the characteristic stretch and bend of their functional groups (Table.1 (a – f)).

Compounds like aromatic ring, aldehyde, quinines, aliphatic primary amines were not detected in the embryonal axis of the seeds at the critical threshold water level phase (Table.1 –e). This may be a reason for the delayed or poor germination, when the days of desiccation prolonged.

All the three stages analysed showed the presence of esters and lactones, alcohols, carbohydrates, ether, nitriles, sulphides, organic phosphate, aromatic phosphates, aromatic nitro compounds, anthroquinone, isothiocyanate, cardiac glycosides, saponins, steroids, anhydride, phosphine oxide, thiocarbynol, phosphor amide, silane, benzaldehyde and halides (CF and CBr). (Table.2). These compounds are found to be essential for germination and also for the prevention of microbial infection (Ajith Kumar *et al.*, 2016)

Compounds like ketones, ammonium salts, peroxides, aliphatic phosphates, halides (CCl and CI), aliphatic nitro compounds, aliphatic secondary amine, heterocyclic amine, cyanogenic glycoside, sulphon amides, alkaloids, hexane, thioamide and thiourea were not detected in the cotyledons of seeds at threshold water content stage (Table.2). This can also be a reason for loss of viability and subsequent poor germination.

Alkanes were not detected in the cotyledons

Table 2. Phytochemicals identified, through FTIR Spectroscopic analysis, in different stages of V.indica seeds.

SL NO	COMPOUND	Freshly Fallen Seed (Axis)			Freshly Fallen Seed (Cot.)			Plumule			Radicle			Critical water level stage (Axis)			Critical water level stage (Coty)		
		1M	5M	C	1M	5M	C	1M	5M	C	1M	5M	C	1M	5M	C	1M	5M	C
1	ALKANES			+									+						
3	AROMATIC RING			+						+									
4	ALDEHYDE									+	+								
5	KETONES	+	+		+					+	+			+	+				
6	QUINONES	+	+	+	+	+	+	+	+	+	+	+	+						
7	ESTERS AND LACTONES			+		+				+			+			+			+
8	CARBOXYLIC ACIDS AND THEIR SALTS	…			…						…		+						
9	AMMONIUM SALTS			+	+		+	+	+	+	+	+		+	+	+		+	
10	AMIDES			+			+								+	+			+
11	ALCOHOLS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+
12	$1^0,2^0,3^0$ ALCOHOLS	+		+			+											+	+
13	PHENOLS	+	+	+	+	+	+	+	+	+	+	+	+				+	+	+
14	CARBOHYD RATES	+		+	+	+	+			+	+		+	+	+	+			+
15	ETHER	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16	ALIPHATIC PRI.AMINES			+	+			+		+		+							
17	NITRILES															+	+		
18	SULPHIDES		+		+	+	+	+	+		+	+	+	+	+	+	+		
19	DISULPHIDES				+		+		+		+								
20	POLYSULPH IDES								+				+	+	+	+			
21	SULPHUR –OXY COMPOUND									+					+		+	+	
22	PEROXIDES	+		+	+		+	+		+	+			+	+	+			
23	ORGANIC PHOSPHATE			+	+			+		+			+	+	+	+	+	+	
24	AROMATIC PHOSPHATES	+	+	+	+			+	+		+	+		+		+	+		+
25	ALIPHATIC PHOSPHATES	+	+	+		+	+	+	+	+	+	+		+	+	+			
26	HALIDES CF	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	CCl			+	+		+	+			+	+		+	+	+			
	CBr		+		+	+	+	+	+		+	+		+	+	+	+		
	CI		+	+	+	+		+	+		+	+	+	+		+			
27	SILICON OXY COMPOUNDS							+	+					+			+		
28	AROMATIC NITROCOM POUND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
29	ALIPHATIC NITROCOM POUND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

SL NO	COMPOUND	Freshly Fallen Seed (Axis)			Freshly Fallen Seed (Cot.)			Plumule			Radicle			Critical water level stage (Axis)			Critical water level stage (Coty)		
		1M	5M	C	1M	5M	C	1M	5M	C	1M	5M	C	1M	5M	C	1M	5M	C
30	ALI. SEC. AMINE	+	+	+	+	+		+		+	+	+			+	+			
31	HETEROCYCLIC AMINE	+	+	+	+	+		+	+	+	+	+	+		+				
32	ALKYNE											+							
33	ANTHERO QUINONE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
34	TERPENES			+						+			+		+	+			+
35	CYANOGENIC GLYCOSIDES															+			
36	ISOTHIOCYA NIDE															+	+		
37	CARDIAC GLYCOSIDES	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
38	SAPONINS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
39	SULPHON AMIDES	+	+	+	+			+		+	+	+			+	+			
40	ALKALOIDES	+	+	+	+	+		+		+	+	+			+				
41	TANNINS	+	+	+	+	+	+	+	+	+	+	+	+				+	+	+
42	STEROIDES	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+
43	FLAVANOIDES	+	+	+	+	+	+	+	+	+	+	+	+				+	+	+
44	ANHYDRIDE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
48	SULPHONIC ACID	+	+	+	+	+		+	+	+	+	+	+				+	+	+
49	THIOAMIDE&THIOU REAS	+	+	+	+	+		+		+	+	+				+			
54	PHOSPHINE OXIDE	+	+	+		+	+	+	+	+	+		+	+	+	+	+	+	+
55	PHOSPHINE															+			
56	IMINE			+			+			+					+	+	+		+
57	THIOCARBONYL	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
58	ARENES	+	+		+	+					+			+	+	+			
59	PHOSPHORAMIDE	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+		+
60	SILANE															+	+		
62	BENZALDEHYDE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
63	ALLENEC=C=C															+			
64	HEX-1-YNE	+		+	+					+	+				+	+			

(1M is 100% Methanol, 5M is 50% Methanol and C is Chloroform)

of just fallen seeds and also in the plumule and seeds at the critical threshold water level (Table.2). This indicates that alkane is synthesised in the embryonal axis and is completely getting utilised during the emergence of plumule. This clearly shows the role of alkanes in seed germination (F Perriello., G DiCesare., 2004).

Most of the compounds detected by FTIR spectroscopy in the seeds of *V. Indica* were detected in many medicinal plants. Ashok Kumar and Ramaswamy (2014) analysed the secondary metabolites, by FTIR spectroscopy of leaf extracts of selected Indian medicinal plants and documented amide, alcohol, phenols, alkanes, carboxylic acids, aldehydes, ketones, primary amines, aromatics, esters, ethers, halides and aliphatic amines. This clearly throws light upon the medicinal properties of *V.indica* seeds.

CONCLUSION

Just fallen fully mature seeds show immediate germination without any embryo dormancy and all the seeds germinated. But on desiccation, a drop in the rate of germination was observed. The critical threshold water level in the embryonal axis was found to be 47% and in the cotyledon to be 30%. The seeds remain viable for about a week, under natural conditions, after that viability is steeply reduced. FTIR Spectroscopy showed variations in the biochemical composition during seed germination and subsequent embryo desiccation. Delayed germination and loss of viability of the seeds as a result of desiccation may be due to the lack of synthesis of aldehydes, quinines, aliphatic primary amines, aromatic ring. As a result of desiccation, the membrane stability and permeability is getting lost due to which, some compounds present in the cotyledons are not getting transported from the cotyledon to axis. Desiccation induced the production of germination inhibiting compounds like allene. FTIR studies also threw light upon the pharmaceutically active compounds present in the seeds of *V. indica*.

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Citrus Fruit Peel Crude Extract - A Natural Source of Antioxidant

S. JAMUNA*, S. SATHISKUMAR AND M. SWAMYNATHAN

Department of Botany, Kongunadu Arts and Science College, Coimbatore-641029.
**email: sjmaunaphd@gmail.com*

ABSTRACT

Plant phenolics are one of the most important groups of bioactive compounds that act as prime antioxidant free radical terminators. This paper reports the phytochemical screening and antioxidant activity of various solvent extracts such as petroleum ether, chloroform and ethanol of certain *Citrus* fruit peels *viz., Citrus limon, C. sinensis* and *C. aurantium*. Preliminary phytochemical analysis of the crude extracts of revealed the presence of different kinds of chemical groups such as alkaloids, cardiac glycosides, flavonoids, glycosides, phenols, resins, saponins, steroids, tannins, terpenoids and triterpenoids. Among the three fruit peels the *Citrus limon* had highest amount of total phenolics and flavonoids contents (61.65mg GAE/100 g extract and 38.08mg QE/100g extract). The species, *C. sinensis* has evident extensive antioxidant activity with the IC_{50} value of 158.13µg/ml.Our findings provide evidence that the peel extract of *Citrus*is a potential source of natural antioxidants, and this justify its uses in folkloric medicines.

Keywords *Citrus fruit peels, crude extract, phytochemcials, antioxidant*

Medicinal plants are the local heritage with global importance, world is endowed with a rich wealth of medicinal plants have made a good contribution to the development of ancient material medica (Suriyavathana *et al*., 2010). It has been used as sources of food and medicinal purposes for centuries

Citrus sinensis

Citus medica

Citrus lemon

Citrus aurantium

Fig. 1. Citrus fruits

Table 1. Percentage yield of various solvent extracts of *Citrus* fruit peels.

Name of the plant	Yield (%)		
	Petroleum ether	Chloroform	Ethanol
Citrus limon	7.69	6.83	2.56
C. aurantium	3.26	3.64	3.23
C. sinensis	2.10	1.40	0.46
C. medica	7.56	11.35	13.51

and knowledge has been passed from generation to generation and also has good antioxidant properties without any side effects (Aiyegoro and Okoh, 2010). Fruits are considered as an important part of a good diet. Besides their delicious taste and flavor,they are known to reduce rite of several chronic diseases. It contains significant amount of phytoconstituents which are negatively associated whit the morbidity and from cerebrovascular, cardiovascular and certain types of cancer.

Fruit wastes and their by-products are formed in great amounts during industrial processing and hence represent serious problems, as they exert harmful impact in environment. Growing knowledge about antioxidants, that assumption that a number of common synthetic preservatives may have hazardous effects has led to multiple investigations in the natural antioxidants.The present study was aimed at to evaluate phytochemical constituents and free radical scavenging potential of various fruit peels *viz.,Citrus*

Table 2. Qualitative phytochemical analysis of petroleum extract of *Citrus* fruit peels.

	Phytochemical test	Petroleum ether extract			
		Citrus limon	*C. auranitium*	*C. sinensis*	*C. medica*
1.	**Alkaloids** b) Meyer's Test	++	+	+	++
2.	**Cardiac Glycosides** a) Keller killiani Test	+	+	+	+
3.	**Flavonodis** a) Shinoda Test	-	-	-	-
	b) Lead Acetate Test	+	++	++	++
4.	**Glycosides** a) Keller Kiliani Test	+	+++	++	+
	b) Legal's Test	-	-	+	-
5.	**Phenols** a) FreeicChloride Test	+	-	++	+
6.	Resins Test	+	++	++	+
7.	**Saponins** a) Frothing/Foam Test	+	+	+	+
8.	**Steroids** a) Libermann-Burchard's Test	++	+	+	++
9.	**Tannins** a) Braember's Test	+	+	+	-
10.	**Terpenoids** Salkowski Test	++	-	+	++
11.	**Triterpenoids**Salkowski Test	++	++	++	-

(-) – Not available; (+) – present; (++) –moderately present; (+++) – highly present.

Table 3. Qualitative phytochemical analysis of chloroform extract of *Citrus* fruit peels.

Phytochemical tests		Chlorofrom extract			
		Citruslimon	*C. auranitium*	*C. sinensis*	*C. medica*
1.	**Alkaloids** b) Meyer's Test	+	+	+	-
2.	**Cardiac Glycosides** a) Keller killiani Test	-	-	-	++
3.	**Flavonodis** a) Shinoda Test	-	-	-	-
	b) Lead Acetate Test	++	+	++	-
4.	**Glycosides** a) Keller Kiliani Test	+	+	-	-
	b) Legal's Test	-	-	-	-
5.	**Phenols** a) FreeicChloride Test	-	-	-	+
6.	Resins Test	++	+	+	-
7.	**Saponins** a) Frothing/Foam Test	++	++	++	+
8.	**Steroids** a) Libermann-Burchard's Test	+	++	+++	+++
9.	**Tannins** a) Braember's Test	-	+	-	+
10.	**Terpenoids** Salkowski Test	+++	+	-	+++
11.	**Triterpenoids**Salkowski Test	-	+++	+++	-

(-) – Not available; (+) – present; (++) –moderately present; (+++) – highly present.

*limon, C.aurantium, C. sinensis*and *C. medica.*

MATERIALS AND METHODS

Collection of plant materials

The ripenfruit peel samples of the species*, Citrus limon, C. aurantium, C. sinensis*, and*C. medica* (Fig. 1) were collected at local merket. Coimbatore. The peels of the fruits were shade dried and powdered.

Extracts preparation

Plant samples were extracted using successive solvents *viz.,* petroleum ether, chloroform and ethanol by cold extraction (20g/200ml). Then the extracts were filtered and evaporated under room temperature. The yield of the fruit peel extracts was analysed by following formula;

Percentage yield = [Amount of residue taken/ Amount of plant powder taken] X 100

Phytochemical analysis

The ethanolic extract was subjected to preliminary phytochemical analysis as described by Harborne (1998) and Trease and Evan (2002). The total phenolics and flavonoids content were evaluated and expressed as gallic acid equivalents (GAE) mg/ 100g (10 to 50µg/ml; R^2 = 0.996) (Siddhuraju and Becker, 2003) and rutin (RE) mg/100g equivalents (10 to 200µg/ml; R^2 = 0.991) (Zhishen *et al.,* 1999) respectively.

DPPH radical scavenging activity

The ability of pomegranate fruit peel extract to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radicals was assessed by using Blois (1958) method with some modifications. 0.2mM solution of DPPH• in methanol was prepared and 500µL of this solution was added to different concentrations of the extracts

Table 4. Qualitative phytochemical analysis of ethanolic extract of *Citrus* fruit peels.

S.No.	Phytochemical tests	Ethanolic extract			
		Citruslimon	*C. auranitium*	*C. sinensis*	*C. medica*
1.	**Alkaloids** b) Meyer's Test	+	+	+	+
2.	**Cardiac Glycosides** a) Keller killiani Test	+++	++	+	++
3.	**Flavonodis** a) Shinoda Test	-	-	-	-
	b) Lead Acetate Test	++	++	++	-
4.	**Glycosides** a) Keller Kiliani Test	+	++	+	-
	b) Legal's Test	+	-	+	-
5.	**Phenols** a) FreeicChloride Test	+	-	+	++
6.	Resins Test	+	++	+++	-
7.	**Saponins** a) Frothing/Foam Test	++	+	+++	++
8.	**Steroids** a) Libermann-Burchard's Test	-	+	+	++
9.	**Tannins** a) Braember's Test	+	++	++	+++
10.	**Terpenoids** Salkowski Test	-	-	-	++
11.	**Triterpenoids**Salkowski Test	++	++	++	+

(-) – Not available; (+) – present; (++) –moderately present; (+++) – highly present.

(50-300µg/mL). The mixture was shaken vigorously and allowed to stand for 30min at room temperature. Control was prepared as above but without the sample extracts and methanol was used for the baseline correction. Then changes in the absorbance of the plant samples were measured at 517nm using spectrophotometer. A lower absorbance value indicates the higher radical scavenging activity. Results were compared with the standard antioxidants (rutin, quercetin, BHA and BHT). The ability of DPPH radical scavenging activity was calculated by using the following formula:

Table 5. Quantitative phytochemical analysis of ethanolic extracts of *Citrus* fruit peels.

Name of the Plant	Total phenolics (mg GAE/100 g extract)	Total flavonoids (mg QE/100 g extract)
Citrus limon	61.65 ± 0.38^{b}	38.04 ± 0.07^{b}
C. aurantium	31.32 ± 0.50^{cd}	26.12 ± 0.30^{d}
C. sinensis	36.69 ± 0.07^{c}	18.37 ± 0.03^{e}
C. medica	25.35 ± 0.03^{e}	29.51 ± 0.03^{c}

GAE - Gallic Acid Equivalent, QE - Quercetin Equivalent.

Values are performed in triplicates and represented as mean ± SD (standard deviation).

Mean values followed by different superscripts in a column are significantly different ($p<0.05$).

Table 6. DPPH radical scavenging activity of various solvent extracts of *Citrus* fruit peels.

Sample concentration (µg/ml)	% of Inhibition			
	Citrus limon	***C. auranitium***	***C. sinensis***	***C. medica***
50	19.58±0.20^{e}	22.66±0.03^{e}	47.79±0.09^{c}	44.30±0.08^{d}
100	38.97±0.02^{d}	42.66±0.12^{d}	52.87±0.08^{bc}	46.56±0.22^{cd}
150	41.64±0.11^{c}	46.15±0.13^{cd}	52.51±0.03^{bc}	47.07±0.24^{c}
200	51.48±0.01^{b}	57.07±0.06^{c}	57.53±0.02^{b}	53.23±0.13^{b}
250	62.20±0.02^{ab}	63.12±0.14^{b}	68.05±0.03^{a}	66.61±0.11^{ab}
300	73.43±0.12^{a}	76.01±0.11^{a}	78.76±0.12^{a}	77.23±0.13^{a}
IC_{50} (µg/ml)	198.87±0.42^{a}	197.13±0.23^{ab}	158.13±0.34^{e}	178.45±0.67^{d}

Values are performed in triplicates and represented as mean ± SD (standard deviation).

Mean values followed by different superscripts in a column are significantly different (p<0.05).

Table 7. DPPH radical scavenging activity of standard antixodants.

Standard antioxidants	IC_{50} (µg/ml)
Rutin	42.07±0.00^{b}
Quercetin	50.82±4.00^{c}
ButylatedHydroxy toluene (BHT)	52.97±8.23^{d}
ButylatedHydroxy Anisole (BHA)	38.47±1.03^{a}

Values are performed in triplicates and represented as mean ± SD (standard deviation).

Mean values followed by different superscripts in a column are significantly different (p<0.05).

DPPH$^{\cdot}$ scavenging effect (% of inhibition) = $[(A_0-A_1)/A_0]$ X100

Where, A_0 is the absorbance of the control, and A_1 is the absorbance of the extracts. The IC_{50} (the microgram of extract to scavenge 50% of the radicals) value was calculated using linear regression analysis. Lower IC_{50} value indicates greater antioxidant activity.

Statistical analysis

Analysis was carried out in triplicates and mean±SD (Standard Deviation) using Duncan's Multiple Range Test (DMRT) (Duncan, 1955). Statistical significance (p<0.05) were subjected to one way analysis of variance (ANOVA) by using a statistical Package for Social Science (SPSS) (Version 9, SPSS, Inc., Chicago, USA).

RESULTS AND DISCUSSION

Percentage yield

Extractive yield of *Citrus* fruit peels were depicted in Table 1. The highest extractive yield was shown by three species, *Citrus limon, C. aurantium* and *C. sinensis*(7.69, 3.26 and 2.10%) inpetroleum ether extract than the other two solvents. Interestingly, *Citrus medica* has revealed higher extractive yield in ethanolic extract, 13.51%. It may be due to high polarity of the solvent which can draw high variety of plant constituents than the other solvents (Paulsamy and Jeeshna, 2011).

Phytochemicalanalysis

Preliminary phytochemical analysis

Tables 2, 3 and 4showed the phytochemical analysis of different solvent extracts of various *Citrus* fruit peels such as *Citrus limon, C. aurantium, C. sinensis* and *C. medica.* The petroleum ether extract of all fruit peel showed the presence of phytochemicals*viz*., alkaloids, cardiac glycosides, flavonoids, glycosides, phenols, resins, saponins, steroids, tannins, terpenoids and triterpenoids. The results revealed that higher solubility of secondary metabolites such as steroids, terpenoids and triterpenoids in the chloroform extract. Among the fruit peels, the ethanolic extract of *Citrus aurantium* and *C. medica* exhibited higher solubilityof phytochemicals except the glycosides. The results showed plausible antioxidants such as phenolics, flavonoids and tannins are major contributors of this citrus fruit peels (Jamuna *et al.,* 2014)

Quantitative phytochemical analysis

The total phenolics and toal flavonoids content ofethanolic extract of *Citrus* fruit peels were given in Table5. From the study, the highest total phenolics and total flavonoidscontents were exhibited by *Citrus limon* (61.65mg GAE/100 g extract and 38.08mg QE/ 100g extract) than the other studied species. The phenolics and flavonoids are known as most important free radicals quenchers (i.e., OH, ROO) (Altemimi *et al.,* 2017). Furthermore, it proves the antioxidant potential of *Citrus* fruit peels

Invitro antioxidant activity

The percentage of inhibition of DPPH radicals is perceived in all the fruit peels increased with the increasing concentration of extracts from 100,150,200,250 and 300µg/ml (Table 6). *C. sinensis* has pronounced substantial antioxidant activity by having lower IC_{50} value 158.13µg/ml. The free radical scavenging activity was compared with standard (natural and synthetic) antioxidants (Table 7). Antioxidant molecules are excellent choice and basic for the development of important lead molecules in pharmaceutical preparations (Rahman *et al.,* 2017).

CONCLUSION

The phytochemical analysis showed that the various *Citrus* fruit peel extracts contains a mixture of phytochemicals as flavonoid, cardiac glycoside, phenolic compounds, tannins, terpenoids, tripernoids and alkaloids. The DPPH assay indicated that the *Citrus* fruit peel extracts has potent antioxidant activity which can be an excellent option for biological and chemical analysis and can be further subjected for the isolation of the therapeutically active compounds.

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Difference in Agar Content of Major Agarophytes from Coast of Kerala

JAYA LEKSHMI. R

Department of Botany M.M.N.S.S College, Kottiyam
email: jayalekshminilamel@gmail.com

ABSTRACT

Seaweeds are the only source for the production of Agar, Alginate and Carrageenan. These phytochemicals are extensively used in varies industries such as food ,confectionary ,textile, pharmaceutical ,dairy and paper mostly as gelling, stabilizing and thickening agents. Agar is a water soluble, gel forming polysaccharide showing *hysteresis*. The amount of agar production from *Gelidium* and *Gracilaria* was the largest in the world when compared to *Gelidiella* and *Pterocladia* . Agar consist of D-galactose, 3, 6-anhydro-L-galactose and sulphate .Gel property depend on the amount and position of sulphate groups as well as the amount of 3,6anhydrogalactose fraction of the polysaccharide. The gel strength of agar decreases with an increase in sulphate and a decrease in 3, 6 anhydrogalatose concentration. In this study the agar yield of both NA and ATA were examined. Physical characteristics like gel strength, gelling temperature and melting temperature where studied. Chemical features like 3,6- AG and sulphate content were calculated. *G.corticata* showed poor agar yield when compared to other Indian species. *G.verrucosa* had higher agar yield than species from other countries .The yield of agar in species of *Gelidium* was examined then *G.micropterum* showed better results than *G.pusillum*. The agarophytes grown on the coast of Kerala can be utilized as a good source of agar. *G.micropterum* can be cultivated on a commercial scale as a source of bacteriological agar.

Key words *Difference, Agar Content, Major Agarophytes, Kerala*

Seaweed are the only source of agar, alginate and carrageenan. These phytochemicals are extensively used in varies industries such as food, confectionary, textile, pharmaceutical,dairy and paper mostly as gelling ,stabilizing and thickening agents .The Indian coastal regions are geographically divided in to The West Coast Region and South East Coast Region. Rocky coastal places of Kerala are rich in species diversity and they are restricted to places of Thiruvanthapuram, Kollam, Kozhikode, Kannur, and Kasargod districts of Kerala .The red algal species are rich on the Kerala coast due to unique climatic conditions of the state. Agar is a water soluble, gel forming polysaccharide showing *hysteresis*. The major Rhodophycean alga *Gelidium* yield best quality of aga. The amount of agar production from *Gelidium*and *Gracilaria* was the largest in the world when compared to *Gelidiella* and *Pterocladia*.Agar consist D-galactose, 3, 6-anhydro-L-galactose and sulphate. Gel property depend on the amount and position of sulphate groups as well as the amount of 3,6unhydrogalactose fraction of the polysaccharide. The gel strength of agar decreases with an increase in sulphate and a decrease in 3, 6 anhydrogalatose concentration

MATERIALS AND METHODS

Collections were made from Thirumullavaram coast (*Gelidium* species and *Gracilariacorticata*) and also from the Ashtamudy Lake (*Gracilariaverrucosa*) from the intertidal regions during low tide. All the collected materials were washed in freshwater, cleaned and spread over old newspaper for drying overnight. The agar extraction method was performed as described by Chirapart *et al* (1995). Native agar and alkali treated agar were studied separately. Dried agar was powdered for the analysis of physical properties like Gel strength, gelling and melting temperature and chemical characteristics like 3,6anhydrogalactose and estimation of sulphate content

RESULTS AND DISCUSSION

The agar yield of *Gracilariacorticata*both, NA and ATA and characteristics are presented in table 1. Table 2 for *Gracilariaverrucosa*, table 3 for *Gelidiumpusillum* and table 4 for *Gelidiummicropterum*. A gradual decrease in agar yield was reported for all alkali concentration and at 15%, agar yield was reduced to optimum.All other contents excluding sulphate were increasedwith increasing alkali concentration.*G.corticata* showed poor agar yield when compared to other Indian species like *G. edulis, G.dura and G.folifera. G.verrucosa* had higher agar yield than species from other countries. The yield of agar in species of*Gelidium* was examined then *G.micropterum* showed better results than *G.pusillum*.

CONCLUSION

Alkali treatment dramatically improved the quality of agar. The agar yield and gel strength of *G. corticata* and *G. verrucosa* suggested that it may have potential as a commercial source of agar. This

Table 1. Mean± SD, ANOVA (F-value, p-value), agar yield, gel strengh, gelling temperature,melting temperature, 3,5-AG andsulphate content of *Gracilaria corticata*

NaOH	Yield(% weight)	Gel Strength (g/cm^2)	Gelling Temperature (^{0}C)	Melting Point (^{0}C)	3,6 - AG(g/100g)	sulphate content
Native	21.27±1.94	75.37±9.61	29.4±1.1	70.97±0.8	16.13±0.97	9.1± 1.08
2%	17.47±0.91	101.77±1.78	33.47±1.18	73.77±0.51	16.77±1.19	8.8±0.66
4%	15.77±0.45	105.73±1.29	35.63±0.55	75.2±0.44	19.83±1.14	7.1±0.8
6%	14.23±0.51	109.07±1	36.97±0.61	76.2±0.36	22.93±1.25	5.27±0.5
8%	12.37±0.55	113.4±0.62	37.93±0.15	76.8±0.6	27.3±0.5	4.3±0.26
10%	10.1±0.3	115.17±0.32	38.47±0.31	77.53±0.85	28.43±0.96	3.83±0.31
15%	8.5±0.56	116.77±0.71	38.9±0.62	79.6±0.66	29.13±1.08	2.83±0.45
F-value	70.23	42.92	63.67	59.30	84.49	44.94
p-value	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**

**significant at 1% level of significance

Table 2. Mean± SD, ANOVA (F-value, p-value) of agar yield, gel strengh, gelling temperature,melting temperature, 3,6-AG and sulphate content of *Gracilaria verrucosa*

NaOH	Agar Yield	Gel Strength (g.cm2)	Gelling Temperature (0C)	Melting Point (0C)	3,6 - AG(g/100g)	sulphate content
Native	30.73± 3.92	188.53±31.57	34.83±1.07	64.8±0.56	23.93±1.03	5.97±0.59
2%	25.5±0.56	176.27±7.6	35.87±0.59	66.87±0.45	25.97±1.47	4.77±0.35
4%	23.77±0.55	215.13±15.56	41.67±0.67	68.27±0.45	29.9±1.3	3.77±0.42
6%	22.07±0.47	243.53±12.67	37.4±0.8	69.17±0.87	32.37±0.45	2.75±0.47
8%	19.67±0.8	264.87±5.41	39.8±0.61	71.4±0.66	35.63±0.7	2.11±0.2
10%	16.67±0.75	276.53±9.49	40.93±0.46	72.83±0.4	38.9±0.7	1.7±50.12
15%	14.2±0.95	275.1±4.65	42±0.53	75.23±0.55	42.63±1.72	1.42±0.13
F-value	35.54	22.56	51.17	114.34	106.72	64.14
p-value	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**

**significant at 1% level of significance

Table 3. Mean± SD, ANOVA, F-value, p-value, ANOVA test of Agar yield, gel strengh, gelling

NaOH	Agar yield(% by wt)	Gel strength(g/cm2)	Gelling temperature(°C)	Melting point(°C)	3,6-Anhydrogalactose(g/100g)	Sulphate content(% by wt)
Native	18.1±3.21	212.87±33.87	29.67±0.58	90.67±0.58	22.83±0.75	4.11±0.28
2%	18.47±0.93	302.13±19.9	30.33±0.58	91±0	24.27±0.4	3.11±0.22
4%	17.03±0.31	356.1±44.39	31.67±0.58	91.33±0.58	25.97±0.59	2.43±0.27
6%	15.63±0.74	442.65±38.68	32.33±0.58	91.67±0.58	28.13±0.25	1.9±0.21
8%	13.47±0.78	508.98±11.82	33±0	92±0	28.8±0.6	1.2±0.29
10%	11.43±0.65	550.5±22.79	32.67±0.58	92.33±0.58	30.8±0.36	0.89±0.06
15%	9.73±0.47	592.88±15.71	33.67±0.58	92.67±0.58	32.23±0.4	0.73±0.03
F-value	18.46	68.71	22.06	6.53	136.99	97.12
p-value	0.000**	0.000**	0.000**	0.002**	0.000**	0.000**

**significant at 1% level of significance

Table 4. Mean SD, ANOVA, F-value, p-value, ANOVA test of agar yield, gel strengh, gelling temperature, melting temperature, 3, 6-AG and sulphate content of *Gelidium micropterum*

NaOH	Agar yield(% by wt)	Gel strength(g/cm2)	Gelling temperature (°C)	Melting point(°C)	3,6-Anhydrogalactose(g/100g)	Sulphate content(% by wt)
Native	23.6±3.6	429.7±141.21	38.67±0.58	92±0	33.49±0.53	3.71±0.34
2%	21.5±0.92	1061.2±18.2	39.67±0.58	93±1	34.18±0.74	3.03±0.44
4%	19.73±0.35	1144.47±58.52	40±0	93.67±0.58	35.38±0.68	2.07±0.23
6%	17.83±0.87	1356.37±84.05	40.67±0.58	93.67±0.58	37.31±0.56	1.14±0.15
8%	15.97±0.8	1491.97±13.66	40.67±0.58	94.33±0.58	39.16±0.61	0.84±0.11
10%	13.43±1.2	1542.57±39.58	40.33±0.58	94.33±0.58	40.69±0.6	0.53±0.09
15%	10.8±0.56	1566.83±122.33	40.67±0.58	95.33±0.58	42.05±0.52	0.19±0.06
F-value	25.0	75.65	5.72	8.96	87.46	90.34
p-value	0.000**	0.000**	0.003**	0.000**	0.000**	0.000**

**significant at 1% level of significance

result indicated that the environmental condition found during dry season are more optimal for growth and gel strength of Rhodophyceanalgae .The agarophytes grown on the coast of Kerala can be utilized as a good source of agar. The large Indian species of *Gelidium, G. micropterum* gives good results than the well-known *G. pusillum*, *G. micropterum* can be cultivated on a commercial scale as a source of bacteriological agar.

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Preliminary Analysis of Nutritional factors in the Rhizome of *Anaphyllum wightii* Schott.

LEKSHMI. S AND T.S. SWAPNA*

Department of Botany, University of Kerala, Kariavattom.
**email: swapnats@yahoo.com*

ABSTRACT

The plant *Anaphyllum wightii* Schott., commonly called as 'Keerikizhangu', belongs to the family Araceae. It is an ethnomedicinal plant endemic to the Southern Western Ghats. *Anaphyllum wightii* is a tall herb with rhizomatous stem and pinnatisect leaves. The inflorescence is a spadix with characteristically twisted spathe. The rhizome of *Anaphyllum wightii* is the most useful part and is used in tribal medicine to cure various diseases like eczema, scabies etc. Due to the edible character, nutritional analysis of the rhizome is essential. The present study was aimed to evaluate the various nutritional and antinutritional factors in the rhizome of *Anaphyllum wightii*. The nutritional factors like moisture content, total carbohydrates, proteins, lipids, reducing sugars, total aminoacids, chlorophylls and carotenoids, vitaminB2(Riboflavin) and antinutritional factors such as phenols and oxalates were quantified by standard estimation methods.The study revealed that the rhizome of *Anaphyllum wightii* has much higher carbohydrate content (228.36 mg/g) than the other nutritional factors. Also the vitamin B2 (Riboflavin) was found in comparatively higher quantity (0.06 mg/g). But the rhizome showed relatively low levels of anti-nutritional factors. Hence the rhizome of this plant can be used as a good energy source.

Keywords *Nutritional factors; Antinutritional factors; Anaphyllum wightii; ethnomedicinal plant*

Natural plant products like wild leafy vegetables or tuber crops are the major food source of tribal people. The rhizome of *Anaphyllum wightii* Schott. is used as food by the tribal population of India. *Anaphyllum wightii* is an endemic plant of Southern Western Ghats, belonging to the family Araceae. Many common edible tuber crops have been reported from Araceae, that are rich in various nutritional factors. *Anaphyllum wightii* is having an edible rhizome which is also reported to have some pharmacological properties.The rhizome is used as food and also as an antidote against snakebite by tribal people like Kani tribes, Kadars, Madhuvars etc. (Dominic, 2012) and has antibacterial(Manesh *et al.*, 2015), anti-inflammatory(Dharsana *et al.,*2014), anthelmintic (Rajagopal *et al.,*2013), antioxidant, hepatoprotective(Dharsana *et al.,*2014). and antidiabetic activities (Dharsana *et al.,*2013).

The present study was intended for preliminary evaluation of nutritional components like carbohydrates, reducing sugars, proteins, total aminoacids, riboflavin,etc and antinutrients in the rhizome of *Anaphyllum wightii*, which can be used as a valuable energy source for human consumption.

MATERIALS AND METHODS

Collection of plant material

Fresh rhizomes of *Anaphyllum wightii* Schott. were collected from the Kallar region , Trivandrum of Kerala state in India. Fresh samples of rhizome were used for the analysis of the nutritional and antinutritionalfactors. These factors were quantitatively estimated using standard procedures.

Moisture content

Five grams of sample was weighed and taken in a pre-weighed petriplate.The sample was dried at 80^0C in a hot air oven. After 24 hours, the sample was weighed again and the difference in weight was determined. The percentage of moisture was calculated by the following formula:

$$\% \text{ of moisture} = \frac{(\text{Fresh weight} - \text{Dry weight}) \times 100}{\text{Weight of sample taken}}$$

Total carbohydrates

The amount of total carbohydrates present in the sample was estimated by Anthrone method (Hedge &Hofreiter, 1962). One gram of fresh tissue was homogenized in 10 ml distilled water.The homogenate was filtered and centrifuged 10,000 rpm for 10 minutes. The supernatant was collected and made upto a known volume by using distilled water. An aliquot was pipetted and made upto 1 ml by distilled water. 4 ml of anthrone reagent was added and kept in a boiling waterbath for 10 minutes. It was then cooled and the absorbance was read at 620 nm against the blank. Amount of total carbohydrate was calculated.

Total protein

The amount of total protein was estimated by Lowry's method(Lowry et al., 1951). 1g of fresh tissue was homogenized in phosphate buffer (pH 7). The homogenate was filtered and centrifuged at 5,000 rpm for 10 minutes. The supernatant was collected and made upto known volume by buffer. 1 ml of this solution was taken, added with equal amount of 10% trichloroacetic acid and kept in refrigerator for 15 minutes after thorough shaking. This mixture was then centrifuged at 10,000 rpm for 10 minutes.The upper layer was decanted to collect the pellet and was dissolved in a known volume of 0.1N NaOH. Then an aliquot was pipetted and made upto 1 ml using 0.1 N NaOH. Then 5 ml of copper sulphate solution (reagent C) was added to it followed by the addition of 0.5 ml FolinCiocateau reagent(reagent D).Mixed well and kept for 30 minutes in dark at room temperature. Absorbance was measured at 670 nm against the blank. Amount of total protein was calculated by drawing a standard graph.

Lipids

The lipid content in the sample was estimated by the method of Bligh &Dyer(1959). 1g of fresh tissue was extracted with chloroform:methanol (2:1 v/v) mixture. The extraction was repeated three times, the extract was pooled and centrifuged. The supernatant was made upto known volume by chloroform:methanol and transferred to a separating funnel by adding few drops of chloroform, distilled water and 1 ml of saturated NaCl. The mixture was shaken well and allowed to settle. The lower layer was collected in a clean pre-weighed petridish. Solvent was evaporated in a hot air oven at 60^0C overnight until a constant weight was obtained. Thepetridish with lipid was weighed and the lipid content was expressed in percentage.

Reducing sugars

Estimation of reducing sugars was done by Dinitrosalicylic acid method(Miller1972). 1g tissue was homogenised in 10 ml distilled water.The homogenate was filtered and centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and made upto known volume. An aliquot was taken, made upto 3 ml and 2 ml of DNS reagent was added.The mixture was kept in a boiling waterbath for 10 minutes and then cooled. The absorbance at 540 nm was measured against appropriate blank.

Chlorophyll and carotenoids

Chlorophylls and carotenoids were quantified by using Arnon's formula(Arnon, 1949). 1g tissue was homogenised in 10 ml of 80 % acetone. It was then filtered and centrifuged at 5,000 rpm for 5minutes.The supernatant was collected and made upto known volume. 1 ml aliquot was taken and made upto 5 ml by adding 4 ml acetone. Absorbance was read at 645, 663, 652 and 490 nm against 80% acetone as blank. The chlorophylls and carotenoids were determined using Arnon's formula.

Total aminoacids

Estimation of total aminoacids was done by the method of Moore and Stein(1948). 1g tissue was refluxed in 10 ml of 80 % methanol for 10 minutes. The tissue was then ground thoroughly with mortar and pestle. The homogenate was filtered and centrifuged at 7,500 rpm for 10 minutes. The supernatant was collected and made upto known volume by 80% methanol. 0.1 ml of extract was taken and 4.9 ml of ninhydrin reagent was added to it. This mixture was shaken well and heated in a boiling waterbath for 10 minutes. The tubes were then cooled under the running water and the absorbance was read at 570 nm against the blank.

Riboflavin

Riboflavin content was determined according to the method of Indian Pharmacopeia (1996). To 5g of powdered sample, 150 ml of water and 5 ml of glacial acetic acid were added. The solution was boiled for 5 minutes and then cooled. After that, 30 ml of 0.1 M NaOH solution was added and diluted to 500 ml with distilled water.The solution was filtered and the absorbance was measured at 444 nm in shimadzu UV-1201-spectrophotometer.

Total phenol

Estimation of phenol was done by Folin-Ciocalteau method (Malick, and Singh. 1980). 1g tissue was refluxed in 80% methanol and homogenized. The homogenate was then filtered and centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and made upto a known volume by 80% methanol. An aliquot was taken and made upto 3 ml by 80% methanol. Then 0.5 ml of Folin-Ciocalteau reagent was added followed by the addition of 2 ml of 20% Na_2CO_3 solution. The mixture was then kept in a boiling waterbath for 2 minutes. A white precipitate

was formed and the mixture was centrifuged at 5,000 rpm for 5 minutes. The supernatant was collected and the absorbance was taken at 650 nm against the blank.

Oxalates

The oxalate content of the rhizome was determined by the method of AOAC (1984). 1g of homogenate was weighed into a crucible. 10 mlof distilled water was added followed by 1 ml concentrated H_2SO_4. The mixture was allowed to stand for 1 hour. The volume was then made upto 50 ml using distilled water. About 5 ml of this solution was pipetted out and it was titrated against 1% $KMnO_4$.The colour change to red was noted and calculated using the formula:

Concentration of oxalate (mg/g) = Burette reading x 11.5

RESULTS AND DISCUSSION

The intention of the present study was the quantitative analysis of the major nutritional and antinutritional factors in *Anaphyllum wightii* **(**Table 1**)**. The results of the study revealed that the rhizome of *Anaphyllum wightii* has higher level of carbohydrates (228.3 mg/g) and moisture content (22.9%) compared to that of rhizome of *Maranta arundinacea* (72 mg/g and 7.6 % respectively), which is a commonly used edible rhizomatous plant **(**Amrutha and Suganthi, 2017). But it has a relatively lower amount of proteins (39.9mg/100g) compared to that of rhizome of *Maranta arundinacea*. Since the carbohydrate content of rhizome of *Anaphyllum wightii* is higher in quantity, it can be used as a good energy source.Also the vitamin B2 (Riboflavin), total aminoacids, reducing sugars and lipids are present in sufficient quantities in the rhizome. The vitamin B2 is a water soluble and photosensitive vitamin. The sample contained relatively lower amount of chlorophyll and carotenoid pigments since it is a rhizome.

Table 1. Nutritional factors in the rhizome of *Anaphyllum wightii.*

Parameter	Average value
Moisture content	22.9 %
Total carbohydrates	228.3 mg/g
Proteins	39.9 mg/100g
Lipids	1.7 %
Reducing sugars	0.045 mg/g
Chlorophyll a	0.00065 mg/g
Chlorophyll b	0.00132 mg/g
Total chlorophylls	0.00198 mg/g
Carotenoids	0.00298 mg/g
Total aminoacids	6.69 mg/g
Riboflavin (vitamin B2)	0.06 mg/g

Anti-nutritional factors such as phenols and oxalates present in the rhizome were also estimated (Table 2). Antinutritional factors are those substances generated in natural food stuffs by the normal metabolism of the species which may lead to inactivation of some nutrients or, may affect digestive process or metabolic utilization of the food **(**Kumar 1983). Phenols are aromatic compounds with hydroxyl groups and they occur in all parts of the plants. In the rhizome of *Anaphyllum wightii*, the amount of phenols was found to be 0.0002 % which is much lower compared to that of rhizome of *Curcuma longa* (0.08 %) , the most commonly used and highly medicinal plant of *Zingiberaceae* family **(**Ikpeama *et al.,* 2014). The oxalate content of the rhizome was found to be higher (1.07 g/100g) than that of yam (*Dioscorea alata*) (0.58 g/100g) which is a widely used tuber crop **(**Shajeela et al., 2011).

Table 2. Anti-nutritional factors in *Anaphyllum wightii*- rhizome.

Parameter	Average value
Phenols	0.0002 %
Oxalates	1.07%

CONCLUSION

The findings of the present study revealed that the rhizome of *Anaphyllum wightii* contains different nutritional factors like moisture content,carbohydrates, total aminoacids, vitamin B2,etc in sufficient amounts while the antinutritional factors were found in lower quantities. Hence it may be concluded that the rhizome of this endemic plant is of good nutritional value.

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Therapeutic Effect of Betulinic Acid and Fluvastatin on Collagen Induced Arthritis- an *Invivo* Study

LIMI ELIZABETH MATHEW[1], CHITHRA. K PUSHPAN[1], VRINDA RAJAGOPAL[1] AND A. HELEN[1*]

[1]*Department of Biochemistry. University of Kerala, Kariavattom Campus, Thiruvananthapuram 695581, Kerala, India.*
email : limi.mathew87@gmail.com

ABSTRACT

Rheumatoid Arthritis (RA) is a systemic autoimmune disease causing synovial hyperplasia, destruction of articular cartilage and marginal bone that lead to joint destruction. Above half of deaths in RA patients are the result of heart disease and cardiovascular-related fatalities, brought on by the complications of rheumatoid arthritis. The present study was carried out to evaluate the synergistic effect of betulinic acid, a triterpenoid and fluvastatin, a HMG CoA reductase inhibitor in arthritic rats in order to study the mechanism for preventing the occurrence of atherosclerosis in arthritis. Arthritis was induced by bovine type II collagen emulsified in equal volume of incomplete Freund's adjuvant. Betulinic acid (2mg/kg) and fluvastatin (5mg/kg) alone and in combination was administered orally from day 14 to 60. Significant ($p<0.05$) decrease in MCP-1, IFN-ã, MMP-2, 9 and iNOS expression in the aorta was observed in combination therapy using betulinic acid and fluvastatin when compared to collagen induced rats. Administration of both betulinic acid and fluvastatin showed significant ($p<0.05$) decrease in the receptors CD-36 and CD-40 expression. Also, supplementation of betulinic acid or fluvastatin showed significant ($p<0.05$) decrease in MyD 88, TRIF, IRF3 and TRAF-6 expression in the aorta, but the effect was more in the combination therapy. Moreover combination therapy using betulinic acid and fluvastatin showed significant ($p<0.05$) decrease in p38 MAPK, ERK and JNK protein expressions in the aorta. This study provides a novel approach into the probable mechanisms underlying the therapeutic efficacy of betulinic acid and fluvastatin mediated by MyD88 dependent and independent pathways through MAPK signaling pathway.

Keywords *Betulinic acid; fluvastatin; rheumatoid arthritis; MAPK; cardiovascular risk.*

RA is a chronic disease affecting over 1.3 million Americans and as much as 1% of the worldwide population. The annual mortality rate per 1,00,000 people from rheumatoid arthritis in India has increased by 13.6% since 1990, an average of 0.6% a year, is the health impact of rheumatoid arthritis in India over time. Many recent studies in humans revealed that RA patients are predisposed to atherosclerosis as given in the introduction that 40% of death cases in RA is due to cardiovascular dysfunction (Maradit-Kremers *et al.,* 2005; Skeoch and Bruce, 2015). The naturally occurring triterpenoid, betulinic acid is widely distributed throughout the plant kingdom– *Bacopa monniera* (L.), *Ziziphus mauritiana, Triphyophyllum peltatum* and *Ancistrocladus heyneanus* etc, which are exhibiting many pharmacological properties such as anti-cancer (Ren *et al.,* 2010), anti-malarial (Santos *et al*., 2009) and anti-inûammatory properties (Viji *et al*., 2011). Fluvastatin is a water-soluble cholesterol lowering agent that comes under the class of statins whose principle action involves the inhibition of the enzyme HMG CoA reductase. Previous works in our laboratory revealed that fluvastatin has potent anti-inflammatory effects in adjuvant induced arthritis and other studies provide additional scientific datas for the use of statins to reduce cardiovascular mortality in patients with rheumatoid arthritis (Limi *et al*., 2013; Yoshisuke *et al*., 2007). Recent studies also showed that statin therapy using fluvastatin reduces endothelial cell adhesion molecules both in animal models as well as in clinical trials including hypercholesterolemic patients (Romano *et al*., 2000; Yoshisuke *et al*., 2007). The present work aims to evaluate the protective therapeutic effect of betulinic acid and fluvastatin as a combination therapy to reduce the risk of cardiovascular disease in rheumatoid arthritis focusing mainly on CD40/CD36 receptors, MyD88-TRAF6 adapters, TRIF signaling and MAPK signal transduction pathways.

CD-36 and CD-40 are other receptors involved in inflammatory responses. CD36 is another scavenger receptor whose expression is induced by ox-LDL and oxidized fatty acids that may lead to the formation of foam cells (Tabares *et al.,* 2017). CD40 also triggers signaling cascades important in the pathogenesis of arthritis and atherosclerosis (Kristina and Martin, 2015). MyD88 is the canonical adapter for inflammatory signaling pathways downstream of

members of the TLR and IL-1 receptor families. MyD88-dependent pathway includes TIRAP whereas TRIF is associated with MyD88-independent pathway (Takumi Kawasaki and Taro Kawai, 2015). TRIF-dependent signaling is a prerequisite for TLR-mediated production of type-I IFN and several other pro-inflammatory mediators (Ullah MO *et al.,* 2016). Also, regulatory mechanisms shows the role of TRIF pathway in different pathologic states and the potential for manipulating TRIF-dependent TLR signaling for therapeutic benefit as demonstrated by Nilsen *et al.,* 2015. TRAF-6 pathway was recently shown to be reported for LPS induced endothelial cell death through TRAF-6 mediated JNK activation (Ping, 2013).

The MAPK, p38 activation contributes to inflammation, apoptosis, cell differentiation and cell cycle regulation (Cuadrado and Nebreda 2010). The classic ERK1/2 responds primarily to growth factors and mitogens to induce cell growth and differentiation (Shaul and Seger 2007). All these kinases lead to the synthesis of proteins that mediate inflammatory and immune responses which include inflammatory cytokines such as IL-1, IL-6, TNFá, MCP-1, IL-12, IFNs, chemokines, adhesion molecules, co-stimulatory molecules, growth factors, MMPs and enzymes that generate inflammatory mediators such as COX-2 and iNOS. These inflammatory signaling pathways have been well established in atherosclerosis, however the mechanism underlying the progression of atherosclerosis in arthritis is not elucidated so far. So the aim of our study is to elucidate these inflammatory signaling pathways along with the synergistic effect of BA and FLU in combination or alone on the progression of atherosclerosis induced in collagen induced arthritis.

METHODS

Experimental Animals

Female albino rats (Sprague-Dawley strain) of body weight 150-200g which were breed and reared in the department animal house were used for this study. They were provided Laboratory chow (Hindustan Lever Lab diet) and water *ad libitum* throughout the experimental period. The rats were housed in polypropylene cages in a room with temperature maintained at 26 $\pm 1^0$C and a 12 hr light and dark cycle. All experimental protocols were approved by the institutional animal ethics committee [IAEC- KU- 16/2013- 14- BC- AH (25)].

Reagents and drugs

Betulinic acid was provided by Sigma - Aldrich Chemicals, Fluvastatin (FLU) was provided by Novartis Pharma (Basel, Switzerland). Reagents were purchased from Sigma- Aldrich Chemicals, Merck, Eppendorf India Ltd and Spectro Chem. Pvt. Ltd. India.

EXPERIMENTAL PROCEDURE

Type II Collagen induced arthritis

Rheumatoid arthritis was induced by type II collagen (CII) from bovine nasal septum which was dissolved in 0.1 M acetic acid at a concentration of 4 mg/ml and emulsified in equal volume of incomplete Freund's adjuvant. Each rat was given intradermal injections of 100 µl in divided doses at the base of the tail in two sites on day 0 and then received booster injection on day 7. Paw edema was peaked on day 14. Normal non-immune rats were used as negative control. Betulinic acid and Fluvastatin were dissolved in 1% normal saline and fed orally. The dose of Betulinic acid and fluvastatin were chosen as 2mg and 5mg /kg body weight as per the following reference [19, 21] respectively. Duration of the experiment was 60 days. Rats were then sacrificed after overnight fasting by euthanasia. At the end of 60 days, the tissues and blood were isolated for evaluation of biochemical parameters.

Rats were divided into 5 groups each consists of 6 rats:

Group I- Normal rats

Group II- Collagen induced arthritic rats (CIA)

Group III- Rats were given collagen + BA (2mg/ kg body weight)

Group IV- Rats were given collagen + FLU (5mg/ kg body weight)

Group V- Rats were given collagen + BA (1mg/ kg body weight) + FLU (2.5mg/ kg body weight).

Enzyme Linked Immunosorbant Assay (ELISA)

The tissue was lysed in a buffer containing 50mM Tris pH 8.0, 150mM NaCl, 1% Triton X 100, 1mM Na_3VO_4, 2.5mM sodium pyrophosphate, 1mM NaF, 1mM DTT, 1mM PMSF, 1µg/ mL leupeptin. Multiwell ELISA plates coated with the lysates served as the

antigen and incubated for 24 hours at 37^{0}C. Washed the wells with the blocking buffer containing 0.2% gelatin in PBS (0.025M, pH 7.4, 0.15M NaCl) and 0.05% Tween 20. The primary antibodies were diluted in PBS Tween in the ratio of 1:500, were added to the wells (100ìL) and incubated for 1h at room temperature. After washing with PBS-Tween 20, the wells were treated with HRP conjugated anti rabbit IgG that served as the secondary antibody (100ìL) for 1h at room temperature. This was followed by washing with PBS-Tween 20 and PBS. Then the wells were treated with the substrate solution (0.5mL of O-dianisidine 10mg/mL methanol + 60mL of 0.1M citrate –phosphate buffer pH5.0 + 12ìL of 30% H_2O_2) and incubated for 30 minutes. The reaction was stopped by the addition of 100ìL 5N HCl. The optical densities of the samples were determined using microplate (Thermo Multiskan Spectrum) reader set at 405 nm.

Estimation of protein

Protein is estimated by Lowry *et al.* method (1951) using Folin phenol reagent with bovine serum albumin as the standard. The specific activity of the enzyme was expressed as activity/mg protein. 0.1mL of enzyme source was made up to 1mL with distilled water. 1mL of 10% ice cold TCA was added. Placed in ice for 10 minutes and centrifuged for 10 minutes. Supernatant was discarded and the precipitate obtained was dissolved in 1mL of 0.1 N NaOH. From this 0.1mL aliquot was taken as test. 0.1mL of protein solution was taken as standard and 0.1mL distilled water was taken as blank. All the tubes were made upto 1mL with distilled water. To all the tubes 5mL of alkaline copper reagent was added and incubated at room temperature for 10 minutes, followed by the addition of 0.5mL Folin's reagent (2:1 with water). The tubes were mixed and the absorbance was read at 670nm after 30 minutes.

Statistical analysis

The results were analyzed using a statistical program SPSS/PC+, version 17.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA employed for comparison test of significant differences among groups was determined. Pair fed comparisons between the groups was made by Duncan's multiple range tests and $p< 0.05$ was considered significant.

RESULTS

Effect of BA and FLU on the expression of scavenger receptor, CD36 in the aorta

CD36 contributes in atherosclerotic lesion

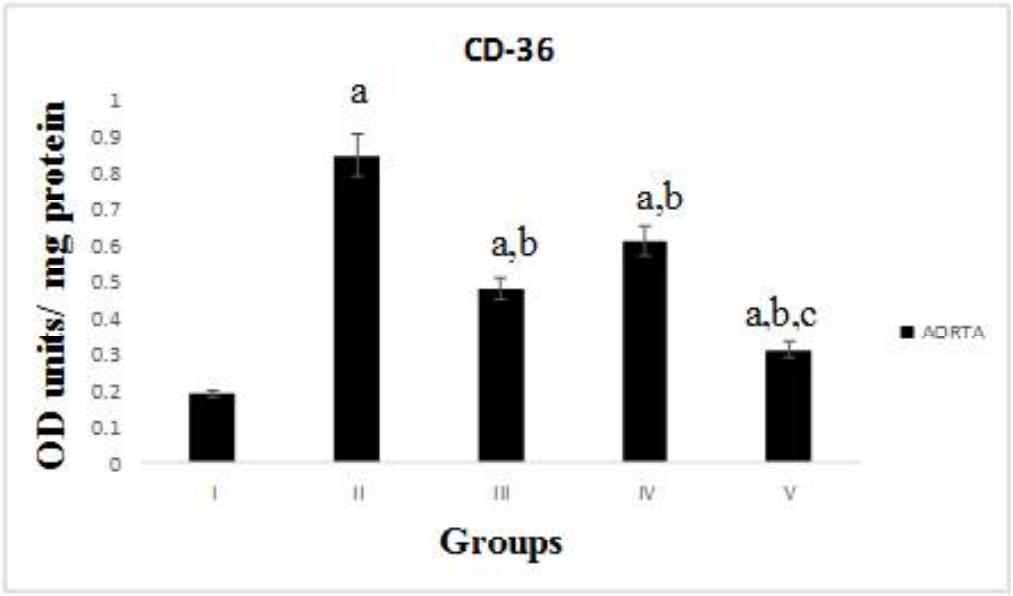

Fig.1. Effect on the expression of CD36 in the aorta: Group I- Normal, Group II- CIA, Group III- CIA+ BA, Group IV- CIA+ FLU, Group V- CIA+ BA + FLU. CIA- collagen induced arthritis, BA- betulinic acid and FLU- fluvastatin. Values expressed as average of 6 values ± SEM in each group, a- Significant difference when group II compared with group I at $p<0.05$, b- Significant difference when group III, IV, V compared with groups II at $p<0.05$, c- Significant difference when group V compared with group III and IV at $p<0.05$.

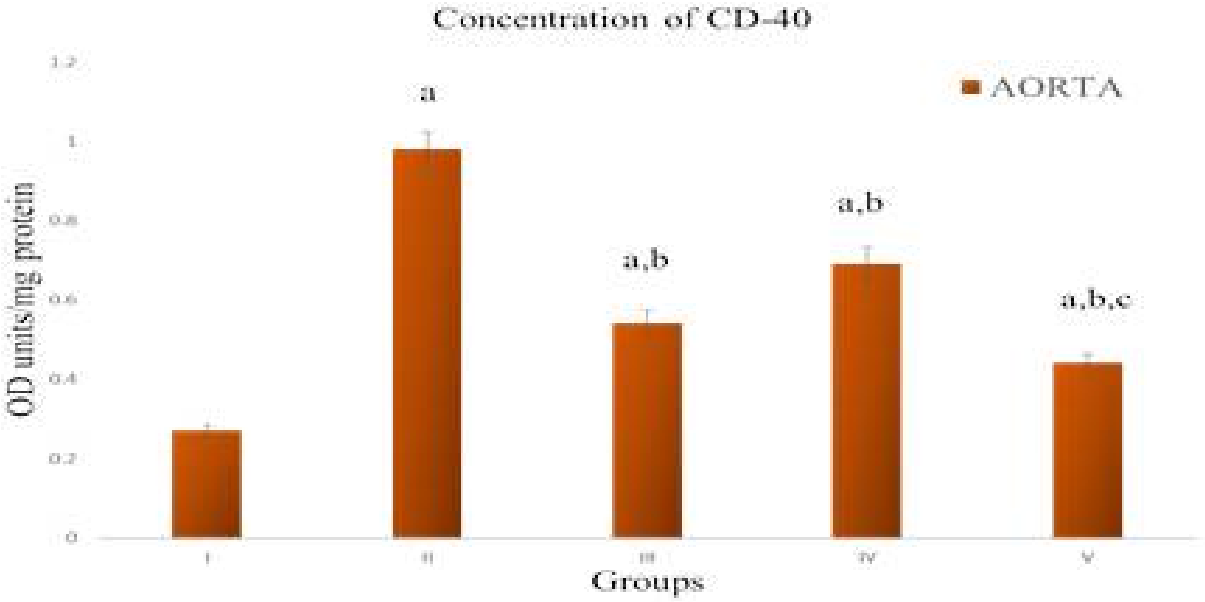

Fig.2. Effect on the expression of CD40 in the aorta: Protein expression done by ELISA. Group I- Normal, Group II- CIA, Group III- CIA+ BA, Group IV- CIA+ FLU, Group V- CIA+ BA + FLU. CIA- collagen induced arthritis, BA- betulinic acid and FLU- fluvastatin. Values expressed as average of 6 values ± SEM in each group, a- Significant difference when group II compared with group I at $p<0.05$, b- Significant difference when group III, IV, V compared with groups II at $p<0.05$, c- Significant difference when group V compared with group III and IV at $p<0.05$.

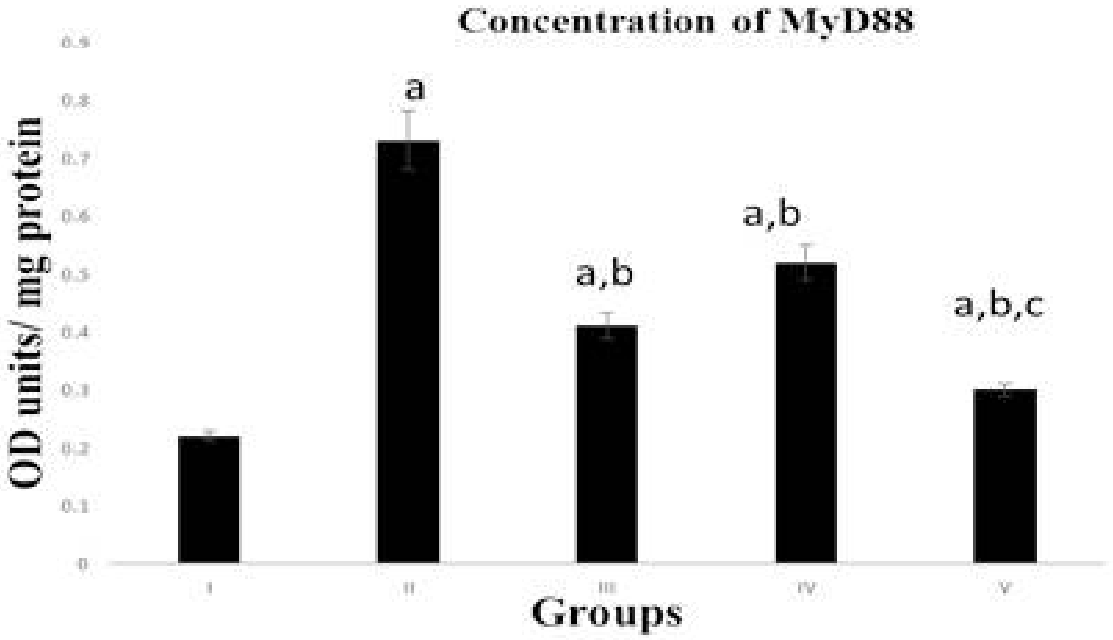

Fig.3. Effect on the expression of MYD88 in the aorta: Group I- Normal, Group II- CIA, Group III- CIA+ BA, Group IV- CIA+ FLU, Group V- CIA+ BA + FLU. CIA- collagen induced arthritis, BA- betulinic acid and FLU- fluvastatin. Values expressed as average of 6 values ± SEM in each group, a- Significant difference when group II compared with group I at $p<0.05$, b- Significant difference when group III, IV, V compared with groups II at $p<0.05$, c- Significant difference when group V compared with group III and IV at $p<0.05$.

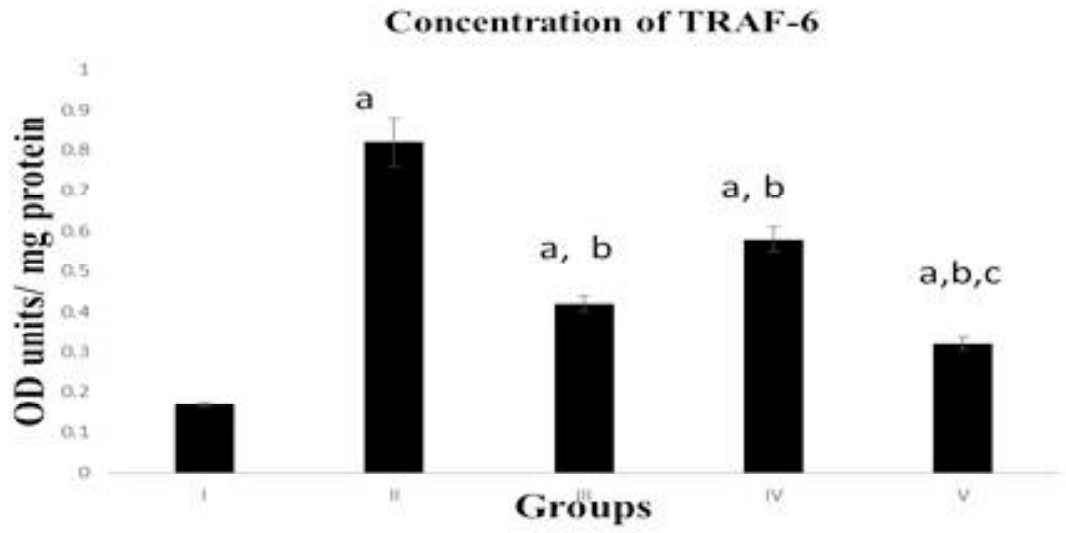

Fig.4. Effect on the expression of TRAF-6 in the aorta: Group I- Normal, Group II- CIA, Group III- CIA+ BA, Group IV- CIA+ FLU, Group V- CIA+ BA + FLU. CIA- collagen induced arthritis, BA- betulinic acid and FLU- fluvastatin. Values expressed as average of 6 values ± SEM in each group, a- Significant difference when group II compared with group I at $p<0.05$, b- Significant difference when group III, IV, V compared with groups II at $p<0.05$, c- Significant difference when group V compared with group III and IV at $p<0.05$.

formation through its contact with oxLDL, which elicits the signaling cascades for inflammatory responses. CD36 functions in oxLDL uptake and foam cell formation, which is the early critical phase of atherosclerosis. Here, the expression of CD-36 was significantly ($p<0.05$) increased in collagen induced rats when compared with normal rats. Administration of both betulinic acid and fluvastatin showed significant ($p<0.05$) decrease in CD-36 expression when compared to collagen induced rats (Fig.1).

Effect of betulinic acid and fluvastatin on the expression of CD40

CD-40 has a major role in the pathogenesis of atherosclerosis. The expression of CD-40 in the aorta was also determined by ELISA, which was significantly ($p<0.05$) increased in collagen induced rats. Combination therapy showed significant ($p<0.05$) decrease in CD-40 expression in the aorta when compared to collagen induced rats as shown in Fig.2.

Effect of betulinic acid and fluvastatin on the expression of MyD 88 in the aorta

To determine whether betulinic acid and fluvastatin had a regulatory role on the TLR-4 downstream pathway, the expression of the adaptor molecules MyD 88 was analyzed. The expression of MyD 88 was significantly ($p<0.05$) increased in collagen induced rats when compared with normal rats. Administration of either betulinic acid or fluvastatin showed significant ($p<0.05$) decrease in MyD 88 expression in the aorta, but the effect was more in the combination therapy (Fig.3).

Effect of betulinic acid and fluvastatin on the expression of TRAF-6 in the aorta

The expression of the adaptor molecule TRAF-6 was significantly ($p<0.05$) increased in collagen induced rats when compared with normal rats. Supplementation of either betulinic acid or fluvastatin alone or in combination showed significant ($p<0.05$) decrease in TRAF-6 expression in the aorta of collagen induced rats and the effect was maximum in combination therapy (Fig.4).

Effect of BA and FLU on the activity of p38 MAPK, ERK and JNK in the aorta

To evaluate the regulatory role of betulinic acid and fluvastatin on MAPK signaling pathway, the protein expressions of p38 MAPK, ERK and JNK were determined by ELISA. Combination therapy using betulinic acid and fluvastatin showed significant ($p<0.05$) decrease in p38 MAPK, ERK and JNK protein expressions in the aorta than the drugs given

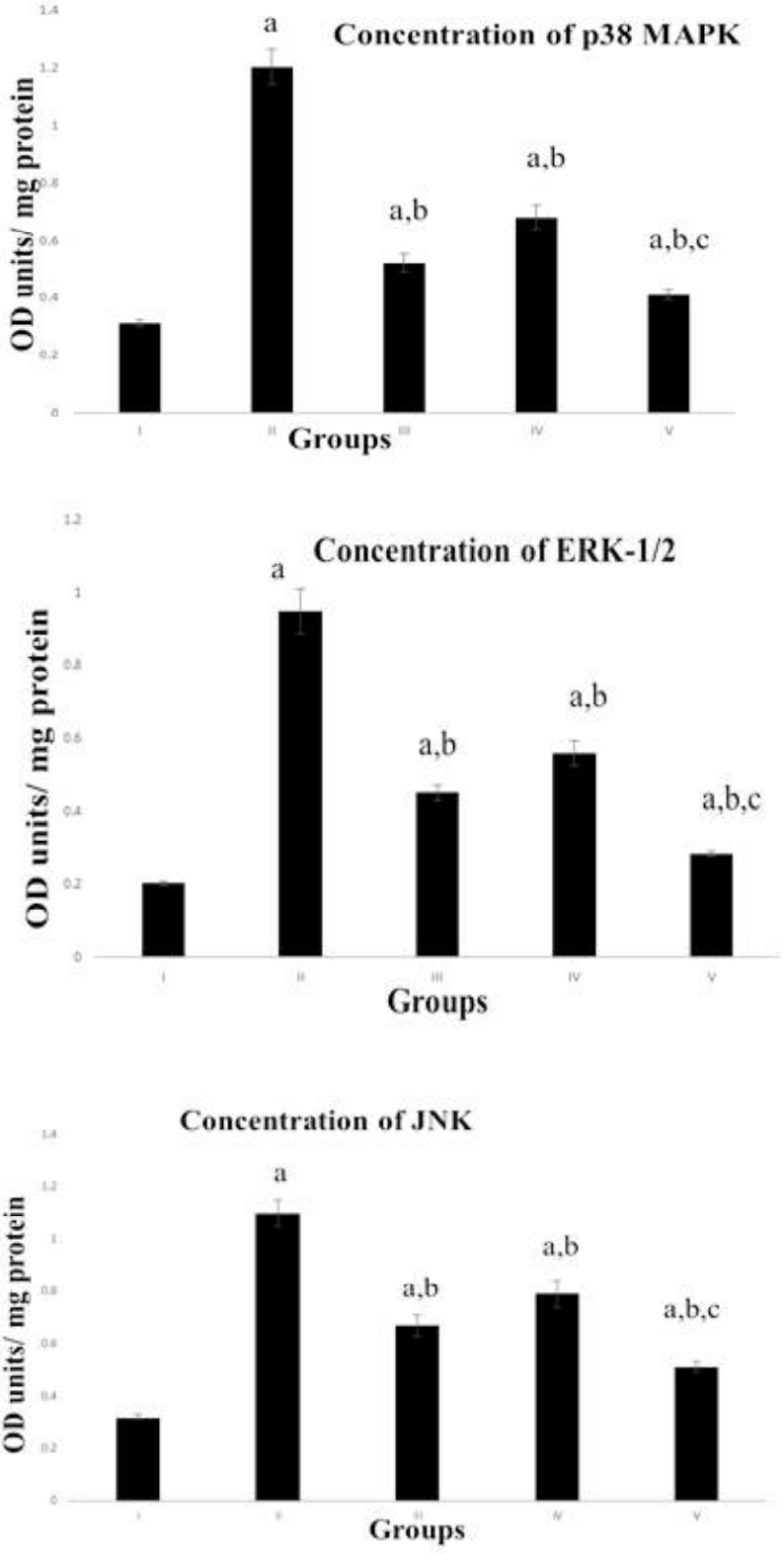

Fig.5. Effect of BA and FLU on the expression of p38 MAPK, ERK and JNK in the aorta: The level of activation of p38MAPK, ERK-1/2 and JNK was estimated by measuring the protein expression by ELISA using specific antibodies against p38mapk, ERK and JNK. Group I- Normal, Group II- CIA, Group III- CIA+ BA, Group IV- CIA+ FLU, Group V- CIA+ BA + FLU. CIA- collagen induced arthritis, BA- betulinic acid and FLU- fluvastatin. Values expressed as average of 6 values ± SEM in each group, a- Significant difference when group II compared with group I at $p<0.05$, b- Significant difference when group III, IV, V compared with groups II at $p<0.05$, c- Significant difference when group V compared with group III and IV at $p<0.05$.

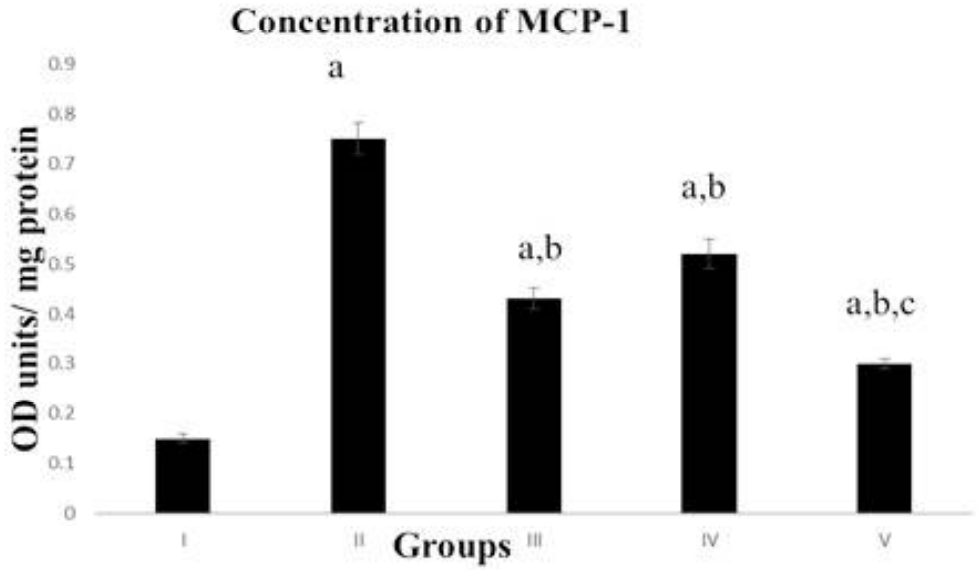

Fig.6. Effect of betulinic acid and fluvastatin on MCP-1 in the aorta: Group I- Normal, Group II- CIA, Group III- CIA+ BA, Group IV- CIA+ FLU, Group V- CIA+ BA + FLU. CIA- collagen induced arthritis, BA- betulinic acid and FLU- fluvastatin. Values expressed as average of 6 values ± SEM in each group, a- Significant difference when group II compared with group I at $p<0.05$, b- Significant difference when group III, IV, V compared with groups II at $p<0.05$, c- Significant difference when group V compared with group III and IV at $p<0.05$.

alone whereas the expressions of p38 MAPK, ERK and JNK was significantly ($p<0.05$) increased in collagen induced rats when compared with normal rats. (Fig.5)

Effect of BA and FLU on monocyte chemoattractant protein-1 (MCP-1) in the aorta

Monocyte chemoattractant protein-1 (MCP-1)

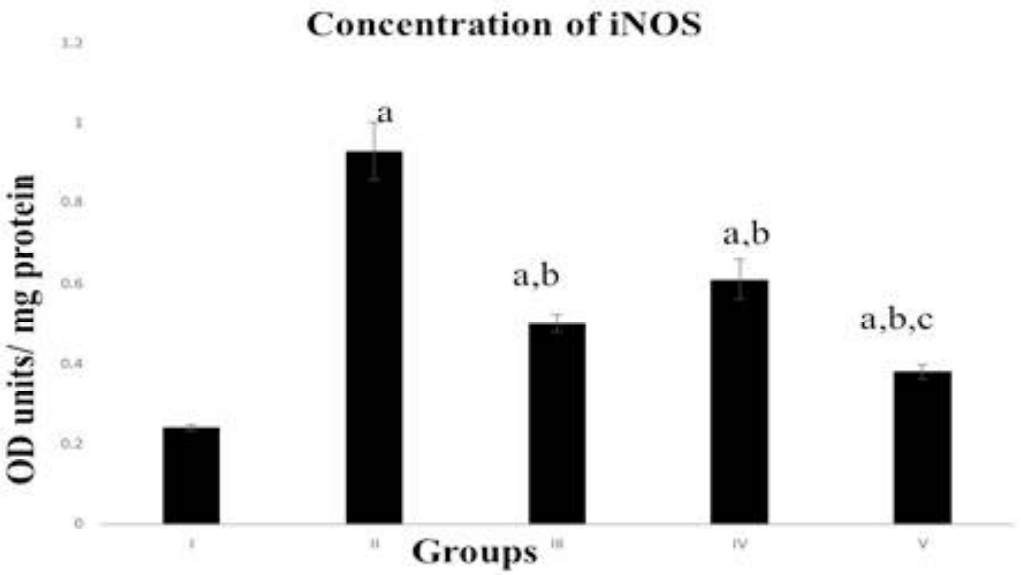

Fig.7. Effect of betulinic acid and fluvastatin on i-NOS in the aorta: Group I- Normal, Group II- CIA, Group III- CIA+ BA, Group IV- CIA+ FLU, Group V- CIA+ BA + FLU. CIA- collagen induced arthritis, BA- betulinic acid and FLU- fluvastatin. Values expressed as average of 6 values ± SEM in each group, a- Significant difference when group II compared with group I at $p<0.05$, b- Significant difference when group III, IV, V compared with groups II at $p<0.05$, c- Significant difference when group V compared with group III and IV at $p<0.05$.

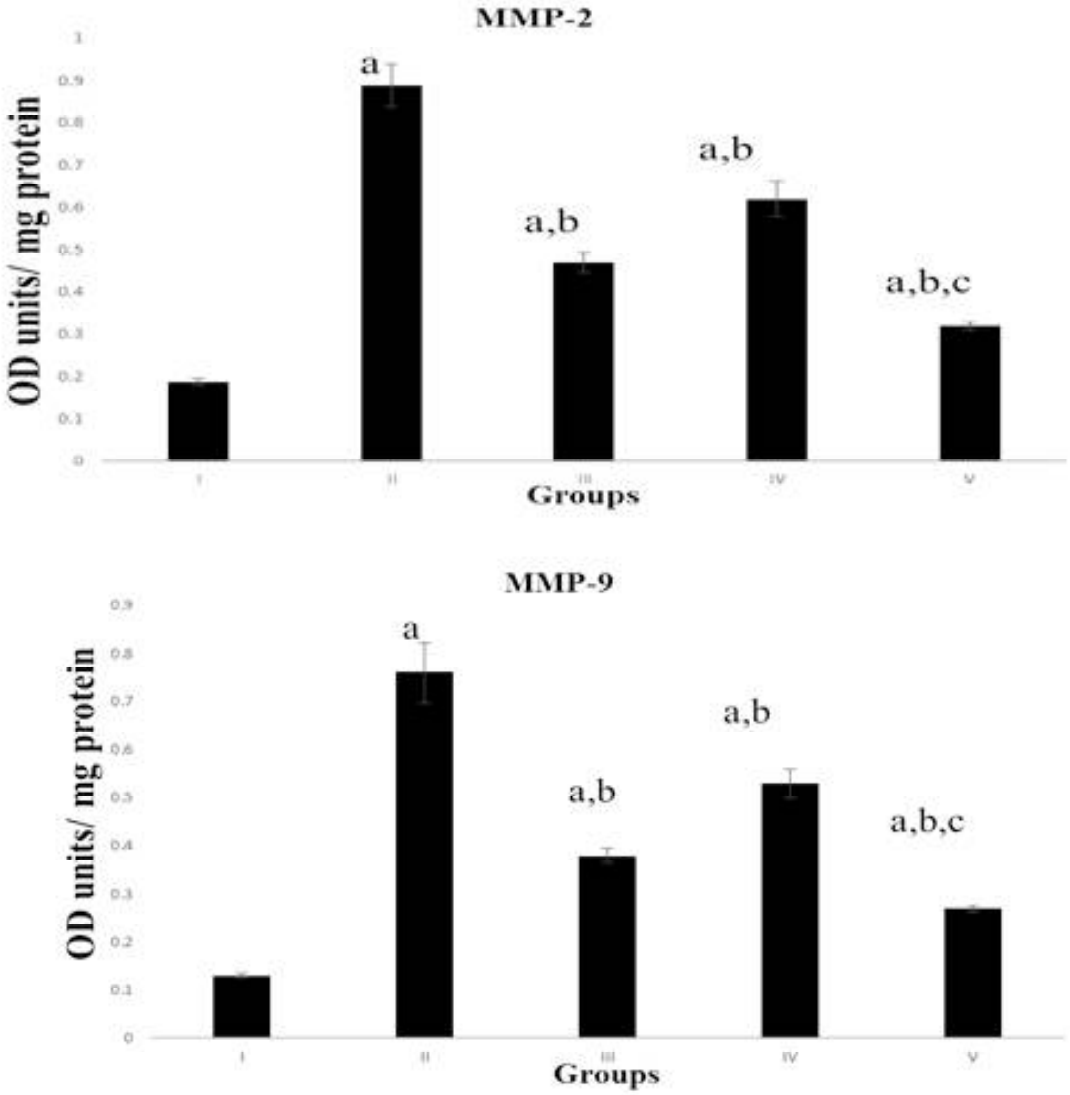

Fig.8. Effect on matrix metalloproteinases, MMP-2 and MMP-9: Group I- Normal, Group II- CIA, Group III- CIA+ BA, Group IV- CIA+ FLU, Group V- CIA+ BA + FLU. CIA- collagen induced arthritis, BA- betulinic acid and FLU- fluvastatin. Values expressed as average of 6 values ± SEM in each group, a- Significant difference when group II compared with group I at $p<0.05$, b- Significant difference when group III, IV, V compared with groups II at $p<0.05$, c- Significant difference when group V compared with group III and IV at $p<0.05$.

is one of the important chemokines that regulate migration and infiltration of monocytes the expressions of monocyte chemoattractant protein-1 (MCP-1) was examined in the aorta. Significant ($p<0.05$) decrease in MCP-1 expression in the aorta was observed in combination therapy using betulinic acid and fluvastatin when compared to collagen induced rats (Fig.6).

Effect of BA and FLU on inducible nitric oxide synthase (iNOS) in the aorta

Since iNOS plays a critical role in mediating inflammatory immune response, the expressions of inducible nitric oxide synthase (i-NOS) was examined in the aorta. Combination therapy using betulinic acid and fluvastatin showed significant ($p<0.05$) decrease in i-NOS expression in the aorta when compared to collagen induced rats (Fig.7).

Matrix metalloproteinases, MMP-2 and MMP-9 in the aorta

Since the activation of matrix metalloproteinases triggers the inflammatory process in atherosclerosis, the protein expression of MMP-2, 9 in the aorta was analyzed by ELISA. The expression of MMP-2 and MMP-9 was significantly ($p<0.05$) increased in collagen induced rats when compared with normal rats. Administration of betulinic acid and fluvastatin showed

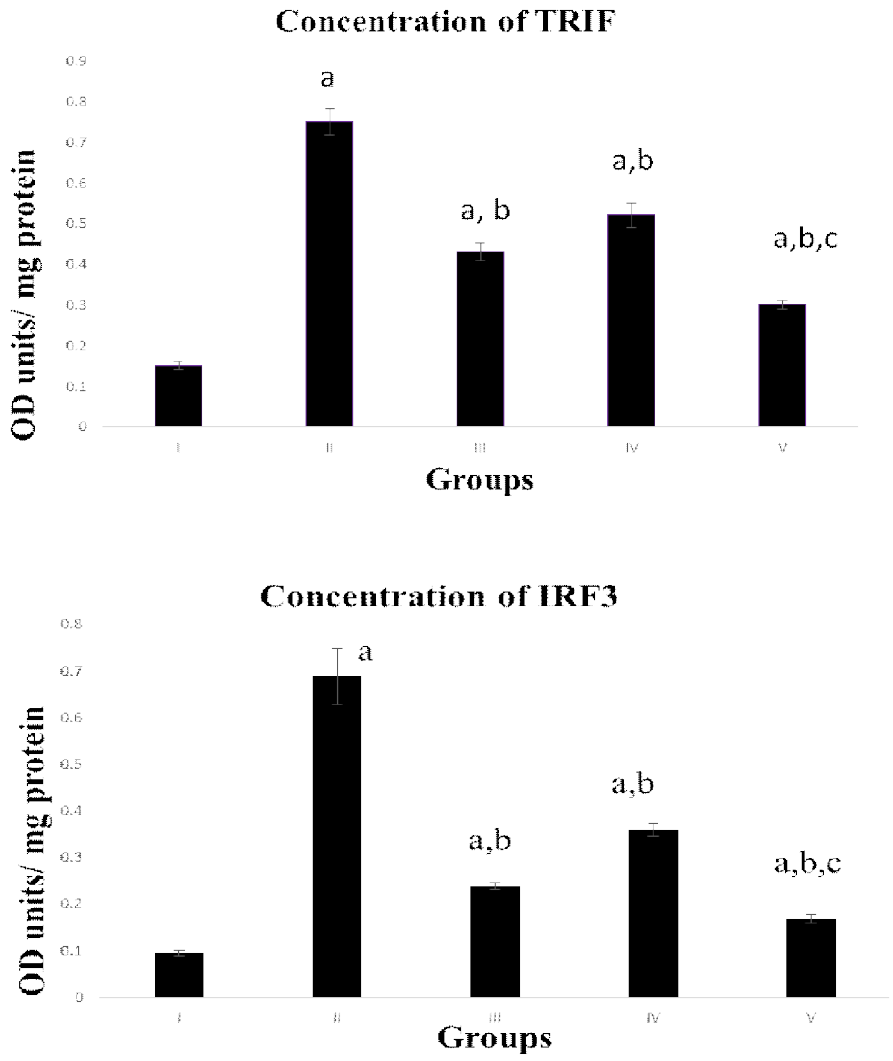

Fig.9. Effect on the activation of TRIF and IRF3: Group I- Normal, Group II- CIA, Group III- CIA+ BA, Group IV- CIA+ FLU, Group V- CIA+ BA + FLU. CIA- collagen induced arthritis, BA- betulinic acid and FLU- fluvastatin. CIA- collagen induced arthritis, BA- betulinic acid and FLU- fluvastatin. Values expressed as average of 6 values ± SEM in each group, a- Significant difference when group II compared with group I at $p<0.05$, b- Significant difference when group III, IV, V compared with groups II at $p<0.05$, c- Significant difference when group V compared with group III and IV at $p<0.05$.

significant ($p<0.05$) decrease in MMP-2 and MMP-9 than the drugs given individually (Fig.8).

TRIF and Interferon regulatory factor-3 (IRF3) in the aorta

To examine the role of combined effect of betulinic acid and fluvastatin on TRIF dependent signaling pathway. The expression of TRIF and interferon regulatory factor-3 (IRF-3) was significantly ($p<0.05$) increased in collagen induced rats when compared with normal rats. Combination therapy showed significant ($p<0.05$) decrease in the expression of TRIF and IRF3 when compared to the collagen induced rats (Fig.9).

Effect of BA and FLU on the expression of IFN-ã

The regulatory role of betulinic acid and fluvastatin on TRIF dependent signaling pathway was confirmed by the expression of interferon-ã. The expression of interferon-ã was significantly ($p<0.05$) increased in collagen induced rats when compared with normal rats. Administration of either betulinic acid or fluvastatin showed significant ($p<0.05$) decrease

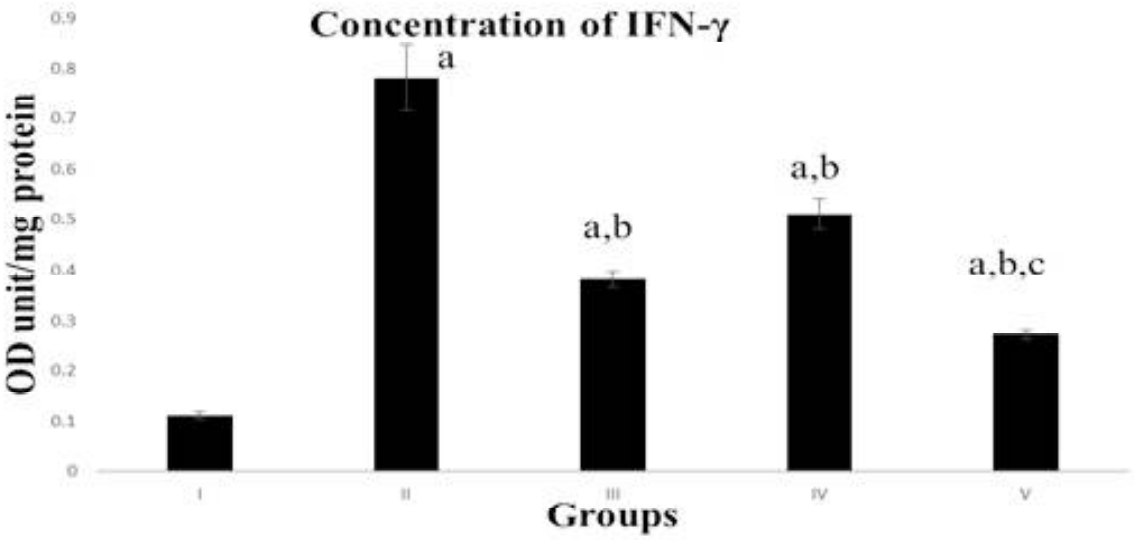

Fig.10. Effect on the activation of IFN-ã: Group I- Normal, Group II- CIA, Group III- CIA+ BA, Group IV- CIA+ FLU, Group V- CIA+ BA + FLU. CIA- collagen induced arthritis, BA- betulinic acid and FLU- fluvastatin. CIA- collagen induced arthritis, BA- betulinic acid and FLU- fluvastatin. Values expressed as average of 6 values ± SEM in each group, a- Significant difference when group II compared with group I at $p<0.05$, b- Significant difference when group III, IV, V compared with groups II at $p<0.05$, c- Significant difference when group V compared with group III and IV at $p<0.05$.

in interferon-ã but the effect was noticed more in combination therapy (Fig.10).

DISCUSSION

In our study we evaluated the decreased arthritic index and serum lipids in combination therapy which was found to be elevated upon collagen induction (Limi et al., 2017). We found that MyD88 dependent and independent pathways were activated in CIA rats including the adaptors TRIF and TRAF-6. This is consistent with the recent study that TLR-3 and 4 signaling through the TRIF and TRAM adaptors in haematopoietic cells promotes atherosclerosis (Anna *et al.,* 2013; Emina *et al.,* 2015) and the expression of TRIF was increased in TLR2-induced foam cell formation (Huang *et al.,* 2016). Our findings showed that co-administration of betulinic acid and fluvastatin inhibited MyD88 dependent and independent signaling pathways involving TRAF6 and TRIF. Downregulated activity of TRIF by the combination therapy, consequently blocked the phosphorylation of IRF3. Thus lead to the decreased synthesis of type I IFN, particularly IFN-ã. Here also, IFN-ã was significantly decreased in the aorta which was seen upregulated in CIA rats. Thus combination therapy using betulinic acid and fluvastatin blocked the activation of both MyD88 dependent and independent pathways which further repressed the adaptors TRAF6 and TRIF thereby regulated the expression of inflammatory genes in the aorta.

Moreover, CD36 is a multi-ligand scavenger receptor present on the surface of a number of cells such as platelets, monocytes/macrophages, endothelial and smooth muscle cells plays a critical role in atherosclerosis (Sophie *et al.,* 2007). The monocyte or the macrophage CD36 has been displayed to perform an important role in the development of atherosclerotic lesions as it has the ability to engulf ox-LDL and it is implicated in the formation of foam cells. In the present study, we found that collagen induction increased expression of CD36 which may cause upregulation of TLR, this is also in co-relation with the report that CD36 has diverse downstream signaling pathways and functions by interacting with other membrane receptors such as integrins, TLR etc (Akira *et al.,* 2003) and CD36 accelerated foam cell formation induced by ox-LDL (Wong *et al*., 2016). Moreover, CD36 was increased in the peripheral blood mononuclear cells and aorta of hypercholesterolemic rabbits reflects the onset of atherosclerosis (Yazgan *et al.,* 2017). Also, our findings showed that the expression of scavenger receptor CD-36 in the aorta was significantly decreased in combination therapy.

In association to this result, the recent report showed that natural biflavonoids modulate macrophage ox-LDL interaction via CD36 receptor expression *in vitro* and promote atheroprotection *in vivo* (Tabares *et al*., 2017). Besides CD36 protein expression was downregulated in arginine fed hypercholesterolemic rats (Xin Wang *et al.,* 2015). Recent findings also showed that simvastatin downregulates CD36 mediated inflammation and atherosclerosis in Apo E knockout mice (Yin *et al.,* 2017).

CD40 signaling is important in the upregulation of chemokines, proinflammatory adhesion molecules, cytokines, MMPs and procoagulants. Studies have supported that CD40 has a major regulatory role in the pathogenesis of atherosclerosis (Lievens *et al.,* 2009; Gocmen *et al.,* 2013). Besides, CD40 activation on endothelial cells triggers the release of various inflammatory mediators of critical importance in the initiation and development of atherosclerosis. In CIA rats, upregulated expression of CD40 was observed in the monocytes and aorta. These results are linked to the current studies that CD40 played a crucial role in inflammation by stimulating activation of leukocytes and endothelial cells, thereby promoting atherosclerosis (Norbert *et al.,* 2016) and high levels of soluble CD40 ligand (sCD40L or CD154) in the circulation was observed in atherosclerosis (Yuan *et al.,* 2015). In the present study, CD40 expression in the aorta and monocytes were significantly decreased in combination therapy. In consistent to these results, reports demonstrated that atherosclerosis can be prevented by the inhibition of CD40 signaling in mice (Mach *et al.,* 1998). Therefore combination therapy have a regulatory role on CD36 and CD40 receptors suggest that it possess therapeutic potential in the treatment of arthritis.

The p38 MAPK pathway activation involves the production and activation of inflammatory mediators to initiate leucocyte recruitment and activation. The p38 MAPK positively regulates expression of many genes involved in inflammation such as those coding for TNF-á, IL-1, IL-6, IL-8, COX-2 (Cuadrado and Nebreda, 2010). In collagen induced rats, increased CD40-TRAF6 leads to the activation of MAPK pathway by the phosphorylation of p38, ERK and JNK in TLR mediated signal transduction leading to activation of NF-êB which further causes the synthesis of pro-inflammatory cytokines. This is in support of Ricci *et al.,* 2004 demonstrated the major role for JNK2 in association with scavenger receptor in hypercholesterolemic mice. Administration of BA and FLU leads to the inhibition of MAP kinases such as p-38 MAPK, ERK and JNK thus regulates the genes involved in the inflammatory response. Thus TRAF-6 and MAPK expression was tightly regulated by combination therapy which could evidently alleviate the inflammatory cause for disease condition such as arthritis.

Increased expression of the chemokine MCP-1 was observed in the aorta of CIA rats which plays an important role in the pathogenesis of atherosclerosis. This is in agreement to the reports that MCP-1 was increased in the CIA mice and atherosclerotic lesions (Hansel and Bruckert, 2010). Earlier study showed that MCP-1 is a major monocyte chemotactic factor induced by modified-LDL in endothelial cells that may trigger firm adhesion of monocytes to vascular endothelium under blood flow (Javier Mestas and Klaus Ley, 2008). Our findings showed decreased expression of MCP-1 was observed in rats administered with both BA and FLU. We also found that the expression of the pro-inflammatory enzyme iNOS was increased in the aorta of CIA group. Increased expression of iNOS was observed within the human atherosclerotic lesions that promotes lipid peroxidation and vascular damage (Buttery *et al*., 1996). Combination therapy with BA and FLU decreased the expression of iNOS in the aorta of treatment group. In agreement to this result, recent report suggested that betulinic acid regulates the expression of iNOS that contribute to the anti-atherosclerotic effects of BA (Jin *et al*., 2016).

In CIA rats, the expression of MMP-2 and 9 in the aorta were increased which is in support with the recent report that MMP-2 and 9 are increased in the synoviocytes that contribute to the progression of arthritis (Meng *et al.,* 2014). Also, the expression of MMP-2 and 9 were decreased in the aorta of treatment group thereby decreasing the degradation of extracellular matrix. So the present study reveals that in arthritis, type II collagen progress atherogenesis which is very similar to that of studies related to atherosclerosis. The mechanism of progression of atherosclerosis in arthritis is via CD36, CD40, MAPK signaling pathways through MyD88 dependent and independent pathways by the activation of NF-êB

which translocates into the nucleus that inturn transcribe the genes responsible for the synthesis or production of iNOS, MMP, COX-2 and MCP-1. This contribute to the worsening of the arterial wall in arthritis. This may be due to the formation of immune complex by collagen induction.

CONCLUSION

Synergistic effect of BA and FLU therapy suppress or inhibit the activation of CD40, CD36 expression, MAPK signaling pathways through MyD88 dependent and independent pathways in collagen induced rats, thus establishing a potential mechanism that helps in the production of inflammatory mediators, pro-inflammatory cytokines and chemokines that may contribute to the reduced pro-inflammatory state of atheromatic lesions thereby inhibiting atherosclerotic progression in experimental arthritis.

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Evaluation of Biochemical Changes and its Mitigation in Sesame- *Alternaria* Interaction

A.S. LUBAINA[1], MURUGAN. K[2]

[1]*Department of Botany, Christian College, Kattakada*
[2]*Plant Biochemistry and Molecular Biology Laboratory, Department of Botany, University College, Thiruvananthapuram, Kerala 695 034, India.*
email id: lubainanizam@gmail.com

ABSTRACT

***Alternaria* leaf spot disease in *Sesamum orientale* L. caused by *Alternaria sesami* ranked as a major threat to sesame cultivation and production. Analysis of the host parasite relationships reveals the pattern of pathogenesis and the defence mechanisms exhibited by the plants challenged by the pathogen. Perception of a pathogen by a plant triggers rapid defense responses via multiple signalling pathways that lead to the production of reactive oxygen species (ROS). Sesame *Alternaria* interaction remarkably increased ROS production - hydrogen peroxide (H_2O_2) and were substantiated by histochemical localization data. Lipid peroxidation measured in terms of malondialdehyde (MDA) content, showed progressive increase in the infected sesame when compared with the control. Ascorbate, glutathione content and the related key enzymes of ascorbate-glutathione cycle viz., ascorbate peroxidase (APX), glutathione reductase (GR) showed high activities up to 9th day followed by a decrease. Monodehydroascorbate reductase enzyme was active to an extent to regenerate the reduced ascorbate, the substrate for APX to scavenge H_2O_2. Cellular burst in sesame is due to the less efficient defense system in the form of ascorbate – glutathione cycle. The antioxidant defense ability is not achieved properly in sesame by increasing the amounts of ascorbate and glutathione as well as antioxidant enzymes under biotic stress.**

Keywords *Sesamum orientale L.; Alternaria sesami; H_2O_2; Ascorbate; Glutathione; Ascorbate peroxidase; Glutathione reductase*

Sesame (*Sesamum orientale* L.) of Peadaliaceae is one of the traditional oil seed crops of India. The cultivated sesame derives from wild populations originated from South Asia, especially the western Indian peninsula. Its tolerance towards drought and high temperatures make sesame well suited to land where other crops fail to survive. However, compared with other oilseed crops, sesame seed production is not consistent, as it is susceptible to pathogens. *Alternaria* leaf spot of sesame, is one of the important biotic stresses that results in small, irregular brown spots mainly on the leaf lamina as well as on stem and pods. Research has revealed that plants and pathogens communicate with each other in a conversation through reactive oxygen species (ROS) signalling network. Induction of ROS is one of the earliest observable manifestations of a plant defence strategy orchestrated by ROS gene network. The interplay between ROS production and scavenging knowledge of plant pathogen interaction appears to be more and more complex. In this scenario, the ROS cycle seems to have an increasing importance. Despite this significance, there is still a lacuna to dissect the identities, activities and relative importance of the ROS generating system in host pathogen interaction. The present study aims to unravel the biochemistry of ROS cycle during this particular plant pathogen interaction.

MATERIALS AND METHODS

Sesamum orientale L. variety Thilarani seeds were collected from Regional Agricultural Research Station (RARS), Kayamkulam as the study material. Plantlets were raised from seeds in healthy conditions at the green house, Department of Botany, University College, Thiruvananthapuram. For *in vitro* fungal inoculation studies, sesame plants were inoculated with 20 µl of *Alternaria sesami* conidial suspension (1×10^3 conidia ml^{-1}) prepared from pure culture by drop method. Sterillised water (20 µl) was used for mock inoculation. The inoculated plants, along with their respective healthy controls, were maintained at room temperature and covered with a transparent polyethylene bags for providing high humidity to facilitate successful penetration of the pathogen into sesame. Leaves from control and pathogen inoculated plants were harvested from 1st to 13th days after inoculation for all analytical and biochemical analysis.

Quantification of hydrogen peroxide (H_2O_2)

H_2O_2 concentrations of the experimental tissues were estimated as per the procedure of Bellincampi *et al*. (2000) with some modifications. It was based

on the peroxidase mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange. 1 g tissue was homogenized in 10 ml cold 10 mM phosphate buffer (pH 7) using a prechilled mortar and pestle. Filtered the homogenate and centrifuged at 10000 rpm for 10 min. The supernatant was collected and made up to a known volume using the buffer. 1.5 ml of the extract was added to an equal volume of assay reagent containing 500 mM ammonium ferrous sulphate, 50 mM H_2SO_4, 200 mM xylenol orange and 200 mM sorbitol. The assay mixture was incubated for 45 min and the absorbance of the Fe^{3+} xylenol orange complex at 560 nm was observed. Control was performed by eliminating the H_2O_2 in the reaction mixture without extract.

Quantification of lipid peroxidation (LPX)

The level of lipid peroxidation in the cells was measured in terms of malondialdehyde (MDA) content determined by the thiobarbituric acid (TBA) reaction as described by Zhang and Kirkham (1996). 0.4 g tissue of the samples was homogenized in 4 ml 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 rpm for 10 min. 1 ml of the supernatant was diluted 1:5 (v/v) with 20% (w/v) trichloroacetic acid containing 0.5% (w/v) TBA. The mixture heated at 95°C for 30 min and cooled in an ice bath, followed by centrifugation at 10,000 rpm for 10 min, and read the absorbance of the supernatant at 532 nm. The nonspecific absorption value at 600 nm was subtracted from the 532 nm reading. The malondialdehyde concentration was calculated using the extinction coefficient of 155 mM cm^{-1}.

Histochemical localization of hydrogen peroxide (H_2O_2)

Histochemical localization of H_2O_2 was done by staining the tissues with Tetramethyl benzidine (TMB) reagent, as per the method of Ros Barcelo (1998), with slight modifications. Fresh, thin sections of infected and healthy leaf tissues were incubated in 50 mM Tris-acetate buffer (*p*H 7), containing 0.1 mg/ml TMB-HCl, for 15 to 20 min. The sections observed under light microscope and H_2O_2 localization detected as blue deposits. Controls were performed in the presence of catalase (200 U/ml), 1.0 mM ascorbate and by blanching the sections in 80°C hot water before staining with TMB.

Quantification of ascorbate (Asc) and dehydroascorbate (DHA)

Asc and DHA were measured according to Logan *et al*. (1998) with minor modifications. Briefly 1 g of experimental materials was ground in 1 ml of ice cold 6% (v/v) $HClO_4$. The extract was centrifuged for 10 min in 10,000 rpm at 4°C and supernatant was immediately assayed for Asc and DHA. 100 ìl of the extract was neutralized with 30 ìl 1.5 mM Na_2CO_3. Asc was assayed spectrophotometrically at 265 nm (€ = 14 mM cm^{-1}) in 100 mM potassium phosphate buffer (pH 5.6), before and after 15 min incubation with 5 units of ascorbate oxidase. For total ascorbate, 100 ìl of extract was neutralized with 30 ìl 2 mM Na_2CO_3 and incubated for 30 min at room temperature with equal volume of 20 mM GSH in 100 mM Tricine–KOH (pH 8.5).

Estimation of reduced glutathione (GSH) and oxidized glutathione (GSSG)

The levels of reduced and oxidized glutathione were estimated fluorimetrically (Hissin and Hilf, 1976). Plant material (1 g) was ground in 1 ml 25% H_3PO_3 and 3 ml 0.1 M sodium phosphate–EDTA buffer (pH 8.0). The homogenate was centrifuged for 20 min at 10,000 rpm and the supernatant was used for the estimation of GSH and GSSG in a spectroflourimeter. The supernatant was further diluted five times with sodium phosphate–EDTA buffer (pH 8.0). The final assay mixture (2.0 ml) contained 100 ìl of the diluted supernatant, 1.8 ml phosphate–EDTA buffer and 100 ìl *O*-phthalaldehyde (1 mg ml^{-1}). After thorough mixing and incubation at room temperature for 15 min, the solution was read at 420 nm after excitation at 350 nm.

An aliquot of 0.5 ml of the supernatant was incubated at room temperature with 200 ìl 0.04 M *N*-ethylmaleimide for 30 min to interact with the GSH present in the supernatant. To this mixture, 4.3 ml 0.1 N NaOH was added. A 100 ìl of this mixture was taken for the measurement of GSSG, using the procedure outlined for GSH assay, except that 0.1 N NaOH was used as the diluent rather than phosphate–EDTA buffer (Hissin and Hilf, 1976).

Extraction and assay of antioxidant enzymes

Extraction of enzymes was carried out at 4°C. All the experimental samples (0.5 g) were ground and

Table 1. H_2O_2 and TBARS content (µg/g tissue) in control (C) and infected (I) Sesame variety Thilarani from 1-13 days after infection

Days	H_2O_2		TBARS	
	C	I	C	I
1	4.58	9.30	197.80	292
3	4.24	10.08	204.20	526.40
5	4.28	13.16	210.60	979.40
7	4.38	15.22	204.40	1145.40
9	4.32	18.74	210.60	1281.80
11	4.60	19.70	212.80	1300.80
13	4.04	20.41	215.80	1312.18
F ratio I ID	2166.229 ** 156.0995 **		6937.072 ** 240.938 **	
$CD_{(0.05)}$ I ID	0.498 0.606		24.27958 30.06501	

homogenized in 20 ml ice-cold extraction buffer (100 mm KH_2PO_4 /K_2HPO_4 (pH 7.8), 300 mg polyvinyl pyrrolidone (PVP), 1% (v/v) Triton X-100 and 5 mM ascorbate (Schwanz *et al*., 1996). The homogenate was centrifuged at 25,000 rpm (20 min, 4°C). The supernatant was used for the determination of APX, GR and MDHAR.

Ascorbate peroxidase (APX)

In ascorbate peroxidase assay, the reaction mixture consisted of 100 mM K-phosphate buffer (pH 7.0) (0.25 ml), 1 mM ascorbate (0.25 ml), 0.4 mM EDTA (0.25 ml),0.19 ml distilled water, 10 mM H_2O_2 (0.01 ml) and enzyme extract (0.05 ml). The reaction was started by adding H_2O_2 and the oxidation rate of ascorbate was measured by the initial rate of decrease of absorbance at 290 nm. The molar absorption coefficient of ascorbate (2.8 mM cm^{-1}) was used to calculate the activity of enzyme Yanagida *et al*. (1999).

Monodehydro ascorbate reductase (MDHAR)

In MDHAR assay, the reaction mixture included of 100 mM K-phosphate buffer (pH 7.0), 0.1 mM NADH, 4 mM ascorbate and 0.05 ml enzyme extract. The reaction was started by adding NADH and the oxidation rate of ascorbate was measured by the initial rate of decrease of absorbance at 340 nm. The molar absorption coefficient of NADH (6.2 Mm cm^{-1}) was used to calculate the enzyme activity (Song *et al*. (2005).

Glutathione reductase (GR)

In glutathione reductase assay, the reaction mixture consisted of 100 mM K-phosphate buffer (pH 7.8) (0.25 ml), 10 mM oxidized glutathione (GSSG) (0.05 ml),0.48 ml distilled water, 1 mM NADPH (0.12 ml) and enzyme extract (0.1 ml). The assay was started by addition of GSSG. GR activity was determined from the rate of NADPH oxidation measuring the absorbance decreased at 340 nm. GR activity was monitored by A_{340} for NADPH oxidization as GSSG reduction according to Chen and Wang (2002).

Statistical analysis

ANOVA in CRD was done with two factors namely I and D. The 1st factor I has two levels, I_0(control) and I_1 (infected). Since the observations were recorded from 1st to 13th day(D) after infection for alternate days. Thus D has seven levels which

were denoted as $D_1, D_2, D_3, \ldots D7$(Gomez and Gomez,1984). In inoculation trials after confirming the significance of F values, the significance of the differences between the mean values of 5 replications were tested using ANOVA. Significant differences were considered at $P < 0.01$ probability levels.

RESULTS AND DISCUSSIONS

Estimation of hydrogen peroxide (H_2O_2)

ROS are used by plants as signalling molecules in a variety of process ranging from defense against pathogens to developmental process. The interplay and the balance between ROS production and its scavenging will determine the defense signaling output as well as damage and cell-death responses. H_2O_2, is a versatile ROS at low concentrations can induce stress acclimatization, while at high concentration it can trigger cell death (Torres, 2010). First signs of H_2O_2 generation were observed at 24 h after pathogen inoculation (Table 1). A two fold H_2O_2 generation began at1st day after pathogen infection and it became five fold on 13thday in treated plants compared to the control. Do *et al.* (2003) reported high profile of H_2O_2 formation in *Capsicum annuum* due to hypersensitive response to *Xanthomonas campestris*pv. Vesicatoria. Comparatively, in the present study the level of H_2O_2 formation is less. The reason for the lower H_2O_2 generation during *A. sesami* infection in Thilarani, the cultivar may be because of the presence of quenchers of ROS, known to be produced by fungi during infection and pathogenesis. *A. alternata* is reported to produce mannitol, a potent quencher of ROS during its infection on tomato (Prasad and Upadhyay, 2010).

Histochemical localization

Hydrogen peroxide (H_2O_2)

Tetra methyl benzidium (TMB) stain was employed to localize H_2O_2 in the leaf tissues of inoculated and control sesame. Leaf sections incubated in the TMB medium showed tremendous deep blue deposits on the mesophyll and vascular tissues of the infected sesame than the control (Fig. 1a and b). The blue colour deposits in the cells suggest the oxidation of TMB by the ROS- H_2O_2 accumulated in the cells. Apart from the synthesis of H_2O_2 in cell system as a normal metabolic event, the upregulation of H_2O_2 in the diseased leaves of infected plants is possibly a manifestation of oxidative stress. Further, it can be a part of defense strategy contributed by the host sesame against infection. It can be proposed that $O_2^{\bullet-}$ and H_2O_2 play key role in plant host to act as first line of defense against pathogen. ROSs generating system might kill pathogens or induce a hypersensitive response (HR) within the cells, leading to incompatible plant pathogen reactions (Mendoza, 2011).

Lipid Peroxidation (LPX)

Induction of lipid peroxidation (LPX) was assessed by determining the accumulation of thiobarbituric acid reactive species (TBARS) at different time interval following inoculation of *A. sesami* suspensions adjusted to a concentration of 10^3 conidia mL^{-1} in intact healthy leaves of sesame. Increasing levels of TBARS were detected from 1st to13thday of the experiment (i.e., 292 to 1312.8 µg/g tissue) (Table 1). The profile of LPX strongly corroborate with ROS

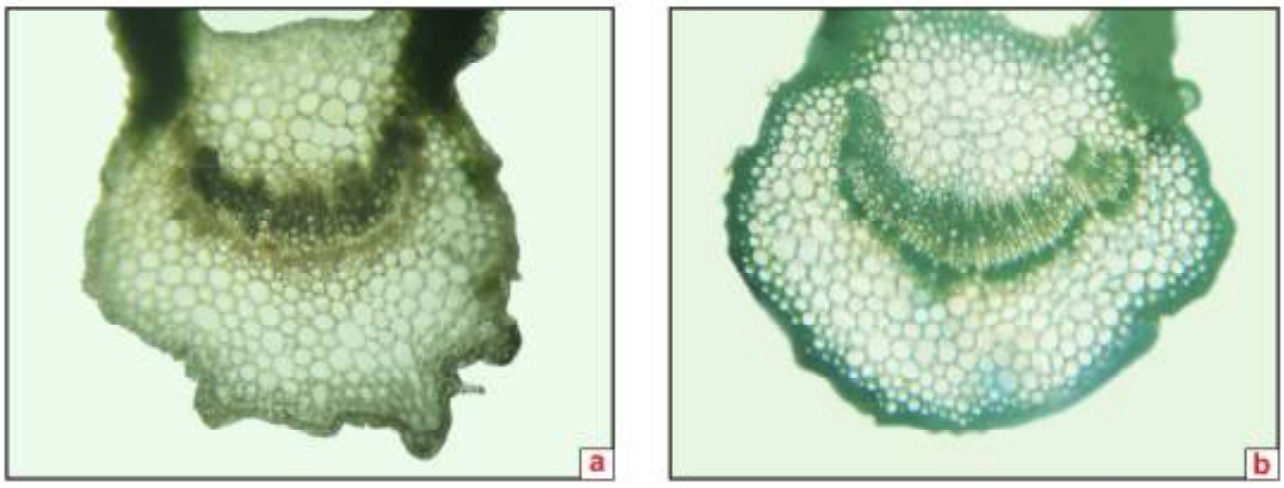

Fig 1a & b:- Histochemical localization of H_2O_2 (a) control and (b) infected sesame

Table 2. Quantification of ascorbate (AsA), dehydro ascorbate (DHA), reduced glutathione (GSH), oxidised glutathione (GSSG) in µg/g tissue in the control (C) and infected (I) Sesame variety Thilarani from 1-13 days after infection

Days	AsA		DHA		GSH		GSSG	
	C	I	C	I	C	I	C	I
1	46.36	71.20	6.7	10.3	9.38	17.00	1.9	3.2
3	47.84	77.00	7.3	10	8.26	19.56	2.2	4
5	47.40	83.84	8.5	11	9.14	21.46	2.4	5.3
7	48.46	90.48	7.9	10.9	9.02	24.02	1.86	6
9	47.80	96.40	8.2	11	9.74	26.86	2	7
11	46.60	86.26	7.8	12.5	8.74	20.62	1.94	7.6
13	46.60	81.28	8	12.8	9.44	16.48	1.79	7.9
F ratio I ID	4478.349 ** 29.01712 **		2536.587 ** 28.4376 **		3888.324 ** 22.5163 **		689.235 ** 31.2586 **	
$CD_{(0.05)}$ I ID	1.248 3.005		0.835 1.732		0.435 1.482		0.303 0.561	

level in the infected tissues. As a consequence of lipid peroxidation, the membrane loss its integrity and subsequently leads to oxidative burst in cell systems. This phenomenon can be initiated directly by ROS and indirectly through the involvement of lipoxygenase (Torres, 2010).

Ascorbate and glutathione content

Ascorbate and glutathione contents were analyzed to test whether the water soluble antioxidants are involved in the defense system against *A.sesami* during oxidative stress in the cultivar Thilarani of *S.orientale*. The contents of total AsA, DHA and the ratio of AsA/DHA were significantly influenced by pathogenicity. AsA and DHA increased remarkably in the cultivar Thilarani from 1st (71.20 and 10.3 µg/g tissue) to 9th day (96.40 and 11 µg/g tissue) after infection when compared to the control plants (Table 2). To analyze the regeneration rate of AsA, the ratio of AsA/DHA was calculated. Interestingly, AsA/DHA increased with to maximum at 9th day after infection followed by a drop.

The glutathione(GSH), glutathione disulphide contents (GSSG) and glutathione/glutathione disulphide ratios were increased from initial stages of pathogen treatment followed by a marginal decrease. Glutathione/glutathione disulphide ratio is an indicator of glutathione regeneration rate which decreased with pathogenicity. This may be due to the fact that glutathione di-sulphide increase was higher than that of glutathione (Table 2).

The activities of major enzymes in the ascorbate-glutathione cycle were also affected upon infection. APX, MDHAR and GR activities increased upto 9th day and then decreased gradually, but the activities were still higher than the control. MDHAR, the enzyme involved in regeneration of reduced ascorbate also increased proportionally with time intervals (Table 3). Lipid peroxidation in terms of TBARS and H_2O_2 level strongly corroborates with the antioxidant machinery in the infected sesame (Table 1). APX, MDHAR and GR activities from 1st to 9th day after infection were 16.44 to 27.9; 27.22 to 36.62; 10.38 to 18.32 U/mg proteins respectively. Post inoculation from 11th day onwards the activities showed decline.

This part of the study was aimed for a better

Table 3. Activity of Ascorbate peroxidase (APX), Monodehydro ascorbate reductase (MDHAR), and Glutathione reductase (GR) (U/mg protein) of control (C) and infected (I) Sesame variety Thilarani from 1-13 days after infection

Days	APX		MDHAR		GR	
	C	I	C	I	C	I
1	7.26	16.44	14.72	27.22	6.48	10.38
3	7.46	18.96	16.04	29.72	7.28	11.52
5	7.12	21.66	15.48	32.00	7.06	14.12
7	6.90	24.72	16.0	34.98	7.44	16.36
9	8.70	27.90	16.64	36.62	7.66	18.32
11	8.60	22.90	16.10	30.26	8.26	14.02
13	8.28	15.64	16.14	25.32	8.74	10.26
F ratio I ID	692.2286 ** 11.10505 **		1738.68 ** 25.42663 **		455.0494 ** 23.71727 **	
$CD_{(0.05)}$ I ID	1.001 3.051		0.829 1.505		0.649 1.294	

understanding of the regulation of the antioxidant defense system in *S.orientale* in response to *A.sesami* infection. Using lipid perox-idation as indicator, the tolerance limit has been identified. Increased TBARS contents in cultivar – Thilarani suggest the occurrence of oxidative damage. Further, in the cultivar Thilarani defense against pathogen and repairing systems are operating at a low pace suggesting its susceptible nature. A survey of the pathogen tolerance of crops from tropical habitats showed that pathogenicity caused pronounced impact in cultivar species (Jensen *et al*., 2008). Similarly, *Citrus* cultivars responded differently to the antioxidant enzymes and metabolites of the ascorbate glutathione pathway to oxidative stress caused by biotic stress (Peroni *et al.,* 2007). The present study suggests that the antioxidant defense system has evolved in plants to protect them from oxidative stress induced by pathogens. Induction of ROS with infection reflects the H_2O_2 generation in the plants, may be considered as signal cascades which in turn trigger the antioxidant system in the cells.

CONCLUSION

Phytopathogenic fungi release premeditated molecules during invasion that contribute to the establishment of the infection. These compounds account for the development of host cell wall degradation enzymes for favouring penetration and creating oxidative stress via reactive oxygen species (ROS). Activity profile of antioxidant enzymes substantiates the quantified data of ROS - H_2O_2 content. Ascorbate and glutathione contents corroborate with enzymes of ascorbate-glutathione cycle which in turn participate in recycling the reduced ascorbate to scavenge the H_2O_2.

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Cytokinin Benzyl Adenine Overrides the Inhibitory Effects of Gibberellins and Low Sucrose Concentrations During Microtuberisation of Potato

K.K. NISHA*[1] AND M G PURUSHOTHAMA[2]

[1]*Assistant Professor of Botany, All Saints' College, Thiruvananthapuram, Kerala, India.*
[2]*Director of Research (Biotechnology), Indus Seeds, 9/3, Pattanagere, Noojibai Compound, Srinivasapura Kengeri-Uttarahally main Road, Bangalore- 560 098*
**email: nishabot@gmail.com*

ABSTRACT

Potato tuberisation is a complex developmental process involving biochemical and physiological changes. Sucrose is a critical stimulus for *in vitro* tuber formation and high concentrations (6-8%) are required for microtuberisation. Gibberellins inhibit microtuber formation by promoting stolon elongation. Cytokinins have a promotory effect in microtuberisation. The effect of cytokinin benzyl adenine (BA) on microtuber formation was studied both under inductive conditions in medium containing 8% sucrose and under non-inductive conditions – in medium containing 1.5% sucrose and medium containing giberrellin, 5.8µM GA_3. Under inductive conditions, larger and increased numbers of tubers were obtained in medium containing high levels of BA. Under non-inductive conditions, higher concentrations of BA could over ride the inhibitory effect of giberellins as well as complemented the lack of induction at low sucrose concentrations. In both cases small microtubers were produced.

Keywords *Microtuberisation, potato, cytokinin, gibberellins, benzyl adenine, sucrose*

Microtubers provide a uniform and convenient experimental model for tuberisation studies (Banfalvi et al, 1997; Xu et al, 1998). *In vitro* tubers resemble field grown ones with respect to structure, proteins and starch composition (Paiva et al, 1982). This approach has been successfully used for the elucidation of biochemical aspects involved in tuber formation (Ulloa et al, 1997) and also as a potential screening method for heat stress as well as salinity stress tolerance assessment on nodal explants of potato cultivars (Nowak and Colborne, 1989; Silva et al, 2001). Now it is becoming established as an effective means for rapidly multiplying new or existing cultivars in disease-free conditions (Piao et al, 2003).

Microtuber formation is sensitive to a wide range of controllable factors comprising the hormonal and gaseous environment of cultures together with the light and temperature conditions. Low temperature, low nitrogen levels, ratio of photoperiod, nutrient compositions and high sucrose concentration influenced microtuberisation (Stallknecht and Farnsworth, 1979; Forti et al, 1991; Akita and Ohta, 1998; Coleman et al., 2001; Tugrul & Samanci, 2001).

The most critical stimulus for tuber formation *in vitro* is attributed to sucrose. Forti et al (1991) have reported that an increase in sucrose concentration from 2% to 8%, regardless of growth regulators, enhanced microtuber formation. Studies on two potato varieties, 'Hunde' and 'Ararsa', indicated that microtuber induction was highly dependent on sucrose and genotype interaction. At 40 g/L sucrose, both varieties did not produce microtubers. However, when 60 g/L sucrose was added to growth media, 'Hunde' produced microtubers in 36 days, while tuberisation initiated in 'Ararsa' only after 43 days (Fufa and Diro, 2014).

Gibberellins (GA) inhibit tuberisation and appear to play a role in the photoperiodic control of tuberisation by preventing tuberisation in long days. The addition of GA consistently inhibited tuber formation and promoted the growth of thin leafy shoots under *in vitro* conditions (Lovell and Booth, 1967). High levels of GA in the stolon tip favoured elongation of stolon meristems, whereas decreasing levels were required for the initiation of tuberisation (Xu et al, 1998). A decrease in GA level was observed when the stolon tips started to swell in cultured single-node cuttings grown in a high-sucrose tuber-inducing medium (Xu et al, 1998). Furthermore, tuberisation was improved by the application of inhibitors of GA synthesis such as paclobutrazol or ancymidol (Menzel, 1980; Hussey and Stacey, 1984; Jackson and Prat, 1996).

Cytokinins are necessary at very early stages of potato tuber formation, probably because of their role in stimulating cell division and radial cell growth, and later in stimulating starch synthase activity (Smith and Palmer, 1970). Cytokinins are considered to be tuber-inducing factors due to the promoting effect of exogenous cytokinins in microtuber formation (Kumar and Wareing, 1974; Hussey and Stacey, 1984) and the presence of high levels of endogenous cytokinins in induced tissue (Mauk and Langille, 1978; Obata-Sasamoto and Suzuki, 1979). Cytokinins in the medium promoted stolon formation and tuberisation under *in vitro* conditions (Palmer and Smith, 1969; Mauk and

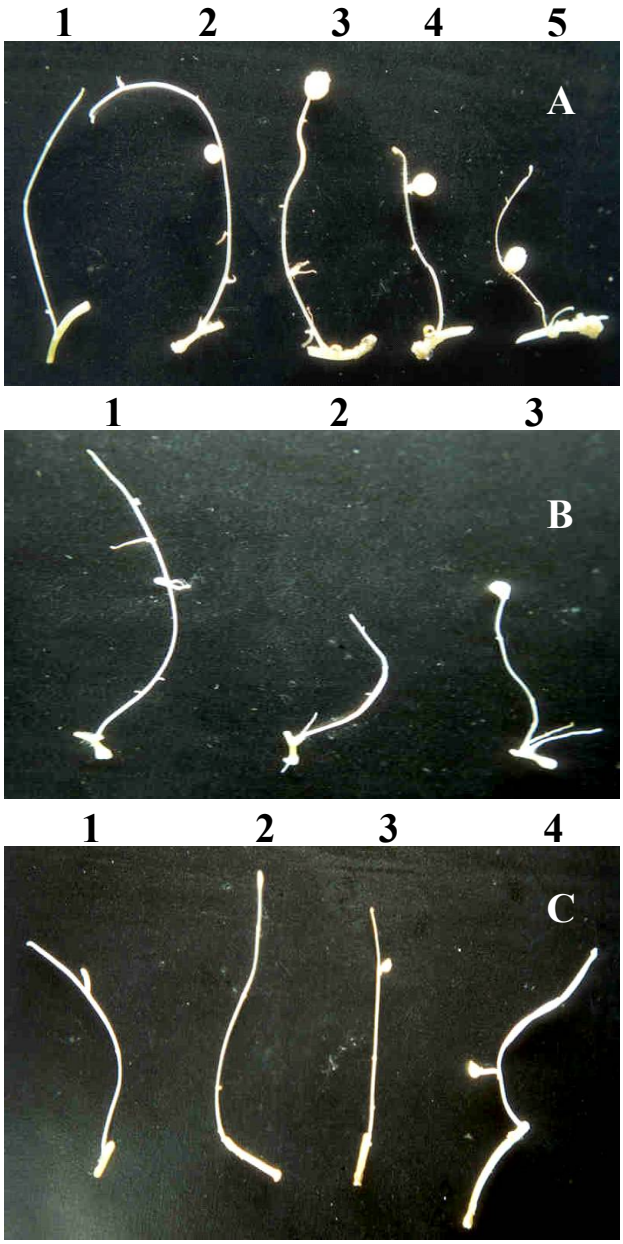

Fig.1 Microtuber induction studies under *in vitro* conditions using single nodes in various media: **A.** MS medium supplemented with 8% sucrose and varying amounts of BA (1- 0µM; 2- 8.9µM; 3- 17.8µM; 4- 26.6µM; 5- 35.5µM) **B.** MS medium supplemented with 8% sucrose, GA3 (5.8µM) and varying amounts of BA (1 - 8.9µM; 2 - 17.8µM; 3 - 26.6µM) **C.** MS medium supplemented with 1.5% sucrose and varying amounts of BA (1- 0µM; 2- 8.9µM; 3- 17.8µM; 4- 26.6µM)

Langille, 1978). MS medium supplemented with 6% sucrose and 4 mg/L Kinetin was found to promote microtuber formation and increased the average weight of microtubers produced (Hoque, 2010). However, Hussey and Stacey (1984) found that the addition of GA to nodes cultured on medium containing cytokinin overrode the tuber promoting effects of cytokinin.

In the present study the effect of cytokinin benzyl adenine (BA) on microtuberisation was studied under non-inductive conditions of low sucrose concentration (1.5%) and presence of GA_3 in the medium.

MATERIALS AND METHODS

Plant material

Tubers of *Solanum tuberosum* cv. Kufri Jyoti was obtained from the Central Potato Research Station, Ooty, Tamilnadu. *In vitro* cultures were established by *in vitro* germination of tubers in $1/4^{th}$ strength of Murashige-Skoog (MS) basal medium (Murashige and Skoog, 1962) without sucrose. The germinated plantlets were transferred into MS basal medium supplemented with sucrose (30g/L).

Microtuber induction studies under inductive conditions

Single nodes were inoculated into MS basal medium supplemented with 8% sucrose and varying concentrations of BA (0, 8.9, 17.8, 26.6 and 35.5µM). Cultures were grown in complete darkness at 20 ± 1^0C in a growth chamber. After 20 days growth of stolons, tuberisation pattern was recorded.

Microtuber induction studies under non-inductive conditions

Single nodes were inoculated into MS basal medium supplemented with 8% sucrose, 5.8µM GA_3 and varying concentrations of BA (8.9, 17.8 and 26.6µM). To study the effect of BA on low sucrose concentrations, single nodes were inoculated into MS basal medium supplemented with 1.5% sucrose and varying concentrations of BA (0, 8.9, 17.8 and 26.6µM). Cultures were grown in complete darkness at 20 ± 1^0C in a growth chamber. After 20 days growth of stolons, tuberisation pattern was recorded.

RESULTS AND DISCUSSION

Microtuber induction under inductive conditions

After four weeks of incubation, only elongated stolons were formed in explants in medium without BA. Very small microtubers were observed on the elongated stolons from explants in medium containing 8.9µM BA. As the concentration of BA increased, larger microtubers were produced in all the explants

(Fig. 1A). Banfalvi et al (1997) have reported that the addition of BA to the medium with high sucrose further promoted *in vitro* tuber formation. Our results also confirmed the tuber inducing ability of cytokinins under *in vitro* conditions.

Microtuber induction in the presence of GA_3

The inductive ability of cytokinins on tuberisation in the presence of negative regulator GA was studied by adding 5.8µM GA_3 to the media. Different concentrations of BA (8.9, 17.8 and 26.6µM) were added to the medium and *in vitro* tuber formation was studied. Lower concentrations of BA could not overcome the inhibitory effects of GA_3 in the medium and all the explants produced thin elongated stolons. But 26.6µM BA produced very small microtubers on elongated stolons (Fig. 1B).

GA_3 has been reported to prevent *in vitro* tuber formation even when other factors (high sucrose, low N_2, BA, short days) were optimal for tuberisation (Vreugdenhil et al, 1998). Lovell and Booth (1967) reported that the addition of GA_3 (2.9µM and 0.29µM) to the medium led to the growth of thin elongated leafy shoots with no tuberisation evident even after 3 months. The addition of 2.9µM GA_3 to nodes cultured on medium containing cytokinins (8.9µM BA) led to the reversal of the inductive effects of BA and upright shoots were formed, completely inhibiting tuberisation (Hussey and Stacey, 1984). We also obtained similar results with BA at 8.9 µM and 17.8µM. But, when the concentration of BA was increased to 26.6µM, it led to initiation of microtubers (Fig. 1B). Thus, cytokinins at higher concentration could overcome the inhibitory effects of GA_3, thereby establishing their inductive role in tuber initiation. The results also suggest that the balance between the factors which either favours or inhibits tuberisation appears to be crucial for tuberisation under *in vitro* conditions.

Microtuber induction under low sucrose concentrations

The inductive potential of cytokinins under very low sucrose concentrations (1.5%) was studied by adding the cytokinin BA at varying levels to the medium. BA at concentrations 0, 8.9, 17.8 and 26.6µM were used and tuberisation response was studied. No tuberisation was observed in explants on medium without BA and with BA 8.9µM. However, tuber initiation started in explants at 17.8µM BA after 4 weeks, while the explants on medium with 26.6µM BA formed small tubers. Addition of BA at high concentrations favoured tuberisation *in vitro* even under low sucrose concentrations (Fig. 1C).

Earlier reports have used sucrose concentrations from 2-8% for *in vitro* tuber induction (Hussey and Stacey, 1984; Machackova et al, 1997). Xu et al (1998) did not observe any tuberisation when the medium contained less than 2% sucrose. But, we obtained microtuber induction in 1.5% sucrose when high concentrations (17.8 and 26.6µM) of BA was added (Fig. 1C). Thus BA was effective in inducing tuberisation *in vitro* even under non-inductive conditions of low sucrose concentration.

Our results strongly support the positive role for cytokinins in tuber initiation under *in vitro* conditions. The positive effect of cytokinins in tuber initiation has already been reported (Palmer and Smith, 1969; Mauk and Langille, 1978), but our experiments proved that high concentrations of cytokinins could override the inhibitory effects of GA_3 and also could complement the lack of induction at low sucrose concentrations.

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The Effect of Aqueous Fruit Extract of *N. cadamba* on Nephrolithiasis Induced Oxidative Stress

P.V. PRATHIBHAKUMARI AND G. PRASAD

Department of Zoology, University of Kerala, Karyavattom, Kerala
email : prathibio@gmail.com

ABSTRACT

The oxidative stress (OS) is developed in an organism by the imbalance between free radical production and antioxidant defence. The over production of reactive oxygen species (ROS) in oxidative stress enhances the structural and functional modifications of biomolecules. Free radical production causes damage to membrane lipids, proteins and nucleic acids, which leads to the modification of structural and functional properties of cellular constituents (Kagon *et al*., 1990). Antioxidants are the micronutrients which have the capacity to neutralize free radicals and protect the body against the damaging effect of free radicals (Mishra *et al*., 2006). Antioxidant activity in human body is carried out by the action of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, glutathione reductase (GST) and non enzymatic antioxidants include hydrophilic and lipophilic antioxidants. Ascorbic acid, uric acid, bilirubin, albumin and flavanoids are the hydrophilic non enzymatic antioxidants. Alpha tocopherol, ubiquinol and carotenoids are classified as lipophilic non enzymatic antioxidants (Galli *et al*., 1999). The antioxidants react with ROS and produce non reactive weak radical type compounds. Mitochondria and mitochondrial cytochrome oxidase enzyme (cyto P450) are mainly account for the production of 90% of oxidant production.

Key words *Oxidative stress, reactive oxygen species, antioxidants*

Kidney stones are produced by the deposition of the mineral salts within the kidney, ureters and urinary bladder. Patients with kidney diseases (CKD) are found to have significant oxidative stress due to their declined renal functions (Himmelfarb *et al*., 2002). *In vivo* measurement of ROS is difficult because of its low concentration, short half life and high reactivity. Thus measurement of antioxidant levels and oxidative byproducts of ROS is considered as biomarkers of oxidative stress.

OBJECTIVE

To evaluate the potential of aqueous fruit extract of *N. cadamba* on nephrolithiasis induced oxidative stress in wistar rats.

MATERIALS AND METHODS

Collection and preparation of fruit extract

The fruits of *N. cadamba* were collected from the botanical garden, University Campus, Kariavattom ($8^0$37'36N, 76^o50'14E), Thiruvananthapuram. The plant material was authenticated by the Department of Botany, University of Kerala, Kariavattom and the voucher specimen was kept in the same department for further identification (voucher number: KUBH 5811). The aqueous fruit extract was prepared by the soxhlet extraction method

Experimental design

Healthy adult male albino rats of wistar strains (150- 250g) were used for the present study. The ethical clearance has been obtained from the institutional animal ethical committee prior to the experiment (number: IAEC -KU-23/2011-12-ZOOL -GP (3)). Calcium oxalate nephrolithiasis induced in rats by supplementing 0.75% ethylene glycol (EG) and 2% ammonium chloride (AC) The healthy albino rats were weighed and randomly divided into control and experimental groups.

Study protocol for AFNC

Group I: Normal control group – Received standard rat feed and water at *ad libitum*

Group II: Antilithiatic control group – Received standard anti urolithiatic drug, cystone (750mg/kg b.wt.)

Group III: Lithiatic control group – Received EG (0.75%) and AC in drinking water till 28^{th} day.

Post treatment regimes (PR)

Group IV: received EG+AC *ad libitum* from 1-28 days and AFNC to next 28 days (400mg/kg b. wt.).

Group V: received EG+AC *ad libitum* from 1-28 days and AFNC to next 28 days (200mg/kg b. wt.).

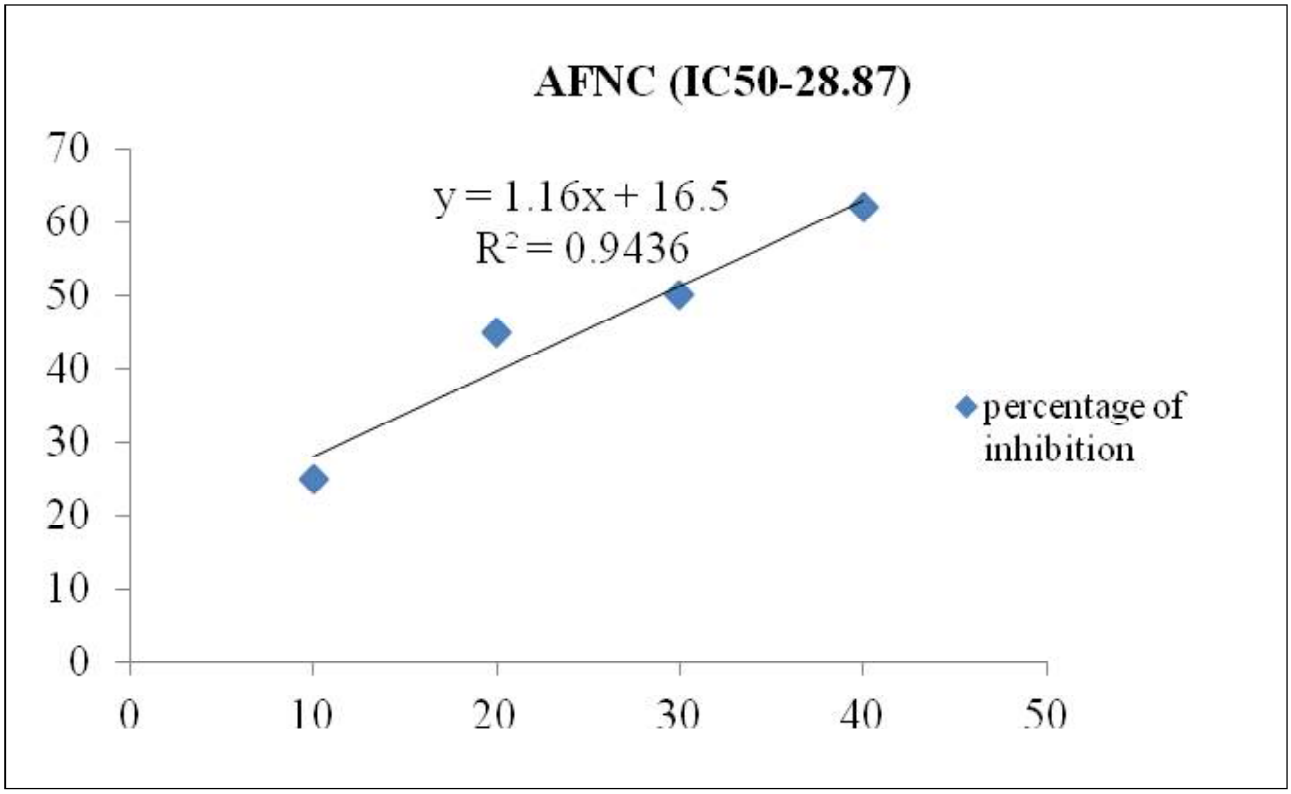

Fig 1. DPPH scavenging activity of AFNC

Assessment of oxidative stress

In the present study, lithiasis induced oxidative stress was assessed by measuring the antioxidant levels in serum was analyzed for serum marker enzymes like serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT). Kidney was used for the estimation of antioxidant activity by estimating lipid peroxidative markers (TBARs, CD and hydroperoxides), non enzymatic antioxidant (GSH) and antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GST)).

DPPH photometric radical scavenging assay

DPPH (2, 2, diphenyl 1-picryl hydrazyl) assay was based on the measurement of loss of DPPH colour after reaction with test compounds (Espin *et al*., 2000). The decrease in absorbance of the reaction mixture was monitored at 517nm in UV-visible spectrophotometer and calculated the percentage radical scavenging capacity (RSC).

Statistical analysis

The results were expressed as mean ± SEM and analyzed using one way ANOVA followed by Tukey test. Differences between data sets were considered significant at $P<0.05$.

RESULTS

In the present study, antioxidant activity of methanol and aqueous fruit extracts were investigated by DPPH scavenging assay and reducing power of the extract. The DPPH radical scavenging activity of the fruit extract is based on the ability of DPPH to decolourise solution in the presence of antioxidants. The aqueous extract of *N. cadamba* exhibited a significant inhibition of DPPH radicals. Aqueous extract of fruit has profound antioxidant activity than methanol extract (Fig 1).

The significant ($p<0.01$) increase in TBARS value (40.48±0.72) was observed in lithiatic control rats when compared with normal control rats and post treatment regimes showed significant ($p<0.01$) decrease in TBARS value when compared with group III. Group IV of post treatment regimes showed TBARS value of 17.66±0.54, which is lower than the value of group V. The observed value of conjugated diene (CD) in group I was 25.26±0.39 and was significantly higher in group III administered with ethylene glycol (198.41±0.69). In curative regimes group IV of post treatment also reduced the value of CD (79.17±0.69)

In the case of hydroperoxides (HP), normal

Table 1. Effect of AFNC on antioxidant activity in urolithiatic rats

Treatment groups	Thiobaraturic acid reactive substances (TBARs)	Conjugated diene (CD)	Hydroperoxides) (HP)	Reduced glutathione (GSH)
Group I	12.09±0.35	25.26±0.39	7.59±0.27	69.23±0.39
Group II	15.84±0.29a**c*	79.80±0.95c**	15.76±0.50	61.48±0.53c**
Group III	40.48±0.72a**	198.41±0.69a**	16.60±0.63a**	40.44±0.54a**
Group IV	17.66±0.54c**	79.17±0.54c**	10.25±0.69b*c**	61.51±0.56c**
Group V	36.14±0.20c**	138.88±0.45c**	15.31±0.53	65.01±0.57c**

Each value is the mean ± SE for 4 animals. a-indicates significant difference with normal control groups, b- indicates significant difference with antilithiatic group, c-indicates significant difference with lithiatic groups, *- P<0.05, **-P<0.01

control rats were reported with HP value of 7.59±0.27, which was significantly increased by the supplementation of stone inducing agents and the value was 16.60±0.63. AFNC reduced the HP value in all the groups. When comparing between groups of post treatment regimes, low HP value of 10.25±0.69 was recorded from group IV treated with 400mg/kg of fruit extract. Reduced glutathione value of 40.44±0.54 was observed in group III rats. AFNC administration enhanced the reduced glutathione value towards normal. The reported GSH value of from group IV and group V was found to be higher than group III (lithiatic control rats).

The elevated kidney SGOT value was observed from lithiatic control rats and the value was highly significant (P<0.01) with group I and group II. The post treatment regimes, group IV and group V exhibited decreased SGOT levels in kidney but the values were not statistically significant. Similar to SGOT, the elevated SGPT value (504.86±43.57) was reported in lithiatic control rats (group III). The increased SGPT level in lithiatic control rats lowered significantly (P<0.01) in 400mg/kg extract administered rats in group IV.

The animals in group III (lithiatic control) exhibited a remarkably significant (P<0.05) decrease in SOD activity when compared with group I. A significant (P<0.05) increase in superoxide dismutase was observed in extract treated group (group VI) when compared with lithiatic control group. The normal level of catalase activity in group 1 (7.18 ±0.36) was decreased in group III (3.19 ±0.45) and the values were highly significant (P<0.01). In the post treatment regime of rats, group V exhibited an elevated catalase level than in group III. The increased GST activity may be due to the potency of the extract in lowering CaOx induced oxidative stress.

DISCUSSION

DPPH is stable nitrogen centered free radical and accept an electron or hydrogen radical to become stable diamagnetic molecule. When DPPH radical react with reducing agents, corresponding hydrazine will form and thus the solution loses colour (Rajesh and Natvar, 2011). DPPH radical is commonly used for fast evaluation of antioxidant property of a compound. The earlier studies showed that the antioxidant activity of herbal extract reduced the incidence of calcium oxalate calculi formation.

The kidney stone related oxidative stress was studied in wistar albino rats by the administration of ethylene glycol (EG) and ammonium chloride (AC) for 28 days. Evidences in previous studies indicated that 28 days period of ethylene glycol administration to young male albino rats developed calcium oxalate renal calculi and oxidative stress (Selvam *et al*., 2001; Atmani *et al*., 2003). Similar results were obtained in the present study when the rats were treated with EG and AC.

Under hyperoxaluric condition, oxalate has been reported to induce lipid peroxidation and to cause renal damage by reacting with polyunsaturated fatty acids in the cell membrane (Gandhi *et al*., 2013). The enzyme, alcohol dehydrogenase convert EG into oxalic acid and hipuric acid (Khan and Hackett, 1985; Bruijn *et al*., 1994) and are the main cause of formation of renal stones. AC induces induces urinary acidifications, which in turn favour the retention and aggregation of calcium oxalate (CaOx) crystals in kidney (Touhami *et al*., 2007). The increased lipid peroxidation (LPO) and decreased antioxidant level have been reported in calculogenic rats (Karadi *et al*., 2006) and similar results were obtained in the present study also. The administration of ethylene glycol increased the oxidant level and decreased the

Table 2. Effect of AFNC on antioxidant enzyme activity in urolithiatic rats

Treatment groups	SOD activity	CAT activity	GST activity	Kidney protein
Group I	1.8±0.10	7.18±0.36	0.04±0.001	5±0.39
Group II	2.5±0.14	4.3±0.10	0.03±0.003	5.3±0.03
Group III	0.56±0.16a**	3.18±0.45a**	0.01±0.003	4.73±0.29
Group IV	3.47±0.09c**	1.78±0.332	0.87±0.028a**c**	4.66±0.328
Group V	1.55±0.16c**	4.44±0.231	0.16±0.032c**	5.03±0.09

Each value is the mean ± SE for 4 animals. a-indicates significant difference with normal control groups, b- indicates significant difference with antilithiatic group, c-indicates significant difference with lithiatic groups, *- P<0.05, **-P<0.01

cellular antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GST) (Purnima *et al*., 2006). The increased value of TBARs, CD and HP and decreased GSH in group III rats might be due to the activation of the lipid peroxidation system and decreased antioxidant defence mechanism (Malini *et al*., 2011). The decreased values ($p<0.01$) of lipid peroxidative markers in AFNC administered rats are because of the inactivation of LPO system in the curative and preventive regimes.

Free radicals damage cells and provide a favourable environment for the development of crystals, while antioxidants prevent its deposition in renal tissues and could be ascribed to the decreased GSH value in hyperoxaluric animals (group III). However, the hyperoxaluric rats when subjected to AFNC, non enzymatic antioxidant concentration in their kidney was increased significantly ($p<0.01$) and exhibited a tendency to reach the concentration as in control animals.

Oxidative damage is related to crystalluria and renal tissue crystal depositions. The increase in tissue membrane damage increased with progressive crystal deposition by the production of TBARs. These observations strengthen the view that oxalate and CaOx exerts more effect on the production of lipid peroxides (Selvam, 2002) and enhance the accumulation of H_2O_2, which is highly toxic to renal cells. Antioxidant enzymes are involved in the elimination of the peroxides. Enhanced peroxidation increases the oxalate binding activity and promotes crystal nucleation and aggregation property of stone matrix and peroxidation can be treated as a causative factor for the initial stage of stone formation (Vasavidevi *et al*., 2006).

Exposure of calcium oxalate increased the production of reactive oxygen species (ROS) and reactive nitrogen species. ROS exert their effects through the reversible oxidation of active sites in transcription factors such as nuclear factor-kappa B (NF-kB) and activator protein 1 leading to gene expression and cell growth. ROS can also cause indirect induction of transcription factors by activating signal transduction pathways (Amit and Priyadarsini, 2011). The increased concentration of free oxalate ions and insoluble calcium oxalate crystals directly cause oxidative stress by combining with inflammatory events (Sandhya *et al*., 2010). This could be the reason of increased oxidative stress in group III. Excess production of ROS leads to the reduction of cellular antioxidant enzymes (SOD, peroxidase, GSH, CAT and GST) and radical scavengers, which develop the oxidative stress (Khan, 2005). The decreased antioxidant activity is because of excess production of ROS in EG received group. Oxidative stress enhances the renal epithelial injury and inflammation in renal cells. The oxidative stress modified the Tamm Horsfall glycoprotein, which was detected in stone patients (Pragasam *et al*., 2006) and also resulted in the regulation of key transcription factors, cell proliferation and necrosis (Hensley *et al*., 2000). Mitochondrial malfunction inhibits the membrane Na-K+ ATPase activity and abnormalities of sodium, potassium and calcium. The free radicals produce cytotoxic metabolite peroxynitrite, which is capable of causing lipid peroxidation and protein modification (Sandhya *et al*., 2010).

The polyunsaturated fatty acid content of kidney is mainly involved in ROS attack. Both hydrogen peroxide and superoxide anions promote the production of arachidonic acid by the activation of cytosolic phospholipase A2. It could be the mechanism behind eliciting renal epithelial cell injury. The oxalate elicits the pathogenesis of nephrolithiasis through a lipid signaling pathway (Kohjimoto *et al*., 1999). The reduced level of SOD activity was observed from the

Table 3. Effect of AFNC on SGOT and SGPT on urolithiatic rats

Treatment groups	SGOT	SGPT
Group I	54.72±15.8	129.93±8.05
Group II	113.94±5.91	152.10±3.06
Group III	346.83±24.2a**b**	504.86±43.57a**
Group IV	242.78±28.006	155.62±3.89c**
Group V	283.06±27.06	380.83±35.60c**

Each value is the mean ± SE for 4 animals. a-indicates significant difference with normal control groups, b- indicates significant difference with antilithiatic group, c-indicates significant difference with lithiatic groups, *- P<0.05, **-P<0.01

lithiatic control rats. The decreased SOD level attributed to the opening of the mitochondrial PT pores and the disruption of the osmotic equilibrium of the matrix (Fridovich, 1975). Another way of decreased SOD activity in group III may be because of the rupture of outer mitochondrial membrane which leads to the leakage of SOD into the cytoplasam. SOD is involved in the cytoprotection of cells against free radical damage by catalytically scavenges the superoxide radicals (Fridovich, 1975). Cells with high oxidative metabolism are distributed with SOD which protect against the deleterious effect of superoxide anions (Table 2).

The increased level of CAT activity and GST activity in the AFNC treated animals substantiate the decreased oxidative stress (OS) and free radicals. Relationship between increased lipid peroxidation and decreased antioxidant level has been reported in rat kidneys (Sumathi *et al*., 1993; Saravanan *et al*., 1995). The present study also reports the enhanced level of antioxidant enzyme activity and decreased lipid peroxidation in AFNC received groups.

The antioxidant enzymes such as catalase and reduced glutathione are involved in the elimination of hydrogen peroxide. Here, lower levels of reduced glutathione have been observed in lithiatic animals and similar results were observed by Muthukumar and Selvam (1998). Lower LPO could be the reason of decreased crystal deposition in curative regimes. The increased SGOT and SGPT levels in serum of ethylene glycol administered animals have observed in the present study

This is probably because of the damaged structural integrity of the renal and hepatic cells, which releases the enzymes that are located in the cytoplasm into the circulation. The formed renal stone exerts pressure on renal pelvis and ultimately damage the renal tubular cells (Arunachalam and Chinnaraju, 2011). The aqueous fruit extract restores and maintains the levels of serum SGOT and SGPT near normal which indicate the renoprotective action and antinephrolithiatic activity of AFNC.

The present study proved that the fruit extract can prevent the free radical production in CaOx induced lithiatic rats, thus protecting the renal cells from oxidative injury by renal stones. The study also reported that the AFNC increased the activity of antioxidant enzymes either equal or higher than the normal control animals and able to reduce the oxidative stress in kidneys. This enlightens the antioxidant effect of fruit extract in lowering CaOx induced lithiasis and free radical production. The imbalance between the oxidant and the antioxidant enzyme could be the reason for the enhanced crystal deposition and renal tissue damage and the study clearly demonstrates that AFNC can cure nephrolithiasis induced oxidative stress in stone induced wistar albino rats.

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Angiosperm Diversity with Elevation Gradients in a Semi-Evergreen Forest Tract of Dhoni Hills of Southern Western Ghats - A Case Study

A. REKHA VASUDEVAN[1,2] AND MAYA C. NAIR[2]

[1]*Department of Botany, Mercy College (University of Calicut), Palakkad, Kerala, India – 678 006*
[2]*Post Graduate and Research Department of Botany, Govt. Victoria College (University of Calicut), Palakkad, Kerala, India - 678 009*
email : vasudevanrekha94@gmail.com

ABSTRACT

Exploration and documentation of angiosperm diversity in three hill tracts of semi-evergreen forest of Dhoni hills along an elevation gradient revealed the presence of 403 species belonging to 79 families, of which 64 are dicotyledonous and 15 are monocotyledonous families. As Dhoni hills lie near the Palghat pass, one of the widest and low elevation mountain passes in the whole of Western Ghats, it has tremendous influence on the macro and microclimatic features of the region. *Rubiaceae* and *Acanthaceae* are the most abundant families in the predominantly semi-evergreen vegetation, but the generic and species composition showed variation at different elevations. Higher rate of inter-specific diversity is recorded for genera like *Strobilanthes* with variable species composition at different elevation gradients. Diversity of species and endemism peaks at the mid elevation. Plant associations of *Utricularia -Eriocaulon –Smithia* is restricted to the upper zone while the lower zones show the *Tectona – Dillenia- Lagerstroemia lanceolata- Terminalia paniculata* association. Insectivorous plant *Utricularia albo-caerulea*, saprophytic orchid *Epipogeum roseum* are indications of the adaptive diversity of the vegetation and phytogeographically vegetation is predominantly of Indo- pacific.

Key words *Palghat gap; Dhoni hills; Angiosperm diversity; Elevation gradient.*

Palghat gap of southern Western Ghats has remarkable influence on climate and vegetation of the state of Kerala and is distinguished from other passes in the Western Ghats viz. Goa and Shencottah by the relatively low elevation and extreme width (Soman *et al*., 1990; Santosh *et al*., 1992). There have been very few studies examining the impact of these gaps on the distribution or population genetic structure (Deshpande *et al*. 2001, Vidya *et al*. 2005) of different species in the Western Ghats. Palakkad Hills run along the northern lip of the Palghat Gap in the southern Western Ghats and the windward side of the ranges is home to luxuriant evergreen forests, while the leeward side of the ranges is much drier and is vegetationally much less diverse. The edges of the Palghat gap face the climatic conditions of both the windward and leeward regions and are therefore very different from the rest of the area on the Western Ghats. Dhoni hills of the Southern Western Ghats lying between 10° 45' and 10° 55' North latitude and 76° 10' and 76° 50' East longitude fall within the Chenath Nair Reserved Forest, consist of four parallel, steep, high crescent shaped ridges running from the northwest to south and southeast in the Palamala-Dhoni-Sappal Hills of the Palakkad Hills.

The floristic diversity of Palakkad district was documented by Subramanian *et al.* (1984), Manilal (1988) and Vajravelu (1990). Subramanian *et al.* (1984) reported 72 species belonging to 50 families from the Dhoni hills and Vajravelu (1990) has listed 1355 plants from the entire district of Palakkad and 74 of these were cited from Dhoni Reserve forest. Recent additions of new species of *Chlorophytum palghatense* (Prabhukumar *et al.* 2014), *Zingiber sabuanum* (Prabhukumar *et al,* 2016a), *Justicia gambleana (*Prabhukumar *et al.* 2016b) from Dhoni hills and rediscovery of *Impatiens concinna* Hook.f (Prabhukumar *et al*. 2015) from the Palamala hills of Dhoni forests are indications that the floristic diversity of Dhoni hills is yet to be completely revealed and thorough exploration is necessary to evaluate the existing diversity in the area. This study attempts to understand the diversity of angiosperm vegetation of different elevation zones of Kallipara, Mullamala and Neelipara peaks of Dhoni hills.

MATERIALS AND METHODS

The sample plots at differential elevation gradient for the present study are located between 10°86'32" - 10°89'02" N latitudes and 76°61'4" – 76°62'70" E longitudes at Kallipara, Mullamala and Neelipara peaks of Dhoni Hills. The documentation of angiosperm diversity between the peaks was made at the lower zone with elevation under 500ft, the middle zone with elevation 500 – 1000ft and the upper zone above 1000ft elevation. All species occurring in the study area were identified with the help of regional floras, including Flora of Palghat (Vajravelu, 1990), Flora of Presidency of Madras (Gamble and Fischer, 1915-1936) along

Table 1. Distribution of common and endemic taxa in different zones

Species	Distribution			Endemic taxa		
	I	II	III	I	II	III
Aerva paniculata	+	+				
Allmania nodiflora	+	+	+			
Argostemma courtallense		+	+			
Atalantia monophylla	+	+				
Baccaurea courtallense	+	+		+	+	
Bosenbergia pulcherrima	+	+	+	+	+	
Cipadessa baccifera	+	+				
Colebrookea oppositifolia	+	+		+	+	
Curculigo orchioides	+	+				
Dioscorea oppositifolia	+	+				
Glycosmis macrocarpa	+	+		+	+	
Gomphandra tetrandra	+	+				
Humboldtia unijuga var.unijuga	+	+	+			
Impatiens minor	+	+		+	+	+
Kunstleria keralensis	+	+		+	+	
Mallotus philippensis	+	+	+			
Nothapodytes nimmoniana	+	+				
Pothos scandens	+	+	+			
Pupalia artopurpurea	+	+	+			
Putranjiva roxburghiana	+	+	+			
Rhynchoglossum scabrum		+	+			
Syzygium munronii	+	+	+			
Terminalia paniculata	+	+		+		+
Thunbergia mysorensis	+	+	+	+	+	
Utricularia albo-cerulea				+		+
Actinodaphne lawsonii				+		
Aglaia barberi					+	
Amomum ghaticum				+	+	
Anaphylum wightii				+	+	
Arisaema nilamburense					+	
Arundinella ciliata						+
Callicarpa tomentosa				+		
Canscora perfoliata						+
Ceropegia thwaitesii						+
Chionanthus mala-elangi ssp. mala-elangi				+		
Cinnamomum palghatensis				+		
Crotalaria heyneana				+		

Species	Distribution			Endemic taxa		
	I	II	III	I	II	III
Croton malabaricus				+		
Curcuma ecalcarata				+		
Dendrobium ovatum						+
Diospyros candolleana				+		
Dysoxylum malabaricum				+		
Geophila repens				+		
Gymnostachyum canescens				+		+
Holigarna arnottiana				+		
Hopea parviflora				+		
Hymenodictyon obovatum				+		
Impatiens dasysperma				+		
Impatiens sasidharani						+
Ixora brachiata				+		
Ixora elongata				+	+	
Justicia simplex						+
Knema attenuata				+		
Litsea oleoides				+		
Luvunga eleuterandra					+	+
Memecylon heyneanum				+		
Memecylon deccanense						+
Momordica silentvalyensis						+
Munronia pinnata						+
Neonauclea purpurea				+		
Nothopegia travancorica					+	
Oldenlandia herbacea						+
Olea dioica					+	
Polyalthia fragrans				+		
Pterocarpus marsupium				+		
Pterospermum rubiginosum				+		
Rungia sisparense					+	
Saprosma glomeratum					+	
Sonerila rheedei					+	
Soneritia sahyadrica					+	
Strobilanthes barbatus						+
Strobilanthes pulneyensis					+	
Strobilanthes rubicundus						+
Syzygium laetum				+		
Thottea sivarajanii				+		
Utricularia lazulina				+		
Xanthophyllum arnottianum				+		
Zingiber anamalayanum						+

+ present; Zone I- 0-500ft, II- 500-1000ft,III >1000ft

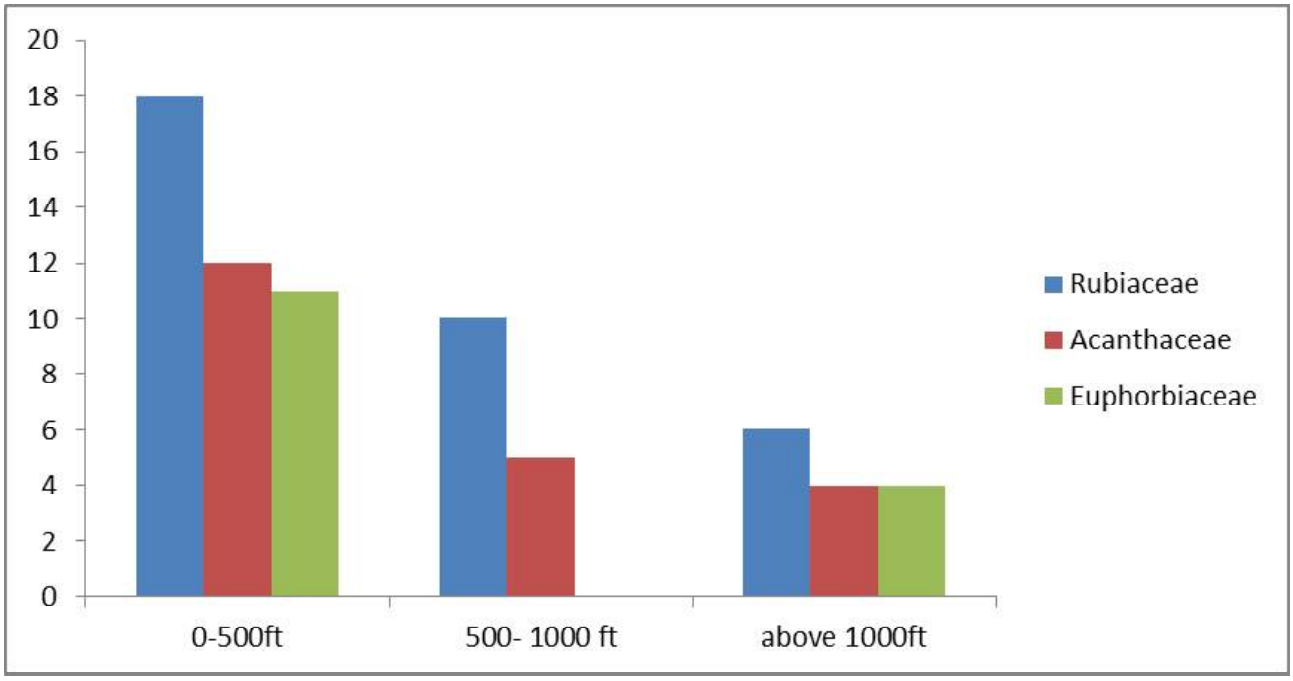

Fig. 1. Abundance of families in the three elevation zones

with reference to local herbaria as MH and CALI. Collected taxa were classified on the basis of APG IV (Chase *et al.* 2016) and phytogeographical affinities were also worked out (Cox, 2001). The database sources, International Plant Name Index (http://www.ipni.org), the Plant list (www.plantlist.org) and Kew digital herbarium were referred to update the nomenclature of plant species and to confirm identity of plant specimens. The endemic status of the floral elements was ascertained based on checklists (Sasidharan, 2010, Ahmedullah and Nayar, 1987) and status of the species was assigned as per IUCN (2017). The specimens were identified using standard floras and herbarium was prepared.

RESULTS

The angiosperm vegetation in this case study with the three zones in the study area, viz. Kallippara, Mullamala and Neelippara consists of 403 species belonging to 79 families of which 64 are dicotyledonous and 15 are monocotyledonous families. The lower zone reaching upto 500ft elevation has 241 species belonging to 73 families, of which 62 are dicotyledonous and11 are monocotyledonous. The middle region between 500 ft - 1000ft has 80 species belonging to 39 families, of which 32 are dicotyledonous and 7 are monocotyledonous. The upper zone from 1000ft and above has 82 species belonging to 39 families, of which 32 are dicotyledonous and 7 are monocotyledonous. Diversity in terms of number of families relative to the total number of species is therefore highest in the middle zone and the least in the lower zone.

The most abundant family in the lower and middle zones is *Rubiaceae* followed by *Acanthaceae*. In the lower and middle zones, *Rubiaceae* is represented by 18 and 10 species respectively, followed by *Acanthaceae* (12sp. and 5 sp.). In the lower elevation, *Euphorbiaceae* has 11sp. and in the upper zone *Rubiaceae* with 6 species is the most abundant, followed by *Euphorbiaceae* and *Acanthaceae* with 4 species each (Fig.1)

Higher rate of inter-specific diversity is recorded for few genera like *Strobilanthes*. The genus is present in all the three altitudinal zones but the species constitution showed differential distribution pattern with *S. heyneana* and *S. zeylanica* in the lower zone, while *S. anamalaica* and *S. pulneyensis* in the middle zone and *S.barbatus* and *S. rubicundus* in the upper zone.

Endangered taxon *Hopea parviflora* and vulnerable taxa *Actinodaphne lawsonii*, and *Xanthophyllum flavescens* are present in the lower zone (IUCN, 2017- 3). Endemism is the highest in the middle zone with 26% of plants being endemic to Western Ghats or Peninsular India. The upper zone and lower zones have 20% and 16% endemism respectively (Table 1).

The vegetation shows characteristic ecological associations in different zones. The well established

Table 2. Additions to Flora and extended distribution in Palakkad district

Species	Floristic Addition						Extended distribution		
	Flora of Palghat District (Vajravelu, 1984)			Flowering Plants of Kerala (Sasidharan, 2010)					
	Zones			Zones			Zones		
	I	II	III	I	II	III	I	II	III
Abrus pulchellus		+							
Actinodaphne lawsonii	+								
Ailanthes excelsa	+		+				+		+
Amomum masticatorum		+							
Amomum pterocarpum		+							
Amomum ghaticum	+			+	+			+	
Amomum subulatum	+								
Amorphophallus poenifolius	+								
Artocarpus incisus	+	+							
Atalantia monophylla	+								
Barlaria courtallica						+			
Bosenbergia pulcherrima	+	+							
Caryota urens	+								
Cinnamomum palghatensis	+		+						
Croton caudatus	+								
Curcuma ecalcarata	+								
Cyanotis cucullata	+					+			
Cyathula tomentosa				+		+			+
Dalbergia paniculata	+								
Diospyros ebenum	+		+						
Elatostemma lineolatum var. linearis						+			
Eleutheranthera ruderalis	+								
Fimbristylis microcarya						+			
Glycosmis microcarpa	+								
Heliotropium rottleri	+								
Hibiscus micranthus	+								
Hibiscus sabdarifolia	+						+		
Humboldtia unijuga var. unijuga	+								
Impatiens minor	+								
Impatiens dasysperma	+								
Impatiens sasidharanii					+				+
Impatiens trichocarpa	+			+		+	+		+
Jasminum flexile var. ovatum						+			
Kunstleria keralensis	+	+							
Ochroma lagopus	+			+		+			
Orophea erythrocarpa	+								

Species	Floristic Addition						Extended distribution		
	Flora of Palghat District (Vajravelu, 1984)			Flowering Plants of Kerala (Sasidharan, 2010)					
	Zones			Zones			Zones		
	I	II	III	I	II	III	I	II	III
Osbeckia truncata	+								
Phaeanthus malabaricum						+	+		
Phrynium pubinerve						+			
Polygala persicariifolia	+								
Putranjiva roxburghiana	+	+							
Rhopalophora scaberrima	+								
Sida alnifolia	+								
Thottea siliquosa	+								
Thottea sivarajanii						+			
Utricularia albo-cerulea	+		+	+		+	+		+
Utricularia lazulina	+								
Vitex pinnata						+			
Zingiber anamalayanum		+							
Zingiber nimonii		+							
Zingiber sabuanum		+							

+ present; Zone I- 0-500ft, II- 500-1000ft,III >1000ft

plant association of *Utricularia -Eriocaulon – Smithia* is restricted to the upper zone while the lower zones show the *Tectona – Dillenia- Lagerstroemia lanceolata- Terminalia paniculata* association. Epiphytic species are more common in the upper zone while terrestrial orchids are seen in the middle zone. Insectivorous plant *Utricularia albo-caerulea*, saprophytic orchid *Epipogeum roseum* are indications of the adaptive diversity of the vegetation.

The vegetation in the area is of mixed phytogeographical affinities. Plants with Indo-pacific affinity dominates the lower zone constituting 30%, while species of Indo-Pacific –African affinity is 6% , that of Indo-Pacific –South American- African affinity is 4% with lesser percentages of Indo-Pacific-Australian, Indo-Pacific-African-Australian, Indo-Pacific-South American, South American and South American- African.

Among the species in the lower zone 35 are additions to the Flora of Palghat District (Vajravelu, 1990), while 10 and 8 species respectively are additions from the middle and upper altitudinal zones (Table 2). Among the species in the lower zone, as per Flowering plants of Kerala, 2010, 14 are new records to Palakkad district, while the middle and upper zones have 2 and 6 additions respectively (Table 2).

Several taxa are showing an extended distribution in the district apart from the localities so far reported. The lower zone has 9 taxa with extended distribution, the middle and upper zones have 2 and 6 species respectively (Table 2).

DISCUSSION AND CONCLUSION

The species distribution of the three zones is constitutionally very different and clearly reveals the influence of altitude on the structure and composition of vegetation in the area. Altitudinal gradient is well known to be one of the decisive factors shaping the spatial patterns of species diversity (Szaro 1989, DeBano and Schmidt 1990, Lieberman *et al*. 1996, Zimmerman *et al*. 1999, Brown 2001, Lomolino 2001).

The diversity in terms of number of families relative to the total number of species is highest in the middle zone and the least in the lower zone. Several studies reported that diversity peaks at intermediate elevations, in tropical rain forests (Lieberman *et al*. 1996, Vazquez and Givnish 1998, Zimmerman *et al*. 1999). Lomolino (2001) argued whether the diversity-

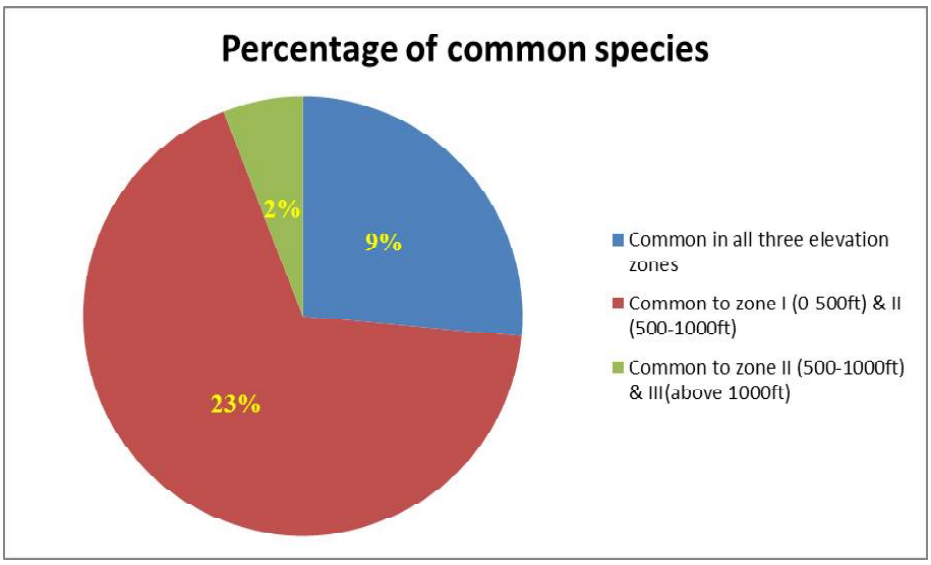

Fig. 2. Percentage of species common to the different elevation zones

elevation gradient is increasing or decreasing, or modal with a peak at intermediate elevations, will depend largely on patterns of co-variation and interaction among the geographically explicit variables. Although a clear and accurate description of the pattern of elevation gradients in diversity was put forward nearly two centuries ago, the issue still remains uncertain (Lomolino, 2001).

The semi evergreen elements show a decreasing gradient along the elevation as the higher altitudes have more grassy slopes. In the Western Himalayas tree layer diversity was found to be higher in the middle part of the gradient (Saxena *et al*. 1985) but the decrease in shrub layer diversity was also observed with increasing attitude (Bhandari *et al*. 1997).

Colebrookea oppositifolia, Haldina cordifolia, Kingiodendron pinnata, Naringi crenulata etc. recorded from the study area are indicators of the concept that India and Sri Lanka are part of the same continental shelf which does not exceed 70m and both areas are united in the Western Ghats –SriLanka biodiversity hotspot. They are construed as forming a community of species that fit together as a biogeographic unit (Bossuyt *et al*. 2004).

The predominantly semi-evergreen forest vegetation shows clear elemental differentiation along an elevational gradient in the three peaks of Dhoni hills. The families *Rubiaceae, Acanthaceae and Euphorbiaceae*are the most abundant in the three zones but the species diversity constitution differs remarkably in the three zones (Fig 2).

The endemic species seen in the three zones are not common. Ecological associations are elevation dependent, being restricted to specific zones. Multiple physical factors associated with the different altitudes certainly seem to have contributed to the diversity along the elevation gradient. Further studies at higher elevation levels are essential to disclose the speciation strategies that might have been evolved at sky – island zones isolated from other eco-geological disturbances.

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Effects of pH Stress on the Morphological Physiological Activity and Anatomy of Cow Pea Plant (*Vigna unguiculata*)

S. SWATHY LEKSHMI AND AYONA JAYDEV

Department of Environmental Science, All saints' college TVM, Kerala, India
email : lekshmiswathi03@gmail.com ; sureshayona@gmail.com

ABSTRACT

In this study 21 days old seedlings of Cow pea plant (*Vigna unguiculata* L) were subject to different pH stress levels (pH 2 and pH 8) at germination and early seedling growth stage of plan development. Data were analyzed for growth parameters such as plant height, fresh and dry weight, leaf water content (LWC), and length of radicle and plumule during germination period, and biochemical parameters such as proline content, membrane stability index (MSI), malondealdehyde (MDA) content, chlorophyll content, and antioxidant enzyme activity Catalase (CAT) and Peroxidase (POD). In this study it was seen that the effect of salt stress reduced plant height, fresh and dry weight, LWC, radicaland plumule length. Salt stress reduced the biochemical activities and also chlorophyll a, chlorophyll b and total pigment content. The decrease was 2.14(control), 0.19(pH 2) and 0.17(pH 8) respectively. The result showed an increase in the activity of CAT enzyme in leaves and root with increasing different pH level concentration. An increase CAT activity were found with pH treatment which represented values of relative increasing of 12.58,g/L, 15.88 g/L, a19.2nd in leaves and7.16 g/L, 9.48 and 13.2g/ L in root respectively. There was increase in the activity of POD enzyme in leaves and root with different pH concentration.

Key words *Vigna unguiculata; pH; catalase; peroxidase.*

In this study the 21 days old seedings of Cow pea plant (*Vigna unguiculata* L) were subject to different pH levels (pH level 2 and pH level 8) at germination and early seedling growth stage of plant development. Data were analyzed for growth parameters such as plant height, fresh and dry weight, leaf water content (LWC), and length of radicle and plumule during germination period, and biochemical parameters such as proline content, membrane stability index (MSI), malondealdehyde (MDA) content, chlorophyll content, and antioxidant enzyme activity Catalase (CAT) and Peroxidase (POD). In this study the effect of pH stress reduced plant height, fresh and dry weight, LWC, radical and plumule length. Stress reduced the biochemical activities and also chlorophyll a, chlorophyll b and total pigment content.

Effects of Acidic and alkaline stresses on growth and physiological changes in pea seedlings were explored. Result showed that biomass, water content and chlorophyll content decreased while cell membrane permeability significantly increased under acid and alkaline stress. These stress is obvious effect on pH value in tissue fluids of shoot and root.Acid and

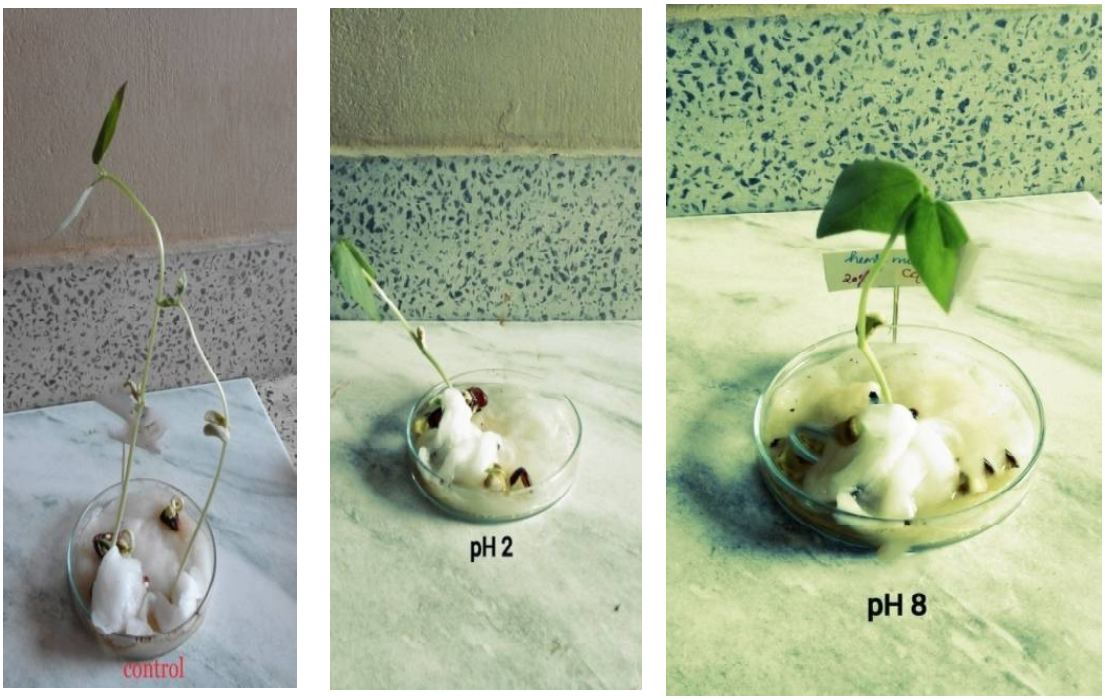

Plate 1, 2 and 3 Study material showing pH stress induced

alkaline stress increased pH value in root tissue fluid(Shi and Sheng, 2005). The increments of proline and organic acid were both greater under pH stress. Acid and alkaline stress caused more harmful effects on growth and physiological changes in oat seedlings especially broke the pH stability in root tissue fluid. Physiological adaptive mechanisms of pea seedlings under acid stress and alkaline stress were different.

MATERIALS AND METHODS

The present study is done to analyze the effect of the abiotic environmental stress (pH) response on plants. The plant material selected for the study is Cow pea plant (*Vigna unguilata* L) considering the ease in the growth of the plant

Study material

Sample Collection

Cow pea plant (*Vigna unguiculata* L) seeds were collected from Kerala Agricultural College, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala, India.

Morphological Parameters

Determination of plant height

The plant height (centimeters) was measured with the help of scale at the time of harvest. The length was measured from the point where the root and shoot joins to the end of root for root length and to the top of shoot for shoot length.

Determination of fresh and dry weight of shoot and root

After harvesting the seeding, the shoot was cut from root at the point where they joined together. The fresh weight was recorded for each part separately. And the sample was dried in an oven at 70°C up to constant dry weight.

Determination of length of radicle and plumule

The radicle and plumule length (centimeter) were measured with the help of a centimeter scale at time of germination. The length was measured from the point where the root tip and shoot joints to the end of root.

Determination of leaf water content

Leaf water content was estimated according to the method described by Smart and Bingham (1974). Leaf discs were punched from each treated plant and the fresh weight was determined. The same leaf discs were kept on water for 4 hrs and turgid weight recorded. The leaf sample was dried in oven at 85°C for dry weight.

Determination of pigment content

The chlorophyll content was determined according to Dere et al., (1998). Leaf fresh materials (1g) were ground properly in 50ml of 100%acetone then centrifuged for 10min at 2500g, absorbance was read spectrophotometrically at 662,645 and 470nm.Pigment content was estimated following formula;

Chl a = (0.0127xODx663)-(0.00269xODx645)

Chl b= (0.0229xODx645)-(0.00468xODx663)

Total Chl = (0.0202xODx645)+(0.00802xODx663)

Biochemical Parameters

The study materials were analyzed for the following biochemical parameters.

Enzymatic Antioxidants

Determination of Catalase (CAT)

The enzyme extract of V. *unguiculata* was prepared in phosphate buffer. The homogenate was centrifuged and supernatant was used for enzyme assay. H_2O_2 - phosphate buffer was taken in an experimental cuvette, followed by the rapid addition of 0.1ml of enzyme extract and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units recorded at 240 nm in a spectrophotometer (UV-1800 Shimadzu).The enzyme solution containing H_2O_2 - free phosphate buffer was kept as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240nm by 0.05units.

Determination of Peroxidase (POD)

Enzyme extract A (20% homogenate) was prepared in 0.1M Phosphate buffer (pH 6.5) from the various parts of the plant, clarified by centrifugation and supernatant was used for the assay. To 3.0ml of pyrogallol solution, 0.1ml of the enzyme extract was added and the spectrophotometer was adjusted to read zero at 430nm.To the test cuvette, 0.5ml of H_2O_2 was added and was mixed. The change in absorbance was recorded every 30 seconds up to 3minute minutes in a spectrophotometer (UV-1800 Shimadzu). One unit of peroxidase is defined as the change in absorbance/

minute at 430nm.

Non enzymatic Antioxidant

Determination of proline contents

Dry weight (0.5g) was extracted by homogenization in 3% (w/v) aqueous sulphosalicylic acid. After the 20 min of centrifugation at 3000xg supernatant collected was mixed with acetic acid and ninhydrin. The mixture was boiled for 1 hour and then absorbance was read spectrophotometrically at 520 nm using toluene as blank.

Determination of malonaldehyde content

Fresh leaves were ground in 1% (10 ml/g fresh weight) trichloro acetic acid (TCA). The homogenate was centrifuged at 10,000 rpm for 5 minutes. Reaction mixture containing 1.0 ml of supernatant and 4.0 ml of 0.5% (w/v) thiobarbituric acid (TBA) was heated at 95°C for 30 min, cooled on ice bath and centrifuged at 5000 rpm for 5 min for clarification. Absorbance of the supernatant was taken at 532 and 600 nm.

Determination of membrane stability index

Fresh leaf samples (0.1 g) were taken in test tubes in two sets containing 10 ml of double distilled water. One set was kept in water bath for half an hour at 40°C and the electric conductivity was recorded (C1). Another set was kept in water bath at boiling temperature (100°C) and EC was recorded (C2). MSI was calculated as per the formula: (MSI) = [1 - (C1/C2)] × 100

Anatomy of stem

Two to three centimeter long pieces of the material were taken. Thin sections were taken using a razor. The thinnest section of the material was taken with the help of delicate brush. For staining, the sections were left for 3 – 5 minutes in a watch glass with stain. Leave 3-5 minutes. The stained sections were mounded on a watch galss and were viewed under a compound microscope with a camera attached.

RESULT AND DISCUSSION

Morphological parameters

The effect of pH stress on plant growth parameters is shown in Table1. Exposure of cow pea plant to various pH (acidity and alkalinity) conditions reduced fresh and dry weight of plant, plant height, leaf water content, length of plumule and radical during germination period with increasing pH Concentration treatment. Acidity and alkalinity induced reduction in weight was reported to be 1.70g (pH 2), 1.86g (pH 8), 0.99g (pH 2), and 0.63g(pH 8), in fresh and dry weight of plant against 2.12 g of fresh weight of control plant and 1.88g of dry weight of control plant. The length of the plumule was measured to be 3.6cm (pH 2), 3.9 cm (pH 8), against 8.5 cm (control) and the length of the radicle were 3.3cm (pH 2), and 2.5 cm (pH 8), against 5.63cm in control plant, respectively at various treatments. Leaf water content (LWC) is 68.7 (pH 2), 69.2 (pH8) against 92.11 in the control plant

In this result acidity and alkalinity reduced the plant growth. Alkalinity and acidic treatment pH2 and pH8 in significant effect on the vegetative growth where the concentration of acidic and alkalinity resulted in severe reduce on the corresponding parameters in study. Similar results were reported in potato (Kerkeni, 2002) for root length, in canola (Byubordi, 2010) for leaf water content (Mensah et *al.* 2006) for number of leaves. Al Thabet et *al.* (2004) working on sprout, Singh et al. (2007) on peanut, Farhoudi and Tafti (2011) in soybean and Keshavarzi (2011) on savory found also that under different pHlevel stress plant growth is inhibited due to the low water potential, ion toxicity and imbalance excreted by salinity (Greenway and Munns, 1980).

Chlorophyll content

The effect of acid and alkalinity on the production of plant pigment is presented in Table 2. It is seen that acidity and alkalinity reduced chlorophyll a, chlorophyll b and total pigment content in the experimental plant. The values obtained with respect to the pHlevel stress given were as follows. Chlorophyll a showed values such as 0.19(pH2) and 0.17 (pH8), where the corresponding values for the control plant was 2.14. The content of chlorophyll b was 0.03 (pH2) and 0.02(pH8) as compared to 0.84 in control and total pigment content showed values 0.04(pH2)and 0.02(pH8), when the control plant showed a total chlorophyll content of 2.98 mg/g of fresh weight.

As consequence, a decrease in photosynthetic pigment content was observed with increasing salt concentration in all cultivars. Reduce of 93% and 97% respectively in chlorophyll b and chlorophyll a content compared to control. This result is in agreement with those of Tewari and Singh (1991) in lentil, Beinsan et *al.* (2003) in bean, Iqbal et *al.* (2006) in wheat, Chen and Yu (2007) in *Glycine max* seedlings, Moussa Helal

Table 7.1. Influence of pH stress on growth parameters of Cowpea plant

SL No	Treatment	Plant height(cm)	Fresh weight (g)	Dry weight(g)	Leaf water content (%)	Length of plumule (cm)	Length of radical (cm)
01	Control	30±1.00	2.12±0.03	1.88±0.036	92.11±2.43	8.5±0.14	5.63±0.05
02	pH2	20.2±0.22	1.70±0.014	0.99±0.01	68.7±0.09	3.6±0.15	3.3±0.25
03	pH8	22.4±0.15	1.86±0.35	0.63±0.33	69.2±0.03	3.9±0.27	2.5±2.5

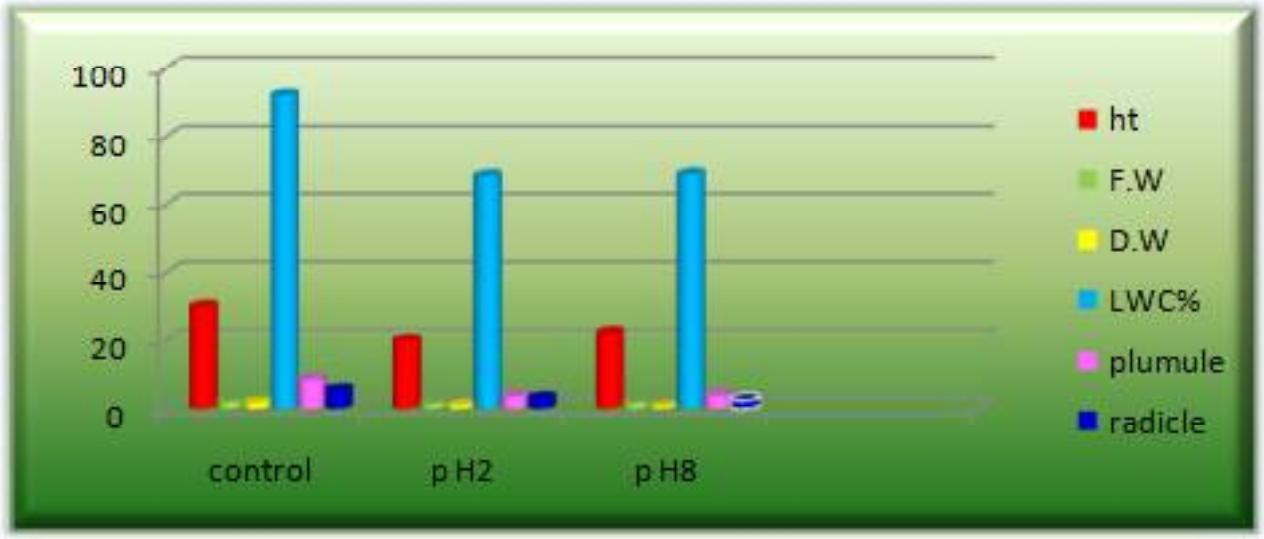

Fig 1. The change in the growth parameters

Table 7.2Influence of pH stress on pigment system of *Vigna unguiculata*

SL No:	Treatment	Chlorophyll a (mg/g fresh weight)	Chlorophyll b (mg/g fresh weight)	Total chlorophyll content (mg/g fresh weight)
01	Control	2.14±0.16	0.84±0.06	2.98±0.07
02	pH2	0.19±0.004	0.03±0.01	0.04±0.02
03	pH8	0.17±0.07	0.02±0.005	0.02±0.03

(2006) in maize, Molazem et *al.* (2010) in corn, Malik et *al.* (2010) in cucumber, El Iklik et *al.* (2011) in tomato and Rahdari (2012) in Purslane Parida and Das (2005) suggested that decrease in chlorophyll content in response to acidity and alkalinity is a general phenomenon which led to disordering synthesizing chlorophyll and appearing chlorosis in plant. According to Rao and Rao (1981).Different pHlevel stress decreased total chlorophyll content of the plant by increasing the activity of the chlorophyll degrading enzyme.

Biochemical parameters

Enzymatic activity

Catalase (CAT)

Result of antioxidant content in this study showed that CAT activities increased with level of pH level 2, pH level 8 stress. Increase in CAT of alkalinity and acidic enhancement was 15.88 (pH level 2) and 19.2 (pH level 8) as compared to 12. 58 in control plant in leaf samples. The activity was recorded as 9.48 (pH level 2) and13.2 (pH level 8) as compared to a 7.16 in control in root samples.

The CAT is one of the highest turnover rates for all enzymes with the potential to directly dismutate H_2O_2 into H_2O and O_2 and is indispensible for ROS detoxification in peroxisomes during stress condition (Sairam and Srivastava, 2001). This indicated plants will produce more CAT, SOD and POD under drought conditions to remove the extra ROS in cells. In this study, CAT SOD and POD activities increased markedly in the drought tolerant varieties, while they reduced in the sensitive one. This showed that drought-

Fig. 7.2 Shows pH level of pigment system

tolerant varieties were efficient scavenger of H2O2, which may result in better protection against H2O (Athar *et al*., 2002).

Peroxidase (POD)

Table 7 shows the result of peroxidate enzyme activity in the study plants. High levels of peroxidase enzyme (POD) activity in leaves were found with pH level 2 and pH level 8 treatment (83.59 and 89.14 respectively) with respect to 71.14 mg in leaf tissues of control plant. The values of peroxidase in root tissue in control plant was found to be 79.2 and the stressed

Table 7.3. Catalase content in spHstress in Cow pea plant (*Vigna unguiculata* L)

SL No:	Treatment	Catalase(CAT)	
		Leaf	Root
01	Control	12.58±0.106	7.16±0.029
02	pH2	15.88±0.051	9.48±0.042
03	pH8	19.2±0.06	13.2±0.25

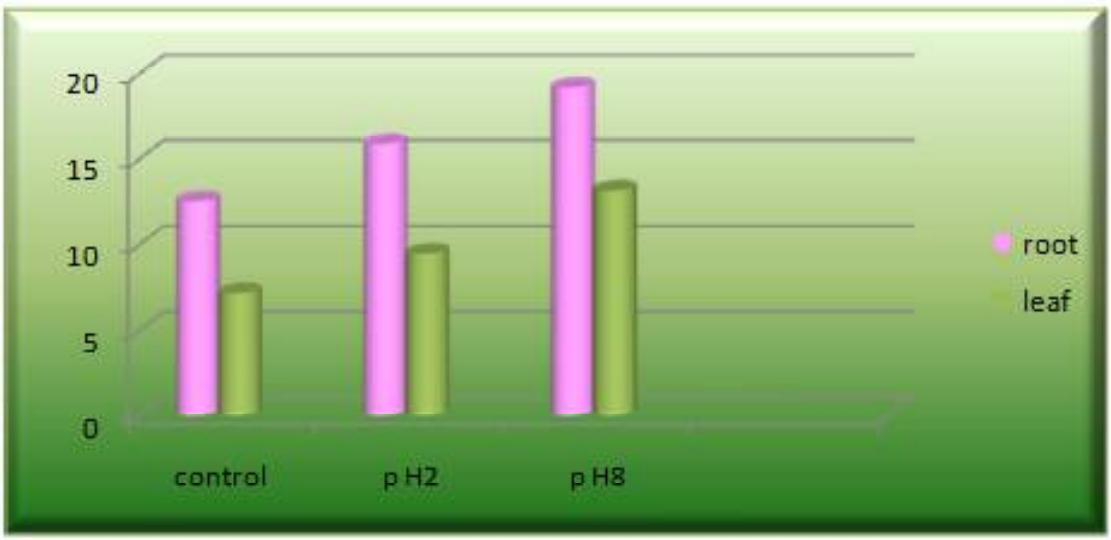

Fig. 7.3. Influence catalase of pHlevel of cow pea plant

Table 7.4. Peroxidase content in pH stressed *Vigna unguiculata* L

SL No:	Treatment	Peroxidase (POD)	
		Leaf	Root
01	Control	71.14±0.056	79.2±0.33
02	pH2	83.59±0.010	87.2±0.1
03	pH8	89.14±0.04	91.4±0.32

Fig. 7.4. Influence of pH level of cow pea plant

plant showed values 87.2 and 91.4 for pH level 2 and pH level 8 concentrations respectively. POD also involved in various plant processes, including lignifications, (Hendriks *et al*., 1991), oxidation of phenolics, (Largrimini, 1991), regulation of cell elongation (Mohammadkhani and Heidari, 2008) and Lum *et al* 2014 detoxification of toxic compounds such as H2O2 (Chaparzadeh *et al*., 2004). The tolerance of some genotypes to environmental stresses has been associated with higher activities of antioxidant enzymes. For example, the pH level -tolerant species of pigeon pea (*Cajanus cajan*) (Kumar *et al*., 2011), wheat (*Triticum aestivum*) (Hasheminasab *et al*., 2012; Omar, 2012) and black gram (*Phaseolus mungo*) (Pratap and Sharma, 2010) had higher activities of POD in the acidity and alkalinity soil.

Non enzymatic activity

Proline content

As seen in Table 5, increase in proline content was conspicuous in different pH level stressed plant. pH stress pH 2 level and pH 8 level induced treatment showed a proline content of 6.01 and 4.76 respectively, where in the control plant showed 1.93 g only.

A significant variation in proline content in leaves of plant treated with different pH level was observed. There was a gradual increase in Proline concentration. Maximum Proline content was observed pH 2 level in plant treated. The results of the study are in agreement with Zahra *et al*., 2010 in *Plantago ovata* under pH stress. Proline is an organic solute known to be involved in osmoregulation which reduces the cell osmotic potential to a level to provide high turgor potential for maintaining growth (De Lacerda *et al*. 2005; Ashraf and Harris, 2004; Chaum *et al*. 2004).

Table 7.5. proline content in pH level of *Vigna unguiculata*

SL No	Treatment	Proline content
01	Control	1.93 ±0.25
02	pH2	6.01±0.32
03	pH8	4.76±0.24

Malondialdehyde (MDA)

In given table 4, Lipid peroxidation measured in terms of MDA content was reported to increase by 4.12(pH 2 level) and 5.5(pH 8 level) treated cow pea plant. The control plant showed only 4.12 g of MDA. The lipid peroxidation level, as indicated by MDA

Fig. 7.5. shows pH level in proline content

accumulation, increased significantly under the heavy metal stress.

The results from another study indicated that the content of MDA significantly increased in various pH level stressed pea seedlings in comparison to the control plants, which is consistent with the findings of Seckin et al. (2008) in Pea. Thereafter, the content of MDA significantly increased after pea seeds were soaked in the suspension of pH stress. Taken together, results are consistent with data from Rawat et al. (2011), who demonstrated that wheat seed biopriming with aacidity or alkalinity tolerant isolates of *T. harzianum* Th-14, Th-19,andTh 13 increase the accumulation of MDA content, where as,it increased the proline content in pea Seedlings under both acidic and alkalinity.

Table 7.6. Influence of pH level Malondealdehyde

SL No:	Treatment	Malondialdehyde (MDA) (g)
01	Control	4.12±0.42
02	pH2	4.2±0.021
03	pH8	5.5±0.028

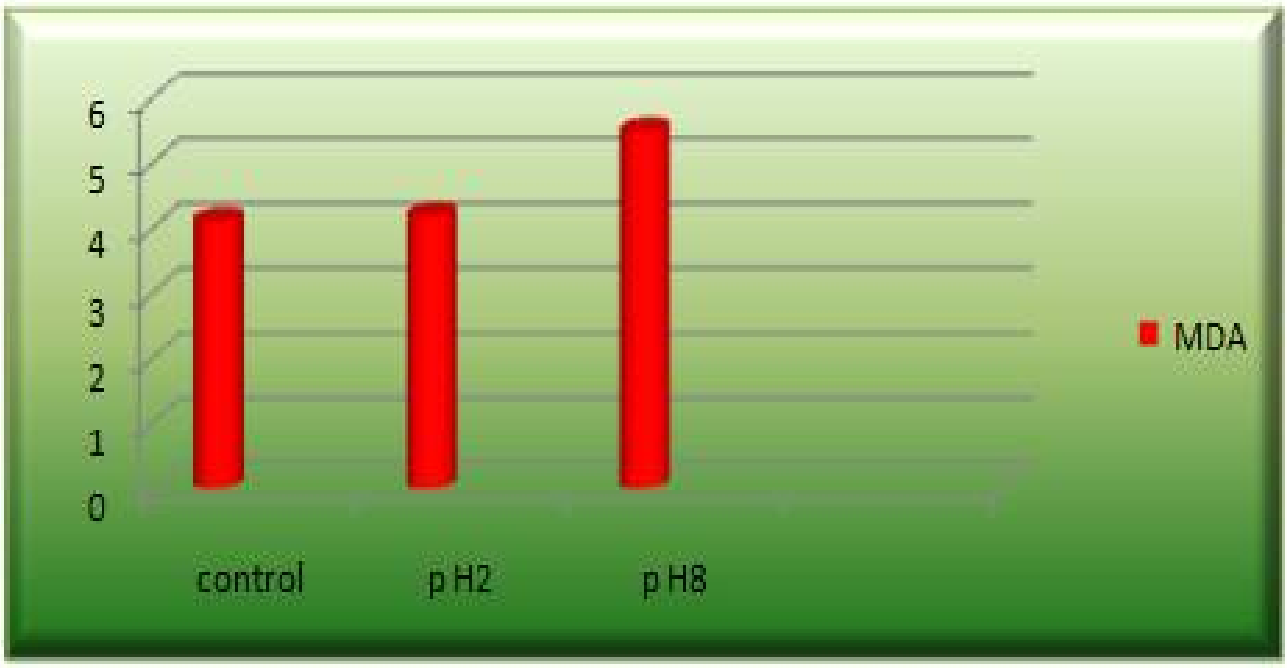

Fig. 7.6. Shows pH level of MDA

Membrane stability

The results of MSI are given in table 3. It can be seen that both level of salinity stress induced reduced MSI in comparison to control. Salinity induced reduction is reported to be 56.12%(pH 2),54.1%(pH 8) is compared to control 87.12%.

Acidic and alkalinity stress reduced the membrane stabilitydepicted in our results corroborates with the results of Ahmad (2010) who in mustard demonstrated that exposure to pH 2 level and pH 8 level increases membrane leakage and hence reducing the stability of membranes. Tuna *et al*., (2008) confirmed that pH (acidity and alkalinity)enhances membrane leakage.

Table 7.7. Influence of of pH level on Membrane Stability

SL No:	Treatment	Membrane stability Index (MSI) (%)
01	Control	87.12±2.11
02	pH2	56.6±0.02
03	pH8	54.1±0.05

Anatomy of stem

The cross section (anatomy) of stem of *Vigna unguiculata* was analyzed to assess the effect of pH level and the anatomical adaptation of this plant to be acclimatized under acidity or alkalinity stress. There was significant alteration in anatomical feature of stem of cow pea seedings imposed to various level of pH. Transverse section of stem *V. unguiculata* showed decrease in thickness of upper epidermal layer pH 2 level compared to control. Ph 8 level has small variation compared to control. The thickness of cortex layers of stem was reduced by different concentration level. The thickness of hypodermal layer and pith area of stem were increased by various pH levels treated plants. The pith cell diameter increased significantly in stem of acidity and alkalinity treated seedling as compared to control. There was significant changes observed in the xylem vessels diameter of the stem of cow pea plant xylem vessels are contracted.

SUMMARY AND CONCLUSION

A plant's response abiotic stress strongly depends on its developmental Stage and the environmental conditions to which it is subjected. Many stress such as Salinity, Drought, Temperature, UV radiation, pH level and heavy metal. Here pH stress combinations lead to phenotypic damage and, as mentioned above, the expression of defense is affected according to the type of abiotic stress involved. Overall, the complex response of the plant stems from the interplay of specific signaling pathways involved in abiotic stress. The stress types leads to an increased accumulation of a large number of signaling compounds that, in an ideal case, will be expressed as cross-tolerance. pH resulted in altered growth of cowpea due to its effects on the various physiological and biochemical parameters studied.pH level and heavy metal increased lipid peroxidation leading to membrane imbalance then decrease the membrane stability index

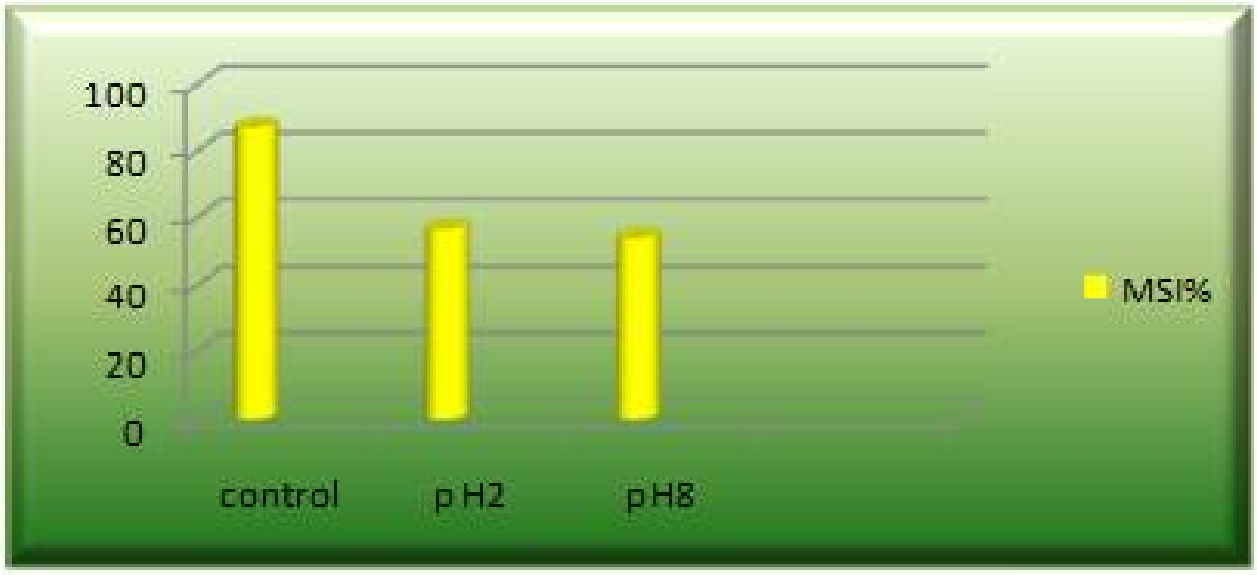

Fig. 7.7. Shows the effect of pH level in MSI

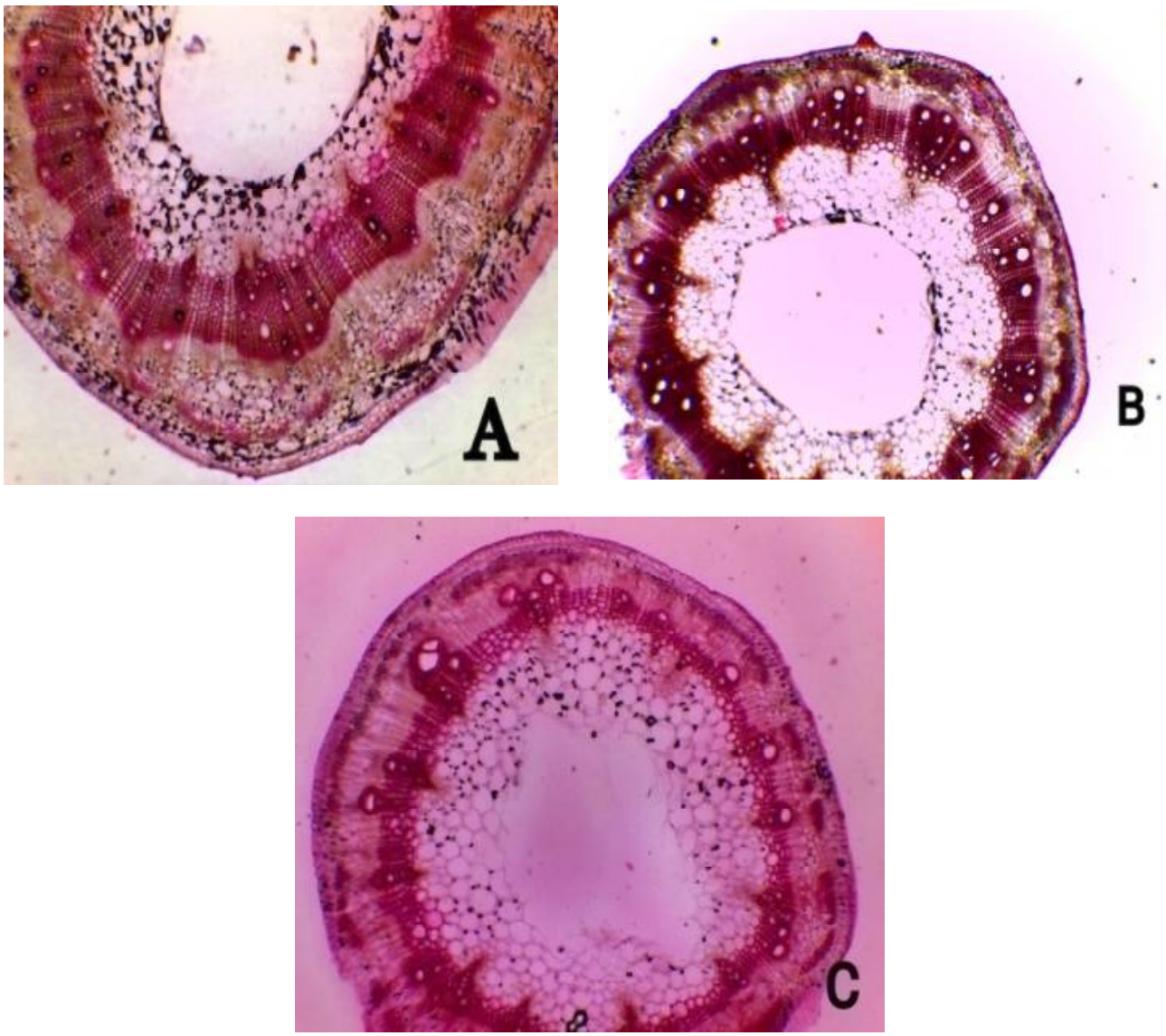

Plate 4: Effects of drought on stem anatomy (10x magnification) Figure(A) Control) Fig. (B) pH level 2 Fig.(C) pH level 8

and at the same time also reduced the uptake of important mineral elements and increase MDA and proline content in different abiotic stress compared to control, increase the enzymatic antioxidants activity such as POD and CAT.

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Biological Properties of Volatile Oils of *Heracleum hookerianum* Gamble (Apiaceae) Leaves, Fruits and Rhizome

S. KARUPPUSAMY

Department of Botany, Centre for Botanical Research, The Madura College, Madurai – 625 011, Tamil Nadu, India.
email : ksamytaxonomy@gmail.com

ABSTRACT

The aim of the present study was to investigate antioxidant and antimicrobial effects of essential oils from *Heracleum hookerianum* Gamble leaves, fruits and rhizomes. The antimicrobial activity was determined by agar dilution and disc diffusion methods. The free radical scavenging activity was studied by using 1,1-diphenyl -2-picryl hydrazyl (DPPH) antioxidant model of screening using vitamin E (5mM) as standard. The essential oils of *H. hookerianum* rhizome was found to be effective against both antibacterial and antioxidant activities. Rhizome oils showed active against Gram positive bacteria than Gram negative bacteria. Similarly, fruit oils exhibited significant antioxidant activity in 1,1-diphenyl -2-picryl hydrazyl (DPPH) radical scavenging model. The radical scavenging capacity of the fruit oil was determined with an IC_{50} value of 4.3 ± 0.88 ìg/ml. The study confirms the possible antibacterial and antioxidant potentiality of the essential oils of *H. hookerianum*. Presence of monoterpenes or in combination with oxygenated hydrocarbons with its other components could be responsible for the activities.

Key words *Biological Properties, Volatile Oils, Heracleum hookerianum, Gamble (Apiaceae), Leaves, Fruits and Rhizome*

Free radicals are potentially important in a number of ailments states that can have severe effects on the cardiovascular system, either through lipid peroxidation or vasoconstriction (1). Although the antioxidant defense systems include both endogenously and exogenously derived compounds, dietary plants based antioxidant have recently received a great attention (2). Hence many studies have been performed to identify antioxidant compounds with pharmacological activity and a limited toxicity from medicinal plants. In this context, ethnopharmacology plays a significant part in the search for interesting and therapeutically useful plants. In order to contribute to the knowledge of plants from Western Ghats of India, in the present study, *Heracleum hookerianum* (Apiaceae) essential oils were screened to determine their free radical scavenging and antibacterial activities.

Nowadays, there is great world-wide interest in finding new and safe antioxidants from natural sources, to prevent oxidative deterioration of foods and to minimize oxidative damages of living cells. Traditionally, chemically synthesized compounds, such as butylated hydroxy-anisole (BHA) and butylated hydroxytoluene (BHT), are used as antioxidants in oil products. However, some of these compounds have been questioned for their safety (3). The use of BHA and BTH is proved to be carcinogeneic. Therefore, there is an increasing interest in the antioxidative activity of natural compounds (4). Higher and aromatics plants have traditionally been used in folk medicine as well as to extend the shelf life of foods (5). Most of their properties are due to essential oils produced by their secondary metabolism (6). Several of them are qualified as antioxidant and are proposed to replace synthetic antioxidants used in food industry where they do not affect the organoleptic characteristics of the final product. Also, numerous scientific reports have highlighted an important antioxidant activity of essential oils (7). These biological activities depend on the chemical composition (8) which varies according to the geographical origin, the environmental and agronomic conditions, the stage of development of the plant material and the extraction method (9). Therefore, the evaluation of the biological activity of an essential oil should be supplemented with the determination of its chemical composition.

Researchers have reported the antimicrobial activity of several herbal plants. In recent years, multiple drug resistance in human pathogenic micro organisms has developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. This situation has forced scientists to search for new antimicrobial substances from various sources as novel antimicrobial chemotherapeutic agents [9]. The cost of production of synthetic drugs is also high and they produce adverse effect compared to plants derived drugs. Hence much attention has been paid recently, to the biologically active compounds derived from plants used in herbal medicine[10].

Heracleum L. (Apiaceae) includes more than 70 species in the world. In India there are 15 species and 5 are endemic to Peninsular India (10). *Heracleum hookerianum* is growing wildly in grass lands of

Nilgiri hills of Western Ghats at 2000 m (msl). The rhizome and seeds are used as folk medicines. They are reported to be effective in indigestion, sunburn, skin diseases and external tumors (11). Many studies reported on the biological activities and essential oil composition of the genus *Heracleum* (12-15). As part of our on-going studies on the genus *Heracleum* from Western Ghats of India, we now report on the composition of the volatile oils of the rhizome, leaves and seeds of *H. hookerianum* for first time, together with its antioxidant and antibacterial properties.

MATERIALS AND METHODS

Plant material

The leaves, seeds and rhizomes of *Heracleum hookerianum* Gamble were collected, during December 2009, form the Nilgiri hills, Western Ghats of Peninsular India. The identification of the specimens authenticated with comparison of herbarium sheets deposited in Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu. The voucher specimens have preserved in the Department of Botany, The Madura College, Madurai, Tamil Nadu.

Isolation procedure

The essential oils of leaves, mature seeds and rhizomes of *H. hookerianum* were isolated from fresh plant material (200 g) by hydrodistillation, for 4 h, using a Clevenger-type apparatus.

Gas chromatography

The GC analysis was carried out with a Hewlett-Packard HP6890, equipped with a HP-Innowax silica capillary column (60 m x 0.25 mm, film thickness 0.25 ìm) and a flame ionization detector. Nitrogen was used as carrier gas with a flow rate of 0.8 ml/min. Injector and detector temperatures were both set at 250^0C. Column temperature was programmed to 60^0 C for 10 min, gradually increased to 220^0C at 4 0C/min, held for 10 min and then increased to 240^0 C at 10C/min. Split ratio was 50:1 and one microliter of sample (dissolved in hexane as 20 % v/v) was injected into the system.

Gas chromatography-mass spectrometry

The GC-MS analysis of the oil was carried on an Agilent 6890N Network GC system combined with Agilent 5973 Network Mass Selective Detector (GC-MS). The capillary column used was an Agilent 19091N-136 (HP Innowax Capillary; 60.0 m x 0.25 mm x 0.25 ìm). Helium was used as carrier gas at a flow rate of 1.0 ml/min with 1ìl injection volume. Samples were analyzed with the column held initially 60 ºC after injection with 10 min hold time, then increased to 220ºC with 4 ºC/min heating ramp and kept at 220ºC for 10 min. Then final temperature was increased to 240 ºC with 1 ºC/min heating ramp. The injection was performed in split mode (50:1). Detector and injector temperatures were 230°C and 280°C, respectively. Run time was 80 min. MS scan range was (*m/z*): 35-450 atomic mass units (AMU) under electron impact (EI) ionization (70 eV).

Identification of the volatile oil components

The components were identified by comparing their relative retention times with those of authentic samples and mass spectra with the data from the Baser library of essential oil constituents as well as Wiley and Nist Library. Relative content of % components were determined with area under peaks using Agilent software. The results are expressed as an average of three determinations in all cases. GC and GC/MS analysis were both conducted at the Sophisticated Analytical Instrument Facility (SAIF), Central Drug Research Institute, Lucknow, India.

Antioxidant activity measurement

The hydrogen donating ability of extracts was examined in the presence of DPPH stable radical (Mensor *et al.,* 2001). One milliliter of 0.3 mM DPPH ethanol solution was added to 2.5 ml sample of different concentrations of essential oils from *H. hookerianum* and allowed to react at room temperature. After 30 min, the absorbance values were measured at 517 nm. Ethanol (1.0 ml) with essential oil of *H. hookerianum* (2.5 ml) was used as a blank. DPPH solution (1.0 ml, 0.3 mM) with ethanol (2.5 ml) served as negative control. The positive control was the one using the standard anti-oxidant (Vitamin E) solution. The percentage of antioxidant ability was calculated by comparing the results of the test with those of controls as per the following formula:

Inhibition (%) = [(control " test) ×100] / control.

Antimicrobial activity

Bacteria

A total of 5 bacterial strains belonging to different genera were tested in this study. The test organisms were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India. Gram-positive strains

Table 1. Volatile oil constituents of *H. hookerianum* leaves, fruits and rhizome.

Component	Constituents (%)		
	Leaves	Fruits	Rhizome
α-Thujene	0.3	1.5	1.3
α-Pinene	6.0	0.6	0.1
Camphene	5.5	0.1	0.3
β-Pinene	7.0	0.2	1.5
Myrcene	1.2	1.7	1.8
α-Phellandrene	11.8	12.8	11.5
β-Terpinene	10.3	11.2	13.1
Limonene	2.6	0.2	4.1
Sabinene	0.3	0.2	0.1
Ocimene	0.1	--	0.2
1-8, Cineole	31.0	15.8	30.4
ρ-Cymene	17.8	18.3	6.2
3-Octanol	0.2	1.6	0.8
Terpindene	0.2	1.3	--
Sabinene hydrate	--	0.1	0.2
α-Terpinolene	--	0.2	1.3
Linalool	3.9	0.7	1.8
trans-Pinocarveol	5.8	6.2	1.9
Camphor	6.9	7.2	1.2
Borneol	1.5	2.6	1.2
Varveol methyl ether	0.1	--	1.0
Isoborneol	0.1	0.2	--
Myrtenal	--	--	2.2
Pinocarvyl acetate	0.1	--	--
Terpinen-4-ol	0.1	0.1	0.1
Caryophyllene	1.1	1.3	--
Carvacrol	1.2	0.2	0.6
Phellandral	0.1	0.2	--
Caryophyllene oxide	0.1	--	0.1
Nerolidol	0.1	--	0.1
Cumin alcohol	0.2	0.2	0.3
Hexadecanoic acid	0.1	--	0.1
Total identified			

% of constituents calculated from FID data.

were grown in nutrient broth (NB, Oxoid brand) and Gram-negative bacteria were grown in peptone water (PW, Oxoid brand, bacteriological peptone plus Nacl 0.5%) for 18 h before use.

Determination of antimicrobial activity

Sensitivity tests were performed by disc diffusion method[18]. The nutrient agar plates (Oxoid brand), containing an inoculum size of 10^5-10^6 cfu.ml^{-1} of bacteria were used. Previously prepared essential

Table 2. DPPH radical scavenging activity of essential oils of *Heracleum hookerianum.*

Sample	Concentration (µg/ml)	Absorbance at 517 nm	% inhibition	IC_{50} (µg/ml)
Control		0.882		
Leaf oil	3.125	0.772 ± 0.090	16.54 ± 0.06	13.1 ± 0.57
	6.25	0.663 ± 0.070	29.58 ± 0.061	
	12.5	0.445 ± 0.090	54.45 ± 0.03	
	25	0.250 ± 0.065	76.86 ± 0.09	
	50	0.115 ± 0.041	92.47 ± 0.03	
Fruit oil	3.125	0.739 ± 0.003	47.90 ± 0.02	4.3 ± 0.88
	6.25	0.574 ± 0.009	61.07 ± 0.30	
	12.5	0.340 ± 0.002	76.83 ± 0.011	
	25	0.259 ± 0.001	91.90 ± 0.32	
	50	0.091 ± 0.002	98.62 ± 0.03	
Rhizome oil	3.125	0.796 ± 0.005	11.97 ± 0.04	16.0 ± 0.57
	6.25	0.661 ± 0.003	28.00 ± 0.06	
	12.5	0.556 ± 0.001	41.25 ± 0.006	
	25	0.330 ± 0.001	67.13 ± 0.01	
	50	0.111 ± 0.003	93.06 ± 1.01	
Vitamin E (standard)	1	0.584 ± 0.003	42.98 ± 0.003	3.2 ± 0.15
	2	0.549 ± 0.002	52.72 ± 0.006	
	4	0.454 ± 0.003	65.64 ± 0.002	
	8	0.310 ± 0.006	81.97 ± 0.003	
	16	0.231 ± 0.009	95.46 ± 0.005	

Values shown are mean ± SEM for four tests. $p<0.001$, as compared to control.

loading discs (Concentration 128-2000 ìg.ml^{-1}) were placed aseptically on sensitivity plates. The discs containing known antibiotics (Gentamicin) served as positive controls. All the plates were then incubated at 37°C±2°C for 18 h. The sensitivity was recorded by measuring the clear zone of inhibition on agar plate around the discs.

The MICs were determined by the standard agar dilution method [19]. All the test oils were dissolved in hexane (85%). These were then individually added at each final concentrations of 0-2000 ìg.ml^{-1}, to molten agar (Oxoid brand), mixed thoroughly, pH adjusted to 7.2 to 7.4 and poured into sterile Petri dishes. Bacterial cell suspensions were spot inoculated on the plates using a bacterial planter (10 ìl). The final number of cfu inoculated onto the agar plates was 10^4 for all strains. The inoculated plates were then incubated at 37°C±2°C for 18 h. The lowest concentration of the plate, which did not show any visible growth after incubation, was considered as MIC. The agar plate containing only hexane and Gentamicin was served as negative and positive control.

STATISTICAL ANALYSIS

The analytical values of the antioxidant activity measurement represent means of three replicates, done in two different experiments. Data obtained were subjected to one-way analysis of variance and Tuckey's test analysis. Significance was assumed at $P< 0:05$.

RESULTS AND DISCUSSIONS

The oil yields obtained from the different parts of *H. hookerianum* varied considerably (Table 1).

The highest oil yield was obtained from leaves and fruits (1.3% and 1.1% v/w), while the lowest one was obtained from rhizome (0.9%). These yields were relatively lower than the average oil yields reported in related species of *H. candolleanum* from Western Ghats of India (18). The different harvesting period of the samples could partly be responsible for these differences because both the oil yield and the proportions of the several constituents of essential oil may vary greatly according to the developmental phase of the plant (19).

Twenty five to thirty two components could be identified, representing 87–98% of the total oils, which are listed in Table 1. Although monoterpenes were dominant in all oils (87–96%), the importance of the oxygen-containing or monoterpene hydrocarbons

Table 3. Antibacterial activity and minimum inhibition concentration of essential oils of *Heracleum hookerianum*.

Test organisms	Diameter zone of inhibition (mm)			
	Fruit oil (MIC - µg/ml)	Leaf oil (MIC)	Rhizome oil (MIC- µg/ml)	Gentamicin (MIC - µg/ml)
Bacillus subtilis	26.6 ± 0.5 (11)	13.8 ± 0.3 (16)	16.6 ± 0.4 (15)	31.0 ± 0.2 (8)
Staphyloccocus aureus	22.0 ± 0.1 (12)	12.1 ± 0.1 (14)	14.8 ± 0.2 (18)	34.2 ± 0.2 (8)
Enterobacter aerogens	16.8 ± 0.4 (13)	10.8 ± 0.2 (18)	15.6 ± 0.2 (13)	36.3 ± 0.4 (9)
Escherichia coli	4.0 ± 0.1 (NR)	1.0 ± 0.0 (NR)	2.0 ± 0.0 (NR)	30.0 ± 0.0 (9)
Salmonella typhi	3.0 ± 0.0 (NR)	5.2 ± 0.1 (NR)	4.2 ± 0.0 (NR)	34.3 ± 0.6 (10)

(Values expressed in M ± SE; MIC values are in ìg/ml; NR - Not resulted)

varied. The essential oils of *H. hookerianum* leaves were dominated by monoterpenes (91.5%), whereas in seed oils, monoterpenes (87%) and the rhizome oils, monoterpene hydrocarbons (90%) were present in more approximate amounts.

1,8-Cineole was the dominant component in leaves (31.0%), fruits (15.8%) and rhizome (30.4%) of *H. hookerianum* essential oils, which is in accordance with the report by Saraswathy and Sasikala (20) for the *Heracleum* spp. oils, although different from most of the Indian species of *Heracleum* chemotypes. Predominant components in all the parts of *H. hookerianum* are 1,8-cineole, á-Phellandrene, ñ-Cymene and Terpinene. It is showing similarities to some previously studied populations of related species *H. candollenaum* and *H. concanense* (21, 22). 1,8-Cineole and â-Pinene was the main component in all the parts of *Heracleum* oils, which is also in agreement with previous reports for other species (23).

All the essential oils showed some antioxidant capacity, in the absence of the radical inducer, increasing over the concentration range tested (Fig. 1). *H. hookerianum* rhizome oils showed, at 25 and 50 µg/ml, the highest antioxidant index, comparable to or higher than that of Vitamin E. The essential oils of *H. hookerianum* leaf showed much lower antioxidant indices than that of fruit and rhizome oils. The decrease in concentration of the oils produced a drastic reduction in their activity, and at the lowest concentration (3.125 µg/ml) the oils were scarcely active.

Our results showed considerable antibacterial activity both the zone of inhibition and minimum inhibition concentration (MIC) of the essential oils of *H. hookerianum*, especially against gram positive strains (Table 3).

This is probably due to many monoterpenes present in the essential oils, which was well documented for many compounds. Due to the continuous emergence of antibiotic-resistant strains there is continual demand for new antibiotics. In many developing countries about 80% of available drugs come from medicinal plants and in industrialized countries plants make up the raw material for processes, which synthesize pure chemical derivatives [24].

The fact that *H. hookerianum* oils, which has no phenolic compounds, showed the highest antioxidant activity demonstrates that the presence of this type of compound is not obligatory for the antioxidant activity.

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Studies on the Biochemistry and Allelopathy of *Salvinia molesta* D.S. Mitchell

LATHA SADANANDAN, CHITHRA CHANDRAN AND GEETHU C.B.

Post Graduate and Research Department of Botany and Biotechnology,
Sree Narayana College, Kollam – 691 001
email: sadanandanlatha@gmail.com

ABSTRACT

Biochemical investigation on *Salvinia molesta* for it's total carbohydrates, proteins, phenol content, chlorophyll content and enzyme assay tests were carried out using standard procedures. Studies on the allelopathic effects of aqueous leaf extract of *Salvinia molesta* on *Vigna radiata* L. were carried out under laboratory conditions. Parameters such as germination percentage of seeds, shoot length and root length of seedlings, fresh weight and dry weight of the seedlings were studied. Biochemical investigation showed protein 15.5 mg/g tissue, reducing sugar 0.22 mg/g tissue, phenol 0.79375 mg / g tissue, amylase activity 0.13 Eu/ g tissue/min, peroxidase activity 4.56 Eu/g tissue, polyphenol oxidase activity 6.2 Eu/ g tissue, chlorophyll 0.0022583 mg/g tissue. Allelopathic studies showed that the germination of *V. radiata* seeds, hypocotyl and epicotyl growth, fresh weight and dry weight of *V.radiata* seedlings decreased with increase in concentration (75% and 100%) of aqueous leaf extract of *S. molesta*. The biochemical studies on *S. molesta* reveals it's potential as a source of vegetable or feed, high phenol content reveals it's antioxidant property. The allelopathic studies on *V.radiata* shows that the weed can be exploited as organic fertilizer in lower concentrations for the cultivation of the crop. The study addresses the need for further study of this weed for it's utilisation that paves way for the conservation of wetland ecosystem.

Key words *Salvinia molesta*, *Vigna radiata*, Biochemistry, Allelopathy

Salvinia molesta, (Water Moss) belonging to the Family Salviniaceae, is an aquatic floating fern. *Eichhornia crassipes* and *Salvinia molesta* are ranked first and second places respectively on a list of the world's most problematic aquatic weeds (Holm *et al*., 1977). *S. molesta* exhibits unique adaptive features in morphological, anatomical, physiological and reproductive characters that together help the plant for its wide distribution. It forms a thick mat in slow moving water bodies (Plate 1) that dramatically impacts water flow, blocks sunlight from reaching native aquatic plants, and starves the water of oxygen, often killing fishes. The plants also create a prime habitat for mosquitoes, the classic vectors of diseases. Utilization of this plant to produce eco-friendly products can help to reduce such environmental problems to some extent. A preliminary knowledge on the chemistry of the plant and it's interaction with environment paves way to the discovery of new potential drugs in pharmaceutical companies, eco-friendly biofuel, or eco-friendly products.

Plate 1.*Salvinia molesta* D.S. Mitchell

Plate 2.*S.molesta* leaf extract X *V.radiata* seeds

MATERIALS AND METHODS

Fresh samples of *S. molesta* were collected from water bodies of Kollam district. They were shade – dried and powdered or used fresh as required for biochemical studies. They were analysed for total reducing sugar, protein content, phenol content and enzymatic activity of amylase, peroxidase and polyphenol oxidase using standard procedures (Sadasivam and Manickam, 1997). The allelopathic effect of different concentrations (25%, 50%, 75% and 100%) of aqueous leaf extract of S.molesta on *Vigna radiata* (green gram) were studied. The aqueous extract was prepared by boiling 5g powdered

leaf samples in 100ml distilled water for 30 minutes and filtering. Distilled water was maintained as control. Three replicates were maintained for each treatment. Five seeds of surface- sterilized (95% ethanol) *V. radiata* were sown in each replicate. The treated samples were incubated at room temperature (30± 2°C) for 7 days. Results were taken on the 7th day. The parameters studied were germination percentage of *V.radiata* seeds, hypocotyl and epicotyl growth of seedlings, fresh weight and dry weight of the seedlings. The observations were noted and subjected to statistical analysis.

RESULTS AND DISCUSSION

BIOCHEMICAL EVALUATION

Extraction and estimation of protein by Bradford method

The total soluble protein was calculated using the formula:

$$\frac{Concen.of\ standard \times OD\ of\ sample \times Total\ vol.of\ extract}{OD\ of\ standard \times vol.of\ sample \times weight\ of\ tissue}$$

$$= \frac{0.06 \times 0.065 \times 15}{0.189 \times 0.02 \times 1}$$

=15.47619 mg / g tissue

Estimation of reducing sugar by DNS method

Total soluble sugars =

$$\frac{Concen.of\ standard \times OD\ of\ sample \times Total\ vol.of\ extract}{OD\ of\ standard \times vol.of\ sample \times weight\ of\ tissue}$$

$$= \frac{0.04 \times 0.011 \times 10}{0.098 \times 0.2 \times 1}$$

= 0.22448 mg / g tissue

Estimation of total phenol

Totalphenol =

$$\frac{conc\ of\ std \times OD\ of\ sample \times total\ vol\ of\ distilled\ water}{OD\ of\ Std \times aliquot\ taken\ /\ weight\ of\ tissue}$$

$$= \frac{0.04 \times 0.635 \times 10}{0.160 \times 0.2 \times 1}$$

= 0.79375 mg / g tissue

Isolation and Assay Of Amylase

Total enzyme activity =

$$\frac{conc\ of\ Std \times OD\ of\ sample \times Total\ Vol.Of\ extract}{OD\ of\ Std \times Vol.of\ sample \times Wt\ of\ tissue \times Incubation\ time}$$

$$= \frac{0.04 \times 0.405 \times 10}{0.062 \times 1 \times 1 \times 20}$$

= 0.1306 Eu / g tissue/ min

Isolation and Assay of Peroxidase

$$\text{Enzyme activity} = \frac{\Delta\ OD \times total\ vol\ of\ enzyme}{Vol.of\ sample \times wt.of\ tissue}$$

Δ OD = finalvalue – intial value

=0.352 – 0.238

= 0.114

$$\text{Peroxidase activity} = \frac{0.114 \times 20}{0.5 \times 1}$$

= 4.56 Eu /g tissue

Isolation and Assay of Polyphenol Oxidase

$$\text{Enzyme activity} = \frac{\Delta OD \times total\ vol.of\ enzyme}{Vol.of\ sample \times Wt\ of\ tissue}$$

OD = final OD – initial OD

=0.280 -0.125

=0.155

$$\text{Activity of PPO} = \frac{0.155 \times 20}{0.5 \times 1}$$

= 6.2 Eu /g tissue

ALLELOPATHIC STUDIES

The effect of aqueous extract of *S. molesta* on *V. radiata* seeds showed that it inhibited the germination of the seeds at higher concentrations (75% and 100%) while lower concentrations did not affect the germination of the seeds. The germination percentage were 92% and 86% at 75% and 100% concentrations of the extract while it was 100% germination at 25% and 50% concentrations of the extract which was same in Control (Plate 2).

Epicotyl and hypocotyl growth of *V.radiata* seedlings

Studies on the effect of aqueous extract of *S. molesta* on the length of epicotyl and hypocotyl of *V. radiata* seedlings showed that it affected them at higher concentrations through inhibition (Table 1).

Effect on biomass of *V.radiata* seedlings

The effect of aqueous extract of *S. molesta* on *V. radiata* in terms of their biomass showed that the fresh weight and dry weight of the *V. radiata* seedlings decreased with increase in concentration of the extract (Table 2).

Table 1. Effect of aqueous leaf extract of *S.molesta* on the growth of *V. radiata* seedlings

Treatments	Average epicotyl length (cm) ± S.D	Average hypocotyl length (cm) ± S.D
Control	14.5 ± 0.02	8.02 ± 0.01
25%	13.96 ± 0.11	5.653 ± 0.02
50%	12.83 ± 0. 05	5.413 ± 0.03
75%	12.54 ± 0.03	4.850 ± 0.01
100%	11.69 ± 0.01	4.330 ± 0.04

Table 2. Effect of aqueous leaf extract of *S.molesta* on the biomass of *V. radiata* seedlings

Treatments	Average fresh weight(g) ± S.D	Average dry weight (g) ± S.D
Control	2.578 ± 0.001	0.1920 ± 0.010
25%	2.512 ± 0.011	0.1636 ± 0.010
50%	2.087 ± 0.010	0.1443 ± 0.014
75%	1.889 ± 0.012	0.1413 ± 0.012
100%	1.656 ± 0.011	0.1343 ± 0.010

Pandey (1994), has studied on the relative effect of parthenium (*Parthenium hysterophorus* L.) plant residue on growth of *Salvinia* and paddy seedlings. The inhibitory activity of the residue as shown by its effect on the number of healthy fronds (HFN) and biomass was in the order: flower and leaf > stem and root. The flower (FP) and leaf (LP) residue was lethal at and above 0.75% (w/v, the convention used throughout), and inhibitory at lower doses. The stem (SP) and root (RP) residue supported growth of *Salvinia* at lower doses and were slightly inhibitory at higher (1.25%) dose. The study shows that *Salvinia* is more sensitive to allelochemicals released by FP and LP into the aqueous medium. The results are discussed with reference to the possible role of allelopathy by parthenium on the population dynamics of aquatic weeds in natural ecosystems.

SUMMARY AND CONCLUSION

Salvinia molesta D. Mitchell commonly known as water moss is an invasive aquatic weed native to Brazil. It poses serious threat to environment. This weed can be exploited for various purposes which demands correct understanding of the plant. With this in view, the work aims at biochemical screening and to study it's allelopathic effects on a common pulse *Vigna radiata* (green gram) since it is often used as organic fertilizer to crops.

Collection of *S. molesta* was done from different localities of Kollam district. Biochemical investigations were done using Standard procedures. The preliminary investigation of the allelopathic effect of aqueous leaf extract of the weed on a common pulse *Vigna radiata* were carried out under laboratory conditions.

Biochemical investigation showed protein 15.5 mg/g tissue, reducing sugar 0.22 mg/g tissue, phenol 0.79375 mg / g tissue, amylase activity 0.13 Eu/ g tissue/min, peroxidase activity 4.56 Eu/g tissue, polyphenol oxidase activity 6.2 Eu/ g tissue. The weed showed allelopathic inhibition at higher concentrations of aqueous leaf extract (75% and 100%) on the germination, growth and biomass of *Vigna radiata.* This shows that the leaf extract is non-toxic to the crop at lower concentrations. Similar studies has to be carried out on other crops to use them as biofertilizer. It's high protein content and phenol content addresses the need for further study on this weed for it's bioprospecting and exploitation for a sustainable environment and thus reduce pollution in wetland ecosystem.

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Phytochemical Profile and Radical Scavenging Activity of Alcoholic Extract of *Ophiorrhiza radicans* Gardner (Rubiaceae) – a Rare Plant of Southern Western Ghats of India

G. PRABHA[1] AND S. KARUPPUSAMY[2*]

[1]*Department of Biotechnology, Annai Velankanni College, Tholayavattam, Kanyakumari District – 629 157, Tamil Nadu, India.*
[2]*Department of Botany, The Madura College (Autonomous), Madurai – 625 011, Tamil Nadu, India.*
**email: ksamytaxonomy@gmail.com*

ABSTRACT

The presence of diverse secondary metabolites have been reported from *Ophiorrhiza radicans* by preliminary screening and GC-MS analysis of ethanolic plant extracts. From the qualitative screening, alkaloids, flavonoids and triterpenes are reported positively. GC-MS analysis showed that the presence of 11 different phytoconstituents with 91.6% of extracts and Methyl-E,E-2,13-Octadecandien-1-ol is a major constituents with 36.9%. DPPH radical scavenging *in vitro* model resulted the moderate radical scavenging capacity of ethanol extract of *O. radicans* and IC_{50} value is comparable with ascorbic acid standard. Further investigation on isolation, purification, charaacterization of phytochemicals and clinical studies of the consitituents will support to develop the novel and safe medicine for postrity.

Key words *Ophiorrhiza radicans; endemic plant; phytochemicals; antioxidant.*

The genus *Ophiorrhiza* L. is comprised about 307 species (The Plant List, 2013) distributed in wet tropical forests of South-East Asia and extends from Sri Lanka and Eastern India to China. In India, it is represented with 49 species (Deb and Mondal, 1997). Peninsular India, especially the Western Ghats is one of the diversity centre of *Opiorrhiza* species following the Western Himalayas. Nearly about 22 taxa are distributed in the evergreen forests of Western Ghats (Sasidharan, 2013). Among these, 18 are reported from Tamil Nadu state, and 14 are endemic to Western Ghats. Some species of *Ophiorrhiza* namely *O. mungos* and *O. pumila* are reported to have medicinal properties both in traditional and conventional medicine for treating snake bite, stomatitis, ulcers and wound healing (Kirtikar and Basu, 1975). Recently *Ophiorrhiza* species has reported as a source of the anticancer drug camptothecin in particularly from Western Ghats (Gharpure et al., 2010: Rajan et al., 2013), which has drawn great attention world wide as an anticancer drug and potential inhibitor of DNA topoisomerase-1 (Bodley *et al*., 1998; Uady and Kondapi, 2010; Krishnakumar et al., 2012). Camptothecin is also possessed clinical applications against anticancer, HIV-1 (Priel *et al*., 1991; Li et al., 2010), HSV-2 (Liu et al., 2010), parasitic trypanosomas and leishmania (Bodley and Shapiro, 1995). It is an indole alkaloid group of phytochemical reported from taxonomically unrelated families of Apocynaceae, Gelsemiaceae, Icacinaceae, Loganicaceae, Nyssaceae and Rubiaceae (Kurtan *et al*., 2014).

Antioxidants, molecules with a radical-scavenging capacity, are thought to exert a protective effect against free radical intercellular damage. Several phytomolecules may contribute to the prevention of many chronic diseases such as cancer, cardiovascular diseases, atherosclerosis, diabetes, asthma, hepatitis and arthritis (Formagio et al., 2014). The species of the genus *Ophiorrhiza* are not well explored so far that to establish the phytomedicine for challenging diseases like cancer and diabetes. In order to evaluate the phytochemical investigation and radical scavenging property of an endemic *Ophiorriza radicans* is aimed to carry out in the present study.

MATERIALS AND METHODS

Plant materials

Plant materials of *Ophiorrhiza radicans* was collected

From Megamalai Wildlife Sanctuary of Tamil Nadu and botanical identification authenticated from Botanical Survey of India (BSI), Southern Circle, Coimbatore, Tamil Nadu, India. The voucher specimen (SK368) was kept at the Sriganesan Herbarium (SGH), Department of Botany, The Madura College, Madurai, India. Aerial parts of the plant materials were air-tried in room temperature for two weeks before extraction.

Preparation of extracts

Shade tried plant materials was ground with electronic grinder and prepared coarse powder. 250 g

Fig. 1. Plant twig of *Ophiorrhiza radicans* collected from the Western Ghats

of plant powder filled with Soxhlet's thimble and 250 ml of ethanol as solvent taken in bottom flask for extraction. The Soxhlet's apparatus runs with 50^0C for 8 hr continuous reflux. The extracts removed from the Soxhlet's apparatus and concentrated under reduced pressure using a rotary evaporator with a temperature set a 40^0C. The crude plant extract then air-tried and stored in refrigerator for 15 days. After the crude extracts were reconstituted with 95% ethanol, and filtered through a Whatman No. 1 paper for further bioassay.

Qualitative phytochemical profile

Ethanolic extracts derived from whole plant of *O. radicans* were subjected to phytochemical screening for the preence of alkaloids, flavonoids, tannins, sterols, triterpenes, saponins and glycosides by standard methods (Evans, 2002).

GC-MS analysis

GC-MS analysis of the ethanol extract of *O. radicans* was performed using a Perkin–Elmer GC Clarus 500 system comprising an AOC-20i auto-sampler and a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with a Elite-5MS (5% diphenyl/95% dimethyl poly siloxane) fused a capillary column (30 × 0.25 ìm ID × 0.25 ìm df). For GC-MS detection, an electron ionization system was operated in electron impact mode with an ionization energy of 70 eV. Helium gas (99.9%) was used as a carrier gas at a constant flow rate of 1 ml/min, and an injection volume of 2 ìl was employed (a split ratio of 10:1). The injector temperature was maintained at 250 °C, the ion-source temperature was 200 °C, the oven temperature was programmed from 110 °C (isothermal for 2 min), with an increase of 10°C/min to 200°C, then 5 °C/min to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 0 to 2 min, and the total GC/MS running time was 36 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The mass-detector used in this analysis was Turbo-Mass Gold-Perkin-Elmer, and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass ver-5.2.

Identification of phytocomponents

Interpretation on mass-spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library. The name, molecular weight, and structure of the components of the test materials were ascertained.

DPHH – radical scavenging activity

The DPPH radical scavenging activity of *O. radicans* extract was performed following the method of Chigayo et al., (2016) with a few modifications. A volume of 500 mL of test extract at various concentrations (1–30 mg/mL) was mixed with 375 mL of 99.5% ethanol and 125 mL of DPPH solution (0.02%) as a free radical source. After that, the preparation was incubated for 1 h in the dark at room temperature. At the end, scavenging capacity was estimated spectrophotometrically by controlling the reduction in absorbance at 517 nm. In its radical form (purple color), DPPH has an absorption band at 517 nm which disappears upon reduction by an antiradical molecule (yellow colour). A good radical scavenging activity has been interpreted by decreasing it in mixture absorbance. Synthetic antioxidant, BHA was used as positive reference. DPPH radical scavenging activity was calculated as:

DPPH radical scavenging activity % = [(Ac"As)/Ac] × 100

Where, Ac is the absorbance of the control reaction, As is the absorbance of *O. radicans* ethanol extract. Tests were performed in duplicate. IC_{50} values were estimated by a linear regression.

RESULTS

The phytochemical profile of the ethanolic extracts of *O. radicans* are presented in Table 1.

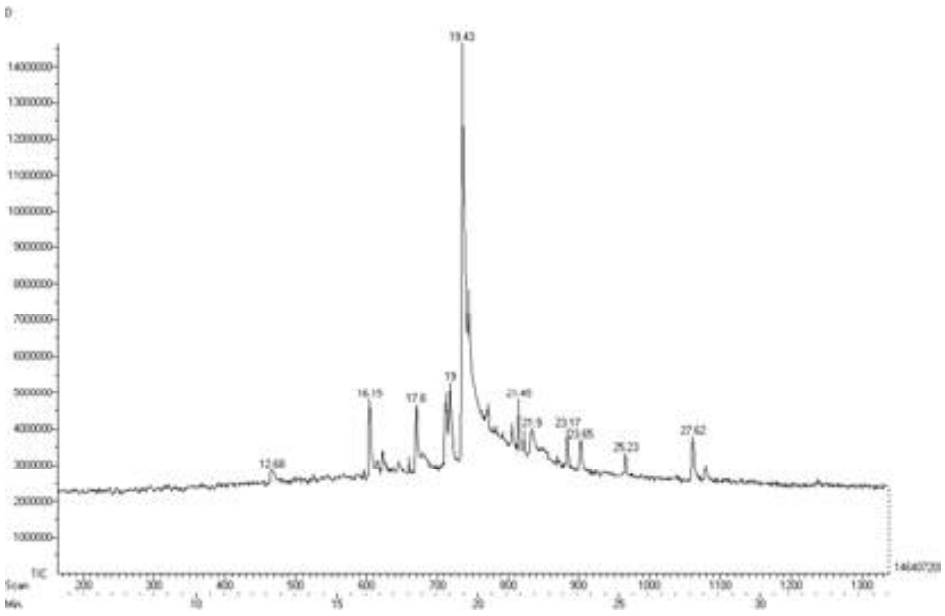

Fig. 2. Gas chromatogram of ethanol extract of *Ophiorrhiza tirunelvelica*

The plant extract was found to contain a greater number of secondary metabolite chemical groups. Alkaloids and flavonoids are represented in abundance whereas triterpenes were detected modereate amount. Sterols and tannins are resulted in little amount. Saponin and glycosides were not detected in the test extract.

A total of 11 constituents, representing 91.6% of the extract were identified by GC-MS analysis (Fig. 2).

It showed 12-Methyl-E, E-2, 13-Octadecadien-1-ol (36.9%), E-2-Tetradecen-1-ol (22.3%) and Cyclopropanedecanoic acid (12.3%) as major constituents. Moreover Phenol-2, 6-bis (1,1-diemthylethyl) (5.5%), 4-Methoxy-5,7-dihydroxy isoflavone (3.8%) and Ethyl oleate (2.9%) were reported in significant concentrations (3-6%). Whereas in other five phytochemical compounds were noted trace amounts in the extract (Table 2).

Table 1. Phytochemical profile of ethanol extract of *O.radicans*.

Phytochemicals	Sample extract
Sterols	+
Triterpenes	+
Flavonoids	++
Alkaloids	+++
Saponins	-
Glycosides	-
Tannins	+

+ present in little amout; ++ present in moderate amount; +++ present in abundance: - constituents not detected.

Radical scavenging activity of the ethanol extract of *O. radicans* were tested in free radical scavenging DPPH assay. The results are expressed as IC_{50} vlaues in µg/ml, the concentration of sample required to scavenge 50% free radicals (Table 2). The values exhibited a concentration dependent antiradical activity by quenching DPPH radicals and this activity was comparable to that of ascarbic acid standard. The marked radical scavenging activity of plant extract seemed to be due to the presence of phytochemicals.

DISCUSSION

Preliminary phytochemical screening revealed the presence of alkaloids and flavonoids whihc is similar to that of its related plant species *Ophiorrhiza mungos* (Madhavan et al., 2015). These secondary metabolites have been reported to exhibit several medicinal and physiological properties. GC-MS analysis provides significant additional information on the chemical composition of plant extract which are showing major chemical constituents possessed to have potential alkaloid and flavonoid precursors (Table 2). Many other species of *Opiorrhiza* have already been identified camptothecin yielding plants in Western Ghats as *O. mungos* and *O. prostrata* (Krishnakumar et al., 2012), *O. pumila* (Roja, 2006) and *O. rugosa* (Gharpure et al., 2010). The present study is also provided possible occurence of camptothecin in *O. radicans*. The divergent bioactivities of plants belonging to the same genus may due to a difference in phytochemical profiles across geographical regions.

Table 2. Chemical composition of ethanol extract of *O. radcans* analysed by GC-MS

Sl.No.	Name of compound	RT	%	Chemical formula
1.	E-2-Tetradecen-1-ol	1615	22.3	$C_{14}H_{28}O$
2.	12-Methyl-E, E-2,13-Octadecadien-1-ol 1443	36.9	$C_{19}H_{36}O$	
3.	Ethyl oleate	1228	2.9	$C_{20}H_{38}O_2$
4.	Cyclopropanedecanoic acid	1556	12.3	$C_{17}H_{15}O_4$
5.	9-Ocatadecanoic acid, 2 hydroxy ethyl	1394	1.6	$C_{20}H_{20}O_4$
6.	Isopropyl stearate	1541	1.3	$C_{21}H_{42}O_2$
7.	Docosa-Pentaen-22-al	1475	1.0	$C_{21}H_{40}O$
8.	Phenol-2,6-bis (1,1-diemethyethyl)	1441	5.5	$C_{14}H_{22}O$
9.	2,3-Dihydroxyprophyl elaidate	1394	1.6	$C_{21}H_{14}O_4$
10.	Eicosanoic acid ethyl ester	1452	2.4	$C_{22}H_{44}O_2$
11.	4-Methoxy-5,7-dihydroxy isoflavone	1402	3.8	$C_{16}H_{12}O_5$

It is known that variations in climatic conditions and soil types are major contributors to the differences in secondary metabolite profile of the plant species. Climatic and edaphic variations of Western Ghats served the habitat for potential metabolite producing plant species.

DPPH is a stable free radical at room temperature, which produces a violet solution in ethanol (Orech et al., 2005). It is widely used to evaluate the free radical scavenging effects of natural antioxidants. DPPH shows a strong absorption band at 517 nm in visible spectrum (deep violet colour). As the electron became paired in the presence of free radical scavenging, the absorption vanishes and the resulting discoloration stochiometrically coincides with the number of electrons taken up.

The bleaching of DPPH absorption is representative of the capacity of the test drugs to scavenge free radicals independently. It has been found that tocopherol, flavonoids, tannins and aromatic amines reduce and decolorize DPPH radicals by their hydrogen donating ability (Yokozawa et a*l*., 1988). In the present study, the DPPH radical scavenging activity of plant extract were dose dependent (Table 3). Based on the mechanism of reduction of DPPH radical described in the literature, that is correlated with the presence of hydroxyl groups on the antioxidant molecule, the present investigation can infer that *O. radicans* extract significantly scavenge the free radicals comparable to ascorbic acid (Table 3).

Table 3. DPPH radical scavenging activity of the ethanolic extracts of *Ophiorrhiza radicans*

Sample	Concentration (µg/ml)	Absorbance at 517 nm	% inhibition	IC_{50} (µg/ml)
Plant extract	3.125	0.739 ± 0.003	47.90 ± 0.02	4.3 ± 0.88
	6.25	0.571 ± 0.009	61.07 ± 0.30	
	12.5	0.340 ± 0.002	74.83 ± .011	
	25	0.219 ± 0.001	91.91 ± 0.32	
	50	0.091 ± 0.002	98.62 ± 0.03	
Ascorbic acid (standard)	1	0.584 ± 0.003	41.98±0.003	3.2 ± 0.15
	2	0.549 ± 0.002	52.72±0.006	
	4	0.454 ± 0.003	65.64±0.002	
	8	0.310 ± 0.006	81.91±0.003	
	16	0.231 ± 0.009	95.46±0.005	

Antioxidants with high scavenging activity should have a low IC_{50} value. This is supported by lowest value being exhibited by ascorbic acid, a well known antioxidant. This result is to be expected as crude extracts were used before purification. Results for purified extracts are expected to be much more closely related to those of ascorbic acid.

CONCLUSION

In our present investigation revealed that extracts of *O. radicans* are rich in phytochemicals particularly alkaloids and flavonoids. The results of radical scavenging activity that this plant have a powerful radical scavenging property. Our findings reinforce the potentials of *O. radicans* as a valuable source of natural antioxidants and support its medicinal uses in the treatment of many diseases.

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Antibacterial Activity of Ethanol Extract of *Aplidium multiplicatam* from Vizhinjam, South West Coast of Kerala

DIVYA. T. DHARAN[1] AND G. PRASAD[2]

[1]*Department of zoology Christian college, Kattakada*
[2]*Department of Zoology, University of Kerala,*
Kariavattom Campus, Thiruvananthapuram
email: dvyremya@yahoo.co.in

ABSTRACT

Ascidians are rich source of bioactive agent which could be used for novel antimicrobial drugs. Ascidians are belongs to phylum chordata and class ascidiacea. In the present study a compound ascidia *Aplidium multiplicatum*, collected from Vizhinjam, southwest coast of India was assayed for their antibacterial activity against six human bacterial pathogens. The antibacterial activity of ethanol extract of ascidians showed inhibitory activity against all six species. In the present study *A. multiplicatum* extract has showed promising source of antibacterial activity. From the tested bacteria, *P. aureus* was most sensitive against ethanol extract. In the screening of the antibacterial activity the highest activity and it was fractionated by normal phase silica gel column chromatography by employing a step gradient solvent system with increasing polarity. The solvents were concentrated by rotary evaporator with reduced pressure and used for further chemical investigations like GC-MS analysis. GC- MS study is the first step towards understanding the nature of active principles. Further studies are needed to the isolation, purification and structural determination of the chemical compounds responsible for the biological activities which may be lead to the discovery of drug molecules as chemotherapeutic agents in combating various diseases of mankind.

Key words *Ascidian; Antibacterial activity; Ethanol extract; Isolated pathogen; column chromatography*

The marine environment is a rich source of both biological and chemical diversity. The ocean cover more than 70% of the earth surface and the marine environment supports rich faunal diversity. Marine organisms would be wonderful sources of biologically active molecules (Kijjoa and Sawangwong, 2004). Because of their extreme environmental conditions like salinity, temperature, pressure etc. and biological factors like competitive and aggressive conditions demands the production of novel and potent active molecules (Srinivasan and Jayaprabha, 2013). Several molecules isolated from various organisms such as algae, fungi, bryozoans, molluscs and ascidians are currently under study at an advanced stage of clinical trials and some of them have already been marketed as drugs (Proksch *et al*., 2002). Marine invertebrates, especially ascidians are most prominent sources of new compounds with antimicrobial, anti-viral and cytotoxic potential.

Ascidians, commonly called sea squirts (subphylum: Urochordata, Class: Ascidiacea) are a prolific source of diverse bioactive metabolites and also interesting organisms from the view point of chemical ecology (Hongwei *et al*., 2004). The number of natural products isolated from marine organisms increases rapidly and now exceeds with hundreds of new compounds being discovered every year (Proksch *et al.*, 2002; Jain *et al.,* 2008). A large portion of these natural products have been extracted from marine invertebrates, especially from colonial ascidians and some of them are currently in preclinical and clinical trials (Proksch *et al.,* 2002).

The antibacterial activity of the compounds isolated from the colonial ascidian *Didemnum psammathodes* was reported by Ramasamy and Murugan (2003). An investigation was carried out by Ali *et al*., (2008) to analyse the bioactive compounds of *P. nigra* and *H. pallida* and also their possible antagonistic effects against several bacterial pathogens. Amutha *et al*., (2010) investigated the antimicrobial properties of marine biofouling ascidian extracts. A preliminary study was carried out by Ganesan *et al*., (2011) to analyse the antimicrobial activity of the tissue extracts of *P. indica* and *P. arabica* and were tested against ten human pathogenic bacterial strains. Bragadeeswaran and Thangaraj (2011) examined the haemolytic effecters in ascidians *P. madrasensis* and *P. nigra* collected from Tuticorin coast of India.

The antibacterial and cytotoxicity effectors in crude extract of ascidians from Tuticorin waters were investigated by Bragadeeswaran *et al*., (2010). According to their observation the crude extract of *P. nigra* showed highest antibacterial activity against *S. aureus* than other bacteria. The cytotoxic activities of *P. madrasensis* and *P. nigra* have been tested at

various concentrations and the results showed highest cytotoxic assay conducted, indicating the presence of cytotoxic compounds. Abourriche *et al*., (2003) analyzed the antibacterial activity from the crude extract of Atlantic ascidian *C. savignyi* against human pathogens. Biological activities are frequently observed in ascidian crude extracts include antibiotics against both human microbial pathogens and microorganisms (Mayer *et al*., 2007). Hence a broad spectrum screening of ascidians for bioactive compounds is necessary. The present study was carried out to investigate the antibacterial activity in crude extracts of ascidian *Aplidium multiplicatum* collected from Vizhinjam bay, south west coast of India.

Sack like filter feeder tunicates have been reported to be an important source in drug discovery. Dehydrodidemnin B and Didemnin B a cyclic depsipeptide isolated from *Tridemnum solidum* and *Ecteinascidia turbinate,* Didemnin B was the first marine compound to enter human cancer clinical trial as a purified natural product (Carte, 1996). Halocydiene was isolated as an antimicrobial peptide (3443Da) from the haemocytes of the solitary ascidian *Halocynthia aurantium* (Jang *et al*., 2002). A microbial antibiotic plicatamide, an octapeptide was isolated from the ascidian *Styela plicata* (Tincu *et al*., 2003). Styelin D is an antimicrobial compound isolated from the ascidian *Styela clave* reported by Taylor *et al.,* (2000). Biard *et al*., (1994) isolated a cytotoxic diterpenes called lissoclimetis from *Lissoclinum voeltzkowi*.

Selva Prabu *et al*., (2013) studied the isolation and purification of bioactive compounds from ascidians *Phallusia mammalian* from Andaman Nicobar Islands. Iyappan *et al.,* (2013) also studied the isolation and characterization of bioactive compounds from *Polyclinum madrasensis* from Palk bay, south west coast of India. Ascidians are renowned for their overwhelming bias towards the production of nitrogenous secondary metabolites. However, with the continued chemical interest in ascidians, an increasing number of non-nitrogen containing metabolites are being isolated. The objective of the present investigation is to identify the possible chemical constituents in the extract of ascidian with the aid of GC-MS spectral analysis.

MATERIALS AND METHODS

Specimen collection and identification

Ascidians were collected from the cement blocks, pilings and pearl oyster cages of Vizhinjam Bay (lat 8Ú22’35.95"N-76Ú59’16.40E”), by SCUBA diving at depth ranging from 4 to 6 m between June to November 2013. The samples were thoroughly washed with sea water, cleaned of sand, mud and overgrowing organisms at the site of collection and transported to laboratory and identified by standard keys of Kott (1985, 1989, and 2002) .

Extraction

The freshly collected samples (20g) were weighed and soaked in ethanol for one week and filtering through Whatman No.1 filter paper and the solvents were concentrated by rotary evaporator with reduced pressure to give a dark brown gummy mass. The resultant residues were stored at 4ÚC for further analysis. The extraction process was carried out by the method of Chellaram *et al*., (2004).

Microbial strains used

Antibacterial activity of tissue extract was determined against six different bacterial pathogens, viz*., Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi* and *Proteus mirabilis*. The clinical strains were obtained from MTCC microbial culture collection, Chandigarh.

Antimicrobial susceptibility assay

The antibacterial activity of the crude extract and concentrations of 25, 50, 75 and 100 mg/ml was done using disc diffusion method (Kirby and Bauer, 2000). Diameter of zone of inhibition was measured for determining the antibacterial activity. Streptomycin was used as a control (Sri Kumaran *et al*., 2011) and the extracts were tested in triplicate.

GC-MS analysis was carried out on a GC Clarus 500 Perkin Elmer system comprising a AOC-20i auto sampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument Identification of components

Interpretation on mass spectrum of GC-MS was conducted using the data base of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrums of the unknown

components were compared with the spectrum of the known components in the NIST library.

Statistical analysis

The results are expressed as mean ±SD of the three independent values.

RESULTS

Antibacterial activity of crude extract of *A. multiplicatum* against six human pathogenic bacterial strains was presented in Table1. Ethanol showed more antibacterial activity against all tested pathogens than acetone extract. In the present investigation, ethanol extract of *A. multiplicatum* showed high antimicrobial activity against both gram positive and gram negative bacteria. Among the tested bacteria *P. aeruginosa* (12.05±0.10mm) was the most sensitive bacteria against ethanol extract of *A. multiplicatum* and minimum zone of inhibition (2.16±0.13mm) was observed against *S. typhi.* The corresponding zones of ethanol extract produced a maximum inhibition zone of 10.35±0.72mm in *P. mirabilis* and minimum zone of 1.06± 0.12 mm was observed in *K. pneumoniae.*

In the present investigation, the antibacterial activity of ethanol extracts at different concentrations of *A. multiplicatum* against gram negative and gram positive bacteria was shown in Tables 5. 2 .High concentration of ethanol extract of *A. multiplicatum* showed strong antimicrobial activity against tested pathogens.

Table 1. Antibacterial activity of *A. multiplicatum* against human pathogens

Pathogens	Ethanol
Staphylococcus aureus	3.98±0.03
Salmonella typhi	2.14±0.13
Klebsiella pneumonia	0
Pseudomonas aeruginosa	12.05±0.10
Escherichia coli	5.15±0.14
Proteus mirabilis	4.07±0.22

GC-MS analysis of ethanol extract of *A. multiplicatum*

GC-MS chromatogram of the ethanolic extract of *A. multiplicatum* (Fig. 3) showed 17 peaks indicating the presence of 17 chemical constituents figures 6. 26 to 6. 42. The 20 active principles with their retention time (RT), molecular formula, molecular weight (MW) and peak area (%) in the ethanolic extract of *A. multiplicatum* are presented in tables 6. 23 to 6. 36 . The prevailing compounds are 4-Butylbenzoic acid, tridec-2-ynyl ester (41.7%), 5,8,11,14,17-Eicosapentaenoic acid, meth (20.8%), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahy(13.6%), Phenol, 3,5-dimethoxy-, acetate(10.0%), Vinylbital(8.3%), 4- and Methylimidazole-5-[1,1-dimethylethanol(6.0%) which may be involved in various biological activities like anti-microbial anti-

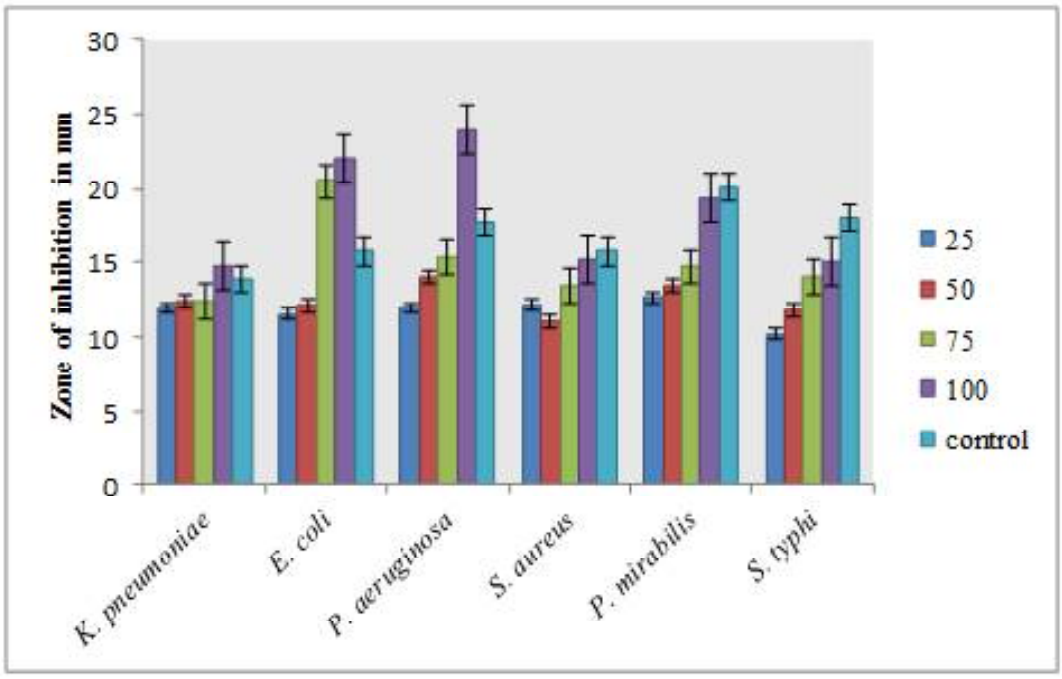

Fig. 2. Antibacterial activity of ethanol extract of *A. multiplicatum* against bacteria

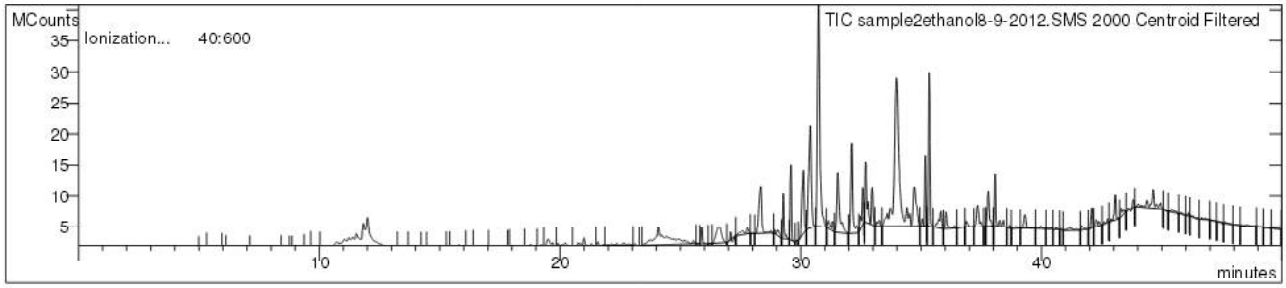

Fig. 3. GC-MS spectrum of ethanolic extract of *A. multiplicatum*

inflammatory, anti-parasitic, anti-carcinogenic etc. Of the 17 chemical constituents 16 are first reported from ascidians especially from *A. multiplicatum*.

DISCUSSION

Marine organisms have been found to produce a great diversity of novel bioactive secondary metabolites and are potential source of drug discovery. Extensive investigations of ascidians in chemical and pharmacological studies have been already reported. Several drug discovery projects have screened ascidians for antibiotic activities. Overall, ascidian extracts caused growth inhibition in gram positive and gram negative bacteria, indicating that these extracts do not selectively inhibit one group of microorganism (Thompson *et al.,* 1985). Here we have examined antibacterial activity ethanol extract of *A. multiplicatum* against gram positive and gram negative bacteria. In the present study *A. multiplicatum* extract has showed promising source of antibacterial activity. It showed high and moderate antibacterial activity against six pathogens assayed. From the tested bacteria, *P. aureus* was most sensitive against ethanol extract (12.05±0.10mm. Antibacterial activity has previously been detected in methanol and dichloromethane extracts of the ascidians *H. pyriformis* and a mixture of two Styela species (Lippert *et al*., 2003). Anand and Patterson (2002) reported that the ascidians *D. psammathodes* seems to be the promising source of antibacterial compound.

In ethanol extract at 100mg /ml concentration produced a maximum zone of inhibition against *P. aeruginosa* (24±0.035) followed by *E. coli* (22.03±0.035), *P. mirabilis*, (19.38±0.020) *S. typhi* (15.23±0.001) and *S. aureus* (13.106±0.060) and minimum zone of inhibition was exhibited by *S. typhi* (11.91±0.037) in 25mg/ml. Similar results were reported by Ravi Kumar *et al*., (2002). They observed that the crude diethyl ether extract of seaweed showed good antibacterial activity against both gram positive and gram negative bacteria. Regarding ethanol extract, maximum antimicrobial activity of 18.0±0.08 mm was found against *P. aeruginosa*.

. Bhosale *et al*., (2002) has reported the antibacterial property of marine organisms against biofilm bacteria isolated from test panels. The crude extracts of *Stichodactyla haddoni* showed good antibacterial activity against gram negative bacteria (Sureshkumar *et al.,* 2002). *P. aeruginosa* was the most susceptible bacteria for all tested extracts. Amutha *et al*., (2010) reported that the *P. aeruginosa* was the susceptible bacteria for all fractions of ascidian extracts with maximum zone of inhibition. This study corroborates with the previous report of Amutha *et al*., (2010). They reported that *P. aeruginosa* was most susceptible bacterium after treatment with all fractions tested.

Dowzicky and Park (2008) also reported that, UTI (Urinary Tract Isolates) bacterial pathogens have exhibited decreased susceptibility rates to tigecycline over the years. From the present results the ascidian extracts showed hopeful source of antimicrobial compounds towards isolated pathogens. The observed results strongly suggest that the *A. multiplicatum* extracts can be used as antimicrobial agent. These results indicate that ascidians exhibits remarkable antibacterial activity against microbes (Srikumaran *et al*., 2011).

Meenakshi *et al*., (2013) have been identified 20 different compounds using GC-MS analysis from the ethanolic extract of *Microcosmus exasperatus* compared to nine compounds from methanolic extract of *M. exasperatus*. The solvent selected for extraction plays an important role in identifying the chemical compounds. In this study ethanoilic extract of *A. multiplicatum* is rich in many bioactive chemical components with biological activities. A study of Pearce *et al*., (2007) reported anti-inflammatory activity of *Aplidium* species and a wide variety of biologically active compounds have been isolated

exhibiting anti -proliferative activity. Again Pearce *et al.,* (2007) isolated orthidines a dimeric product of tubsterine from the ascidian *Aplidium orthium.* A previous study of Pramitha and Sreekumari (2013) reported that GC-MS analysis of *Sargassum glilter* extract of ethyl acetate contain seventeen compounds and exhibited biological activities such as anti- tumour, anti- cancer and antimicrobial.

A. multiplicatum contains various bioactive compounds in ethanolic extract with various activities like antimicrobial, anti-cancer, diuretic, anti-inflammatory, anti- fungal, antioxidant, pesticide and chemo protective. The continuing and overwhelming contribution of ascidian metabolites to the development of new pharmaceuticals are clearly evident and need to be explored. Thus the current study revealed the presence of antibacterial activity from ascidians of Vizhinjam bay. Hence, further purification may lead to the discovery of novel antimicrobial compounds. Activities found in crude and purified extracts showed promising activities and with enormous potential for discovery, development and marketing of novel marine bioproducts and methods by which these products can be supplied in a way that will not disrupt the ecosystem or deplete the resources may be considered. It is worthy to note that the product from nature source is good for health and devoid of side effects. However, further investigations involving application of the extracts as drug for human administration need more research.

GC- MS study is the first step towards understanding the nature of active principles. Further studies are needed to the isolation, purification and structural determination of the chemical compounds responsible for the biological activities which may be lead to the discovery of drug molecules as chemotherapeutic agents in combating various diseases of mankind.

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Pro-antimetastatic Effect of Essential Oils from *Pogostemon benghalensis* (Burm.F.) and *P. cablin* (Blanco) Benth

D.P. PRADEEP[1*] AND MURUGAN K[2]

[1]*Plant Biochemistry & Molecular Biology Lab, Department of Botany, University College,*
[2]*Department of Biotechnology & Botany, Govt. Arts College, Trivandrum, 695 014, Kerala*
[*]*email : prempradeep358@gmail.com*

ABSTRACT

Biologist attempts in the search of novel antimetastatic agents of plant origin. Many studies have reported that plant based phytochemicals possess anti-proliferative activities against different malignant cell lines. Essential oils are proven for their therapeutic potential. In the present study, the anti-proliferative effects of of EOs from *Pogostemon benghalensis* and *P. cablin* on human colon cancer cell lines (SW 480) and human breast cancer (MCF-7) was carried. Essential oil was extracted from the fresh leaves of the selected species using Clevenger apparatus. The components were fractionated by gas chromatography-mass spectroscopy (GC-MS). Cytotoxic and viability of the essential oil against SW 480 and MCF-7 tumor cell lines were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Trypan Blue Dye Exclusion Method respectively. The GC-MS analysis revealed 41 and 36 volatile compounds from the leaf oil of *Pogostemon benghalensis* and *P. cablin* respectively. The essential oil from *P. beghalensis* showed cytotoxicity of 58.42% and 59.22 % against SW480, MCF 7 respectively at concentration of 100 µg. 57.47 and 58.77 % cytotoxic effect was noticed in EO from *P. cablin*. Similarly a significant reduction of the viability among SW 480 and MCF-7 cells was also noticed in a concentration dependent manner. The mechanism of action may be via induction of Caspase-3 activity as noticed in terms of µM PNA liberation/min/g protein by the oil. The volatile components of essential oil were probably responsible for cytotoxicity. Therefore, *Pogostemon* species might have a good potential for active antimetastatic agents.

Key words *Pogostemon benghalensis, P. cablin,* human colon cancer cell lines, human breast cancer cells, essential oil

Cancer is a multifaceted genomically complex disease and researchers have gradually showed that basic biological mechanisms involved were cell cycle arrest and apoptosis. The components of essential oil from different plants have got appreciation as antimetastatic data obtained from various cancer cell lines. Essential oil (EO) is the concentrated hydrophobic liquid with specific aroma produced by aromatic plants (Celiktas *et al*., 2007). These are also called as volatile oils and the constituents may be terpenes, aromatic compounds and other derived compounds of various origins. EOs are considered more potent than their constituents due to their synergistic and more selective effect (Ozka and Erdogan, 2011). EO was widely used in pharmaceutical, sanitary, cosmetic, agriculture and food industries for their bactericidal, fungicidal, virucidal, insecticidal and antiparasitical properties. Similarly their anticancer activity is well documented. Terpenoids, phytosterols, flavonoids, organic acids, lignins, alkaloids, glycosides, alcohols, aldehydes contains in the EO were responsible for its biological properties. In spite of much progress made in synthetic drug research, plants and their products are still considered to be the major sources of medicaments and have extensive use in the pharma industry (Harvey,2008) Most modern medicines were derived from plants and their products obtained by applying modern technologies to traditional practices (Sucher and Carles, 2008) *Pogostemon cablin* also known as patchouli is an important herb which possesses many therapeutic properties and is widely used in the fragrance industries. In traditional medicinal practices, it is used to treat colds, headaches, fever, nausea, vomiting, diarrhea, abdominal pain, insect and snake bites. In aromatherapy, patchouli oil is used to relieve depression, stress, calm nerves, control appetite and to improve sexual interest. In this scenario, the present study was undertaken to validate the pro-antimetastatic potential of EOs from *Pogostemon benghalensis* and *P. cablin.*

MATERIALS AND METHODS

Plant material

The plant material, *Pogostemon benghalensis* is collected from the Munnar hills of Kerala. *P. cablin* was from the medicinal garden of University College, Thiruvananthapuram.

Essential oil extraction

Fresh leaves of plants were cut into small pieces and were subjected to hydrodistillation in a Clevenger type apparatus for continuous 4 h to extract the

essential oil. The oils were dried over anhydrous sodium sulphate and stored in amber coloured glass bottles in deep freezer at -4^{0}C for further studies .

GC-MS Analysis

Gas Chromatography – Mass Spectrometry (GC-MS) analysis of the oil was performed by on a Hewlett Packard 6890 gas chromatograph fitted with HP-5MS capillary column coupled with a model 5973 mass detector. GC-MS operations conditions: injector temperature 220^{0}C, carrier gas- helium at 1.4 mL/ min. Individual components was identified by Wiley 275.L database.

Prometastatic studies

Cells and culture conditions

The cell lines used for the present study were Human colon cancer cell lines (SW480) and human breast cancer (MCF-7) were initially procured from National Centre for Cell Sciences (NCCS), Pune Cell lines were maintained Dulbecco's Modified Eagle's Medium, supplemented with 10% FBS and antibiotics.

Cell proliferation assay (MTT Method)

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a simple non-radioactive colorimetric assay to measure cell cytotoxicity and proliferation. Cells were seeded into a 96-well microtitre plate (5,000 cells/well). Cisplatin treated Cells were taken as positive control and another set maintained without any treatment as control. At specific time interval, the medium was removed and equal volumes of fresh medium were added along with 10 ìL MTT (2.5 mg/ml) to each well. The yellowish MTT is reduced to dark coloured formazan by viable cells only. The formazan crystals formed were washed out with MTT buffer. The colour developed was quantified with an ELISA plate reader .

Cell viability Assay using Trypan Blue Dye Exclusion Method

The cell lines were washed thrice times with ice cold PBS and checked the viability using Trypan Blue dye exclusion method .The cells were incubated at 37^{0}C with different concentrations of the EOs. For cytotoxicity assessment, 0.1 ml of Trypan Blue was added and the number of dead cells was determined using a haemocytometer.

Measurement of Caspase-3 Activity

Caspase-3 was estimated by Caspase-3 Assay Kit. Cells were treated EOs from both the plants species. Positive Control was treated with cisplastin. After the treatment, pelleted the induced cells and the control cells by centrifugation at 600× g for 5 min at 4º C. The supernatant was removed by gentle aspiration. The cell pellets were washed with 1ml of Phosphate Buffered Saline. The cells were centrifuged and the supernatant was removed completely by gentle aspiration. Suspended cell pellets in 1× lysis buffer at a concentration of 100 µl per 10^7 cells. Incubated the cells on ice for 15-20 min. Lysed cells were centrifuged and the supernatants were taken for assay .

Statistical Analysis

All the data were mean ±SD of six replication. The significance was considered as $P < 0.01$ and $P < 0.05$.

RESULTS AND DISCUSSION

Extraction and GC MS Analysis

The continuous hydro distillation of leaves of *P.benghalensis* and *P.cablin* yields 0.37% and 0.22% (v/wt) EOs with characteristic medicinal aroma. 41 and 36 volatile compounds were identified from the leaf essential oil of *P. benghalensis* and *P. cablin* through the GC-MS analysis. The major components of *P. benghalensis* were 1,8 cineole (7.14%) and aromadendrene (4.16%) followed by bornyl acetae (2.15%), longicyclene (2.74%), â –elemene (1.56%), trans-á-farnesene (1.56%), á patchoulene (2.39%), gurjunene (2.86%), valencene (2.21%), epi cubedol (1.03%), bicyclogermacrene (2.78%), trans â guaiene(1.26%), delta-cadinene (2.45%), spathulenol (1.16%), guaiol (1.79%), isolongifol (1.20%), cubenol-1-epi (1.56%), á murolol (1.45%), bulsenol(1.51%) and cadalene 8,9 epoxide (2.95%) . The predominant molecule in the the essential oil of *P.cablin* were â-patchoulene (8.45%), á-patchoulene (6.18%). The other major volatiles were á-pinene (2.38%), 1,8-cineole (4.21%), allo-aromadendre (2.17%), seychellene (1.84%), cis-â-guaiene (1.37%), á-selinene (4.36%), trans â-guaiene (1.03%), germacrene A (5.48%), á-bulnesene (1.42%), globulol (2.37%) and guaiol (2.47%).

Sundaresan *et al*., (2009) reported that the essential oil of *P. cablin* was rich in a-patchoulene (3.3%), â-patchoulene (4.2%), patchouli alcohol (23.2%) and á-guaiene (14.6%). Similarly Wu *et al*., (2004) also reported â-pinene, â-phellandrene, ä-

elemene, limonene, eucalyptol, 3-octanol, heptanal, nonanal, camphor, â-bourbonene, â-elemene, á-guaiene, 4-terpinenol, á-terpineol, á-patchoulene, â-patchoulene, á-caryophyllene, ä-guaiene, â-selinene, ä-cadinene, myrtenol, caryophyllene oxide, elemol, á-elemenone, globulol, epiglobulol, patchoulol, spathulenol, ledene oxide-(I), *cis*-farnesol and pogostone from patchouli oil. The patchouli plants collected from different cultivation regions and harvested at different times shows differences in their volatile oil compositions (Hu *et al*., 2006 and Silva *et a*l., 2004).

Hu et al., (2006) suggested that the chemical profile of patchouli oil from different regions were identified with nine compounds namely á-guaiene, â-guaiene, ä-guaiene, â-patchoulene, caryophyllene, seychellene, spathulenol, patchouliol and pogostone. The mentioned compounds were also present in *P. benghalensis,* but different concentrations.

Cell proliferation assay (MTT Method)

The MTT assay is deemed to be a versatile method and accordingly the viability of the cells could be evaluated upon various treatments. The MTT assay is a colorimetric assay for assessing cell metabolic activity. NADP (H)-dependent cellular oxido-reductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes were capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which yields a purple color. The EOs from both species of *Pogostemon* showed remarkable cytotoxic activity against the cell lines. The EO from *P.beghalensis* showed cytotoxic activity of 58.42% and 59.22 % against SW480, MCF 7 respectivly at concentration of 100 µg. 57.47 and 58.77 percentage cytotoxic effect was noticed in EO from *P. cablin.*

According to the reports of Greay *et al*. (2010) and Doll-Boscardin *et al*. (2012), terpinen-4-ol (IC50 = 50.2 ìg/mL), exhibited a cytotoxic efficiency in Jurkat cell line while á-pinene and ã-terpinene (IC50 192.4 and 136.6 ìg/mL, respectively) exhibited very low inhibitory effects against the cell line. The essential oil from *D. ammoniacum* showed cytotoxic effect on SW480 and MCF-7 cancer lines were with 625 and 312 µg/m L (IC50 value) respectively (Morteza *et al*., 2011). Essential oil from *Teucrium pseudochamaepitys* was found to possess cytotoxic effects on the HEp-2 cell line at 653.6 µg/mL concentration (Saoussen *et al*., 2015).

EO isolated from the *Neolitsea variabillima* exhibited significant cytotoxic activity against human colon cancer cell lines (Su et al., 2013). Similarly EOs from *Satureja khuzistanica* (Yousefzadi *et al*., 2014) *Casearia sylvestris* (Bou *et al*., 2013) *Cedrelopsis grevei* (Afoulous *et al*., 2013) and *Solanium spirale* (Keawsa-ard et al., 2012) significantly increased cytotoxicity of MCF7 cells in a dose-dependent manner. The MTT cytotoxicity test against HT-29 by Ginger and Thyme essentilal oil has revealed that 60µl/mL and 77l/mL concentrations showed significant toxic effect on HT-29 (Mohammad *et al*., 2016). The review showed a positive correlation was noticed from the EOs from *P. benghalensis* and *P. cabli* on cytotoxic effects against SW480 and MCF- 7 cell lines.

Cell viability assay using Trypan blue dye exclusion assay

The cell viability of SW 480 (colon cancer cells) and MCF 7(human breast cancer cells) were assessed by trypan blue method. Trypan blue is an energy-dependent dye exclusion viability testing method; the dye is being excluded from live cells. As trypan blue is a weak acid, its affinity is increased for basic proteins; nuclei uptake is generally higher due to the presence of histones, yielding marked blue intensity, whereas the cytoplasm remains faintly stained. This method helped to determine the percentage of viable and dead cells in the treated cells. The results are presented in Table 1. There was a dose dependent decrease in viability of the treated cells. In the positive control, the cells showed 44.56 % and 42.89 % viability for SW480 and MCF 7 respectively, whereas essential oil of *Pogostemon benghalensis* treated cells showed a range from 85.37 to 42.64% for SW480 and 81.2 to 41.25 for MCF7 at concentration 10 to 100 ìg/ml. Meanwhile *P. cablin* EO treated SW480 and MCF7 cell lines showed the respective values lies in between 88.82 to 45.78% and 86.47 to 43.87 for the similar range of concentration.

The decrease in percentage of viability becomes significant with all different concentrations of essential oil from *Pogostemon benghalensis* and *P. cablin* ($P < 0.01$). The net loss of viability at the higher concentrations in trypan blue assay indicated the cytotoxic effect of essential oil towards malignant cells. No other studies have been published previously revealing the influence of essential oil on viability of SW 480 and MCF 7. Similar results were also observed

Table 1. Cell proliferative assay using MTT and Trypan Blue Dye Exclusion Methods using the EOs from *P.benghalensis* (PB) and *P.cablin* (PC) against SW480 and MCF 7 cell lines.

Volume (µg/ml)	PERCENTAGE OF VIABILITY(MTT Method)		PERCENTAGE OF VIABILITY(Trypan Blue)	
	SW480	MCF 7	SW480	MCF 7
Control (Cisplatin)10µM	43.25	42.15	44.56	42.89
PB				
10	87.43	86.46	85.37	84.12
20	81.07	80.24	80.43	79.47
40	76.40	73.78	74.69	72.76
60	61.78	58.24	60.41	58.47
80	56.14	55.15	53.93	51.78
100	41.58	40.78	42.64	41.25
PC				
10	89.14	87.46	88.82	86.47
20	85.27	83.14	84.19	81.79
40	78.91	76.14	76.89	75.14
60	65.21	61.25	63.52	60.87
80	57.64	55.78	55.39	51.41
100	42.53	41.23	45.78	43.87

in *Rubus occidentalis* (black raspberry) extracts, they suppressed the proliferation of colon (HT-29), prostate (LNCaP), oral (KB, CAL-27), and breast (MCF-7) tumour cell lines and the cell viability decreased in a dose dependent manner The extracts of some *Rubus* species such as *R. jamaicensis*, *R. rosifolius, R. racemosus*, *R. acuminatus*, and *R. idaeus* also exhibited great potential to inhibit cancer cell growth in colon, breast, lung, and gastric human tumour cells (Jeong, 2010).

Measurement of Caspase-3 Activity

Caspase-3 is a caspase protein that interacts with caspase-8 and caspase-9. It is encoded by the CASP3 gene. The CASP3 protein is a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a key role in the execution-phase of cell apoptosis. As it is either partially or totally responsible for the proteolytic cleavage of many key proteins, such as the nuclear enzyme poly (ADPribose) polymerase (PARP) (Fernandes-Alnemri, 1994). There is significant level of Caspase-3 activity was noticed when compared to standard drug Cisplatin. The maximum value was 0.71 was reached at concentration of 100 µg for SW480 and 0.69 was noticed against MCF7 for the EOs from *P. benghalensis.* Similarly value of 0.68 and 0.65 were shown by the EO from *P. cablin* while drug gives only 0.64. The result in turn satisfies the prometastatic potential of EOs by stimulating the apoptotic activity in malignant cell lines (Table 2).

Caspase 2, 8, 9, and 10 have been classified as apoptotic initiator caspases and caspase 3, 6, and 7 as apoptotic effector caspases. On receipt of death signal, apoptotic initiator caspases are activated and results in the cleavage of downstream effector caspases. *P. benghalensis* and *P. cablin* essential oils up-regulated the levels of caspase-3 activity. Pancreatic cancer cell lines treated with essential oil of *Boswellia sacra* also exhibited similar patterns of the time-dependent caspase 3 activation (Xiao Nil *et al*., 2012). Lee (2016) also reported significant caspase and cytotoxicity activity of essential oil from *Zingiber officinale. Verbena officinalis* essential oil also induced significant apoptosis and activated caspase 3 profile in chronic lymphocytic leukaemia cells (Laura *et al*., 2011).

CONCLUSION

The present study revealed the Clevenger extraction method of essential oils from the selected *Pogostemon* species. This extract was found to exert

Table 2. Caspase 3 activity of the EOs from *P.benghalensis* (PB) and *P.cablin* (PC) against SW480 and MCF 7 cell lines.

Volume (µg/ml)	Caspase 3(µM PNA liberation/min/g protein)	
	SW480	MCF 7
Control(Cisplatin) 10µM	0.64	0.62
PB		
10	0.43	0.41
20	0.49	0.48
40	0.56	0.55
60	0.61	0.59
80	0.68	0.65
100	0.71	0.69
PC		
10	0.41	0.41
20	0.46	0.44
40	0.52	0.50
60	0.59	0.56
80	0.64	0.61
100	0.68	0.65

significant cytotoxic and non-viavility effects. Caspase-3 activity data substantiate the pro-antimetastatic potential of the oil. Overall, the biological activities observed in the study could be attributed to the versatility of the essential oil constituents of the species. Based on the findings of the present study, it was suggested that these EOs may be promising candidates for a novel chemo preventive or chemotherapeutic formulation of, perhaps, minimal side effects. Further studies are in progress to investigate the toxicity profile of EOs and it's in *vivo* anticancer activity.

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Physicochemical Analysis and Conservation Strategies for A Pond Emerged By Pamba Irrigation Project In Vazhakunnam, Pathanamthitta District

SAM ALEX[1] AND LEENA ABRAHAM[2]

[1]*Department of Botany, University of Kerala*
[2]*Department of Botany, St. Thomas College, Kozhencherry, Pathanamthitta, Kerala*
email : samapril30@gmail.com

ABSTRACT

Ponds have a small catchment size than rivers and lakes; this physical feature confers both benefit and disadvantage to ponds with respect to their protection. The objective of this study was to estimate the physicochemical characteristics of the selected pond and to suggest various conservation strategies based on the results. The collected water samples were subjected to various physicochemical analysis as per the standard methods prescribed in APHA, 1998. Some of chemical constituents such as pH (4.5±0.1) and Iron (0.49±0.3mg/l) content of the pond water exceeded the permissible limits as prescribed by World Health Organization (WHO) and the Bureau of Indian Standards (BIS). Microbial analysis showed the presence of pathogenic organisms like faecal Coliform (FC) and faecal Streptococcus (FS). All physicochemical investigations proved that the Vazhakunnam pond was under stress. And also this work proposes various reclamation and conversation strategies for this particular ecosystem.

Keywords *Pond ecosystem, APHA, Physicochemical analysis, Conservation.*

Water is one of the most abundant compounds in earth approximately covering three-fourth of the earth's surface. Majority of water available on earth is saline in nature; only a small quantity exists as fresh water. Fresh water has become a scarce commodity due to over exploitation and pollution (Ghose and Basu, 1968; Gupta and Shukla, 2006; Patil and Tijare, 2001; Singh and Mathur, 2005). Uncontrolled discharge of domestic waste water into the ponds has resulted in eutrophication of ponds (Pandey and Pandey, 2003). Physico-chemical properties (pH, conductivity, free CO2, COD, alkalinity, chlorinity-salinity, ions such as Na+ and K+) of water in any aquatic system are largely governed by the existing meteorological condition, and are essential for determining the structural and functional status of natural water. Presence of bacteria in the water body affects turbidity, taste, odour, and colour of the aquatic body. And also water quality clearly affects the primary productivity of any aquatic water body. Gupta & Day, 2012 opined that phytoplankton productivity is often the prime source of all the organic matter in an aquatic ecosystem.

The study of different physico-chemical parameters is very important for understanding the metabolic events in aquatic ecosystem. The parameters influence each other and govern the distribution and abundance of flora and fauna (Shinde et al, 2011). Therefore the aim of the present work is to analyze physico-chemical characteristics and microbial status of the pond and then to propose various reclamation and conversation strategies based on the results. It is also an initiative to fill the gap on dearth of studies on ponds.

MATERIALS AND METHODS

Study area

The Pamba Irrigation Project (PIP) aims the utilization of tail-race water of Sabarigiri Hydro Electric Project for irrigation purposes. For the construction of canal, many artificial ponds were created, of which present selected pond is one among them.

The selected pond ecosystem comes under Cherukole Panchayath and Cherukole Village of Kozhencherry Taluk in Pathanamthitta district (resurvey number 205/5). The study pond has a surface area of 93.86cent with an average depth of 5.36 feet. Presently, the pond is about 35 years old (Source: Revenue Department, Cherukole).

Sample collection

Water samples were collected from pre-decided locations of the pond in plastic container as prescribed by APHA (American Public Health Association,1998) during pre-monsoon season, and brought in the laboratory for analysis.

Physico-chemical analysis

Temperature, Turbidity, Electrical conductivity, Total Dissolved Solids (TDS), pH, Salinity was measured at sampling sites itself while the rest of the parameters were analyzed in the laboratory. The various physico-chemical parameters were analyzed as per standard procedures (APHA,1998).

Table1. Physico Chemical Quantification of Pond Water

Sampling Sites	Temperature (^{0}C)	Turbidity (NTU)	Electrical conductivity (µm/s)	TDS (ppm)
I	30.5	2.06	32.3	21.1
II	31.0	1.15	32.2	22.7
III	37.8	0.90	32.1	22.6
IV	36.7	4.08	30.2	20.6
V	38.4	3.01	27.5	20.6

Table 2. Chemical Quantification of Pond Water

Sampling Sites	Chloride (mg/l)	Phosphate (mg/l)	Nitrate (mg/l)	Iron (mg/l)	Sulphate (mg/l)	pH	Salinity (ppt)
I	30.7	0.03	0.1	0.06	1.0	4.64	24.8
II	18.4	0.01	0.3	0.7	1.5	4.63	23.5
III	21.3	0.01	0.5	0.9	1.3	4.47	24.5
IV	11.3	0.01	0.3	0.6	1.3	4.80	22.7
V	8.5	0.01	0.5	0.2	1.2	4.39	23.5

Table 3. MPN Index of Faecal Indicator Bacteria in the Selected Pond

Bacteria	MPN per 100ml of sample water					Mean MPN Index per 100ml
	I	II	III	IV	V	
Faecal Coliform (FC)	2400	11000	2400	2400	4600	4560
Faecal Streptococcus (FS)	90	11000	0	30	0	2224

Bacteriological analysis

The presence of faecal coliform (FC) and faecal streptococcus (FS) in the pond was done by MPN test.

Recommendations and suggestions

Based on the physical, chemical and biological investigations of Vazhakunnam pond, recommendations and suggestions were proposed. The conservation strategies were proposed after having detailed discussions with District Soil Conservation Office, Revenue Department and environmental experts of School of Environmental Sciences, Mahatma Gandhi University, Kerala

RESULTS AND DISCUSSION

Pond is a neglected ecosystem which needs immediate initiatives for its conservation. Among the physical parameters, only the turbidity levels were about to exceed the limits as prescribed by WHO and BIS Table 1 . All the chemical constituents were found to be within the limits as prescribed by WHO and BIS except pH and iron Table 2. Highly acidic pH of the pond water is believed to be stressful for the life forms of that ecosystem. The high Fe concentration was attributed to anthropogenic activities and land runoff. The presence of high concentration of Fe may also increase the hazard of pathogenic organisms; since most of them need Fe for their growth. The bacteriological study showed that the pond was highly contaminated with Faecal Coliform (FC) and Faecal Streptococcus (FS) Table 3.

All the physical, chemical and biological investigations cumulatively prove that the Vazhakunnam pond is under stress and need immediate planned conservation strategies in make it potable for human consumption or for pond fish culture.

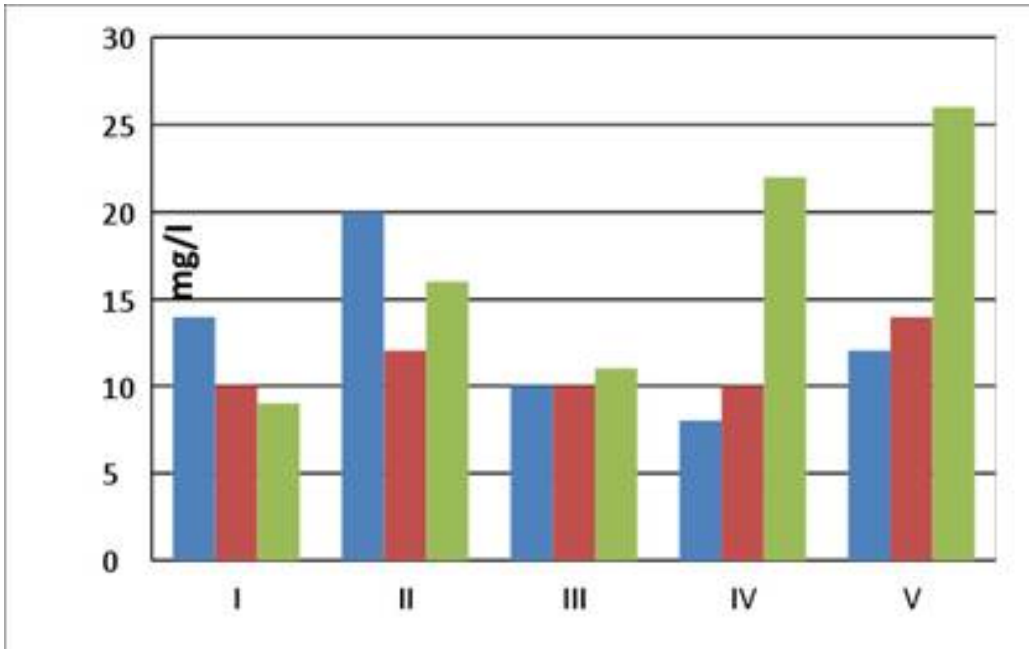

Fig. 1. Total Alkalinity, Total Acidity and Hardness of water

Strategy to make water potable

Physical/Chemical Methods include -Copper sulphate. Phosphorus inactivation products such as Aluminum sulfate (alum), sodium aluminate, calcium hydroxide, carbon dioxide. Common mechanical methods include-pumping of surface algal scums, aeration, barrier systems etc.Water Lily, Iris, Stratiotes, Scirpus (club-Rush), Sweet flag are capable of removing heavy metals, harmful bacteria, parasites, radioactive isotopes, chemicals etc.

Strategy to convert this pond to a fish culture pond

Fishes become stressed in acidic pH. The pH of the study pond was found to 4.5 which is stressful for the fishes. (Ideally aquaculture pond should have a pH between 6.5 to 9). Study pond showed lower levels of DO. Usually 5.0mg/l is essential to sustain the fish for long time. Some remedies to maintain the DO levels are artificial or manual beating of water and avoid overstocking of fishes.

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Microbicidal Potentialities of Purified Anthocyanin from *Osbeckia reticulata* Bedd; An Ethnic Medicinal Plant

BOSCO LAWARENCE[1], ARATHY R[1], MANOJ GS[2]& MURUGAN K[3]

[1]*Plant Biochemistry & Molecular Biology Laboratory, University College,*
[2]*Department of Botany, NSS College, Nilamel,*
[3]*Dept. of Biotechnology & Botany, Govt. Arts College, Thiruvananthapuram, 695 014*
email: boscotvm@gmail.com

ABSTRACT

Numerous studies have revealed that the flavonoid group of compounds like anthocyanins displays a variety of biological features such as antioxidants, antiproliferative, antiinflammatory and antimetastatic. In fact, antimicrobial potentiality related with anthocyanin is poorly documented. This study was attempted with an aim to evaluate the antimicrobial potential of purified anthocyanin from *O. reticulata*. Antimicrobial analyzes includes zone of inhibition, MIC, MKC and mode of action of anthocyanin on selected bacterial and fungal strains. The microbicidal potentiality was determined in the anthocyanin using agar disc diffusion method. The antimicrobial activities of anthocyanin (0.25, 0.5, 1, 1.5 and 2.0 mg/ml) of *O. reticulata* was tested against bacteria - *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa* and fungal species such as *Candida albicans* and *Aspergillusflavus*. Zone of inhibition of anthocyanin from *O. reticulata* was compared with standard chloramphenicol for bactericidal activity and griseofulvin for fungicidal activity. The results showed that the remarkable zone of inhibition of the bacterial growth with optimal MIC and MKC was noticed against the tested microbes. Depolarizing of cell membrane was revealed by significant efflux of potassium and the influx level of PI into cytoplasmic membrane explicit the antimicrobial mechanism of anthocyanin against the tested pathogens. Thus, the anthocyanin of *O. reticulata* may be used as bioactive natural products that may serve as lead molecules in the development of new pharmaceuticals research activities.

Key words *Osbeckia reticulata, in vitro antimicrobial activity, anthocyanin*

Plants are reservoirs of secondary metabolites such as saponins, tannins, phenols, terpenoids, alkaloids, flavonoids, and glycosides etc., which have been provedto have in *vitro* microbicidal potentialities. Herbal drugs have been used by man from the aboriginal periods. Therapeutic power of many indigenous species for diverse disorders has been documented by traditional practitioners (Walsh and Toleman, 2012). Antimicrobial features of medicinal herbs are being increasingly reported from different parts of the world. WHO revealed that ethnic plant species or their bioactive components were used as aboriginal crude drugs in local treatments of nearly 80% of the world's population in under developed countries (Awouafack *et al.,* 2013). The harmful controlled microbes with synthetic drugs resulted into the emergence of multiple drug-resistant bacteria (MDRB) and it has created adverse clinical issues in the treatment of diverse infections. The pharmaceutical industries have produced many novel antibiotics resistance drugs which indirectly increased the resistance pathogens number also. In general, bacteria have the genetic ability to transform and acquire resistance to synthetic drugs which are utilized as therapeutic agents. These MDRB enhance the morbidity and mortality rate due to drastic mutations in the related diseases (Srivastava *et al.*, 2013; Santiago *et al.*, 2015). Thus, the treatment options available are remarkably reduced. Certain strains of MDRB have also acquired sound virulence and increased transmissibility (Adamu *et al.*, 2014). For example, streptomycin and isoniazid drugs have previously proved effective against bacterial disease, but parallel the development of total drug resistance was also evolved rapidly (Makhafola and Eloff, 2012). Similarly, *S. aureus* became resistant to penicillin treatment relatively. Methicillin was introduced in the early 1960 in the defense against penicillinases, with the outbreak of methicillin-resistant *S. aureus* (MRSA) (Santiago *et al.*, 2015). In addition, the origin of MRSA within the community may be due to the massive use of bactericidal cleaning products in household and hospital. Likewise, the unscientific usage of microbicidal agents has led to the formation of many such super pathogens, and this need urgent care before MRSA like pathogens becomes more difficult to control.

The rising prevalence of antibiotics resistant pathogenic microbes in the last decades raises the need for finding new alternative antimicrobial agents with

less side effects. Therefore, the aim of current study was to evaluate the antimicrobial activity of some local *Osbeckia* species which have potential of treating infectious diseases. No studies have been carried out to evaluate the antimicrobial properties of these plant species till this date. The current study appears to be the first antimicrobial screening of anthocyanin from *Osbeckia* species. In general, these plants were used in traditional medicine in the treatment of skin diseases, gastrointestinal tract diseases and respiratory problems (Sujina*et al* 2012). The present study targets isolation, purification and fractionation of anthocyanin from cell suspension culture of *O. reticulata* followed by its antibiotic potentialities against selected pathogens.

MATERIAL AND METHODS

Osbeckia reticulata stocks were collected from Illikkal hill of Kottayam Dstrict, Kerala India. They were planted in pots maintained in shade house with drip irrigation facilities. It was difficult to establish the plants in green house, but once acclimatised, the new branches were formed frequently and were used for initiating culture.

The explants were subjected to surface sterilisation using 10% teepol solution, ethanol 70% (v/v) for 30 s and, mercuric chloride.After sterilisation inter nodal fragments and leaf cuttings were used for callus induction. During the entire process of*in vitro* culture, the plantlets were kept in 2.5 × 15 cm test tubes. The MS culture media was supplemented with vitamins, sucrose (30 gm /L), and agar (7 gm /L). The culture media pH was adjusted to 5.7 ± 0.1. Media were sterilized by autoclaving for 15 min at 120°C and 15 lbs of pressure

Protocol by Sutharut and Sudarath (2012) was used for the estimation of anthocyanin content in flowers. Aqueous acidified methanol and ethanol have been most commonly used in the extraction of anthocyanins. LC- MS/MS analyses were performed on an Agilent 6410A Triple Quad LC-MS/MS system.

Microorganisms

Fresh cultures of *Staphylococcus aureus* -ATCC 25923, *Enterococcus faecalis* -ATCC 29212, MRSA, *Escherichia coli* -ATCC 25922, *Pseudomonas aeruginosa* -ATCC 27853, and *Candida albicans* -ATCC 66027, *Aspergillusflavus* -ATCC 16888 were brought from Institute of Microbial Technology, Chandigarh.All test strains were successively re-isolated thrice on Mueller Hinton agar or potato dextrose agar medium to purifyand identity was confirmed by standard manuals (Ishaku Leo Elisha *et al.*, 2011). The size of the inoculum of each test strain was standardized according to the Committee for Clinical Laboratory Standards (Kiehlbauch*et al*2000). The strains were incubated for 3–6 h at 35 °C in a shaker water bath until the culture attained a turbidity of 0.5 McFarland Unit. The final inoculum was adjusted to 5×10^5cfu/ mL

Antimicrobial Activity

Determination of zone of inhibition method

In vitro antimicrobial activities were examined for purified anthocyanin extracts of *O. reticulata* against *Staphylococcus aureus*, *Enterococcus faecalis*, MRSA, *Escherichia coli* and *Pseudomonas aeruginosa* . The fungal species were *Candida albicans* and *Aspergillusflavus* were investigated by the agar disk diffusion method (Alastruey-Izquierdo*et al* 2015). Purified anthocyanin was dissolved in dimethyl sulfoxide, sterilized by filtration using sintered glass filter, and stored at 4°C. The sets of five dilutions (0.25, 0.5, 1.0, 1.5, and 2.0 mg/ml) of anthocyanin extract and standard drugs were prepared in double-distilled water. Mueller-Hinton sterile agar plates were seeded with indicator bacterial strains (10^8 cfu) and allowed to stay at 37°C for 3 h Control experiments were carried out under similar condition by using chloramphenicol for antibacterial activity and griseofulvin for antifungal activity as standard drugs. The zones of growth inhibition around the disks were measured after 18 to 24 h of in incubation at 37°C for bacteria and 48 to 96 h for fungi at 28°C. The sensitivities of the microbes to the anthocyanin extracts were determined by measuring the sizes of inhibitory zones (including the diameter of disk) on the agar surface around the disks, and values < 8 mm were considered as not active against microbes.

To determine MIC and MKC values, the methods of Dulger and Aki (2009) and Nakamura *et al.* (1999) were used with minor modification (Obeidat, 2011). The MIC was considered the lowest concentration of the sample that prevented visible growth. The MKC was defined as the lowest

concentration yielding negative subcultures or only one colony. All samples were examined in triplicate.

Potassium (K+) leakage

The efflux of potassium was analyzed using flame emission and atomic absorption spectroscopy following Edris *et al.*, method (2013). The solution was filtrated after contact with the phytochemicals. The samples were analyzed in a GBC AAS 932 plus device using GBC Avante 1.33 software.

Membrane integrity analysis using propidium iodide uptake

Simoes *et al.*, (2005).protocol was employed to assess membrane integrity of the selective stain by exclusion method using Live/Dead BacLight kit (Invitrogen). This is a rapid method employed to screen viable and total counts of bacteria (Sun *et al.*, m,2014).

Statistical analysis

The mean values were recorded as the Mean ± Standard Deviation (SD) and were analyzed using one-way ANOVA (SPSS 19.0 for Windows). Significance level was noticed as $p < 0.05$.

RESULTS AND DISCUSSION

Surface sterilization

O. reticulata sterilization protocol was optimized with minor modification using five different sets by adjusting the time and concentrations of sodium hypochlorite (1-5% for 5-10 min) and mercuric chloride (0.05 – 0.2% ,1-5 min). 1- 5% sodium hypochlorite for 5 min had no sprouting but further, gradually the duration of treatment was increased, which showed, 39.7% success rate. In contrast, $HgCl_2$ treatment yielded the highest number of viable cultures during surface sterilization. Among the different duration of treatments with 0.1% $HgCl_2$, the treatment for 5 min produced 97.7% contamination free cultures and yielded 96% aseptic plantlets. When treatment duration was more than 5 min, the survivability of the culture was decreased. Irrespective of minor drawbacks, $HgCl_2$was emerged as a potent surface sterilizing agent in modern plant tissue culture protocols.

Callus induction

Callus was induced on MS medium with different concentrations and combination of PGRs using leaf and inter nodal explants. The callus could be evaluated by biomass, the colour and nature of the callus. Suitable callus induction was on MS medium supplemented with 0.5-1.4 mg/L NAA and 1.0 mg/L to 1.2mg/l BA and yielded green (Fig.1a) and red compact calli (Fig.1b). Most of the friable callus were obtained from leaf explants. Taha and Wafa (2012) also produced different coloured calli in *Celosia cristata*. All the explants were able to produce reddish brown callus on MS medium containing 0.5 mg/L BA and 0.5 mg/L NAA.

Anthocyaninwas extracted from the callus tissues using a mixture of ethanol and water in the ratio 70:30 acidified with 1% HCl. Anthocyanins were isolated and purified by column chromatography in silica gel and subsequently using amber lite. Amber lite XAD7HP had the highest capacity and desorption ratio. As free sugar was the degradation factor for anthocyanins, the free sugar concentration before and after purification were tested and it was found decreased significantly.

LC-MS/MS analysis of purified anthocyanin from *Osbeckia reticulata* contain unique fractions of anthocyanins, *i.e*, malvidin, cyanindin and cyanidinaglycone.

Microbial activity

The antimicrobial activity was analyzed using purified anthocyanin extract of *O. reticulata* against *Staphylococcus aureus*, *Enterococcus faecalis*, MRSA, *Escherichia coli, Pseudomonas aeruginosa, Candida albicans* and *Aspergillusflavus*. Initially, the antibacterial and antifungal potential of purified anthocyanin extract was assessed in terms of zone of inhibition of microbial growth. The results of the inhibition were presented in the Table-1.

The antimicrobial activity of purified anthocyanin extract increased linearly with increase in concentration (mg/ml). As compared with standard drugs, the results revealed that *S. aureus* was more sensitive as compared with *E. coli* and *P. aeruginosa*, and for fungal activity, *C. albicans* showed sound result as compared with *A. flavus* with *O. reticulata*. The growth inhibition zone measured at 2.0 mg/ml concentration ranged from 14.9 to 26 mm for the bacteria, and 18.5 to 26.7 mm for fungal strains.

Table 1. Microbicidal activity of purified anthocyanin of *O. reticulata* determined by agar disk diffusion assay. Mean ± SD, $p< 0.05$

Conc. (mg/mL)	Inhibition Zone Diameter (mm)						
	E. coli	***P.aeruginosa***	***E. faecalis***	***S.aureus***	**MRSA**	***A. flavus***	***C.albicans***
0.25	2.54 ± 0.23	6.4 ± 0.12	1.75 ±0.08	9.31 ± 0.09	5.4 ± 0.07	2.2 ± 0.03	11.8 ± 0.51
0.5	4.5± 0.03	10.6 ± 0.9	4.5 ± 0.08	12 ± 0.05	9.5 ± 0.21	6.5 ± 0.20	13.2 ± 0.4
0.75	9.2 ±0.02	12.1 ± 0.07	6.5 ± 0.11	13.2 ± 0. 4	11.2 ± 0.23	9.3 ± 0.05	13.9 ± 0.02
1.0	13.7 ±0.05	19.5 ± 1. 5	11.3 ± 0.35	24.6 ± 0.47	15.3 ± 0.04	17.3 ± 0.26	25.4 ± 0.06
1.5	14 ±0.32	20.3 ± 0.12	11.7 ± 0.09	25.1 ± 1.25	15.6 ± 0.01	17.8 ± 0.22	25.8 ± 0.67
2.0	14.9 ±0.16	21.5 ± 0.09	12.9 ± 0.02	26 ± 1.48	16 ± 0.07	18.5 ± 0.94	26.7 ± 0.28

Significant antimicrobial effects, expressed as MIC and MKC of promising purified anthocyanin extract against test microorganisms are shown in Table-2.

Purified anthocyanin extract of *O. reticulata* was most active with the MIC values for bacterial strains ranging from 0.625 to 4 mg mL^{-1}. Among the tested strains, *E. faecalis* showed more resistance as compared to others with the MIC value 4 mg mL-1. The MKC values of the purified anthocyanin extracts ranged from 1.25 to 4 mg mL^{-1}; the lowest MKC for the tested bacteria and fungi were revealed by *S. aureus*and *C. albicans* respectively and was 1.25 and 1.0 mg mL^{-1}, respectively. Whereas, the highest MKC for *E. faecalis* was 4 mg mL^{-1}.

Effects of anthocyanin on intracellular potassium leakage:

The K^+ leakage analysis is used to pinpoint alterations of the membrane permeability of cells. The effects of purified anthocyanin on K^+ release among bacterial strains were shown in Table -3.

Table 3. Concentration of k+ (5ØßG/mL) in solution of selected bacteria after 1 h of exposure to MIC of anthocyanin. Mean ± SD, $p< 0.05$

Pathogens	K+ (??g/mL)
Staphylococcus aureus	0.297 ± 0.06
P.aeruginosa	0.468 ± 0.05
MRSA	0.57 ± 0.09
Escherichia coli	0.60 ± 0.03
Enterococcus faecalis	0.2 ± 0.01

0.297 ± 0.06 loss of intracellular K^+ was seen for *Staphylococcus aureus*cells treated with anthocyanin at the tested concentration. For *Enterococcus faecalis*, K^+ leakage was found as 0.2 ± 0.01.

Table 2. MIC and MKC of purified anthocyanin of *O. reticulata* (mg/ml) using disc diffusion assayMean ± SD, $p< 0.05$

Organism	MIC	MKC
E. coli	1.5 ± 0.04	3.0 ± 0.05
P.aeruginosa	0.75 ± 0.13	1.5 ± 0.07
E. faecalis	2 ± 0.49	4.0 ± 0.25
S.aureus	0.625 ± 0.01	1.25 ± 0.04
MRSA	1.0 ± 0.09	2.5 ± 0.46
A. flavus	1.0 ± 0.09	2.0 ± 0.5
C.albicans	0.5 ± 0.01	1.0 ± 0.06
Chloramphenicol	0.25 ± 0.04	0.5 ± 0.02
Griseofulvin	0.25 ± 0.07	0.5 ± 0.03

The viability and integrity of plasma membranes can be analyzed based on the ability of PI to intrude via cytoplasmic membrane. Commonly, PI only intrudes the cells with damaged cell membrane. In this manner, the potential of purified anthocyanin to interfere with membrane integrity after 1 h exposure was analyzed (Table 4). The PI uptake results suggest that anthocyanin reveal differential reaction with the integrity of the cytoplasmic membrane of the tested bacteria ($p< 0.05$). For *Staphylococcus aureus*the percentage of cells stained with PI after 1 h of treatment (at corresponding MIC) was 79.5 ± 0.19%. For MRSA exposed to anthocyanin the damage in cytoplasmic membrane was about 57.4± 1.2% of the total cells (Table 4).

Table 4. Permeability of bacteria to propidium iodide (PI) after 1 h of exposure to anthocyanin at their MIC. Mean ± SD, $p< 0.05$.

Pathogens	Permeability to propidium iodide (%)
Staphylococcus aureus	79.5 ± 0.19
P.aeruginosa	64.7 ± 0.15
MRSA	57.4± 1.2
Escherichia coli (-)	45 ± 0.08
Enterococcus faecalis(+)	32 ± 0.07

Mean values ± SD for at least three replicates are illustrated. $P< 0.05$.

Sun *et al.*, (2014) reported the plausibility of multi drug resistance (MDR) strains that cause antibiotic resistance via target alteration, drug inactivation, decreased permeability and increased efflux, drug extrusion by the multidrug efflux pumps. In the present study, anthocyanin effectively alters the membrane permeability and therefore scope for further analysis among MDR strains.

The test microorganisms exhibited optimal or high susceptibility to purified anthocyanin extract of *O. reticulata*. Therefore, the MIC and MKC were determined on purified anthocyanin extract of *O. reticulata* expecting promising antimicrobicidal activity. Interestingly, the antifungal MIC and MKC values of purified anthocyanin extract of *O. reticulata* was comparable with the synthetic drug. Similarly, the difference in susceptibility between the eukaryotic cells of *C. albicans* and the prokaryotic cells of bacteria might be attributed to their difference in cell type. This finding is in agreement with Awouafack *et al.*, (2013) who reported that *C. albicans* MIC and MMC values were higher than that obtained with bacteria. Lower MIC and MMC values and higher zones of inhibition for purified anthocyanin extract of *O. reticulata* implies the remarkable potentiality of the molecule.

Microbicidalpotentialioties of herbals are being increasingly reported from different parts of the earth. The WHO estimates that herbal extract or their active constituents were used as folk medicine in traditional therapies of 80% of the world's local population. In the present work, the purified anthocyanin extract obtained from *O. reticulata* show sound activity against most of the tested bacterial and fungal strains. The results were comparable with antibiotic drugs. In this screening work, extract of *O. reticulata* was found to be differential in activity against Gram-positive, Gram-negative, and fungal strains.

Nayan *et al.*,(2011) analyzed antimicrobial activities from leaf extracts of *Cassia fistula*. Alzoreky and Nakahara (2003) reported antibacterial activity of extracts from seleced edible plants commonly consumed in Asia. Maher Obeidat *et al.*, (2012) compared the crude extracts of some plant leaves used as edible. Leaf extracts of *Ocimumgratissimum* was analyzed for bactericidal effect on selected diarrhoea causing bacteria in Southwestern Nigeria by Adebolu and Oladimeji, (2005). Antibacterial, cytotoxic and phytochemical screening of traditional medicinal plants in Brazil was reported by Bouzada *et al.*, (2009). Leaf extyract of endemic *Stachyspseudopinardii* in Turkey was proved as antimicrobial by Dulger. and Aki, (2009). Elisha *et al.*, (2017) correlated antibacterial activity of nine plant extracts against *Escherichia coli.* Aliero and Ibrahim (2012) reviewed antibiotic resistance and the prospects of medicinal plants in the treatment of salmonellosis. Makhafola *et al.*, (2012) isolated ochnaflavone and ochnaflavone 7-O-methyl ether two antibacterial biflavonoids from *Ochnapretoriensis*. DieudonnéLemuhNjimoh *et al.*, (2015) reviewed antimicrobial activities of medicinal plant extracts and hydrolates against human pathogens to reverse antibiotic resistance. Lee and Choi HJ (2015) reported

antiviral and antimicrobial activity of medicinal plant extracts. Opinde *et al.*, (2016) evaluated antimicrobial power of crude methanolic leaf extracts from selected medicinal plants against *Escherichia coli*. Bipul Biswas *et al.*, (2013) analyzed antimicrobial activities of leaf extracts of guava against two Gram-negative and Gram-positive bacteria. Ncube *et al.*, (2008) assessed techniques of antimicrobial properties of natural compounds of plant origin. Cheesman *et al.*, (2017) designed new antimicrobial therapies by synergistic combinations of plant extracts/compounds with conventional antibiotics. Paulo *et al.*, (2010) studied the effects of resveratrol on human pathogenic bacteria. Taylor *et al.*, (2014) also demonstrated the effect of resveratrol as antimicrobial against *Propioni bacterium acnes*.Mahboubi and Bidgoli (2010) essential oil of. *Zatariamultiflora* and its synergy with vancomycin. Santiago *et al.*, (2015) confirmed the inhibition of penicillin-binding protein 2a (PBP2a) in methicillin resistant *Staphylococcus aureus* (MRSA) by combination of ampicillin and a bioactive fraction from *Duabangagrandiflora.*.

CONCLUSION

In the current study, the purified anthocyanin extract obtained from *O. reticulata* yielded higher MIC and MKC values. Thus, the present study justified the claimed uses of floral extract in the traditional system of medicine to treat various infectious disease caused by the microbes. However, further studies are warranted to better analyze the possible effectiveness of the extracts as antimicrobial agents. The present result requires further investigation in the potential discovery of new natural bioactive lead compounds.

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Seasonal Variation and Developmental Changes in the Biochemical Composition of Coconut Kernel in *Mohachao narel*, A Sweet Endosperm Coconut (*Cocos nucifera* L.) Population from Maharashtra

P. AJITH KUMAR[1] AND K. SAMSUDEEN [2]

[1]*Government Arts College, Thiruvananthapuram*
[2]*Central Plantation Crops Research Institute, Kasaragod*
email: ajithkumargck@gmail.com

ABSTRACT

***'Mohachao Narel',* a coconut variant characterized by sweet and soft kernel with less fibre content, has been reported from Guhaghar taluk of Ratnagiri district of Maharashtra State in India. Farmers of the area get a premium price for sweet endosperm nuts and the sweet kernel is mainly used for raw consumption. A total of 27 mother palms possessing nuts with sweet kernel have been identified in these areas. The number of nuts with sweet endosperm per bunch varies from 10 to 77 percent in these 27 palms. Thus two types of nuts (sweet and normal) have been developed in these palms. The solid endosperm, kernel, undergoes biochemical changes as it matures during its 12- month growth period after fertilization. The objective of this work was to study these biochemical changes in the coconut endosperm in relation to the maturity of the nuts and season of harvesting between and within the sweet and normal type nuts. The dried kernel or copra was analyzed for its oil, total sugars, reducing sugars, total protein, total free amino acid and fibre contents. Both the sweet and the normal nuts harvested in the post monsoon season had higher oil, total sugar and fibre contents, but lesser kernel protein and amino acid contents than the nuts harvested during pre-monsoon and monsoon seasons. Sweet nuts had higher levels of total sugar and reducing sugar contents, but less fibre and oil contents than the normal nuts. Total protein and amino acid contents were found to be similar in both types of nuts. Percentage of oil content of kernel, in both types of nuts, was increasing steadily from seventh to twelfth month of maturity. Both types of nuts were found to have a significant increase in soluble sugar with nut maturity, to a maximum in the tenth month, after which it fell significantly in the twelfth month. In both type of nuts, harvested at different seasons, reducing sugar was maximum in the seventh month, after which it decreased to a minimum by the tenth month, it again increased by the twelfth month. In both the types of nuts, there was a significant decrease in protein and free amino acid contents as the nut matured. But both type of nuts showed the same trend of increasing in fibre content as the endosperm matured.**

Key words *sweet kernel coconut; copra; season; maturity; oil; biochemical characters.*

Coconut is a versatile and unique plant. It bears fruit all year round. Due to its multifarious uses, coconut (*Cocos nucifera* L.) symbolizes an important plant for the rural communities in developing tropical countries since it provides the basis for food production and by-product utilization in addition to its uses in industrial processing (Persley, 1992). Every part of this 'tree of life' is beneficial to mankind in one manner or other, the most extensively used part being the endosperm and its derivatives. Coconut endosperm, which is hard and white in colour, is rich in proteins, amino acids, sugars, vitamins, minerals and growth factors. The endosperm is mostly used for extraction of coconut oil and culinary purposes. Studies show that every coconut cultivar has its own unique characteristic that may explain for the variances in the oil and other metabolites like carbohydrates, protein etc. in the endosperm and also the percent composition of fatty acids in the oil. This is attributed to several factors such as location and varietal differences (Laurels *et al.,* 2000) as well as maturity of the nuts (Balleza and Sierra 1976; Repellin *et al*., 1994) and time of the year the nuts are harvested.

Certain coconut palms produce nuts containing soft, jelly-like endosperm, called *Makapuno* in Philippines (Torres, 1937), which has been commercially exploited for product diversification especially in confectionary industries. Mutants similar to *Makapuno*-type have also been reported from other coconut-growing regions: Coco Gra (Seychelles), Kopyor (Indonesia), *Thairu* or *Nei Thengai* (India), Dikiri Pol (Sri Lanka), Mapharao Khati (Thailand), Sap (Vietnam), Niu Garuk (Papua New Guinea) and Pia (Polynesia) (Arunachalam and Rajesh, 2008). This *Makapuno* trait results in abortion of embryo and is known to occur because of the effect of lethal recessive gene (Zuniga, 1953). Similar types have been sporadically reported from India (Arunachalam and Rajesh, 2008).

Recently, another variant with sweet and soft endosperm, named *'Mohachao Narel'* has been reported from Guhaghar taluk of Ratnagiri district (N17° 28'55" to N17° 29'50" and E73° 11'03" to E73° 19'50") of Maharashtra State in India (Anitha Karun *et al*., 2010; Samsudeen *et al*., 2010). Twenty seven

Table 1. Percentage of oil content in sweet and normal nuts at various maturity stages, harvested in different seasons.

Nut Type	Maturity (month)	Season of harvest			Mean
		Pre monsoon	Monsoon	Post monsoon	
Normal	7	45.15	41.10	45.73	43.99
	8	48.75	42.35	51.65	47.58
	10	57.45	53.48	61.65	57.53
	12	63.46	62.23	68.04	64.58
	Mean	53.70	49.79	56.77	53.42
Sweet	7	15.60	18.65	19.15	17.80
	8	18.65	20.60	24.65	21.30
	10	23.58	25.10	28.20	25.63
	12	29.47	26.70	36.64	30.94
	Mean	45.15	41.10	45.73	43.99
Mean		37.76	36.28	41.96	38.67

$CD_{0.05}$ season = 4.48; Maturity = 10.22

such palms are there. These 27 tall palms, with the stem height ranging from 7 m to 27 m, are aged between 40 to 80 years and found randomly distributed in the population. The number of nuts with sweet endosperm per bunch varied from 10 to 77 percent in different palms and the sweet endosperm nuts get a premium price and are mainly used for raw consumption (Samsudeen *et al*., 2010). One cannot differentiate the sweet nuts from the normal type nuts without organoleptic test of the kernel. Studies on fruit component traits of sweet and normal nuts of this population revealed that nuts possessing sweet endosperm types had slightly less fruit and husked fruit weight compared to normal nuts. Shell thickness was similar in both, but the shell weight was more in normal types. Likewise, endosperm thickness was similar in both, but the endosperm weight was more in normal types. Copra weight and copra recovery was more in normal nuts compared to sweet endosperm types. Total soluble sugars (Brix values) were same in both type of nuts, but organoleptic test showed that water in sweet endosperm nuts was poor in taste (Samsudeen *et al*., 2012).

The solid endosperm of coconut undergoes biochemical changes as it matures during its 12- month growth period after fertilization. Proteins and carbohydrates that accumulate during seed development are not only essential reserves that support germination and early seedling growth in plants, but are critical to human and animals as a major source of food (William H. Vensel *et al*., 2005). The objective of this work was to study these biochemical changes in the coconut endosperm in relation to the maturity of the nuts and season of harvesting, between and within the sweet and normal type nuts.

MATERIALS AND METHODS

A field survey was conducted in the initial stage of the study, in Ratnagiri region of Maharashtra. A total of 27 mother palms possessing nuts with sweet kernel ('NSD') have been identified in this area. The identified palms were marked for further studies. In this study, as many mature nuts (12 months) as possible from each of the 27 palms and four nuts, each from two selected palms (NSD 2 and NSD 28) which showed consistently higher percentage of sweet nuts in all seasons, were harvested at 7, 8 and 10 months after fertilization, for three seasons: pre monsoon (March 2011), monsoon (July 2012) and post monsoon (October 2011). They were dehusked, broken and categorized into sweet and normal types, on the basis of organoleptic test. Nut collected from the individual experimental palms processed to copra samples. Two replicates of each treatment, each a composite of two nuts, were analyzed for oil, total sugar, reducing sugar, total protein, total free amino acid and fibre contents.

Percentage of oil was estimated gravimetrically, following the method of Sadasivam and Manikam

Table 2. Total soluble sugars (mg/gm dry weight) and reducing sugars (mg/gm dry weight.) in sweet and normal nuts at various maturity stages, harvested in different seasons.

Nut Type	Maturity (Months)	Season of harvesting					
		Pre monsoon		Monsoon		Post monsoon	
		Total soluble sugars	Reducing sugars	Total soluble sugars	Reducing sugars	Total soluble sugars	Reducing sugars
Normal	7	35.29	8.56	35.47	12.92	45.08	12.66
	8	42.98	6.85	41.57	10.35	60.38	8.31
	10	55.21	3.71	63.45	5.37	79.69	4.39
	12	49.22	5.14	46.56	8.73	70.67	6.33
Sweet	7	87.07	21.02	54.75	21.96	109.07	18.03
	8	61.41	14.43	56.56	20.13	119.73	18.44
	10	87.60	15.98	90.62	17.45	113.55	16.25
	12	90.24	17.99	103.53	23.11	109.57	20.89

(2008). Defatted (oil extracted) copra samples were kept in the oven, 70^0C for 1 hour and ground with mortar and pestle. From the powdered sample 250 mg. were taken for biochemical analysis. Spectrophotometric quantification of total soluble sugar was done by the phenol-sulphuric acid method (Dubois *et al*., 1956), reducing sugar by the method of Nelson - Somogyi (Somogyi, 1952), total soluble protein by using Bradford (1976) method as standardized by Naresh Kumar *et al*., in 2007; total free amino acids by the method of Lee and Thakahashi (1966) and crude fibre was calculated gravimetrically by the method described by Sadasivam and Manickam (2008). The mean, standard deviation and coefficient of variation were computed and the data was subjected to three- way ANOVA by using SAS.

RESULTS AND DISCUSSION

Oil content: The oil content of nuts harvested in the post monsoon season was maximum, followed by the pre monsoon and the monsoon seasons respectively, in both type of nuts. Oil content was much higher in normal type while very low in sweet type nuts. The difference in oil contents of nuts differing in maturity was significant at 5% level. Percentage of oil content of kernel, in both types of nuts, was increasing steadily from seventh to twelfth month of maturity.

Total soluble sugars and reducing sugars: Sweet type nuts had greater total soluble sugar content than the normal type in all seasons. Total soluble sugars of nuts harvested in the pre monsoon and monsoon seasons were on par, while that of nuts harvested in the post monsoon season was significantly higher. Both types of nuts were found to have a significant increase in soluble sugar with nut maturity, to a maximum in the tenth month, after which it fell significantly in the twelfth month. The reducing sugar content was more in nuts harvested in monsoon, followed by those in post monsoon season. Of the two types of nuts, sweet type had the highest reducing sugar concentration than the normal type nuts. In both type of nuts, harvested at different seasons, reducing sugar was maximum in the seventh month, after which it decreased to a minimum by the tenth month, it again increased by the twelfth month(Table 2).

Thus there was a significant inverse relationship in the trends of total soluble sugar and the reducing sugars in both types of nuts in all three seasons. It can be concluded that for maximum total soluble sugar content, the tenth month kernel is the best. The same trend of variation was reported in some selected cultivars/ hybrids of coconut by Shamina Azeez and John George (2004).

Protein, Amino acid and Fibre contents: Nuts harvested in pre monsoon season had greater protein and free amino acid contents, followed by those harvested in monsoon. In sweet and normal type nuts the concentration of both the metabolites was on par, irrespective of other variables. In both the types of nuts, there was a significant decrease in protein and free amino acid contents as the nut matured (Table 3). The decrease in protein and free amino acid contents with nut maturity reflects on their utilization for cell division and proliferation in the maturing of

Table 3. Total protein (mg/gm dry weight), total free amino acid (mg/gm dry weight) and fibre (% dry wt.) in sweet and normal nuts at various maturity stages, harvested in different seasons.

Nut Type	Maturity (Months)	Season of harvesting								
		Pre monsoon			Monsoon			Post monsoon		
		Total Protein	Total free amino acid	Fibre	Total Protein	Total free amino acid	Fibre	Total Protein	Total free amino acid	Fibre
Normal	7	93.42	12.29	14.25	88.91	11.97	12.92	47.37	12.19	16.39
	8	79.25	11.91	16.67	79.63	11.47	18.22	38.07	9.93	26.41
	10	71.93	10.71	20.19	63.04	10.07	22.38	27.20	8.83	28.42
	12	56.56	11.23	22.03	50.15	10.47	21.97	22.92	8.45	29.21
Sweet	7	73.15	12.67	10.72	77.49	12.44	11.40	34.69	11.44	9.99
	8	86.29	11.62	11.00	56.19	13.23	10.24	27.47	10.05	12.44
	10	69.95	12.40	9.78	54.02	11.51	10.44	28.22	9.35	12.30
	12	55.14	11.68	10.63	49.73	10.21	10.33	23.51	9.10	12.50

the kernel.

Nuts harvested in post monsoon season had greater fibre content in the solid endosperm than the other two seasons. Maximum fibre content was noticed in normal type nut than the sweet type one, irrespective of the seasons. But both type of nuts showed the same trend of increasing in fibre content as the endosperm matured (Table 3). Balleza and Sierra (1976), Repellin *et al*., (1994) who observed the same trend, are of the opinion that as the solid endosperm matures, more of the sugars are transformed into cellulose.

The results of the present study revealed the existence of wide variability in oil, total sugar, reducing sugar and fibre contents between the sweet and normal type of nuts , produced in the same palm. Concentration of total sugars and reducing sugars were greater but percentage of oil and fibre contents were much lower in sweet type of nuts, compared to that of the normal type nuts. The total protein and free amino acid contents of both types of nuts were found to be on par. A significant observation made in the present study was that both the sweet and the normal type nuts followed the same trend of variation in all the metabolites- total sugar, reducing sugar, protein and free amino acid – oil and fibre contents with respect to seasons and maturity. The oil content of nuts harvested in the post monsoon season was maximum, followed by the pre monsoon and the monsoon seasons respectively, in both type of nuts. Percentage of oil content of kernel, in both types of nuts, was found to be increasing steadily from seventh to twelfth month of maturity. Total soluble sugars of nuts harvested in the pre monsoon and monsoon seasons were on par, while that of nuts harvested in the post monsoon season was significantly higher. Both types of nuts were found to have a significant increase in soluble sugar with nut maturity, to a maximum in the tenth month, after which it fell significantly in the twelfth month. The reducing sugar content was more in nuts harvested in monsoon, followed by those in post monsoon season. In both type of nuts, harvested at different seasons, reducing sugar was maximum in the seventh month, after which it decreased to a minimum by the tenth month, it again increased by the twelfth month. Nuts harvested in post monsoon season had greater fibre content in the solid endosperm than the other two seasons. Maximum fibre content was noticed in normal type nut than the sweet type one, irrespective of the seasons. But both type of nuts showed the same trend of increasing in fibre content as the endosperm matured.

Seed nuts from identified palms were collected and a population was conserved at CPCRI, Kasaragod for further evaluation and possible utilization in the coconut improvement programme. Exploitation of sweet kernel trait will help in product diversification in coconut which will lead to profitability of coconut industry. The results can pave the way for devising strategies for conservation and management of the sweet kernel coconut population and their use in future breeding programmes.

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Accurate Selection of Reference Genes for the Normalization of Gene Expression Analysis by Real Time PCR during Organogenesis in *Nicotiana tabacum* L.

ANUJA T.J.[1], HAREESH.P.S.[1], A. GANGAPRASAD[1, 2]*, A. JAYAKUMARAN NAIR[1], P. R. SUDHAKARAN, G. M. NAIR[1]

[1]*Inter University Centre for Genomics and Gene Technology, Dept. of Biotechnology, University of Kerala, Kariavattom Campus, Thiruvananthapuram-695 581*
[2]*Department of Botany, University of Kerala, Kariavattom.*
**email: agangaprasad@yahoo.com*

ABSTRACT

***Nicotiana tabacum* serves as an excellent model system for studying complex biological processes in modern plant science. Organogenesis is an efficient technique for large-scale propagation and popularization of selected genotypes. A thorough knowledge about the molecular mechanisms and gene regulatory networks involved in organogenesis is essential in order to achieve improved *in vitro* plant regeneration and genetic transformation frequencies. This complex molecular machinery can be well studied through real-time reverse transcription PCR. The selection of appropriate reference genes as internal control for normalization during qPCR is a prerequisite for the accurate gene expression analysis. In this study we examined the expression stability of twelve housekeeping genes such as GAPDH, beta-tubulin, alpha-tubulin, HisH4, 18sRNA, L25, EF-1alpha, Actin, PP2A, CDC6, MCM3 and Ntubc2 were analyzed with respect to their use as normalizer in tobacco. The data were analyzed using Excel based softwares such as BestKeeper, geNorm, NormFinder etc. The analysis was done using the tool, GenEx and GraphPad Prism5 softwares. Organogenesis was induced and samples were collected during callus induction, shoot induction and root induction. The systematic analysis of qPCR data using different statistical techniques could identify the most stable internal reference gene for gene expression analysis. After the analysis of all the data using different statistical techniques, Ntubc2 was found to be the most stable internal reference gene for expression studies during organogenesis in *N. tabacum*.**

Key words *Nicotiana tabacum,* Organogenesis, mRNA, Reference gene, Normalization, Quantitative real time PCR

Nicotiana tabacum L. is a member of the solanaceae family which is used as a model plant and an extremely versatile system for all aspects of cell and tissue culture research. Majority of the discoveries in the field of plant cell tissue culture and molecular biology have originated from the experimentation with tobacco plants. Tobacco is widely grown across the world, but within the United States, not only is the production of tobacco a major contributor to the economy due to the production of tobacco products such as cigarettes and cigars, but tobacco has recently been considered as a potential biofuel crop. As a result, any genetic studies that are performed to help elucidate pathways in which tobacco can be genetically modified to grow in more extreme environments or with increasing oil production for biofuel use would be beneficial.

Organogenesis is the development of adventitious organs from undifferentiated cell mass in tissue culture by the process of differentiation. Plant organogenesis in *vitro* is an efficient technique for large-scale propagation and popularization of selected genotypes and has been extensively applied in plant genetic engineering, double haploid or polyploid breeding, and asexual reproduction of mutants or threatened species (Engelmann, F., 1991, Benson, E. E., 2000). However, this technique shows species dependence and genotype specificity (Barbier de Reuille et al., 2006; Dewan, A et al., 1992). So a thorough knowledge is required for understanding the molecular mechanisms and gene regulatory networks involved in organogenesis in order to achieve improved *in vitro* plant regeneration and genetic transformation frequencies.

qRT-PCR is one of the most sensitive and accurate method, that enables reliable detection and expression analysis of RNAs. Sensitivity of qRT-PCR depends on various factors of which the selection of best endogenous control or reference gene is an important strategy. For accurate and reliable gene expression analysis using quantitative real-time reverse transcription PCR (qPCR), the selection of appropriate reference genes as an internal control for normalization is crucial. Normalization requires the use of one or more reference genes (also called internal control genes) for which expression is constant and stable at different developmental stages, nutritional conditions or experimental conditions (Dheda, K. et al. 2005). Several studies have shown that the expression of commonly used HKGs varies considerably in different cells (Radonic, A. et. al. 2004; Czechowski, T. et al.,

2005, Tong, Z. et al., 2009); Meng, Y. et al., 2009) and tissues under different experimental conditions and, in these comparisons, they become unsuitable for qPCR data normalization (Rapacz, M. et al., 2012; Zhong, H. et al., 1999). So the present work was designed to identify and evaluate suitable reference genes for expression studies during organogenesis in tobacco.

MATERIALS AND METHODS

Plant Material

For the induction of callus, leaf explants of tobacco were aseptically placed on petri dishes containing basal MS medium (Himedia) supplemented with 2.0mg/L NAA and 0.2mg/L Kinetin and 0.2mg/L 2, 4-D (Husin, MA et al., 2005; Vinod, Kumar et al.,1988). Caulogenesis and rhizogenesis were further done with M. S medium supplemented with 0.3mg/L IAA and 2mg/L IAA respectively using callus as explants (Kavi Kishore, P. B. and Metha, A. R., 1983). Leaf explants from normal plants were taken as control samples. All the harvested tissues were immediately frozen by immersion in liquid nitrogen and stored at -80°C until further use.

Reference gene selection and primer designing

In this study, twelve mRNA reference genes such as GAPDH (Glyceraldehyde 3-phoshate dehydrogenase), beta-tubulin, alpha tubulin, HisH4 (Histone H4), 18sRNA, L25, EF-1alpha, Actin, PP2A (Protein phosphatase 2A), CDC6 (Cell division cycle 6), MCM3 (Minichromosome maintenance complex component 3), Ntubc2 (Ubiquitin-conjugating enzyme 2) were selected to evaluate the most stable internal reference gene for qRT-PCR normalization in tobacco. The reference genes for mRNA expression studies were chosen based on their previous use in tobacco (Dambrauskas et al., 2003; Gregor, W. S. and Sven, K. D. 2010). The transcript abundance of these genes using specific primers (Table 1) was analyzed during organogenesis in *N.tabacum*.

qPCR Analysis

Frozen samples were ground to a fine powder in liquid nitrogen using a mortar and pestle. Total RNAs were extracted from the respective samples of *Nicotiana tabacum* using the RNA Isolation Kit (Qiagen) according to the manufacturer's protocol. From each developmental stage, total RNAs were isolated with three biological replicates including control samples and complementary DNA (cDNA) for mRNA was synthesized using the cDNA synthesizing kit (NEB) following the manufacturer's protocol. Quantitative PCR was performed with the real-time PCR system, BioRad MJ Mini Thermal Cycler, using SYBR Green I double strand DNA binding dye. Reactions were performed in duplicate and in three biological triplicates.

Data analysis

Primer efficiency (E), correlation coefficient (R^2) and PCR amplification efficiency were calculated for all the selected primers. All PCR products were examined by melting curve analysis and in 2% agarose gel electrophoresis The Ct values from each of the candidate genes were input directly in the softwares and were used to calculate the most stable reference genes. The packages such as geNorm, NormFinder and Heatmap are incorporated to use in a web-tool (GenEx ver 6), [genex.gene-quantification.info] which provides the assessment of the most stable reference gene. The BestKeeper provide the necessary information about data processing were done from http: //www.wzw.tum.de/gene-quantification/bestkeeper.html. These web-tools use only raw Cq values as raw input. In addition to this box plot analysis of data was performed using prism5 software.

Results

Samples, which kept in specific M.S medium, were collected in days such as 20th day during callus induction, 12th day during shoot induction and 8th day during root induction (Fig.1). The integrity of the isolated RNA samples was assessed by 1% Agarose Gel Electrophoresis and quantified using biophotometer (Eppendorff).

Amplification efficiency of candidate reference genes

PCR amplification products were obtained for all genes but CDC6 and MCM3, which were excluded from analysis as it displayed consistently high Ct values (> 30) and failed to amplify in some samples in which all other genes amplified, suggesting it is not expressed in sufficient quantity to be used as an effective housekeeping gene in these specimens. Each candidate reference genes were selected for normalization of mRNA expression studies in different samples of tobacco. The PCR amplification efficiency

Table 1. List of real time primers used for this study

Gene	Sequences (5'……3')
GAPdH	F- GCACTACCAACTGCCTTGCACCT R- GGCAATTCCAGCCTTGGCATCG
PP2A	F- GTGAAGCTGTAGGGCCTGAGC R- CATAGGCAGGCACCAAATCC
CDC6	F- GAGAAGCAGCAGCCAGCAGGCACG R- GCACATAGTTCCAATGCTTGAGGC
Ntubc2	F- CTGGACAGCAGACTGACATC R- CAGGATAATTTGCTGTAACAGATTA
Histone H4	F- GGCACAGGAAGGTTCTGAGGGATAACA R-TAACCGCCGAAACCGTAGAGAGTCC
L25	F- CCCCTCACCACAGAGTCTGC R- AAGGGTGTTGTTGTCCTCAATCTT
EF-1alpha	F- TGAGATGCACCACGAAGCTC R- CCAACATTGTCACCAGGAAGTG
Actin	F-CCTGAGGTCCTTTTCCAACCA R-GGATTCCGGCAGCTTCCATT
Alpha- tubulin	F-CAAGACTAAGCGTACCATCCA R-TTGAATCCAGTAGGGCACCAG
Beta- tubulin	F-GCATCTTTGCGTACACTTTGCT R-ACATAAGCCCAAACTAGCTGGA
18srRNA	F-GGTGGAGCGATTTGTCTGGT R-CAGGCTGAGGTCTCGTTCGT
MCM3	F-CTGCGTCTGGTCAGCTCCCAAGAAC R-CTGTCAGCAAGAACCATTGCCCCAGC

for each pair of primers was determined by qRT-PCR as described above. In this test, efficiency values were in the range of 91% to 108% and R^2 values > 0.980 for each primer pairs (Table 2). Based on melting curve analysis and agarose gel electrophoresis, the specificity of all the primers was detected. All primer pairs amplified with a single band on a 2% agarose gel after 45 cycles of amplification and a single major peak on melt curve analysis in all samples of tobacco. This result showed the high quality and specificity of the PCR reaction.

Expression profiling of the candidate reference genes

The candidate reference gene displayed quite different expression levels from each other during organogenesis, with Ct values spanning between 21 & 30.21 (Fig.2). HisH4, Ntubc2, PP2A, etc showed minimum expression variation with standard deviation 0.41, 0.52, 0.47 respectively. Expression of the candidate genes varies considerably during organogenesis.

Table 2. Amplification efficiency of each candidate reference genes

Reference genes	PCR amplification efficiency (%)	R^2
actin	93.07	0.988
18srRNA	92	0.99
Alpha- tubulin	91	0.979
Beta- tubulin	92	0.987
EF- 1alpha	91	0.9941
L25	93.1	0.988
HisH4	91	0.979
PP2A	92.5	0.9876
GAPdH	108	0.9893
Ntubc2	91.2	0.99

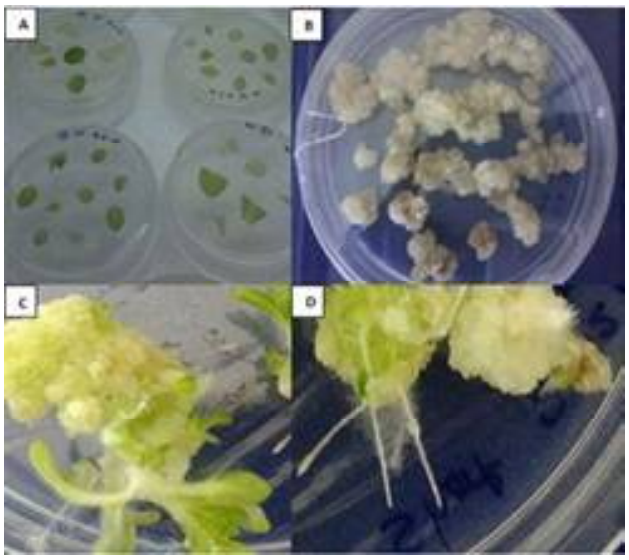

Fig.1. Induction of organogenesis. A- Tobacco leaf explants inoculated on callus induction medium (0th day). B- Callus induction (20th day post inoculation). C- Shoot induction from callus (12th day post inoculation). D- Root induction from callus (8th day post inoculation).

Stability Ranking of Candidate Reference Genes

GeNorm anaysis

Using GenEx software geNorm analyses were done which ranked genes based on their average expression stability (M). The candidate gene possessing the lowest M value is the most stably expressed gene in that set. In the case of mRNA reference gene set Ntubc2 and PP2A have the lowest stability value or M value (0.312) followed by actin (M value, 0.421) while GAPdH (M value, 1.09) and beta- tubulin (M value, 0.851) have the highest M values (Fig.3). Candidate reference genes were ranked using the M values of genes and hence from geNorm analysis it was found that Ntubc2 can be used as best reference genes for qRT-PCR during organogenesis in tobacco.

Analysis by NormFinder

NormFinder calculates stability values for each reference gene according to inter- and intra-group variation in expression. As in geNorm, NormFinder ranked genes based on their stability value (SD), in which the candidate gene possessing the lowest SD value is considered as the most stably expressed gene. Results obtained from this algorithm showed that Ntubc2 had the lowest SD value (0.0.156) followed by actin (SD value, 0.189) and PP2A (SD value 0.244) while GAPdH (SD 2.17) and beta- tubulin (SD 1.607) were having the highest SD values (Fig.4). From this analysis, it was found that Ntubc2 followed by PP2A can be used as best reference genes for qRT-PCR based expression analysis during organogenesis in tobacco.

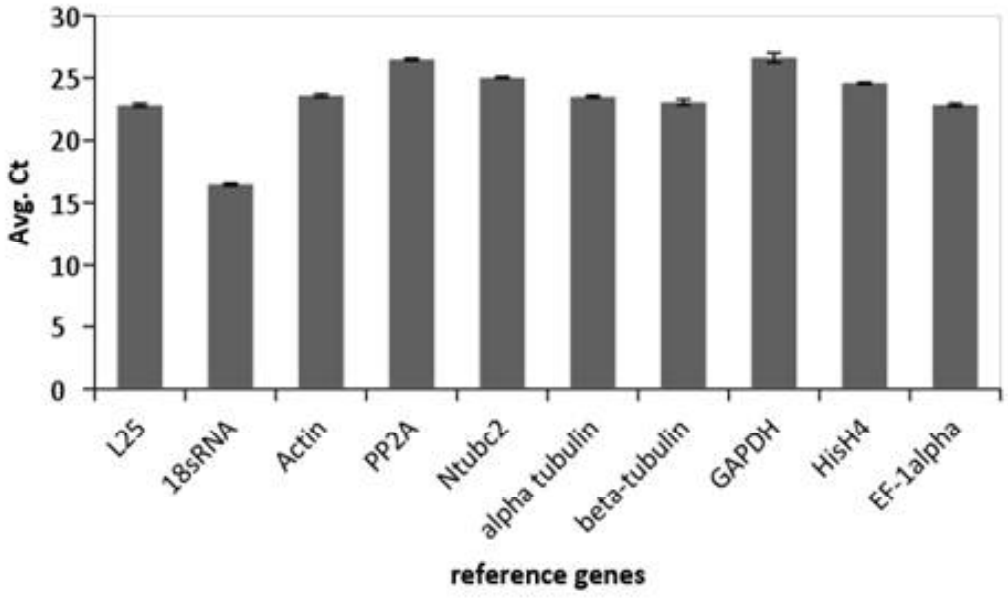

Fig.2. Expression profiling of reference genes based on average Ct values. The bars indicate the standard error (<0.5, n=3).

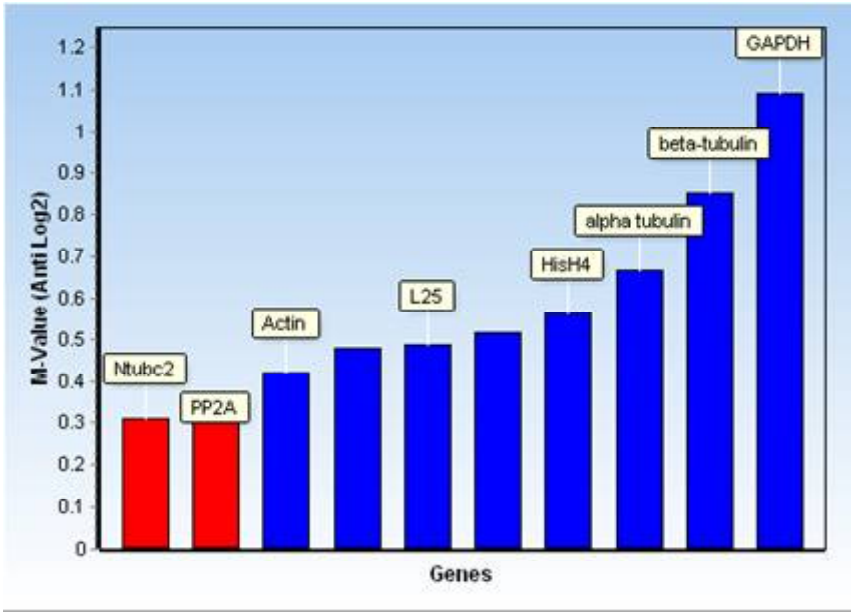

Fig.3. Expression stability and ranking of reference genes as calculated by GeNorm in all samples, done using GenEx software. A lower value of average expression stability indicates more stable expression (n=3).

Best Keeper analysis

The algorithm calculates BestKeeper correlation coefficient (r) and predicts gene stability based on high BestKeeper correlation coefficient. The candidate genes were ranked according to their 'r' values. Here Ntubc2 have highest Bestkeeper correlation coefficients (0.908, 0.873) while alpha- tubulin and beta- tubulin have the lowest values (0.079, 0.076) (Fig.5). Results showed that like in geNorm and Normfinder Ntubc2 can be used as best stable reference gene.

Box plot

The range of Ct for each reference gene is shown in the boxplot (Fig.6). The boxplots highlight the variation in expression levels of the reference genes investigated under organogenesis. The genes with low Ct values or high expression and comparatively short range of box plots are considered as most stable reference gene. Result analysis from the box plots inferred that Ntubc2 can be used as reference genes during organogenesis in tobacco.

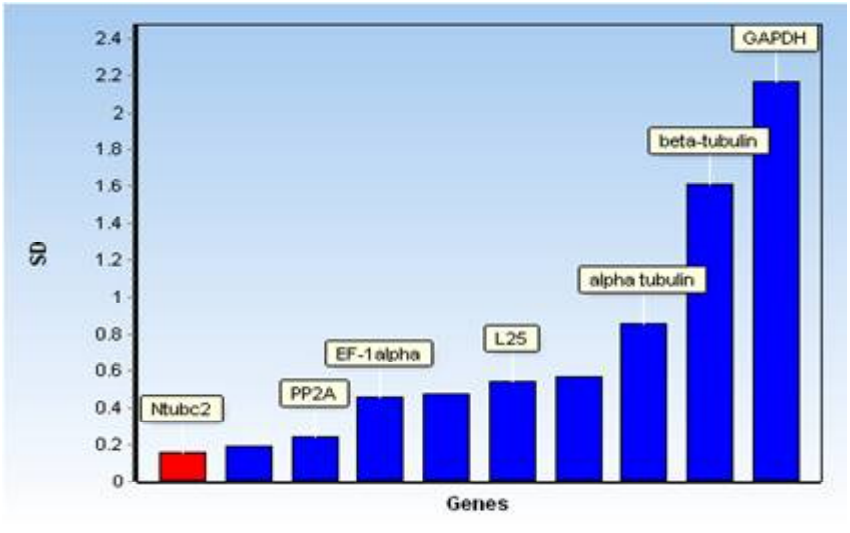

Fig. 4. Expression stability and ranking of reference genes based on stability value, SD as calculated by NormFinder in all samples done using GenEx software. A lower value of stability indicates more stable expression (n=3).

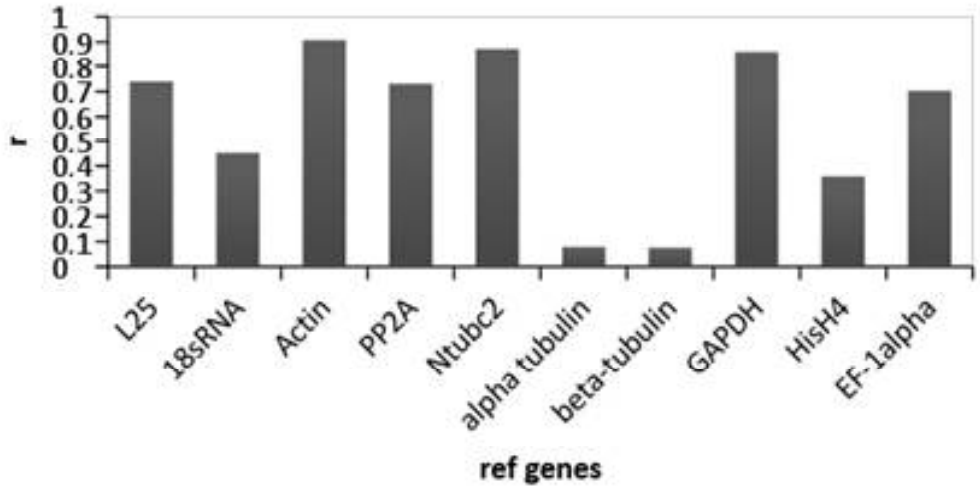

Fig.5. Expression stability and ranking of reference genes based on coefficient of correlation, r as calculated by BestKeeper in all samples. A higher value of r indicates more stable expression (n=3).

Validation of reference genes using different softwares indicates that Ntubc2 is the most appropriate reference genes that can be used as normalizer for qRT-PCR during organogenesis in *N. tabacum* (Table.3).

DISCUSSION

Selection of appropriate reference gene is an important factor for gene expression studies using qRT-PCR. Usually the expression of commonly used reference gene may vary with each set of experimental conditions. So the random selection of controls may lead to the misinterpretation of qPCR results (Ohl, F. et al., 2006; Brattelid, T. et al., 2010). A suitable reference gene should be stably expressed regardless of the experimental conditions and it is difficult to find a single reference gene especially in plants as its expression varies with biotic and abiotic stresses, during developmental stages, etc (Schmidt, G. W. and Delaney, S. K. 2010). In this study the selected reference genes were evaluated to determine the best one instead of choosing arbitrarily.

GeNorm calculates measurement of variation, Normfinder calculates stability deviation whereas Best

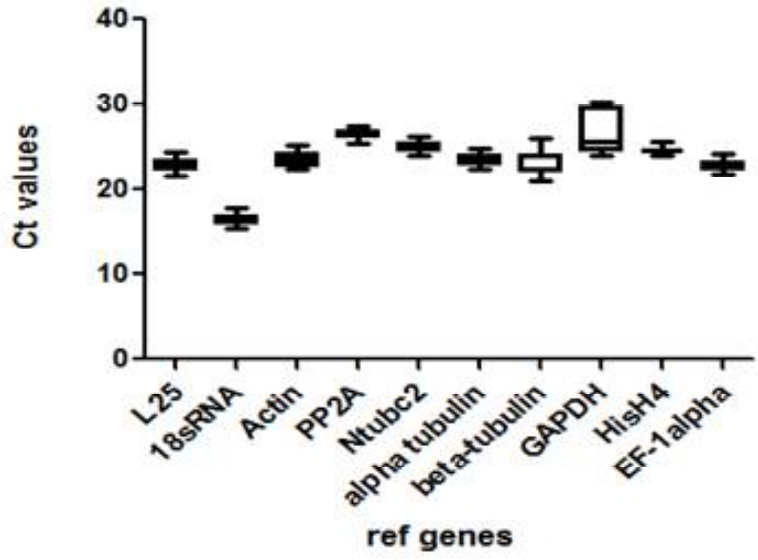

Fig.6. Box-plot diagram showing Ct-value range of the reference genes. Box plot analyses were done using Graph Pad Prism6 software (n=3).

Table 3. Stability ranking of reference genes based on each softwares

Sl. No.	GeNorm	NormFinder	BestKeeper
1	Ntubc2	Ntubc2	Actin
2	PP2A	Actin	Ntubc2
3	Actin	PP2A	GAPdH
4	EF-1alpha	EF-1alpha	L25
5	L25	HisH4	PP2A
6	18sRNA	L25	EF-1alpha
7	HisH4	18sRNA	18srRNA
8	Alpha-tubulin	alpha tubulin	HisH4
9	beta-tubulin	beta-tubulin	Alpha- tubulin
10	GAPDH	GAPDH	Beta- tubulin

keeper calculates coefficient of correlation.Vandesompele et al. (Vandesompele, J. et al., 2002) defined the stability measure Mj of a given gene (j), as the mean of all pairwise variations Vjk, between gene j and all other examined genes and lower M values represent genes with more stable expression across different experimental conditions. Using of 3 or 4 of the most stable genes were also suggested for accurate normalization. The geometric mean is better able to control for outliers and abundance differences than the arithmetic mean (the sum of the individual Ct values divided by the n - the total number of values).

Assessments of results were done with these different algorithms which were recoded to select the best one. GeNorm provides multiple reference genes as output in which Ntubc2 & PP2A pair for mRNA expression studies. However the Normfinder provides more reliable information on stability of single reference genes according to its developer. NormFinder also suggested the same reference genes like that of geNorm. The other excel based tool, BestKeeper, was used to test the accurate reference genes. This software was able to compare expression levels of maximum ten reference genes with up to hundred biological samples. Raw data input in the BestKeeper software were on Excel tables, where calculation and results proceeds in the background. Results of best keeper obtained as Pearson correlation coefficient, r. The analysis showed a strong correlation ($0.076 < r < 0.903$) for all candidates, thereby Ntubc2 can be used as the most stable single reference gene during organogenesis.

CONCLUSION

This study emphasizes the use of an appropriate reference gene(s) for qRT-PCR studies and identifies reference genes with stable expression during organogenesis in *N. tabacum*. Analyses of our data using different statistical techniques indicated that Ntubc2 is the most stable reference gene for expression studies during organogenesis in the model plant, *N.tabacum*.

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The Impact of Substrate Variation on the Growth, Yield and Nutrient Composition of *Pleurotus ostreatus* Kumm.

ANUSHA S BRITTO[1] AND PREETHA P S[2]

[1]*Department of Botany, Kariavattom Campus, University of Kerala, Thiruvananthapuram.*
[2]*P G department of botany and Biotechnology, SreeNarayana College, Kollam.*
email :anushasbritto2016@gmail.com,preethapremjith@gmail.com

ABSTRACT

A mushroom is the fleshy , spore bearing fruiting body of a fungus. It plays an important role in industrial and agricultural field. Mushroom of *Pleurotus* genus are popularly consumed all overthe world due to their taste andflavour. The climate of kerala is very suitable for the cultivation of *Pleurotus ostreatus*kumm. Mushroom cultivation is one of the efficient way by which residues can be recycled. This high nutritional value suggest that these are important functional foods. This paper aims to elucidate the cultivational process as well as nutritional aspects of *Pleurotus ostreatus* in different substrates. For this study *Pleurotus ostreatus* is cutivated on a wide range of plant waste such as paddystraw,sawdust,coconut petiole,fibrous husk of coconut. Fast mushroom growth was observed in coconut petiole and least growth was observed in sawdust.The first harvest takes few days in fibrous husk of coconut and maximum days for harvest in sawdust substrate. Maximum yield obtained from the paddy straw substrate. The nutrient analysis shows that cooked oyster mushroom contain less amount of protein, fat and ash compared with fresh ones.

keywords *Mushroom; Pleurotus ostreatus; Harvest; Substrates.*

Edible mushrooms are the fleshy edible fruit bodies of several species of macrofungi. *Pleurotus ostreatus* Kumm. (Basidiomycota) of the pleurotaceae family, is one of the edible mushroom and it is commonly known as oyster mushroom. The common name “oyster mushroom” comes from a white shell like appearance of the fruiting body, not from the taste. It varies texture from very soft to very chewy, depending on the strain and what time of the year you pick it.They tend to be chewier during the colder months of the year. This oyster mushroom is found in chinese, korean and japanese menus. It is cooked in soups,soy sauce and other dishes. It is picked young for the kitchen because when it becomes tougher as it grows and its taste and smell deteriorate.With the recent focus on locally sourced food and foraging,the importance of the mushrooms doesnot be slowing down. If anything, mushrooms are now more popular than ever.It can also be easily cultivated on a variety of substrates,so it is making its way on to many supermarket shelves.

In nature *Pleurotus* species are found mainly as saprophytes growing on wood trunks,endowed with the capacity to secret wide spectrum of hydrolyzing enzymes (Hong and Namgung, 1975 a,b; Hong,1976; Toyama and Ogawa,1976 ; Daugulis and Bone, 1977; Molitoris,1979; Rajarathnam *et al*,1979; Madan and Bisaria, 1983). Cultivation of *Pleurotus* species on their natural habitat was first described at the beginning of 20th century(Flack,1917) and on a sawdust cereal mixture by Kaufert (1935). The foundation for the industrial production of *Pleurotus*on different substrates was laid by several workers(Kalberes and Vogel,1974; Zadrazil,1974 and Kurtzman,1979).

Cultivation of oyster mushroom has increased tremendously throughout the world because of their abilities to grow wide range of temperature. *Pleurotus ostreatus* demands few environmental controls, and their fruiting bodies are not often attacked by diseases and pests,and they can be cultivated in a simple and economic way (Kues andLiu,2000).

In India, and particularly Kerala,the *Pleurotus ostreatus* is farmed extensively and the people there make a wide range of dishes from it. Agricultural waste products are used for mushroom cultivation.It is said to be easiest to grow.Among all the cultivated mushrooms *Pleurotus* has maximum number of commercially cultivated species suitable for round the year cultivation.The nutrition in *Pleurotus ostreatus* is very high.These have a very low calorific value so making them an ideal part of the healthy diet.The present work was undertaken with a view to carrying out morphological and nutritional aspects and cultivation method of *Pleurotus ostreatus* Kumm.

MATERIALS AND METHODS

Cultivational method

Pleurotus ostreatus can be cultivated in a room.The room is arranged with little ventilation and light.But for large scale production, thatched sheds are preferred for cultivation.For the cultivation process

Table 1. Effect of different substrate on mycelium spread & days of spawn running completed

Substrate	Completion of mycelium formation	Daysofspawnrunning completed
Paddy straw	9th day	21
Saw dust	20th day	27
Fibrous husk of coconut	7th day	22
Coconut petiole	6th day	16

Fig:1 A -Spawn Used For Mushroom Cultivation :B-saw Dust:C-fibrous Husk Of Coconut D- Paddy Straw : E-coconut Petiole

Table 2. Number of days & yield of first and second harvest in different Substrates

Substrate	Day sand yield in first harvest	Days and yield second harvest
Paddy straw	28 days & 585 g/bed	34 days & 465g/bed
Saw dust	43 days & 150 g/bed	55 days & 110 g/bed
Fiboushuskofcoconut	24 days & 475 g/bed	27 days & 286 g/bed
Coconut petiole	30 days & 278 g/bed	32 days & 203g/bed

NUTRITIONAL ANALYSIS

TABLE-3 :COMPOSITION OF FRESH *Pleurotus ostreatus*

SUBSTRATE	TOTAL ASH (%)	TOTAL PROTEIN (%)	TOTAL FAT (%)	CARBOHYDRATES (%)
PADDY STRAW	0.84	2.98	0.4	6.6
SAW DUST	0.82	2.9	0.35	7.1
FIBROUS HUSK OF COCONUT	0.84	2.93	0.39	6.9
COCONUT PETIOLE	0.8	2.89	0.38	5.95

TABLE-4 :COMPOSITION OF COOKED *Pleurotus ostreatus*

SUBSTRATE	TOTAL ASH (%)	TOTAL PROTEIN (%)	TOTAL FAT (%)	CARBOHYDRATES (%)
PADDY STRAW	0.81	2.97	0.36	6.6
SAW DUST	0.8	2.9	0.3	7.1
FIBROUS HUSK OF COCONUT	0.82	2.91	0.34	6.9
COCONUT PETIOLE	0.8	2.87	0.33	5.94

seeds collected from krishivigyankendra, sadanandhapuram in kottarakara.Seeds are also known as spawn.

Substrate

The material on which the mycelium of the mushroom grows is called substrates.The properties of substrates determine which mushrooms and microbes can grow in it.

The substrates used for the present study are:

1. Paddystraw
2. Sawdust
3. Fibrous husk ofcoconut
4. coconut petiole

Pre treatment of substrates

About 750gram of each substrate is cut into small pieces of 3 to 5cm long and soaked in water overnight. The substrates is then cooked in a drum containing boiling water for 30 to 45 minutes at 75^0C to 100^0C .Add 2 % lime while cooking the substrate to increase the yield of mushroom. After cooking excess water is

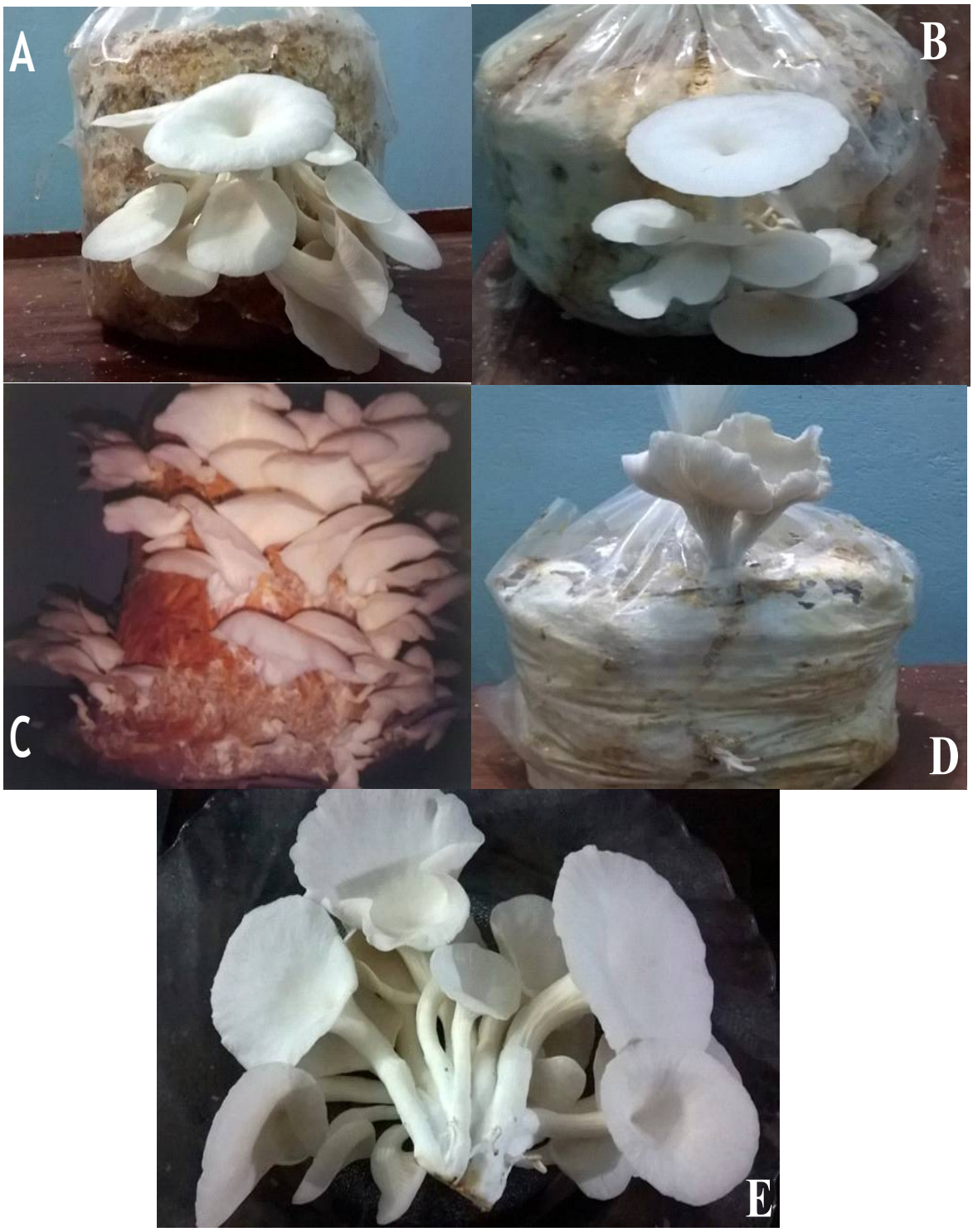

Fig 2. Production of Fruitingbodies A-sawdust:B-fiboushusk of coconut: C-paddystraw D-coconut petiole: E-harvested mushroom

drained by spreading the substrates over a gunny cloth.The correct water content of the substrate is checked by squeezing the substrate in between hands and so that no drop of water should ooze from the substrate.This sterilized substrate wasused for cultivation.

Spawning

About 500gram of spawn is divided into four equal parts. Take a polythene bag of 60x30cm and bottom portion of the bag is tied with a thread and make two holes at the base of the bag. The substrate added into the polythene bag to a height of 5cm. 175gram spawn

is spread over the substrate. Again substrate is placed over the first layer for about 10cm height. Then second part of the spawn is spread over it. Third layer of substrate is placed over the second layer followed by spawn over it. Again fourth layer of substrate is place over the third layer and fourth part of spawn is spread over it. Then substrate added over the fourth layer to a height 5cm.The mouth of the bag is then tied with a thread.Small holes are made on the surface of the bag to facilitate good aeration and to reduce rise intemperature.

Maintenance of Mushroom beds

Polythene bag containing spawned substrate is called mushroom bed. The mushroom beds are kept on racks in a mushroom shed. Sprinkling of water on the floor helps to keep a suitable moisture condition inside the mushroom shed. After 12 to 15 days of spraying, the fungal mycelium grows and forms a compact bed inside the polythene bag. The mushroom beds are watered twice a day to maintain the temperature inside the room. Many small fungal knots appear on the surface of the mushroom bed. These are pin head stage ,later develops into mature mushroom within two to fourdays.

Harvesting

The matured mushrooms were harvested by hand picking. The base of the stipe is cleaned with a sharp knife and packed well aerated containers. The first harvest usually done in 20 to 22 days after spawning. The subsequent harvest is done at weekly intervals. From a single bed 4 to 5 harvest can be done.

Nutritional studies

For the study of nutritional value of *Pleurotus ostreatus*, the quantity of carbohydrate,protein,fat,ash present in the fresh and cooked mushroom grows on different substrate are find out in the laboratory test and other nutrient information collected from available resources systematically arranged. Total fat analysis of cooked and fresh mushroom done by AOAC, method number:948.22,total protein analysis of cooked and fresh mushroom done by using AOAC,method number:950.48,955.04C,total ash content analysis of cooked and fresh mushroom done by AOAC,method number:950.49 and the carbohydrate analysis of carried out by anthrone method.

RESULT AND DISCUSSION

Pleurotus ostreatus (Jacq) P. Kumm. belonging to the family pleurotaceae and grows in shelf like clusters on dead logs and living trees.It have three distinct parts-a fleshy shell shaped cap,a short or long lateral stalk called stipe and ridges and furrows underneath the pileus called gills. The mycelium of *Pleurotus* is pure white in colour. The present study revealed that *Pleurotus ostreatus* is a delicious edible mushroom. This is the second most important cultivated mushroom for food purposes through outworld wide (Chang,1991).

At present, edible mushrooms can be used as a weapon against starvation because of its high protein content. Oyster mushroom cultivation also provides employment generation. There is ample scope to earn more from mushroom cultivation using some innovation like attractive packaging for longer shelf life, processing units and new products such as biscuits, pickles, soup powder etc..

In the study *Pleurotus ostreatus* cultivated in four kinds of substrates gives varies in yielding,out of which more yield(585g/ bed and 465g/bed in first and second harvest) obtained from paddy straw. In the cultivational process the coconut petiole recorded maximum mycelia spread in minimum period of time (16 days) and the mushroom harvested firstly from the fibrous husk of coconut. *Pleurotus ostreatus* grown on sawdust take longer days (43 days) for harvest. The nutritional test shows that average amount of carbohydrate,protein,fat and ash content of fresh and cooked *Pleurotus ostreatus* are 6.64%, 2.92%, 0.38%, 0.83% and 6.64%, 2.91%,0.33%,0.8% respectively. Protein, fat and ash showing less variation after cooking and carbohydrate remains same.It contain low amount of carbohydrate compared with other food substance. The carbohydrate content is different in each oyster mushroom cultivated from various substrates. The nutrient variation of fresh mushroom occurs due to the environmental condition and substrate used for the cultivationmethod.

CONCLUSION

Edible mushrooms are popularly consider as a boneless vegetarian meat or white vegetables. *Pleurotus ostreatus* cultivation is a profitable agribusiness. Artificial cultivation of mushroom help to improve social as well as economic status of small farmers. *Pleurotus ostreatus* requires a shorter growth time in comparison to other edible mushrooms. Almost all produces use paddy straw for mushroom cultivation

but now a days availability of paddy straw is difficult, the use of variety of substrate is essential. This study reveals that fibrous husk of coconut and coconut petiole can be used alternative substrate for mushroom cultivation and also heating can affect the nutritional value of *Pleurotus ostreatus*.

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Removal of Pollutants from Pestilent Water using Selected Hydrophytes

APARNA SREEKUMAR[1] AND JOSE JOHN[2]

[1]*Department of Botany, University of Kerala, Kariavattom campus,Thiruvananthapuram.*
[2]*Center for post graduate studies and research,Department of Botany,Sacred Heart College,Thevara, Kochi.*
email: aparnaavani@gmail.com

ABSTRACT

The toxicity and bioaccumulation of two heavy metals Zinc and Cadmium in selected hydrophytes from synthetic heavy metal solution was carried out. The study examines the efficiency of *Eichhornia,Salvinia,Lemna* and *Pistia*to remove heavy metals such as Zinc and Cadmium.Water is collected from a river and determined the water quality parameters. The study confirmed the efficiency of *Eichhornia,Salvinia,Pistia* and *Lemna* to remove heavy metals such as cadmium and zinc. The study revealed that the BCF value of *Pistia* is higher than *Eichhornia* with respect to zinc.However the percentage efficiency of *Eichhornia* is greater than *Pistia*. Higher BCF value for *Salvinia* indicates that it is a better hyper accumulator of Cadmium compared to *Lemna.* It is found that in this case the percentage efficiency is in conformity with the BCF value. Anatomy of roots, leaves and stem of *Eichhornia*, *Pistia, Salvinia* and *Lemna*were compared before and after adding heavy metals in water. However analysis did not reveal any observable changes for this limited period of treatment time.

Key words *Phytoremediation; Heavy metals; Percentage efficiency;Bio Concentration Factor.*

The problem of heavy metals pollution is becoming a more serious problem with increasing industrialization. The accumulation of heavy metals in soils and water possess a threat to the environment and human health.

The study of Dipu and Kumar (2012) revealed the presence of Nitrtes, Phosphates andpresence of Mercury, lead, cadmium, Copper, Zinc, Chromium and Manganese in the Cochin estuary. The media reports point out that water bodies in Kerala is facing threat from heavy metal pollution. Phytoremediation which is an ecofriendly technology involves efficient use of aquatic plants to remove, detoxify or immobilize heavy metals.The idea of using metal accumulating plants to remove heavy metals and other compounds were first introduced in1983, but the concept was actually implemented for the last 300 years (Henry *et al*., 2000).In our country most of the phytoremedial studies have been conducted on aquatic plants which have proved potential bio sorbents for various heavy metals such as Cu, Zn, Cr, Co, Sr, Ni, Cs, Fe and Pb (Srivastavet al.,1993).*Eichhorniacrassipes* has the unique property to accumulate heavy metals such as Cd, Cu, Pb and Zn from the root tissues of the plant (Muramoto and Oki, 1983 andNor 1990).Aquatic plants such as *pistia, Lemna*can have remediatry effects on lead removal fromwastewater(Singh *et al*.,2012). The studies of Prasanakumari *et al*., (2014) analysed the absorption potential for heavy metals by various ferns associated with Neyyarriver. The present study aims to evaluate the efficiency of *Pistia, Eichhornia, Salvinia* and *Lemna* to absorb nutrients, cadmium and Zinc when exposed to contaminated waters.

MATERIALS AND METHODS

The plants used for the study was collected from a river. The collected plants were thoroughly washed several times under tap water to remove any impurities from fibrous roots. Plants were acclimatized in normal water for a period of 1 week. Healthy mature plants of similar size, shape and height were selected and kept separately in 5L capacity trays. The physical and chemical parameters such as P^H, Electrical conductivity , Total Dissolved Solids (TDS), Dissolved oxygen (DO), Biochemical oxygen demand (BOD), Chloride, phosphate, nitrate, sulphate, Zinc, Cadmium were analysed (APHA 1992). Water for experimentation was prepared by adding 1.2g, 2.4 g $ZnSO_4$ in500g of each *Pistia* and *Eichhornia* grown in 5L Of water in tray.0.005 g , 0.01g $CdCl_2$ was added to tray with 500 g *Salvinia* and *Lemna* with 5L of water for each .

Controls were kept for each plants (adding initial amount of heavy metals in water without growing plants). Plants that were not exposed to metals served as control. The test duration was kept for 2 weeks. (Lu *et al*., 2004).

Tap water was added daily to compensate for water loss through plant transpiration, Sampling and evaporation. At first water with growing plants and added heavy metals were analysed. At the same time roots of the plants were also analysed after adding

Table 1. Physical and Chemical Characteristics of water.

Parameter	Values
pH	6.8
Turbidity(NTU)	5
EC (micromhos/cm)	200
TDS (mg/l)	334
Nitrate (mg/l)	0.2
Phosphate (mg/l)	0.02
Sulphate (mg/l)	1.92

heavy metal**.** Plant samples were cleaned and separated into root, stem and leaves. Prior to heavy metal analysis, plant samples were dried in the oven at 80 degree Celsius for 48 h. Dried samples were pulverized into powder and approximately 0.5 g samples were subjected to acid digestion process. Nitric acid followed by perchloric acid (2:1) was added to powdered plant samples.

Digested samples were further diluted and aliquots were used for the estimation of metal concentrations. The measurements of these metal elements were conducted using ICPMS.

Bio Concentration Factor (BCF) was determined by dividing the concentration of heavy metal in plant to the initial concentration of heavy metal in water. Only root of the plant was taken for the analysis. For the calculation of efficiency of plant the initial concentration of heavy metal and final concentration in the test solution is considered. Cross sections of hydrophytes *Eichhornia* and *Pistia* grown in 1.2g and 2.4g $ZnSO_4$and *Salvinia* and *Lemna* grown in 0.005g and 0.01g $CdCl_2$weretaken for the anatomical studies. 80 sections of plant parts before and after adding heavy metals were analysed. The best 12 transerse sections were selected for anatomical study. The sections were stained using saffranine and mounted in glycerine on glass slides. The sections were examined under the microscope and photograhs were taken using Leika with a resolution of 10X.

RESULT

The results of the physical and chemical characteristics of water sample is presented in table 1.

Percentage efficiency of the plant was calculated from the initial and remaining concentration of metal. Percentage efficiency is expressedby the equation, (C0 - C1/ C0) × 100. Where C0 is the initial concentration, C1 is the remaining concentration in the medium (mg/l). It is shown in table 2 and 3.

The BCF provides an index of the ability of the plant to accumulate the metal with respect to the metal concentration in the substrate. BCF is the ratio of Concentration of metal in plant tissue to the initial concentration of metal in external solution (Bokhari et al., 2016).The BCF and Percentage efficiency of the plants were determined with respect to Zinc and Cadmium and presented in table 2 and 3 respectively.

Table 2. Percentage efficiency of plants with respect to Zinc

Plants	*Eichhornia*	*Pistia*
Concentration of heavy metal in water (Initial)	240 Ppm	240 ppm
Concentration of heavy metal in water (Final)	11.71 Ppm	17.67 ppm
Concentration of heavy metal in plant	503.70 ppm	988.51ppm
BCF	2.09	4.12
Percentage efficiency	95.12	92.64

Table 3. Percentage efficiency of plants with respect to Cadmium

Plants	*Salvinia*	*Lemna*
Concentration of heavy metal in water (Initial)	0.6ppm	0.6ppm
Concentration of heavy metal in water (Final)	0.05 ppm	0.32 ppm
Concentration of heavy metal in plant	22.32 ppm	11.31 ppm
BCF	37.2	18.85
Percentage efficiency	91.16	46.58

Anatomy of roots, leaf and stem of *Eichhornia, Pistia*, *Salvinia* and *Lemna* were compared before and after adding heavy metal in water. The photographs of sections of root, stem , leaves and petiole of each plant is taken and closely examined. Anatomical analysis of plants did not reveal any observable changes for this limited period of treatment time.

DISCUSSION

The study confirmed the efficiency of *Eichhornia, Salvinia, Pistia*and *Lemna* to remove heavy metals such as cadmium and zinc. Both percentage efficiency and BCF of these plants were determined with respect to zinc and cadmium. The study concluded that the BCF value of *Pistia* is higher than*Eichhornia* with respect to zinc.This indicates that the root of *Pistia* is a better hyperaccumulator, however the percentage efficiency of *Eichhornia* is greater than *Pistia*. Higher BCF value for *Salvinia*indicates that it is a better hyper accumulator of Zinc compared to *Lemna*.It is found that in this case the percentage efficiency is in conformity with the BCF value.

The study carried out by Qaisar *et al*., (2005); Melo *et al*., (2007); Srighar *et al*., (2005); Vollenweider *et al*.,(2006) showed many hydrophytic adaptations with higher heavy metal concentrations in water. However this study did not reveal any observable changes because of the limited period of time exposure.

CONCLUSION

Different water quality parameters of the river was analysed. The deleterious health effects upon exposure to toxic heavy metal in the environment is a matter of serious concern and global issue. Huge variation for heavy metal tolerance which is present in nature may be used to select and breed heavy metal tolerant plant. (Rajib and Jayalekshmi, 2015). The study confirmed the efficiency of *Eichhornia, Salvinia, Pistia* and *Lemna* to remove nitrate, phosphate and heavy metals such as cadmium and zinc. The study concluded that the BCF value of *Pistia* is higher than *Eichhornia* with respect to zinc. However the percentage efficiency of *Eichhornia* is greater than *Pistia* indicating the efficiency of *Eichhornia* in removing the heavy metal Zinc when the whole plant is considered. The study proved that 500g of *Eichhornia* plant could tolerate 1.2 g Zinc for a period of two weeks. Higher BCF value for *Salvinia* indicates that it is a better hyper accumulator of Cadmium compared to *Lemna*. It is found that in this case the percentage efficiency is in conformity with the BCF value. Here the efficiency of *Salvinia* is proved and 500 g plant can withstand 0.005 g Cadmium for a period of two weeks. Anatomical characteristics of root, stem and leaves of these hydrophytes were compared before and after adding heavy metals. Anatomical analysis did not reveal any observable changes for a period of one week.

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Nutritional Evaluation of *Rubus glomeratus* Blume - A Wild Edible Fruit Consumed by *Kani* Population in Agasthyamala Biosphere Reserve

R.D. ARUN RAJ[1], R.D. ANPIN RAJA[2], M. REGINALD APPAVOO[3]

[1]*Department of Botany,Christian College Kattakada, Thiruvananthapuram*
[2]*Department of Botany, Nirmalagiri College, Kuthuparamba, Kannur*
[3]*Department of Botany & Research Centre, Scott Christian College (Autonomous) Nagercoil*
email: arunrajrd@gmail.com

ABSTRACT

***Rubus glomeratus* Blume (Rosaceae) is an indigenous fruit widely consumed by *Kani*s, an indigenous tribal community residing in the Agasthyamala Biosphere Reserve (ABR), Southern Western Ghats. The present investigation aimed to evaluate the nutritional and mineral profiling of this lesser known aggregate fruit, mostly seen in moist deciduous forests, as thickets in grass lands in the Biosphere reserve. Results indicated a high moisture content of 76.9 g /100g. while total carbohydrate content was 18.45g in 100g of edible portion. Its energy value was 81 kcal/100 g. Results indicates the presence of antioxidant vitamins C and E which had values of 22.77mg/ 100 g and 0.96 mg/g respectively. Potassium and sodium were the most abundant mineral elements analyzed. The current study emphasized the fact that the fruit of *R. glomeratus* Blume is one of the highest sources of the water soluble, flavonoid pigments anthocyanin, and the mean value observed was 304.26 mg/100 g which is well comparable with the many commercial fruits which are reported to be rich sources of this antioxidant pigment.**

Key words *Rubus glomeratus Blume; Wild edible fruit; Nutritional composition; Kani; Agasthyamala Biosphere Reserve.*

Fruits are generally known to be an excellent source of nutrients and supplement for food in the world that faced with problem of food scarcity. Mostly fruits are rich in rapidly absorbed sugars which are very sweet and have relatively high calorific values. The nutritional potential of fruits lies mostly in their micro nutrient content and dietary fibers. They supply several minerals including potassium, iron, calcium, manganese, phosphorous and magnesium. Fruits generally contain a large quantity of vitamins such as vitamin C, provitamin A in the form of â-carotenoids and to a lesser extend vitamin E and B group of vitamins. Fruits also act as an optimal mix of antioxidants as they contain the phenolic compounds, anthocyanins, carotenoids and other flavonoids along with antioxidant vitamins C and E, which can modify the metabolic activation of carcinogens (Wargovich, 2000) and act as anti-aging compounds (Pandey & Rizvi, 2009). Scientific evidences indicate intake of fruits has been associated with a reduced risk of cardiovascular disease and cancer at several sites (Bazzano *et al.*, 2002; Liu *et al.*, 2000; Crawford *et al.*, 1994). They can also maintain the normal pH of the body as they are rich in precursors to bicarbonate ions which serve to buffer acidity.

Dependence of man on fruits is as old as the genesis of Human race. Being the rich source of macro and micronutrients, they can form an essential part of a healthy and balanced life style. In the culinary sense, fruit is defined as the fruit, infructescences or the fleshy portions of floral organs that have achieved appropriate degree of maturity and are fit for human consumption (Hurtado *et al.*, 2008). Even though, fruits account for a substantial fraction of the world's agricultural output, many fruit plants still remain unnoticed. Wild fruit plants are categorized as neglected crops which grow in the wild or are grown in small scale such that their economic potential is poorly addressed and restricted to traditional and local use (Misra and Misra, 2016). Mainly depending on forest and forest product for livelihood, the indigenous communities inhabited in wild all over the planet hold enormous knowledge on these plants.

Currently, increasing attention has been paid by consumers to the lesser known wild fruits which have unusual flavours and attributes, and many of which are rich in health beneficiary compounds like anthocyanins. Studies reveal that even very small fruits are nutritionally dense and can deliver big benefits when the rest of the diet is deficient in vitamins and minerals, because of the overly dependent on starchy staples (Martin *et al.*, 1987). Analytical studies from various parts of the world prove that many of these fruits are nutritionally superior to the cultivated ones in several constituents (Magaia *et al.*, 2013; Mahapatra *et al.*, 2012; Darnet & da Silva, 2011; Battino & Mezzetti, 2006). Wild fruits, aside from being used by aboriginal communities, are recommended today as a supplement for healthy diets in even the developed countries (Redzic, 2006). However, the

genetic resources of many of these plants used by various tribal populations across the world have not acquired much exposure they deserve due to scant and dispersed documentation and limited research on their nutritive composition. The coverage of wild fruits in all existing nutrient databases is very limited, compared with the numbers of fruits consumed.

The plant selected for the present study *Rubus glomeratus* Blume belongs to the family, Rosaceae is an underexploited fruit plant widely consumed by *Kani*s, an indigenous tribal community residing in the Agasthyamala Biosphere Reserve (ABR), Southern Western Ghats (Fig. 1). Plant native to Sri Lanka, India, Myanmar, Philipines and Malaysia is now distributed in India, Sri Lanka, Southeast Asia, China, New Guinea, Fiji and Australia. This scrambling shrub bears fragrant pinkish white flowers and is commonly called, '*Mullu munthiri* or *Kattu munthiri*' among *Kani*s (Raj & Appavoo, 2012). In the Biosphere Reserve, this is mostly seen in moist deciduous forests, as thickets in grass lands upto the elevation of 2000m. The flowering and fruiting season is between November and June. It is a round aggregate fruit with many drupelets easily detaching from the receptacle. Drupelets are single seeded, 2–2.8 mm long, 0.7–1.5 mm wide, 0.5–1 mm thick. Style is often persistent. A fruit weighs 2 – 4 g and is composed of around 40 drupelets. It is bright red at maturity and turns purplish black when fully ripen. It is juicy, sweetish tart in taste and has a distinct pleasant aroma. The seed is discarded and fleshy portion eaten. Literature perusal reveals that there have been no systematic studies concerns with detailed evaluation of nutritional composition of this lesser known fruit used by *Kani*s, the oldest tribe in south India, until now. The present study, focusing on this aspect therefore deserves utmost significance.

MATERIALS AND METHODS

Specimen collection and documentation

The participatory rural appraisal (PRA) method (Martin, 1995) was used for collecting the ethno botanical information regarding the plant. During field work, tribal informants were accompanied to identify and collect the fruits. Length and diameter were measured as an average of randomly selected 10 fruits

Fig. 1. Fruits of *Rubus glomeratus* Blume

with the help of a vernier calliper and expressed in centimetres. Ten randomly selected fruits were also weighed using an electronic weighing balance; range and the average of weights are expressed in grams. Unblemished fully ripened fruits collected were cleanly washed and taken immediately to the laboratory and stored at 4°C for for further analysis. Approximately 200 gram of fruit was collected and the edible portions were separated for the evaluation of total carbohydrate, total protein, and vitamins. One portion was dried in a hot air oven for 24 hours at 45°C. Then the dried fruits were ground well and kept in an air tight bottle for the evaluation of mineral profiling.

Nutritional profiling

Nutritional composition of fruits samples was determined on fresh weight basis. The sample was scrutinized for a total of 15 parameters which include proximates, vitamins, minerals and other phytonutrients. The proximate composition including the moisture content was estimated as per the gravimetric method (AOAC, 2000). Total carbohydrates were estimated by the method given by Hedge & Hofreiter (1962). Total protein content was quantified using the method by Hartree - Lowry assay (Hartree, 1972). Crude fat (Total lipids) was determined by Bligh and Dyer's method (Bligh & Dyer, 1959). Total energy was estimated by Atwater specific factor (ASF) system (FAO, 2003a), a more refined energy conversion system based on the Atwater general factor (Atwater & Woods, 1896). The vitamins C, E and the pigment, anthocyanin were evaluated by as per various methods given by Association of Analytical Chemists (AOAC, 1990; 2005). The minerals; Potassium (K), Magnesium (Mg), Calcium (Ca), Iron (Fe), Copper (Cu) and Manganese (Mn) in the sample prepared by either dry ashing or wet digestion, are quantitatively measured by atomic absorption spectrophotometer (AAS) at specific wavelengths.

RESULTS AND DISCUSSION

Indigenous communities across the globe are well aware of seasonal availability of most of the wild fruits they consume. Since these fruits are freely accessible within natural habitats, *Kani*s in ABR also have acquired knowledge from practical experience on how to gather fruits and where to collect a particular species from. The present ethno botanical scrutiny reveals the fruits of *R. glomeratus* Blume is widely consumed by *Kani*s especially in the Kerala part of the Biosphere reserve. There are reports on its edibility from the various tribal groups across the country. Udayan *et al.,* (2008) report, this fruit is consumed by *Kurichyar* tribe in Tirunelli forest in Wayanad district of Kerala where it is called '*Chorapazham*'. *Malappandaram*, *Urali*, *Mala-arayan*, and *Ulladan*, the tribes in Pathanamthitta district of Kerala also eat this fruit (Binu, 2010). Kumar *et al.*, (2012) has reported this plant from Kanuvai and Madukkari forests in Nilgiri Biosphere Reserve, where also it is called, '*Kattu munthiri*'. The nutritional analysis has revealed that these are excellent source of food constituents that necessary in the regular diet.

Nutritional composition

The nutritional analysis results of *R. glomeratus* Blume were presented in Table 1. The moisture content in fresh fruits was relatively high with value of 76.9 ± 0.39g/100g. The high content of moisture in fruits suggested that they require to be preserved well as they are highly perishable in nature (Lim & Rabeta, 2013). Carbohydrates are one of the most important components in many fruits. The total carbohydrate was noted in the fruit was 18.45 ± 0.55g/100g of edible portion. A comparative analysis of the data also suggested that the this fruit qualifies as reasonable amount of carbohydrate comparable to most of the cultivated counterparts as banana (22.84 g), guava (14.32 g), orange (12.54 g) and grapes (13.93 g) (USDA, 2013). The fruit showed the protein content of 2.74 ± 0.195 g/100 g. The result also shows this fruit is richer in protein content than the popular cultivars like mango (0.82 g), grapes (0.81 g), orange (0.70 g) and apple (0.26 g). The fat content of the fruit was very low (0.62 ± 0.05g /100g) as generally observed in most of the popular cultivated fruits (FAO, 2003b). The results showed that total ash, which is an index of mineral contents (Coimbra & Jorge, 2011), for the fruit was 1.53g/100g. The total energy provided by this wild edible fruit was 81kcal per 100 gram of edible portion. As per the Atwater specific factor (ASF) system, an increase in any of the three proximate principles, carbohydrate, protein and fat or these factors together proportionately increases the energy value.

The amount of mineral elements present in a plant depends to a large extend on the genetics, climate, soil nutrient content, time of harvest and growing location (Kruczek, 2005). The present study estimated

Table 1. Nutritional Composition of *Rubus glomeratus* Blume fruits

Nutrient	Unit	Value/100 g
Proximates		
energy	kcal/kJ	81/338
Protein	g	2.74 ± 0.195
Total lipid (fat)	g	0.62 ± 0.05
Carbohydrate	g	18.45 ± 0.55
moisture	g	76.4 ± 0.39
ash	g	1.53
Minerals		
Potassium	mg	177
Calcium	mg	27
Magnesium	mg	31
Iron	mg	0.52
Copper	mg	0.671
Manganese	mg	1.610
Vitamins		
Vitamin C (Ascorbic acid)	mg	22.77 ± 1.32
Vitamin E (α-tocopherol)	mg	0.96 ± 0.063
Other		
Anthocyanin (CGE)	mg	304.26 ± 1.54

the composition of six mineral nutrients; three macro elements (K, Ca and Mg) and three microelements (Cu, Mn and Fe) present in the fruit. The level of K was 177 mg/100 g which is well comparable to the concentration observed in commercial fruits like mango (168 mg), orange (169 mg), grapes (203 mg) and pomegranate (236 mg/100 g). In the tissue of many fruits, Ca is one of the minerals believed to be an important factor governing fruit storage quality (Lechaudel *et al.,* 2005). Ca content recorded for the fruit was 27 mg/100 g, which is relatively a common concentration in most of the domesticated fruits. Mg concentration observed was 31 mg/100 g. This result is at par with some of the tropical counterparts like Banana (27 mg/100g) and Papaya (30 mg/100g). Iron is an important trace element required in numerous essential proteins (Arredondo & Nuñez, 2005). Manganese is a cofactor in function of antioxidant enzymes, such as the mitochondrial superoxide dismutase (Vicente *et al.*, 2009). Copper is an essential mineral required by the body for bone and hemoglobin formation, and for coding various specific enzymes (Arredondo & Nuñez, 2005). Iron, Manganese and Copper composition noted for the fruit was 0.52 mg, 1.610 mg and 0.671 mg/100 g respectively per 100 gram of edible tissue.

The vitamins in fruits make an important contribution to human nutrition, as they involve in specific metabolisms in normal body functions. Fruits, particularly tropical species, are the main dietary sources of vitamin C with value normally ranged 20-60 mg/100 g of edible portion (FAO, 2003b). The ascorbic acid composition noted was 22.77±1.32 mg/ 100 g which is in agreement with this observation. Vitamin E content registered in the fruit was 0.96 ± 0.063 mg/100g which is well comparable to the values observed in some popular commercial fruits, guava (0.73 mg/100 g), peaches (0.73 mg/100 g) and pomegranates (0.60 mg/100 g) known to be rich source of tocopherol (USDA, 2013).

Apart from major constituents like proximates, minerals and vitamins, fruits contain several health beneficiary phytonutrients. The current study emphasized the fact that the fruit of *R. glomeratus* Blume is one of the highest sources of anthocyanin, the water soluble, flavonoid pigments with great relevance due to their contribution to the strong antioxidant capacity of fruits (Blando *et al.*, 2004).

The value observed was 304.26 ± 1.54mg/100 g which is well comparable with the fruits of raspberry (214-589 mg/100 g), black currants (254.49-810mg/100 g) and grapes (497-842 mg/100g) which are reported to be rich sources of this pigment (Oancea *et al.*, 2011; Stanciu *et al.*, 2010).

CONCLUSION

The result of the nutritional analysis of the *R. glomeratus* Blume, eaten as raw by *Kani* tribals in ABR, indicates that this fruits is either superior or have identical status to the popular commercial fruits. The study portrays the status the macro and micronutrients present in this underutilized edible fruit. It was also observed that, compared to the large sized cultivated fruits, this small minor fruit, even though tart in taste, were more nutritional in terms of biologically active substances such as ascorbic acid, tocopherol, and anthocyanin (CGE). The consumption of small fruits, which are often referred to as "natural functional products" (Kondakova, 2009; Joseph *et al.*, 2000), has been associated with diverse health benefits such as prevention of heart disease, hypertension, certain forms of cancer and other degenerative or age-related diseases (Viskelis *et al.*, 2012). The present study showed that the fruit of *R. glomeratus* Blume, ssp *sundanum* fruits could also be used as a potential source of protein, vitamin, minerals and monomeric anthocyanins. Considering no cost of production, easy availability, this underutilized and neglected species need to be popularized as a source for better health and nutritional status of the rural communities of the country. Research is also required on producing value added food products using these fruits as such knowledge will ensure sustainable harvest of these edible resources, which in turn can contribute to the economic well-being of the people and make them involved in the conservation of biodiversity.

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Drought Tolerance Efficacy of *Phaseolus vulgaris* L. And *Vigna radiata* L.

R.V. CHINTHU*[1] AND SHALCHANDRAN[2]

*[1]*Department of Botany University of Kerala, Kariavattom*
[2]*Dept of Botany, S N College Cherthala*
email:chinthukailas@gmail.com

ABSTRACT

Drought is one of the most severe problems in worldwide agricultural production. Seed germination is critical and governs the later growth and yield of the plant. The drought stress studies were carried out in two species of Pea plants viz. *PHASEOLUS VULGARIS L.* AND *VIGNA RADIATA L.* which make the food more proteinaceous. In addition, *PHASEOLUS VULGARIS L.* AND *VIGNA RADIATA L.* have manymedicinal properties. The *in vitro* drought was induced in these plant seeds by using different levels of polyethylene glycol (PEG) i.e., 5%, 10%, 15% and control. Observations on the seed germination parameters were made after 48 hours of sowing. The seed germination percentage of both *Phaseolusvulgaris* L and *Vignaradiata L.* was not much affected upon drought stress as compared to their control. But the embryonic root growth was much affected in the case of. *Vignaradiata L.* as compared to *Phaseolusvulgaris* L. This showed the high drought withstanding potential of *Phaseolus vulgaris* L. The proline and total sugar content was increased significantly in *Phaseolusvulgaris* L. as compared to *Vigna radiata L.* upon drought stressed condition. Thus the result of this study denotes the drought tolerance potential of *Phaseolus vulgaris* L.

Key words *Drought stress, PEG, Seed germination, Proline, Sugar*

As a result of various abiotic and biotic stresses there is a heavy loss in crop reported as in various vegetable crops, the scientific community is always look out for stress tolerant crop species / variants. These stress tolerant crop species are generated through various techniques, of which the biochemical approach plays a major role. So it has become essential to have information regarding the drought stress tolerance potential of various Pea species.This will ensure that the cultivation of these species will not turn out to be a failure in the middle of the growing season and thus can ensure productivity.

MATERIALS AND METHODS

Seeds of two pea plants viz., and *Phaseolus vulgaris* and *Vignaradiata* were collected from Cherthala. The seeds were sown in soil mixed with cow dung. after 30 days the plantlets were transferred to polythene bags containing sand, soil, cow dung in a ratio of 1:2:1.

Induction of drought by using PEG

The different concentration of poly ethylene glycol (0, 5%,10%,15%) were prepared. These medium were poured in to appropriately labelled petriplates. Pea seeds from each species (10 in number) and controls were transferred to corresponding concentrations of PEG in aseptic condition on to a Whatman filter platform. After 24 hour of incubation the seedling were screened for germination properties as well as for their growth rate . Investigations on germination were carried out with reference to the following parameters.

Germination percentage and root growth

The germination percentage was calculated using the formula.

$$\text{Germination percentage} = \frac{\text{No of seeds germinated X 100}}{\text{Total no of seeds sawn}}$$

Embryonic root growth was measured by using a standard scale.

Induction of Drought

Drought was imposed to one set of 12 plants by withholding irrigation where as the other set of 12 plants were kept as control.After acclimatization to this condition for 10 days, the leaves were excised and collected in labelled polythene bags for various analysis.

Estimation of Total Sugar

Total sugar content was estimated by using the method of Dubois *et.al* (1956). 0.5 g of tissue was homogenized in 10 ml of 70% ethanol and centrifuged at 10,000 rpm for 15 minutes. From each sample 1 ml of the supernatant was taken in to test tubes. To this 1ml of 5% phenol solution was added followed by 5 ml con.H2SO4. The reagent blank was also prepared by taking the entire reagent except sample.1 ml ethanol was taken as blank instead of sample. The mixture was then kept at 30 f c for 20 minutes. Read the color of the mixture at 490nm using spectrophotometer.

Estimation of Proline

Estimation of proline content was according to Bates method (1973). 1g. of leaf tissue was weighed &homogenized in 3 ml of 3% sulphosalicylic acid. The homogenate was then of centrifuged at a speed of 10000rpm for 15 minutes. The supernatant was saved & used for the estimation.From each sample 1 ml. of the supernatant was taken in to test tubes & then 1ml. of glacial acetic acid & 1ml of ninhydrin was added to it. The test tubes were then covered with round marbles & further kept in boiling water bath for 1h. To terminate the reaction, the tubes were dipped in cold water. The reagent blank was also prepared by taking the entire reagent except the sample. Instead of sample 1ml. of 3% sulphosalicylic acid was taken. 4 ml of toluene was then added to each test tube &shaken well using a vortex mixture.The red chromophore was collected & the absorbance was measured against a reagent blank at 520nm using a spectrophotometer.

RESULTS AND DISCUSSION

Germination percentage and Root Length

In this investigation it was found that the seed germination of *Phaseolus vulgarisL. and VignaradiataL.* is not much inhibited even at high concentration of PEG (Table.1). The growth of embryonic root was found to be decreased at 10 % of PEG in the case of *Phaseolus vulgaris* but in the case of *Vignaradiata*it was not inhibited even at 10 % of PEG.

Total sugar content

Total sugar s may help the plants to bring about the osmotic adjustment (Hare *et.al* 1998).In this present investigation,*Phaseolus vulgaris L.* has showed greatest potential to accumulate sugars at enhanced level as compared to *Vignaradiata L.* When there was 62.2 % (2120µg/g fr.wt) increase in the total sugar content in *Phaseolus vulgaris L* on subjecting to drought stress as compared to their control plants, it was only 32% (**2120** µg/g fr .wt) increase in the case of *Vignaradiata L.*

Total proline content

It is a compatible solute and its play an important role in the osmoregulation plant when plants are exposed to various abiotic stresses (Bohnert*et.al*, 1995). It can also prevent membrane damage and protein denaturation by scavenging free radicals. Therefore increasing proline content can help the plants to tolerate salinity stress. In this present investigation *Phaseolus vulgaris* was recorded to accumulate proline at enhanced level as compared to *Vignaradiata*(Table.3). *Phaseolus vulgaris* showed 59.5% (1018 µg / g Fresh weight) increase in the proline content on subjecting to salinity stress as compared to their control plants whereas in *Vignaradiata* it was only 8% (998 µg / g Fresh weight).

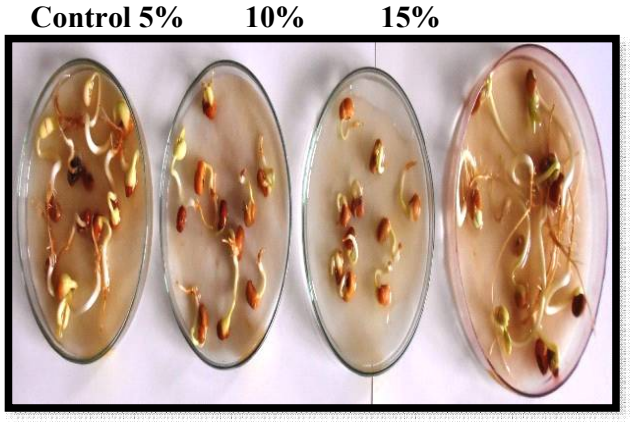

Fig. 1(a). Effect of the concentrations of P E G on seed germination ofPhaseolus vulgaris

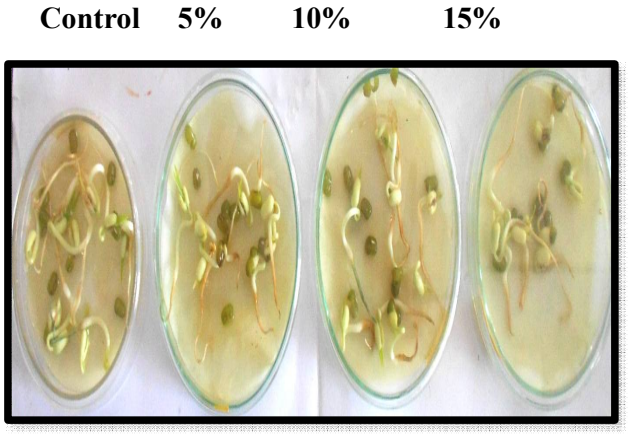

Fig.1(b). Effect of the concentrations of P E G on seed germination of*Vignaradiata*

Table 1. Germination percentage

Concentration of P E G (%)	*Phaseolus vulgaris*	*Vignaradiata*
0 %	100	100
5%	100	100
10%	100	100
15%	100	100

Fig. 2(a). Effect of stress on Phaseolus vulgaris

Fig. 2(b). Effect of stress onVignaradiata

Table 2. Root growth

Plants	Root length (cm)			
	Control	5% P E G	10% P E G	5% P EG
Phaseolus vulgaris	24.2	24.5	9	3
Vignaradiata	7.8	7.3	7.5	4

Table 3. Total sugar content

Name of the plant	Total sugar content (µg / g Fresh weight)		Percentage of difference
	control	Drought	
Phaseolus vulgaris	800 µg	2120 µg	62.2%
Vignaradiata	1400 µg	2120µg	32%

Table 4. Total Proline content

Name of the plant	Proline content (µg / g Fresh weight)		% of difference
	Control	Drought	
Phaseolus vulgaris	12	1018	59.5
Vigna radiata	917	998	8

CONCLUSION

In the present study various parameters have been made to analyze the drought tolerance potential of *Phaseolus vulgaris L.* and *Vignaradiata L.* and to compare its drought tolerance capacities. For this purpose experiments to find out the germination capabilities of these two species at different concentration of P E G (0%,5%,10% 15%) and various non enzymatic mechanisms (proline and total sugar content) of drought tolerance under water deficit condition.

The seed germination percentage of both *Phaseolusvulgaris* L and*Vignaradiata L.* was not much affected upon drought stress as compared to their control. But the embryonic root growth was much affected in the case of.*Vignaradiata L.* as compared to *Phaseolusvulgaris* L. This showed the high drought withstanding potential of *Phaseolusvulgaris* L. The proline and total sugar content were increased significantly (59.5 & 62.2% respectively) in *Phaseolus vulgaris L* .under drought stressesed conditionas compared to *Vignaradiata L.* These results again pointed out the drought tolerance potential

of *Phaseolus vulgaris L.* and this data is very much correlated with the result of root length studies in seed.

From this present study it was well clear that *Phaseolus vulgaris L* .is a drought tolerant species in comparison with *Vignaradiata L.* Its salt tolerance nature has been characterized by its ability to maintain the embryonic root growth at different concentration of PEG and efficient osmoregulation capabilities (accumulation of proline and total sugars).

In general the result of this study indicates the higher drought tolerance potential of *Phaseolus vulgaris L.,* as compared to *Vignaradiata L.,* and it can be recommended for cultivation in drought affected area.

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Green Synthesis of Silver Nanoparticles Using *Gleichenia dichotoma*

H. DHANYA[1], RESHMI.P[2] AND BOBY. T. EDWIN[*3]

[1]*Department of Biotechnology, CEPCI, Kollam, Kerala*[1]
[2]*Department of chemistry, TKM College of Arts and Science, Kollam, Kerala*
[3]*Department of Botany, TKM College of Arts and Science, Kollam, Kerala*
email: bobytedwin2003@gmail.com

ABSTRACT

The present study reports the use of *Gleichenia dichotoma*, a pteridophyte, for the synthesis of nanoparticles followed by its characterization using UV-Vis spectroscopy, SEM-EDX and XRD analysis. The phytochemical screening evidenced the existence of reducing bioactive compounds. The UV-Vis spectrum revealed the formation of silver nanoparticles by exhibiting Surface Plasmon Absorbtion maxima at 435 nm. SEM analysis confirmed the existence of silver nanoparticles with average size of 98.20 nm. EDX spectrum showed peaks that validated the presence of elemental silver.The crystalline nature of synthesized nanoparticle is confirmed by XRD analysis. Silver nanoparticles showed bactericidal activity against *Escherichia coli* but no activity was observed against *Staphylococcus aureus*.

Key words *Gleichenia dichotoma , nanoparticles, UV-Vis spectroscopy, SEM-EDX analysis, XRD*

Over the past few decades synthesis of nanoparticles is one of the active areas of research. According to American Society of Testing and Materials, nanoparticles are those which have two or more dimensions and are in the size range of 1-100 nm possessing unique thermal, electronic, electrical, optical, mechanical, magnetic and chemical properties that are different from their bulk material counterparts(Khan *et al*., 2014; Firdhouse *et al*., 2012; Pantidos *et al*., 2014). The special and enhanced properties of nanoparticles are due to their large reactive and exposed surface area and quantum size effect as a result of specific electronic structures finding wide applications in various fields ranging from medical to physical needs. The routinely used metals for the synthesis of nanoparticles include silver, aluminium, gold, zinc, palladium, platinum, titanium, iron and copper (Kulkarni *et al*., 2011; Vadlapudi *et al*., 2014). Owing to their wide range of applications silver nanoparticles have become the focus of much research interest. Silver nanoparticles find its application in photography, catalysis, biosensor, biomolecular detection, as antimicrobial agents, drug delivery and tissue or tumor imaging. The physical and chemical methods that are available for the synthesis of nanoparticle include chemical and photochemical reactions in reverse micelles, laser ablation, phase transfer processes, microemulsion, microwave treatment, gamma radiation, sonochemical, electrochemical and thermal decomposition of silver compounds (Guzmán *et al*.,2009; Van Dong *et al*., 2012; Bhor *et al*., 2014). But these methods are expensive, labour intensive and are potentially hazardous to the environment and living organisms, hence biosynthetic methods employing the use of microorganisms or plant extract emerged as a simple and viable alternative to physical and chemical methods (Makarov *et al*., 2014). Use of plant extract for synthesis of nanoparticle is advantageous over the use of microorganism as maintenance of microbial culture is a complex and elaborative process (Nalwade *et al*., 2013). Green synthesis of nanoparticles using plant extract is rapid, economical, ecofriendly, easy to scale up and is a single step technique and can provide nanoparticles of controlled size and morphology.The plant assisted reduction of silver ions is mediated by phytochemicals which include terpenoids, alkaloids, phenolics and coenzymes(Kulkarni and Muddapur, 2014). There are many reports of synthesis of silver nanoparticles using the extracts of *Azadirachta indica, Aloe vera, Acalypha indica, Ceratonia siliqua, Datura alba, Jatropha curcas, Helianthus annus, Murraya koeinji, Ocimum sanctum, Piper nigrum* and so on (Namratha and Monica, 2013;Chandran *et al*.,2006;Krishnaraj *et al*., 2010; Awwad *et al*., 2013; Nalwade and Jadhav,2013; Bar *et al*., 2009; Leela and Vivekanandan,2008; Christensen *et al*.,2011; Singhal *et al*.,2011).

When compared to angiosperms, much lesser studies are conducted using Pteridophytes for the synthesis of nanoparticles. Ferns and their allies belong

to the major division of plant kingdom, Pteridophyta. Gleichenia, a P.teridophyte belonging to the family Gleicheniaceae is a large genus consisting of about 130 species. It is distributed in tropical and sub tropical regions of world. In India it is found in southern India, Western Ghats, Eastern Himalayas and Kumaon Hills. They are diploid sporophyte and usually form bushy growths. After extensive literature survey and to the best of our knowledge no studies reported the biosynthesis of nanoparticle using *Gleichenia*. Hence the present study was aimed at the phytochemical analysis, synthesis of silver nanoparticles using the aqueous leaf extract of *Gleichenia dichotoma* and the characterization of synthesized nanoparticles using UV-Spectrophotometer, X-Ray diffraction (XRD) and Scanning electron Microscopy (SEM). The antibacterial activity of the nanoparticles against *Staphylococcus aureus* and *Escherichia coli* is also studied.

MATERIALS AND METHODS

Collection of plant material

Gleichenia dichotoma were collected from in and around the areas of Punalur , Kollam district, Kerala, India.

Preparation of leaf extract

Fresh and healthy leaflets were harvested and rinsed several times with distilled water to remove dust and unwanted visible particles and then dried. The leaves were finely cut in to small pieces. Precisely weighed leaves were dispersed in 100 ml of sterile distilled water and boiled for 20 minutes at 100 ^{0}C. The extract was cooled to room temperature and was filtered using Whatman No. 1 filter paper to remove particulate matter and to get clear solutions. The extract was then refrigerated and used within a week.

Synthesis of silver nanoparticles

10 ml of leaflet extract was added drop by drop into 90 ml of 1 mM silver nitrate solution while stirring. The mixture was stirred magnetically and maintained at 80 ^{0} C for few minutes. It was kept for 6 hours at room temperature. The colour change of reaction mixture from colourless to dark brown was checked periodically. The colour change indicates the synthesis of silver nanoparticles.

UV-Vis Spectra analysis

The reduction of pure Ag^+ ions was monitored by measuring the UV-Vis spectrum of the reaction medium. A small aliquot was drawn from the reaction mixture and spectrum was taken at a wavelength ranging from 300 to 800 nm using a UV-Vis Spectrophotometer 117, Systronics.

Purification of Silver nanoparticles

The silver nanoparticle solution thus obtained was purified by frequent centrifugation at 5000rpm for 20 minutes followed by redispersion of the pellet of (Ag) nanoparticles into 10ml of deionized water. After air drying, the purified nanoparticles were characterized using XRD and SEM-EDX analysis.

XRD Analysis:

XRD measurement was carried out on films of the dehydrated pellet powder drop coated onto glass substrates using Xray Diffractometer (Bruker AXS D8 Advance)operating at a voltage of 20 kV and a current of 30mA with Cu k á 1 radiation. The crystallite domain size was calculated from the width of the XRD peaks, using the Scherrer formula.

$$D = 0.94\ ë / â \cos è$$

where D is the average crystallite domain size perpendicular to the reflecting planes, ë is the X-ray wavelength, â is the full width at half maximum (FWHM), and è is the diffraction angle.

SEM –EDX analysis of silver nanoparticles

Scanning Electron Microscopic analysis was done using Nova Nano SEM (NPEP 252). Thin films of sample were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and then the films on the SEM grid were allowed to dry by putting under a mercury lamp for 5 min. The peak obtained from EDX gives the elemental composition of the sample.

Antibacterial assays

The comparative antibacterial activity of silver nanoparticles, silver nitrate and plant leaf extract was assayed by disc diffusion method.Two test bacteria *Escherichia coli (ATCC 25922*), a Gram negative bacteria *and Staphylococcus aureus (ATCC9144),* a Gram positive bacteria was used. A 100 µl of bacterial

culture (1 x 10^6cfu/ml) was swabbed on Muller-Hinton agar plates. Three sterile paper discs of 6 mm diameter each was loaded with 30 µl of silver nanoparticles 1mM silver nitrate and plant extract was placed over bacterial lawn and incubated at 37 ^{0}C for 24 hours. After incubation period the plates were examined for zone of inhibition, which appear as a clear area around the disc. The diameter of each zone of inhibition was measured.

Phytochemical Screening:

Phytochemical screening of the extract was carried out according to standard procedure (Harborne, 1978).

RESULTS AND DISCUSSION

Synthesis of silver nanoparticles:

The colour change after addition of phytoextract due to the reduction of silver ions is evident in the Fig (1). The change in colour from colourless to dark brown in aqueous condition was observed. The colouration was due to the excitation of surface plasmon vibration in the silver nanoparticles (Nagati*et al*., 2012). After initial intensification the colour remained constant for several weeks indicating the stability of synthesized nanoparticles. After one hour there was no significant colour change which indicates the completion of reduction reaction. Previous reports suggested the effectiveness of protein, carbohydrate, flavonoids and phenols in reduction of siver ions to silver nanoparticles (Anuradha*et al*., 2014).

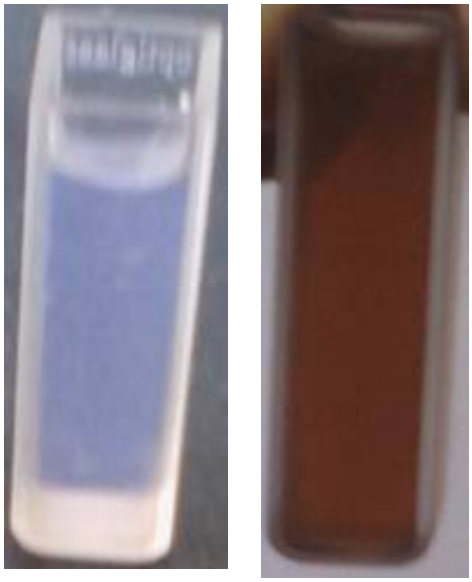
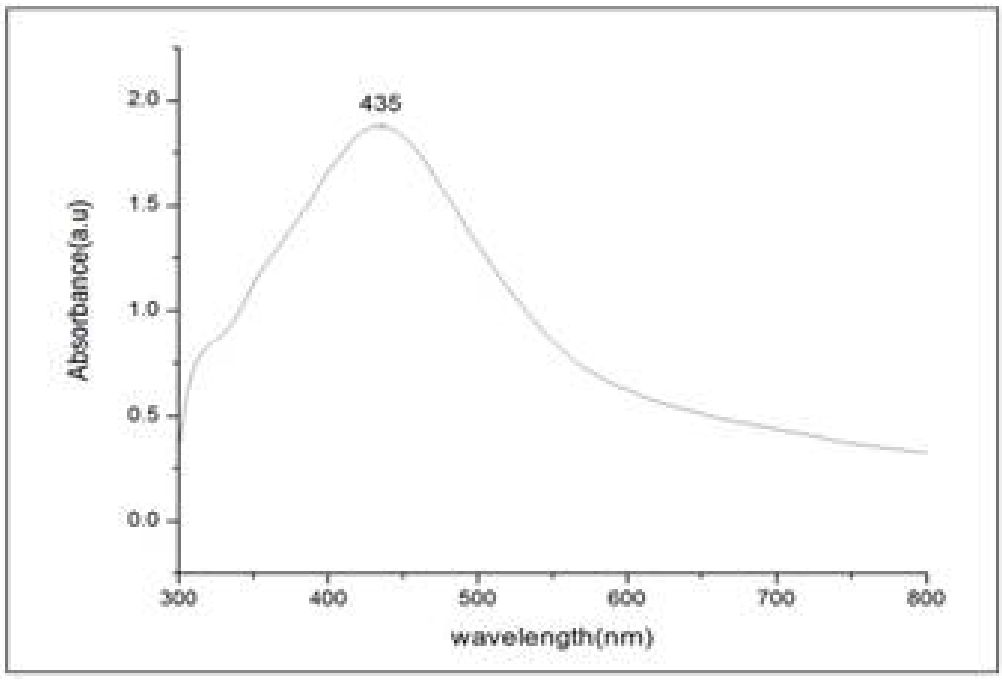


Fig. 1. Photograph of silver nitrate solution (a) Before reduction to nanoparticles (b) After nanoparticle formation

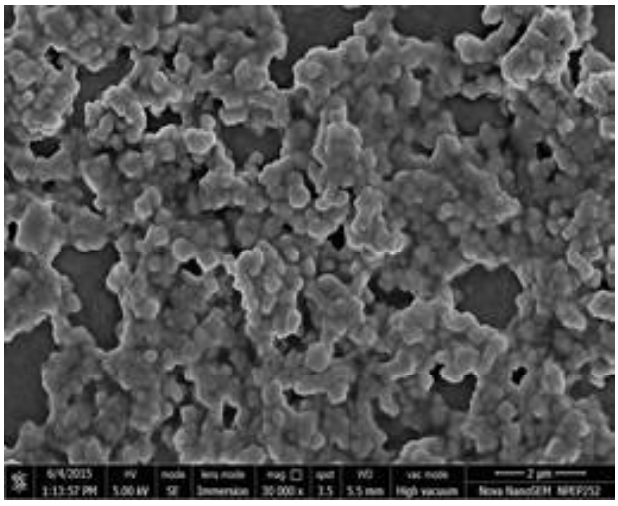
UV-Visible Spectra Analysis:

Nanoparticles have optical properties specific to their size, shape, concentration, agglomeration state and refractive index. So UV/ Visible spectroscopy can be used for identifying, characterizing and studying these materials. The optical spectrum of the synthesized silver nanoparticle exhibited a single absorption band at 435 nm (figure 2) .The broad



spectra indicated that the silver nanoparticles were poly dispersed. The single SPR band indicated that the

particles were spherical. According to Mei's theory spherical nanoparticles result in a single SPR band in the absorption spectra. Similar surface plasmonic absorption band at 435 nm was reported for the silver nanoparticles synthesized using *Murraya koeinji*and *Hevea brasiliensis (*Desai*et al.*, 2012;Ponarulselvam*et al.*, 2012). In metal nanoparticles like silver the conduction band and valence band lie very close to each other in which electrons move freely. These free electrons give rise



to a surface plasmon resonance (SPR) absorption band due to collective oscillation of electrons of silver nanoparticles in resonance with the light wave. The SPR of nanoparticles is influenced by size, shape, inter particle interactions, free electron density and surrounding (Ahmed*et al.*, 2016).

Fig. 2. UV Vis Spectra of synthesized Silver nanoparticles

SEM-EDX analysis:

The morphology of silver nanoparticles was observed under Scanning Electron Microscope (SEM). The SEM image of synthesized nanoparticles showed well dispersed nanoparticles with mere agglomeration. The average particle size was found to be 98.20 nm. The EDX spectrum of nanoparticles revealed peaks around 3keV corresponding to the binding energies of silver(Figure 4). The results indicated that the reaction product was composed of pure silver nanoparticles with fewer amounts of other elements.

Fig. 3. SEM image of synthesized AgNPs

XRD analysis

X - ray powder diffraction is a rapid analytical technique primarily used for the phase identification of a crystalline material which can provide information on all unit cell dimensions(Hachet *et al.*, 1996). The crystalline nature of the biosynthesized nanoparticle was confirmed from the analysis of the x-ray diffraction pattern (Fig 5). The XRD peaks at two theta values

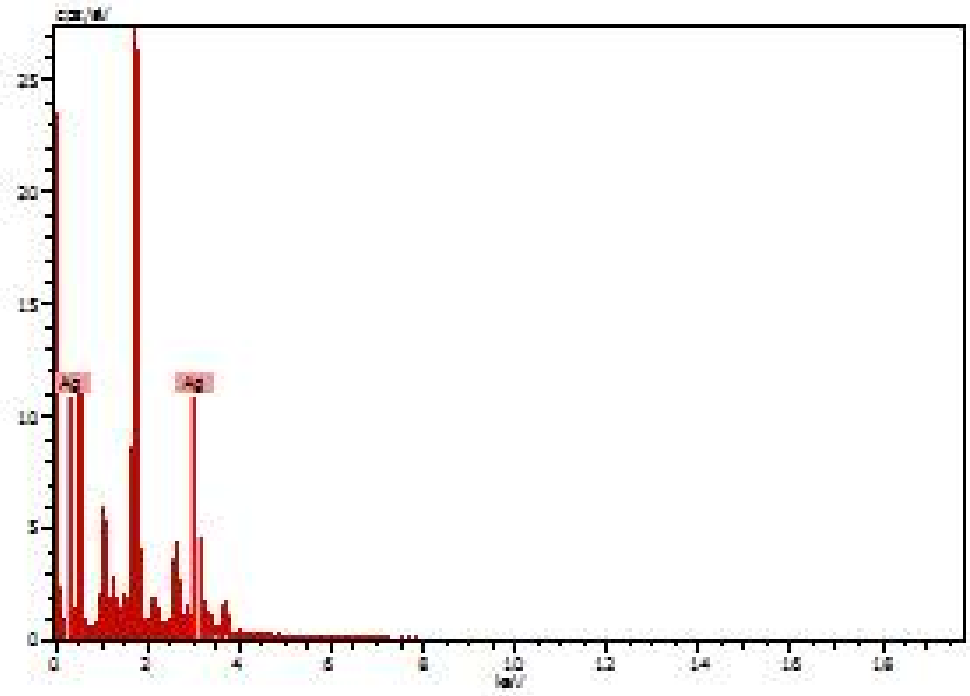


Fig. 4. EDAX spectrum of Silver nanoparticles

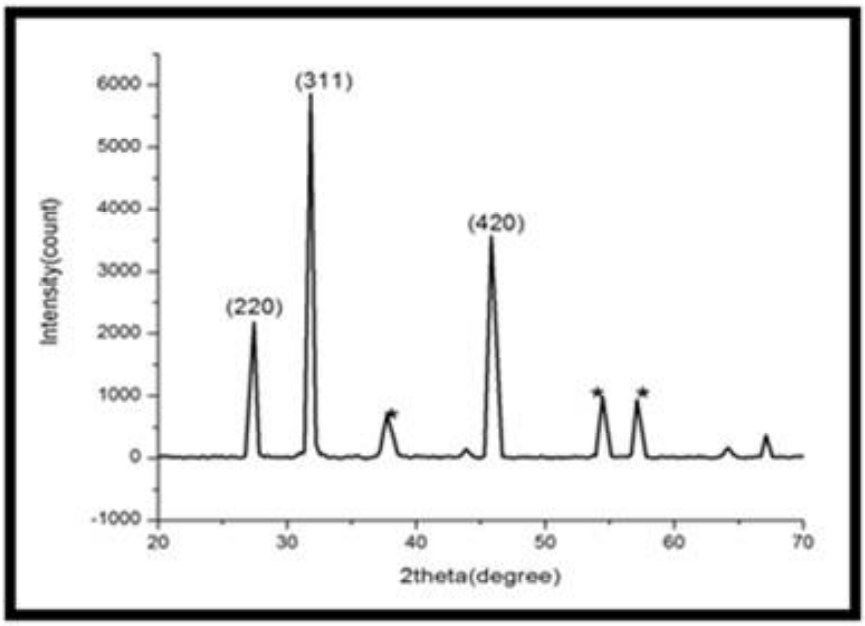


Fig. 5. Xray diffraction Spectra of synthesized Silver nanoparticles

Table 1. Phytochemical analysis of aqueous extract of *Gleichenia dichotoma*

Phytochemical	Result
Alkaloid	-
Tannins\Phenols	+
Flavonoids	+
Saponins	+
Cardiac glycosides	+
Steroids	+
Quinones	+
Phytosterols	-
Anthroquinones	+
Terpenoids	+
Aminoacids	-

of 28º, 32º and 46ºrepresenting the (220), (311) and (420) Bragg reflectios respectively which may be indexed based on the face centered cubic structure of silver nanoparticles. X- ray Diffraction data confirmed that the silver nanoparticles synthesized using *Gleichenia dichotoma* extract are crystalline in nature. The unassigned peaks observed suggest the crystallization of bio-organic phase occurs on the surface of silver nanoparticles. The average particle size of nanoparticles is calculated using Debye-Scherer equation and was found to be 28.56 nm.

Antibacterial assay

Antibacterial activity of synthesized nanoparticles, plant extract and silver nitrate was evaluated using disc diffusion method. The nanoparticles and aqueous silver nitrate solution showed zone of inhibition against *E.coli* but no zone of inhibition was observed against *S.aureus*. The plant extract alone has no activity against both the pathogens. The diameter of the zone of inhibition is summarized in Table 2.

The exact mechanisms of antibacterial activity by silver nanoparticles are still under investigation. A variety of reasons are given in literature for the antimicrobial activity of silver nanoparticles. The antibacterial ability of silver nanoparticles may be due to their ability to anchor to the bacterial cell wall thereby altering the membrane permeability, generation of free radicals, interaction with the thiol groups of many vital enzymes and inactivating them. It was also suggested

Table 2. Antibacterial activity of silver nanoparticles

Organism	1mM$AgNO_3$	Plant extract	AgNP
E.coli	7mm	No zone 13mm	
Staphylococcus aureus	No zoneNozone	No zone	

that silver nanoparticles interact with phosphorus and sulphur containing compounds such as DNA thereby act against bacteria (Guidelli *et al*., 2011).

Phytochemical screening:

The preliminary phytochemical screening of aqueous extract of *Gleichenia dichotoma* revealed the presence of various bioactive compounds as shown in Table 1.The presence Tannins\Phenols, Flavonoids, Saponins, Terpenoids ,Steroids, Quinones, Anthroquinones and Cardiac glycosides ensured that the plant extract had bioactive compounds that had reducing ability.

CONCLUSION

The utilization of *Gleichenia dichotoma* for the synthesis of nanoparticles has not been reported so far. In the present study the silver nanoparticles were synthesized using aqueous extract of *G.dichotoma* and were characterized. Phytochemical screening revealed the presence of bioactive compounds and the synthesized nanoparticles showed activity against *Escherichia coli*.

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Effect of *Ocimum sanctum* Supplemented Diet on Selected Parameters of Giant Fresh Water Prawn, *Macrobrachium rosenbergii.*

JASMINE ANAND[1]* AND C. BIJU[2]

[1]*Department of Zoology, S.N. College, Punalur,*
[2]*Department of Botany, S.N.College Punalur.*
**email : ja7210@gmail.com.*

ABSTRACT

A study was undertaken to assess the efficiency of methanolic extract of medicinal herb-*Ocimum sanctum* on the general metabolic levels and thereby on the biochemical, hematological and microbiological indicators of freshwater prawn *Macrobrachiumrosenbergii* post larvae (PL 30). The post larvae were fed with an experimental diet prepared in the laboratory with different concentrations of methanolic extracts of *Ocimum sanctum* @ 250 mg, 500mg and 1gm per kg normal feed. On the termination the total protein, lipid and glycogen in the tissue analyzed showed significant improvement in muscle and hepatopacrease compared to the control values. T Significant decreases in the total microbial load at the non-specific immune sites were recorded in the experimental prawns. Differential haemocyte counts of the experimental prawns recorded an elevation in hyaline cell populations compared to the controls. Thus preliminary observation indicates positively a growth promotion and immunomodulation on *Macrobrachiumrosenbergii*fed with medicinal herb *O.sanctum*supplemented diet.

Key words *Macrobrachiumrosenbergii, Ocimum sanctum* and immunomodulation.

To meet global viable standards, intensive aquaculture is normally practiced. This creates a highly stressful environment for the fish, triggering a suppression of the immune response coupled with increased susceptibility to diseases. Traditionally, antibiotics have been used as one of the most important chemotherapeutics in aquaculture for the prevention and treatment of bacterial diseases and also to avoid an acute infection. Antibiotic therapy is undesirable as it leads to the development of antibiotic- resistant bacteria, immunosuppression and destabilization of the helpful bacterial populations.

Prawns and shrimps are known for their high nutritive value. However, the intensive culture methods adopted in prawn cultivation frequently cause physiological stress to the animals and consequently predispose them to infections. The need to reduce the lethal and debilitating effects of pathogens is stimulating a renewed interest in the defense mechanisms of immune system of crustaceans for consolidating a sustainable shrimp culture sector. Thus upgrading the non-specific immune response of aquaculture candidates has become an active area of research in Disease Management of Aquaculture Systems (Murthy, 2000).

Immunostimulants are substances, which enhance the non-specific defense mechanism and provides resistance against the invading broad spectrum pathogenic microorganism. Many substances from different sources (bacterial components, chemical agents, animal or plant extracts etc) have been studied as prospective immunostimulants for fish (Sakai, 1999). Immunomodulation by 'Immuplus' was reported in *Macrobachiumrosenbergii (*Kumari Jaya, 2004)Oral administration of peptidoglycan derived from *Bifidobacteriumthermophilum* had found to increase the disease resistance of *Penaeusjaponicus* (Itami, 1998). Levamisole was also found to be an effective immunostimulant in *Macrobachiumrosenbergii* (Baruah, 2001). But there are major limitation to the general use of these agents such as increased risk of infection and generalized effect throughout the immune system (Diasio, 1996). Many herbals have been used for millennia as home remedies and some of these have potent anti-viral properties. Among them a few have been found to have anti-viral activity against shrimp viruses (Direkbusarakom, 1996).

Plants are the store houses of rich resources of safer and cheaper secondary metabolites. These natural plant products have been reported to have various activities like antistress, growth promoters, appetizer, tonic, immunostimulants and antimicrobials (Citarasu, 2002). Moreover, the substances are obtained from nature sources, besides possessing other interesting properties like non – toxic, biodegradable and are biocompatible (Citarasu, 2003).

Table 1. Fluctuations in the Differential Cell Count of *Macrobrachium rosenbergii* Subsequent to 4 Months of Treatment with *Ocimum sanctum*

Hemocytes	Control	TD 1	TD 11	TD 111
Hylinehemocytes	75.%	76%	78%	78%
Large Granular hemocytes	13%	13%	12%	13%
Semi granular hemocytes	10%	11%	10.%	10%

The methanolic extract of the botanical candidate of current interest *Ocimum sanctum* leaf was prepared. The test substance was administered orally. Assessments have been attempted on the basis of general biochemical, hematological and microbiological observations.

MATERIALS AND METHODS

M. rosenbergii (30 post larval stage) were obtained from a commercial farm in Varkala (ADAK) and acclimated in the laboratory for two weeks at 30 + 2.1^{0}C before experimentation. Water quality parameters such as salinity (28 + 1.5%), pH (8.2+0.1) were maintained every day. Prawns were fed with the basal diet prepared in the laboratory (Boonyaratpalin, 1993). Three sets of experiments were carried out in glass tanks of 100L capacity and proper aeration was provided. 50% of water was renewed daily during removal of waste feed and faecal matter. In each set, 15 prawns fed at 4 % body weight / day was maintained. The medicated feed was prepared incorporating methanolic extract of *Ocimum sanctum* as follows: 0.25 gm extract / kg feed (Test

Table 2. Biochemical Parameters of *Macrobrachium rosenbergii* Treatment with *Ocimum sanctum* Supplemented Diet

Duration	Sites (mg%)			Test dose		
			Control	TD 1	TD 11	TD 111
2 month	Protein	Muscle	8.2±1.50	9.3± 0.12	$^{11.12}$± 1.10	13.7± 0.03
		Hepatopancrease	4.12±1.50	4.34 ± 1.6	4.46 ± 1.7	4.49 ± 1.8
4 month	Protein	Muscle	9.5± 0.12	11.05± 1.10	12.35± 0.04	16.19± 1.50
		Hepatopancrease	6.57±0.12	6.61 ± 0.13	6.68 ± 0.14	6.69 ± 0.15
2 month	Glycogen	Muscle	0.289±0.12	0.328± 0.21	0.431± 0.31	0.47± 0.32
		Hepatopancrease	0.372±0.31	0.421± 0.38	0.453± 0.41	0.492± 0.43
4month	Glycogen	Muscle	0.31 ± 0.21	0.47 ± 0.24	0.62±0.31	0.68± 0.33
		Hepatopancrease	0.42±0.14	0.49 ± 0.15	0.68± 0.16	0.71 ± 0.17
2 month	Lipid	Muscle	0.038±0.12	0.045± 0.11	0.059± 0.01	0.062± 0.01
		Hepatopancrease	0.045±0.13	0.052± 0.11	0.065± 0.14	0.068± 0.21
4 month	Lipid	Muscle	0.048 ± 0.31	0.052 ± 0.01	0.085± 0.12	0.092± 0.21
		Hepatopancrease	0.057±0.12	0.063 ± 0.13	0.088± 0.14	0.124 ± 0.16

*Mean + Standard Error

Table 3. Total Bacterial Load in the Water, Exoskeleton and Gut of *M. rosenbergii* After 2 and 4 Months of *Ocimum sanctum* Treatment

Non specific immune sites	Treatment	Sample tested			
		Control	TD 1	TD 11	TD 111
WATER	2 month	7.0×10^6	6.4×10^6	6.4×10^6	6.3×10^6
	4 month	7.5×10^6	6.4×10^6	6.3×10^6	6.2×10^6
EXOSKELET0N	2 month	7.0×10^6	6.2×10^6	6.1×10^6	6×10^6
	4 month	8.2×10^6	5.1×10^6	5.1×10^6	5×10^6
GUT	2 month	6.5×10^6	5.4×10^6	5.4×10^6	5.3×10^6
	4 month	6.4×10^6	4.4×10^6	4.3×10^6	4.3×10^6

dose – I, abbreviated to TD-I) 0.50 gm extract / kg feed (Test dose – II, abbreviated to TD-II) 1gm extract / kg feed (Test dose – III, abbreviated to TD – III)

The experiments were terminated after 2 and 4 months subsequent to TD-I, TD-II and TD- III doses. Simultaneously a set of control prawns are cultured by giving normal feed. Total protein (Lowry *et.al*; 1961) muscle and hepatopancreas, the glycogen content (Sifter et.al, 1950).of muscle and hepatopancreas and the total lipid content (chloroform methanol method.) in the muscle and hepatopancreas were estimated. The total bacterial load in the water, gut and exoskeleton was assayed as per the techniques outlined by Kannan*et.al* (2002) with slight modification.

RESULT AND DISSCUSSION

The results obtained according to the experimental design are represented in Table 1-3. Among the physiological parameter studied protein, glycogen and lipid content of muscle tissue and hepatopancreas exhibited wide variations. Haematological aspects also present remarkable variation. Marked decreases were recorded in microbial count after the treatment.

DISCUSSION

The parameters studied indicate a positive effect in these areas. An increase of protein content in various tissues (muscle and hepatopancreas) indicates better biochemical turn over. Elevation in the glycogen and lipid content of both muscle tissue and hepatopancreas also indicate a healthy energy profile. After the treatment, the bacterial load at the sites of non-specific immune show a marked decrease which is reflected in the better growth and survival percentage of the prawn. This can be comparerable with our previous work (Jasmine Anand, 2011, Salini.M.P*et, al.* 2014, Rebecca *et, al.* 2014,Muralisankar*et, al.* 2017.).

Haemocytes were observed and classified using RogerioGargioni and Margherita Anna Barraco (1998). Here we can see an increase in the hyline cells which accounts for the improvement in the phagocytic activity in the treated ones with respect to control.

To summaries the present study it can be stated conclusively that *O.sanctum* has a growth promoting as well as immunomodulatory effect on *M.rosenbergii*. This preliminary work says little about the physiological mode of action of the plant extract but is the tip of an iceberg of an ecofriendly area for future scientific work.

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Morpho-Anatomical Study of *Gelidium micropterum* (Gelidiaceae, Rhodophyta) from Kollam Coast of Kerala

JAYA LEKSHMI. R

Department of Botany, M.M.N.S.S College, Kottiyam
e-mail: jayalekshminilamel@gmail.com

ABSTRACT

Marine algae are popularly known as seaweeds. They are autotrophic small filamentous or large parenchymatous highly advanced forms and are available largely in shallow coastal waters wherever there is substratum on which they can grow and flourish. Rocky coastal places of Kerala are rich in species diversity and they are restricted to places of Thiruvananthapuram, Kollam, Kozhikode, Kannur and Kasargod districts of Kerala. The red algal species are rich on the Kerala coasts due to the unique climate conditions of the state.

Key words *Morpho-Anatomy, Gelidium micropterum, Gelidiaceae, Rhodophyta, Kollam*

Hommersand & Fredericq (1988) conducted detailed morphological studies of vegetative and reproductive development and redefined the order. Despite the economic importance of some *Gelidium* species, solid vegetative characters for recognizing the species are generally lacking (Rodriguez & Santelices1988, Satelices 1990), and reproductive structures are often not available on specimens collected in nature.

Identification of the species of *Gelidium* are notoriously difficult. This resulted from a high degree of morphological variation, particularly in the smaller and medium-sized species (Dixon and Irvine, 1977). *Gelidium* species are larger in size only in temperate countries.

The common Indian *Gelidium species* include *G.comeum* (Hudson) Lamouroux, and G. *corneum* var. *subrigidum,G. micropterum* Kuetzing, G. *pusillum* (Stackhouse) Le Jolis, G. *pusillum* var. *pulvinatum* (C. Agardh) Grunow. Among *these, Gelidium pusillum* is cosmopolitan in distribution. The plants of G. *pusillum* is very small 1.0-2 cm long, exhibit a lot of morphological variations. Since the plants are very difficult to collect and difficult to get raw materials this species is not exploited as a source material for agar in any part of the world. *Gelidium micropterum* is the largest *Gelidium* species reported from India. Its distribution is restricted to South Indian coast, especially throughout the coast of Kerala. It can be utilized as a source of bacteriological grade of Agar. Its agar yield varied from 23 to 50%. So far studies on the structural vegetative and reproductive morphology of the Indian species are not available. The Indian species of *Gelidium* are not documented.

MATERIALS AND METHODS

Plants of *G. pusillum* were collected from, exposed rocky areas of intertidal regions. Plants were very small and firmly attached on the rocks by means of rhizomatous expanded disc. Plants of *G.micropterum* were collected and kept in plastic bags. Seawater of about 10 to 20 liters was also collected in plastic containers. After reaching the laboratory the plants were observed under a dissection microscope and species were sorted for tetrapsporic, male and female and preserved separately in 10% formalin. The tetrasporic plants were more than 75% when compared to sexualplants. The collected specimens were preserved in 5% formaldehyde in seawater and in large plastic containers for future use. Both vegetative and reproductive parts were selected and fixed in 1 % chrome-acetic fixative for taking microtome sections. Paraffin blocks were prepared by TBA method (Johansen, 1940) and sections were taken at 5 to 10 µm, by using an ERMA rotary microtome. Sections were critically examined under a trinocular research microscope, Olympus CX41, fitted with a Canon SLR Camera 6000 by using an adaptor, photographs were taken and transferred to a computer. All the photographs were suitably arranged by using Photoshop software CS3.

RESULTS AND DISCUSSION

Vegetative morphology

The plants are 5-8 cm tall attached to a rocky substratum, with several primary axes arising from a branched creeping portion. The basal creeping portion is more conspicuous during the early stages and later on it becomes irregular and spreads over the base of the erect axis forming a holdfast with one or many distichously branched erect axes arising from holdfast of entangled stolons. The erect axes are purplish-red and flat; while the prostrate axes, cylindrical and pale red in colour. The basal portions of erect axes are narrow and cylindrical, with, while the upper portions expand up to 3 mm wide with smooth margins.

The erect axes are invested with many oppositely or alternately arranged lateral branches, and they reach a length of 4 mm to 6 mm .The primary branches are of four or five orders with regularly or irregularly arranged alternate or opposite branches. The lateral branches are similar in shape and appearance to the primary axis.

Growth occurs by means of a transversely dividing apical cell. It is lenticular, 4.0 - 5.0 µm in length and 9.9 µm in width and lies in a notch overtopped on both sides by more rapidly growing thallus margins

Vegetative anatomy

The transactional outline of the different regions shows much variation. The basal part of the primary axes is terete, with distinct cortex and medulla .The outer cortical cells are compactly packed. The medullary cells are colourless, more or less rounded, and slightly larger than the inner cortical cells. Rhizoidal cells are small, elongated and dense in the medulla region.

The cortex is three or four layered with pigmented cells. The inner cortical cells are large, ovoid and 6.6 - 11.0 µm in diameter. The size of the cortical cells diminishes towards the surface and the outermost assimilatory layer consist of deeply pigmented photosynthetic cells.

Medullarycells are ovoid to spherical, colourless with thick walls. Rhizoidal cells small, densely packed in the medulla and the inner cortex. Anatomically, the fertile pinnules differ from vegetative pinnules. The area adjacent to the fertile central furrow contrasts sharply with the sterile portion.Tissues on either side of the fertile furrow resemble vegetative cortex and medulla. The rhizoidal filaments are present only in the medulla of the vegetative tissue, but totally absent in the fertile central region.

Reproductive morphology and Anatomy

The gametangial and tetrasporic generations are isomorphic, although the tetrasporophytes occur in varied size and they are slightly larger up to 8 cm. The male thalli are comparatively smaller and irregularly branched with light reddish in colour.

Tetrasporangial plants

The tetrasporophytes usually reach a height of 3-8 cm .Tetrasporangia are formed in terminal stichidia. Each stichidium is about 2 - 5 mm long and 0.75-1 mm board with a notched apex

In a branch ,the development of fertile stichidia are in an acropetal order .Two types of stichidia are found, stichidia short broadly rounded with a width of 600-800 µm and an elongated, with a length of 800-1000 µm .Initially the tetrasporangia are compactly arranged and later on scattered over the stichidium, surrounded by a sterile region .

The sporangial initials are cut of laterally from the cortical cells by anticlinal divisions. At maturity they become embedded in the inner cortex, as a result of continued growth of surrounding cortical filaments. Tetrasporangia are broadly ellipsoid to spherical in shape, 35.0 - 49.5 µm long and 23.0 - 25.0 µm broad, cruciately divided into four tetraspores.

Cystocarpic plants

The mature female plants are about 3-6 cm high, fertile branchlets once or thrice compound and crowded with pinnately or irregularly arranged or densely branched pinnules .The fertile area is initiated by the formation of a longitudinal groove which extends down the middle of the pinnule on both sides for about 2/3 of its length .This fertile area contains the developmental stages of several carpogonia. Later, the reproductive area became

dark and slightly swollen with a small terminal sterile tip.

At maturity,the cystocarps are raised, oval to circular in outline and bilocular with a central partition of two locules. The mature carposporangia are ovoid, 50.0 - 66.5 µm long, and 20.5 -29.0 µm broad .The two cystocarpic locules develop at a similar rate with ostiolar openings on opposite sides. After the liberation of the carpospores, the fertile areas of the pinnules are provided with conspicuous holes

Spermatangial plants

The male plants are comparatively smaller than tetrasporic and cyctocarpic ones. They usually reach a height of about 2-4 cm, and often difficult to distinguish from some sterile plants. They are readily distinguished from tetrasporic plants in having a fading colouration and the absence of conspicuous fertile stichidia. The male thalli are characteristically compact and bushy. The spermatangial sori occur superficially as regular, elongated or irregular patches on the main axes, lateral branches and ultimate branchlets. They are pale yellowish and somewhat translucent. A gelatinuous cuticle over the spermatangia imparts a mucilaginous texture to the frond. In the fertile areas, the cortical cells elongate and divide anticlinally to produce a group of spermatangial mother cells. The spermatia are cut off from the mother cells by transverse divisions, each with a diameter of 2.0 - 3.5 µm Boergeson (1937) reported *G.micropterum* from India, Ceylon, and Mauritius. In India its distribution is restricted to Cape Comerin, Quilon, and Veravel (Sreenivasa Rao, 1970). . The taxonomy and the structural morphology of this important *Gelidium* species have been neglected and not properly studied. In India and many other parts of the world, it can be utilized as a source of good quality agar. In nature all the three kinds of plants are readily available. The tetrasporic plants are large in size when compared to male and female. They are not strictly isomorphic. Tetrasporic plants are more in number, purple red in colour. The male and female plants are smaller in size, the male plants are not deeply coloured with irregular branched, the branch tips are more whitish at the time of liberation of spermatia. The female plants are irregularly branched deeply pigmented with dark red in colour.

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Long Term *In vivo* Response to Silicone Tissue Expander Material in Rabbit Model

JOSNA JOSEPH[1, 2], SABAREESWARAN ARUMUGHAM[1]*, MIRA MOHANTY[1]

[1]*Histopathology Laboratory, BMT Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram*

[2]*Advanced Centre for Tissue Engineering, Department of Biochemistry, University of Kerala, Thiruvananthapuram*

**email:asw@sctimst.ac.in*

ABSTRACT

The formation of extensive fibrosis around silicone implants that lead to capsular contracture and implant failure is a multifaceted problem in which several factors are engaged. The major aim of this study is to evaluate the long term *in vivo* response to silicone biomaterial with special reference given to the leaching out of silicone to peri prosthetic tissue and subsequent fibrous capsule formation. Commercially available Silicone tissue expander material was implanted in the paravertebral muscle of rabbits and the long term *in vivo* response over 180, 270, 365 days was studied. Ultrahigh molecular weight polyethylene (UHMWPE) was implanted as the control material in contra lateral leg. Tissue response to silicone expander material was examined by retrieving the implant, after the implantation term along with peri-prosthetic tissue and by histology and stereomicroscopy. SEM-EDAX and ICP-AES studies were done to detect the presence of silicone release products in the peri-implant tissue. The data from this study shows that the continuous leaching of silicone from the surface of Tissue expander material leads to the laydown of a thick peri-implant ECM tissue around the silicone material leading to the failure of the implant.

Key words *Silicone; tissue expander; capsular contracture; implant-tissue interaction*

Within the last decade, there has been a tremendous increase in both the variety and the number of implants and biomaterials available for use in the human body. Silicone is a biomaterial widely used in medical device industry. Although there had been several attempts to define the biological response to silicone implants, the situation is still not clearly understood. In contrast to early presumption that silicone is inert, Kossovsky *et al.,* suggested the bioactivity theory in 1995 which states that silicones interact with their biological environment, perturb that environment and are acted upon by that environment (Kossovsky, and Frieman, 1995). The polymeric and hydrophobic characteristics of silicone make it potentially immunogenic. Reports indicate that the rates of localized complications and repeat surgeries following breast implantation are high with patients developing clinical symptoms in later periods (Shanklin, and Smalley, 1999). In addition to the local phenomena, several case reports of systemic connective tissue diseases have been published (Baldwin, and Kaplan, 1983, Bronzena, *et al*., 1988). Hence, this study aims at studying the long term *in vivo* response to silicone biomaterial with special reference given to the leaching out of silicone to periprosthetic tissue and subsequent fibrous capsule formation.

The implantation of a biomaterial itself causes an injury, which could result in acute and chronic inflammation. The end stage of healing response to biomaterials is fibrosis or fibrous encapsulation which is the formation of a dense connective tissue scar. The net balance between production of growth promoting factors and destructive proteolytic factors should be viewed as an extremely delicate balance that could favour fibrous capsule formation and/or degradation. While some degree of fibroblast contraction is necessary for wound closure, excessive proliferation of fibroblasts may lead to scar tissue formation. Rudolph *et al.,* 1978 found contractile fibroblasts in fibrous tissue capsules around silicone breast implants. Myofibroblasts are transdifferentiated form of fibroblasts, which exert contractile forces to nearby Extra Cellular Matrix (ECM) and deposit collagen at wound site. They are the intermediate cell types between fibroblast and smooth muscle cell (Phan, 2002, Eyden, 2003).

The risk of implant rupture increases with implant age. Studies in animal model have shown that capsule forms in a relatively short time of two months (Piccha, and Goldstein, 1990). It was reported that there was a significant positive correlation between capsule thickness, presence of chronic inflammation and macrophage presence (Eltze *et al*., 2003).

The role of macrophages in fibroblast modulation have been addressed by our earlier studies (Josna *et al*., 2010). *In vivo*, when an implant is placed into

body, the circulating monocytes from the blood reach the injury site and get activated to macrophages secreting a myriad of cytokines that play a pivotal role in the subsequent wound healing. An *in vitro* study was designed to probe the role of macrophage secreted cytokines in fibroblast activation and the signaling pathways involved in such transformation (Joseph *et al*., 2013). This work is thus a sequel to the earlier studies to probe the long term *in vivo* response to the silicone tissue expander material, the findings of which would pave way for elucidation of newer drug targets for preventing the failure of clinically implanted silicone materials.

MATERIALS AND METHODS

Implantation and retrieval

Long term implantation study of silicone expander material was carried out in the para vertebral muscle of New Zealand white rabbit models. Six female rabbits were implanted with tissue expander (SE) material and the control material, Ultra High Molecular Weight Poly Ethylene (UHMWPE) was implanted in the contra lateral side. Both SE and UHMWPE were cut into pieces with dimensions of 1.5cm x 1.5cm x 1 mm, cleaned and sterilized with ethylene oxide. The skeletal muscle was selected as an ideal site for implantation, because in the clinical situation the silicone expander material is in contact with skeletal muscle of breast. The skin was swabbed with 70% alcohol and a 1.5 cm incision was made in the gluteus muscle. The SE was inserted into the muscle. The wound was closed with catgut and the skin sutured externally with a nylon suture. The implantation procedure was done based on ISO-10993-6 .

At the end of study, Animals were euthanized with an overdose of sodium thiopentone. Material with the surrounding tissue were retrieved and fixed in 10% buffered formaldehyde for Haematoxylin &Eosin staining and in 3% buffered gluteraldehyde for Scanning Electron Microscopy-Energy Dispersive X-Ray spectroscopy (SEM-EDAX). One cm^2 cross sections of implant site from all time periods with surrounding muscle were dehydrated in iso propyl alcohol in ascending grades of dilution, cleared in chloroform and impregnated in paraffin wax. 5 µm thick paraffin sections were taken using the Automatic microtome (Leica RM 2155) and stained with Hematoxylin & Eosin in Leica Autostainer XL. The stained sections were examined by bright field microscopy (Nikon Eclipse E600).The fibrous capsule formed around the implant was quantitated by measuring the average thickness from five equidistant sites. The data obtained was analysed statistically by one way ANOVA and represented graphically.

Scanning electron Microscopy-Energy Dispersive X-Ray spectroscopic (SEM-EDAX) analysis

The explanted material fixed in 3% buffered gluteraldehyde were dehydrated in ascending grades of ethanol and dried in liquid [phase with iso amyl acetate. Critical point drying was carried out (Critical point Dryer, Model HCP-2 Hitachi Science systems,

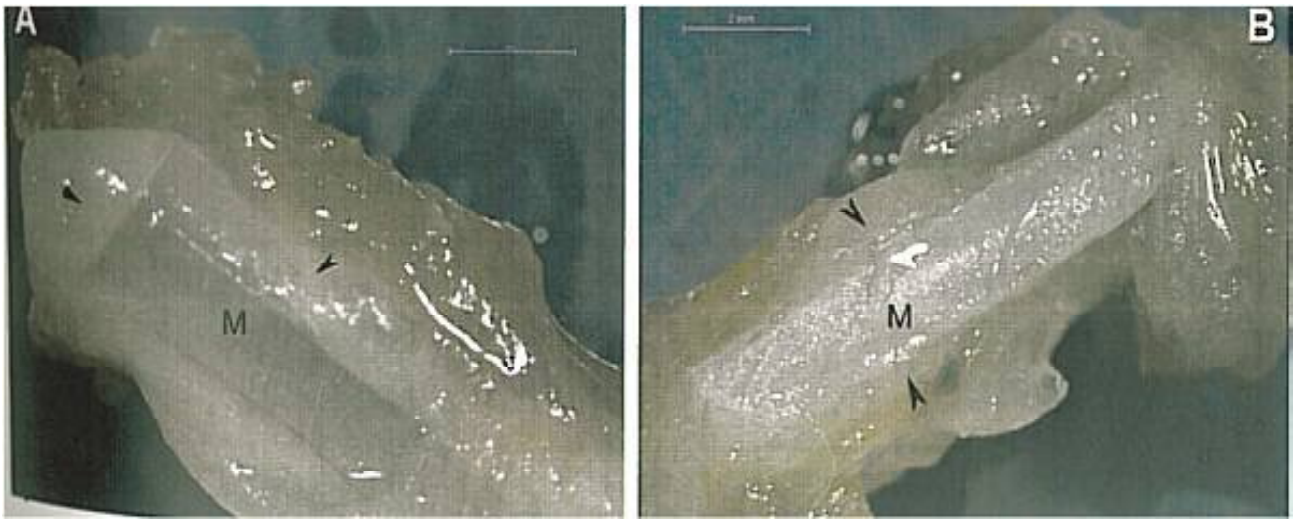

Fig. 1. Stereo microscopic images of the retrieved implant material with (M) surrounding peri-prosthetic tissue: A) Silicone Tissue expander B) UHMWPE after one year of implantation, showing a layer of ECM deposition

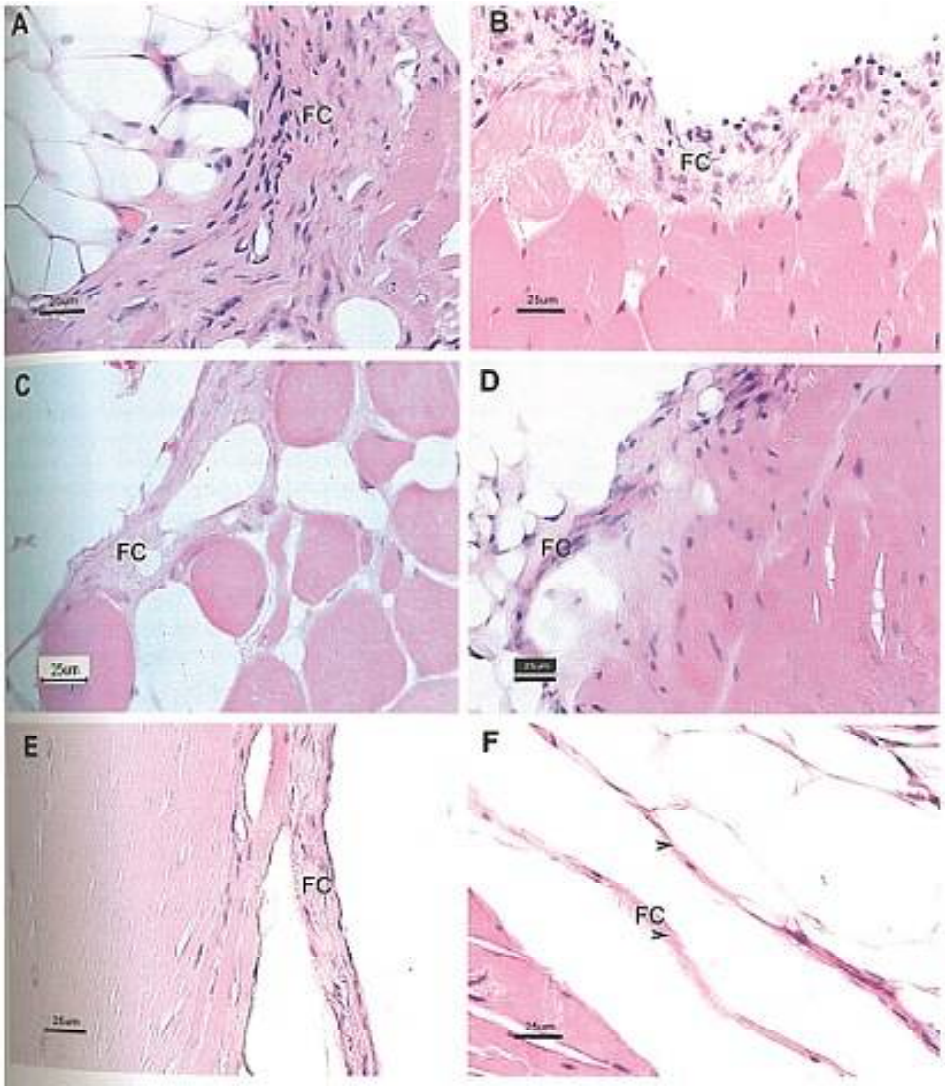

Fig. 2. Light micrographs of Haematoxylin & Eosin staining in sections of peri implant tissue around Silicone expander: 180 days; 270 days; 365 days (A, C, E) and around UHMWPE(B,D,F) FC-fibrous capsule, 40X magnification

Japan) in liquid Co2 at 150 Kg/cm^2. The samples were sputter coated with gold (Ion Sputter Model E101, Hitachi, japan) and observed using a scanning electron Microscope (S2400 Hitachi) at an accelerating voltage of 15 kV. Spectrum was analyzed using the program ICIC link.

Inductively coupled plasma atomic emission spectroscopic (ICP-AES) analysis

The tissue samples which were fixed in 3% buffered gluteraldehyde were washed well with deionised water, dried at 56^0 C for 2 hours. The tissues were digested by dissolving with 5 ml HNO3 and 2 ml HClO4 and made upto 50 ml using HPLC grade water. The filtered sample was analyzed with ICP-AES system (Thermo electron IRIS Intrepid II XSP DUO).

RESULTS

Histology and Histomorphometry

Upon retrieval at 365days post implantation, both the implants were deeply embedded in extra cellular matrix (Fig.1).The stereo microscopic images showed that the matrix deposition was thicker around SE (Fig. 1A) when compared to UHMWPE (Fig. 1B).

These results correlated well with Hematoxylin & Eosin staining which showed a thick fibrous capsule formation around silicone implants (Fig. 2A,C,E) when compared to the biocompatible control material, UHMWPE (2 B,D,F).

Histomorphometric analysis of fibrous capsule around the retrieved implant

Thick fibrous capsule formation and persistence

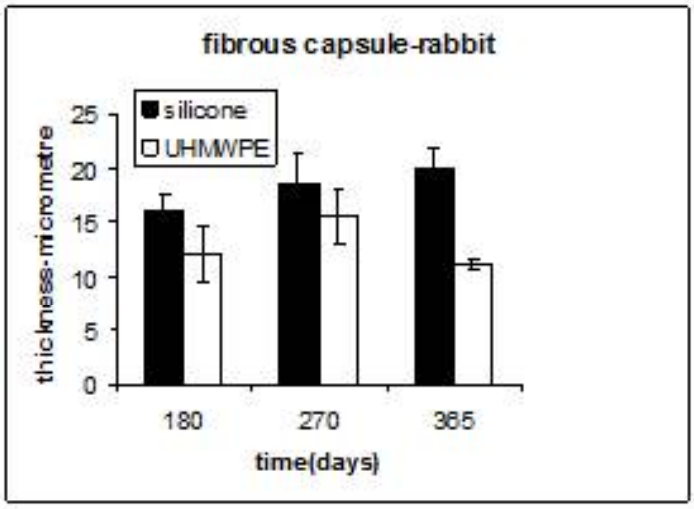

Fig. 3. ECM deposition at tissue material interface around SE and UHMWPE.

around SE even at the end of 365days when observed by Hematoxylin & Eosin staining as compared to the UHMWPE (Fig. 2E). These results corroborated well with the findings of our earlier *in vivo* studies in rat (Josna *et al*., 2010). The inflammatory response at 180 days resolved to fibrous encapsulation at 365 days around both implants (Fig. 3). There was significant difference in the thickness of fibrous capsule between the two materials, silicone and UHMWPE (Fig. 3). The fibrous capsule formation around SE showed an increasing trend over time, whereas around UHMWPE there was a decrease at 365 days.

SEM-EDAX analysis of retrieved peri implant tissue

Results from this study showed only negligible amounts of silicone content in peri-implant tissue both at 180 days and 365 days post implantation (Fig. 4A and B).

ICP-AES of retrieved tissue for silicone content

ICP-AES analysis of silicone in peri-implant tissue long term corroborated with SEM-EDAX results. It has been reported that ICP –AES detects precise amount of silicon in body fluids and tissues and they are more sensitive than NMR measurements (Peters *et al*., 1999). No difference was observed in the silicone content of peri-implant tissue between 180 day and 365 day samples (Table 1).

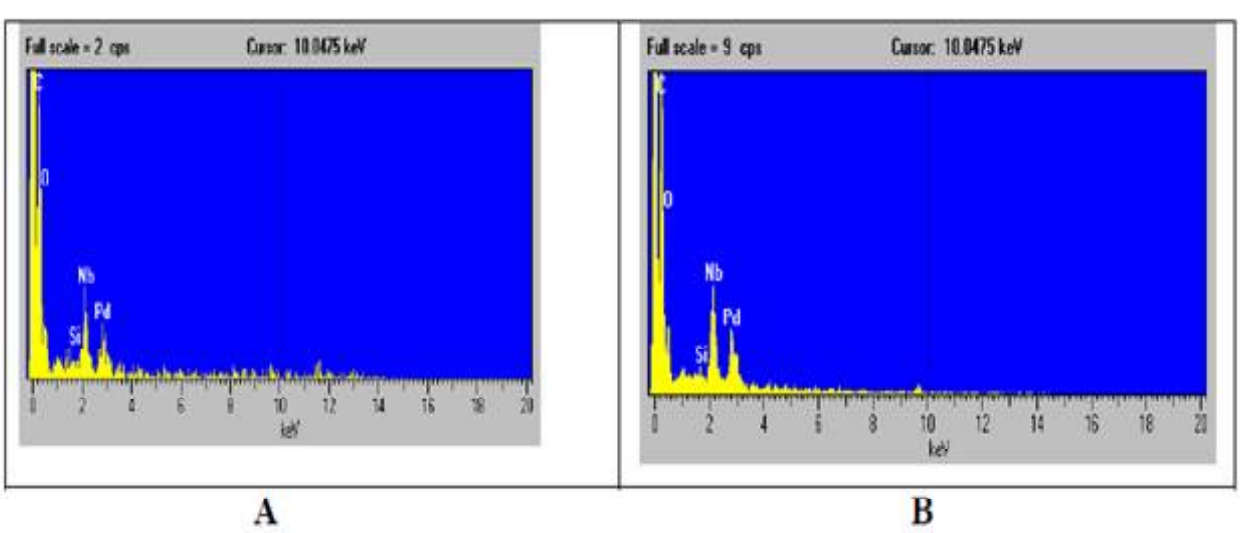

Fig. 4. Elemental distribution of silicone expander (SE) at peri implant tissue at 180 days (A) and 365 days (B) post implantation

Table 1. Silicone distribution in peri-implant tissue

Time period	SE (ppm)
180 days	0.05
365 days	0.05

DISCUSSION

The use of different types of polymers as components of medical devices has increased over the years. Polymers have physical properties that most closely resemble those of soft tissues and therefore this class of materials is used extensively to replace the functions of soft tissues including skin, tendons, cartilage, vessel walls, lens, breast and bladder. Silicones now represent the most widely used implantable synthetics. However, they are also the most controversial of the implantable materials.

Silicone can diffuse through the shell of the implant (Mancino *et al.*, 1984) and is then phagocytized by macrophages present in the surrounding tissue. Macrophages can degrade silicone in vivo, and hydrolysed silicone compounds as well as coordinated silicon complexes and silica can be formed. The ingestion of silicone particles by the macrophages induces a state of activation leading to the release of cytokines with high fibrogenic activity. This process is thought to trigger fibroblast proliferation and capsule formation around the implant. Moreover, chronic inflammation such as a foreign body reaction and silicone granulomas will develop.

A major unanswered question is whether local inflammatory and hyper plastic cellular reactions are silicone-specific, ie caused by specific components, additives, degradation products unique to silicone or whether these responses reflect a nonspecific Foreign Body reaction (FBR). Silica has been reported in the lymph nodes in saline implant patients. The silastic envelope of saline implants and tissue expanders is also a silicone polymer that may breakdown in the body into silica, possibly eliciting an immune response (Weinzweig *et al.,* 1998). This may be due to extra capsular migration of silicone to distant organs. The SEM-EDAX results are in agreement with Barnard *et al.,* 1997 stating that solid silicone elastomer implants shed particulate material through fatigue and surface abrasion and such particles can be transported to remote sites (Barnard *et al.,*1997, Hunt *et al.*, 1989).

The formation of extensive fibrosis around silicone implants that lead to capsular contracture and implant failure is a multifaceted problem in which several factors are engaged. Continued release of leachants from silicone implant by spalliation into the surrounding milieu, stimulates macrophages to release fibrogenic cytokines that are involved in a thick fibrous capsule formation around the implant. T cell activation around silicone implants occurs as early as 1 year after implantation and can persist for as long as nine years (Katzin *et al.*, 1996). Peri-implant connective tissue capsule may represent a possible site of antigen processing and presentation (Millonig *et al.*, 2001).

However, most of the earlier studies were carried out on clinically retrieved prosthesis and the results were inconclusive. Experimental studies have been few and mostly related to either material characteristics or *in vitro* response to material (Ziats *et al.*, 1988). This implies the relevance of this study in addressing a major clinical problem by simulating the *in vivo* situation in experimental animal models.

CONCLUSION

This study throws light on role of material surface and degradation products in the activation of fibroblasts and collagen deposition resulting in the extensive fibrosis around silicone implants leading to clinical contracture and pain. There exists a dynamic cycle of silicone release, activation of macrophages, release of cytokines and increased collagen deposition along with formation of myofibroblasts. This provides a plausible mechanism behind long term failure of silicone implants due to peri-implant contracture leading to severe pain. Identification of molecule/s involved would aid in devising newer ways to prevent the occurrence of excessive fibrosis around otherwise useful silicone implants.

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A Comprehensive Epigenomic Analysis of A3B Gene in Breast Cancer-A pharmacogenomics Approach

S. KARTHIKEYAN[1], GILU FRANCIS[2], P K KRISHNAN NAMBOORI*

Center for Computational Engineering & Networking (CEN),
Amrita School of Engineering, Amrita Vishwa Vidyapeetham, Coimbatore.
Dept of Electrical and Electronics Engineering, Amrita School of Engineering, Amrita Vishwa Vidyapeetham, Coimbatore
**Amrita Molecular Modeling and Synthesis (AMMAS) Research Lab, Center for Computational Engineering & Networking (CEN), Amrita School of Engineering, Amrita Vishwa Vidyapeetham, Coimbatore.*
email : n_krishnan@cb.amrita.edu

ABSTRACT

Breast cancer is one of the most prominent types of cancer. Genetic and epigenetic modifications in the genes are the major reasons behind development of breast cancer. Although numerous genes are associated with breast cancer, A3B gene is considered as an endogenous mutagen, which mutates other genes and is considerably up regulated in breast cancer tissues. In this paper, the epigenomic studies have been conducted for A3B gene towards proneness of breast cancer. The presence of genetic signatures in the regions of epigenomic variations has been observed. The methylation possibilities of A3B gene have been studied using the CpGIsland regions of A3B and 250 genetic signatures have been identified to be present in the CpG island regions suggesting the possible deregulation of methylation in A3B gene due to the presence of meSNPs. 300 breast cancer associated genetic signatures have been observed in the histone modifying genes. 16 SNPs in the DNA sequence of miRNAs of A3B have also been identified. A population wise analysis of the SNPs in the CpG islands and SNPs in the histone modifying genes suggest that CEU, YRI, CHB and JPT ethnic groups are more prone to epigenomic deregulations. This analysis of identifying the genetic signatures behind the epigenomic alterations of A3B gene would suggests the involvement of epigenomic impairments in A3B are also responsible for the origin of disease.

Key words *Comprehensive Epigenomic Analysis, A3B Gene, Breast Cancer, Pharmacogenic Approach*

Cancer is one of the major diseases affecting people around the world. Traditionally, cancer has been viewed as a disease caused due to mutations in the genes (1). However, this idea was changed and the epigenomic mechanisms were also considered as a reason of variations in the gene expression(2), (3). Epigenomics include a range of chemical compounds that can alter the gene expression without a change in the nucleotide sequence of the gene. Such impairments causes silencing of an active gene or expression of an inactive gene which is known as epimutation(4). This could be tagging of functional groups such as methyl group to the DNA(5). The major epigenetic modifications are DNA methylation(6), microRNAs and histone posttranslational modifications(7). Variations in the DNA methylation like hypermethylation and hypomethylation of tumor suppressive genes and oncogeneswere a major concern in cancer research(8).The residues of the histone undergo methylation, acetylation, phosphorylation, ubiquitylation and sumoylation(7). These modifications enable the cells to perform distinctly even with the presence of same genetic material(9).Although epigenomic processes are naturally occurring, inappropriate occurrences may result in disadvantageous health conditions(2). The inappropriate epigenomic variations can be found by the presence of disease markers like SNPs (10) in the genes responsible for epigenomic processes.

Various studies have demonstrated the association of methylation, acetylation, phosphorylation, ubiquitylation and sumoylation with cancer(11). Studies shows that there is an association with CPG island DNA methylation and tumor genesis(12),(13).Computational methods are widely used to determine the CpG islands and the methylation profile of genes. DataBase of CpG islands and Analytical Tool (DBCAT) identifies the methylated regions of a gene(14). The Single Nucleotide polymorphisms (SNP)s of the methylated regions are known as meSNPs(15) and the presence of meSNPs are likely to have effect on the methylation status(16).

MicroRNAs (miRNAs) are noncoding RNAs which plays a major role in determining a number of biological activities including cancer development and

Table 1. miRNAs of A3B gene with target scores greater than 90.

Target Score	miRNA Name	UTR sequence	Chromosome start positions	chromosome end position	SNPs
96	hsa-miR-4261	**TGT TTC**	38988867	38988873	rs911605532
			38985891	38985897	
			38988532	38988538	
			38988090	38988096	
			38991327	38991333	rs746966270, rs758237218, rs780014617
			38988876	38988882	rs192115582
			38992764	38992770	rs548087996
91	hsa-miR-4314	**TCCCAGA**	38983200	38983207	
			38992680	38992687	rs928013419, rs930052363

Table 2. Population analysis of HiSNPsXEthnic Group

	SNPs			
CEU	rs20554	rs2230139	rs535586	rs608118
YRI	rs20554	rs2230139	rs535586	rs608118
CHB	rs20554	rs2230139	rs535586	rs608118
JPT	rs20554	rs2230139	rs535586	rs608118
ASW	rs20554	rs535586	rs608118	
CHD	rs20554	rs535586		
GIH	rs535586			
LWK	rs20554	rs535586	rs608118	
MEX	rs535586			
MKK	rs20554	rs535586		

progression through regulation of gene expression(17). SNPs present in the miRNA gene sequence referred as mir-SNPs tend to affect the generation and functioning of miRNAs(18).The miRNA targets which are predicted using high throughput sequencing experiments, are available online in miRDB database(19).

Histone protein modifications are associated with deregulation of cancer associated genes and thus play an important role in cancer progression(20).Numerous post translational modifications occur on the N terminal tail of histone(21).For example,Lysines and arginines are subjected to histone methylation(21). A group of proteins are involved in histone modification includingacetylation by histone acetyl transferases (HATs), deacetylation by histone deacetylases (HDACs), methylation by histone methyltransferases (HMTs), demethylation by Histone Demethylase (KDM), Phosphorylation by kinases RPS6KAS, RPS6KA4 and BAZ1B and dephosphorylation by PPP. The SNPs present in the histone modifying genes influence the functioning of histone modifying proteins.

In the present study, the epigenomic analysis of breast cancer have been taken up.There are numerous genes associated with breast cancer.However, APOBEC3B gene is a master gene which induces mutations in cancer associated genes. In a study conducted by Burns et al, APOBEC3B gene has been

SUPPLEMENTARY DETAILS:

Table 1. Me SNPs

Gene Region	SNPs
38983951-38984253	rs143126732,rs752732848,rs761719120,rs774966499,rs779435424,rs111887063,rs112326825,rs113972364,rs114628569,rs115649149,rs116171368,rs117739457,rs139242988,rs139257374,rs139823580,rs145424338,rs149211753,rs150835370,rs186448053,rs199817842,rs200682782,rs367829367,rs367990393,rs369407878,rs371593494,rs372003442,rs372240785,rs374911938,rs375587851,rs375891493,rs377626351,rs532907341,rs547057305,rs549284303,rs551433639,rs557715283,rs573552790,rs751661633,rs751751478,rs755159659,rs756453471,rs757665891,rs757755950,rs758435695,rs759424680,rs760254937,rs762885055,rs763803605,rs766399491,rs766494496,rs767518498,rs768160451,rs770176181,rs771441000,rs773140102,rs773141905,rs773807764,rs774383486,rs776032853,rs778031662,rs778142544,rs779270247,rs896414568,rs903708981,rs960359836,rs972199750,rs1009321485,rs1034049000,rs1040360982
38985816-38986036	rs373745509,rs748111072,rs766117548,rs770721127,rs2076109,rs2076111,rs17000697,rs17000716,rs17854845,rs17857431,rs57140217,rs61178845,rs79053565,rs2076110,rs17000717,rs138362043,rs138673527,rs141704515,rs142365295,rs144579352,rs150187552,rs151303359,rs200746401,rs370497287,rs370960629,rs374615605,rs375596163,rs377648242,rs377714302,rs544553826,rs572927411,rs746852661,rs746860264,rs749960311,rs751972538,rs753367208,rs753457369,rs754901889,rs755548779,rs758071876,rs760019133,rs760107103,rs760682686,rs761293067,rs761961858,rs763279372,rs763642285,rs765496184,rs766792256,rs768591176,rs769828037,rs769985060,rs771243865,rs772014221,rs772683650,rs773262081,rs773599359,rs774574932,rs776693485,rs777459217,rs778838185,rs779929390,rs780469922,rs868628109,rs922256810,rs1025766300,rs58754121,rs59708943
38991193-38991725	rs138093253,rs368969177,rs536389154,rs748302883,rs752602030,rs756948955,rs761434816,rs761366083,rs766101518,rs770453697,rs770365670,rs775208296,rs774871602,rs779417531,rs1065184,rs13054219,rs17000560,rs57217289,rs62230502,rs73419957,rs4821851,rs34124580,rs138624531,rs142595375,rs144185478,rs144471703,rs149274572,rs149639159,rs192825139,rs199755827,rs200037182,rs200377379,rs367853574,rs368233569,rs372336066,rs373097436,rs374260464,rs375984210,rs376711822,rs376905026,rs377377948,rs527744108,rs528299145,rs530161764,rs532429881,rs541904248,rs544320686,rs544481700,rs550646291,rs554725216,rs560180606,rs560387218,rs562540315,rs568849992,rs574507100,rs746272740,rs746966270,rs747498277,rs748848992,rs749464336,rs749504588,rs750163214,rs750297692,rs751412009,rs752235701,rs753366575,rs753448371,rs754881364,rs756019959,rs757613037,rs758237218,rs758749682,rs760109051,rs760125354,rs760658380,rs761934686,rs763640667,rs763728440,rs763980775,rs764981113,rs765439037,rs767250819,rs768678900,rs769889025,rs770123452,rs771448638,rs771842710,rs772434543,rs772607602,rs772702945,rs772771449,rs772876371,rs774114207,rs776015983,rs778557319,rs779324810,rs779852798,rs780014617,rs780449933,rs781250164,rs865792651,rs887945569,rs902110922,rs910983615,rs918271236,rs970655444,rs1000947215,rs9611066,rs28446188,rs45479903,rs62230501,rs141343194

Supplementary Table 2: SIFT and Polyphene results of MeSNPS

Gene Region	Damaging SNPs	Probably damaging	Possibly damaging
38983951-38984253	rs139823580	rs139823580	rs374911938
	rs139823580	rs372003442	rs372003442
	rs200682782	rs139823580	rs371593494
	rs371593494	rs200682782	rs374911938
	rs139823580	rs372003442	
	rs200682782	rs139823580	
	rs371593494	rs374911938	
	rs139823580	rs372003442	
	rs200682782		
	rs371593494		

Gene Region	Damaging SNPs	Probably damaging	Possibly damaging
38985816-38986036	rs138673527	rs138673527	rs59708943
	rs144579352	rs144579352	rs374615605
	rs151303359	rs151303359	rs375596163
	rs200746401	rs200746401	
	rs374615605	rs374615605	
	rs375596163	rs375596163	
	rs138673527	rs138673527	
	rs144579352	rs144579352	
	rs151303359	rs151303359	
	rs200746401	rs200746401	
	rs374615605	rs374615605	
	rs375596163	rs375596163	
	rs138673527	rs138673527	
	rs144579352	rs144579352	
	rs151303359	rs151303359	
	rs200746401	rs200746401	
	rs374615605	rs374615605	
	rs375596163	rs375596163	
	rs138673527	rs138673527	
	rs144579352	rs144579352	
	rs151303359	rs151303359	
	rs200746401	rs200746401	
	rs374615605	rs2076110	
	rs375596163		
38991193-38991725	rs192825139	rs149274572	rs149274572
	rs367853574	rs376711822	rs192825139
	rs374260464	rs138093253	rs367853574
	rs376711822	rs367853574	rs374260464
	rs377377948	rs376711822	rs377377948
	rs192825139	rs138093253	rs192825139
	rs367853574	rs368233569	rs374260464
	rs376711822	rs138093253	rs377377948
	rs377377948	rs367853574	rs372336066
	rs138093253	rs377377948	rs149274572
	rs138624531		rs192825139
	rs149274572		rs376711822
	rs372336066		
	rs367853574		
	rs374260464		
	rs376711822		
	rs377377948		

Supplementary Table 3: Histone modifying genes SNPs: 300 Breast cancer associated SNPs

SNPs of the histone modifying genes which are associated with breast cancer.

rs111988493	rs143551315	rs142320590	rs138917060	rs147624173	rs371487025
rs112160628	rs143660871	rs142962259	rs139551099	rs147684194	rs372876045
rs113267516	rs143690368	rs143031242	rs139758629	rs147694084	rs375251697
rs114556688	rs143885808	rs143369082	rs139796173	rs148487813	rs375920693
rs11543970	rs144047215	rs143753697	rs140403496	rs149437753	rs377508897
rs138928546	rs144594889	rs144419564	rs140500249	rs149968022	rs61756764
rs139174396	rs144798492	rs144971678	rs140684440	rs150726474	rs111315183
rs140457143	rs144914344	rs145939638	rs140753811	rs150883284	rs111489369
rs141502552	rs144993798	rs147074295	rs140852765	rs183515178	rs112022505
rs141637640	rs145037345	rs147191857	rs141311387	rs199558419	rs112948044
rs374096478	rs145803296	rs376004661	rs141593868	rs199634229	rs113329190
rs374163115	rs146041458	rs376183002	rs141691930	rs200224797	rs11704815
rs374237094	rs146119145	rs376399243	rs141946699	rs200552560	rs11912899
rs374281264	rs146147293	rs377721434	rs142288817	rs200628840	rs12628803
rs374354690	rs146712254	rs535586	rs142330184	rs200766242	rs137986257
rs140044595	rs147244947	rs608118	rs142399679	rs201207803	rs138697580
rs140366295	rs147355603	rs61745377	rs142431552	rs28709553	rs138876937
rs140758887	rs147566983	rs79319571	rs142673005	rs3173328	rs141605983
rs140999480	rs147863486	rs200728751	rs142769483	rs368423097	rs141789379
rs141566932	rs147880144	rs200795114	rs142885571	rs368594784	rs142165631
rs151312506	rs148026063	rs200852894	rs143148170	rs369017168	rs142523755
rs181323053	rs148040990	rs201239346	rs142755148	rs369022743	rs142606173
rs181397127	rs200446648	rs201374098	rs143160804	rs369594083	rs142646230
rs199922790	rs34985152	rs35560602	rs143349295	rs150923476	rs367583060
rs201764747	rs200872777	rs199686578	rs148414681	rs150941761	rs367915829
rs201878531	rs201626054	rs2230139	rs148504625	rs151289849	rs368320273
rs202246033	rs376616624	rs367619046	rs148892028	rs17002307	rs368321182
rs370080653	rs376779611	rs368224312	rs148939960	rs187938527	rs368368055
rs370163685	rs376984096	rs368283587	rs149058635	rs190025023	rs368954293
rs370388081	rs376996214	rs370117714	rs149418364	rs190524661	rs369467364
rs371408787	rs377052238	rs373430125	rs149471059	rs191758012	rs370454302
rs371711210	rs377059599	rs377173053	rs149611960	rs192742421	rs370565196

rs372194372	rs377140865	rs377639461	rs149806482	rs199535477	rs370568560
rs373235986	rs377315704	rs76956103	rs150000512	rs199572356	rs370702933
rs375369228	rs377556812	rs77438668	rs150294103	rs199573217	rs370810144
rs375492253	rs377652798	rs1064180	rs150367859	rs199679406	rs370889984
rs375889124	rs61756763	rs138824010	rs150498069	rs199751606	rs370931638
rs149653262	rs61756765	rs141798641	rs150532830	rs199798718	rs371107766
rs149797556	rs75752076	rs143606442	rs150845823	rs199828971	rs371344329
rs374523754	rs76268515	rs148034083	rs150898820	rs199901345	rs372046370
rs374679651	rs78045947	rs201611653	rs370384292	rs199994113	rs372254991
rs374715858	rs115631257	rs202043101	rs372368219	rs200111935	rs372604824
rs374768898	rs111556716	rs202151090	rs372420639	rs200271896	rs372759477
rs375358513	rs112461962	rs20552	rs373435582	rs200478275	rs373351924
rs375883849	rs1130581	rs20553	rs375586180	rs200552141	rs373428278
rs143439472	rs113973079	rs20554	rs376225175	rs200624744	rs373752539
rs144132028	rs137861688	rs2230110	rs376457490	rs200688710	rs147698124
rs145404362	rs138941874	rs149474546	rs148652525	rs148382906	rs148290302
rs146704002	rs139994475	rs149787986	rs149159079	rs148424397	rs147102570
rs146768560	rs147657889	rs150547961	rs149423729	rs148599245	rs147391445
rs147034415	rs147667874	rs150601623			

found as an enzymatic source of mutations(22). Therefore A3B have been considered very important in tumor genesis and this paper especially focus on the epigenomic analysis of A3B gene. Genetic signatures associated with DNA methylation of A3B gene, histone modifications, miRNAs of A3B have been identified and categorized as damaging, possibly damaging and probably damaging. Moreover the population wise analysis of these genetic signatures have been carried among the global ethnic groups.

MATERIALS AND METHODS

Identification of MeSNPs of APOBEC3B

CpG islands of A3B gene have been identified using DBCAT(14).The chromosome location of these CpG islands have been noted down and the SNPs in these locations have been identified using NCBI database(23).

Genetic variants of the histone modifying genes which are associated with breast cancer:

The Histone modifying genes have been identified from GeneCards (24). 79 genes have been found to be associated with histone modification. The SNPs of these genes have been noted down. The breast cancer correlation of these SNPs were further identified using SNPNexus and Genetic Association Database (GAD)(24).

Population analysis of MeSNPs and Histone modifying genes:

The MeSNPs and SNPs of Histone modifying genes(HiSNPs) associated with breast cancer have been subjected to population analysis using the HAPMAP data from SNPnexus(24). The various ethnic groups like Northern Europeans from Utah(CEU), Yoruba African(YRI), Han

Supplementary table 4: APOBEC3B MiRNA SNPs

Target Score	miRNA Name	UTR sequence	Chromosome start positions	chromosome end position	SNPs
96	hsa-miR-4261	**TGT TTC**	38988867	38988873	rs911605532
			38985891	38985897	
			38988532	38988538	
			38988090	38988096	
			38991327	38991333	rs746966270, rs758237218, rs780014617
			38988876	38988882	rs192115582
			38992764	38992770	rs548087996
91	hsa-miR-4314	**TCCCAGA**	38983200	38983207	
			38992680	38992687	rs928013419, rs930052363
83	hsa-miR-4522	**CAGAGTC**	38992608	38992615	
83	hsa-miR-6833-5p	**TTCCACA**	38992777	38992784	rs539827112
79	hsa-miR-505-5p	**TGGCTCC**	38982589	38982596	
77	hsa-miR-3135b	**CT CCAGC**	38989089	38989096	
			38983285	38983292	
			38992545	38992552	
70	hsa-miR-4297	**GGAAGGC**	38986879	38986886	
			38992468	38992475	
			38983935	38983942	
62	hsa-miR-4772-5p	**CCTGA TC**	38992582	38992589	rs191905604, rs568294892
			38991108	38991115	rs565440710
60	hsa-miR-4646-5p	**TTCCCAG**	38988223	38988230	
			38992679	38992686	rs928013419, rs930052363, rs982501127
60	hsa-miR-204-3p	**TTCCCAG**			
55	hsa-miR-3591-5p	**AC ACTAA**			
55	hsa-miR-1207-3p	**CCAGCTG**	38985872	38985879	rs370960629
			38992547	38992554	
54	hsa-miR-4307	**AAAACAT**	38987782	38987789	
			38992740	38992747	
			38983168	38983175	
50	hsa-miR-3614-5p	**CCAAGTG**	38992657	38992664	

Chinese(CHB), Japanese Tokyo(JPT), African ancestry in Southwest USA(ASW), Chinese in Metropolitan Denver, Colorado(CHD), Gujarati Indians in Houston, Texas(GIH), Luhya in Webuye, Kenya(LWK), Mexican ancestry in Los Angeles, California(MEX), Maasai in Kinyawa, Kenya(MKK) and Toscani in Italia(TSI) ethnic groups have been considered for population analysis(25).

Identification of miRNA

The miRNAs associated with A3B gene have been excavated using miRDB database(19). The DNA sequence corresponding to the miRNAs of A3B have been identified the 3' UTR sequence of the corresponding miRNAs.

RESULTS AND DISCUSSION

MeSNPs

The CPG islands have been found in the locations 38983951-38984253, 38985816-38986036, 38991193-38991725 of APOBEC3B gene. A total of 250 SNPs have been identified in these regions (supplementary data 1). Sorting Intolerant from Tolerant (SIFT) predicts whether an SNP is damaging or tolerant based on the structure of protein and Polyphene predicts possibly damaging and probably damaging based on the amino acid substitution (26),(27). The SIFT and Polyphene analysis of the MeSNPs have been carried out. 51 damaging SNPs by SIFT, 41 probably damaging SNPs and 19 possibly damaging SNPs by Polyphene have been identified (supplementary table 2).

Histone modification:

The SNPs of histone modifying genes (HiSNPs) have been excavated and 300 of them have been identified to be associated with breast cancer.The SNP rs377508897 has been identified to be damaging by the SIFT prediction. The SNPs rs115631257, rs115631257, rs115631257, rs115631257 have been identified as possibly damaging, rs61756764 as probably damaging by Polyphene.

MiRNA

The miRNAs of A3B gene with a target score greater than 90 have been given in table 1. 2 and the wholw set of miRNAs of A3B excavated using miRDB database is given in supplementary table 4. MiRNAs hsa-miR-4261 and hsa-miR-4314 with target scores 96 and 91 have been in table 1.

Population analysis:

The SNPsrs17000717 and rs2076109 are conserved in CEU, rs17000717 and rs2076109 in YRI, rs17000717 and rs2076109 in CHB, rs17000717 and rs2076109 in JPT, rs2076109 in ASW, rs2076109 in GIH, and rs2076109 in MKK ethnic groups. The population wise analysis of the histone SNPs are given in table 2.

CONCLUSION

Cancer is not merely caused due to aberrations in the genomic sequences, it is also due to the epigenomic abnormalities. The major epigenomic factors like DNA methylation, histone modification have been taken up to analyze the genetic signatures that hints the epigenomic aberrations of the gene APOBEC3B. The involvement of microRNAs in disease development have been identified by finding out the genetic variants present in the corresponding DNA sequence of A3B miRNAs. The ethnic groups in which MeSNPs and HiSNPs of A3B gene are present have been spotted suggesting increased proneness of these ethnic groups towards epigenetic alterations. Thus identifying such SNPs in a patient helps to discover that epigenomic aberrations are also one of the reasons of origination of the disease and thus enhances personalization of the treatment.

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Antioxidant Potential of Anthocyanins from Selected Cultivars of *Impatiens balsamina* L.

R. ARATHY[1], G.S. MANOJ[2] AND K. MURUGAN[3]

[1]*Department of Botany, Mahatma Gandhi College, Thiruvananthapuram.*
[2]*Department of Botany, NSS College Nilamel, Kerala.*
[3]*Plant Biochemistry and Molecular Biology Laboratory, Dept. of Botany, University College, Thiruvananthapuram.*
email: manojmalackal@gmail.com

ABSTRACT

Anthocyanins are water soluble pigments and have been used to colour food from time immemorial. The major function of anthocyanins is to provide colour to flowers, fruits and there by facilitate pollination and also help dispersal of seeds. Anthocyanins are also protecting leaves from ultraviolet radiation and deterring herbivores. Balsam is one of the species show wide variation in terms of colour and shape of flower. Six cultivars of *Impatiens balsamina* L.were collected from open field and used for estimating anthocyanin of flowers. The cultivars screened were violet, red, light lavender, dark lavender, purple red and pink. They were named CV-1, CV-2, CV-3, CV-4, CV-5 and CV-6 respectively. Purple red cultivar displayed the highest anthocyanin content at open field when compared with other cultivars of balsam. DPPH and FRAP assays showed significant antioxidant potentiality when compared with the standards (BHT and ascorbate). This basic work suggests that species of Impatiens may be used for extraction of anthocyanin as organic colour of plant origin and further studies are needed to use them as natural food colourants.

Key words *Anthocyanin; antioxidant; balsam; DPPH; FRAP*

Plants rich in anthocyanins are *Vaccinium* species, such as blueberry, cranberry, and bilberry; *Rubus,* berries, including black raspberry, red raspberry, and blackberry; blackcurrant, cherry, eggplant (aubergine) peel, black rice, , muscadine grape, Concord grape,red cabbage, and violet petals. Red-fleshed peaches and apples contain anthocyanins. Anthocyanins are less abundant in banana, asparagus, pea, fennel, pear, and potato, and may be totally absent in certain cultivars of green gooseberries. Content of anthocyanins in the leaves of colorful plant foods, such as purple corn, blueberries or lingonberries, is about 10 times higher than in the edible kernels or fruit. The color spectrum of grape berry leaves can be analysed to evaluate the amount of anthocyanins. Fruit maturity, quality and harvest time can be evaluated on the basis of the spectrum analysis.

Anthocyanins are considered secondary metabolites as a food additive with E number E163 (INS number 163); they are approved for use as a food additive in the EU, (UK Food Standards, 2011) Australia and New Zealand (Australia New Zealand Food Standards, 2011)

In this scenario the present study aims to investigate the total anthocyanin content of six balsam (*Impatiens balsamina* L.) varieties, a less utilized ornamental plant and their antioxidant potentiality.

MATERIALS AND METHODS

Plant material

The plant material selected for the study was flowers of six cultivating varieties of *Impatiens balsamina* L. The cultivars were named CV-1, CV-2, CV-3, CV-4, CV-5 and CV-6 based on their flower colour i.e. violet, red, light lavender, dark lavender, purple red and pink respectively.

Quantification of Anthocyanin

Anthocyanin was extracted and quantified by the methodology of Moreira *et al*.,(2008).

Analysis of antioxidant potentiality

Evaluation of DPPH radical scavenging activity

The free-radical scavenging activity of anthocyanin was measured with the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) in terms of hydrogen- donating or radical-scavenging activity according to the procedure of Rajesh and Natvar (2011). Butylated hydroxy toluene (BHT), and ascorbic acid were used as reference compounds.

Ferric reducing antioxidant power (FRAP)

The reducing capacity of anthocyanin was measured following the method of Peiyuan *et al.,* (2011).

Table 1. Total anthocyanin content in different cultivars of *I. balsamina*

Sl. No	Flower colour	Variety number	Total anthocyanin content (mg/L)
1	Violet	CV-1	44.97
2	Red	CV-2	101.95
3	Light lavender	CV-3	2.62
4	Dark lavender	CV-4	5.59
5	Purple red	CV-5	132.09
6	Pink	CV-6	5.92

RESULTS AND DISCUSSION

Quantification of total Anthocyanin

The analysis of total anthocyanin in balsam flowers revealed significant differences among the anthocyanin content among the flowers of the cultivars (Table 1). The anthocyanin content in the flower was noticed to be significantly maximum in purple red (CV-5) i.e., 132.1 mg/g tissue (Fig. 2). Lowest amount of anthocyanin was noticed in lavender (CV-3) i.e., 2.63 mg/g. Hamouz *et al*., (2011) also reported that total anthocyanin content of potato tubers varies with flesh colour. They also suggested the possibility of effect of environment in variation of anthocyanin content.

Analysis of Antioxidant Potentiality

DPPH Scavenging Potentiality

Anthocyanin of CV-5 showed varying level of scaven-ging activities over DPPH free radicals (92% at 1000 µg/ml). Values were marginally equal to ascorbate and BHT (93 % and 92 at 1000 µg/ml respectively) (Table 2).The present results are higher than that of DPPH free radical scavenging assay reported by Lima *et al*.,(2011) in 12 Acerola genotypes. In Acerola genotypes the DPPH scavenging potentiality varied from 25.58 to 47.04%.

Kahkonen *et al*., (2003) reported the DPPH scavenging activity in blackberry, bilberry and cowberry using anthocyanin isolates. Comparison of bilberry (*Vaccinium myrtillus* L.) and blueberry (*Vaccinium corymbosum*) fruits for their anthocyanin composition and antioxidant activity is reported by Deividas *et al*., (2009).

Significant differences between berry and skin extracts were not established. Studies with fruits showed that the strongest antioxidant activity possesses blueberry cultivar (82%). Meanwhile, the amount of quenched free radicals in bilberry samples was 63%. The results corroborates with the radical scavenging property of CV-5 (*I. balsamina*) anthocyanin extract. The DPPH radical scavenging activities of Purple-Fleshed Sweet potato purees were 47.0 to 87.4 ìmol trolox equivalent (TE)/g FW (Steed and Truong, 2008).It has been also reported that chokeberry (*Aronia melanocarpa*) extracts, which are rich in cyanidin glucosides, exhibited stronger antiradical effect when determined by the DPPH method.

Table 2. DPPH radical-scavenging assay of anthocyanin extracted from *I. balsamina* cultivar (CV-5)

Conc. of anthocyanin (µg/ml)	DPPH assay (% Inhibition)
50	8
100	17.8
200	36
400	54
800	79.9
1000	92
IC_{50} (µg/ml)	380
Ascorbate (500 µg)	93
BHT (500 µg)	92

Ferric reducing antioxidant power (FRAP)

Higher absorbance indicates higher ferric reducing power. Anthocyanin of CV-5 showed significant ferric reducing power, i.e. 287 ìM/g at 1000 µg/ml concentration (Table 3).

Sudarat and Sudarat (2012) reported the total anthocyanin and antioxidant activity of germinated coloured rice and emphasized the need of protecting anthocyanin in rice seed husk. This data support the

Table 3. FRAP assay of anthocyanin extracted from *I. balsamina* cultivar (CV-5)

Conc. of anthocyanin (µg/ml)	FRAP assay (µM/g)
50	25
100	89
200	145
400	179
800	232
1000	287
IC_{50} (µg/ml)	-
Ascorbate (500 µg)	100
BHT (500 µg)	125

necessity of present study. Total anthocyanin and their antioxidant properties of various coloured fruits and leaves are reported by various researchers. For example antioxidant properties of anthocyanin rich extracts from Blueberry and Blackcurrant juice by Zoriþa *et al*., (2015), anthocyanin pigments in grape varieties (*Vitis vinifera* sp.) and their antiradical activity by Kallithrakaa *et al.,* (2005).

In some small fruits (Moyer *et al*., 2002), raspberries (Mullen *et al.,* 2002), sweet potatoes (Oki *et al.,* 2002), the antioxidant capacity has been correlated to a significant degree with anthocyanin content, indicating that anthocyanins may govern to ascertain extent the antioxidant capacity of several plant tissues. The findings on grape skins are consistent with observations reported for red wines, where in spite of examinations supporting the high antioxidant efficiency of purified fractions rich in anthocyanins (Ghiselli *et al*., 1998; Saint-Cricq de Gaulejac *et al.,* 1999).

It has been strongly substantiated that the antioxidant efficiency is very significantly correlated with the flavanol fraction, composed of monomeric catechins and proanthocyanidins (Landrault *et al*., 2001). Anthocyanins in general have been claimed to exhibit various activities of biological importance, i.e., protection against DNA oxidative damage (Sarma and Sharma, 1999), antioxidant and anti-inflammatory function (Wang *et al*., 1999) and peroxynitrile scavenging (Tsuda *et al.,* 2000).Overall, all findings in this study comprise an important contribution to anthocyanin research in biological studies.

CONCLUSION

The anthocyanin content in flower of balsam cultivars were found significant when compared to other anthocyanin yielding plants. Variation in anthocyanin content among flowers of cultivars was also found. The cultivars purple red and red has been screened out as high anthocyanin content cultivars and may be used to exploit for extraction of anthocyanin as organic colour of plant origin. Growing conditions influence the anthocyanin contents of flowers of different cultivars i.e., an increasing trend of colour of the flower was noticed among open field grown cultivars. Antioxidant potential of various fruits, flowers and leaves are already reported. But studies in Impatiens flowers are limited. The study presented here provided valuable data with regard to anthocyanin composition of *I. balsamina* and the results give way to use purple red flowers as good source of anthocyanin for preparation of natural food colourants.

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Effect of Coconut Water and Activated Charcoal on Seed Germination in an Endemic Orchid *Rhynchostylis retusa Blume.*

S. NISHA RAJ

SAS SNDP Yogam College, Konni, Pathanamthitta, Kerala
E mail: nishsek@yahoo.co.in

ABSTRACT

The present study on *in vitro* seed germination of *Rhynchostylis retusa* obtained on nutrient medium made clear pathway for the *in vitro* propagation of orchids by seed culture. The presence of coconut water in the medium enhanced seed germination and growth of seedlings. For enhancing the growth of seedlings further the individual seedlings were cultured on MS medium supplemented with 3 mg/l BAP and 1g/l activated charcoal. These seedlings were sub cultured on rooting medium (IBA) supplemented with activated charcoal. The plantlets were transferred to soil with 90% success. Hence the present investigation made possible for the large scale multiplication of *R.retusa* and thus to conserve this endemic orchid.

Key words *Effect of Coconut Water, Activated Charcoal, Seed Germination, Endemic Orchid, Rhynchostylis retusa, Blume.*

In vitro culture of orchids is of much importance since the percentage germination in nature is extremely low. Conventional vegetative propagation is very slow and there are chances of virus transfer. The method help to get virus free seedlings at a faster rate. Besides, it is useful in propagating rare and endangered species and conserves them by reintroducing to the natural habitat. Essentially, the orchids are out breeders and they produce very small seeds. The seeds are produced in large numbers ranging from 1,300 to 40, 00,000 per capsule. The great majority of species have relatively undifferentiated seed, no cotyledons and no endosperms. Less than 5% of the seeds germinate in their natural environment.

Orchid embryo is a mass of undifferentiated cells, some with and others without suspensor (Manilal and Sathish Kumar, 1985). Swamy (1949) described 5 types of embryos within the group of suspensorised embryos. Two fundamental types of embryo development occur in Orchidaceae. They are: (1) Zygote divides to form an undifferentiated mass of cells from where the suspensor cells are formed earlier and filamentous proembryo. (2) Zygote divides transversally to form a linear row of four cells. The majority of orchids show the latter type of development. The sub tribes *Cymbidiinae,* and *Crytopodiinae*, where first type is seen (Vijayaraghavan, *et al*.,1986).

Formation of orchid seedling from the seeds involves 3 sub sequential phases namely germination, protocorm formation and seedling development (Mitra, 1976). The germination of orchid seed begun by the inhibition of water through the testa of seed. Early response of germination was noted by observing the colour and shape of the seed. First visible sign of germination is the swelling of embryo followed by their turning green and emergence of the breasted seedcoats. This is called the spherical stage which subsequently develops into protocorm stage and later into the swelling stage (Mitra, 1976).

In the protocorm stage it develops rhizoids confined only to the basal region of protocorm or covers the whole except the meristamatic region. Rhizoids may be simple or branched. The formation of rhizoids in the protocorm is a unique feature of Orchidaceae. Subsequent cell divisions occur both in the apical and basal regions of the protocorm and leaf primordia, morphogenesis is started from the shoot meristem. Later first roots are formed endogenously (Mitra, 1971).

The *in vitro* culture of orchid seeds reveals that the seeds obtained from green pod 8-12 weeks of anthesis, germinate readily in a large number of species. The mature seeds on the other hands are hard to germinate due to some dormancy factors (Mitra, 1976).

Of the various media tried for seed germination the one formulated by Burgeff (1936), Knudson (1946), Vacin and Went (1940), Raghavan and Torrey (1964) and Mitra et al (1976) with lesser amounts of ammonium, nitrates, calcium, potassium and phosphate ions along with several vitamins have been found to be most suitable for a large number of orchids. The additional presence of amino acids, urea, casein hydrolysate, yeast extract, coconut milk, auxins, cytokinins, adenine and gibberellins in the medium has

yielded a better germination of embryos and protocorm formation. (Swamy, 1949). Vitamins also play an important role in the germination and in maintaining the development of germinating seeds (Anderson 1967; Arditi 1967). Apart from seed germination and meristem culture, other parts of plants viz. leaves, leaf tip and inflorescence are also used as explants.

Plant tissue culture offers several advantages over conventional propagation vegetative and sexual methods, for large scale propagations. Shoot multiplication can be achieved in small space because miniature plantlets are produced

MATERIALS AND METHODS

Culture media

Murashige and Skoog's (1962) ½ strength basal media was used for the present *in vitro* studies of *Rhyncostylis retusa.* Half strength of medium (Table 1) with coconut water were used for the germination of *R. retusa* seeds.

Preparation of the media

Chemicals of high purity were used for the media preparation. The stock solutions of macro and micro elements were prepared separately and stored in glass bottles under refrigeration at 5o-10oC. All the stock solutions for MS media were prepared and stored separately. Appropriate quantities of the various stock solutions (Here half strength MS medium used), growth regulators and other supplements were mixed and the final volume of the medium was made up with double distilled water.

PH of the media were adjusted to 5.8 after adding plant growth regulators and coconut milk using O.1N. NaOH and O.1N HCl. Medium was solidified by adding agar. About 15 ml of the medium is dispensed to each culture tube and sterilized in an autoclave at 15 lbs, for 20 minutes. Half strength MS Medium with 20% coconut water was added.

For rooting of the germinating seedlings ½ MS medium supplemented with IBA (1-3 mg) were employed. The media were also supplemented with 0.5-3 gm/l of activated charcoal. Activated charcoal added along with IBA enhanced.

Preparation of the explant and inoculation

The green immature undehised capsules of *Rhyncostylis retusa* were collected from the field. The capsules were washed in tap water and brought to the inoculation chamber. The capsules were soaked in rectified sprit and surface burned. They were then opened longitudinally with a sterile blade. Seeds were immediately transferred to MS medium under aseptic conditions. The cultures were incubated at 25±2oC temperature conditions.

The percentage of germination and frequency of plant emergence were recorded initially from 45 days and then continue every 15 days interval from the day of inoculation. The changes were observed, studied and recorded with the help of a dissection microscope.

Germination rooting and transfer of the plant

Immature sterile seeds were cultured on MS media supplemented with coconut water (10 - 20%). Cultures were observed initially after 45 days and then continue observation at every 15 days interval. Germination begins from 45 days of culture and on 120th day of inoculation full plantlets developed. After 120 days of culture the plantlets were isolated and cultured on MS media supplemented with BAP at 3 different concentrations (1, 2, 3 mg/l) and three different concentrations of activated charcoal (1, 2, 3 gm/l).

After four weeks the plantlets attained a length of about 3 to 4 cm with 2 to 3 leaves each. These seedlings were sub cultured on to MS medium containing four different concentration of IBA (1, 2, 3 and 4 mg/l) supplemented with four different concentration of activated charcoal (0.5, 1, 2 and 3 gm/l).

After six weeks, plantlets with well developed roots were transferred to soil and grown under green house conditions. The rooted plantlets were thoroughly washed in distilled water to remove the agar and transferred to garden soil mixed with wood charcoal (1 : 1) in plastic cups (under diameter 6 cm × length 8 cm) and placed in a glass house under high humidity (90%). The plantlets were kept under sterile conditions in plastic containers for 2 months after which they were transplanted to field.

RESULTS AND DISCUSSION

The present investigation was carried out with the objective of rapid multiplication through *in vitro* seed germination of *Rhynchostylis retusa,* an endemic wild and rare orchid.

The seeds from the fruits collected from the field were cultured on MS medium supplemented with various concentrations of coconut milk after

sterilization. The various events occurred during the germination period of the seed were studied with a dissection microscope and is shown in the Table II. The initial sign of seed germination was observed after 45 days of culture. Within 120 days the seeds were germinated up to a length of 1cm (Fig. 1A and 1B).

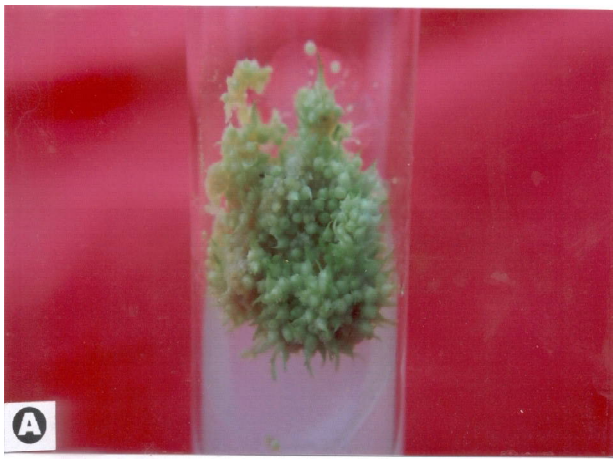

Fig. 1(A). An 80 days old seed culture of *R. retusa* on MS medium supplemented with 15% coconut water. The seeds have started germinating and produced prophyll primordium.

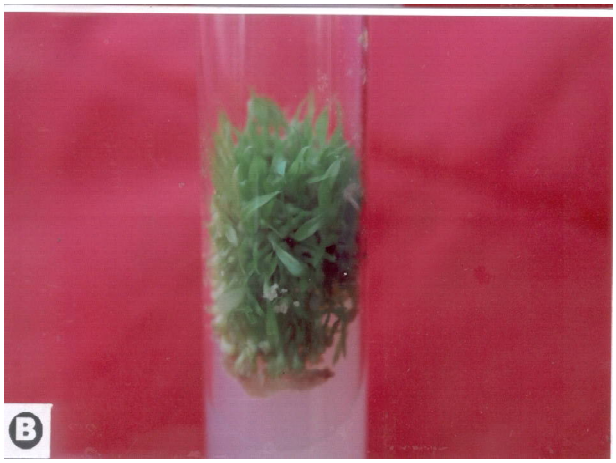

Fig. 1(B). A 100 days old seed culture on MS + 15% coconut milk. The prophylls have elongated and each plantlets can be distinguished.

The effect of various concentrations of coconut milk on seed germination was studied. The earlier reports suggest that coconut milk plays a very crucial role in seed germination of orchid (Hegarty, 1955; Nimoto and Sagawa, 1961; Cheng and Chua, 1980; Mitra, 1976). An optimum concentration of 15% coconut milk gave maximum response after 120 days of culture (Table III). Hence it was confirmed that coconut milk enhances the seed germination in *R. retusa*.

After 120 days of culture the seedlings were about 1cm in length without roots. For enhancing the growth of the seedlings, the individual seedlings were culture on MS medium supplemented with different concentrations of BAP (1, 2, 3 and 4 mg/l) and activated charcoal (1-3 gm/l), 3 mg/l BAP and 1 gm/l activated charcoal gave optimum results. On this medium seedlings reached an average height of about 2.8cm (Fig. 2A and 2B; Fig. 3A and 3B).

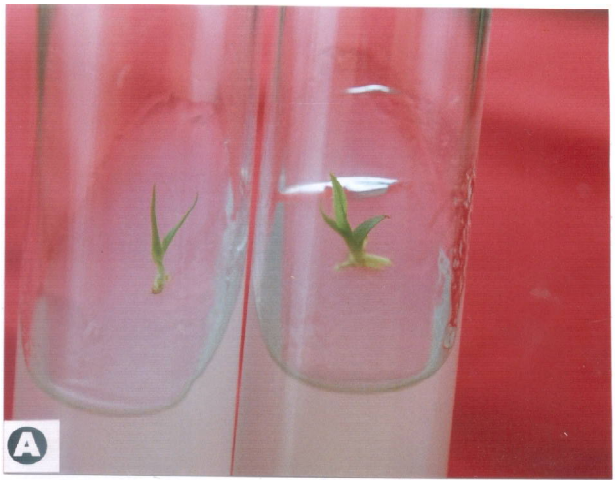

Fig. 2(A). The growth of the seedlings on MS medium supplemented with BAP mg/l without activated charcoal.

Fig. 2(B). An isolated seedling on MS medium supplemented with BAP (3 mg/l + activated charcoal 1 gm/l) for further growth, one week after inoculation. Comparatively the presence of activated charcoal on the media enhances the growth of the seedlings.

Fig. 3(A). A 45 days old seedling on MS medium supplemented with BAP 3 mg/l + activated charcoal 1 gm/l. The seedlings have reached a size of about 2.5 cm length and rudimentary roots have developed at the base.

Fig. 3(B): Seedling on MS medium supplemented with BAP 3 mg/l + activated charcoal 3 g/l for germination. The growth was poor as compared to 1 gm/l activated charcoal.

The root development was very poor with the seedlings. Later, for further growth of seedlings, the seedlings were sub cultured on half MS supplemented with different concentrations of IBA (1 to 4 mg/l) and activated charcoal. Maximum response was observed on MS+IBA (3 mg/l) and activated charcoal (2 mg/l) on the medium 98% of the shoots rooted with an average number of 3.8 roots per shoot (Table IV) (Fig. 4A and 4B).

Plantlets were transferred to soil with 90% success (Fig. 5A and 5B). The rooted plantlets were thoroughly washed in distilled water and transferred to plastic cups containing garden soil mixed with charcoal. They were placed in a glass house under high humidity. After two months the plantlets were transferred to the field.

Fig. 4(A). Induction of roots on ½ MS medium supplemented with IBA 3 mg/l and activated charcoal 2 g/l. Well developed healthy roots have developed from the seedling.

Fig. 4(B). Four seedlings showing the various stages of root development on ½ MS medium supplemented with IBA 3 mg/l and activated charcoal 2g/l after 45 days of sub culture. The roots have reached an average length of 1.7 cm.

The procedure developed here is helpful in rapid multiplication of *R.retusa* and thus to conserve this rare orchid.

Several investigators employed immature seeds from unripe green pods for *in vitro* germination experiments especially in orchids like *Cymbidium, Dendrobium, Epidendum, Oncidium, Vanda* etc. Normally the fertilized ovules were collected 8 to 16 weeks after pollination were used for invitro seed germination on a suitable medium. The difficulty with these experiments is that identifying the critical stage of immature seeds before they pass on to the dormant stage. As the mature and immature seeds can be hardly distinguished morphologically, some biochemical

Table 1. Various changes observed during the seed culture in *R. retusa* on three different concentrations of coconut milk

Days after inoculation	Medium with 10% coconut water	Medium with 15% coconut water	Medium with 20% coconut water
45 days	Embryo enlarged and become globular	Embryo become enlarged; seed coat broken & embryo came outs	Seed coat broken and embryo came out
80 days	Embryo become enlarged; seed coat broken and embryo came out	Prophyll primordium initiated at one direction of the protocorm	Protocorm enlarged in size. Starch grains are uniform in distribution
100 days	Prophyll primordium initiated	Prophyll elongated. Second prophyll formation started at the base of the first	Seed desiccated
120 days	Prophyll elongated	Plantlets with 2-3 cotyledonary leaves were formed	Seed desiccated

Table 3. Effect of three concentrations of coconut milk (10, 15 and 20%) on seed germination 45, 90 and 120 days after inoculation of seed.

Days	Growth of the Seedling			
	*Control	10%	15%	20%
45	-	**+	+ +	+ +
90	+	+ +	+ + +	+ +
120	+	+ +	+ + + +	+ + +

Basal medium: MS.

* Medium without coconut milk.

** The progressive '+' signs indicate an increase in pace of seed growth.

experiments are needed for identifying the right type of immature seeds. Raghavan (1976) described the changes in enzyme compliments as the orchid seed changes from immature to mature stage.

The culture of ovules or seeds from fruits can be extended to study *in vitro* pollination and fertilization leading to hybrid production. The propagation of orchid via. seed germination include three stages ie.

Fig. 5(A). A hardened plant one week after transfer to soil and wood charcoal.

Fig. 5(B). Five plantlets transferred to soil growing at various stages of development 2 week after transfer.

Table 4. Effect of different concentrations of IBA (1-4 mg/l) and activated charcoal (0.5 - 3 g/l) on root induction from the germinated shoots of *R. retusa*. Observations were taken after 45 days of culture.

½ MS IBA mg/l	Charcoal gm/l	% rooting	Average number of roots	Average length of roots (cm)
*Control	0	-	-	-
1	0.5	55	1	1.0
2	1	72	3	1.1
3	2	98	3.8	1.7
4	3	78	3.2	1.4

* Control: ½ MS basal medium

germination, protocorm formation and seedling development. The development of a germinated seed via. protocorm into seedlings may be called as "direct" method. In other cases the embryos may form spontaneous callus formation during germination (Curtis and Nichols, 1948; Withner, 1959; Goh, 1970; Mitra et.al, 1976; Vij *et al*., 1981). Here the multiplication pathway is via. organogenesis and it depend upon medium and plant growth regulators. Hence the seed callus via. protocorm formation developing into seedling can be referred to as "indirect" method. This is basically used for obtaining hybrid orchids from embryos.

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DNA Barcoding – A Rosetta Stone To Understand Biodiversity

RAMYA R PRABHU

Department of Biotechnology, Government Arts College Trivandrum
email : ramyarprabhu@gmail.com

ABSTRACT

Identification and classification of organisms holds a key position in the field of biology. There are various methods adopted for the same, of which the use of genetic sequences as markers for identifying a particular trait (popularly known as Molecular markers) is gaining importance. This short review discusses about the use of such molecular markers with emphasis to the technique of DNA Barcoding. Use of DNA barcoding pragmatically, has revolutionised the field of taxonomy. It acts as a Rosetta stone, a breakthrough in the area of identifying and classifying the organisms which in turn, contributes to the understanding and assessment of biodiversity.

Key words *Species, Molecular markers, DNA Barcoding, Biodiversity*

In Biology, a species is defined as a group of potentially interbreeding population of natural origin which are reproductively isolated from other groups (de Quiroz, 2005). The definition was put forward by one of the most distinguished evolutionary biologists of 20th century, Ernst Walter Mayr. The speciesis considered as the largest group of organisms who can produce a fertile offspring by means of sexual reproduction. This is why species is the basic unit of biological classification and enjoys the lowermost rank in taxonomic hierarchical order (although we adopt the usage of subspecies under certain circumstances). This emphasises the importance of identifying an organism upto species level. It is of utmost importance because it also helps in understanding the biodiversity of living organisms. In turn, a knowledge of biodiversity is essential as it helps in conservation of organisms and gives an idea of their existence status whether they are endangered, threatened, extinct etc., especially in the context of accelerating habitat destruction.

The organisms are mainly identified based on the morphology. Taxonomy is the science of identifying, naming, describing and classifying an organism. Taxonomists have started relying on gene sequences present on the DNA for identification of organism. Such sequences which serves as tag for identification are known as Molecular markers.

MOLECULAR MARKERS

The use of molecular markers is gaining wide acceptance in species identification. A molecular marker is a sequence in DNA in the genome of an organism which can be identified. Molecular markers are reliable and represent the genetic makeup of an organism. They are consistent and remain unaffected or unaltered by environmental factors. They are stable and are inherited variations exhibiting DNA polymorphism responsible for observed phenotypic variability (Mitra et al., 1999). They will be associated with useful traits which help us to determine the linkage between them as well as inheritance.Therefore, molecular markers are landmark in the genome that represent the variations at the genomic level (Khan et al., 2015). They can be easily tracked and quantified in a population.

DNA BARCODING

A wide range of molecular markers are in use today, of which DNA Barcoding is gaining attention. We all are familiar with the Universal Product Code used in supermarkets where a scanner distinguishes a code present on products shown as black and white stripes.This is popularly known as 'barcode'. Although barcodes look similar to naked eyes, upon microscopic scrutiny, we can see that they are different. It aids in identifying a particular item.

The method of using a short sequence from a known part of DNA, showing variability which helps in identification of organisms is known as DNA Barcoding. DNA Barcoding uses a very short gene sequence from DNA for identification of species. Usually the sequence is universal so as to make the identification across species, easily. One of the choices of gene from animals is a short sequence, about 600

bp of the gene Cytochrome oxidase (CO1) present in mitochondrial genome. (Hebert et al., 2003). The choice of the gene is based on the criteria that it harbours sufficient variation between species to discriminate them. The technique of barcoding is based on PCR amplification, so the chosen gene should be short enough to be amplified using PCR (Vijayan and Tsou, 2010). CO1 has the property of 'barcode gap' as it varies a lot between the species, but not too much among the individuals of the species. However, CO1 as candidate gene for barcoding did not work out for plants and fungi. This was mainly because of slower rate of evolution of CO1 gene in plants. (Kress et al., 2005). This problem is also enhanced by the presence of hybridisation and polyploidy in plants (Fazekas et al., 2009). Various searches were conducted towards this direction and finally, a possible candidate proposed was an intergenic spacer present in chloroplast genome which is trnH-psbA, a short 450 bp region (Kress et al., 2005). It had sufficient variation between the species and regions flanking the proposed barcode gene for designing universal primers. Many other regions were also proposed for plants, of which matK,rbcLand nuclear internal transcribed spacer region (ITS) gained much acceptance. Still, DNA Barcoding for plant identification is facing certain issues, so enhancing currently available barcodes and establishing more thorough and widely usable target loci with the help of emerging technologies like genome skimming (Hollingsworth et al., 2017). Another target gene used in DNA Barcoding is 16S rRNA which is a potential forensic tool that helps in biosecurity, food augmentation, investigations against illegal trading of endangered species etc. (Ferri et al., 2009).

DNA Barcoding has been employed in identification of various species and the list would be endless. To name a few, it has been used in the identification of most non-chordates and chordates including fishes, insects, birds; algae, fungi and in vascular plants including angiosperms (Kress at., 2014).It has been used for the identification of previously known but unrecognised animals (Smith et al., 2008). It helps to demarcate the species boundaries and thereby adds to the knowledge of species diversity. DNA Barcoding also helps to associate the different stages of life cycle in organisms especially insects (Garcia-Robeldo et al., 2013).

The consortium for the barcode of life (CBOL) and International barcode of life (IBOL) are two main bodies dedicated to coordinating, communicating and effecting researches and all findings relating to DNA Barcoding. There is an online workbench for barcoders which also is a barcode repository and interface for submission and comparison of sequences known of the Barcode of Life Data systems (BOLD) (Vijayan and Tsou, 2010)

CONCLUSION

DNA Barcoding is really a boon as it complements various other identification techniques used in taxonomy. It assists in deciphering the phylogenetic relationships between organisms. With the support of new molecular techniques like next generation sequencing (NGS), DNA Barcoding helps taxonomists to recognise species reliably, cheaply and quickly (Schindel and Miller, 2005) when compared to conventional methods. There are some advantages of this technique like it can work with fragments, it works in all stages of life, helps identifying look alikes, can be used for analysing even degraded material etc(Hartwig et al., 2015). Moreover it helps in understanding the biodiversity of nature which in turn facilitates improvement and implementation of the conservation strategies.

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Assessment of Genetic Variability in *Rauwolfia serpentina* Benth. : An Endangered Medicinal Plant Using RAPD.

S. REMESHKUMAR[1] AND G.M. NAIR[2]

[1]*Department of Botany, VTM NSS College, Dhanuvachapuram.*
[2]*Kerala State Council for Science , Technology and Environment, Thiruvananthapuram*
email : remeshsurya70@gmail.com

Genetic variation among seven accessions and one *invitro* regenerated plant of *Rauwolfia serpentina* Benth. collected from different parts of Kerala (India) were analyzed using 21 decamer primers by RAPD. The analysis generated a maximum of 185 products of which 110 were polymorphic. The primer OPX- 17 generated a maximum of 14 products while OPX-13 generated only one. The low level of polymorphism and close clustering with small molecular distances indicated a narrow genetic base among the cultivars with a mean coefficient of similarity of 0.95. The Unweighted Pair Group method using Arithmetic Averages (UPGMA) permitted cluster analysis of data from 21 primers on 7 accessions and one regenerated plant of *Rauwolfia serpentina*, which showed that closely related cultivars can clearly be differentiated.

The genus *Rauwolfia* is represented by 110 species which are distributed widely in the warm regions (Porter,1959). *Rauwolfia serpentina* Benth. is an evergreen perennial and erect shrub of high medicinal value belonging to the family Apocynaceae. The plant parts, especially the roots are enriched with therapeutically active indole alkaloids such as ajmalicine, reserpine, serpentine and ajmaline which are used in the treatment of circulatory disorders (Tyler *et al*,1989). These highly medicinally active compounds are accumulated in the roots over a period of 1-3 years and the total content varies from 1-3% dry weight (Virmani *et al*,1979). The restricted distribution and indiscriminate over exploitation of this plant for medicinal purpose has led the plant to the brink of extinction. The cultivation of this species through conventional method is difficult due to the formation of non viable seeds . Consequently, propagation by tissue culture is an easy way to create a large number of plants within a short span of time.

The PCR based technique RAPD, is being routinely used for finger printing and estimation of genetic variability in several plant species like barley (Russel *et al*, 1997) maize (Pejic *et al* 1998), sorghum(Bohn *et al* 1999), wheat (Dje *et al* 2000), tobacco (Arsalan and Okumus, 2006),*Ralstonia solanacearum*(Grover *et al*. 2006) and *Mammilaria haworth*.(Mattagajasingh *et al* 2006) . The main advantages of this technique are its simplicity and rapidity, its ability to detect extensive polymorphisms and the requirement of a very small amount of DNA for analysis. In the present paper, we have tried to determine the molecular relatedness among *Rauwolfia serpentina*, growing in different parts of Kerala. Seven accessions of *Rauwolfia serpentina* from different regions and one *invitro* regenerated plant were randomly sampled for the analysis.

MATERIALS AND METHODS

Seven distinct populations and one *in vitro* regenerated plant of *Rauwolfia serpentina* Benth. (Apocynaceae) collected randomly from different parts of Kerala (India) and maintained in the green house of Department of Botany, Karyavattom served as the experimental material for DNA analysis.

DNA was extracted from the fresh leaves of *Rauwolfia serpentina* by a modified CTAB procedure of Murray and Thompson (1980) with necessary optimization. A 2% (m/v) CTAB, 20 mM Na_2 EDTA, 1.4 M NaCl,100 mM Tris pH 8.0 and 1% β mercaptoethanol (v/v) form the main ingredients of the extraction buffer. The DNA was sedimented as a pellet in the solution of ethanol. The DNA buried up in the ethanol was collected and resuspended in TE buffer consisting of 10 mM Tris of pH 8 and 1mM Na_2EDTA. Amplifying reactions were performed in 0.025 cm^3 vol.0.0025 cm^3 10× amplification buffer, 0.5 µ Taq DNA polymerase (Promega USA), 0.2 mM each of dATP, dGTP, dCTP and dTTP, 0.025 cm^3 of $MgCl_2$,15 pM of decamer primers(Operon Technologies Inc.USA) and 50 µg of the isolated DNA form the major components of the reaction mixture.

Amplification was carried out in a Perkin Elmer DNA thermal cycler(DTC 480). The present study was performed by using 21 decamer primers. The successive steps involved were : First cycle of 2 min. at 93º C,2 min. at 35º C and 2 min..at 72º C followed by 38 cycles of 1 min. at 93º C, 1 min. at 36 º C and 2 min. at 72 º C. The last cycle lasts about 10 min. extension at 72º C. The amplified products were resolved in 1.2 % agarose gel using ethidium bromide stain. The bands were detected using Alpha Chemiimager (Alpha Innotech Corporation, San Leandro, CA,USA).Bands on RAPD gels were scored as present (1) or absent (0) for all cultivars studied. Amplified products that were reproducible and consistent in performance were alone chosen for data analysis.

The data was analyzed using population genetic analysis software POPGENE,ver.1.0(Yeh and Boyle,1997) .Dendrogram was prepared based on Nei's genetic distance(Nei, 1972).

RESULTS AND DISCUSSION

RAPD analysis of *Rauwolfia serpentina* using 21 decamer primers generated a total of 185 products of which 110 were found to be polymorphic. The data shows a mean value of 8.8 products and 5.2 polymorphisms per primer. The primer OPX-17 generated a maximum of 14 products while OPX-13 produced only one. The maximum polymorphism per primer was also exhibited by OPX-17 (Table:1) The data further shows that total number of products generated by the individual accessions range from 112 to 140 with an average of 125 per accession. Two of the accessions (5 and 1) from Kallar and Pattambi produced 133 bands each while the accession number 2 from Kunduthodu (Kozhikode District) produced only 112. Analysis of RAPD data indicates that the extent of intra specific variation in this species is relatively less as evidenced by the coefficient of similarity values that ranges from 0.81 to 0.99, with a mean value of 0.95.

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Cluster analysis of the data shows grouping of accessions into one single cluster with tightly linked sub groups. Cluster I a with four accessions (7,5,4&1) represents those collected from the Kulathupuzha, Kallar, Kariavattom and Pattambi respectively. The mean coefficient of similarity of the cluster is 0.95. Accessions 5 and 4 from Kallar and Kariavattom shares high genetic similarity in this group as they are tightly linked with a mean GS of 0.99. Cluster I b has 3 accessions (6,2&3) from Seethathodu, Kunduthodu and Pala with a mean coefficient of similarity of 0.95. Accessions 2 and 3 from Kunduthodu and Pala are genetically close in this cluster showing a mean GS of 0.99. The *in vitro* member of this group is found to be genetically distant from its counterparts. The mean GS of this tissue cultured plant is 0.88 with the rest of the accessions.

Rauwolfia serpentina Benth. is an endangered medicinal plant which contains therapeutically active indole alkaloids as secondary metabolites. At present, the plant is on the brink of extinction due to indiscriminate over exploitation of its root for the extraction of several types of alkaloids. The genus

Nei's Original Measures of Genetic Identity and Genetic Distance

[See Nei (1972) Am. Nat. 106:283-292)]

pop ID	1	2	3	4	5	6	7	8
1.	******	0.9632	0.9497	0.9490	0.9492	0.9452	0.9562	0.9038
2.	0.0375	******	0.9880	0.9286	0.9304	0.9059	0.9175	0.8876
3.	0.0516	0.0120	*******	0.9215	0.9214	0.8955	0.9180	0.8651
4.	0.0523	0.0740	0.0817	*******	0.9196	0.9112	0.9226	0.8108
5.	0.0522	0.0722	0.0819	0.0838	******	0.9364	0.9443	0.8975
6.	0.0563	0.0988	0.1103	0.0930	0.0657	*******	0.8697	
7.	0.0448	0.0861	0.0856	0.0805	0.0573	0.0110	*******	0.8705
8.	0.1012	0.1193	0.1449	0.2098	0.1082	0.1396	0.1387	********

Table 1. List of primers used for RAPD analysis and their results

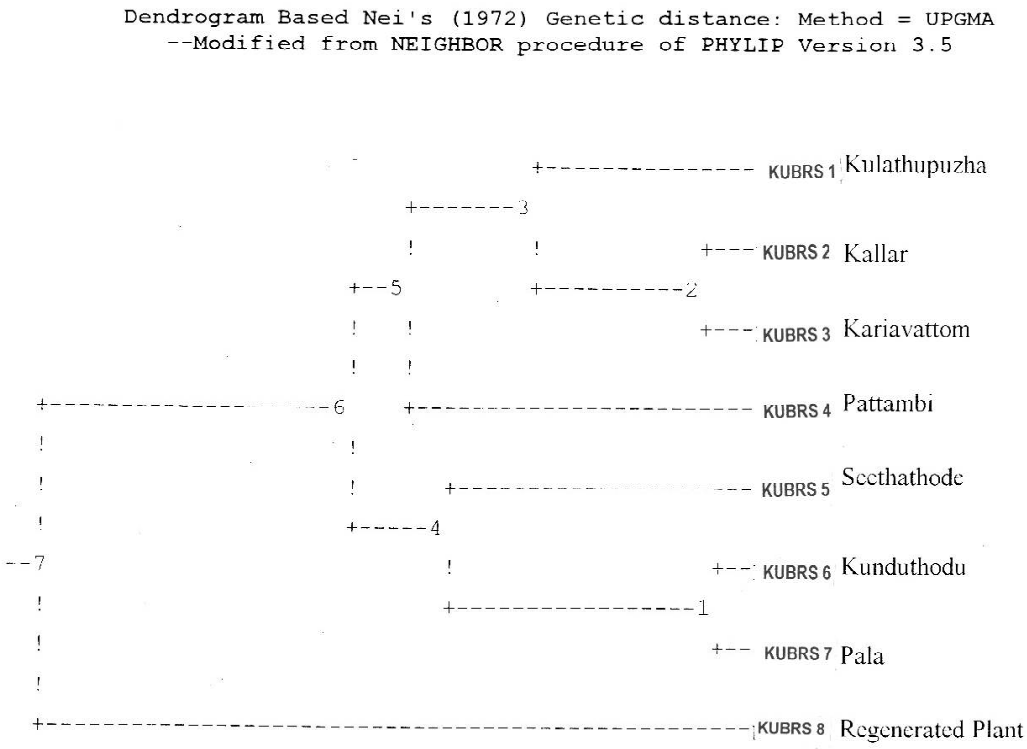
% Polymorphism	59.45
Total No. of products per primer	8.8
No. of polymorphism per primer	5.2
Maximum No. of products	OPX 17 = 14
Minimum No. of products	OPX 13 = 5

Serial No.	*Primer*	*Number of bands*	*Number of polymorphic bands*
1.	OPX – 01	9	3
2.	OPX – 02	9	9
3.	OPX – 04	11	8
4.	OPX – 05	11	10
5.	OPX – 06	6	5
6.	OPX – 12	8	5
7.	OPX – 13	5	1
8.	OPX – 16	6	5
9.	OPX – 17	14	13
10.	OPX – 18	11	5
11.	OPX – 19	11	6
12.	OPX – 20	9	8
13.	OPP – 01	8	3
14.	OPP – 02	8	5
15.	OPP – 03	8	3
16.	OPP – 04	9	3
17.	OPP – 05	7	5
18.	OPP – 16	8	4
19.	OPP – 17	9	4
20.	OPW – 07	7	3
21.	OPW – 08	11	6
Total Number of bands		185	110
Mean per primer		8.8	5.2

Rauwolfia is pantropical with very wide distribution extending from tropical America through Africa, Asia and several of the oceanic islands. The distribution pattern of the sections of the species suggests three or four centers of differentiation; with each of the major land areas having one or two widely distributed species. Significantly, these are also widely variable and have broad tolerances to varied environment. In Asia, the species having the widest distribution are *Rauwolfia densiflora* and *Rauwolfia verticillata,* closely followed by *Rauwolfia serpentina* (Sahu, 1979).

Rauwolfia serpentina Benth. belong to the section Ophioxylon of the family Apocynaceae with distribution in India,Ceylon,Burma,Thailand and Java(Sahu,1979) .This species is distributed almost all over India. In Kerala it is found in Wayanad, Kozhikode, Palakkad, Trichur, Kollam, Kottayam and Trivandrum districts.

In spite of such wide distribution, the diversity is not reflected at the genetic level as revealed by RAPDs. RAPD technique has emerged as a means of estimating genetic variation at the population level(Welsh and McClelland,1991). These markers



have been successfully used to assess the genetic diversity within several groups of plants. RAPD markers were used for determining genetic diversity among *Avicinnia marina* (Balakrishna, 1995), Papaya (Stiles *et al* 1993) etc. Useful polymorphism has been found at a variety of taxonomic levels ranging from populations and cultivars to subspecies, species and above species level. In the present data, the total number of products generated by the individual accessions ranged from 112 to 140 with an average of 125 per accession. Analysis of RAPD data indicates that the extent of intra specific variation in the *Rauwolfia serpentina* from seven different places within Kerala are showing high degree of similarity coefficient which ranged from 0.81 to 0.99 with a mean value of 0.95. The most diverse one was the *in vitro* plant used for this study, which showed a GS of 0.88 which reveals a very narrow genetic base for this species. Similar results were observed by (Iqbal *et al* 1997) in cotton where, in spite of a high level polymorphism, it showed a narrow genetic base. Similarly, a number of studies have reported a low genetic diversity in rare and endangered plants (Waller *et al* 1987), Gustaffson and Gustaffson, 1994) and (Swensen *et al* 1995).

This low genetic diversity among the *Rauwolfia serpentina* accessions studied might be due to the fact that they are not growing in their natural conditions. This is in conformity with Kaul (1955) who proposed that the sporadic distribution of this species, its year round flowering habits and high sterility of fruits are indications that the plant is not growing in its natural conditions. According to Adam Charles (1902) maximum polymorphism can be seen in the region of origin because of the presence of forms such as biotypes, species, subspecies etc. in which a prolonged time is required for evolution in the steadily increasing number of different kinds. Species concentration, species differentiation and distribution pattern of related genera all indicate the possibility of tropical America being the birth place of the genus *Rauwolfia* .Rao Aragule Satyanarayana (1956).

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Coloured Edible Plants As Livelihood And Nutritional Security: A Search

ROSHNI S, SANGEETHA M S, SNEHA CHANDRAN S, SONA SALIM S, ADITHYA VIJAYAN, SARANYA SASIDHARAN, RESHMA T L, ATHIRA R, ANJU KRISHNA S, LEKSHMI SS, LEKSHMI A V, SWATHY R S, K MURUGAN

Department of Chemistry, Govt. Arts College, Thiruvananthapuram, Kerala

email : imthiazahamed1234@gmail.com

ABSTRACT

Natural food colour is any dye, pigment or other phytochemicals obtained from plant products capable of colouring food, cosmetic or any part of human body. These colours were produced from a variety of sources such as seeds, fruits, vegetables, algae, and insect. Natural colour exists in the plants at extremely low concentrations and as a result their extraction is expensive and highly sensitive to change. Due to its safety and general availability, food colouring is also used in a variety of non-food applications including cosmetics, pharmaceuticals, home craft projects and medical devices. The present study is the documentation of coloured edible plants from nature. 62 plants were screened for their edible nature and were described. Most of them were not validated scientifically.

Key words *Colour, edible plants, economic values, pigments*

Natural colours are extractants from fruits, vegetables, seeds, roots and also from microorganisms which are biocolours. Plant pigment, by virtue of their natural occurrence in plants is generally treated to be harmless. Nature produces diverse brilliant coloured pigments such as water soluble anthocynanins, betanins and fat soluble pigments carotenoids and chlorophylls used for colouring foods. Anthocyanins, a group of water soluble pigments are responsible for the attractive red to blue colours in many fruits and vegetables including cherries, grapes, black berries and have high colour intensity at pH values less than 4.0. Anthocyanins absorb UV and visible light in a range 250-650 nm. They are used worldwide as a source of edible colours in beverages and desserts which have an acidic pH. In this scenario, a survey was attempted related with coloured edible plants in nature.

MATERIAL AND METHODS

The method employed in this study was designed with the purpose of providing base line information on the use of plant species in local system, through literature survey and field visits to various institutes like CPCRI, NIIST, RGCB, CTCRI, ARI from Thiruvananthapuram districts (Balasingh et al., 2000).

RESULTS AND DISCUSSION

Dandelion

Taraxacum, or more commonly referred to as "Dandelion", in fact, the entire plant is edible. This means that its flowers, leaves, and even roots, are safe for consumption. The leaves of a Dandelion are generally 5-25 cm long or longer and their flower heads are yellow to orange coloured which are open during the daytime, but closed at night (Aberoumand, 2009).

Chickweed

Stellaria media, can be used as a cooling herbal remedy and a safe, nutritious edible. Chickweeds can be identified by fine hairs located on only one side of its stem in a single band and fine hairs on its sepals. Similarly, a prime salad ingredient in food for Western people.

Curly Dock

Rumex Crispus, commonly referred to as "Curly Dock". Although the leaves of this plant can be described as appetizing and a prime source of both vitamin A and vitamin B, it is best to be consumed moderately as it can cause urinary tract irritations.

Asparagus

Asparagus, easily identified by its fleshy green spears which are luscious and delicate, can be eaten raw as opposed to boiled. It is a good source of vitamin C and potassium. Harvest length of these plants is usually 6-8 inches (Aberoumand and Deokule, 2009).

Chicory

This member of the Dandelion family is a bushy plant with typically bright blue flowers. The leaves of this plant can be eaten raw, along with the flowers but the roots aren't edible unless they are boiled.

Wood Sorrel

This medium-sized plant is an effective thirst

quencher and exhilarating snack. The flowers can range from green to bright yellow in colour, and the leaves are a great source of vitamin C. The leaves, flowers and other parts have a flavour that is slightly sour which may be comparable to lemons (Addis *et al*., 2005).

Bull Thistle

The firm and thorny stem and leaves that end in sharp, extended thorns. This plant looks like one should be sceptical to touch, let alone eat it. However, the Bull Thistle roots as well as its young leaves are edible. The prickles must be removed from the leaves before consumption.

Alfalfa

A flowering plant in the pea family, Alfalfa is nutritious and may be used for a variety of benefits including; treatment for alcoholism and drug dependency. It has deep roots and can grow quite tall making it very resilient. The leaves and young shoots are the only parts that you can eat raw (Adera, 2013).

Broadleaf Plantain

A species of the plantain family, Broadleaf Plantain is rich in vitamins A, C and K. The leaves are green and oval-shaped with thick-stemmed leaves that meet at a base. The entire plant is edible but it recommended that the leaves be eaten raw while they are young and tender (Afolayan and Jimoh, 2009).

Creeping Charlieo

The plant which is an excellent source of vitamin C, has been commended for centuries for its nutritious value. It is covered with fine hair and its main root is thick. The young leaves of this plant can be consumed raw.

Forget Me Not

Scientifically referred to as *Myosotis;* the Forget-Me-Not flowers are minuscule, tender and usually blue in colour. They are more common during Spring. The flowers are the edible part of the plant and are indeed a safe choice for a snack.

Garlic Mustard

All parts of this plant emit a strong odour. Its flower is only visible from the month of May until June. The Garlic Mustard plant has broad, kidney-shaped leaves, small flowers and slender pods that contain the seeds. It is classified as an invasive species, in North America, where you are more likely to commonly see it (Ahmed *et al*., 2014).

Wild Black Cherry

Prunus serotina, more commonly referred to as wild black cherry, is an edible plant that is wise to be consumed in a very small proportion. This is due to the fact that their seeds contain compounds that can be converted into cyanide (Ajayi *et al*., 2011).

Harebell

This beautiful plant is relatively small in size and can be identified by its slender stems, the white sap discharged by the stems, and its sagging blue flowers. Harebell's can range from 30 to 60cm in height. The leaves are edible. This should be nutritious as the Harebell contains vitamin C (Alencar *et al*., 2014).

Elderberry

Found mostly in temperate to subtropical regions of the world, many species are widely cultivated to be used as ornaments. However, the Elderberry's flower, as well as its fruit, is edible, although, the raw fruit does not yield a favourable taste. The fruit, when ripe, is a purplish-black colour. All other parts of this bush are toxic.

Field Pennycress

An annual plant with round, flat, winged pods with a deep apical notch. They can grow up to 60 cm. The plant offers little in terms of food as only its leaves can be eaten raw. Unfortunately, the leaves must be harvested before the plant flowers, and even then they yield a bitter taste.

Coneflower

This member of the sunflower family is a heat and drought resistance plant. It has purple or pink flowers that display a prickly seed cone in the center. The leaves are of a rough texture. The leaves and flower petals are edible.

Kudzu

A yellow-green vine with large leaves which are shed annually. The speedy growth rate of these vines leaves them with the tendency to be invasive and they are considered noxious weeds and is entirely edible.

Meadowsweet

"Queen of the Meadow", "Lady of the Meadow", or "Bridewort", this plant is mostly

recognized as an herbal medicine. It is a large clump-forming plant that usually grows relatively tall. The young leaves are edible.

Mallow

The Mallow, or *Malva*, is a low growing weed with a deep fleshy tap root. The plant is totally edible, its leaves can be used as a substitute for lettuce and are an excellent source of vitamins A and C, calcium and iron.

Peppergrass

Common substitute for black pepper. It is easily identified by its raceme which comes from the plant's branched stem. It also grows between 15 and 60 cm long.

Pineapple Weed

Pineapple weed's flowers and leaves are, in fact, appetizing finger foods for hikers. The plant is commonly mistaken for chamomile. It does resemble chamomile with the exception of the flower petals. It grows up to 30 cm tall.

Pickerelweed

This plant grows in shallow freshwater including lakes and streams. Although tastier when roasted, Pickerelweed seeds can be eaten raw and will surely help out a famished hiker. Its leaves are glossy green with fine parallel veins. It can be recognized by its violet-blue flowers that bloom during the summertime.

Mullein

An attractive plant with a tender, velvet-like physique. It is a tall, erect stem with soft leaves and yellow flowers at the top. They are capable of reaching heights of two meters. Its leaves and flowers are not a bad choice for a snack, although, you would be doing your taste buds more of a favour if they were used to make tea instead.

Red Clover

Along with its many medicinal uses, Red Clovers are considered to be the tastiest of all clovers and will surely make a good find. It has a reddish flower head made up of different tubular-shaped flowers. The leaves are edible and the flower too.

Partridgeberry

A low trailing shrub with fuzzy white flowers that grow in pairs that form one berry per pair. The flowers of this plant emit a pleasant smell when blooming. The leaves and berries are edible, although they are not very tasty. The stems are typically light green to light brown.

Sheep Sorrel

Commonly found in acidic, sandy soils and grasslands, this plant typically grows in areas where blueberries can be found. Sheep Sorrel leaves can be eaten raw and have a pleasant lemony or tangy flavour. Be sure to look out for its reddish upright stems.

Shepherd's Purse

It was given its name due to its triangular flat fruits which are purse-like. The leaves of this plant can be eaten raw, which is the healthier option. It is a common weed of cultivated soil in many countries.

Sunflower

Helianthus annus and grow an average height of 1-2 m tall. Wild Sunflower seeds are small and can be consumed raw. The wild seeds are much smaller than those used for commercial production.

Spring Beauty

*Claytonia carolinianas*is also called Spring Beauty. It can be identified by its small flowers produced in the spring which have pink petals, or white petals with darker pink veins. The root, leaves and stem of the Spring Beauty can be eaten raw.

Tea Plant

Its flower blossoms are white and fragrant, have both male and female organs, and are pollinated by bees. The leaves and flowers can be eaten raw.

Toothwort

This plant is a member of the mustard family which grows best in moist soils preferably in wooded areas. Its flowers have four petals which are white to pinkish or light purple. The leaves and roots of this plant are totally edible.

Teasel

Originally brought to North America from Europe, an average Teasel plant produces over 3000 seeds that birds feed on. It is a self-fertile plant noted for attracting wildlife. The flowers bloom by first forming in a ring around the plant's head. A Teasel's young leaves are edible after avoiding its spiny, stout hairs.

Wild Grape Vine

A vining plant with no solid, upright trunk. They grow thicker and higher than most other native vines. A great choice for a trail snack, although, it tastes better after the first frost. Grape vine leaves are green and lobed with long, pointed tips and a smooth surface.

Wild Bee Balm

Identified by its thick lavender flowers, this plant prefers dry and sunny locations to avoid developing mildew. Its fragrant flowers begin to bloom in the centre of the flower head and can be pink in colour depending on its location. The leaves of the Wild Bee Balm are edible and make a safe trail snack for you.

Vervain Mallow

This plant prefers sunny or partially shaded areas and mostly grows in thickets and in waste areas. They can grow from 50 cm to over 1 m tall. Their flower has five white to pink petals with five green sepals. The flowers, leaves, and seed are edible, although, it is recommended that only the leaves be eaten raw.

Prickly Pear Cactus

The Prickly Pear Cactus is a palatable as well as nutritious plant that is typically found in the deserts of North America. Its fruit strongly resembles a red or purplish pear.

Herb Robert

Its many medicinal uses include: toothache and nosebleed remedy, and healing wounds. Freshly picked leaves release a strong odour when crushed, and is said to repel mosquitoes if you rub it on your skin. The entire plant is edible and fresh leaves can be used in your salad or to make tea.

Mayapple

Woodland plants usually growing in colonies derived from a single root. Their stems grow to 30-40 cm tall and they have large, deeply cut leaves. All of their parts are poisonous, including the fruit which is covered by the large leaves. However, once the fruit is ripe, and has turned yellow and soft as opposed to green and firm, you can safely consume it.

Joe Pye Weed

This plant can be described as an herb, a wildflower, and a butterfly plant. Purple flowers at the end of the stems. It can be found in moist woods and along streams. The entire plant, including the root, is edible.

Knapweed

The flowers of these plants are diverse in colour, ranging from deep reds, blues and yellows to any mixture of these. The stems are ribbed and may become hairless as they age. Only the flowers are edible.

Wild Leek

A North American species of wild onion that grows deep in the woods. It is bulb-forming with broad, smooth, light green leaves. Similar to that of an onion. The leaves and bulbs are edible but it is best if you consume them in small quantities.

Cleavers

An annual plant with small hooked hairs growing out of their leaves and stems. It is also known as "stickyweed", "sticky willow", or "sticky jack". The entire plant is eaten raw, however, its small hooked hairs will make it much less appetizing.

Cattail

Easily recognizable by its brown, cigar-shaped head, you can find the cattail in open wet areas as well as swamps and ditches. Most of the cattail is edible. The leaves are better off boiled.

Blue Vervain

It is mostly found in moist conditions with full to partial sun. Places it is commonly found include: moist meadows, riversides and pastures. Edible leaves and flowers raw but the seeds must be roasted.

Common Yarrow

A flowering plant that is considered by many to be an aggressive weed. The leaves can be eaten raw, although, they are bitter in taste.

Common Sow Thistle

A plant of the dandelion tribe within the daisy family. These plants have hollow stems, a short taproot and deeply lobed leaves. The flowers are yellow and the seeds are light with white silky hairs. The leaves can be eaten raw and will prove to be good for your body as they contain vitamin C, protein and carbohydrates.

Coltsfoot

Another member of the daisy family, this plant

has bright yellow flowers that bloom early in the spring before any leaves are even present. Its flowers can be eaten raw as well as its leaves in small quantities.

Fern Leaf Yarrow

Fern Leaf Yarrow grows as a single, stout stem. It has fern-like leaves that are typically 5 to 20 cm long. Its aerial parts are safe to eat. Although bitter in taste, the leaves of this plant can be eaten raw, but best to do so in small quantities despite its nutritional value.

Henbit

Soft, finely hairy stems. The leaves have a lobed margin and the flowers are pink to purple in colour. Its name is derived from the observation that chickens are fond of it. It is more likely to grow in light, dry soil as well as cultivated soil. Henbit is very nutritious as it is high in vitamins, iron and fibre.

Crimson Clover

A species of the clover family, the Crimson Clover has leaves and stems that resemble those of the Red Clover. It grows in fields, meadows and lawns and can reach heights between 30 and 50 cm. The seeds and flowers are edible and can be eaten raw.

Evening Primrose

The Evening Primrose is an herbaceous forb that flowers only late in the day and into the evening, hence its name. It can grow between 30 and 150 cm tall and has a bright yellow flower with a mild lemony aroma. The young shoots can be eaten raw as well as the slightly sweet-tasting flowers.

Downy Yellow Violet

Divisible into two varieties, the Downy Yellow Violet is a soft and hairy violet that can grow up to 9-12 inches tall. The bright yellow flowers and hairy appearance of this plant make it unique and easy to identify. Although it is not particularly nutritious, it is safe for you to eat the flowers and leaves of this plant.

Daisy Fleabane

Another plant with a hairy nature; the Daisy Fleabane is an attractive plant with composite flowers. It has hairy leaves and stems and is best grown in areas exposed to full sun. Only the leaves of this plant are edible, although, you might frown while eating them as its hairy nature might result in an unpleasant taste.

Japanese Knotweed

A large plant that has been classified as an invasive species by many countries, the Japanese Knotweed has hollow stems and broad oval leaves. They have a rapid growth rate with stems reaching a maximum height of 9.8-13ft each growing season. It is best to eat the young shoots of this plant-which some say has a lemony flavour.

Milk Thistle

Red to purple flowers and shiny pale green leaves with white veins. Apart from being a source of food, this plant has medicinal uses such as: increasing appetite, digestion aid, liver cleansing and poisoning treatment. The root, leaves and stems can all be consumed raw.

Lambs Quarters

This is a dusty looking plant from a distance due to a white coating on its leaves. It is common near rivers, streams, and in gardens with an average height of 1 m. The leaves, shoots, seeds and flowers of this plant are all edible. Due to potentially toxic components, it would be best if you consumed these in small quantities, especially if consuming them raw.

Queen Anne's Lace

The legend tells of Queen Anne pricking her finger and a drop of blood falling on a lace that she was sewing. Similarly, this plant's flower resembles a lace, and the single purple dot that is often seen in the middle of the flower resembles the drop of blood. The leaves and roots are edible but it is advised to use first-year plants.

Purple Deadnettle

Lamium purpureum, it translates into English as "the devouring purple monster." Scary! The plant, in fact, has many medicinal purposes including the use of its leaves for treatment of external wounds or cuts. It has pink flowers that only last about six weeks after they bloom. Only the leaves of this plant are edible.

New England Aster

Although considered an aggressive weed by some, the New England Aster is praised by others for its extravagant flowers. The flowers are large and rose-purple in colour with many rays. The root, flowers and leaves are edible and are said to provide health benefits (Ahmad et al., 2006).

Supplejack Vine

A vine found only in New Zealand, Australia and New Guinea, Supplejack Vine produces leafy stems that bear flower and fruits once it reaches sunlight. It can grow extremely long and is likely to grow towards the sunlight through the forest. The berries it produces, once ripe, can be eaten either raw or cooked as well as its young shoots and leaves.

CONCLUSION

All these edible wild plants are considered as nature's mother for food. Future studies are warranted to validate their nutraceutical potentials.

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Retrometabolic Approach for Cardio Protective Anti Cancer Drug Design

SANJAY KUMAR P.[1] AND KRISHNAN NAMBOORI.P.K[2]

[1]*Amrita Molecular Modeling and Synthesis (AMMAS) Lab, Amrita School of Engineering, Coimbatore, Amrita VishwaVidyapeetham, India*
[2]*Amrita Molecular Modeling and Synthesis (AMMAS) Lab, Amrita School of Engineering, Coimbatore, Amrita VishwaVidyapeetham, Amrita, India*
Email: n_krishnan@cb.amrita.edu

ABSTRACT

Anti-cancer drugs have been reported as causing side effects resulting in cardiovascular and neurodegenerative disorders. The current research focuses on minimizing cardiovascular disorders reported during the use of a number of anti-cancer drugs. There are various mechanisms induced by the anticancer drugs leading to this condition and most of the FDA approved cancer drugs are found to be having these side effects. A comprehensive study of the mechanisms corresponding to cardio vascular disorders associated with the use of anti-cancer drugs has been carried out. The study revealed that the FDA approved drugs RDns (drug name codified to safeguard research interests) considered showed considerable effective drug response towards cardiovascular side effects. Among the drugs RD5 showed maximum anti-cardiovascular drug response. The drug RD5 acts as ErbB antagonist and ErbB-2 antagonist and can disrupt the regulation of receptor tyrosine kinase, an ErbB family of receptors. These receptors enable the cells interacting with the external environment by transducing signals to the nucleus, thereby promoting heart morphogenesis and function. A retro-metabolic drug design approach which uses nontoxic biocompatible metabolites as parent compound to design a cardio protective drug was used in this research. The potential cardio protective molecule designed with a high level of personalization has been found to have minimal anti-cardio side effects and this approach can be recommended in designing personalized drugs for cancer with minimum cardiovascular side effects.

Key words *Anti-cancer; FDA; RD5; retro-metabolic drug*

It has been reported that, along with the chemotherapeutic treatment for cancer, complications such as diabetes, Alzheimer's disease, cardiovascular disorders etc. may follow in most of the cases as side effects. The drug induced cardiovascular side effects are always a major concern of anticancer drugs. In few cases, this may become highly complicated and sometimes causing death of the patient. The side effects may be experienced as a consequence of the secondary effects of the drugs or disease. The accommodation of cardiovascular system towards disorders and the extensive energy reserve carried by the organ may lead into late diagnosis of the side effects. Moreover, the modern treatment regimens with multiple agents and functionalities may result in deleterious cardiac side effects. The drug induced cardiac effect varies from person to person, necessitating a pharmacogenomics approach in drug design. The patient related variants include genetics, individual variation, previous cardiac history, metabolism disorders etc. A greater understanding of the mechanisms associated with the process and the genetic signature behind proneness of the side effects would help in designing new promising molecules with lesser cardiovascular toxicities. The drug related attributes responsible for the variation include the dose, route of administration, drug combinations, mechanisms associated with the drug action etc. (Edward *et al*, 2004). The anticancer drug Trastuzumab, Lepatinib (HER2 antagonist) etc. are reported as not completely free from cardio-toxicity. A phase three trial report submitted in 2006 mentioned cardiac events such as symptomatic heart failure and reduction in the 'left-ventricular-ejection-fraction' while a combination of capecitabine and Lepatinib was administered to treat cancer (Nicholas and Peter 2012).

The importance of personalization and administration of P4 - (predictive, participatory, preventive and personalized) pharmacogenomic technique become inevitable, while deciding the right drug to the right patient. In the design phase of drugs, the P4-technique is introduced to minimize side effects and to provide maximum efficiency. The retro-metabolic approach, designing potential drugs from the bio-compactable metabolites, has become a major technique in the P4-strategy. The process helps in the design of a 'soft drug'- 'drug delivery' complex with an active metabolite as the soft drug and an inactive metabolite counterpart as the chemical drug delivery system (CDS) (Bodor and Buchwald 2010). The aim of the present research is to design a cardio-protective anti-cancer drug considering all the above mentioned factors.

Table 1. The table shows the Potential Soft drug-CDS complex (SFD), its drug response along with docking and complementarity scores.

Sl. no.	IUPAC of Soft drug-CDS complex	Effective Drug Response	Docking score With BRCA1protein Target		Complementarity score with BRCA1 protein target	
			3EU7	3PXB	3EU7	3PXB
SFD	2-Amino-4-carbamoyl-2-[2-hydroxy-4-(7-hydroxy-4-oxo-3-chromenyl)phenoxy]-3-propylheptanoic acid	11.145	33.61	31.043	5.616	4.123

MATERIALS AND METHODS

The strategy employed in this research is to study the mechanisms involved in causing cardiovascular disorders as a side effect during cancer therapy. Out of 4000+ mechanisms happening in our body, the cardiovascular mechanisms responsible for side effects were streamlined into 12 which include Angiogenesis stimulant, ErbB antagonist, ErbB-2 antagonist, Fibrillation atrial, Histamine release stimulant, Myocarditis, Thromboxane A2 antagonist, Thromboxane antagonist, Thromboxane synthase inhibitor, Thromboxane synthase stimulant, Transcription factor NF kappa B inhibitor and Cardio depressant. The mechanism, angiogenesis stimulant can induce neo-vascularization contributing to the growth of atherosclerotic lesions which has been considered as the major factor for plaque destabilization and rupture. A cancer drug which has this property can induce cardiovascular related side effects (Rohit *et al*, 2005). Other important mechanisms which can cause cardio side effect are ErbB antagonist and ErbB-2 antagonist. These agents can disrupt the regulation of receptor tyrosine kinase, an ErbB family of receptors. These receptors enable the cells interacting with the external environment by transducing signals to the nucleus, thereby promoting heart morphogenesis and function (Wieduwilt and Moasser, 2008). Progressive fibrosis in atria leads into fibrillary atria, certain anticancer drugs can induce fibrosis as a side effect (Everett and Olgin, 2007). If there is an elevation in blood histamine levels the chance of getting stable coronary artery disease is high, some anticancer drugs possess Histamine release stimulant property which can again lead into cardiovascular side effects (Sanda *et al*, 2002). Inflammation of heart muscles which can lead into myocarditis can be a side effect of cancer therapy as well (Jay, 2003). Thromboxane A2 is an arachidonic acid metabolite, is required for platelet function stimulation, vasoconstriction and secretion of 5-HT, PDGF etc. which are the major platelet derived storage products. A cancer drug acting as a Thromboxane A2 antagonist can decrease this activity which may cause acute coronary disorders (Karsten, 1992). Being a redox sensitive transcription factor which regulates many inflammatory genes, Nuclear factor kappa-B (NF êB) plays a significant role in various pathological conditions. This transcription factor acts as a cardio protective and pharmacological inhibition may cause cardiovascular disorders (Guro, 2001).

The most popular anti-breast cancer drugs (RD1-RD18) anti-cardio vascular mechanisms was studied to compare it with the newly designed anti-breast cancer molecules using a retro-metabolic design strategy. A condensation process of design was implemented in this research, where an active metabolite or its modified derivative was condensed with an inactive metabolite within our body. After condensation the active metabolite acts as a soft drug and the inactive metabolite acts a safe wandering molecule as a chemical drug delivery system (CDS). The Soft drug was designed as a personalized drug for breast cancer. The active metabolite, 3-(3,4-Dihydroxyphenyl)-7-hydroxy-4-chromenone (3'-Hydroxydaidzein) is associated with anti-cancer drug genistein's parent compound, Hydroxyisoflavonoid which was condensed with L-Glutamine an inactive metabolite to construct a modified Soft drug - CDS complex. Pass online tool was used to study anti-cardio mechanisms of cancer drugs, an SAR based tool which predicts the activation *(Pi^a)* and non-activation *(Pi^{a*})* of a molecule. Multi-level neighborhood of atoms (MNA) descriptors is used here to predict the same (Lagunin *et al*, 2000; Iyer *et al*, 2017). Effective drug

response or EDR is calculated by;

$$EDR = \sum_i \left(P_i^a - P_i^{a^*} \right)$$

To study the interaction between the target and the analogues CDOCKER algorithm was used of the Accelrys (BIOVIA) .

RESULTS AND DISCUSSION

Interactions with the Breast Cancer targets

The Soft drug-CDS complex designed (SFD) (Fig. 1) was found to be having high interaction with the hotspot of the breast cancer target proteins of the genes BRCA1 (3PXB) and BRCA2 (3EU7). This proves the high level of personalization of the designed drug (Table 1).

The anti-cardio properties of the SFD designed were studied exclusively and it was found to be much lower (Fig. 2) when compared to RD5, the FDA approved popular breast cancer drug. RD5 was also compared with other similar FDA approved non personalized breast cancer drugs which were also found to have a lesser anti-cardio property when compared with RD5 (Fig. 3). Most of the non-personalized anti-cancer drugs showed anti-cardiovascular properties. Among the anticancer drugs, RD5 has been found to be having maximum effective drug response towards cardiovascular disorders. The drug RD5 acts as an ErbB and ErbB-2 antagonist and can disrupt the regulation of receptor tyrosine kinase, an ErbB family of receptors. These receptors enable the cells interacting with the external environment by transducing signals to the nucleus, thereby promoting heart morphogenesis and function.

Fig. 1. The structure of cardio protective anticancer SFD designed through Retrometabolic drug design strategy.

The inclusive study of anti-cardio properties of FDA approved non-personalized anti-breast cancer drugs and personalized SFD, it reveals the importance of personalization in cancer drug design. The SFD designed in a condensed retro metabolic method was found to be more personalized and cardio-protective when compared to RD5 and other breast cancer drugs.

CONCLUSION

It has been observed that most of the Cancer drugs have cardiovascular side effects which may lead into undesired complications after cancer therapy. The present research compared the level of anti-cardio properties of non-personalized anti-cancer drugs and highly personalized SFDs. The SFDs designed were found to be having lesser anti-cardio properties when compared with popular anticancer drugs. Personalization of drugs has become inevitable in the present scenario as many anticancer drugs are seen undesired for many patients due to genetic variations. The condensed retro-metabolic drug design has been found to be beneficial as the cardio-protectiveness and personalization of the drugs designed was found to be

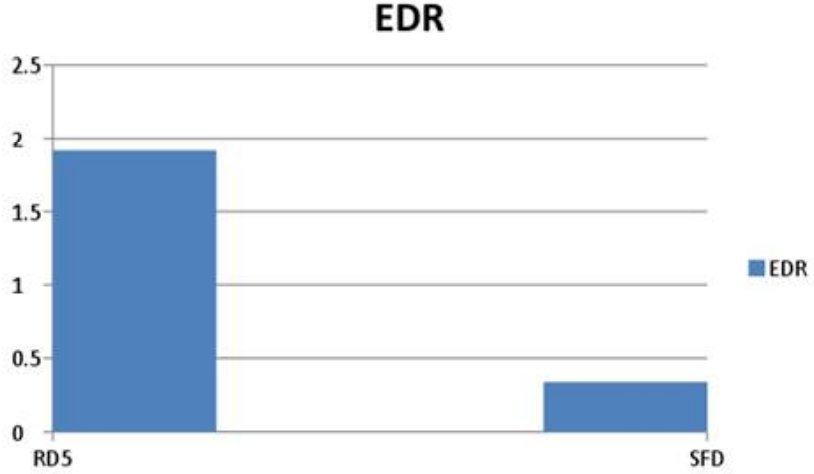

Fig. 2. Cardiovascular side effects measured as EDR of RD5 vs SFD

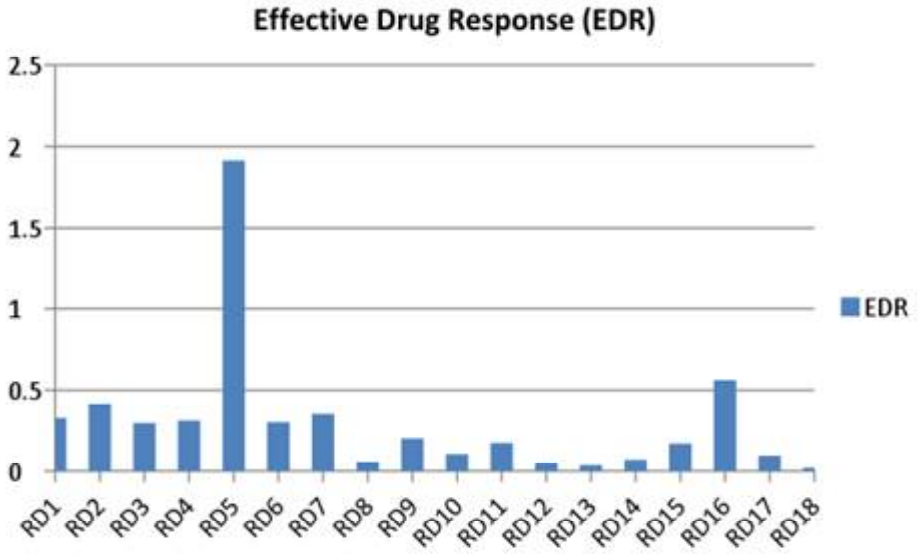

Fig. 3. EDR of Non-personalized anti-breast cancer drugs towards cardiovascular side effects.

much better when compared to popular FDA approved anticancer drugs.

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Seed Development and Germination Studies of Two True Mangrove Species *Rhizophora mucronata* Poir and *Bruguiera cylindrica* (L) Blume

S. SEKARAN

Sree Nayana College for Women,
Kollam, Kerala
email: drsekhar72@gmail.com

ABSTRACT

The present study was carried out on phenological observations and reproductive characteristics including seed development, maturation and number of days taken for produce mature propagules/seeds by the selected two important mangrove species *Rhizophora mucronata* . Prior *Bruguiera cylindrical* (L) Blume of the family Rhizophoraceae. An interesting adaptation noticed in true mangroves is that or those belonging to the family Rhizhophoraceae reproduce through a unique biological phenomenon called vivipary. In this mode of reproduction in the post fertilization the zygotes stay on the mother plant for a period 3-7 months until they mature in to seedlings or commonly called as propagules.

Key words *Seed Development, Germination Studies, True Mangrove Species, Rhizophora mucronata Poir and Bruguiera cylindrica*

The physiological maturity of seeds generally determined on the basis of accumulation of higher dry weight with maximum germination. In *Rhizophora macronata* physiological maturity of seed determined as 14th weeks after anthesis. The moisture content of the seed was decreased to with increase of dry weight. In *Brugeiera cylindrica*, the harvestable maturity can be fixed on 12th weeks after anthesis. It was based on the maximum dry weight. 2.4 gm with minimum fresh weight of 4.09 gm. The germination percentage of seeds was also maximum during that period.Seed maturation studies of *Rhizophora mucronata* indicate that the best collection time prevails from April to June and in *Bruguiera cylindrica* the best seed collection time prevails from May to July.

Mangroves are halophytes occurring in saline marshy places. The word "mangroves" is considered to be a combination of the Portuguese word "mangue" and the English word "grove". Mangroves are salt tolerant forest ecosystems of the topical subtropical inter tidal regions of the word. Mangrove ecosystem is a group of numerous plants and animal interacting with each other and their surrounding. In India, Vegetation formation also termed as 'Tidal forests'. Macnae (1968) coined a new term to the mangroves i.e.; "mangal" for mangrove community and "mangrove" for individual species. Mangroves are prominent component of coastal vegetation occupying flood plains, margins of bays and tidal river in addition of shores. Uniqueness of mangrove ecosystem is that

Rhizophora mucronata

Bruguiera cylindrica

the biota is constantly under physiological stress caused by extreme conditions, mangroves have been successfully colonized by developing morphological, reproductive and physiological adaptations like pneumatophores, prop roots still roots and viviparous germination which facilities their growth in aquatic environment .(Tomlinson,1986) Arunprasath and Gomathinayagam(2014) reported the phenology , reproductive biology and storage studies of five true mangrove species of Pichavaram mangrove forests of Tamil nadu. A detailed phytosociological and floristic composition of two natural mangrove vegetation including the study site Ayiramthengu of Kollam district was reported (Sekaran *et a*l.,2015)

The scope of the present study is to analyse the various phonological and reproductive characteristics of two mangrove species in Ayiramthengu mangrove forest of Kollam district for developing a data which could be of help the forest managers in planning to regenerate the species of the mangrove forest.

MATERIALS AND METHODS

The present study was carried out on phenological observations and reproductive characteristics including seed development, maturation and number of days taken for produce mature propagules/seeds by the selected two important mangrove species *Rhizophora mucronata* Prior & *Bruguiera cylindrical* (L) Blume of the family Rhizophoraceae.

Seed Development and Maturation studies

The flowers of the two species were tagged separately considering the time of anthesis as the main criteria for determination of physiological maturity. The propagules/seeds were collected from the tagged flowers at weekly intervals to tag more number of flowers to overcome the problem of heavy flower/ fruit shedding with most care. The results were expressed as weeks (1^{st}, 2^{nd} , 3^{rd} , 4^{th} ….etc) The physical characters namely length, dry weight fresh weight of seed were measured during every sampling time. Physiological characters such as percent germination were studied in the above seeds periodically.

SEED GERMINATION TEST

To obtain germination percentage five replicates of 20 seeds were germinated in sandy media in a plastic germination cover placed under the tidal condition. The number of seeds germinated and the germination percentage was calculated.

Root and Shoot length

Ten seedlings were taken 30 da after sowing for both Rhizophora and Brugueira random from the standard germination test. The seedlings were removed from the germination cover without damaging the root and shoot, washed thoroughly to remove the adhering soil particles. The length of root and shoot was measured individually for the entire seedling selected. The shoot length was measured from collar region to the tip of the leaf and root length from collar region to the tip of the primary root and their means were expressed in centimeters. (cm).

Vigour index

The vigour index was calculated adopting the formula proposed by Abdul Baki and Anderson (1973) and expressed in number.

Vigour index = Germination percent x (Root length + Shoot length cm)

Selection of Water Media for growth of seedlings.

To find out suitable water media for better growth of seedlings of both species. The following media were attempted in salt water and fresh water.

From this, the seedling height shoot height, basal diameter, node number, leaf number root biomass, stem biomass, leaf biomass and total biomass were recorded.

Selection of suitable media for viability Test

To find out suitable seed testing media for viability the following media were attempted in Sand, Sand+Humus +Soil and Clay soil

The observations on germination ,Root length, Shoot length, collar diameter and number of leaves were carried out.

RESULT AND DISCUSSION

Seed development and maturation studies

For the present study the changes in physical characters and germination of seeds/ Propagules of *Rhizophora mucronata* over a period of time from the date of anthesis to propagule / seed maturation at weekly intervals are given in table.1. The seed characters of *Rhizophora mucronata* is noted that the various physical characters such as are steadily

Table 1. Seed development and maturation studies on *Rhizophora mucronata*

Week	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Fresh Weight	0.30 (±0.08)	0.5 (±0.09)	2.2 (±0.15)	3.1 (±0.2)	5.28 (±0.26)	10.5 (±0.3)	14.92 (±0.5)	18.88 (±0.7)	22.86 (±0.8)	26.5 (±0.5)	28.92 (±0.3)	30.1 (±0.2)	34.67 (±0.22)	34.90 (±2.3)	34.90 (±2.3)
Dry Weight (gm)	0.03 (±0.0003)	0.07 (±0.0002)	0.3 (±0.1)	0.6 (±0.03)	1.1 (±0.04)	2.59 (±0.05)	4.1 (±0.29)	6.2 (±0.12)	7.5 (±0.35)	9.5 (±0.1)	11.1 (±0.26)	13.26 (±0.29)	17.2 (±0.17)	28.6 (0)	28.6 (±2.3)
Length (cm)	0.32 (±0.1)	0.5 (±0.05)	1.22 (±0.07)	2.3 (±0.1)	4.0 (±0.23)	6.9 (±0.29)	8.5 (±0.23)	11.25 (±0.25)	13.33 (±0.2)	14.56 (±0.11)	16.70 (±0.23)	17.9 (±0.24)	18.36 (±0.25)	19.85 (0)	19.85 (±2.3)
Moisture Content	90 (±2.5)	86 (±3.4)	86 (±3.4)	80 (±2.3)	79 (±2.3)	75 (±1.6)	72.5 (±1.3)	67.16 (±2.6)	67.10 (±2.1)	64 (±1.3)	61.61 (±1.9)	55.9 (±1.1)	50.38 (±0.5)	48.42 (±2.3)	48.42 (±2.3)
Germination (%)	-	-	-	-	-	-	-	5 (±0.01)	12 (±0.03)	15 (±0.03)	17 (±0.02)	25 (±0.04)	45 (±0.01)	50 (±0.01)	60 (±0.04)

increased during the process of seeds/ propagules maturation. The length, fresh weight and dry weight increased stadily in R. mucronata upto 12th week after an thesis. On the other hand the moisture content of the seed was decreased during the propagules maturation i.e., (90% to 48.42%). The maximum moisture content were recorded in the initial stages of seed development.

Seed development stages of *R.mucronta*

The propagules / seeds maturity of R. mucronata can be identify on the basis of their seed colour (greenish to brownish in colour). In *R. Mucronata* the propagule maturity attain at the period of 13th week.

The in physical characters and germination of seeds of *B. cylindrical* over a period of time from the date of anthesis to seed maturation at weekly intervals are given in table. 2, The physical characters noted that such as fresh weight, dry weight and length were increased steadily up to 11th week after anthesis. On the other hand, the seed moisture content was decreased rapidly throughout the study period i.e., 88.88% to 41.4%. the maximum moisture content were recorded in the initial stages of seed formation.

Table 3 clearly shows that the seed formation starts at 4th week and the physical characters like fresh weight and dry weight of seed increases and the moisture content of the propagules / seeds decreased

Table 2. Seed development and maturation studies on *Bruguiera cylindrica*.

Week	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Fresh weight (gm)	0.09 (±0.002)	0.18 (±0.06)	0.25 (±0.07)	0.33 (±0.57)	0.57 (±0.15)	0.75 (±0.16)	1.12 (±0.19)	1.85 (±0.2)	2.18 (±0.23)	3.4 (±0.2)	4.09 (±0.2)	4.1 (±1.1)	4.1 (±1.2)	4.1 (±1.1)
Dry weight (gm)	0.01 (±0.001)	0.03 (±0.06)	0.05 (±0.003)	0.08 (±0.03)	0.15 (±0.01)	0.24 (±0.03)	0.43 (±0.05)	0.95 (±0.1)	1.10 (±0.19)	1.9 (±0.19)	2.4 (±0.05)	2.4 (±0.03)	2.4 (±0.04)	2.4 (±0.05)
Length (cm)	0.2 (±0.09)	0.5 (±0.1)	0.8 (±0.08)	1.2 (±0.1)	2.35 (±4.67)	4.67 (±0.23)	6.30 (±0.24)	7.92 (±0.3)	10.68 (±1.32)	12.32 (±0.2)	13.2 (±0.3)	13.2 (±1.2)	13.2 (±0.2)	13.2 (±0.3)
Moisture content (%)	88.88 (±2.2)	83.33 (±2.6)	80 (±2.5)	75.75 (±2.5)	73.36 (±1.7)	68 (±2.0)	61.60 (±1.9)	51 (±2.3)	49.54 (±2.1)	44.11 (±2.1)	41.32 (±1.1)	41.4 (±0.5)	41.4 (±1.1)	41.4 (±1.2)
Germination (%)	-	-	-	-	-	-	-	5 (±0.01)	12 (±0.03)	15 (±0.03)	17 (±0.02)	25 (±0.04)	45 (±0.01)	50 (±0.01)

± : Standard Deviation

Table 3. Effect of different seed testing media on germination percentage, shoot length and root length and Vigour index of *Rhizophora mucronata* and *Bruguiera Cylindrica*

	R.mucronata				B.cylindrica			
Seed testing media	**Germination %**	**Shoot length (cm)**	**Root length (cm)**	**Vigour Index**	**Germination %**	**Shoot length (cm)**	**Root length (cm)**	**Vigour Index**
Sand	44	18.3	10.3	1258	40	7.9	12.8	828
Sand + humus + Red soil	61	23.9	12.9	2245	56	10.1	14.5	1378
Clay soil	88	32.6	16.6	5210	75	12.3	18.9	2340

Seed development stages of *B.cylindrica*

throughout the maturation period. The seeds / propagules of *B. cylindrical* were started to germinate at 6th week after anthesis. The propagules of *B. cylindrical* attained physiological maturity at 12th week.

The accumulation of maximum dry weight of seeds at 12th week indicate the physiological maturity in R. Murcronata and B. Cylindirca. The physiological maturity denotes the attainment of maximum dry weight. Such increase in day weight of seed during development and maturation was reported by Hocking *et al.,* (1980) in Nuytisa, Floribunda, Husin *et al*., (1981) in Hevea brassiliensis. The increase in dry weight of seed might be due to decrease in the moisture content coupled with increased accumulation of food reserve material . however , the change in seed dry weight were not related to change in seed dry weight were not related to change in seed quality of marrow (Demir and Ellis,1993).

Effect of seed testing medium on germination, shoot length and root length

The seeds of Rhizophora mucronata were sown on different testing media such as sandy soil, sand + humus + redsoil, clay soil, conditions for the observation on germination, root-length, shoot length were recorded (Table.3). The freshly collected seeds of R. mucronata shows maximum germiability in clay soil ie., 88% and les germination in sandy soil i.e., 43%.

The maximum root length, shoot length and germination percentage of seeds were recorded in muddly soil present in that area. The average shoot length and root length of seeds are 33.6cm and 16.6cm, and it is decreased in other mediums.

The seeds of Bruguiera cylindrical were also sown in three different testing media such as muddy soil, sandy soil and sand soil + humus + Red soil, Conditions for observation on germination, root length and shoot length of seeds were recorded (Table.5). The freshly collected seeds of B. Cylindrica shows maximum germiability in muddy soil, i.e. 75% and less germination in sandy soil. The maximum root length, shoot length and germination percentage of seeds were recorded in muddy soil. The average root and shoot length of seeds are 12.3cm and 18.9cm.

Effect of growth in tidal and land keeping seedlings of *R. mucronata* and *B.cylindrica*

The seeds of *R.mucronata* and *B.cylindrica* are placed in tidal condition and in land, the effect of seeds of both species are different in these conditions. to observe the seedling growth of R.mucronata and B.cylindrica after 30 days,60 days and 90 days, the tidal water dipping seedlings show s higher growth than land keeping seedlings. The characters of seedlings-shoot length ,root length, collar diameter, number of leaves and dry weight of seedlings is higher in tidal water dipping seedlings and lesser in land keeping seedlings.(Table.4)

Table 4. Effect of water condition on growth parameters and biomass of mangrove seedling

Species	Period	Tidal water dipping seedlings					Land keeping seedlings				
R.mucronata		Shoot Length	Root length	Collar Diameter	Number of leaves	Biomass of seedling	Shoot length	Root length	Collar diameter	Numb er of leaves	Biomass of seedling
	30 days	20.5	7.9	4.5	2	23.5	22	8	3.9	2	22
	60 days	29	10.5	6.8	6	28.9	28	10	6	4	26
	90 days	32.8	15.1	9.3	8	35.1	31	12.6	8	6	30
B.cylindrica	30 days	14.5	6.5	3.3	2	6.6	12.2	6.1	3.4	2	5.3
	60 days	19.1	8.6	5.7	4	9.1	16.1	7.3	5.2	4	7
	90 days	22.8	11.4	7.6	6	13.6	20	10.1	7	6	11.3




Growth Rate In Nursery Stages of *Rhizophora mucronata and Bruguiera cylindrical*

Effect of growth of *Rhizophora mucronata* and *Bruguiera cylindrical* in tidal and land keeping seedlings

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In vitro Antioxidant Potentiality *of Andrographis elongata* (Vahl) T. Anderson- an Endemic Medicinal Plant

Y.B. SHEEJA[1], OOMMEN P SAJ[2] AND LAIJA S NAIR[3]

Department of Botany, University College, Trivandrum
email: sjkattakada@gmail.com

ABSTRACT

***Andrographis elongata* is an endemic medicinal plant distributed in the western ghats of South India. It is used for various medicinal purposes. *A. elongata* is used in the treatment of snake bite, hyperglycemia, skin disease, and also veterinary medicines. The various part of the plant is used by the tribal village herbalist, village dwellers, local medicine men and other traditional healers for the treatment of numerous human and liverstock ailments. *A. elongata* is a best medicine for diabetes. The medicinal qualities of the plant lie in their phytoconstituents such as flavonoids, alkaloids and other phenolic compounds. The main objective of this work was to evaluate the antioxidant ability of the methanol extracts of leaf, stem and roots of *A. elongata* by employing DPPH radical scavenging activity, FRAP (Ferric Reducing Antioxidant Power), Super oxide scavenging activity and Hydrogen peroxide scavenging activity with ascorbic acid as standard. The findings of the present study showed that the methanol leaf extract of *A. elongata* possessed high antioxidant activity. So it may be used as the source of natural antioxidant for treating the oxidative stress related diseases.**

Key words *Andrographis elongata; Antioxidant assays; DPPH; FRAP; Super oxide; Hydrogen peroxide.*

Oxidation is essential for living organisms for energy production. Hence reactive oxygen species are continuously produced within the living cells resulting cell death and tissue damage. Oxidative damage caused by free radical may be related to aging and degenerative diseases such as atherosclerosis, diabetes, cancer and liver cirrhosis, immune system decline, brain dysfunction and cataracts (Halliwell *et al* ., 1984 and Ames , 1993).

Lipid peroxidation is another effect of free radicals. A large number of toxic chemicals are produced during lipid peroxidation. Free radical (eg. OH) reacts with carbohydrates to produce carbon-centered radical. This molecule leads to the chain disruption. Oxy-radicals are responsible for the rheumatoid arthritis. The main target of free radical is DNA. Free radical caused heritable mutation in the DNA resulting cancer in somatic cell and germ cell. ROS caused modification of DNA bases, single and double DNA breaks, damage to the deoxyribose sugar, and damage to DNA repair system.ROS alter the protein structure and function. The amino acid side chains are irreversibly modified in to aldehyde or ketone group. This will lead to protein aggregation, inactivation or degradation. These changes lead protein carboxylation process resulting due to oxidative stress. The ROS caused oxidative damage to cellular components and alters the cellular function (Gracy *et al.,* 1999).

Antioxidants are a substance that significantly delays or prevents oxidation of easily oxidisable substrate at very low concentration. The main role of antioxidant is to help the body to protect against damage caused by reactive oxygen species (Shahidi, 1997). Antioxidants acts at the levels of prevention, interception and repair. Preventive antioxidants help to stop the production of reactive oxygen species (ROS).Antioxidant action is fulfilled by the increase of antioxidant enzyme such as glutathione Peroxidase (GPX), glutathione Reductase (GR), catalase (CAT) superoxide dismutase (SOD) and glutathione S transferase (GST). The amounts of ROS (Reactive Oxygen Species) production increased by the presence of xenobiotic (Livingstone, D R , 2001).

Herbal medicines have an important role in prevention and curing of ailments. According to World Health Organisation (WHO), 80% of the rural people depend on plants for their primary health care needs (Sakarkar and Deshmukh, 2011). The active principles derived from plants possess diverse pharmacological activities such as antioxidant, anti-proliferative, antibacterial *etc* (Roja and Heble,1994). *Andrographis elongata* is an endemic medicinal plant (Ahmedulla and Nayar, 1986) belonging to the family Acanthaceae, distributed in the western ghats of South India. It is used for the treatment of snake bite, hyperglycemia, skin disease, and also in veterinary medicines (Cinnappan Alagesaboopathi, 2010) in old Travancore. The present investigation aims to provide a scientific basis of the antioxidant potentiality of the plant that may account on its traditional usage.

MATERIALS AND METHODS

Plant material collection

Andrographis elongata (Vahl) T Anderson .was collected from the home garden in Parassala, Thiruvananthapuram district, Kerala.

Preparation of extracts

The plant material was weighed and dried under the shade for 10 days. The root, stem and leaves were separated from the whole plant. The dried materials (root, stem and leaves) were powdered and 50 gm of powdered samples were packed in a thimble and kept in soxhlet apparatus. Methanol was used as the solvent. Five hundred ml of methanol was taken into the round bottom flask. The whole apparatus was kept over a heating mantle and heated continuously for 8 hrs at boiling point. The extracts were concentrated to dryness (at 37^0C - 40^0C in oven) and weighed, the residues were transferred to a pre weighed sample bottles and stored in a desiccator for further antioxidant studies (Harborne, 1973).

In vitro Antioxidant assays in methanol extracts of *Andrographis elongata*

The antioxidant potentiality of the methanol extracts of root, stem and leaves of *Andrographis elongata* was studied by employing different antioxidant assays such as DPPH radical scavenging activity , FRAP (Ferric Reducing Antioxidant Power), Super oxide scavenging activity and Hydrogen peroxide scavenging activity with ascorbic acid as standard.

DPPH radical scavenging activity

The free radical scavenging activity of different concentrations of three extracts were measured with the stable radical DPPH (2, 2– Diphenyl-1-picrylhydrazyl) in terms of hydrogen donating or radical scavenging activity. 3 ml of 0.2 mM DPPH in methanol was added to 100µl of methanol extract of root stem and leaves at different concentrations (100, 250, 500, 750, 1000 µg/ml). After 30 minutes, the absorbance was measured at 517nm according to the procedure of Soler *et al.,* (2000) with slight modifications. Ascorbic acid was used as reference.

DPPH scavenging activity (%) = $[(A_{Control} \times A_{Sample}) / A_{Control}] \times 100$

Where A'is the absorbance.

FRAP (Ferric Reducing Antioxidant Power)

The ferric reducing capacity of the different concentrations (100, 250, 500, 750, 1000 µg/ml) of the methanol extract of root, stem and leaves were measured by using the method of Benzie and Strain (1996) with slight modifications. The antioxidant react with a ferric 2, 4, 6- *tri*- pyridyl-S-triazine (Fe^3_TPTZ) complex and produced a coloured ferrous 2, 4, 6 - tri-pyridyl-S-triazine (Fe_2-TPTZ). The assay was carried out in a total volume of 1.0 ml containing a suitable aliquot of flavonoid extract in freshly prepared FRAP reagent. FRAP reagent was prepared by mixing 10 ml volume of 22.78 mM sodium acetate buffer (pH 3.6), 1.0 ml of 20mM ferric chloride and 1.0ml of 10mM 2, 4, 6- tri-pyridyl-S-thriazine solution prepared in 40mM HCl in the ratio, 10ml:1ml:1ml. FRAP reagent and reagent blank were pre-incubated for 5 minutes at 30^0 C before starting the reaction. Different concentration of samples were added to the FRAP reagent and incubated at 37^0C for 1 minute. Absorbance was recorded at 593nm against reagent blank. Ferrous sulphate was used as standard for calculating the total antioxidant power.

Super oxide scavenging activity

Superoxide scavenging was determined by Nitroblue tetrazolium (NBT) reduction method by Liu (1997).The reaction mixture consist of 1 ml of NBT solution (1 M NBT in 100mM phosphate buffer, pH 7.4), 1ml NADH solution (1M NADH in 100mMphosphate buffer, pH7.4) and 0.1 ml of different concentrations of methanol extract (100, 250, 500, 750, 1000 µg/ml). The reaction was started by adding 100µl of PMS solution (60µM PMS in 100 mM phosphate buffer, pH 7.4) in the mixture. The tubes were uniformly illuminated with an incandescent visible light for 15 minutes and the OD was read at 530nm before and after the illumination. The % inhibition of superoxide generation was evaluated by comparing the absorbance values of the control and experimental tubes. The ability to scavenge the superoxide radical was calculated by using the following formula,

% scavenging = (1- Ae / Ao) x 100

Where, Ao= Absorbance without sample

Ae = Absorbance with sample

Hydrogen peroxide scavenging activity

The Hydrogen peroxide scavenging activity of the methanol extract of root, stem and leaves of *A.elongata* was determined by the method of Ruch

Table 1. DPPH assay on the methanol root, stem and leaves extract of *A. elongata*

Sample concentration (µg/ml)	% of inhibition ± SD			
	Root	Stem	Leaves	Standard (Ascorbic acid)
100	48.91±0.93	56.29±0.06	71.94±0.12	76.02± 0.34
250	51.31±0.02	63.26±0.10	75.18±0.02	87.21 ± 0.27
500	57.24±0.13	72.14±0.18	79.23±0.04	92±0.02
750	62.17±0.19	80.23±0.89	88.27±0.15	99.43±0.12
1000	69.54±0.56	83.27±0.19	95.35±0.01	108.49± 0.21

et al., (1989). 40 mM Hydrogen peroxide solution was prepared in phosphate buffer, pH 7.4.The aliquots of the different concentrations (100, 250, 500, 750, 1000 µg/ml) of the extracts were added to a 0.6 ml of 40mM Hydrogen peroxide solution and kept for 10 minutes. Absorbance of Hydrogen peroxide was measured at 230 nm against a blank containing phosphate buffer without Hydrogen peroxide. The percentage of Hydrogen peroxide scavenging activity of *A.elongata* was calculated by the formula,

% scavenged Hydrogen peroxide = $[(A_{control} - A_{sample})/ A_{control}] \times 100$

RESULT AND DISCUSSION

The methanol extract of root, stem and leaves of *A.elongata* were tested for its antioxidant property by DPPH radical scavenging activity , FRAP (Ferric Reducing Antioxidant Power), Super oxide scavenging activity and Hydrogen peroxide scavenging activity with ascorbic acid as standard. The reducing properties are vested in the hands of some phytochemical compounds present in the plant system which breaks the free radical chain by donating a hydrogen atom.

DPPH is one of the stable organic lipophilic radical. When the antioxidant reacts with DPPH, the molecule transfers an electron or hydrogen atom to DPPH, by neutralizing it. (Pattanayak *et al.,* 2012). DPPH Scavenging activity has been used to analyze the free radical Scavenging capability of the methanol extract *A.elongata.* The methanol extract of root, stem and leaves showed varying levels of scavenging activities over DPPH free radical. The methanolic extract of root, stem and leaves showed significant DPPH free radical scavenging activities such as 69.54±0.56%, 83.27±0.19% and 95.35±0.01% respectively at 1000 µg/ml.. Among these the methanol leaf extract showed highest DPPH free radical scavenging (Table 1).

In the FRAP assay, the reducing potential of an antioxidant with ferric tripyridyltriazine (Fe3+) to ferrous tripyridyltriazine (Fe2+). The methanolic extract of root, stem and leaves showed significant ferric reducing power such as 0.675±0.03%, 0.984±0.01%, and 1.224±0.01% respectively at 1000 µg/ml.. Among these the leaf extract showed highest ferric reducing power (Table 2).

In the Super oxide free radical scavenging assay, the leaf extract exhibited significant super oxide anion scavenging ability with inhibition rate of 82.82±0.17% at 1000 µg/ml (Table 3).

The methanol leaf extract of *A. elongata* showed higher Hydrogen peroxide radical scavenging. The methanol leaf extract was most effective *ie* 82.16±0.11%, 85.21±0.16% and 88.67±0.21% respectively at 500 µg/ml, 750 µg/ml and 1000 µg/ml. Among these the methanol leaf extract showed highest Hydrogen peroxide radical scavenging activity (Table 4).

The results obtained from the antioxidant study revealed that the methanol extract of *A. elongata*

Table 2. FRAP assay of methanolic extract of *A. elongata*

Sample concentration (µg/ml)	% of inhibition ± SD			
	Root	Stem	Leaves	Standard (Ascorbic acid)
100	0.179±0.05	0.254±0.02	0.659±0.01	0.192±0.01
250	0.221±0.11	0.321±0.01	0.839±0.03	0.321±0.11
500	0.398±0.10	0.552±0.05	0.996±0.02	0.492±2.02
750	0.513±0.01	0.731±0.02	1.121±0.03	0.561±0.11
1000	0.675±0.03	0.984±0.01	1.224±0.01	0.615±0.04

Table 3 Super oxide free radical scavenging assay on the methanolic extract of *A. elongata*

Sample concentration (µg/ml)	% of inhibition± SD			
	Root	Stem	Leaves	Standard (Ascorbic acid)
100	51.87±0.05	58.37±0.35	71.8±0.11	73.14±0.03
250	53.19±0.12	61.92±0.27	74.15±0.03	85.2±0.02
500	54.82±0.32	64.65±0.13	77.12±0.15	94.31±0.06
750	59.05±0.02	68.27±0.51	79.19±0.02	99.21±0.03
1000	63.61±0.21	72.46±0.31	82.82±0.17	105.21±0.03

Table 4. Hydrogen peroxide scavenging activity of methanolic extract of *A. elongata*

Sample concentration (µg/ml)	% of inhibition± SD			
	Root	Stem	Leaves	Standard (Ascorbic acid)
100	48.21±0.25	63.20±0.36	75.45±0.10	71.23±0.11
250	51.97±0.15	66.45±0.21	79.52±0.15	75.25±0.16
500	55.43±0.12	70.74±0.17	82.16±0.11	80 ± 0.15
750	58.27±0.31	73.15±0.11	85.21±0.16	87.5±0.51
1000	60.24±0.22	76.42±0.24	88.67±0.21	89.92±0.21

leaves showed significant antioxidant activity in all assays. The result also showed high activity with increasing concentration of the extract (1000 µg/ml).The earlier results of antioxidant potentiality of *A. paniculata by* Singh, R P *et al* (2001), Kader Ali. S H (2011), Vijayakumar and Kalaichelvan (2011), Ramya and Lakshmi devi (2015), Suparna *et al.,* (2014), Nagaraja and Chandrasekhar (2014) correlated with the present findings. The antioxidant activity may be due to the presence of andrographolide (Shirika K and Mastan., 2013) and phenolic compounds (Aliyu., 2009) which need further investigation.

CONCLUSION

The present study explores the promising antioxidant potentiality of *A. elongata* . The methanol extract of leaves of *A.elongata* showed higher antioxidant property by DPPH radical scavenging activity, FRAP (Ferric Reducing Antioxidant Power), Super oxide scavenging activity and Hydrogen peroxide scavenging activity. The findings of the present study suggest that the leaf extract of *A.elongata* act as a potential source of natural antioxidant and since the plant extract is not a pure compound, further investigations are needed for the structural elucidation of the compounds present in the extract.

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In vitro Cytotoxicity Evaluation of Methanolic Extract of *Catharanthus roseus* Linn.

SHIJINA BEEGUM N & P G BIJU*

Department of Biochemistry, University of Kerala, Kariavattom Campus, Kerala, Thiruvananthapuram – 695581, India.
email: pgbiju75@gmail.com.

ABSTRACT

The present study embraces in vitro cytotoxicity evaluation of methanoilc extract of *Catharanthus roseus* leaves. Sequential extraction of leaves using hexane, chloroform and methanol were taken. The extracts were dried and powder was collected for further analysis. Toxicity was evaluated in macrophage cell line system. Cell morphology, MTT assay, LDH Assay and NRU assays were done for a period of 12H. The results indicated that, the extract could not elicit toxic effect on the macrophage cell line system. Altogether the extracts can be used for further studies using macrophage cell line system in the tested concentration.

Key words *Catharanthus roseus, Macrophage and Toxicity.*

Plants have important role in the human society because of its medicinal values. Medicinal plants are the boon of nature against diseases. Elucidating efficacy of plant extract in disease curing is an important field of research in recent years. Numerous plant products with anti-microbial, anti-oxidant, anti-atherosclerotic and anti-parasitic activities have been reported so far. The use of traditional medicine is widespread and plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs. It is commonly accepted that, in a situation of oxidative stress, reactive oxygen species (ROS) are generated. The ROS plays an important role in the pathogenesis of various serious diseases, such as neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis, cataracts, and inflammation (Ethertone*t al*., 2004). Naturally occurring anticancer drugs are mainly isolated from plants like vinblastine and vincristine, isolated from *Catharanthus roseus* (Bozin *et al*., 2006, Pereira *et al*., 2010, Moorthy *et al*., 2010). *C. roseus*is is a tropical flowering plant widely distributed around the world. The plant has long history of medicinal value in traditional medicine. However, proper understanding of toxicity effects in general and particularly on macrophage cell line system are essential before further exploration of their potential medicinal properties. Since little information is available regarding the toxicity of the extract in the cell line system, this study is aimed at checking the toxic effect of the methanolic extract of leaves of *C. roseus* in macrophage cell line system.

MATERIALS AND METHODS

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), LDH and NRU kits were purchased from Sigma Aldrich and Cayman Chemical Company.Fetal Bovine Serum was purchased from Thermo Fisher Scientific. All other chemicals and solvents used were purchased from SRL, Ranbaxy and Merck, India.

Plant material

The leaves of *C. roseus* was collected from the campus of University of Kerala, Kariavattom, Trivandrum (India) and further identified from the Department of botany University of Kerala, where a voucher specimen has been deposited.

Preparation of Extracts and different fractions

1Kg fresh leaves of *C. roseus* plant washed with distilled water so that the adhering dust particles are removed. They were dried in shaded and the dried leaves were powdered and stored in clean containers. 100g of dried powder was sequentially extracted using solvents in the order hexane, chloroform and methanol. All the three extracts were analysed for its solubility in the biological pH,. Since only methanolic fraction (ME) was soluble, it was investigated for toxicity studies.

Cell culture

Macrophage cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune. Cells were grown in DMEM medium supplemented with FBS (100U penicillin/ml and 100 µg streptomycin/mL) and cultured in 5% CO_2 at 37°C. Cells were passaged regularly and subcultured to 80% confluence before the experiments.

Experimental groups were designed as follows:

Group I -Normal Control, Group II- Toxic Control (Cisplatin 5µg), Group III- 25µg ME, Group IV-50µg ME, Group V- 100µg ME.

Morphological studies

The morphological analysis of cell lines were determined by adding different concentrations of samples and observed under a invert microscope (Carl ZEISS) using wet mounted slides at 10X magnification.

Evaluation of cell viability

Cytotoxicity

Cell viability was determined by MTT assay (Mosmann*et al*., 1983). Neutral Red Uptake (NRU) assay provides a quantitative estimation of the number of viable cells in a culture condition (Lasarow*et al*., 1992). The release of LDH in response to samples treatment was monitored by using a LDH cytotoxicity assay kit purchased from Cayman Chemical Company (Hubert *et al*., 2005). The absorbance was read using a plate reader (Bio Rad, USA).

Statistical analysis

All experiments were performed in triplilicates (n = 3). The values were expressed as the mean ± SD. All statistical calculations were carried out with statistical package for social sciences (SPSS) software program.. The data were statistically analyzed using one-way analysis of variance (ANOVA) and significant difference of means was determined using Duncan's multiple range tests at the level of $p < 0.05$ (Steel *et al*., 1997).

RESULTS AND DISCUSSION

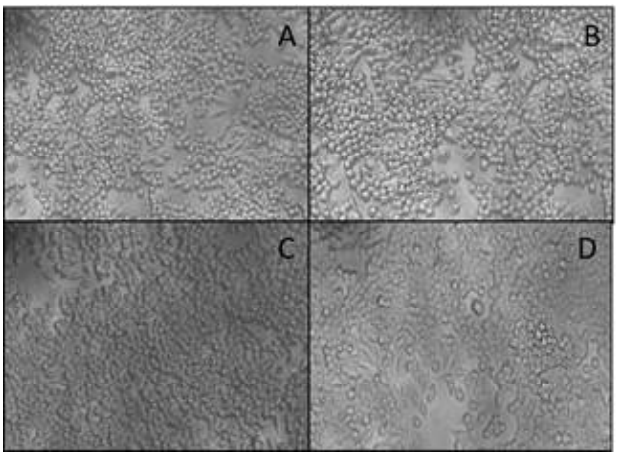

Fig. 1. A-GI, B-GIII, C-GIV, D-GII

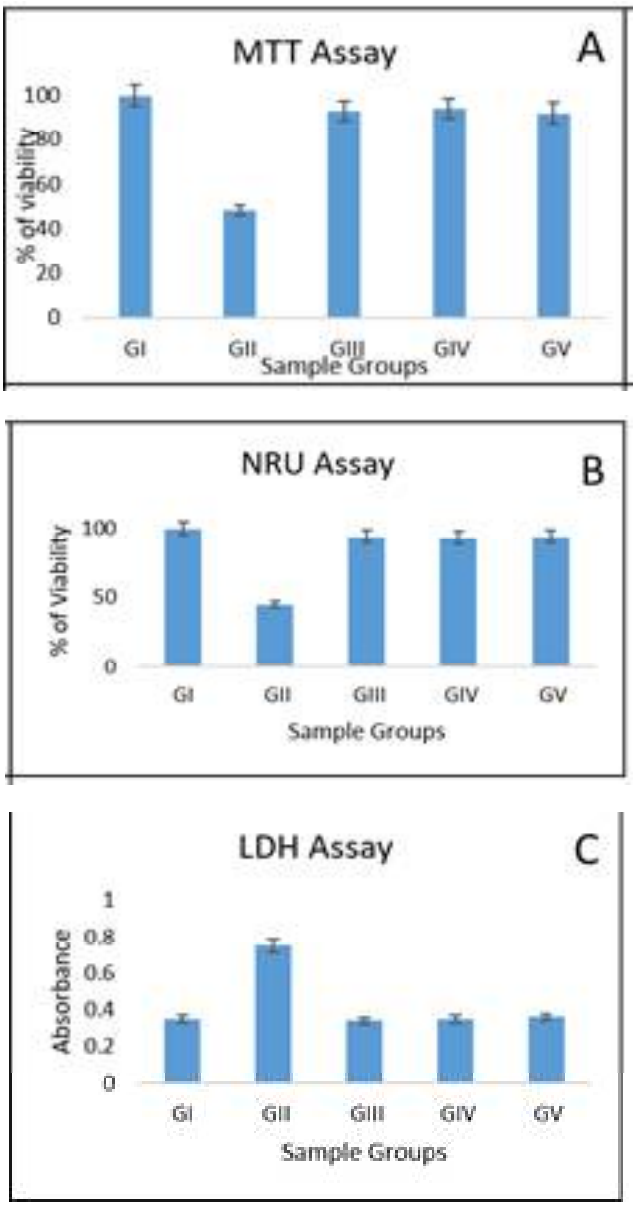

Fig. 2A. MTT Assay, 2B: NRU assay, 2C: LDH assay

RAW cells were treated with different concentrations of plant extract described earlier. Phase contrast microscopic observation of cells after treatment showed that the cells were well spread and there was no distinct change in morphology even after 24 h when compared to negative control. Cells treated with different concentrations of ME were observed to exhibit normal cell morphology (Fig. 2).

In order to estimate the cytotoxicity, the cells were treated with various concentrations of the extract. Results showed that more than 95% of the cells were viable at higher concentration in both MTT assay (Fig. 2A) as well as NRU assay (Fig. 2B) compared to the control. The LDH assay (Fig. 2C)

also showed nontoxic nature of the compound as the enzyme value was very low in comparison with positive control (Fig. 1D). The results showed above 96% cell viability at the highest tested concentration number and at maximum time exposure.

MTT, LDH and NRU results confirmed that the plant extract demonstrated a very low rate of cell death. Previous reports have shown that *C. roseus* extract is non-toxic to living system in a dose dependent manner (Vutukuri *et al*., 2017). The cell morphology analysis and cytotoxity parameters support these reports. The overall results show that the plant extract was safe and can be used for further analysis in the cell line system.

CONCLUSION

The study evaluated the toxicity of the extract in macrophage cells. The Cell morphology analysis reports nontoxic nature of the extract at highest tested concentration. The MTT, LDH and NRU assays also conform withthe previous results. Altogether the study could elucidate the nontoxic nature of the compound at tested concentration and hence it can be used for further biochemical studies using macrophage cell line system.

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Nutrient Characteristics of Groundwater Sources in the Vicinity of Coir Retting Areas in Kadinamkulam Estuary, South India

SONU SASIDHARAN[1] AND JAYA D S[2]

Department of Environmental Sciences, University of Kerala, Kariavattom Campus P.O., Thiruvananthapuram , Kerala.
email: sonuulloor@gmail.com

ABSTRACT

The present study was conducted in the dug wells in the coastal tract of Kadinamkulam estuary. Kadinamkulam lake the major coir-retting area in South Kerala. Retting of coconut husk is basically a biological process involving the release of a verity of biochemical compounds and also produces organic wastes and hydrogen sulphide. The major objective of the study was to assess the variation of nutrient content in ground water samples. For this an extensive field survey was conducted in the study area. The water samples were collected from selected dug wells at different stations in ward 14 near Railway station of Azhoor Grama Panchayath and from the selected stations of Kadinamkulam estuary during monsoon season (June and July 2011) and the concentration of major nutrients were estimated using standard procedures. The nutrient minerals such as calcium, magnesium, sodium, potassium, nitrate, sulphate, phosphate and chloride were analyzed. The results revealed that the phosphate, magnesium and calcium content in wells adjacent to the Kadinamkulam estuary exceeded the permissible limits for these nutrients prescribed by BIS (1991) and WHO Guidelines for Drinking water quality. Therefore the study concludes that the groundwater sources in this area are getting contaminated due to the leaching of pollutants from Kadinamkulam estuary.

Key words *Azhoor Grama Panchayath; coir-retting activity; Kadinamkulam estuary; physico-chemical quality.*

Population explosion together with urbanization and industrialization has created innumerable ecological problems including environmental pollution. Water is an essential requisite for all living organisms. Studies show that no water body is free from pollution (Ahluwalia and Sunitha, 2009). Among the common pollutants that degrade water quality, industrial effluents play a leading role. The chemical composition of ground water is controlled by several factors that include composition of precipitation, anthropogenic activities, geological structure and mineralogy of the watersheds, aquifers and geological process within the aquifer medium (Andre *et al.,* 2005). Water pollution is significant only when it influence living or biological system either directly or indirectly. The presence of toxic pollutants in aquatic ecosystems poses a serious threat to environmental health. Industrialization and growth of human population have led to progressive deterioration in the quality of the earth's environment. Urban, agricultural and industrial activities release xenobiotic compounds that may pollute the aquatic habitat. Schwrzenbach *et al*., (2006) reported that about 300 million tone of synthetic compounds seep annually into water system (rivers, lakes and sea).

Coconut husk retting is the basic process involved in the manufacture of coir. The small scale industry practiced in the backwaters lead to deterioration of water quality. Retting is a biological process that involves the pectinolytic activities of micro-organisms especially bacteria and fungi present in the retting ground that liberate large quantities of organic substance into the medium (Jayasankar *et al*.,1985). Retting of coconut husk present unique and extremely serious problems along the coastal belt, changing the hydro-ecology of the water body. Traditional and conventional method of retting has adverse impact on the ecosystem, including fauna, flora and human being. Lack of dissolved oxygen, very high biological oxygen demand, chloride, hardness, nutrients and low pH with foul smell of hydrogen sulphide is the characteristic features of the retting yard. A vast number of rural people are engaged in retting industry. Hence job related disease likely to occur among these people like filariasis, eye dieses, skin dieses, oedema of lungs and headache due to the inhalation of poisonous gases (Nirmal *et al*., 2002). Review of literature show that no detailed study was conducted to assess the nutrient mineral status in groundwater in the coastal tract of Kadinamkulam estuary. The major objective of the present study was to assess the status of nutrient minerals in the ground water samples of the selected ward in the Azhoor Gramapanchayath near Kadinamkulam estuary.

MATERIALS AND METHODS

Study area

Azhoor is a coastal village in Thiruvananthapuram district in the state of Kerala, India. The location map of the study area is given in Fig. 1. Kadinamkulam Lake (Lat 8^{o} 35' to 8^{o} 40' N

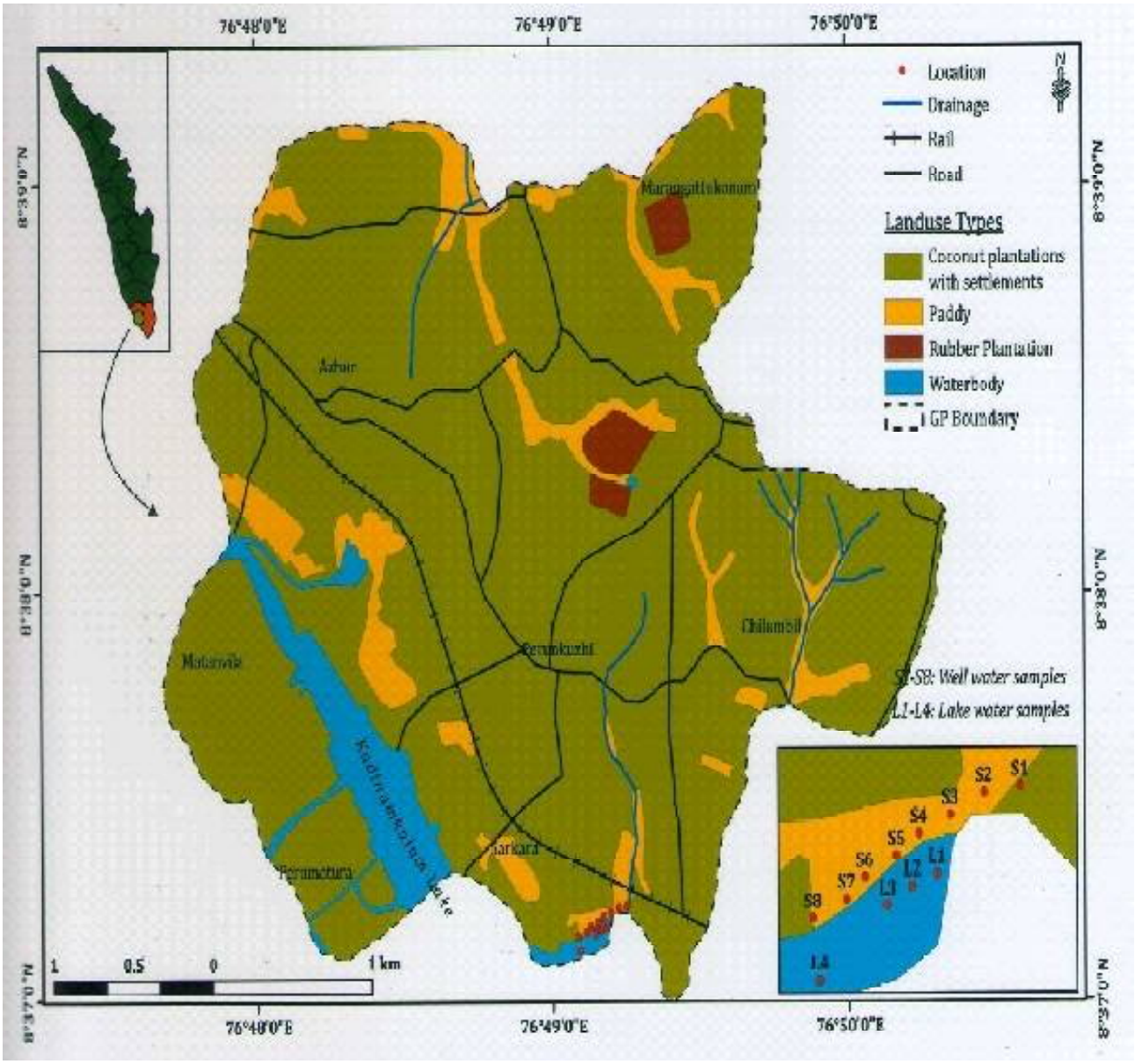

Fig. 1. Location map of Study Area (Azhoor Grama Panchayat)

and Long76° 45to 76°52'E) in the Thiruvananthapuram district, one of the major retting zones dotting the coastal belt of Kerala. Kadinamkulam estuary is connected to the Veli Lake on the south by the Parvathyputhanar canal and to the Anchuthengu estuary on the north through the lower reaches of the Vamanapuram river. This temporary estuary has no direct connection with the Arabian sea, but seasonally it becomes connected through the opening of the sand bar at Perumathura. A detailed survey was conducted in the ward 14, near Railway station of Azhoor Grama Panchayat. For the study eight dug wells were selected (S_1- S_8) in this ward for the study. And surface water samples from four different sites of the Kadinamkulam estuary near Azhoor Grama Panchayat were also collected.

Sample collection

Water samples were collected from the selected eight dug wells and four sites of Kadinamkulam lake during the month of June and July 2011. The samples were collected in clean and dried two litre plastic bottles for analysis. The collection was done between 7.30 am to 9.30 am every month. The dug well samples were analysed to determine the concentration of various nutrient minerals such as nitrates, phosphates, sulphates, chlorides, calcium, magnesium, sodium and potassium according to the standard procedures described in APHA (1995), Trivedi and Goel (1986) and Saxena (1998). The nitrates, phosphates, sulphates were determined by spectrophotometric method and chlorides by argentometric titration method. Calcium and magnesium were measured by complexometric

Table 1. Concentration of Nutrient minerals in Groundwater - June 2011

Parameters	Stations								Average
	S1	S2	S3	S4	S5	S6	S7	S8	
Chloride (mg/L)	106.42	141.9	53.12	159.55	81.55	88.86	124.16	63.85	102.43
Calcium (mg/L)	56.77	75.69	12.61	100.92	12.61	18.92	21.02	21.02	39.95
Magnesium (mg/L)	23.96	27.89	22.54	35.15	6.68	10.02	9.51	10.37	18.27
Nitrate (mg/L)	0.21	0.21	0.27	0.19	0.10	0.17	0.14	0.13	0.18
Sulphate (mg/L)	23.45	5.85	13	64.95	27.25	24.42	32.12	19.29	26.29
Phosphate (mg/L)	0.31	0.43	0.52	0.27	0.25	0.31	0.26	0.32	0.33
Sodium (mg/L)	51.9	84.6	8.7	65.5	6.6	42.7	75.3	25.7	49.06
Potassium (mg/L)	2.0	2.1	2.5	1.8	38.1	1.8	6.9	2.1	3.23

titration. Sodium and potassium content in water samples were estimated by flame photometric method.

RESULTS AND DISCUSSION

The results of various nutrient minerals in the groundwater samples are given in Table 1, 2 and in lake water samples in 3 and 4 respectively.

Nitrates

The concentration of the nitrates in dug wells were recorded in the range of 0.10 mg/L- 0.27 mg/L, 0.16 mg/L - 0.28mg/L during June and July respectively. In normal groundwater, nitrate concentration is typically below 2mg/L and concentrations significantly above this are generally regarded as anthropogenic source (Schivanna, 2008). The desirable limit of nitrates in drinking water is 45 mg/L as per BIS. In this study all well water samples analysed showed the nitrate content within the desirable limit of drinking water standards of BIS (1991). In lake water values ranged from 1.12 mg/L to 2.15 mg/L and 1.82 mg/L to 2.10 mg/L. The consumption of nitrate rich water by young children may give rise to a condition known as methaemoglobinaemia or blue baby syndrome (Kiely, 1997).

Sulphates

In the present study the sulphate content in the well water samples collected in the month of June varied from 5.84mg/L to 64.95 mg/L, and in July the sulphate content varied from 8.46mg/L to 70.79 mg/L. The desirable limit of sulphate in drinking water prescribed by WHO standards is 200 mg/L. Therefore the sulphate content in all the well waters studies are within desirable limits of drinking water quality standards. Sulphate content in lake water varied from 0.30 mg/L to 0.71 mg/L and 0.25 mg/L to 0.66 mg/L during June and July respectively.

Table 2. Concentration of Nutrient minerals in Groundwater -July 2011

Parameters	Stations								Average
	S1	S2	S3	S4	S5	S6	S7	S8	
Chloride (mg/L)	113.52	141.9	49.66	138.35	85.14	92.23	127.71	67.40	101.99
Calcium (mg/L)	65.18	79.89	25.23	103.02	29.43	37.84	27.33	33.64	50.20
Magnesium (mg/L)	31.67	31.74	31.66	38.30	7.45	12.72	16.51	13.75	22.98
Nitrate (mg/L)	0.24	0.28	0.21	0.19	0.18	0.19	0.17	0.16	0.20
Sulphate (mg/L)	25.30	8.46	17.34	70.79	18.76	26.54	28.76	19.64	26.95
Phosphate (mg/L)	0.33	0.41	0.53	0.25	0.27	0.41	0.28	0.20	0.34
Sodium (mg/L)	50.1	79.8	65.9	37.1	42.1	73.4	62.5	20.5	53.93
Potassium (mg/L)	2.1	2.8	2.6	1.8	6.9	2.1	2.9	2.1	2.91

Table 3. Concentration of Nutrient minerals in lake water -June 2011

Parameters	Stations				Average
	L1	L2	L3	L4	
Chloride (mg/L)	3263	318.5	326.5	365.5	334.13
Calcium (mg/L)	23.2	24.5	22.28	21.5	22.87
Magnesium (mg/L)	16.34	14.08	14.24	17.15	15.46
Nitrate (mg/L)	1.92	2.13	2.15	1.12	1.83
Sulphate (mg/L)	0.65	0.71	0.68	0.30	0.585
Phosphate (mg/L)	0.32	0.28	0.43	0.20	0.307
Sodium (mg/L)	17.5	18.2	16.5	14.2	16.6
Potassium (mg/L)	13.8	16.8	15.9	12.1	14.65

Phosphates

The main abiotic source of phosphorus is the igneous rocks whereas the biotic sources of phosphate concentration in freshwater are human wastes and the release of laundry detergents (Kumar, 2002). In the present study high phosphate values were recorded in the well water samples from station S3 during June (0.52 mg/L) and July (0.53mg/L). During the month of July S1, S2, S3 and S6 showed the phosphate value above the limit of drinking water standard (BIS,1991). It may be due to the leaching of effluents containing phosphates from the coir retting stations and domestic waste water into the groundwater. The average value of phosphate in lake water in June was 0.31 mg/L and in July was 0.29 mg/L. Higher concentration of phosphate is indicative of pollution and the major source of anthropogenic phosphorus is sewage, detergents, agricultural effluents and fertilizers (Panday *et al.,* 1979; Sinha *et al.,* 2000). The principal sources of phosphorus to groundwater systems include overlying soils, dissolution of minerals that contain phosphate in aquifer sediments, agricultural fertilizer, animal waste, and leaking septic systems or infiltration of wastewater (Fuhrer et al. 1999)According to BIS (1991) the permissible limit of phosphates in drinking water is 0.3 mg/L.

Chlorides

Most of the chlorides in groundwater is present as sodium chloride, but the chloride content may exceed the sodium concentration due to base exchange phenomena (Karanth, 1987). In the present study, chloride value in ground water samples ranged from 63.85 to 159.55 mg/L during June, and from 67.40 to 138.35 mg/L in July respectively. The permissible limit of chlorides in drinking water as prescribed by ISI (10500) is 250 mg/L, and the values recorded in all the wells in the study area are within the permissible limits. The average value of chloride content in lake water sample during June was 334.13 mg/L, and in

Table 4. Concentration of Nutrient minerals in lake water -July 2011

Parameters	Stations				Average
	L1	L2	L3	L4	
Chloride (mg/L)	312.6	297.7	293	325.6	305.98
Calcium (mg/L)	22.3	24.5	20.48	21.05	22.08
Magnesium (mg/L)	15.38	14.02	17.44	13.12	14.99
Nitrate (mg/L)	1.82	2.10	2.04	1.91	1.97
Sulphate (mg/L)	0.61	0.66	0.62	0.25	0.54
Phosphate (mg/L)	0.24	0.26	0.42	0.22	0.28
Sodium (mg/L)	18.7	16.4	16.7	12.7	16.12
Potassium (mg/L)	13.6	15.8	15.7	13.5	14.65

July the chloride content was 305.98 mg/L. In natural waters, chloride in water results from the leaching of chloride containing rocks and soils with which the water comes in contact. Discharge of agricultural, industrial and domestic waste waters could also be a source of chlorides in natural waters. Human excreta contain about 6 gms of chloride per person per day (Abbasi, 1998). The chloride content normally increases as the mineral content increases.

Calcium

In present study the calcium content in ground water samples ranged from 12.61mg/L to 100.92 mg/L, with an average value of 39.95 mg/L in June, and the calcium content ranged from 25.23 mg/L to 103.02 mg/L with an average value 50.19 mg/L during July. The value of calcium content in well water samples are within the desirable limit of drinking water standards except in stations, S2 and S4 in the month of June. And on July, calcium content in majority of well water analysed are within the desirable limit except in stations S2 and S4, and values were recorded as 79.89 mg/L and 103.02 mg/L respectively. In this study calcium content in lake water samples ranged from 21.5 mg/L to 24.5 mg/L, and 20.48 mg/L to 24.5 mg/L during June and July respectively. Calcium is the second major constituent, after bicarbonate, present in most natural waters, within the concentration range between 10 and 100 mg/L. It is a primary constituent of water hardness, and the calcium level between 40 and 100mg/L are generally considered as hard to very hard. According to WHO (1994), the desirable amount of calcium in drinking water is 75 mg/L. Excess calcium in our body can cause gallstones, kidney stones, bone and joint calcification, arthritis, and hardening and blocking our arteries (WHO, 2011).

Magnesium

Magnesium salts are more soluble than calcium, but they are less abundant in geological formations. In this study, the magnesium content in the water samples collected from different sampling stations ranged from 9.51 mg/L to 35.15 mg/L during the month of June, and in July it ranged from 7.45mg/L to 38.30 mg/L. The desirable limit of magnesium in drinking water is 30 mg/L (BIS, 1991). It was found that the magnesium level in ground water samples in the study area were above the desire limits of drinking water quality. In June, S4 samples recorded high value (35.15 mg/L) and in July the ground water samples from S1, S2, S3, S4 recorded Mg content above the permissible limit of drinking water standards. The average value of magnesium content in lake water was 15.46 mg/L and 14.99 mg/L during the months of June and July respectively.

Potassium

In the present study the average value of potassium content in well water during June was 3.25mg/L and in July was 2.91mg/L. In all ground water samples from the different stations recorded potassium values below the standard permissible limits of drinking water standards (12 mg/L). Most potable groundwater contains less than 10 ppm and commonly ranges between 1.0 and 5.0 ppm (Ramakrishnan, 1998). All natural water contains measurable amounts of potassium. The study showed that in lake water the concentration of potassium ranged from 12.1 mg/L to 16.8 mg/L.

Sodium

The sodium content in ground water samples analysed varied from 8.7 mg/L to 84.6mg/L, and 20.5 mg/L to 79.8mg/L during June and July respectively. The permissible limit for sodium in drinking water is 200 mg/L (WHO, 1994). In the present study all ground water samples collected from the different stations recorded sodium values below the standard permissible limits. In lake water samples sodium content varied from 14.2mg/L to 18.2 mg/L and 12.7 mg/L to 18.7 mg/L during the month of June and July respectively.

CONCLUSION

Nutrient minerals in the groundwater in the vicinity of coir retting areas of Kadinamkulam estuary were analysed during the month of June and July 2011. In the study period the nutrient concentration in majority of the ground water samples are within the permissible limts. The phosphate, magnesium and calcium content in ground water are found above the drinking water standard limits and this may be due leaching of effluents from the coir retting activity in the Kadinamkulam lake and due to disposal of waste water into the surroundings. Therefore effective waste management measures should be undertaken by the Public Health Officials to improve the quality of groundwater in this residential area. Awareness programmers also should be conducted among the residents to do periodic cleaning of wells and surroundings.

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Amino Acid and Gaba Composition of the Seeds of Different Seed Morphoforms of *Dolichos biflorus* L.

S.G. SREELEKSHMI[1,2], SR. AVITA[3] AND K.MURUGAN[4]

[1,3]Department of Botany, St.Teresa's College,Cochi-695002,Kerala;
[2]SCMS Institute of Research and Institute, Kochi, 68203, Kerala;
[4]Plant Biochemistry and Molecular biology Lab, Department of Botany, University College, Thiruvananthapuram, Kerala, India.
email : sreelekshmi80@gmail.com

ABSTRACT

The variability of amino acid levels including gamma-amino butyric acid (GABA) was investigated in three seed morphoforms of horse gram - *Dolichos biflorus* L.: black, cream and brown. Seed morphoforms varied considerably in the amounts of different amino acids. Among them, the cultivar black recorded the highest amounts of six different amino acids and with the content of other amino acids at a satisfactory level. The cultivar cream contained the highest amount of GABA. Meanwhile, the brown morphoform have significantly high levels of only two amino acids. These results demonstrated that horse gram seed morphoforms varied in the amount of different amino acids and GABA and hence have a high potential as a good source of amino acids and GABA in the development of food supplements.

Key words *Amino acid, cultivar, gamma-amino butyric acid, GABA, Dolichos biflorus L.*

Plant proteins provide nearly 65% of the world supply of proteins for humans with 45% – 50% and 10 – 15% from cereals and legumes or vegetables, respectively(Mahe*et al*., 1994).Importance of plant proteins in the average diet varies from the least developed regions to the highly developed regions. Nonetheless there is now an expanding consumption of protein foods of legume and vegetable origin throughout the world. Among the plant species, grain legumes are considered as the major source of dietary proteins. Protein quality in leguminous seeds does not however reach the same level as in animal products. This is due to various factors, among them the well known are the unbalanced amino acid composition, the low true digestibility of protein and the presence of antinutritional factors in the seeds (Norton*et al*., 1985).Characteristically, grain legume seeds have large protein contents, ranging from 20% to as much as 40% of their dry matter, according to species, genotypes within species, and environments(Baudoin, 1991). Storage proteins in legume seeds are mainly located in the cotyledonary tissues. Embryonic axis and test as contribute little to the total seed protein content, mainly because those components represent small proportions of the seed mass.

The major storage proteins in legume seeds are the globulins which usually account for about 70% of the total protein. Glutelins (10–20%) and albumins (10–20%) make up the remainder. The principal storage globulins in most legumes are legumin and vicilin, the latter predominating in common bean(Jansman, 1996). Amino acid profiles of proteins in leguminous seeds are unbalanced. When compared to egg protein, the indispensable sulphur-containing amino acids are at a much lower concentration[1]. Sulphur containing amino acids (i.e. methionine and cystine) are considered as the most critical limiting components of the proteins. The various legume protein sources may differ significantly in the amino acid composition. Compared to soya bean protein, the lysine content in pulses tends to be higher and the content of sulphur containing amino acids (methionine and cystine), and tryptophan tends to be lower(Jansman, 1996). Difference in amino acid composition is also observed among the protein fractions of the seeds. Globulins are relatively poor insulphur-amino acids. Albumins are richer in sulphur-amino acids and other essential amino acids (such as lysine) than globulins. In addition, some antinutritional factors, mainly protease inhibitors, have shown a relatively fair content in cystine and methionine. For example, in common beans between 30 to 40% of the total seed cystine have been calculated to be present in these inhibitors. The objectives of this study were to conduct a comparative analysis of amino acid and GABA content of three forms of horse gram form Kerala.

MATERIALS AND METHOD

Freshly harvested sun-dried seeds of the black,

Table 1. Meandata on the amino acid analysis of the three seed morphoforms of Horse gram

Amino Acids	Black	Cream	Brown	F-Value	P-Value
Aspartic Acid	12.3a ±0.04	12.60b ±0.07	12.42c ±0.03	18.847	0.0026
Threonine	4.61a ±0.04	4.32b ±0.03	4.41b ±0.03	63.815	<0.0001
Serine	9.02a ±0.02	8.57ab ±0.1	8.19b ±0.07	89.507	<0.0001
Glutamic Acid	17.73a ±0.01	18.55ab ±0.01	18.32ab ±0.01	54.768	<0.0001
Proline	1.12a ±0.06	0.73b ±0.01	0.77b ±0.02	84.163	<0.0001
Glycine	8.84a ±0.04	7.57b ±0.05	7.7b ±0.01	874.80	<0.0001
Alanine	6.81b ±0.01	6.84b ±0.01	7.09a ±0.005	934.43	<0.0001
Valine	6.37a ±0.01	6.33b ±0.01	6.56c ±0.08	18.120	0.0029
Methionine	1.33b ±0.005	1.34b ±0.005	1.22a ±0.01	241.80	<0.0001
Isoleucine	4.76a ±0.1	4.26b ±0.06	4.55c ±0.02	36.067	0.0005
Lysine	7.2b±0.02	7.6b±0.10	7.0b±0.05	20.273	0.0021
Tyrosine	1.55b±0.01	1.55b±0.01	1.40a±0.02	92.190	<0.0001
Argenine	2.45a±0.02	2.78b±0.01	2.28c±0.03	346.02	<0.0001
Leucine	9.3b±0.01	9.4b±0.01	8.48a±0.02	2776	<0.0001
Phenyl alanine	7.86a±0.01	7.98b±0.01	7.78c±0.01	204.08	<0.0001
Histidine	3.04a±0.01	2.98ab±0.01	2.87b±0.01	165.31	<0.0001
Tryptophan	1.18a±0.01	1.78b±0.01	2.27c±0.06	11506	<0.0001

(^{abc} =Means in the same column with different superscripts differ significantly (P<0.05) ±—standard deviation.)

cream and brown varieties of Horse gram (*Dolichos biflorus* L.) were obtained from the Agricultural University, Kerala. On the basis of seed colour, they were then grouped into three morphoforms, viz. black, cream and brown. The present investigations were carried out using the seeds raised from pure breeding plants. Kulatha or Horse gram (*Dolichos biflorus* Linn.) is a very hardy crop and also has medicinal utility. It is the "poor man's pulse crop" and one of the hardiest among the legume crops. It is the cheapest source of dietary proteins, amino acids Vitamins, Ca^{2+}, Fe, and rich source of ureases. It is a slender, sub erect low growing common twining creeper, native of most parts of India.

Free amino acid extraction

Amino acids were extracted from freeze-dried seed powder (1 g) with 30 ml of 70% ethanol at 80°C for 20 min. After evaporating ethanol, residue water phase (30 ml) was mixed with ethyl ether (30 ml) in a separation funnel. The water phase was separated and freeze-dried. The extract was resuspended in 3 ml of 0.02 N HCl and filtered using a 0.45 µm syringe filter. The extraction procedure was replicated three times.

Amino acid profiling by RP-HPLC method

In the present investigation the detection and separation of the amino acid composition in the seeds of the three morphoforms of *Dolichos biflorus* L. was made by reverse phase high-performance liquid chromatography (RP-HPLC) method(Ishida*et al.*, 1981).The general procedure was using pre-column derivatizationdansyl chloride (5-dimethylamino-1-naphthalene sulphonyl chloride), producing fluorescent dansyl derivatives that are separated by a reversed

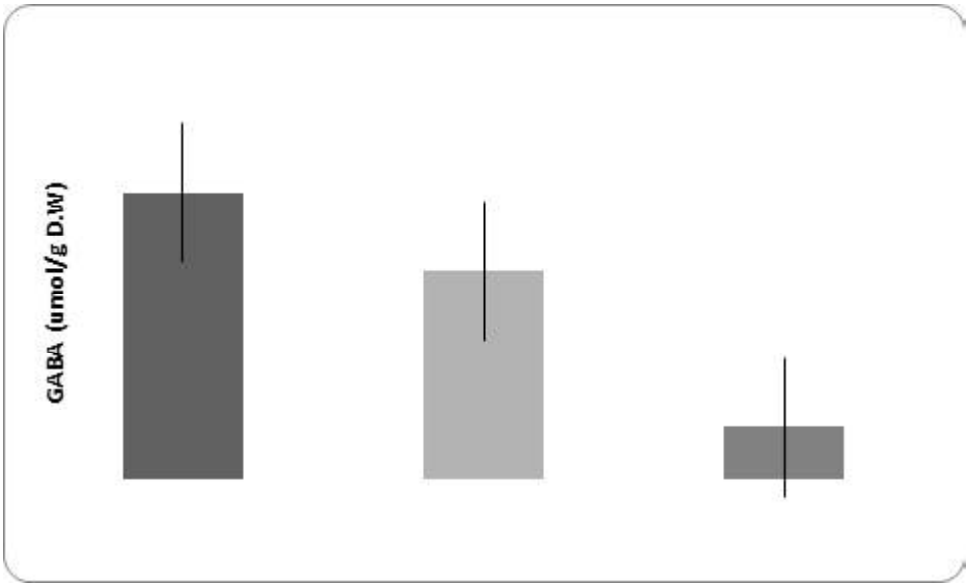

Fig. 1. GABA content in the seeds of different morphoforms of D.*biflorus* L. (The unit of GABA content = umol/g dry weight

phase column chromatographic procedure. The column employs silica gel with attached non-polar hydrocarbon functional groups (e.g., octadecyl moieties) as the stationary phase and uses a multi-step non-linear elusion procedure. Among other eluents, aceto nitrile and water mixtures had been suggested for the separation of dansylated amino acids. These were then detected and measured by a fluorescence detector

The samples were introduced in small volume to the stream of mobile phase. The three seed samples motion through the column was slowed by specific chemical or physical interactions with the stationary phase as it traverses the length of the column The time at which the seed samples elutes (comes out of the end of the column) were called the retention time; the retention time under particular conditions was considered a reasonably unique identifying characteristic of the seed samples.

RESULTS AND DISCUSSION

From the analysis of different seed morphoforms of horse gram collected from Agricultural University, Kerala, seventeen different amino acids were found to be in varying proportions (Table 1). The range of detected amino acid content varied widely with the type of morphoforms tested. Among all the amino acids isolated, glutamate was found to be in remarkably high quantities, while the limiting amino acids were proline, tryptophan, tyrosine and methionine. One of the important amino acids found in large amounts irrespective of morphoform type is glutamate. The mean concentration of glutamate was between 17.7 and 18.5 g/ 16g- N, which was several times significantly higher than any other amino acid found in this study. The amount of each amino acid varied significantly within a particular morphoform. Among the seed morphoforms black variety contained the highest amount of six different amino acids, that is, glycine, isoleucine, threonine, serine, proline and tyrosine. Besides these six amino acids, the same morphoform contained a satisfactory amount of other amino acids also. The cream morphoform contained the highest amount of aspartic acid (16.6 g/ 16g-) and glutamates (18.6 g/ 16g-N) while the amino acids such as proline, threonine, valine and isoleucine represents the lower amounts. The brown morphoform performed the best in terms of two different amino acids, that is, valine and tryptophan whereas it contained the lowest amounts of leucine and arginine.The results obtained was statistically significant.

The seed morphoforms exhibited significant variability in their GABA content, varying between 3.5 - 20 umol/g D.W. (Fig 1). The cream morphoform contained the highest amount (19.3 umol/g D.W.) of GABA followed by the black (14.0 umol/g D.W.) which was around four times more than the other

morphoform, brown ($P<0.001$). The seed morphoforms brown contained as low as 3.5 umol GABA /g dry weight. This work confirms the report on the beneficial effect of vegetable protein (Awadalkareem*et al*., 2008).

Horse gram contained seventeen amino acids at satisfactory levels, except tryptophan and methionine. Among them, the amount of glutamate (18.5 g/ 16g-N), aspartate (12.6 g/ 16g- N) and GABA (19.3 umol/ g D.W.) were quite high irrespective of the type of cultivar. As this pulse contained almost all of the amino acids in satisfactory amounts, especially with regard to gluatmate, aspartate, serine and GABA, which was in high, this crop could be a very good source in the production of gluatmate, aspartate, serine and GABA mean while the other amino acids also found in moderate levels. Thus the medicinal herb *Dolichos biflorus* L. contained amino acids in appropriate quantities that can serve as supplementary potential sources of essential amino acids.

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Ameliorative Potentials of Plant-Derived Phytochemicals against Arthritis

S.G. SREELEKSHMI[1], G.S. MANOJ[2], BOSCO LAWARENCE[3] AND K. MURUGAN[4]

[1]*SCMS, Institute of Bioscience & Biotechnology Research & Development Kalamassery,*
[2]*Department of Botany, NSS College, Nilamel*
[3,4]*Department of Botany, Govt. Arts College, Thiruvananthapuram, Kerala*
email : sreelekshmi80@gmail.com

ABSTRACT

Traditional herbals are practiced from time immemorial for the treatment of arthritis especially in developing countries. Plants inhabiting across the countries especially India, China, Korea and Nigeria were traditionally used by different tribes/ethnic groups for ameliorating arthritis. Herbals/their parts/crude extracts/ polyherbal combinations against arthritis have been accounted reviewed in the study. Plants such as Matricaria chamomilla, Cichorium intybus, Rhododendron campanulatum, Plumbago rosea, Salvadora oleoides, Acacia polyantha were being used traditionally to ameliorate painful inflammatory conditions but not fully explored experimentally in the treatment of arthritis. 124 plant species are documented in the present study as tribal knowledge against arthritis. Similarly, polyphenols are the major phytochemical reported in most of the species. Even though, plant extracts or individual phytochemicals derived from herbals exhibit remarkable potential but the underlying biochemical or molecular mechanism has not been properly elucidated. There is an urgent need to screen the active molecule of these herbals as potential agents and monitor the biosafety of these novel constituents.

Key words *Herbals; phytochemicals; arthriti;, tribal knowledge; polyphenols*

Arthritis is the inflammation in the joints which leads problem in moving around. It can lead to stiffness, pain, swelling and redness in the joints. There are diverse types of arthritis such as osteoarthritis, rheumatoid arthritis, infectious arthritis, gout and psoriatic arthritis. Generally, it is considered as a part of aging posed disease however it occur to others also. The major reasons for the incidence of this disease were injury, autoimmune i.e., immune system starts attacking its own cells and tissues, external infection and excess uric acid accumulation.

Herbals are employed for the treatment of many ailments from historical periods and herbal drugs use is as old as mankind. Herbal drugs are synthesized from ancient system of medicine by the traditional practitioners. Nowadays, pharmacologists shows much interest in those novel drugs derived from herbals because of their safety and less expensive. Nature has empowered with wealth of herbals and is widely distributed all over the world as source of therapeutic drugs for the prevention and cure of many diseases. According to WHO, world's 80% population use plant based drugs for their primary health needs. The medicinally important parts of these herbals are phytochemical constituents that produce a desired physiological reaction on the body and there by curative action.

MATERIAL AND METHODS

The method of screening the data for this work includes those plants: (i) Which are native to India, (ii) used in traditional systems and in various polyherbal preparations, (iii) with reported anti-arthritic activity, (iv) appropriate dosage, (v) mechanism of action, (vi) safety profile, and (vii) models used. Plants/their parts/ extracts used traditionally in acute rheumatic attacks, chronic analgesia, and chronic rheumatism have been considered as anti-arthritic agents.

RESULTS AND DISCUSSION

141 plant species are identified by local interview and questionnaire analysis used by the Kani tribals from Kerala used for curing arthritis. They are listed in the table 1 in terms of binomial, family and parts used.

Major Phytochemicals

Flavonoids

The mode of action of the flavonoids with antiarthritic activity are narrated below:

6-shogaol is identified from the ginger rhizome. 6-Shogaol treatment reduced the concentration of soluble vascular cell adhesion molecule-1 (VCAM-1) in the blood, as well as the infiltration of leukocytes, lymphocytes, and monocytes/macrophages into the synovial cavity of the knee joint [1]. 6-Shogaol also protected morphological integrity of the cartilage lining the femur in CFA-induced monoarthritis of the knee

Table 1. Major plants used in traditional Ayurveda medicine are enlisted

Binomial	Family	Parts used
Acacia polyantha	Mimosaceae	Heart wood
Achillea millefolium Linn.	Compositae	Plant
Adenanthera pavonina Linn.	Mimosaceae	Leaves
Alangium lamarkii Thwaites.	Alangiaceae	Leaves
Alangium salviifolium Wang	Alangiaceae	Roots
Alpinia galanga (L.) Willd.	Zingiberaceae	Rhizomes
Apium graveolens Linn.	Umbelliferae	Seeds
Azima tetracantha Lam.	Salvadoraceae	Root bark, leaves
Barleria courtallica Nees.	Acanthaceae	Roots
Barleria cristata Linn.	Acanthaceae	Roots
Bassia latifolia Roxb.	Sapotaceae	Bark
Bassia longifolia Linn.	Sapotaceae	Bark
Bauhinia racemosa Lam.	Fabaceae	Bark
Bauhinia tomentosa Linn.	Fabaceae	Leaf
Bula alba Linn	Cupuliferae	Bark
Boerhaavia diffusa Linn.	Nyctagineae	Roots
Caesalpinia sappan Linn.	Caesalpiniaceae	Wood
Carthamus tinctorius Linn.	Compositae	Seeds oil
Cassia fistula Linn.	Caesalpiniaceae	Leaves and fruits
Cassia fistula Linn.	Caesalpiniaceae	Fruits
Cichorium intybus Linn.	Compositae	Root
Cinchona officinalis Linn.	Rubiaceae	Bark
Citrullus colocynthis (L.) Schrad.	Cucurbitaceae	Roots
Clerodendron colebrookianum Walp.	Verbenaceae	Roots
Clerodendron inerme Gaertn.	Verbenaceae	Roots
Clerodendron phlomides L.F.	Verbenaceae	Leaves
Clerodendron serratum Spreng.	Verbenaceae	Roots
Clerodendron siphonanthus R.Br.	Verbenaceae	Roots
Clitoria ternatea Linn.	Fabaceae	Roots
Commiphora myrrha (Nees) Engl.	Burseraceae	Gum
*Commiphora wightii (arn.)*Bhandari	Burseraceae	Leaves
Corallocarpus epigeous (Rottler) Hook.f.	Cucurbitaceae	Plant
Cordia dichotoma G.Forst.	Boraginaceae	Fruits
Cotula anthemoides Lour.	Compositae	Plant oil
Dastica cannabina Linn.	Datiscaceae	Root
Delonix elanta (L.) Gamble	Caesalpiniaceae	Plant
Dichrostachys cinerea Wight &Arn.	Mimosaceae	Root
Diospyros candolleana Wight	Ebenaceae	Bark
Diospyros paniculata Dalzell	Ebenaceae	Bark
Drynaria quercifolia (Linn.)	Polypodiaceae	Rhizomes

Binomial	Family	Parts used
Echinopsechinatus	Compositae	Plant
Eclipta prostrata Linn.	Asteraceae	Roots, Leaves
Elaecarpus obolongus Gaertn	Tiliaceae	Flower
Elaeocarpus serratus Linn.	Tiliaceae	Leaves
Euphorbia antiquorum Linn.	Euphorbiaceae	Juice
Eugenia operculata Roxb.	Myrtaceae	Fruit
*Eugenia spicata*Lam.	Myrtaceae	Plant
Euphorbia ligularia Roxb.	Euphorbiaceae	Whole plant
Ferula narthex Boiss.	Umbelliferae	Stem, leaves
Ficus benghalensis Linn.	Moraceae	Aerial root, bark, leaves, buds, fruits, latex
Flacourtia jangomas Rausch.	Flacourtiaceae	Fruits
Fritillaria roylei Hook. F.	Liliaceae	Bulbs
Glycosmis arborea(Roxb.)Dc.	Rutaceae	Whole plant
Gossypium herbaceum Linn.	Malvaceae	Leaves
Grangea maderaspatana (L.) Poir.	Compositae	Root
*Heliotropium indicumn*Linn.	Boraginaceae	Whole plant
Hemidesmus indicus (Linn.) R.br.	Asclepiadaceae	Roots
Hiptage benghalensis (Linn.) Kurz	Malpighiaceae	Bark, leaves, flowers
Holarrhena pubescens Wall. ex G.Don	Apocynaceae	Bark, seeds
Holoptela integrifolia (Roxb.) Planch.	Ulmaceae	Bark, leaves
Hyoscyamus niger Linn.	Solanaceae	Leaves, seeds
Illicium verum Hook. F.	Magnoliaceae	Fruits
Inula racemosa Hook. F.	Asteraceae	Roots
Ipomoea nil (Linn.) Roth	Convolvulaceae	Seeds
Jasminum grandiflorum Linn.	Oleaceae	Root
Jasminum multiflorum (Burm.f.) Andr.	Oleaceae	Flowers
Jatropha curcas Linn.	Euphorbiaceae	Seeds (oil)
Juglans regia Linn. Var. Kumaonia dc.	Juglandaceae	Fruits
Justicia gendarussa Burm.f.	Acanthaceae	Roots, leaves
Kaempferia galangal Linn.	Zingiberaceae	Rhizomes, root-stock, leaves
Lantana camara Linn. Var. *Aculeate* (Linn.)*Moldenke*	Verbenaceae	Fruits
Launaea pinnatifida	Compositae	Plant juice
Lawsonia inermis Linn.	Lythraceae	Leaves
Leucas aspera (Willd.) Link	Lamiaceae	Leaves, flowers
Lilium polyphyllum D.Don	Liliaceae	Bulb
Madhuca longifolia (Koenig) Macbride	Sapotaceae	Seed (oil)
*Mangifera indica*Linn.	Anacardiaceae	Roots, bark

Binomial	**Family**	**Parts used**
Rhazya stricta	Apocynaceae	Roots, stem, leaves, flowers
Rhododendron campanulatum	Ericaceae	Leaves
Ricinus communis Linn.	Euphorbiaceae	Leaves
Rosa alba Linn.	Rosaceae	Flower
Rubia cordifolia Linn.	Rubiaceae	Roots
Ruta chalepensis Linn.	Rutaceae	Leaves
Salvadora oleoides	Salvadoraceae	Seeds(oil)
Salvadora persica Linn.	Salvadoraceae	Leaves
Sarcocephalus missionis	Rubiaceae	Bark
Saussura elappa	Compositae	Root
Schleichera oleosa (Lour.) Oken	Sapindaceae	Bark
Semecarpus anacardium Linn.f.	Anacardiaceae	Fruits
Sesbania grandiflora (Linn.) Poir.	Fabaceae	Root-bark
Setaria italic (Linn.) P.beauv	Poaceae	Grains
Sida cordata (Burm.f.) Borssum	Malvaceae	Roots
Sida rhombifolia Linn.	Malvaceae	Roots, leaves
Solanum surattense Burm.f.	Solanaceae	Whole plant
Spilanthes acmella	Compositae	Leaves
Stereospermum colais	Bignoniaceae	Leaves
Symplocos cochinchinensis (Lour.) Moore	Symplocaceae	Bark
Tabernaemontana divaricate (Linn.)	Apocynaceae	Roots
Trachyspermum roxburghianum (DC.) Craib	Apiaceae	Fruits
Tribulus terrestris Linn.	Zygophyllaceae	Whole plant
Trichodesma indicum (Linn.)R.br.	Boraginaceae	Whole plant
Trichosanthes palmata	Cucurbitaceae	Fruit
Unona narum Dun.	Anonaceae	Leaves
Uraria lagopoides DC.	Papilionaceae	Plant
Urgenia indica Kunth.	Liliaceae	Roots
Urena lobata Linn.	Malvaceae	Bark
Urtica dioica Linn.	Urticaceae	Leaves
Valeriana wallichii	Valerianaceae	Root
Vateria indica Linn.	Dipterocarpaceae	Seeds(oil)
Vitex negundo Linn.	Verbenaceae	Roots
Vitex trifolia Linn.	Verbenaceae	Leaves
Vitis vinifera Linn.	Vitaceae	Stem(ash)
Xylia dolabriformis	Mimosaceae	Seeds
Zingiber officinale	Zingiberaceae	Ginger
Zizyphus jujube Mill.	Rhamnaceae	Roots and Bark

joint of rats. Naringin, a citrus flavanone, reduced arthritis as assessed clinically and histologically and afforded protection against interchondral joints damage in rats with CIA [2].(3) A similar effect was observed with another citrus flavonoid, hesperidin, against CIA [3]..

(4) Total flavonoid of orange (TFO) decreased paw thickness and improved pathological condition of ankle joint in rats with AA. TFO also suppressed elevated TNF-, IL-1, and PGE2 levels in serum and COX-2 expression in the synovial tissue [4].

(5) Genistein is an isoflavone, which suppressed the levels of IFN-, increased the production of IL-4, and normalized the Th1/Th2 balance in CIA. Genistein also inhibited the proliferation of FLS in rats with CIA by inhibiting phosphorylation of ERK and downregulating tyrosine kinase of MAPK signal transduction pathway [5,6].

Triterpenes

Triterpenes possessing antiarthritic properties were Lupeol, a pentacyclic triterpene isolated from the latex of Calotropis gigantea, showed potent antiarthritic activity as evaluated in the rat AA model. Lupeol ameliorated the paw swelling and reduced the levels of proinflammatory cytokines such as TNF-, IL-1, and IL-6 [7]. Celastrol, a triterpene extracted from Celastrus, is a potent antiarthritic biomolecule. Both Celastrus extract and its bioactive component Celastrol suppressed the expression of proinflammatory cytokines (IL-17, IL-6, and IFN-), the transcription factor STAT3 for IL-6/IL-17, and the activity of matrix-degrading enzyme MMP-9. In addition, it inhibited the phosphorylation of ERK. Celastrol also has a bone damage-protective effect. Celastrol inhibits osteoclastic activity via reducing RANKL production and suppressing the elevated RANKL/OPG ratio in rats with AA [8]. Tripterygium wilfordii Hoog-derived trypterine reduced the paw swelling and bone destruction in AA. It also suppressed the expression of IL-1 and TNF- in arthritic rats. Boswellic acid is a pentacyclic triterpene isolated from boswellia plants. It reduced leukocyte infiltration into the knee joint and the pleural cavity as observed in bovine-serum-albumin- (BSA-) induced arthritis in rabbits. 3-Acetyl-11-keto-beta-boswellic acid (AKBA), which is well known for anti-inflammatory activity, also has antiarthritic activity. Topical application of the polymeric nanomicelles of AKBA showed potent anti-inflammatory and antiarthritic activities [9].

Phenols

Several polyphenols have been studied extensively for their beneficial effect against arthritis and the mechanisms involved in that process; for example, green tea prepared from the dried leaves of Camellia sinensis is a commonly consumed beverage in many parts of the world. The polyphenolic compounds from green tea (PGT) possess anti-inflammatory and antiarthritic properties. PGT-induced reduction in clinical and histological features of arthritis was associated with a decrease in proinflammatory cytokine IL-17 and increase in anti-inflammatory cytokine IL-10. In another study using the CIA model of arthritis, PGT treatment reduced the expression of COX-2, IFN-, and TNF-. Epigallocatechin-3-gallate (EGCG) is one of the most well-studied purified plant components against different diseases. EGCG inhibits IL-1-induced inducible nitric oxide synthase (iNOS), nitric oxide (NO), and JNK activity, all of which mediate cartilage degradation. It also suppresses IL-1-induced glycosaminoglycan release from cartilage via inhibiting ADAMTS (A disintegrin and metalloproteinase with thrombospondin motifs), MMP-1, and MMP-13 in chondrocytes. EGCG reduces osteoclast formation by inhibiting osteoblast differentiation without affecting their viability and proliferation. Osteoclast-specific NFATc1 and bone resorption associated with RA are also suppressed. EGCG also inhibits RANKL-induced activation of JNK and NFB pathways, thereby suppressing the expression of c-Fos and NFATc1 in osteoclast precursors. In another study, combination therapy of methotrexate and EGCG inhibited arthritis progression as evidenced by histopathology and radiographical examination. Furthermore, this combination suppressed the expression of TNF- and IL-6 in the joints of rats with AA. Grape seed proanthocyanidin extract (GSPE), an antioxidant derived from grape seeds, showed antiarthritic effect as evident from suppression of clinical signs of arthritis and IL-17 response along with increased Foxp3-expressing CD4+ regulatory T cells. Oligomeric procyanidins

(HOPC) isolated from Jatoba, a South American herb, ameliorated arthritic inflammation and joint pathology in mice with CIA.

Resveratrol, a polyphenolic compound derived from grapes, suppressed swelling and bone erosion in the paws of mice with CIA. This effect was associated with reduced serum levels of proinflammatory cytokines including IL-17 and reduced numbers of Th17 cells. The antiarthritic effect of resveratrol also involves suppression of IL-1, ROS, PGE2, and MMPs, and enhancement of proteoglycan synthesis and chondrocyte proliferation in vitro. Curcumin, a principle component of turmeric, possesses anti-inflammatory and antiarthritic activity. Curcumin treatment downregulated clinical arthritis score, proliferation of splenic T cells, expression of TNF- and IL-1 in the ankle joint, and serum IgG2a levels. In addition, curcumin ameliorated the NF-B transcriptional activity in FLS and inhibited the production of PGE, COX-2 and MMP. A randomized, pilot study conducted to assess the efficacy and safety of curcumin in RA patients revealed an improvement in overall disease activity score (DAS) and American College of Rheumatology (ACR) scores, and the treatment was safe without any significant adverse events. Capsaicin treatment suppressed bone erosion and trabecular damage in osteoarthritis in rats. Ferulic acid isolated from corn germ promotes bone remodeling and prevents bone loss in ovariectomized rats. Oleuropein aglycone, an olive oil-derived compound, improved clinical and histological features of arthritis in the joints of mice with CIA. Quercetin isolated from onion has a bone damage-protective attribute. It inhibited RANKL-induced osteoclast differentiation and RANKL-stimulated osteoclast-related genes in rats with ovariectomy-induced bone loss Silibinin, a major active constituent of silymarin, inhibits osteoclast formation by attenuating the downstream signaling cascades associated with RANKL and TNF-, such as osteoclast-associated receptor (OSCAR), NFATc1, Cat K, and MMP-9. Rosmarinic acid is an active component of Plectranthus amboinicus. It inhibited RANKL-induced formation of TRAP-positive multinucleated cells and suppressed NF-B activation and NFATc1 nuclear translocation in the CIA model [10, 11, 12, 13]. Aleomannan and acemannan found in the species of Aloe vera showed anti-tumor, immune-suppressive and anti-inflammatory properties. The anti-inflammatory property enabled it to inhibit the activity of COX-2 (cyclooxygenase) enzyme which is involved in the inflammatory pathways. Similarly, Tag et al., [14], analyzed potential anti-inflammatory effect of lemon and hot pepper extracts on adjuvant-induced arthritis in mice. Shivaprasad et al., [15] reported about control of autoimmune arthritis by herbal extracts and their bioactive components. Choudhary *et al.,* [16], reviewed medicinal plants with potential anti-arthritic activity.

CONCLUSION

It is increasingly been realized that inflammation and bone damage are generally linked in arthritis and various other disorders. A better understanding of the shared processes would help define the molecules and pathways that can serve as targets for effective antiarthritic therapy. In parallel, it is essential to search for newer therapeutic agents that are both effective but safe. In this regard, herbal products may offer promising alternatives or adjuncts to conventional antiarthritic agents. In this paper, we have elaborated upon both these important aspects of immune pathology and herbal therapy of arthritis.

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Metal Nano Particles Synthesized from *Riccia fluitans* and its Application as Antimicrobial Agent

G. GANGA

Department of Microbiology, Sree Ayyappa College
Eramallikkara, Chengannur
email :gangaprasanth@gmail.com

ABSTRACT

Riccia is a genus of liverworts in the order marchantiales. An attempt was made in this study to synthesis metal nano particle from Riccia plant and comparison for its efficacy as potent antimicrobial agent. The raw material used in this study was a Spp of a *Riccia fluitans*, collected from damp soils from Eranakulam district. The nanoparticles used in this study exhibit potential antibacterial activity against tested organism. The identity of Nanoparticle was confirmed using UV visible spectra SEM and XRD. In addition to that antioxidant property Riccia CnNp nano particle of the nanoparticle also was evaluated in the study.

Key words *Riccia fluitans, Green synthesis, Nano particle, Antimicrobial efficacy*

Bio molecules present in plant extracts can be used to reduce metal ions to nanoparticle in a single-step green synthesis process. The reducing agents involved include the various water soluble plant metabolites(example alkaloids, phenolic compounds, terpenoids) and coenzymes. Silver(Ag) and Gold(Au} Copper (Cu) nanoparticles have been the particular focus of plant based synthesis. Extrtacts of a diverse range of plant specious have been successfully used in making nanoparticles. (Abdul-Rehman Phull *et al*., 2016)

Metallic nanoparticles are being utilized in every phase of science along with engineering including medical fields and are still charming the scientists to explore new dimensions for their respective worth which is generally attributed to their corresponding small sizes. Among several noble metal nanoparticles, silver nanoparticles and copper nano particles have attain a special focus. Conventionally metal nanoparticles are synthesized by chemical methods using chemicals as reducing agents which later on become accountable for various biological risks due to their general toxicity; engendering the serious concern to develop environment friendly processes. Biological approaches are coming up to fill the void for instance green synthesis using biological molecules derived from plant sources in the form of extracts exhibiting superiority over chemical methods. These plant based biological molecules undergo highly controlled assembly for making them suitable for the metal nanoparticle synthesis. Biosynthesis of nanoparticles using plant extract is one of the of green, eco-friendly method for the production of nanoparticles and exploited to a vast extent because the plants and widely distributed, easily available, safe to handle and with a range of metabolites (Archibald and Smith 2-6, Hikmah *et al.* 2016, Atherton, 2010., Chaloupka *et al.* 2010. and Schuster and Rudolf, 1992)

Bryophytes are the second largest group of land plants after angiosperms. There is very less knowledge available about medicinal properties of these plants. Bryophytes are popular remedy among the tribal people of different parts of the world. Tribal people use these plants to cure various ailments in their daily lives. (Chaloupka *et al* 2010) Bryophytes are used to cure hepatic disorders, skin diseases, cardiovascular diseases, used as antipyretic, antimicrobial, wound healing and many more other ailments by different tribal communities of Africa, America, Europe, Poland, Argentina, Australia, New Zealand, Turkey, Japan, Taiwan, Pakistan, China, Nepal and different parts of South, North and Eastern. *Riccia* is a Bryophyte of the genus of liverworts in the order Marchantiales. The plants are small and thallose, that is not differentiated into root, stem and leaf. (*Edwards,2012.,* Mohd and Sayeed, 2013 and Smith, 1997)

Resistance to antibiotics is one of the biggest problems that faces public health. Bacteria have developed resistance to all known antibiotics and, as so, the economic burden associated with these multidrug-resistant bacteria is high. Many reports are available regarding the use of nanoparticle as an alternative to overcome this problem. Keeping this aim in mind the an attempt was made for the green synthesis of nano particle and evaluate its antibacterial activity. So far there was no report on synthesis of Nanoparticle from *Riccia fluitans* a member of Ricciaceae family called floating crystal wort, was the selected as the source material for the study.

MATERIALS AND METHODS

Bio Synthesis of Riccia-metal nanoparticles

Riccia plants were collected at vyttila, ernakulam

Table 1. Results of Agar well diffusion test of AgNp and CuNp particles of Riccia Sp.

Bacteria	Agarwell diffusion test	
	Sample (µg/ml)	Zone of inhibition(cm)
Staphylococcus aureus		
	25µg: 1. Riccia extract	-
	2. Riccia+Cu	1.7
	3. Riccia+Ag	1.3
	4. Riccia+combo	1.4
	50 µg: 1.Riccia extract	
	2. Riccia+Cu	-
	3. Riccia+Ag	2.4
	4. Riccia+combo	1.4
		2
Bacillus subtilis	**25 µg**: 1.Riccia extract	-
	2.Riccia+Cu	1.7
	3.Riccia+Ag	1.3
	4.Riccia+combo	1.5
	50 µg: 1.Riccia extract	-
	2.Riccia+Cu	2.4
	3.Riccia+Ag	1.4
	4.Riccia+Combo	1.9
Escherichia coli	**25 µg:** 1.Riccia extract	-
	2.Riccia+Cu	1.6
	3.Riccia+Ag	2
	4.Riccia+Combo	1.5
	50 µg: 1.Riccia extract	-
	2.Riccia+Cu	2.2
	3.Riccia+Ag	1.9
	4.Riccia+Combo	2
Klebsiella pneumoniae	**25 µg**: 1.Riccia extract	-
	2.Riccia+Cu	1.4
	3.Riccia+Ag	1.5
	4.Riccia+Combo	1.3
	50 µg: 1.Riccia extract	-
	2.Riccia+Cu	1.9
	3.Riccia+Ag	1.5
	4.Riccia+Combo	1.5

50gm extract was Made in 200ml water For this purpose equal volumes of silver nitrate (0.1%) Riccia plant extract (1%) were vigorously mixed in the ratio of 1:1. The mixture was incubated for 3 h at room temperature. Change in color was observed by naked eye and later analyzed by UV-Visible spectroscopy.Similarly of (1:1) $CuSO_4$ and plant extract was mixed and incubated. The sample was then mixed well. It was then incubated for 12 hrs at 72°C. After 12 hrs the colloid was centrifuged at 12,000rpm for 10 minute to separate particle from suspension. The precipitate was suspended in acetone (90%, v/v) and the centrifugation was repeated three times to remove unreacted agents. Finally, the precipitate was dissolved in water, dried under vacuum overnight and stored.

Antibacterial assay

The bacteria used for investigating the

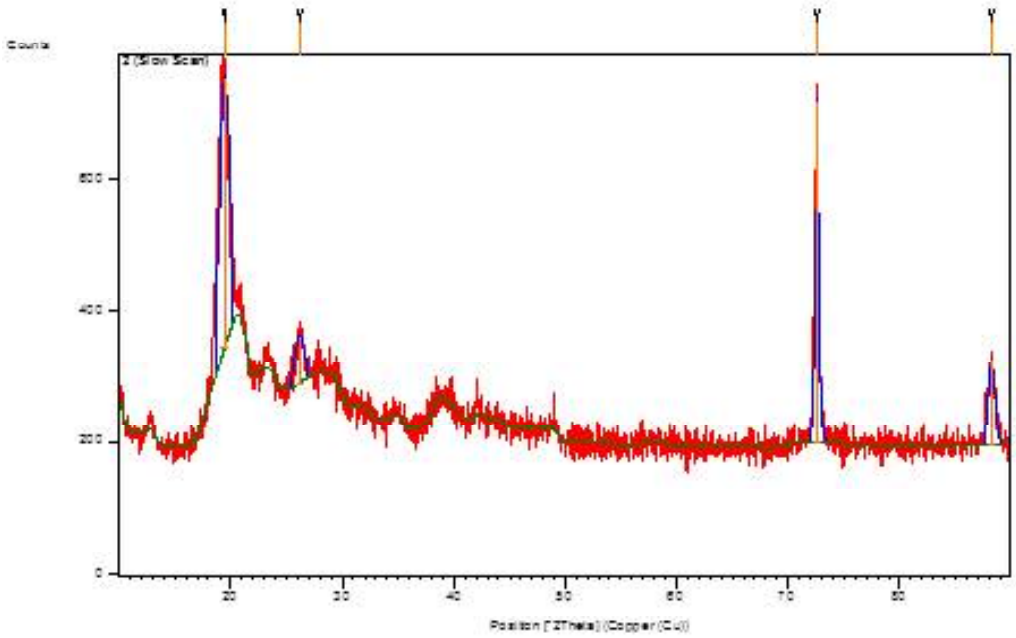

Fig1 XRD Analysis of CuNP

antimicrobial activity of Riccia were collected from clinical laboratory. They were identified by microscopic observation and biochemical characteristics. Pure cultures were obtained from them and they were maintained at 37°C in nutrient agar medium.

A well diffusion method was used to assay the antibacterial activity of synthesized Cu-Riccia NPs, Ag-Riccia NPs and a combination of Cu and Ag Riccia NPs against *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae, Escherichia coli* were tested standard antibiotic against appropriate control were used .Water extract of Riccia was also used separately to check the antimicrobial activity. The plates were incubated for 24 hrs at 37°C and inhibition zones were measured.

Characterization of CuNPs and AgNp

Nano Particle sysnthesised was characterized the nanoparticles using standard protocols: i) Ultraviolet (UV-)visible spectra using a spectrophotometer in the 300–800 nm wavelength range; ii) fourier transform infrared spectroscopy (XRD) analysis using a Spectrum and scanning electron microscope (SEM) analysis using a JFC-1600 instrument (JEOL, Ltd., Tokyo, Japan)

Evaluation of total antioxidant activity

Total antioxidant activities of the samples of synthesized CuNPs and aqueous leaf extract were analyzed . Briefly, 100 mg of the synthesized CuNPs were mixed with 0.05% DMSO in the reaction vial and a 0.1-ml aliquot of the sample was subsequently mixed with 1 ml of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated at 95°C for 90 min. After the samples were cooled to 25°C, absorbance was measured at 695 nm against a blank, which contained 1 ml of the reagent solution without the sample

RESULT AND DISCUSSION

In the present study Riccial metal nanoparticle were sysnthesised and was compared their antimicrobial efficacy. The antimicrobial activity of different AgNP and CuNp was compared for its antimicrobial activity .A combination of both AgNP and CuNp was also tested for antimicrobial effect by well diffusion method. Result of the tests are given in the table.1

Among the four bacteria tested *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae, Escherichia coli* were found to have antimicrobial activity riccia. It was observed that antimicrobial activity was more in gram positive bacteria than in negative bacteria. Riccia CuNp was found to have more antibacterial activity and Riccia AgNp and Riccia water extract. Acombination Riccia CuNp +AgNP was also tested for its antimicrobial property it was observed that CuNp alone is found to have potent antimicrobial effect than in using in combination.

Total antioxidant activity was expressed as the absorbance of the sample. A high absorbance value indicates increased antioxidant activity. Ascorbic acid was also assayed as standard. The antioxidant potential of Riccia AgNPs was expressed as an ascorbic acid equivalent (AAE). *Samples were taken* at the different concentrations of 100 µg/ml to 500 µg/ml. The aqueous extract demonstrated values of 0.22±0.06, 0.33±0.14, 0.38±0.11, 0.49±0.67 and 0.57±0.78 mg GAE/g; standard ascorbic acid values were: 045.5±0.11, 0.53±0.06, 0.65±0.15, 0.73±0.25 and 0.75±0.84 mg GAE/g; and CuNPs values were 0.44±0.19, 0.47±0.67, 0.72±92 0.75±0.56 and 0.86±0.30 mg GAE/g,

Characteristic of nanoparticle was confirmed using UV-spec absorption peak was observed at 565 nm and may be due to the surface plasmon band of Cu colloids formation of non-oxidized CuNPs and characteristic peak at 480 nm showed the presence of AgNp Nano particle. However, CuNp was found to be more effective in antimicrobial analysis hence it was subjected to XRD analysis. Using XRD spectrum analysis, the two different diffraction peaks at 20.1°and 70.3° are shown in Fig. 1. There are three main characteristic diffraction peaks for copper was observed at20.3 (111), 40.3(200), and 70.3(220). This was in accordance with previous reports of Hikmah *et al* 2016.SEM analysis also confirmed the identity spherical shaped particles were observed.

Riccia extract function as an excellent reducing agent of copper ions, and the biosynthesized CuNPs are reported as safer for the environment This the first report on synthesis of nanoparticle from Riccia plant and the studying its antimicrobial potential and antioxidant potential .Hence more study in this area is needed for its other pharmacological application.

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Growth and Metabolic Characteristics of Rauvolfia Micrantha *Hook. F.,* Under Treatment with Paclobutrazol - A Systemic Fungicide

V. DIVYA NAIR[1] AND R. GOPI[2]

[1]*Department of Botany, HHMSPBNSS College for Women, Neeramankara, Trivandrum*
[2]*Stress Physiology Lab, Department of Botany, Annamalai University,*
Annamalai Nagar, 608002, Tamil Nadu, India.
email : divyanair013@yahoo.com

ABSTRACT

The effect of Paclobutrazol (PBZ), a triazole compound was investigated in *R. micrantha* Hook. f., Plants were treated with aqueous solution containing 15 mg/l Paclobutraazol on 50th, 70th, 90th day after planting. Treatment was given by soil drenching. Plants were harvested on 60th 80th and 100th DAP and used for analysis. The growth characters like shoot length, root length, fresh and dry weight were changed significantly due to Paclobutrazol treatment. Photosysnthetic characters, total protein content, proline, total amino acid and glycine betaine were found varying in different parts of the plant under treatment. In stem roots and leaves of *R.micrantha,* Paclobutrazol causes a decrease in lipid peroxidation and electrolyte leakage and proline oxidase activity when compared to control. Protease activity found increased in various parts of PBZ treated *R.micrantha* plants. These findings are of great significance for cultivation of this medicinal plant, as it was previously reported that Paclobutrazol causes an enhancement of antioxidant metabolism and phytochemical constituent in medicinal plants.

Key words *R. micrantha Hook. f., Triazole, Paclobutrazol, Proline, Lipid peroxidation, Electrolytic leakage, Glycine betaine, Amino Acid, Proline oxidase.*

Abiotic stress conditions cause extensive losses to agricultural production worldwide and these have been the subject of intensive research. Water deficit are one of the major abiotic stresses, which adversely affect the crop growth and yield. Coping with water deficit stress is a global issue to ensure survival of agricultural crop and sustainable food production. Conventional plant breeding attempt have changed over to use physiological selection criteria since they are time consuming and relay on present genetic variability. Currently protection of plant through application of plant growth regulators (PGR) attracts more attention. Plant growth regulator play an important role in crop production and are being increasingly yield to manipulate plant growth and yield (Davis *et al.,*1988*).*

Triazole compounds, such as Paclobutrazol, uniconazole and PBZ, have both fungicidal and plant growth regulatory properties, and induces variety of morphological and biochemical responses in plants, including retarded shoot elongation, stimulated rooting, inhibited gibberellin biosynthesis, and protection from several types of abiotic stresses, such as drought, salt, heat, chilling (Rademacher, 1992).Triazoles have been called plant multiprotectants because of their ability to induce tolerance to environmental and chemical stresses in plants (Fletcher *et al*.,1988)

In agricultural, horticultural and forestry industries the commonly used triazole fungicides and plant growth regulators are propiconazole, epoxiconazole, penconazole, Paclobutrazol and triadimefon. Paclobutrazol (PBZ)2S,3S, -1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1yl) pentan-3-ol, triazole fungicide having plant growth regulator properties is reported to inhibit gibberellic acid biosynthesis and increase ABA and cytokinin contents (Fletcher *et al*., 2000) PBZ has been shown to protect plant from water stress (Fletcher and Nath 1984) and heat stress (Asare -Boamah *et al*.,1986).

Rauvolfia is a genus of the family Apocyanaceae (dogbane family) sub-family Plumeroidae and occurs in nearly all habitable tropical and subtropical regions.

R.micrantha Hook. f. is also used especially in the state of Kerala, as a substitute of *R. serpentina* to treat a variety of nervous disorders (Sahu, 1979) and the plant has been reported as a rare endemic south Indian species (Gamble,1953; 1979). The plant grows generally in the region with annual rainfall of 200-250 cm and up to an altitude of 1000 m and favors deep fertile soil rich in organic matter. It is a perennial woody shrub distributed at an elevation of 600 m in the Tinnevelly and Travancore hills of Western Ghats in Southern India also known as Malabar Rauvolfia. The root of the plant is a store house of therapeutically active alkaloids, e.g. reserpine, serpentine, ajmaline, ajmalicine, yohimbine etc (Anonymous, 1969). Further,

Table 1. Effect of Paclobutrazol on growth characteristics (a) Shoot length (b) Root length(c) Fresh Weight (d) Dry weight of *Rauvolfia micrantha* Hook. f. (Values are mean ± SD of 7 samples).

Parameters	Growth Stages	Control	Paclobutrazol
Root length (cm/plant)	60	22 ± 0.08	28.00 ± 0.9
	80	25 ± 0.09	30.00 ± 0.9
	100	29 ± 0.09	33.00 ± 1.1
Shoot length (cm/plant)	60	35 ± 0.12	21.00 ± 0.1
	80	47 ± 0.15	23.00 ± 0.7
	100	50 ± 0.16	27.00 ± 0.1
Total leaf area (Cm^2/Plant)	60	9 ± 0.3	5 ± 0.2
	80	12±0.4	6±0.2
	100	13±0.4	8±0.3
Fresh Weight (g/plant)	60	7 ± 0.2	8±0.1
	80	14 ± 0.1	22 ± 0.1
	100	15 ± 0.1	24 ± 0.1
Dry Weight (g/plant)	60	2 ± 0.1	3 ± 0.1
	80	4 ± 0.2	5 ± 0.2
	100	5 ± 0.1	6 ± 0.1

the plant has antiseptic, antispamodic, antibacterial properties (Gupta and Sahi, 1998), considering the medicinal importance of the *R.micrantha*, the present study has been designed to find out the Paclobutrazol induced changes on growth and metabolic characteristics of *R.micrantha*.

MATERIALS AND METHOD

PBZ (25% w/v BAYLETON-registered trademark, Bayer, India Ltd., Mumbai) and propagules of plants were raised from root cuttings and plants were grown in greenhouse of Botanical Garden, Department of Botany, Annamalai University, Annamalai Nagar, Tamil Nadu. The experiments were conducted in stress physiology laboratory, Annamalai University. Throughout the study, the average temperature was 26°C-32°C and the relative humidity (RH) varied between 60-75%.

The propagules were sown separately in raised seed beds by broadcasting method and covered with paddy straw to ensure proper germination. The nursery beds were watered regularly for the healthy growth of seedlings. Seedlings were transplanted to six cement vials having 1 m diameter with twelve plants in each vial. Prior to transplantation the vials were filled with red soil sand and farmyard manure (FYM) at a 1:1:1 ratio. The vials were irrigated immediately after transplantation and subsequent irrigation was done twice in a day to keep the optimum moisture level required in the soil.

In the preliminary experiment 5, 10, 15 and 20 mg/l PBZ was used for treatment to determine the optimum concentration of PBZ. Among the treatments 15 mg/l PBZ increased the dry weight and higher concentration slightly decreased the growth and dry weight. At lower concentrations, there was no significant change in dry weight and growth of the plant.

Plants in three cement vials were taken for treatment with PBZ and the other three were kept untreated and served as control. 15 mg/l PBZ was given to each plant by soil drenching. The treatment was given on 50^{th}, 70^{th} and 90^{th} days after planting (DAP). The plants were uprooted randomly on 60^{th}, 80^{th} and 100^{th} DAP and separated in to root, stem and leaves and used for the biochemical analyses.

The shoot length was measured from the soil level to the tip of the shoot and expressed in centimeters. The plant root length was measured from the point of first cotyledonary node to the tip of the longest root and expressed in centimeters. The total leaf area of the plant was measured using the LICOR photoelectric area meter (Model LI-3100, Lincoln,

Table 2. Effect of Paclobutrazol on photosynthetic characteristics (a) Chlorophyll a (b) Chlorophyll b (c) Carotenoid content of *Rauvolfia micrantha*. (Values are mean ± SD of 7 samples).

Parameters	Growth Stages	Control	Paclobutrazol
Chlorophyll *a* (mg/g FW)	60	0.724±0.005	0.891±0.001
	80	0.886±0.007	0.913±0.001
	100	0.810±0.004	0.878±0.007
Chlorophyll *b* (mg/g FW)	60	0.624±0.005	0.771±0.021
	80	0.796±0.007	0.823±0.001
	100	0.730±0.004	0.818±0.001
Carotenoid (mg/g FW)	60	0.018±0.037	0.121±0.031
	80	0.261±0.044	0.330±0.058
	100	0.091±0.038	0.235±0.061

USA) and expressed in cm^2 per plant.

After washing the plants in tap water, the fresh weight was determined by using an electronic balance and the values were expressed in grams. After the fresh weight was measured, the plants were dried at 60^o in a hot air oven for 48 hours. After drying the weight was measured and the values were expressed in grams.

Chlorophyll content was estimated by the method of Arnon, (1949) and expressed in milligram per gram fresh weight.500mg of fresh leaf material was ground with 10ml of 80% acetone at 4^0C and centrifuged at 2500rpm for 10min at 4^0 C. This procedure was repeated until residue become colourless. The extract was transferred to a graduate tube made up too 10ml with 80% acetone and assayed immediately.

Three milliliters aliquots of the extract were transferred to a cuvette and the absorbance was read at 645, 663, and 480 nm with a spectrophotometer against 80% acetone as blank. Chlorophyll content was calculated and expressed in milligram per gram fresh weight.

The carotenoid content was measured using the formula of Kirk and Allen, (1965) and was expressed in milligrams per gram fresh weight.

Lipid peroxidation was estimated as TBARS (Heath and Packer, 1968). Fresh sample (0.5g) were homogenized in 10ml of 0.1% TCA, and homogenate was centrifuged at 15,000 rpm for 15min.To a 1.0ml aliquot of the supernatant,4.0ml of 0.5% thiobarbituric acid (TBA) in 20% TCA were added. The mixture was heated at 95^0C for 30 min in electric oven and then cooled in an ice bath. After centrifugation at 10,000 rpm for 10min, the absorbance of the supernatant was recorded at 532nm.The TBARS content was calculated according to its extinction coefficient of $155mM^{-1}cm$ and expressed in units (U). One U is defined as one micromole of MDA formed per minute and per milligram of protein.

Electrolytic leakage (EL) was quantified according to the methodology of Pinhero and Fletcher 1994.One gram of tissue was cut in to 2cm segments, rinsed in deionised water to remove the contents of cut cells, and placed in test tubes containing 15ml of bidistilled water for 24h at 25^0C.The electrical conductivity of the solution in the tube was determined using a conductivity meter .The tubes were placed in a boiling water bath for 20min, and then cooled to 24^0C,the electrical conductivity was then measured .The percentage of leakage of the tissue was calculated as the ratio of the conductivity after 12h to the conductivity after boiling (total ionic conductivity).

The Soluble protein was extracted and estimated following the method of Bradford (1976) using bovine serum albumin (BSA,Sigma,USA) as the standard and expressed in mg g-1 fresh weight (F.W).

Extraction and estimation of the AA content was followed by the method of Moore and Stein (1948).0.5gram of plant material was homogenized with 10ml of 80% boiling ethanol. The extract was

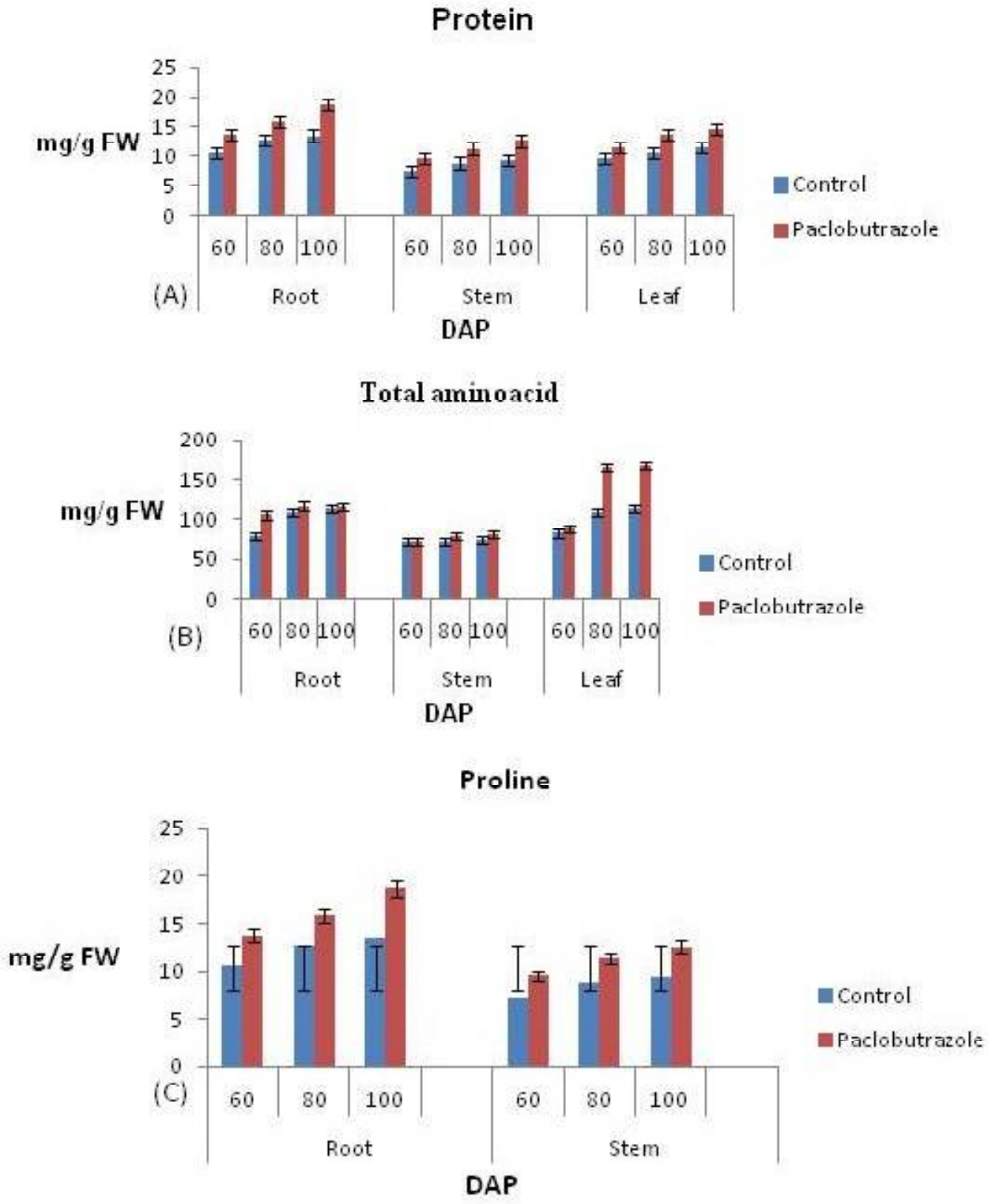

Fig. 1. Effect of Paclobutrazol on protein (a) Proline (b) and Amino acid (c) content of *Rauvolfia micrantha*. (Values are given by means ± SD of 7 samples).

centrifuged at 800g for 15min and the supernatant was made up to 10ml with 80% ethanol and used for the estimation of free AAs. One millilitre of ethanol extract was poured in a 25-ml test tube and neutralized with 0.1N sodium hydroxide using methyl red indicator. One millilitre of ninhydrin reagent was then added. The content was boiled in a boiling water bath for 20min then 5 ml of diluting reagent were added, cooled, and diluted to 25ml with distilled water. The absorbance was read at 570nm in a spectrophotometer. The AA

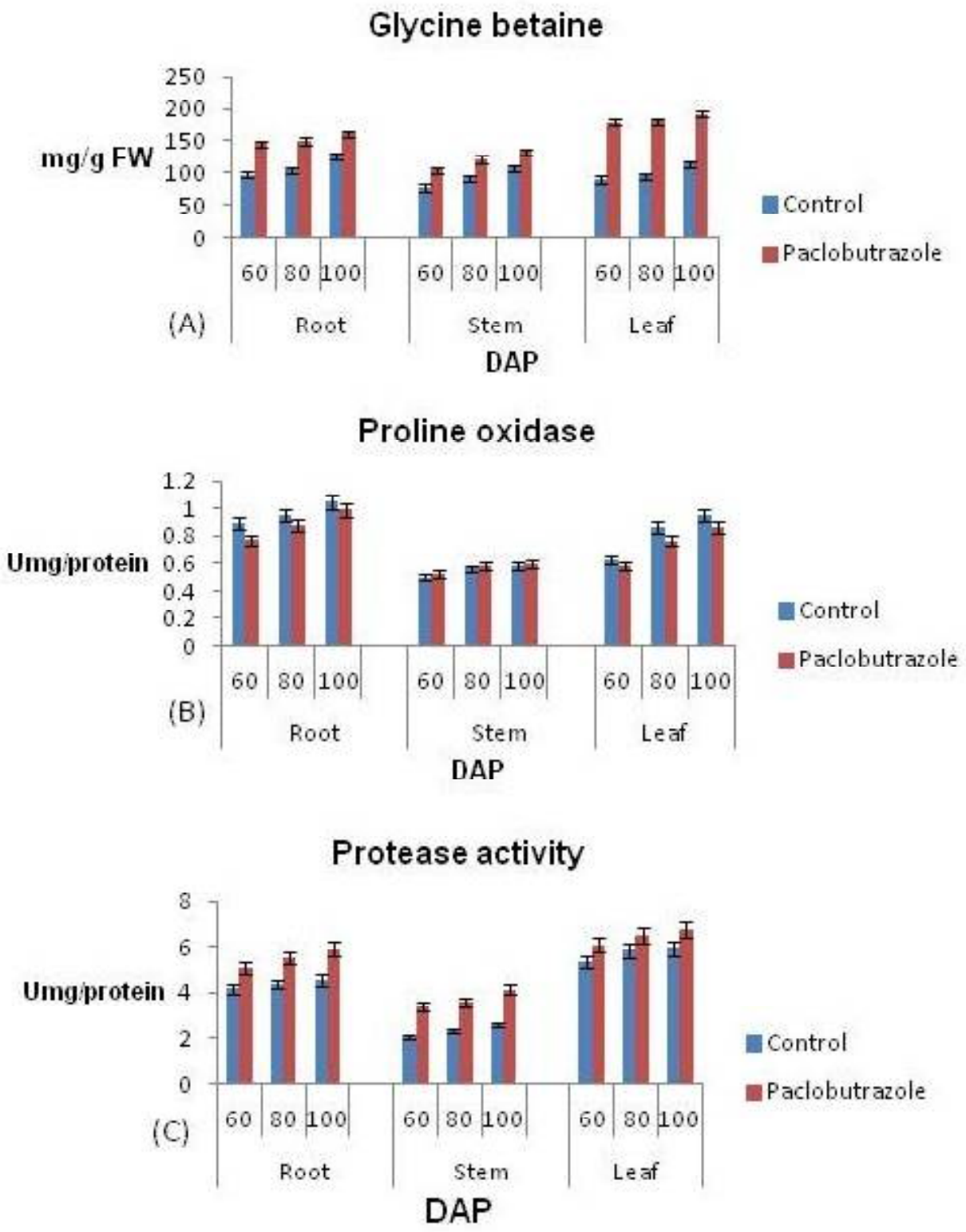

Fig. 2. Effect of Paclobutrazol on glycine bataine (a)Proline Oxidase (b) and Protease (c) content of *Rauvolfia micrantha*. (Values are mean ± SD of 7 samples).

content was calculated using a calibration curve, with glycine as standard. The results were expressed in milligrams per gram of dry weight.

The PRO content was estimated by the method of Bates *et al*., (1973). The plant material was homogenized in 3% aqueous sulfosalicylic acid and the homogenate was centrifuged at 10,000 rpm. The supernatant was used for the estimation of the PRO content.

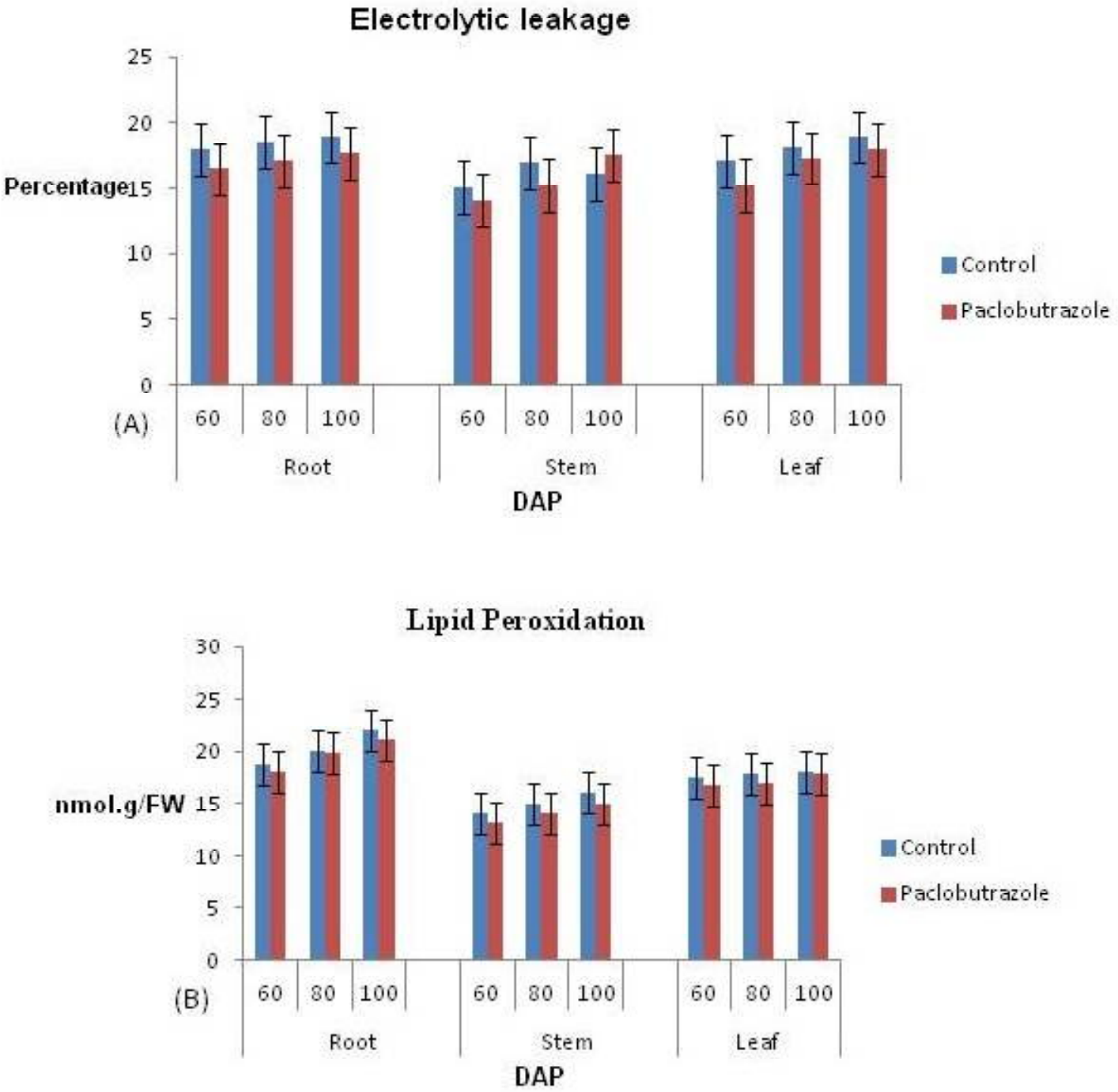

Fig. 3. Effect of Paclobutrazol on electrolyte leakage percent (EL) (a) and Lipid peroxidation (b) of *Rauvolfia micrantha*. (Values are mean ± SD of 7 samples).

The reaction mixture consisted of 2ml of acid ninhydrin and 2ml of glacial acetic acid, which was kept boiling at 100^{0}C for 1 h. The reaction was stopped by cooling in an ice bath, and the reaction mixture was extracted with 4ml of toluene, the absorbance was read at 520nm.

The amount of GB was determined according to the method of Grieve and Grattan (1983). The plant tissue was finely ground, mechanically shaken with 20ml of deionized water for 24 h at 25^{0}C.The samples were then filtered and the filtrates were diluted 1:1 with 2N H_2SO_4.. Aliquots were kept in centrifuge tubes , and cooled in icy water for 1h . A cold KI-I_2 reagent was added and the reactants were gently stirred with a vortex mixture. The tubes were stored at 4^{0}C for 16 h and then centrifuged at 10,000 rpm for 15 min at 0^0 C. The supernatant was carefully aspirated with a fine glass tube. The Per iodide crystals were dissolved

in 9ml of 1, 2 –dichloroethane. After 2h, the absorbance was measured at 365nm using GB as standard and expressed in mg^{-1} g FW.

Protease was extracted and assayed using the method Prisco et al., (1975). One gram of fresh plant tissue was macerated with 10ml of 0.1M sodium phosphatase buffer (PH 7.6) containing 1% NaCl and 7% PVP. The homogenate was centrifuged. The supernatant was made up to 10ml with the extraction buffer and the enzyme extract was incubated with 5ml of 1% casein solution in 0.1M sodium Phosphate buffer (P^H6.0) at 30^0C. The reaction was stopped after 30 min by adding 1ml of 40%TCA. The reaction mixture was centrifuged at 4000rpm for 20min.The supernatant was saved to estimate the TCA soluble amino nitrogen released, by the activity of protease. Lee and Takahashi (1996) method was followed for estimation of the ammoniac nitrogen. Five milliliters of ninhydrin reagent was added to 0.5 ml of supernatant and the tubes were heated in boiling water bath for 12 min with vigorous shaking and they were cooled and measured at 570nm in a spectrophotometer against a blank prepared with 40%TCA. A standard curve was prepared by using glycine as standard. The enzyme activity was expressed in terms of enzyme units. One enzyme unit is equal to micrograms NH_2 released hour $^{-1}$mg^{-1}1 protein.

The PROX (L. proline: O_2 oxidoreductase (EC 1.4. 3.1) activity was determined according to the method outlined by Huang and Cavalieri (1979). Plant samples (1g) were extracted with 5ml of Tris-HCl buffer pH 8.5 and centrifuged at 10,000g for 10 min at 4^0C. The supernatant was again centrifuged at 25,000g at 20 min at 4^oC. Three milliliters of assay mixture were prepared by taking 0.1 ml of extract, 1.2ml of 50 mM Tris HCl buffer pH 8.5, 1.2ml of 5mM $MgCl_2$, 0.1ml of 0.5m M NADP, 0.1 ml of 1 Mm KCN ,0.1ml of 1m M phenazine methosulphate (PMS), O.1 ml of 0.06 m M 2,6 –dichlorophenol indophenol (DCPIP) ,0.1 ml distilled water instead of PRO. The reaction was monitored at 600nm at 25^oC using PRO to initiate reaction; the increase of the OD value was noted after 0,1,2,3,4, and 5min.PROX activity was expressed in $Umg^{-1\ protein}$ (One U = m M DCPIP reduced min^{-1} mg^{-1} protein).

For all enzymatic calculations protein was determined by the method of Bradford (1976) using bovine albumin (BSA, Sigma , USA) as the standard.

Each treatment was analyzed with at least seven replicates and a standard deviation (SD) was calculated; data are expressed in mean ± SD of seven samples.

RESULT AND DISCUSSION

In *R.micrantha*, PBZ a triazole compound having high fungicidal and plant growth regulator properties (Fletcher and Hofstra, 1988) induce drastic changes in growth and metabolism.

Triazole treated plants showed considerable change in morphological characters (Table.1). There is a significant increase in the root length of triazole treated plants when compared to control. In triazole treated plants the root length was 33cm on 100 DAP, while it was 29cm in control plants. PBZ treated plants show considerable decrease in shoot length. On 100^{th} DAP it was 27.11cm in PBZ treated plants and in untreated control plants it was 50.00cm.

The whole plant fresh and dry weight increased with PBZ treatment while compared to control. In PBZ treated plants the whole plant fresh weight was 23.86g on 100 DAP, while in control plants it was 14.92g. On 100^{th} DAP the dry weight in treated and control plant was 4. 6 g, and 6.2g respectively.

The leaf area was increased with age in both control and treated plants. There was a significant reduction in total leaf area in PBZ treated plants (8cm^2 on 100 DAP), when compared to control (12.5 cm^2 on 100 DAP).

There is a significant increase in the root length of triazole treated plants when compared to control. This stimulation of root growth may be related to the increased partitioning of assimilates towards the roots due to a decreased demand on the shoot (Sympson et al., 1990). Barley seedling show reduced growth under treatment with Paclobutrazol (Sunitha et al., 2004).

Shoot length showed moderate decrease in triazole treated plants. The antigibberellin activity of triazoles was already reported. Triazoles inhibit gibberellin and ergosterol bioynthesis in plants and fungi respectively (W.Rademacher, 1992).The increase in root length was reported in Radish due to triazole treatment (Muthukumaraswami and Panneerselvam, 1997).Triazoles inhibit cytochrome p_450 mediated oxidative demethylation reactions ,which are necessary for the synthesis of ergosterol and the conversion of kaurene to kaurenoic acid in the gibberellin biosynthetic

pathway and alter the level of certain plant hormones by inhibiting gibberellin biosynthesis, reducing ethylene evolution and increasing cytokinin level (Fletcher et al., 2000).

Photosynthetic pigment composition increased significantly on PBZ treatment (Table.2). The contents of chlorophyll *'a'*, chlorophyll 'b', and carotenoid in PBZ treated plants shows a considerable increase compared to control plants. In PBZ treated plants the chlorophyll 'a' compositions on 100th DAP was 0.081mg/g fresh weight and in control plants it was 0.878mg/g fresh weight. Chlorophyll 'b' content on 100th DAP was 0.73mg/g FW and 0.7382mg/g FW, in PBZ treated plants and control plants respectively. The carotenoid content was more in PBZ treated plants while compared to control. In PBZ treated plants it was 0.235 mg/g FW on 100th DAP, while in untreated control plants carotenoid content was 0.091 mg/g FW on 100th DAP.

PBZ increased the chlorophyll content when compared to control plants. The increased chlorophyll content under PBZ treatment might be due to the ability of PBZ to increase the cytokinin production and there by stimulate the chlorophyll biosynthesis (Fletcher et al., 2000). Similar observations were also made in winter rape seedlings following treatment with Uniconazole (Zhou and Leul, 1999) and corn seedlings with Paclobutrazol (Pinhero and Fletcher, 1994). These results are in agreement with those reported by Bekheta & Ramadan (2005) and Mahgoub et al., (2006). Increase in photosynthetic pigments due to application of Paclobutrazol was already reported by Navarro et al., (2007).

Treatment with PBZ and Uniconazole significantly increased the free proline content in Muberry (Sreedhar,1991). Similarly, Paclobutrazol increased the free proline content in Mulberry (Mathur and Bohra, 1992). Triazole induced tolerance to low temperature stress has been associated with increased levels of endogenous abscisic acid fletcher et al., 2000 which has increased free proline (Chen and Lin,2003) and scavenging systems (Fath et al., 2001, Zhou et al., 2002).

Plants respond to a variety of stresses by accumulating certain specific metabolites like amino acids (it may provide extra protection to plants against oxygen radical damage arisen from abiotic stresses (Sailerova and Zwiazek., 1993). The amino acid content increased to a higher extend in the leaves stem and root of *R.micrantha* with PBZ treatment. Similar results were observed in Uniconazole treated Phaseolus vulgaris (Mackay et al., 1990).

PBZ treatment increased the protein (figure1.A) and amino acid (figure 1. B) content to a significant level in all parts (leaves, stem, and root) of *R.micrantha* plants than control.The PBZ treatment also increased the proline (figure1.C) content in the leaves, stem and root of *R.micrantha* plants. During this study we noticed a gradual increase in the glycine betaine (figure 2.A) content in *R.micrantha* under PBZ treatment.

PBZ treatment increased the protein content to a significant level in all parts (root, stem and leaf) of *R.micrantha* plants compared to control. The increase in protein content has been previously described in *Echinochloa furmentacea* (Sankhla et al., 1992) and in *Brassica carinata* (Seita, 1995) plants treated with Paclobutrazol and Uniconazole respectively.

The PROX activity (figure 2.B) decreased under PBZ treatment in *R.micrantha* when compared to control plant. Proline oxidase converts free proline in to glutamate. Reduction in PROX activity and simultaneous increase in PRO level were reported in low-temperature –stressed wheat (Charest and Phan,1990). PRO may act as a non –toxic osmotic solute preferentially located in the cytoplasm or as an enzyme protectant, stabilizing the structure of macromolecules and organelles.

In roots, stem and leaves of *R.micrantha* the PBZ treatment show increase in protease activity (figure2.C) when compared to control. The maximum protease activity in the root of PBZ treated plants.

Oxidative damage to tissue lipid (Figure.3) was estimated by the content of total TBARS. The PBZ treated plants showed a lower level of LPO in *R.micrantha* when compared to control. Treatment with PBZ significantly inhibited the EL (Figure.3) in leaf, stem, and root tissue of *R.micrantha.* LPO is a measure of the injury to the membrane. LPO is often used as an indicator of oxidative damage. LPO is measured by the TRBS released, which is a consequence of higher oxidative stress. TBARS, the cytotoxic product of lipid peroxidation, are normally considered as the major TBA reacting compounds that indicate the magnitude of the oxidative stress. Herbicides are known to generate activated oxygen

species, which are likely to contribute to the toxic effects of these herbicides (Qureshi et al., 2007). Uniconazole reduced the EL and MDA accumulation and consequently decreased heat induced LPO in rape plants (Zhou and Leul, 199). Similar results were observed in triazole treated *Egeria densa* leaves (Radice and Pesci, 1991) and Paclobutrazol wheat seedlings (Berova et al., 2002). LPO has been associated with damages provoked by a variety of environmental stresses. Polyunsaturated fatty acids (PUFA) are the main membrane lipid components susceptible to peroxidation and degradation (Elkahoui et al., 2005).

Changes in membrane leakage and injury can be measured by the extent of EL in tissues (Zwiazck and Blake, 1991). Paclobutrazol treated wheat seedlings maintained a high degree of membrane integrity under heat stress (Kraus et al., 1993 and uniconazole treatment inhibited the EL in soybean (Kraus et al., 1993). Paclobutrazol altered the membrane properties and facilitated the removal of damaged areas in the membranes of Maize (Paliyath and Fletcher, 1995). Triazole altered the sterol biosynthesis and changed the composition of sterol in the plasma membrane (Burden et al., 1987). This change in sterol composition may induce changes in cell membrane that may be reflected in increased membrane stability, acclimatization, and frost hardiness, as observed in white spruce (Sailerova et al., 1993).

The ability of plant tissues to mobilize enzymatic defense against uncontrolled lipid peroxidation may be an important feature of their tolerance. The rate of lipid peroxidation in PBZ treated plants was less while compared to the control.

One of the most important mechanisms exerted by higher plants under stress conditions is the accumulation of compatible solutes such as GB. In this study we noticed a gradual increase in the GB content in *R.micrantha*. GB accumulation results from oxidative stress induced by the fungicide application; it is helpful in the stimulation of tolerance mechanism (Sankar et al., 2007).

CONCLUSION

The activation of metaboic processes by fungicide PBZ in medicinal plant *R.micrantha* is evident from the above results. It caused inibition of TBARS and electrolytic leakage in *R.micrantha* when compared to control. Protein, amino acid glycine betaine, and proline contents significantly accumulated in *R.micrantha* under PBZ treatment. PBZ application causes decrease in PROX activity and increase in protease activity. These findings are of great significance in the cultivation of this medicinal plant.

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