COMPARATIVE PHYTOCHEMICAL EVALUATION AND BIOLOGICAL ACTIVITY SCREENING OF *Murdannia nudiflora* and *Tradescantia pallida*

Submitted By

Student ID:11146004

Batch: Spring 2011

Department of Pharmacy

BRAC University

March, 2015

Comparative Phytochemical Evaluation and Biological Activity Screening of *Murdannia nudiflora* and *Tradescantia pallida*

The dissertation is submitted to the Department of Pharmacy of BRAC University in partial fulfillment of the requirement for the Degree of Bachelor in Pharmacy.

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Abstract:

The study was aimed to evaluate the phytochemicals and pharmacological investigation on commelinaceae family which have high rate of growth and found as weeds of notably important therapeutic values. The plants were chosen from different genus, one indigenous, Murdannia nudiflora from the genera Murdannia and the other exotic, native to Mexico and south American countries Tradescantia pallida from the genera Tradescantia which are mainly used as a ornamental plant to find out the presence of notable compounds present and to justify the claims provided in traditional medicine in a more precise and accurate approach of science. Preliminary phytochemical screening shows the presence of carbohydrate, alkaloids, tannins , saponins and flavonoids. Cytotoxic analysis with in vitro brine shrimp lethality assay showed LC_{50} at a higher concentration of 158.638 µg/ml for *Murdannia nudiflora and* 833.85 µg/ml for *Tradescantia pallida*, quite low in comparison to the activity of vincristine sulphate. For toxicokinetic study, the extract shows degree of moderate lethality of more than 60% for Murdannia nudiflora and more than 50% for Tradescantia pallida at a concentration of 640µg/ml.. The percentage of DPPH radical scavenging for antioxidant studies in *Murdannia nudiflora* ranges from 96.825% to 82.539%, while that of *Tradescantia pallida* ranges from 95.238% to 84.127% and that of ascorbic acid ranges from 96.825% to 87.302%. The IC_{50} values for ascorbic acid was $4.209x10^{-13}$µg/ml, for *Murdannia nudiflora was* $1.51x10^{-10}$µg/ml and for *Tradescantia pallida was* $5.48x10^{-10}$µg/ml. Analgesic activity was evaluated using in vivo acetic acid induced writhing method were 0.7% acetic acid used for the induction of pain. This study is used to evaluate both central and peripherial pain and the potential is evaluated in terms of inhibition of writhing. The mean inhibition of writhing by the samples (*Murdannia nudiflora* and *Tradescantia pallida)* were found out to be of statistically significant ($p<0.001$). Dichlofenac sodium (25mg/kg) was used as standard (inhibition of writhing=80.74%).The percentage of inhibition of writhing for *Murdannia nudiflora* was found out to be of 85.67% at 200mg/kg of body weight and 59.57% at 100mg/kg of body weight. While, on the other hand the analgesic potential of *Tradescantia pallida* was found out to be quite less in this regard, the percentage of inhibition of writhing at 200mg/kg was 54.55% and that of 100mg/kg of body weight was below 40%, precisely 36.41%. It also justifies the difference in claims of traditional medicine in relation to the scientific approaches of analysis with more precision and accuracy. This helps in the initial discovery of potential notable pharmacologically active compound of unknown territories of traditional medicine in the burning issues of global healthcare, to minimize the death by different non-communicable and communicable disease aimed in the development of global healthcare.

Declaration

I, hereby, declare that the Project report entitled. 'Phytochemical screening and Biological Activity Evaluation on *Murdannnia nudiflora* and *Tradescantia pallida'* presented to the Department of Pharmacy, BRAC University, Bangladesh, is the outcome of the investigations conducted by me (ID-11146004, 2nd Batch) under the supervision of Professor Dr. Mohammad Shawkat Ali, Chairperson, Department of Pharmacy, BRAC University. I also declare that no part of this Project Report has been or is being submitted elsewhere for the award of any Degree or Diploma

Samin Huq

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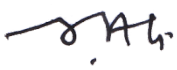
Approval

The Project Report entitled, "Phytochemical Screening and Biological Activity Evaluation of *Murdannia nudiflora* and *Tradescantia pallida*", submitted by Samin Huq, ID-11146004, Batch-2, to the Department of Pharmacy, BRAC University has been accepted as satisfactory for partial fulfilment of the requirements for the degree of Bachelor of Pharmacy (Hons.) and approved as to its style and contents



Professor Dr. Mohammad Shawkat Ali

Chairperson

Department of Pharmacy

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1. Introduction

1.1. General Introduction

Medicinal plants are highly popular in rural areas have been used as remedy of disease without scientific explanation over the claim of remedy of disease. This practice has been passed down to generations for thousands of years. It has taken place by the visual knowledge of the effects of medicinal plants over the cure of a disease. However, they are unknown about the probable mechanism and compound which is actually beneficial in the remedy of disease. It also leaves with the toxicity issues as the same parts can be toxic to the human body if ingested or applied in larger amount. This is the reason of the existence of Phytochemistry giving a core idea of the compounds that have therapeutic benefit in the cure and management of disease.

In addition to their pharmacological and therapeutical importance, medicinal plants also play a highly important role in the economy. Medicinal plants are cheap and easy to access to these people who knows it really well. The bioactive compounds deposited in medicinal plants can serve as important source for raw materials for pharmaceutical manufacturing for commercial purposes. They comprise a precious asset to the country and its healthcare system. A significant initiative in the improvement of stature of public health of the country can be achieved through proper scientific investigation of these therapeutically active compounds. Revenues can be earned even by exporting to other countries as well. Medicinal plant amounting more than 500 with established scientific claim are present in Bangladesh (Ghani, 2003). Plants used in the traditional system of medicine are in extensive set of practice in preparation of unani, ayurvedic and homeopathic medicines of Bangladesh.

According to survey conducted in 1990 in different villages of Bangladesh it was found out that on an average of 14% people suffering from disease consult with qualified doctors, 29% consult with unqualified village doctors, 10% consult with local moulovis, 29% consult with quacks and 19% consult with homeopaths. The statistics clearly indicate the extensive use of medicinal plants, most of them in crude and substandard form. Traditional medicines are still manufactured in our country using the age-old, unscientific methods. Ayurvedic and Unani system of medicine houses a vast number of indigenous plants in different preparations intended for commercial purposes without proper parameters of standardization, quality control, evaluation and determination of chemical nature, investigation of the pharmacological and toxicological aspects of the active components in order to maximum utilization of their therapeutic potential. Innovation and discovery of newer and advanced technologies is bringing the issue of drug safety and toxicity in a more transparent manner as the day goes by. The overall cost of country's healthcare system can be minimized to a great extent by providing the mass with medicaments at

low cost with higher degree of quality and development of standard drug from abundant natural sources can serve as an important initiative to the cause. Thus, priorities should be given on research and development on traditional drugs in order to develop the stature of public health of the country. Another important fact for the emphasis on traditional drug is the import of large number of pharmaceutical raw materials including medicinal plants and semi-processed plant product where a huge amount of money is spent.

1.2. History of Medicinal Plants: Brief Review

Plants have formed the basis of sophisticated approach of traditional approach of medicine existent for over thousands of years, preferably in China (Chang et al, 1986) and in India (Kapoor et al, 1990), being the first of the illustrated book regarding gathering, preparation and use of medicinal plants was written by over 3000 years ago by Chinese emperor shen nung (Summner, 2003). Herbal drug development have been heavily contributed by the Greeks as well. The book named 'De Materia Medica' was written by Dioscorides, the Greek physician (100 AD), describing more than 600 medicinal plants (Samuelsson, 1999). Only Arabs have been preserved the expertise provided by the Greeks in the field of herbal medicine, during the dark and middle ages. Utilizing their own resources, the Arabs have merged together the greek, roman, Chinese and Indian herbs and contributed heavily in the development of the science (Summner, 2003). In India, proven track record of ayurvedic medicines is over 5000 years and forms part of the National Health Service, offered alongside conventional medicine. The Ayurvedic national formulary list contains some 8000 well proven ayurvedic formulation described in dravyaguna (ayurvedic pharmacology). About 25% of the currently used crude drugs with another 25% derived from chemically altered natural product have been provided by herbal medicine (Chitme et al, 2009). They have been utilized to heal ailments from alleviating headache to treating heart diseases (Samuelsson, 1999). Plant based approach in traditional medicines have been extensively documented in many other cultures as well.

Plant based system of medicine play an important role in primary healthcare. A significant role in primary healthcare services to rural people is provided by medicinal plants and used by 80% of the marginal communities of the world as estimated by World Health Organization (Wood-Sheldon et al; Schultes et al, 1990; Prajapati and Prajapati 2002; Latif et al 2003; Shinwari et al 2006). For the remaining 20% population, mainly residing in the developed countries, medicinal products obtained from plants plays a vital role in their healthcare system (Arvigo et al, 1993). Studies shows 119 chemical substances at least obtained 90 plant species are of primary importance in terms of use in the healthcare system of one or more countries. Among them, 74% were discovered as a result of studies directed at the isolation of the active substances from plants used in traditional medicine (Arvigo et al, 1993). Another study shows that nearly 50% of the medicines present in the market are made of natural basic materials (Thomas, 1995).

The shift from using ordinary herbal medicines with the advent of newer technologies to synthesize medicaments for commercial purposes, in brief, modern pharmaceutical was started just in 1800. The transfer was started by isolation of pure compounds from plants. The first important discovery achieved in this approach is the purification of morphine from opium dated in 1805. Another monumental success was the isolation of salicylic acid from the barks of willow, motivated Hoffman to synthesize aspirin in 1897. Another notable discovery was the isolation of Ephedrine from Chinese herb mahaung (Ephedra) in 1887. While the antimalarial sesquiterpene, Artemisine was discovered from a Chinese medicinal herb *Artemisia annua* (Sweet wormwood) (Fan et al, 2006). Many of these drugs were used in traditional remedies since ancient times (Klaymann 1985, Clark, 1996). Forklosin is the anti-hypertensive agent from *Coleus forskholli* Briq. (Labiatae), the plant whose use was mentioned in the ancient Hindu Ayurvedic scripts (Bhat et al, 1977).

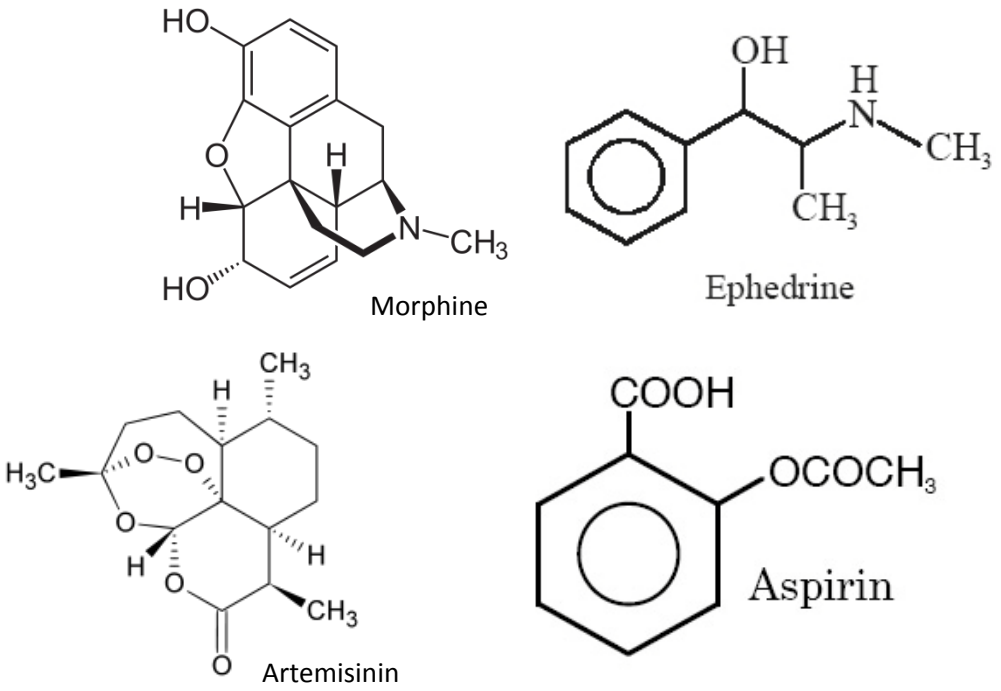


Fig 1.1: Structure of Morphine, Ephedrine, Artemisine, Aspirin

Paclitaxel, a drug isolated from *Taxus brevifolia* is the most recent important example of a natural product having enormous impact on medicine. It interacts with tubulin during the mitotic phase of the cell cycle, preventing the disassembly of microtubules and thereby interrupts the cell division (Wani et al, 1991). The original target disease intended for the compound wee ovarian and breast cancer, now a days it is also used to treat a number of other human tissue proliferating diseases as well. (Strobel et al, 2004). Another milestone discovery in the treatment of cancer is vinca alkaloids, namely vincristine and vinblastine from *Caltharanthus roseus*.

While other notable compounds are etoposide and teniposide which are epimers of podophylotoxin isolated from roots of various species of the genus podophyllum and camptothecin isolated from the Chinese tree *Camptotheca acuminate* (Newman et al, 2000). Natural product were considered as mainstay in cancer treatment owing to the fact that 60% of the worldwide anticancer drugs between 1983 and 1994 were from natural origin (Cragg et al, 1997) while another study shows that paclitaxel and camptothecin were estimated to account for nearly 1/3rd of the global anticancer market or about $3 billion of S9billion in total annually in 2002 (Oberlies and Kroll, 2004). Studies conducted by Eli Lilly & Company shows the potentionally of 40 plants out of 200 plants with antineoplastic activity screened randomly show the presence of these anticancer compounds. Phytochemicals, the pharmacologically important compound is present in each of the medicinal plants in addition to their own nutrient composition. These are nutrients and bio chemicals like carbohydrates, fats and protein playing an important role in satisfying human need for energy and life processes (Novak and Haslberger, 2000).

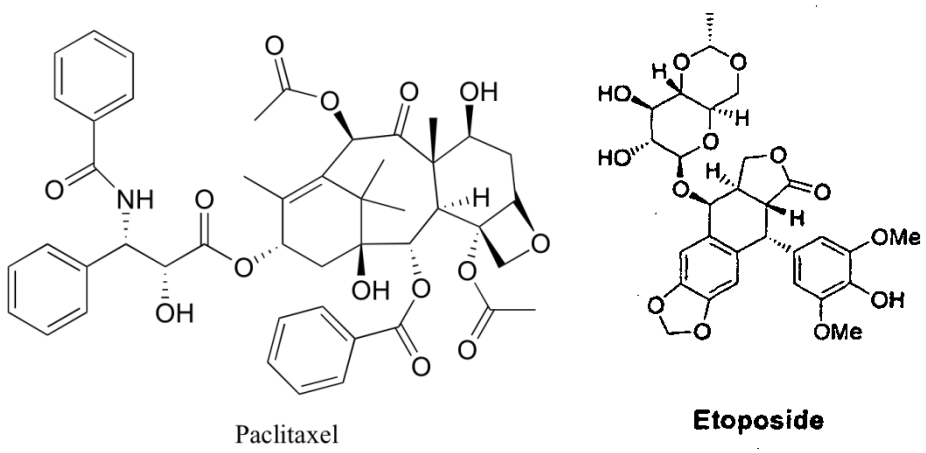


Fig 1.2: Structure of Paclitaxel & Etoposide

The interesting query may run through the mind that even though now-a-days, the isolated compound can be produced synthetically and semi-synthetic drugs are present in the modern healthcare system which can have the possibility of modifying the basic structure of the therapeutically important compound increasing its efficacy and minimizing its side effect, still the interest on the plant based compound has not been decreased rather in some of the cases it has been increased many folds. The increased research in finding plants products that will enhance healthcare systems is due in part to adverse side of conventional drugs (Iwuji and Herbert, 2012), drift towards consuming 'natural products' as opposed to synthetics as well as the increasing awareness of the beneficial effects of the natural products (Duke, 1990; Deug and Graham, 2004; Jacobs et al, 2001). In addition to that emergence of resistant or multiresistant to major class of antibiotics (Karaman et al, 2003), high cost and adverse side effects commonly associated such hypersensitivity, allergic reaction, immunosupression etc. are the major burning

global issues in the treatment of burden of infectious diseases (Schinor et al, 2007; Chandran et al, 2013).

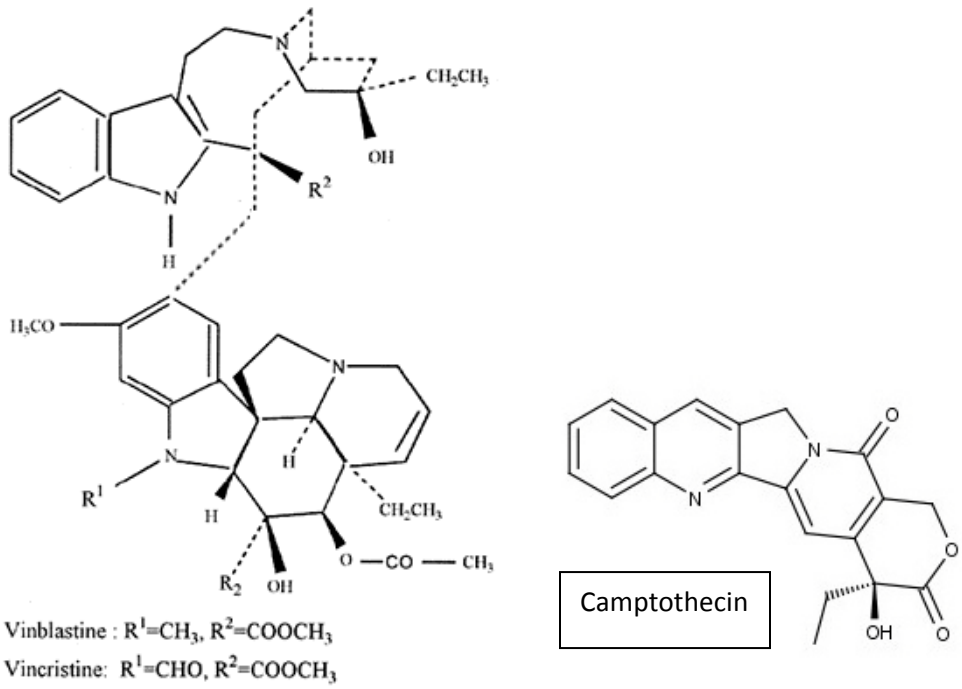


Fig 1.3: Structure of vinka alkaloid and camptothecin

The world is still researching for newer and better ways of treatment with minimized side effect with a higher degree of drug efficacy in order to lower mortality, increase primary healthcare facilities to newer and emerging diseases to the present and advancing world. Even the use & dependency on medicinal plant has been existent for thousands of years, the science of medicinal plants is still a vastly unknown territory. Scholar estimate that 5% of the 2,50,000 species of plants have been investigated (Samuelsson, 1999). The paradigm of healthcare is shifting so rapidly in recent times with breakthrough discoveries, innovation leading to newer and improved product, outdating the present and existent medicaments for treatment, a projection can be made in the increase of isolation of compounds from natural product will be having upward trend in the near future. Thus, the investigation on plant products is still continuing across the globe even today for the generation and isolation of pure compounds to be used for the treatment of both existent and emerging diseases in a degree of higher efficacy and low degree of adverse effects and toxicity.

1.3. Importance of plant derived compounds for therapeutics:

Plants constitute a rich source of secondary metabolites and novel therapeutic compounds naturally to enhance human health with controlled adverse effect (Jin-Mang et al, 2003). Natural products play a vital role in pharmacological and commercial industries, produce a lot of healthcare and medicinal products such as antimicrobial, anti-tumour agent and hepatotoxic, cardiotonic, CNS stimulants, nutraceutical, sweeteners, food additives and animal feed (Gortzi et al, 2008; Verma et al, 2009). Various plants like herbs, trees shrubs and climbers are investigated for their various bioactive compounds for human health (Ezzatzadeh et al, 2012). In addition to that, important bioactive clusters such as alkaloid, flavonoids, saponin, terpenoids, polysaccharides and tannins present in plants are largely contributing to various biological activites in traditional and modern therapeutic principles (Doughari et al, 2012; Mahboobi et al, 2013). The pharmacologically important are separated and isolated by using solvents of polarity of different degrees like methanol, chloroform, ethyl acetate. Studies conducted by Williamson in 2011 finds out that crude total extracted compounds shows significant result in disease management than those of a single isolated active fraction or purified specific constituents. The interaction of various groups of active metabolites in the extract may have amplified the therapeutic effect more than the single ingredient which has a direct link over a particular pharmacological activity (Mohammad et al, 2010).

1.4. Research on medicinal plant: Biological activity guided approach

Natural product, in precise, still serves as the molecular basis in addition to their potentiality for drug discovery and development studies. Compounds present in the plant contain molecular skeletons of high interest for breakthrough discovery. In the present world of healthcare revolution, biological activity guided approach which work on the phytochemical investigation of medicinal plant have the great possibility of yielding new compound of remarkable interest in the cure, prevention and management of new and emerging diseases. The introduction of newer technologies bringing the safety and toxicological issues of using plant extract that is herbal medicine as medicine for the cure and management of disease into light, extensive phytochemical investigation, biological activity screening and isolation of pure compounds have expanded from the position of necessity to requirement for the cure and management of disease with better drug efficacy with the minimization of side effects. The age of healthcare revolution has brought monumental changes in the effective and rapid phytochemical investigation of plant extract and these events have improved the cause of bringing newer molecules into light which serves as the basis of improved and newer classes of molecules.

Bioactivity guided phytochemical approach contain several notable phases of investigation. These are as follows:

a. Firstly, separation of parts which can be utilized as a basis of the presence of compound of high interests

b. Secondly, extraction of selected parts of plant material using various solvent, primarily obtaining the crude extract and secondarily separation of fraction using solvents of different degree of polarity

c. Thirdly, bioassay systems both in vitro and in vivo to be designed in order to determine the potentially in the cure and management of disease and healthcare complications

d. Fourthly, isolation and determination of chemical structure of pure compound defining the predicted mechanism in the cure and management of disease.

However, in some of the cases, chemical constituents of notable therapeutic value can be yielded from extensive phytochemical analysis of plant that have no significant therapeutic interest. The probable theory explaining the events may be due to the having the chemical constituents ineffective for therapy that was used traditionally. For instance, a plant extract of *Vinca rosea* which was traditionally used as anti-diabetic drug was found to contain some hypoglycaemic alkaloid in minute amount but it contain vinca alkaloid useful for anti-cancer treatment in higher yield. Another example, *Rawfolia serpentine*, traditionally used for the ailment of variety of illness, the presence of antihypertensive and tranquilizing agent reserpine has been revealed. Thus, we can come into inference that systemic research on medicinal plants can unveil many mysteries of the unknown therapeutic world.

Thus, from the above discussion, we can understand the need of phytochemical analysis of crude extract as a requirement in search of chemical constituents of significant therapeutic interest. This serves not only the basis for screening the biological activities but also can solve several mysteries of the medicines of the traditional world and unveiling new molecules intended for the cure and management of new and emerging disease.

1.5. Study Protocol

The study was designed with an aim to discover the major groups of natural compound and to evaluate the biological activities of the crude extract of the whole plant, *Murdannia nudiflora* and *Tradescantia pallida* (Family: Commelinaceae) and to explore the unknown territories and opportunity of the plant extract in the benefit of global healthcare. Thus the study protocol consisted of the following steps:

1. Successive cold extraction of the powdered dried plant material with methanol as the choice of solvent

2. Phytochemical Evaluation of the crude methanolic extract of the powdered plant material for the identification of the major group of phytochemicals

3. Evaluation of Cytotoxic activity and determination of LC_{50} value of the crude methanolic extract of the powdered plant material using in vitro brine shrimp lethality bioassay

4. Evaluation and Screening of Antioxidant activity and determination of IC_{50} of the crude methanolic extract of the powdered plant using in vitro DPPH Free Radical Scavenging Method

5. Evaluation and Screening of Analgesic Activity of the crude methanolic extract of the powdered plant using in vivo Acetic acid Induced Writhing method on Swiss albino mice

Chapter-2

Plant Review

2. Plant Review

2.1. The plant family: Commelinaceae

The commelinaceae or in other word the spiderwort family is one of the five families belonging to orders Commelinales are herbs comprising about 40 genera and 640 species(Faden, 1998) that are often somewhat succulent, frequently having cymose terminal and/or auxiliary inflorescences sometimes subtended by a boat-shaped spathe. The leaves are simple, alternate, parallel-veined in terms of general appearanceand usually with a close sheating base that is often enclosed around swollen nodes that produce a 'jointed stem'. The flower are bisexual and actinomorphic or commonly slightly to strongly zygomorhpic, trimerous in nature. There are 6 tepals, in 2 whorls of 3, outer tepals mostly green and uniform while the inner tepals are usually free, equal or unequal, white, pink, blue or purple. Usually the perinath is of two differentiated series. The calyx consists of usually 3 distinct herbaceous sepal while the corolla is usually of 3 equal or sometimes unequal, distinct, deliquescent petals. The androceum typically comprises six distinct stamens but commonly three or sometimes more are reduced to staminodes, the gynaecium consists of a single simple pistil of three carpals, a single style and a superior ovary containing three or occasionally by abortion only two locales, each containing one few axile ovules. Usually, the fruit is a loculicidal capsule or sometimes indehiscent.

A characteristic vegetative 'fleshy' aspect of the plants of this family often includes a mucilaginous slimy sap which upon exposure to air forms a weblike mass upon drying which gives the motivation for this name which is spiderwort family. These plants are distributed widely around the tropical regions of the world. The species are mostly grown horticultural and they are mainly utilized as ornamental plants. These plants are specifically common throughout the Caribbean, North and Latin America, Africa, Asia, the Middle East and parts of Oceania (Faden, 1993; Holm et al, 1977; Standish et al, 2002). Recent data shows that Commelinaceae family contain 23 genera and at least 225 species native to or naturalized in the New World and 23 genera and about 200 species in the Neopetropics (Hong et al, 2009) and 170 species of Commelina in the warmer regions of the world and 50 species of Murdannia occurring in the tropics and warm temperate regions around the world with the greatest diversity being in the Tropical Asia (Fish et al, 2000).

2.2. Taxonomical Classification of Commelinaceae:

Kingdom-Plantae

Subkingdom-Tracheobionta

Super division- Spermatophyta

Division-Magnoliophyta

Class-Liliopsida

Subclass- Commelinidae

Order- Commelinales

Family-Commelinaceae

Fig 2.1: Taxonomy of Commelinaceae

2.3. Role of Commelinaceae plants in traditional medicine:

Sl. No.	Name of the plant	Traditional Uses	References
1.	*Anelima aequinoctiale*	Treatment of cough in Akwa Ibom State, Nigeria	Akpabio et al, 2013
2.	*Calisia fragrans*	Leaves used for skin disease, burns and joint disorder; Used to treat burns, arthritis, skin and oncological diseases, tuberculosis and asthma	Cherenko et al, 2007
3.	*Commelina nudiflora*	The whole green plant extract was used by the Chinese to chill the blood and for blood clotting function	Kaur and Das, 2011
		Used for the treatment of chronic disease like diabetes, skin disease and atherosclerosis; used in the relieve swelling of groin. The plant was used in Sierra Leone for dressing after circumsion, while as tea and medicinal bath, Carribean used it for the treatment of influenza; In Mexico for the ailment of conjunctivitis, dermatitis, dysmenorrhoea.	Wendy & Braithwaite, 2007; Ujowundu et al ,2008
4.	*Commelina diffusa*	Chinese traditional medicine used the plant for the reduction of swelling; used in	Khan et al , 2011

		the treatment of infection occurred urinary and respiratory tract, and other diseases like diarrhoea, enteritis and haemorrhoids; while the crushed leaf is applied on boil, abscesses, wounds and on pain joints; used also in dermatitis, burns, stings of the insects and bites of the snake. Also used in the treatment of tuberculosis, veneral disease, oitits, malaria, leprosy, dysentery and heart problems; Whole plant is used on itches	
5.	*Commelina benghalensis*	Folk medicine utilized the plant for the treatment of leprosy, headache, fever, constipation, jaundice, mouth thrush, snake bite, inflammation of the conjunctiva, psychosis, nose blockage in children, insanity, exophthalmia	Tabuti JR, 2003
6.	*Murdannia loriforms*	Chinese practitioners used it as a remedy for cancer and treating other disease including cold, throat infections, pneumonia, diabetes mellitus, flu and inflammation like inflamed wound	Jiratchariyakul et al, 2006
7.	*Murdannia bracteata*	Used in the treatment of hepatitis, stomatitis, pneumonia, nephritis and many other inflammatory disease	Chiu and Chang, 1995
8.	*Murdannia nudiflora*	Traditionally used for the treatment of asthma, leprosy and piles, stomach complaints, giddiness, astringent. Root paste with the mixture of goat milk is prescribed orally to cure asthma, whole plant paste with common salt is applied on the affected areas of leprosy	Panda and Mishra, 2011
9.	*Palisota hirsute*	In Ghana, whole plant is used for the treatment of stomach pain and while the saps obtained from the roasted leaves are applied over the lumbar region for kidney pains. Leaf infusion or poultice is taken orally or applied locally for piles. In Nigeria, the Igbos of Obkompia prepares an ointment from the plant for gunshot wounds and healing. The leaf is used as aphrodisiac	Dokosi, 1998; Benson et al, 2008
10.	*Tradescantia pallida*	They have the ability to remove volatile organic compound from the air, used as an anti-inflammatory as well as anti-toxic	Yang et al, 2009; Li, 2006

		supplement; used to improve blood circulation	
11.	*Tradecantia zebrine*	Used to treat gastrointestinal disorders	Amaral et al, 2006
12.	*Murdannia edulis*	Dried root is used as aphrodisiac	Alviya et al, 2011
13.	*Tradescantia pendula*	The leaves are used in the treatment of hyperglycaemia	Andrade-Cetto, Heinrich, 2005
14.	*Tradescantia spathaceae*	Leaves are used for the treatment of nervios, in Mexico	Azcarranga-Rosette, 2004
15.	*Commelina erecta*	Whole plant has been used for the treatment of giardiasis; inflorescence used for conjunctivitis	Calzzada et al, 1998b; Agra et al ,2007
16.	*Palisota ambigua*	Leaf is used as anticonvulsant agent	N'gouemeo et al ,1994
17.	*Commelina clavata*	Water accumulated at the base of the bract is collected and administered for eye pain	Sindhu et al, 2012
18.	*Commelina sinesis*	Inflorescence used for conjunctivitis	Klauss and Adala, 1994
19.	*Commelina paludos*	Paste is prepared from roots and applied externally on boil	
20.	*Steptolirion volubile*	Crushed leaves are applied on wound of important organs like ear, nose and navel	
21.	*Zebrina pendula*	Infusion of leaf and stem used in the treatment of diabetes	Madaleno, 2012
22.	*Aneilema conspicuum*	Used as an antifertility agent	De Laszlo and Henshaw, 1954
23.	*Aneilema scapiflorum*	Used as an antifertility agent	De Laszlo and Henshaw, 1954
24.	*Rhoeo discolor/ Rhoeo spathacea*	The decoction of leaves is daily free-consumed as curative of cancer; extracts appear to improve the quality of skin; powder of the aerial part is used in the treatment of arthritis; boiled leaves are used for chronic inflammation	Del Amo, 1979; Argueta and Cano, 1994; Meybeck et al, 1999; Perez, 1996
25.	*Commelina communis*	Used for fever, bleeding, diarrheal and snake bite; plant juice is used in mumps	Handbook of Chinese traditional medicine
26.	*Commelina stephaniniana*	Extracting the creamy sap and used as an ointment for skin fungus around the neck and face	Belayneh and Bussa, 2014
27.	*Aneilema vaginatum*	Used for the treatment of Broken bones	
28.	*Commelina virgina*	Used for Hypertension	Mans et al, 2010

29.	*Palisota tracteosa*	Leaves are used as infusion to stop bleeding	Abondo et al, 1991
30.	*Amischotolype griffithii*	Leaves are used for cleaning the eye	Diba et al, 2013
31.	*Cyantois tuberose*	Tubers are eaten to relieve cough	Murthy, 2012
32.	*Cyanotis villosa*	Stem paste is applied on wounds	Patil et al, 2009
33.	*Cyanotis arachnoida*	Whole plant is used in the treatment of rheumatic disorder	Sharmila et al, 2014
34.	*Callisia monandra*	Leaves and team in used in the treatment of fractured bone and rheumatism	Hoffman and Gallaher, 2007
35.	*Tradescantia pendula*	Boiled aerial part used for diarrhoea	Hernandez et ,2003
36.	*Tradescantia spathacea*	Used in Thai folk medicine to relieve fever, cough and bronchitis	Sriwanthan et al, 2007
37.	*Amischotolype mollissima*	Fresh leaf paste of plants with onion extract is used to treat malaria	Rahman et al ,2007
38.	*Commelina paludosa*	Leaf extract is used to treat dysentery	Rahman et al, 2007
39.	*Commelina Africana*	Used for the treatment of wound	
40.	*Stanfieldiella imperforata*	Powder of leaves are used in wound dressing, headache and as antihelminthic	Jiofack et al, 2010
41.	*Aneilema spekei*	Used in the prevention of fever	Kiringe, 2006
42.	*Commelina Longicaulis*	Used in the treatment of gonorrhoea; Crushed powder applied as dressing for fresh cuts and used in baths for ulcers; Juice is used as an eye lotion; Pounded leaves mixed Piper nigrum and other plants to poultice to swelling in the groin	
43.	*Cyanotis cristata*	Leaf used for the treatment of diabetes	Devi et al, 2011

Table 2.1. : Role of Commelinaceae in traditional medicine

2.4. The plant: *Murdannia nudiflora*

(Kew Bull 7:189. 1952) (Zhu Ye, LHS)

Common Name: Dove weed, Baghnokh.

2.4.1. Taxonomy of *Murdannia Nudiflora*

Murdannia nudiflora (L.) Brenan is one of only two recognized species of the genus Murdannia, the other being *Murdannia spirata* (L.) G. Bruckn. Both of the plants were previously included in the genus Commelina before being transferred. Another species of Murdannia genus, *Murdannia keisak* (Hassk). Hand-Mazz has been transferred to the closely related genus Aneilema (cabi.org). Commelina *nudiflora* is correctly termed as a synonym of *Murdannia nudiflora*, even though it is occasionally used as synonyms of *Commelina diffusa* Burm. F.

Domain: Eukaryota

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Monocotyledonae

Order: Commelinales

Family: Commelinaceae

Genus: Murdannia

Speices: *Murdannia nudflora*

Fig 2.2: Taxonomic Tree of *Murdannia Nudiflora* (cabi.org)

2.4.2. Description of the plant

The herbs are annual. Roots are fibrous, slender; diameter is less than 0.3mm, glabrous or tomentose. Rhizomes are absent. Stems are diffuse, numerous in terms of number, either simple or branched, creeping proximally, 10-50cm, glabrous. Leaves nearly all cauline, sometimes 1 or 2 basal; leaf sheath mostly less than 10mm, hirsute throughout, sometime glabrous except for a hirsute line along mouth slit; leaf blade is either linear or lanceolate, 2.5-10x0.5-1cm, glabrous or sparsely hispid on both of the surfaces, apex is either obtuse or acuminate. Cicinni several, in terminal panicles, or solitary, with several densely arranged flowers; peduncle slender upto 4cm;

proximal involucral bracts leaflike but smaller than leaves, distal ones less than 10mm; bracts caduceus; pedicles slender, straight, 3-5mm. Sepals ovate-elliptic, ca, 3mm. Petals purple in color, obovate-orbicular in terms of shape, Fertile stamens is 2; filaments bearded proximally; staminodes 2-4 in number; antherodes 3-sect. Capsule is ovoid-globose, trigonous in shape, 3-4mm in size. Seed 2 per valve, yellow-brown, deeply pitted or shallowly pitted and radicate white verrucose. Flowers occur in June to August and Fruit occur in September to October.

(a)Whole Plant

(b) Leaves with stem

(d) Roots

(e) Seeds

Fig 2.3: *Murdannia Nudiflora*

2.4.3. Distribution:

The plant is distributed mainly in wet places by water, rarely among grass; low elevation (up to 1500m in Yunnan). Present in Bhutan, Cambodia, Nepal, Pakistan, India, Indonesia, Japan, Laos, Malaysia, Myanmar, New Guinea, Philippines, Sikkim, Sri Lanka, Bangladesh, Brunei; Indian Ocean and Pacific Islands; Angola, Benin, Burkina Faso, Burundi, Cameroon, Congo, Gambia, Guinea, Kenya, Mauritius, Nigeria, Sierra Leone, South Africa, Tanzania, Uganda ; Mexico, USA, Costa Rica, Haiti, Puerto Rico, Trinidad and Tobago; Colombia, Suriname, Guyana, French Guyana, Venezuela (Rojas et al ,2002, Baker & Zettler, 1988, Holm et al, 1977, USDA-NRCS, 2002, Galinato et al, 1999; Waterhouse, 1993; Dangol, 2001; Karim 2003).

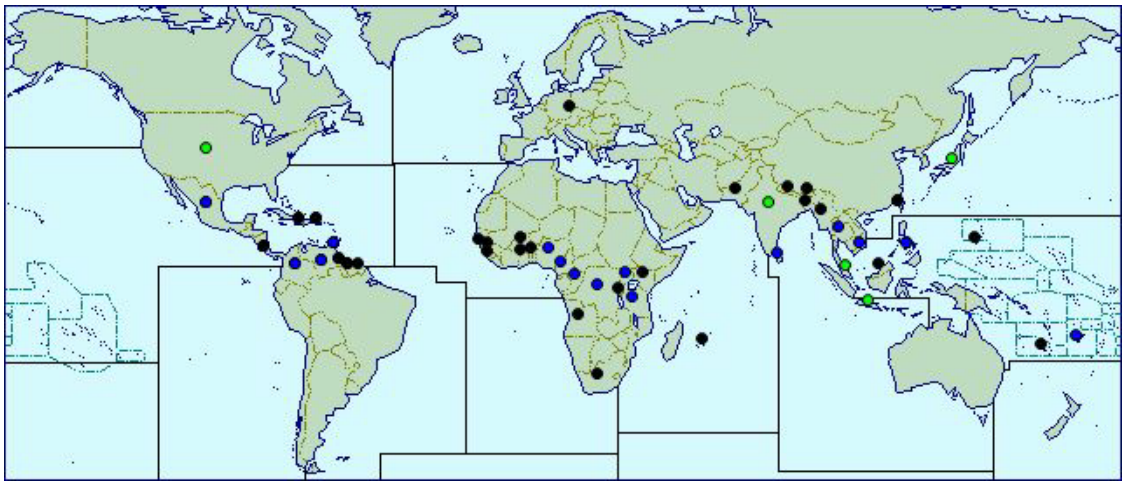


Fig 2.4.: Distribution of Murdannia Nudiflora (cabi.org)

2.4.3. Uses of *Murdannia Nudiflora*

Murdannia Nudiflora has been traditionally used in the cure of asthma, leprosy and piles, stomach complaints, giddiness and as astringent. The root paste mixed with goat milk has been prescribed orally for the cure of asthma, while the paste of the whole plant with common salt is applied on the areas affected to cure leprosy (Panda and Mishra, 2011). The plant has also been used as fodder for animals in several countries, but Holm et al (1977) considered that the presence of high moisture content represent forage of lower value. It is eaten also by the people as a vegetable. In India during famine as it was considered as a palatable vegetable elsewhere and the leaves as poultice in Indonesia (Holm et al, 1977; Soerjani et al, 1987). The leaves are made into paste in order to obtain immediate stoppage of heavy bleeding in the wounded area.

2.4.5. Background of scientific work

Murdannia nudiflora contain significant group of phytochemicals like tannins, flavonoids, saponins, and alkaloids during its preliminary phytochemical studies conducted by Patwari et al, 2014. The same study also conducted the analgesic effect of the plant and was found out to have significant analgesic effect ($P<0.05$,$P<0.01$) in comparison to the standard which is morphine sulphate since the study to evaluate analgesic effect was done using hot plate method of analgesic evaluation.

2.5. The plant: Tradescantia pallida

(Rose) (DR Hunt, 1975)

Common Name: Purple Queen; Purple Heart; spider lily; wandering Jew

2.5.1. Taxonomy of Tradescantia pallida

The genus Tradescantia is native to the New World tropics and includes about 70 species distributed from Canada to northern-Argentina (USDA-ARS, 2012). Many species within the genus are economically important in the nursery and landscape and are widely commercialized as ornamentals and houseplants (Anderson and Hubricht, 1938). First collected in 1907 by E. Palmer in Tamaulipas, Mexico and in 1911, JN Rose formally described it as *Setcreasea pallida*. DR Hunt in 1975 published in the currently accepted name of *Tradescantia pallida* in the new bulletin.

Domain: Eukaryota

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Monocotyledonae

Order: Commelinales

Family: Commelinaceae

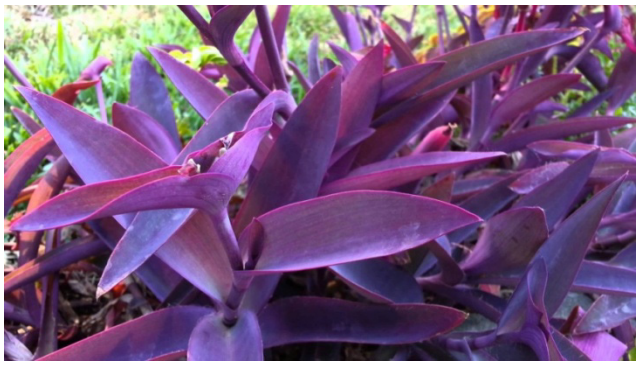
Genus: Tradescantia

Species: *Tradescantia pallida*

Fig 2.5: Taxonomic tree of *Tradescantia pallida* (cabi.org)

4.5.2. Description of plant

Perennial herb with stems ascending with elongate decumbent and with flowering sub-erect brances. Sheaths clasping leaves, ciliate, 1-2.5cm long; oblong blades of 10-18cm long and 2-3.5cm broad, acute at apex where upper one being somewhat smaller. Stems and upper surface of leaves are deep royal in color which becomes suffused with a faint dusty turquoise gunmetal undertone when the foliage grows older while the undersides of the leaves are vivd violet shading towards pink in the place where the petioles clasp and encircle the stem. Inflorescences are present in upper leaf axils and terminal in shape; peduncles mostly 3-10 cm long. Flowers in small densely cymose clusters subtended by 2 or 3 bracts, these similar to leaves but smaller; pedicels umbellate, upto 7mm long, pilose toward apex; sepals being 8-10mm long, oblong in shape while the petals ranges from pink to rose purple sized 15-20mm long; 6 stamens with 3 filaments epipetalous and 3 of them are adherent to petal margines, glabrous or variably pubescent. Fruits are glabrous capsules, 3.5mm. Seeds are very small, 2.5-3mm (Hunt, 1975) (cabi.org).



(a) Leaves with stems

(b) Flower

(c) Leaves with nodes and flower

Fig 2.6: *Tradescantia pallida*

2.5.3. Distribution:

Tradescnatia pallida is a native in Mexcio ((USDA-ARS 2012, Govaerts 2012). It is widely grown as an ornamental and houseplant in many regions of the tropical and subtropical climate including Canary Island, North, South and Central America, West Indies, Iberia, South Africa and Mynamnar (USDA-ARS 2012, Govaerts 2012). In Carribean, Canary Island, South Eastern USA, Argentina, Nicaragua and Honduras, the plant has been naturalized.

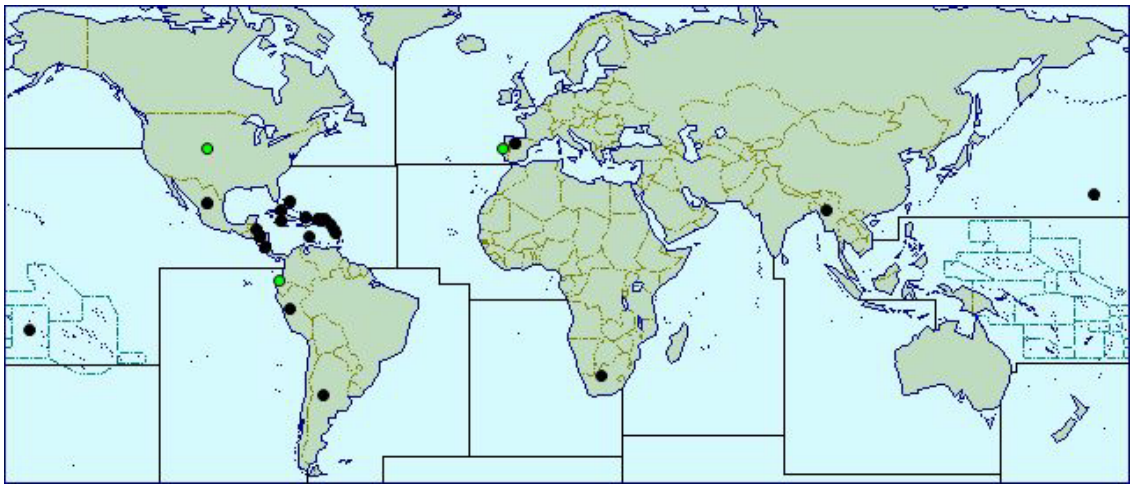


Fig 2.7: Distribution map of Tradescantia pallida (cabi.org)

2.5.4. Uses of the plant

An economically important plant grown in the nursery and landscape trade, it is extensively commercialized as an ornamental plant commonly grown in borders of garden and yards as well as to cover open spaces and used as ground cover in tropical and subtropical regions (Duever, 2006). They are also renowned for their ability of effective removal of volatile organic pollutant from the air (Yang et al, 2009). In Taiwanese traditional medicine, *Tradescantia pallida* has been used as an anti-inflammatory and anti-toxic supplement and used for the improvement of blood circulation (Li, TSC, 2006)

2.5.5. Background of scientific studies

Tradescantia pallida is a plant that has shown high degree of potency in terms of antioxidant potential. Several studies have been conducted in different dimension emphasizing the antioxidant property of the plant. Studies conducted by Lee et al, 2014, shows that the leaf extracts of the plant has 10.6±4mg in terms of Total flavonoids content, 153.1±21.8mg GAE (Gallic acid equivalent)/100mg of total phenolic content, 13.6±2.1 mg TAE (Tannic acid equivalent)/100mg of total tannin content, 103.1±36,9 mg AAE (Ascorbic acid equivalent)/100g of free radical scavenging activity. Another study conducted by Sinha et al, 2014, emphasizing the antioxidant potential in terms of its ability of Chromium accumulation and tolerance, found out that it was able to grow in the presence of chromium concentration 5-20mg of L (01) Cr (VI) in hydrophonic environment and the accumulated chromium content was 408µg/g dry weight after 30 days of culture and 536µg/g dry weight after 60 days of culture. The plant is also a good bioindicator of air pollutant. According to studies conducted by Rhoden et al, 2008, found out the less presence of different trace element and air pollutant in rural areas than in urban areas after 20 days of culture. The study conducted by Lee et al, 2014 also showed anti-bacterial potential of the plant against various micro-organisms having a zone of inhibition ranging 5-10mm in presence of different types of bacteria. However, the same study showed a zone of inhibition of more than 10mm in presence of Staphylococcus epidermis. One study conducted by Shi et al, 1995 showed the isolation of two anthocyanidin, a group of flavonoids, one was cyanidin-g-3,7,3'-triglucoside with 3 molecules of ferulic acid and an extra terminal glucose while the other pigment lack the terminal glucose. Their potential as food pigment was investigated and it was found out to have potential of some significance.

Chapter-3

Materials and Methods

3.1. Materials:

3.1.1. Extraction and purification

3.1.2. Phytochemical Evaluation

3.1.3. Pharmacological Investigation

3.2. Methodology:

3.2.1. Experimental plant:

3.2.2. Collection and Identification of the Plant Sample:

3.2.3. Drying of the plant material

3.2.4. Grinding of plant material and preparation of powdered plant material

3.2.5. Extraction and filtration of the powdered plant material

3.2.6. Evaporation of the solvent and collection of crude plant extract:

3. Materials and Methods

3.1. Materials:

3.1.1. Extraction and purification

A. Materials Required

Sl. No.	Material	Source
1.	Amber Glass Jar (2litres bottle)	Used Reagent Bottle
2.	Beakers (1 litre)	Glassco Laboratory Equipments, UK Borosil, Germany
3.	Beakers (500ml)	Glassco Laboratory Equipments,UK Pyrex, UK
4.	Funnel	Borosil, Germany Glassco Laboratory Equipments, UK
5.	Filter Paper	Whatnam Filter Paper
6.	Beaker (100ml)	Glassco Laboratory Equipments, UK
7.	Beaker (40ml)	Borosil, Germany
8.	Beaker (25ml)	Borosil, Germany
9.	Glass rod	-
10.	Pasteur pipette	-
11.	Dropper	-
12.	Stand with clamp	-
13.	Syringe (3ml)	JMI Industries Limited, Bangladesh
14.	Measuring cylinder (1litre)	Glassco Laboratory Equipment, UK

Table 3.1: Material required for extraction

B. Equipments Required

Sl. No.	Name of the Equipments	Source
1.	Rotary Evaporator	Heidolph Rota-Vap, Germany
2.	Electronic Balance	Shimadzu Corporation Limited, Japan
3.	Oven	MMM, Germany
4.	Grinding Machine	Kenwood Gourmet, Kenwood, UK

Table 3.2: Equipment required for extraction

C. Solvents Required

Sl. No.	Name of the Solvent	Source
1.	Methanol	Merck Germany Limited, Germany Active Fine Chemical Company Limted, Bangladesh

Table 3.3: Solvents required for extraction of plant material

3.1.2. Phytochemical Evaluation

A. Materials

Sl. No.	Material
1.	Test Tube
2.	Volumetric Flask (100ml)
3.	Beaker (100ml)
4.	Beaker (10ml)
5.	Beaker (25ml)
6.	Beaker (40ml)
7.	Syringe (3ml)
8.	Pipette
9.	Measuring Cylinder (100ml)
10.	Measuring Cylinder (10ml)
11.	Test tube stands(18 test tube)
12.	Aluminium Foil Paper
13.	Spatula
14.	Stand with clamp
15.	Test tube with Screw cap
16.	Funnel
17.	Filter Paper
18.	Test tube holder

Table 3.4: Material required for phytochemical screening

B. Equipments

Sl. No.	Equipments	Source
1.	Electronic balance	AMD (Advanced Micro Devices) Inc, USA
2.	Electronic Oven	Ecocell, MMM, Germany
3.	Spirit Lamp	-
4.	Distilled Water Plant	University Laboratory
5.	Automatic Vortex Shaker	-

Table 3.5: Equipments required for phytochemical screening

C. Chemicals

Sl. No.	Name of the Chemical	Source
1.	α-napthol	Merck, Germany
2.	Ethanol	Merck, Germany
3.	Copper Sulphate	Sigma Aldrich Chemicals, China
4.	Sulphuric Acid	Merck, Germany
5.	Sodium Potassium Tartarate	JHD Chemical, Guanghua Sci-Tech Limited, China
6.	Sodium Hydroxide (Pellets)	Merck, Germany
7.	Sodium Citrate	Merck, Germany
8.	Sodium Carbonate Anhydrous	Merck Specialities Private Limited, India
9.	Mercuric Iodide	Qualikems Fine Chemical Private Limited, India
10.	Potassium Iodide	Scharlau Lab Chemicals Limited, Spain
11.	Bismuth Nitrate	JHD Chemicals, Guanghua Sci-Tech Limited, China
12.	Tartaric Acid	JHD Chemicals, Guanghua Sci-Tech Limited, China
13.	Picric Acid	Loba Chemiie Limited, India
14.	Potassium Di Chromate	Active Fine Chemicals Limited
15.	Ferric Chloride	Merck, Germany
16.	Lead Acetate	Active Fine Chemicals Limited, Bangladesh
17.	Hydrochloric Acid (Concentrate)	Merck, Germany
18.	Zinc Powder	Merck, Germany
19.	Distilled Water	-

Table 3.6: List of Chemicals for phytochemical screening

Fig 3.1: Chemical Reagents for Phytochemical Investigation

3.1.3. Pharmacological Investigation

3.1.3.1. Test Material

Sl. No.	Test Materials
1.	Methanolic crude extracts of the whole plant of Murdannia nudiflora
2.	Methanolic crude extracts of the whole plant of Tradescantia pallida

Table 3.7: List of Test Material for Pharmacological Studies

3.1.3.2. Material needed for in vitro cytotoxic activity by brime shrimp lethality assay

Sl. No.	Apparatus and Reagents
1.	Artemia salina leach (brine shrimp eggs)
2.	Sea salt
3.	Small tank with perforated dividing dam to hatch the shrimp
4.	Lamp to attract shrimp
5.	Pumps for the supply of oxygen in the sea water
6.	Artificial sea water
7.	Magnifying glass
8.	4 x Beakers (10ml) Borosil
9.	Screw cap test tubes for the test samples
10.	Dimethyl sulphoxide (DMSO), Fischer Chemical Limited, Switzerland
11.	36 X Test tube for evaluation of the study
12.	1ml (40-unit) insulin syringe
13.	Electronic Balance (AND Balance Limited)
14.	Spatula
15.	Micropipette (Eppendodf Limited, Germany) (0-100µl)

Table 3.8: Apparatus and Reagents for Cytotoxicity study

3.1.3.3. Material needed for in vitro antioxidant studies by DPPH Free Radical Scavenging

Sl. No.	Apparatus and Reagents	Source
1.	27 X Test tube	-
2.	3X Screw Cap Test Tube	-
3.	Electronic Balance (upto 4 digit after decimal point)	Shimazdu Corporation Limited, Japan
4.	Methanol	Active Fine Chemical Limited, Bangladesh
5.	2,2-Diphenyl-1-picrylhyrazyl	Sigma Aldrich Limited, China
6.	Ascorbic Acid	Merck Germany Limited, Germany
7.	Spatula	-
8.	5x 3ml insulin syringe	JMI Industries Limited, Bangladesh

9.	UV-Vis Spectrophotometer	Hitachi Corporation, Japan
10.	Micropipette (50-1000µl)	Eppendrorf Limited, Germany
11.	Test tube stand	-

Table 3.9: Material for Antioxidant study by DPPH Free Radical Scavenging

3.1.3.4. In vivo analgesic activity by acetic acid induced writhing method

A. Reagents, Chemicals and Equipments for screening of analgesic activity

Sl. No.	Reagents and Chemicals	Source
1.	Dicholefac Sodium	Square Pharmaceutical Limited
2.	Acetic Acid	Merck, Germany
3.	Tween-80 (as suspending agent)	Merck, Germany
4.	DMSO (as suspending & solubilising agent	Fischer Chemicals AG, Switzerland
5.	Normal saline solution (0.9% NaCl)	Social Marketing Corporation
6.	Sterile disposable syringe (1ml, 100unit)	JMI Industries Limited
7.	Tuberculin syringe with ball shaped end	-
8.	Disposable syringe	JMI Industries Limited
9.	Electronic and Digital Balance	Shimadzu Corporation Limited, Japan
10.	Industrial Gloves	China
11.	White Board Marker	Red Leaf
12.	Beaker (500ml)	Pyrex Laboratory Equipment, UK

Table 3.10: Materials required for Analgesic activity

B. Animals for pharmacological investigation

Swiss albino mice of either sex, aged 4-5 weeks having a mass of 20-30 gm on an average was obtained from the animal house of the department of pharmacy, Jahangirnagar University was used for the study. They were kept in standard environmental condition and fed with standard formulated mice pellets and water.

Animals	Source
Swiss albino mice (4 weeks of age, weight 25-30gm	Department of Pharmacy, Jahangirnagar University, Savar Dhaka

C. Animal feed

Animal feed	Source
Special 'Rat-pellet'	Department of Pharmacy, Jahangirnagar University, Savar, Dhaka

3.2. Methodology:

3.2.1. Experimental plant:

The species that were collected for this study belong to two different genuses, Murdannia and Tradescantia of the same family, which is Commelinaceae.

3.2.2. Collection and Identification of the Plant Sample:

Plant samples, one of them, *Murdannia nudiflora* was collected from villages located in Savar Area, Dhaka in late August 2014 while the other one, *Tradescantia pallida* was collected from the Urbariculture Department, Dhaka city as the plant is not native and used mostly as ornamental plant with essential values in early September 2014. The plant were identified and confirmed by The National Herbarium, Government of Bangladesh located in Mirpur Area. The DACB Accession Number confirming the proper identification of the plants is as follows:

Sl. No.	Name of the plant	DACB Accession Number
1.	*Murdannia Nudiflora*	40297
2.	*Tradescantia pallida*	40299

Table 3.11: Name of the plant with their DACB Accession Number

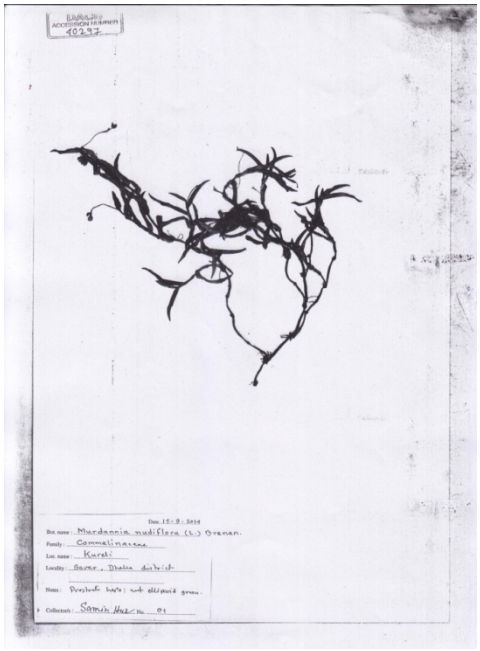


Fig 3.2: Herbarium Sheet of *Murdannia Nudiflora*

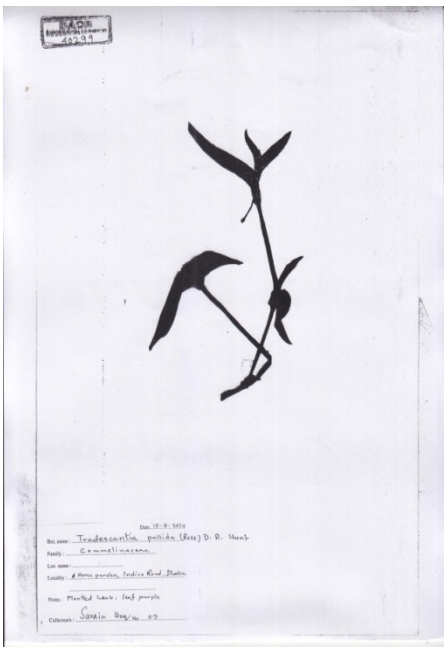


Fig 3.3: Herbarium Sheet of *Tradescantia pallida*

3.2.3. Drying of the plant material

After collection of the plant material, they were divided into small pieces for the facilitation of drying in proper order. They were dried using the process of shed drying for the better identification of the component to be determined for phytochemical screening and to protect the pharmacologically important volatile compound as much as possible. They were exposed to sunlight in a minimum amount required in order to remove their moisture present so that the plants do not dampen under the process of shed drying, The process were continued for about 1.5 months till the plant material were absolutely dried in order to facilitate their proper grinding into powdered plant material as the plant material has been made completely devoid of any water content present.

3.2.4. Grinding of plant material and preparation of powdered plant material

The grinding of the plant material was completed using Kenwood Gourmet Mixing Machine after the plants had been completely dried and visual inspection were completed in order to ensure that there is no bound water and moisture left inside the plant material. The grinding was continued until the plant materials have been completely turned into powder form. The process was continued for 1 whole day in order to complete grinding of the whole plant of the chosen plant specimen

Fig 3.4: Kenwood Gourmet for grinding plant material

3.2.5. Extraction and filtration of the powdered plant material

About 325gm of the powdered plant materials of the whole plant of *Murdannia nudiflora* and 265gm of the powdered plant material of the whole plant of *Tradescantia pallida* was placed in four large amber bottle jar of used reagent (prepared for use after the complete cleaning of the jar in order to remove any scope of contamination of the solvent that was kept) of 2 litres in such a

way that each of the jar can contain not less than 120gm of powdered plant material. Each of the jars were filled with 1 litre of methanol (Merck, GmBh, Germany & Active Fine Chemicals Limited, Bangladesh) in order to facilitate the dissolution of the powdered plant materials and they were kept in such a fashion so that the plant material remain dissolved in the solvent. The jars were kept in this fashion for a week. The whole mixture were filtered through double filtration method, the first filtration process were completed using cotton filter cloth in order to remove the larger solid material and to collect the dissolved plant material while the second filtration process was completed using Whatnam filter paper (Extra Large Size) for the removal of fine particle present in the dissolved plant material in order to collect the purified dissolved plant material. The process was repeated using similar amount of solvent for another three days and same method of filtration to obtain 2nd round of extract.

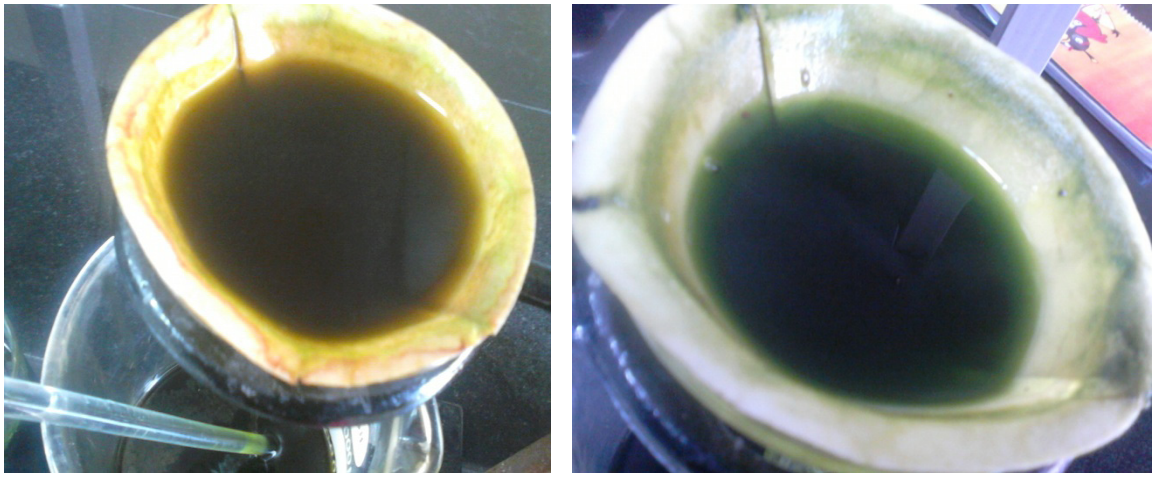


Fig 3.5: Process of filtration of dissolved plant materials

3.2.6. Evaporation of the solvent and collection of crude plant extract:

The concentration of the dissolved plant material after completing double filtration process was carried out using Heidolph Rota-Vap 1500 Rotary Evaporator (Heidolph Laboratory Equipment, Germany) for two hours for each of the plant materials. The water bath temperature condition of the bowl was set at various temperature ranges at 35° to 50°c, while the rotation of the evaporator was set at various speeds ranging between 80 to 100 rpm. The process was continued until the volume of the dissolved plant material was reduced to 150ml.

Then, the concentrated dissolved plant material was left in dried air in order to dry the dissolved methanol and to collect the crude plant extract. In the process, some part of the dissolved liquid was separated into another beaker for the rapid evaporation of the solvent using sterile disposable syringe. Thus, after leaving for open drying for another 10-15, the resultant crude extract of powdered plant material obtained which weighed about 25g.

Fig 3.6: Evaporation of solvent using Rotary Evaporator

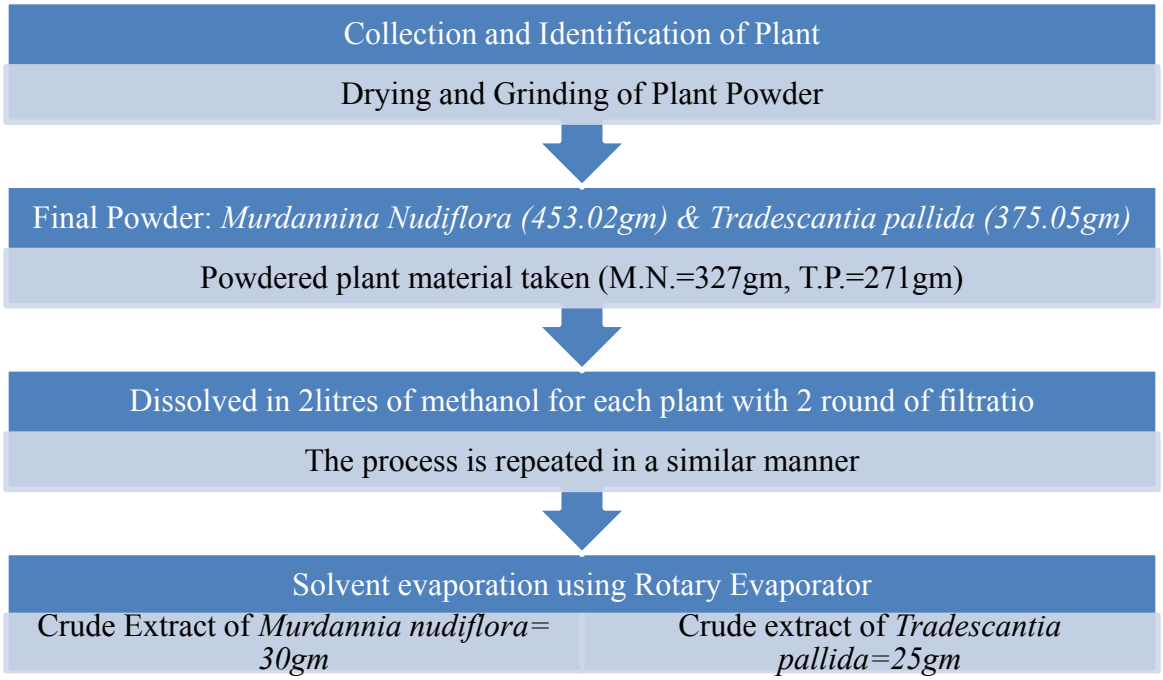


Fig 3.7: Flow chart of process of extraction

Chapter-4

Phytochemical Evaluation of Crude Extract of Plant

4.1.1. Alkaloids

4.1.2. Terpenoids

4.1.3. Flavonoids:

4.1.4.Saponins:

4.1.4. Tanins:

4.2. Principles:

4.3. Preparation of reagent used for different chemical group test

4.4. Procedures for conducting the test of phytochemicals:

4.4.1. Test for Carbohydrates:

4.4.2. Tests for Tannins

4.4.3. Test for Flavonoids

4.4.4. Test for Saponins

4.4.5. Test for Proteins

4.4.7. Tests for Alkaloids

4. Phytochemical Evaluation of Crude Extract of Plant

The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important one among them is the bioactive compounds of plants which are alkaloids, flavonoids, tannins and phenolic compounds (Edoga et al, 2005). These molecules isolated from the plants exhibit various pharmacological actions like antibacterial, antifungal, antioxidant, anticancer, anti-inflammatory, antiviral, analgesic and pain management. Studies considered the importance of compounds from natural sources are better than their synthetic analogues owing to the issue of biodegradability and environmental safety. This is the core reasons behind the expansion of use of natural products in terms of their use as medicament for disease management in the recent years resulting in the shifting of focus from exclusive production of drug molecules synthetically (Khan et al, 2011). However, the choice of special plant materials for treatment of ailments by our ancestors was not based on the knowledge of chemical constituents, rather probably on the observation of the effects caused on certain events in the environments. Phytochemical evaluation comes in this regards as the scientific approach and primary base for the justification of the claims of traditional medicines. The pharmacologically active principles of the medicinal plants can be influenced by the environmental conditions and these can be influenced both qualitatively and quantitatively. Phytochemical evaluation gives us these ideas both quantitatively and qualitatively in a precise manner using sophisticated techniques in the scientific approach. Another importance of phytochemical evaluation is the effective quality control of traditional medicines. In the present age, secondary plant metabolites having the potentiality of unknown pharmacological activities have been investigated as a source of medicinal agents (Krishnaraju and Nigam, 1970). These studies also help with the anticipation of phytochemical with enough pharmacological efficacies to be used in the ailment of disease (Kivcak and Mert, 2002). As per studies of WHO, in order to obtain variety of herbal drugs, medicinal plants are the best possible source. Thus, importance on the studies on the medicinal plants is to be emphasized in order to determine the potentiality of herbal medicine (Ali et al, 2001).

Some important phytochemical and their role in pharmacology are as follows:

4.1.1. Alkaloids

Alkaloids have been reported from various studies as one of the most important groups of phytochemicals obtained from natural source having varieties of potential in terms of pharmacological attributes. Alkaloids contribute to an important role in the defence system against pathogen and animals and the application is not limited to biological control of herbivores rather it has been expanded to various other dimensions in the field of pharmacology, medicine as well as veterinary. Some alkaloids belonging to the beta-carboline group possess antimicrobial, anti-HIV and antiparasitic activities (Bouayad et al, 2011).However, there are

issues of toxicity of serious illness, injury or even death due to the alkaloid obtained from plant ingested intentionally or unintentionally (Beyer et al, 2009).

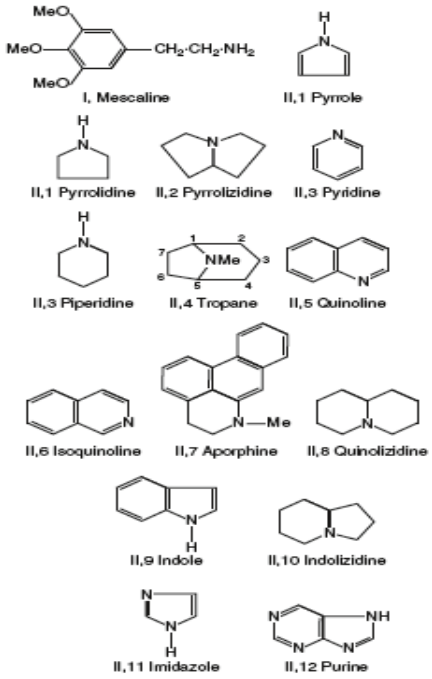


Fig 4.1: Structure of Alkaloids

Interestingly, in the modern age, a lot of commonly used and therapeutically important drugs are obtained from alkaloids (e.g. taxol from *Taxus baccata)*. Their common mechanism of action exerting their biological activities lies with the potentiality of affecting the central nervous system, particularly the action of the chemical transmitters such as acetylcholine, epinephrine, norephinephrine, γ-aminobutyric acid (GABA), dopamine and serotonin. These alkaloids also serve as the role model for the synthesis of newer and better chemical analogues. For example, physostigmine (*Physostigma venenosum*) for parasympathomimetic agent, tubocurarine (*Chondodendron tomentosum*) for skeletal muscle relaxation, cocaine (*Erythroxylon coca*) for local anaesthesia and so on. (Robert and Wink, 1998).

4.1.2. Terpenoids

Terpenoids is one of the major groups of compounds present in alkaloids. Terpenoids are composed of 'isoprenoid' unit constituting one of the largest groups of natural product of accounting for more than 40,000 individual compounds while the number is having an upward trend associated with the discovery of newer molecules every year (Sacchettini and Poulter, 1997; Penuelas et al, 2005, Wither et al, 2007). Terpenoids are mostly plant derived products; however, they can be synthesized by other organism like bacteria and yeast as a part of

secondary metabolism. The synthesis of terpenoids takes place from two five-carbon building blocks known as isoprenoid units. The terpenoids are classified on the basis of their number of building blocks involved, such as monoterpenes like carvone, geraniol, d-limonen and perillyl alcohol, diterpenes like retinol and trans-retinoic acid, triterpenese like betulinic acid, lipeopl, oleanic acid and ursolic acid and tetraterpenes like α-carotene, β-carotene, lutein and lycopene (Rabi and Bishayee, 2009).

Terpenoids have been found to be useful in prevention, cure and management of several diseases that includes cancers, antimicrobial, antifungal, antiparasitic, antiviral, antiallergic, antispasmodic, antihyperglycemic, anti-inflammatory and immunomodulary properties (Rabi and Bishayee, 2009; Wagner and Elmadfa, 2003; Sultana and Ata, 2008; Shah et al , 2009).Additionally, terpenoids can be used as protective substance in the storage of agricultural products owing their insecticidal properties (Theis and Lerdau, 2003).

Epidemiological and experimental studies suggest that monoterpenes may be helpful in the prevention and therapy of several cancers like mammary, skin, lung, fore stomach, colon, pancreatic and prostate carcinomas (Gould, 1997; Reddy et al, 1997; Vingushin et al , 1998; Crowell, 1999; Burker et al, 2002; de Carvalho and de Fonseca, 2006; Kris-Etherton, 2002).

4.1.3. Flavonoids:

4.1.3.1. Antioxidant Activity:

The best described property of flavonoids is its ability to act as antioxidants. The antioxidant potential and the probable mechanism of action of antioxidant activity such as free radical scavenging and metal ion chelation ability is governed by several factors like the configuration substitutions and total number of hydroxyl groups present (Pandey et al ,2012; Kelly et al, 2002). The B ring hydroxyl configuration is the most significant factor in the determination of scavenging of reactive oxygen species ROS and reactive nitrogen species RNS as it donates hydrogen and an electron to hydroxyl, peroxyl and peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoids radical (Cao et al, 1997).

Mechanism of antioxidant action can include as per various studies are as follows (Halliwell and Gutteridge, 1998; Mishra et al, 2013):

a. Suppression of ROS formation either by inhibition of enzymes or chelating trace elements involved in free radical generation

b. Scavenging of ROS

c. Up regulation or protection of antioxidant defences.

A common consequence of oxidative stress is lipid peroxidation. Flavonoids protects lipid against oxidative damage using various mechanism (Kumar et al, 2013). Owing to their lower redox potentials, flavonoids are able to reduce highly oxidizing free radical such as superoxide, peroxyl, alkoxy and hydroxyl radical by hydrogen atom donation thermodynamically. They also inhibit free radical generation (Mishra et al, 2013).

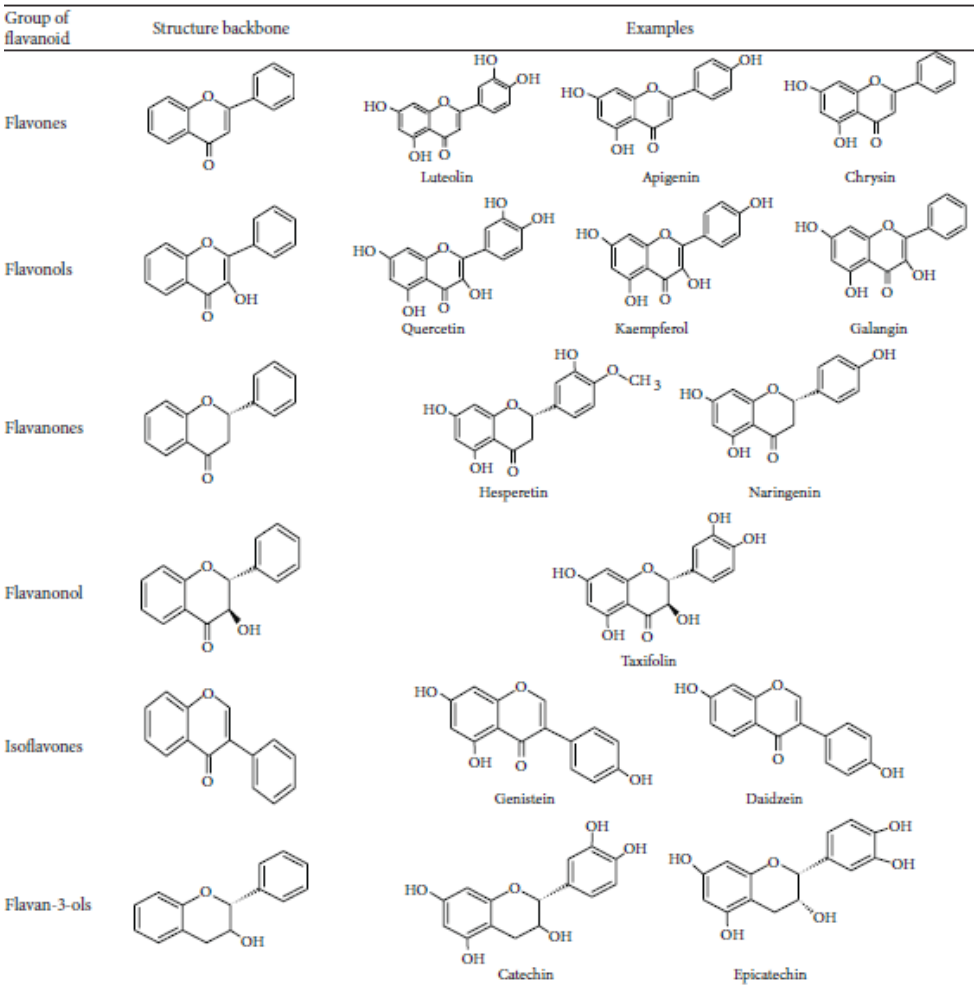


Fig 4.2.: Structure of Flavonoids

4.1.3.2. Hepatoprotective activity:

A lot of flavonoids, namely catechin, apigenin, quercetin, naringenin, rutin and venoruton are reported for their hepatoprotective activities (Tapas et al, 2008). Different chronic diseases like diabetes have the potentiality to the development of hepatic clinical manifestation. Glutamate-cysteine ligase catalytic subunit (Gclc) expression, glutathione and ROS level reported to be decreased in the liver of diabetic mice. Anthocyanins have drawn increasing attention due to their preventive effects against various diseases. Zhu et al demonstrated the increase of Gclc

expression due to anthocyanin cyaniding-3-O-β glucosides causing the increase of cAMP levels to activate protein kinase A, which in turn leads to up regulation of cAMP response element binding protein phosphorylation to promote its binding with DNA.

4.1.3.4. Antibacterial activity:

Some flavonoids including apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones and chalcones have been scientifically reported to possess antibacterial activity (Cushnie and Lamb, 2005). These activities are accomplished by various mechanisms. The principal one involved is the formation of complex with protein through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation. Thus, their mode of action of antimicrobial activity may be related to the potentiality of inactivation of microbial adhesins, enzymes, cell envelope transport protein and so porth. Lipophillic flavonoids may also disrupt microbial membranes (Cowan, 1999).

4.1.3.5. Anti-inflammatory activity:

Flavonoids like hesperidin, apigenin, luteolin and quercetin are reported to possess anti-inflammatory and analgesic effects as they significantly affect the function of immune system and inflammatory cells (Middleton and Kandaswami, 1992). Flavonoids probably affect specifically the function of enzyme systems critically involved in the generation of inflammatory processes, especially tyrosine and serine-protein kinase (Nishizuka, 1998; Hunter, 1995). It has also reported that flavonoids are able to inhibit expression of isoforms of inducible nitric oxide synthase, cyclooxygenase and lipooxygenase, which are responsible for the production of nitric oxide, prostanoids, leukotriense, and other mediators of the inflammatory process (Tunon et al, 2009).

4.1.3.6. Anti-cancer activity:

Dietary factor can play an important role in the prevention and management of cancer. Flavonoids also contain anticancer properties (Ho et al, 1994). Consumption of two most important sources of the flavonoids quercetin is inversely associated with the incidence of prostate, stomach, lung and breast cancers. In addition to that, moderate wine drinkers also have a low affinity to develop lung, endometrium, esophagus, stomach and colon cancer incidence (Koen et al, 2005). Suggestion from studies have been made towards substantial increment of consumption of these foods can achieve major improvements in public health in the ages to come (Block et al, 1992).

Several mechanisms have already been proposed on the effect of flavonoid on the initiation and promotion stages of carcinogenicity including influences on development and hormonal activities (Duthie et al, 2000). The mechanisms includes are the following:

a. Down regulation of mutant p53 protein

b. Cell cycle arrest

c. Tyrosine kinase inhibition

d. Inhibition of heat shock proteins

e. Estrogen receptor binding capacity

f. Inhibition of expression of Ras protein.

Mutation of p53 proteins are among the most common genetic abnormalities that lead to human cancer. Inhibition of it may lead to the arrest of cancer cell in G2-M phase cells. Flavonoids are found to down regulate expression of mutant p53 protein to nearly undetectable levels in human breast cancer cell lines (Davis and Matthew, 2000).

4.1.4. Saponins:

Another important plant derived natural product is saponin which is in brief is the glycosides of steroids, alkaloids and triterpenoids. Saponins are recognized by the unique ability of production of soapy leather on its shaking with water. (Negi et al, 2013). The saponins are widely distributed in 500 genera of plants among them majority of them belong to family Lilliaceae, Solanaceae, Sapindaceae, Agavacaea and Dioscoreacae. The saponins are polar in nature, thus this property refers to their solubility to water and non-soluble to non-polar solvents. Saponins are hydrolyzed into 'sapogenin', an aglycone and the glycon part is known as sugar.

The structural complexity of saponins results in a number of physical, chemical and biological properties. Saponins, however, are usually amorphous in nature having high molecular weight. They are soluble to water and produce foam while, on the contrary, they inhibit their foaming capacity upon dissolution in organic solvents like chloroform, acetone and ether. The solubility is also affected by the parameters of the solvents like temperature, composition, and pH. The common solvents used for the extraction of saponins are water, alcohols like methanol, ethanol. Saponins are surface active compound owing to the presence of lipid-soluble aglycone and water soluble sugar chain in their structure. These properties allow saponin to form micelles above a critical concentration termed as critical micelle concentration. The micelle forming property of saponin is however governed by temperature, concentration and pH of the aqueous phase (Mitra and Dangan, 1997).

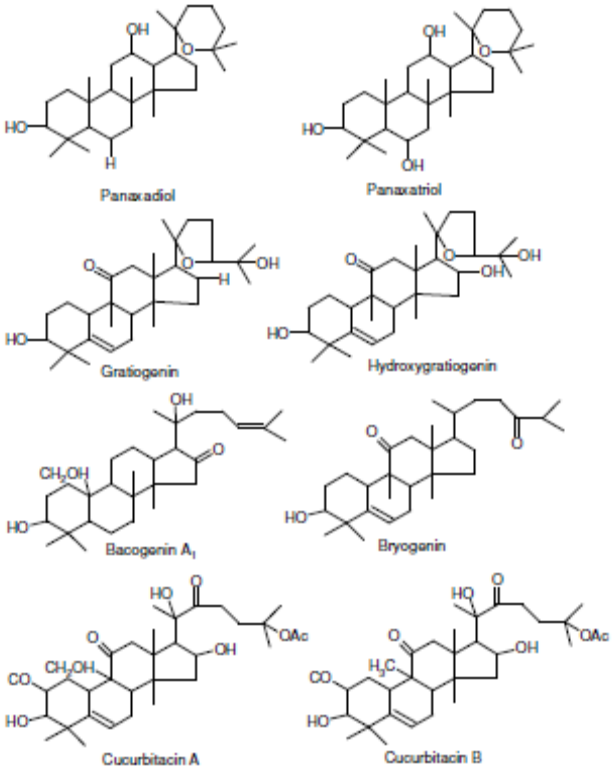


Fig 4.3.: Structure of Saponins

Saponins possess a wide range of biological activities like antioxidant, immunostimulant, antihepatotoxic, anticarcinogenic, antidermal, antiulcerogenic, antioxytoxic, hypocholesterolemic , anticoagulant, hepatoprotective, hypoglycemic, neuroprotective, anti-inflammatory, inhibition of dental caries and platelet aggregation (Gudu-ustundag and Mazza, 2007; Rao and Gurfinkel, 2000). They are useful in diabetic retinotherapy and reproduction. A lot of saponins are reported to have antimicrobial properties to inhibit mould and to protect plants from insects which are considered as a part of defense system and have been placed in a large group of protective molecules found in plant named phytoanticipins or phytoprotectants.

4.1.4. Tanins:

A range of polyphenols (Rompp, 1997) is termed as tannins derived from the French word ‘tanin’ (tanning substance) which is found in higher concentration in nearly every part of the plants, for example bark, wood, leaves, fruit, roots and seed. In most of the cases, the increased production of tannin is often associated with the sickness of the plant. Thus, it is often assumed that the potentiality of tannin in the plant is related to its protective ability against infection, insects or animal herbivore (Haslam, 1989; Porter, 1989). In term of appearance, tannins appears as light yellow or white amorphous powders or shiny, nearly colorless, loose masses with a characteristic strange smell and astringent taste (Falbe and Regitz, 1995)

The tannins are applied widely in the history of traditional medicine. The uses ranges from tanning, known over the millennia (Mediterranean since 1500 BC), including medicinal uses to uses in the food industry. In the world of medicine, especially in Chinese and Japanese natural healing, the tannin-containing plant extracts are often utilized on the basis of their potentionality as astringents, anti-diarrheal agent (Yoshida et al, 1991), diuretics (Okuda et al, 1983; Hatano et al, 1991), stomach and duodenal tumours (Saijo et al, 1989), anti-inflammatory, antiseptic, haemostatic pharmaceuticals (Haslam, 1989). Owing to the ability of heavy metal and alkaloid precipitation, they can also be utilized in poisoning with these substances. Thus, we can have a clear concept on the role of tannin as active principles of plant-based medicine (Haslam, 1996).

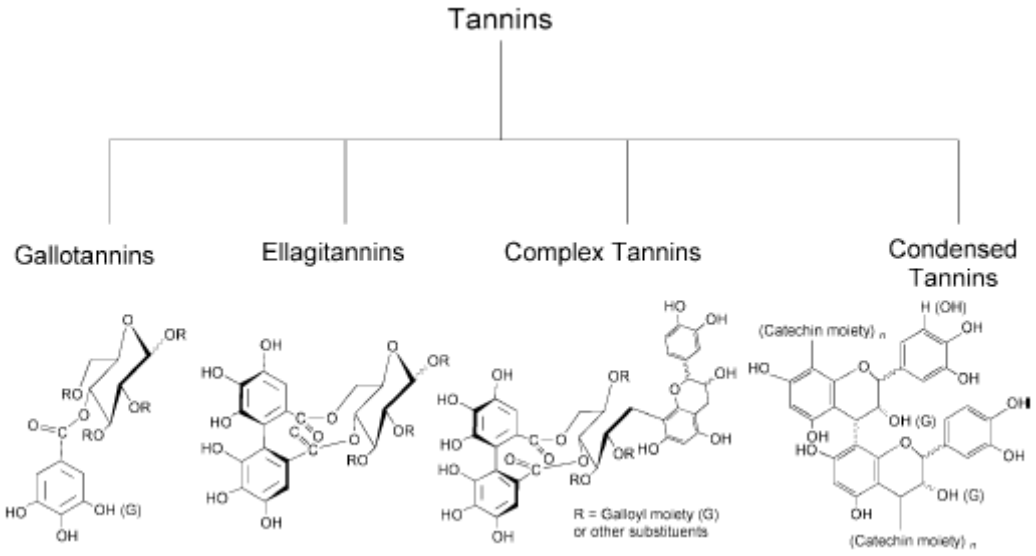


Fig 4.4: Tannins with their classification (Collected from Khanbabee & van Ree, 2001)

In the recent time, owing to the increased incidence of deadly illness like AIDS and various cancers, tannins have attracted scientific interest. The search for new lead compounds for the development of novel pharmaceuticals has become increasingly important and the well documented evidence of tannin-containing plant extract serves as a tool for the search. In the last twenty years many representatives of this class of compound have been isolated and characterized (Xie et al, 1995; Shiota et al, 2000). Currently, known tannins with unambiguously determined structures already far more than 1000 natural products. In extensive biological tests many of representative of this group were found out to have antiviral, antibacterial, specifically, anti-tumour activity (Quideau and Feldman, 1996; Kakiuchi et al, 1986).

4.2. Principles:

The Phytochemical Evaluation was carried out in order to find out the preliminary major group of therapeutically important classes of compound present in the crude plant extract. About 30mg of the crude plant extract was dissolved in 30ml of distilled water with a few drop of DMSO (Di-methyl sulphoxide, Fischer Chemicals Limited) was added as a suspending and solubilising agent to facilitate dissolution. For the evaluation, where alcoholic extract is required, 5mg of the crude plant extract was dissolved in 5ml of ethanol. In order to compare, the presence of other impurities and to identify the presence of pure compound of the plant extract, similar pattern of dissolution was carried out and then the solution was filtrated using activated charcoal. The process was carried out as per procedures described by Ghani, MA (2005). The procedure of the phytochemical evaluation is as follows:

Chemical Groups	Reagents	Test
Carbohydrate	Molisch Reagent Fehling's Reagent	Molisch Test Fehling Test
Tannins	10% Potassium Dichromate 5% Ferric Chloride 1% Lead Acetate	Potassium Dichromate Test Ferric Chloride Test Lead Acetate Test
Proteins	10% Sodium Hydroxide 3% Copper Sulphate	Biurets's Test
Saponins	Water	Frothing Test
Flavonoids	Zinc Ribbon and Concentrated Hydrochloric acid Sodium Hydroxide and Dilute Hydrochloric Acid	Flavonoid Test
Steroids	Sulphuric Acid	Salkowski Test
Alkaloids	Mayer's Reagent Dragendroff's Reagent Hager's Test	Mayer's Test Dragendroff's Test Hager's Test

Table 4.1: Reagent used for different group test

4.3. Preparation of reagent used for different chemical group test

The reagent required for the phytochemical studies were prepared in accordance with the standard procedure as prescribed by Ghani, 2005

Mayer's Reagent: About 1.36gm of Mercuric Iodide was dissolved in 60ml of distilled water and was mixed with a solution containing 5gm of potassium iodide dissolved in 20ml of distilled water. Then, the final volume was adjusted to 100ml

Dragendroff's Reagent: About 1.7gm of bismuth nitrate and 20gm of tartaric acid was dissolved in 80ml of distilled water. The solution was mixed with a solution containing 16gm of

potassium iodide dissolved in 40ml of water. The solution is diluted 10 times with 10% picric acid solution before use.

Hager's solution: 1gm of picric acid was dissolved in 100ml of distilled water to prepare the solution.

Fehling's solution A: About 6.93gm of copper sulphate was dissolved in a mixture containing 0.1ml of concentrated sulphuric acid and sufficient amount of distilled water to prepare the final volume upto 100ml

Fehling's solution B: About 35.2 gm of sodium potassium Tartarate and 15.4 gm of sodium hydroxide was dissolved in sufficient amount of distilled water to produce 100ml. Equal volume of above solution must be present at the time of use

Molisch solution: 2.5gm of pure α-napthol was dissolved in 25ml ethanol to prepare a 10% solution.

10% Potassium Dichromate solution: 10gm of Potassium Dichromate was dissolved in 100ml of distilled water to prepare this solution

5%Ferric Chloride solution: 5gm of Ferric Chloride was dissolved in 100ml distilled water to prepare this reagent

1% Lead Acetate solution: 1gm of Lead Acetate was dissolved in 100ml of distilled water to prepare this reagent

10% Sodium Hydroxide solution: 10gm of sodium hydroxide was dissolved in 100ml of distilled water to prepare this reagent

3% Copper Sulphate solution: 3gm of copper sulphate was dissolved in 100ml of distilled water to prepare this reagent

4.4. Procedures for conducting the test of phytochemicals:

The major chemical groups were identified by observing the characteristics change of colour using the standard procedure mentioned (Ghani, 2003)

4.4.1. Test for Carbohydrates:

a. **Molisch Test**:

2ml solution of crude extract was taken in a test tube and 2 drops of freshly prepared 10% alcoholic solution of α-napthol was added to it. Then, sulphuric acid was added to the mixture to the flown side of the inclined tube so that the acid forms a layer beneath the aqueous solution. A red or reddish violet ring would be formed at the junction of the two layers confirming the presence of carbohydrate. Upon standing or shaking, a dark purple solution would be formed.

The test tube was allowed to stand for 2 minutes; dilution of the sample mixture took place with 5ml of distilled water. A dull violet precipitate would be formed immediately confirming the presence of carbohydrate.

b. **Fehling's Test (Standard Test for Reducing Sugars)**

1ml of a mixture of equal volumes of Fehling's solution A and B was added to a 2ml aqueous solution of the crude extract and was boiled for a few minute. Presence of red or brick-red precipitate would be found immediately which would confirm the presence of carbohydrate.

4.4.2. Tests for Tannins

a. **Ferric Chloride Test**

5ml aqueous solution of the crude extract was taken in a test tube and 1ml of 5% Ferric solution was added. Presence of Greenish black precipitation confirmed the presence of tannins.

b. **Potassium dichromate Test**

1ml of 10% Potassium Dichromate solution was added to 5ml aqueous solution of the crude extract in a test tube. Presence of yellow precipitate confirmed the presence of tannins.

c. **Lead acetate Test**

Addition of few drops 1%solution of lead acetate to a 5ml aqueous solution of the crude extract would show yellow or red precipitate confirming the presence of tannins.

4.4.3. Test for Flavonoids

0.5ml of alcoholic solution of the extract of the sample was taken in a test tube and small piece of zinc ribbon or zinc dust with 5-10 drops of concentrated hydrochloric acid was added. The solution was boiled for a few minutes. Development of red to crimson colours would indicate the presence of flavonoids.

4.4.4. Test for Saponins

Frothing Test

0.5ml of alcoholic solution of the extract of the sample was diluted to 10ml using distilled water and was shaken in a graduated cylinder for 3-5 minutes. Production of persistence frothing would confirm the presence of frothing.

4.4.5. Test for Proteins

1ml of aqueous solution of the crude extract was taken and 5-6 drops of 10% sodium hydroxide and 1-2 drops of 3% copper sulphate solution were added. Development of red or violet colour would confirm the presence of proteins.

4.4.6. Test of Steroids

Salkowski Test

2ml chloroform solution of crude extract and then 1ml of sulphuric acid was added. Emergence of red colour would confirm the presence of steroids.

4.4.7. Tests for Alkaloids

a. **Mayer's Test**

0.2ml of concentrated Hydrochloric acid was added to 2ml aqueous solution of the crude extract. Then 1ml of Mayer's reagent was added. Formation of yellow colour precipitate would indicate presence of alkaloids.

b. **Dragendroff's Test**

0.2ml of concentrated hydrochloric acid was added to 2ml aqueous solution of the crude extract. Then 1ml of Dragendroff's reagent was added. Formation of orange brown precipitate would indicate the presence of alkaloids.

c. **Hager's Test**

0.2ml of concentrated hydrochloric acid was added to 2ml aqueous solution of the crude extract. Then 1ml of Hager's reagent was added. Formation of yellow crystalline precipitate would indicate the presence of alkaloids.

Fig 4.5: Reagents for phytochemical analysis

Chapter-5.

In Vitro Cytotoxicity Evaluation Using Brine Shrimp Lethality Assay

5.1. Brine shrimp Lethality Assay for the Evaluation of Cytotoxic Potential

5.1.1. Immune system and its role in toxicokinetics

5.1.2. Normal Immune Response

5.1.3. Hypersensitivity and Autoimmune Disorders:

5.1.4. Immuno suppression drugs

5.2. Role of Brine Shrimp Lethality Assay in the determination of Cytotoxicity and Immunosuppresion

5.3. Principle (Meyer et al, 1982)

5.4. Procedure

5.4.1. Preparation of sea water

5.4.2. Hatching of Brine Shrimp

5.4.3. Preparation of the Test Solution

5.4.4. Preparation of Control Group

5.4.4.1. Preparation of Positive Control

5.4.4.2. Preparation of Negative Control

5.4.5. Counting of the Nauplii and Analysis of Data

5. In Vitro Cytotoxicity Evaluation Using Brine Shrimp Lethality Assay

5.1. Brine shrimp Lethality Assay for the Evaluation of Cytotoxic Potential

5.1.1. Immune system and its role in toxicokinetics

The immune system is vital for survival, as it protects us from the potentially deadly microbes swarming around in the environment as well as it provides protection against harmful infectious pathogen. Immune deficiencies make the individual susceptible to infections. However, the immune system can play the role of a double-edge sword that causes lethal damage to other with the additional risk of causing self-inflicted damages on the user itself. Though the original intention of the immune system is the capability to provide defence against harmful infections, sometimes hyperactive stimulation of the immune system may cause some disease that can be fatal to the body. These disorders ranges from allergic reaction, anaphylactic shock to the reaction of the immune system against the individual's own tissues and cells, the state commonly termed as autoimmunity.

Any material having pharmacological values at certain dosage can cause toxicological reaction at a higher dose justifying the statement 'All drugs are poisons'. The core reason behind the fact is the over expression of the pharmacological action of the drug material, overcoming the immunological barrier which prevents toxic and adverse reaction associated with the suppression of immunity. Sometimes, this toxicokinetic study provides useful information and motivation in the development of therapies in the prevention of hyper expression of immune systems as well as modulation of the immune systems.

5.1.2. Normal Immune Response

The normal immune response is best understood in the circumstance of the defence against pathogenic infections. The immune response is categorised into two distinct categories. The first one, is termed as innate immunity refers to the defence mechanism present even before infections and that have evolved to specifically recognize microbes and protect the individuals against infections. The components of innate immune response are epithelial barrier blocking the entry of microbes, phagocytic cells (notably neutrophills and macrophages), dendritic cells, natural killer cells and several plasma proteins which includes the proteins of the complement systems. The most important reactions involved in innate immunity are inflammation involving the recruitment of phagocytic leukocytes and activation for the killing of microbes, anti-viral defense mediated by dendritic cells and natural killer cells.

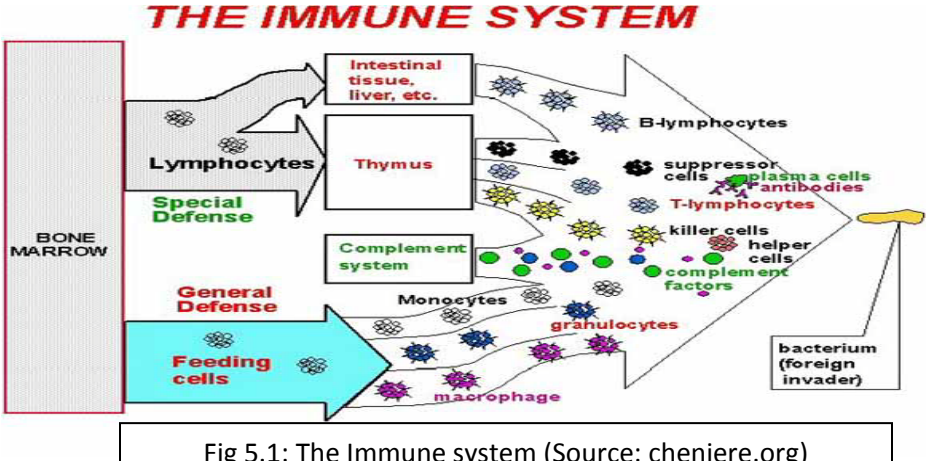


Fig 5.1: The Immune system (Source: cheniere.org)

The other category of immune response termed as adaptive immunity, in other word called acquired or specific immunity consists of mechanism stimulated by microbes and thus are capable of recognizing microbial and nonmicrobial substance. This brand of immune response develops later after the exposure of microbes and thus more powerful than innate immune response owing to the fact that the response is more precise and specific. The adaptive immunity consists of lymphocytes and their products including antibodies. The receptors of lymphocyte are much diverse but not inherently specific like innate immune system as these responses develops after the exposure to microbes. There are two distinct categories which are humoral immunity which protects against extracellular microbes and their toxins and cell-mediated immunity that is responsible for defence against intracellular microbes.

5.1.3. Hypersensitivity and Autoimmune Disorders:

Previous exposure to an antigen makes the individual sensitized. However, repeated exposure sometime may trigger series of pathologic reactions. These reactions take place when the balance between effector mechanism of the immune response and its mechanism of control that are present to limit the immune response to normal level fail and these responses are termed as hypersensitivity. Hypersensitivity is both exogenous and endogenous in category of antigen exposure. Exogenous antigens includes dust, pollens, foods, drugs, microbes, chemicals and some blood product used in clinical practice. The response may vary from annoying but trivial discomforts like itching of the skin to potentially fatal diseases like bronchial asthma and anaphylaxis.

Type of Reaction	Prototypic Disorders	Immune Mechanism	Pathologic Lesion
Immediate (Type I) hypersensitivity	Anaphylaxis; allergies; bronchial asthma	Production of IgE antibody leads to immediate release of vasoactive amines and other mediators form mast cells; later recruitment of inflammatory cells	Vascular dilation, edema, smooth muscle contraction, mucus production, tissue injury, inflammation
Antibody-mediated (Type II) hypersensitivity	Autoimmune haemolytic anaemia; Good pasture syndrome	Production of IgG, IgM leads to binding of antigen on target: cell or tissue that leads to phagocytosis or lysis of target cells by activated complement or Fc receptors; recruitment of leukocytes	Phagocytosis and lysis of cells; inflammation, in some disease, functional derangement without cell or tissue injury
Immune complex-mediated (Type III) hypersensitivity	Systemic lupus erythematosus; some forms of flomerulonephritis; serum sickness; Arthus reaction	Deposition of antigen-antibody complexes leads to complement activation causing recruitment of leukocytes by complement products and Fc receptors causing release of enzymes and other toxic molecules	Inflammation, necrotizing vasculitis (fibrinoid necrosis)
Cell-mediated (Type IV) hypersensitivity	Contact dermatitis; multiple sclerosis; type I diabetes; rheumatoid arthritis; inflammatory bowel syndrome; tuberculosis	Activated T lymphocytes that lead to either release of cytokines leading to inflammation and macrophage activation or T cell-mediated cytotoxicity	Perivascular cellular infiltrates; edema; granuloma formation; cell destruction

Table 5.1: Hypersensitivity reaction and their mechanisms (Collected from Robbins pathological basis of disease, 2010)

Immune system is mediated for the individual's protection against the pathogenic microbes. However, in some of the cases, the immune system can damage the tissues and cells of the body which is commonly termed as autoimmune disorders in other immune reaction against self-antigen. It can be found in individuals particularly in older age groups associated with the formation of innocuous autoantibodies after the damage to the tissues and may serve a physiological role in the removal of tissue breakdown products. The ideal requirements of autoimmunity are as follows:

a. The presence of an immune reaction specific for some self-antigen or self-tissue
b. Evidence of the reaction not being secondary to the tissues rather is of primary pathogenic significance
c. Absence of well defined cause of the disease.

Immune-mediated Inflammatory Disorders	
Disease mediated by Antibodies and Immune Complex	
Organ-specific autoimmune disease	Autoimmune haemolytic anaemia
	Autoimmune thrombocytopenia
	Myasthenia gravis
	Graves disease
	Good pasture syndrome
Systemic autoimmune disease	Systemic lupus erythematosus
Disease caused by autoimmunity or by reaction of microbial antigen	Polyarteritis nodosa
Disease mediated by T cells	
Organ-specific autoimmune disease	Type I Diabetes Mellitus
	Multiple sclerosis
Systemic autoimmune disease	Rheumatoid arthritis
	Systemic sclerosis
	Sjogren syndrome
Disease caused by autoimmunity or by reaction to microbial antigens	Inflammatory bowel disease (Crohn's disease, Ulcerative colitis)
	Inflammatory myopathies

Table 5.2: Autoimmune diseases

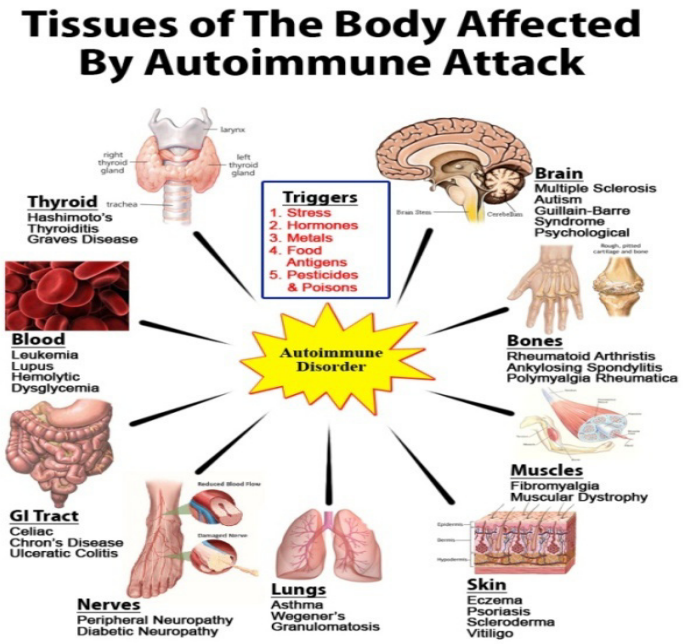


Fig 5.2: Autoimmune diseases (Source: pinimg.org)

5.1.4. Immuno suppression drugs

Immunosuppressive agents are used to dampen the immune response in organ transplantation and autoimmune disease. These drugs have met with a higher degree of clinical success in treating conditions like acute immune rejection of organ transplants and severe autoimmune disease. There are five major classes of immunosuppressive agents. They are explained with their mechanism of actions:

Drug	Site of Action
Glucocorticoids	Glucocorticoids response elements in DNA (regulate gene transcription)
Muromonab-CD3	T-cell receptor complex (blocks antigen recognition)
Cyclosporine	Calcineurin (inhibits phosphatases activity)
Tacrolimus	Calcineurin (inhibits phosphatases activity)
Azathioprine	DNA (false nucleotide incorporation)
Mycophenolate mofetil	Inosine monophosphate dehydrogenase (inhibits activity)
Daclizumab, Basiliximab	IL-2 receptor (blocks IL-2 mediated T cell activation)
Sirolimus	Protein kinase involved in cell-cycle progression (mTOR) (inhibits activity)

Table 5.3: Immunosuppressants (Collected from Goodman & Gilman's Pharmacological Basis of Therapeutics, 12th edition)

5.2. Role of Brine Shrimp Lethality Assay in the determination of Cytotoxicity and Immunosuppresion

Bioactive compounds are tend to be toxic to living body at dose higher than the normal one. Brine Shrimp lethality bioassay (McLaughlin 1990; Persoon 1980) is the rapid and comprehensive bioassay for the bioactive compound of the natural and synthetic origin for the evaluation of toxicity of the bioactive compounds. The method evaluates a wide range of pharmacological activities in addition to toxicokinetic evaluation which are antiviral, pesticidal, immunosuppressant & antitumor etc. Activities of the natural products obtained (Meyer, 1982; McLaughlin 1988). The method is inexpensive and can be completed without the use of serum.

5.3. Principle (Meyer et al, 1982)

Brine shrimp eggs were hatched in artificial sea water for 48 hours using lamp as a source of artificial sunlight and pump as a source of artificial air in the water. Test samples were prepared by dissolving in DMSO and by addition of required amount of DMSO, desired concentration the test sample was prepared. Serial dilution was carried out using equal amount of the test sample and equal amount of DMSO. The nauplii were counted by visual inspection in a unit of 10 per vial and taken into vial containing 5ml of stimulated sea water. Then sample of concentration were added to the premarked vial using micropipette. The vials are then left for 24 hours and then the surviving nauplii were counted again by visual inspection to find out the cytotoxicity of the test samples

Sl. No.	Plant part	Test sample	Amounts Measured(mg)
1.	Whole plant	*Murdannia nudiflora*	5.00
2.	Whole plant	*Tradescantia pallida*	5.00

Table 5.4: Test samples of experimental plant

5.4. Procedure

5.4.1. Preparation of sea water

38gm sea salt (pure NaCl) was weighed, dissolved in 1 litre of distilled water and filtered off to obtain a clear solution.

5.4.2. Hatching of Brine Shrimp

Artemia salina leaches (brine shrimp eggs) were collected from the university laboratory used as the test organisms. Seawater was taken in a small tank and the shrimp eggs were place on one side of the tank while lamp was placed on the opening of the tank. The shrimp were allowed to hatch for two day (48hours) and to be matured as nauplii. Throughout the hatching time, constant oxygen supply was carried out by air pump. The hatched shrimps were attracted to the lamp through the perforated hatch and they were taken for experiment. A Pasteur pipette was utilized to separate 10 living shrimp nauplii which were added in each of the test tube containing 5ml of sea water.

For the experiment, clean test tubes were taken for the facilitation of clear visualization of the surviving nauplii after the completion of required time of the study. The test tubes were used for 9 different concentrations (one for each sample) of test samples in two batches in order to visualize their activity in a more precise and accurate manner, another nine test tube was utilized for the control test.

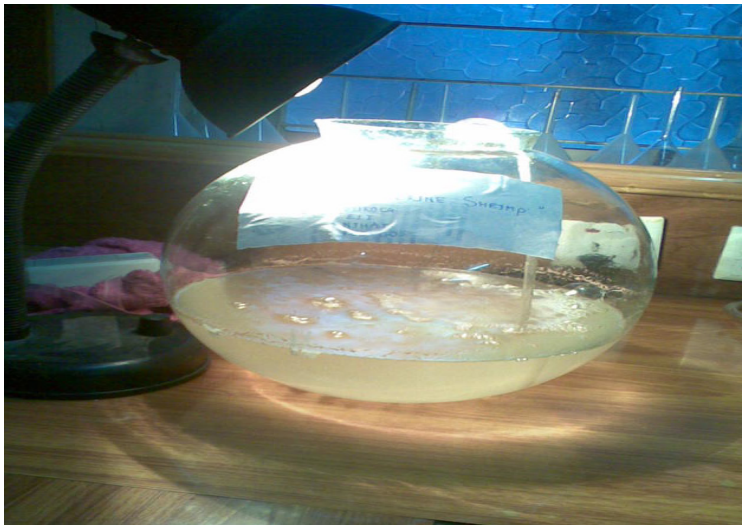


Fig 5.3: Hatching of the Nauplii

5.4.3. Preparation of the Test Solution

About 5mg of test samples was dissolved in sufficient amount of DMSO (Dimethyl sulphoxide) in vial to get the stock solution. The concentration was adjusted in such a way the concentration of the mother solution would be 320µg/ml. Serial dilution of the mother solution has been completed from the mother solution using equal amount of DMSO to obtain a series of solution of lower concentration in a proportionate manner. From each of the test solution, 50µl were added to pre-marked test tube/vials containing 10 shrimp nauplii in 5ml of sea water. Thus, the final concentration of the test samples for the study in the test tube were obtained as 320µg/ml,

160µg/ml, 80µg/ml, 40µg/ml, 20µg/ml, 10µg/ml, 5µg/ml, 2.5µg/ml and 1.25µg/ml. The study was conducted in two-way cross matching approach using the similar concentration for two batches with the same experimental procedure for the two test samples. For toxicity studies, the test solution was prepared by adjusting the concentration to 640µg/ml and 1280 µg/ml in order to visualize their potency at higher concentration.

5.4.4. Preparation of Control Group

5.4.4.1. Preparation of Positive Control

For the study, Vincristine sulphate was used for positive control. 0.32mg of Vincristine sulphate was dissolved in DMSO to get an initial concentration of 40µg/ml from which serial dilution was made using DMSO to obtain 20µg/ml, 10µg/ml, 5µg/ml, 2.5µg/ml, 1.25µg/ml, 0.625µg/ml, 0.3125µg/ml, and 0.15625µg/ml. The control groups containing 10 living brine shrimp nauplii present in 5ml of simulated sea water received the positive control

5.4.4.2. Preparation of Negative Control

As for Negative control, 50µl of DMSO was added to each of the pre-marked test tubes containing 5ml of simulated sea water where 10 living brine shrimp nauplii were placed. The test was considered invalid if the negative control showed a rapid mortality rate and therefore conducted again. The test tube (containing nauplii) were then maintained at room temperature for 24 hours under light for the observation of survival rate

5.4.5. Counting of the Nauplii and Analysis of Data

After 24 hours, the test tubes were inspected using a magnifying glass and the number of surviving nauplii were conducted. The percentage of mortality was calculated for each dilution. The concentration mortality data was analyzed by using probit analysis and linear regression. The effectiveness or the concentration-mortality relationship of plant product is usually expressed as a median lethal concentration (LC_{50}) value. The value represents the concentration of the chemical that produces death is half of the test subjects after a certain period of exposure to the given conditions.

Chapter-6

Antioxidant Evaluation by DPPH Free Radical Scavenging

6.1. Reactive Oxygen Species & Oxidiative Stress

6.2. Antioxidant and their role in oxidative stress:

6.3. Principle:

6.4.1. Preparation of the sample solution

6.4.2. Preparation of the standard & control solution

6.4.3. Preparation of DPPH solution

6.5. Procedure & Method of Analysis:

6. Antioxidant Evaluation by DPPH Free Radical Scavenging:

Biochemical pathways or cellular mechanism have been producing free radicals and reactive oxygen species as an end product (Nantitanon et al ,2007) These unstable chemical compounds are highly dangerous to living cells, owing to their ability to mutate resulting in myocardial infractions, Alzheimer diseases and can also be associated with many other critical diseases. (Adiguzel et al, 2009; Sugumaran and Raj, 2010). The products of reactive oxygen species has been implicated in several scientific claims to play certain role in the etiology of diseases like cancer, diabetes, cardiovascular disease, auto immune disorders, neurodegenerative disorders and ageing. These free radicals reacts with electron acceptor like molecular oxygen very rapidly and they includes superoxide anions, hydrogen peroxide (H_2O_2) and hydroxyl radicals (-OH) (Grisham et al, 1986). Generally, chemical based antioxidants are used to control free radical activity and owing to their adverse effect on human health, the development of natural antioxidant from plant resources draws a lot of attention in the world of healthcare (Rajasekar et al, 2011). Foods from plant based origin contain a lot of bioactive compounds contributing towards antioxidant activities allowing them to scavenge both ROS and electrophiles, inhibit nitrosation, chelate metal ions and modulate certain chemical enzyme activities. These may include vitamins like α-tocophenrol and ascorbic acid, carotenoids, polyphenols and some micronutrient elements like iron, zinc and selenium. There are many commercially available product that contain a large number of such compounds, however, they are almost out of reach for the market owing to their high prices. In order to provide people with economic constraint, the search of novel antioxidant available at a cheaper rate continues.

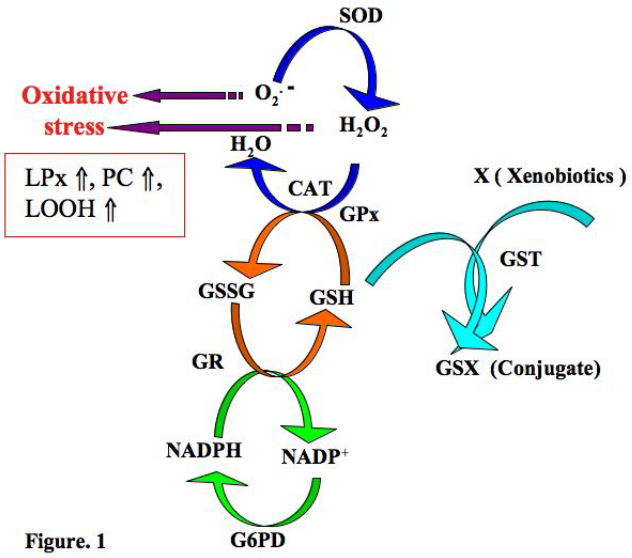


Fig 6.1: Oxidative stress and Free Radical

6.1. Reactive Oxygen Species & Oxidative Stress

The oxygen-derived free radicals are the species that can be released extracelluarly from leukocytes upon exposure to microbes, chemokines and immune complex or by phagocytic challenges (Salvemini et al, 2006). Their production is dependent on the activation of NADPH oxidase system which oxidizes NAPDH and in the process reduces oxygen to superoxide anions (O_2^-). In the case of neutrophills, the rapid oxidative reaction is triggered by activating signals accompanying phagocytosis, commonly termed as respiratory burst. These radical can combine with Nitric oxide (NO) to form reactive nitrogen species. Depending on their nature, ROS react with biomoleucles like lipid, protein and DNA to produce lipid radicals, sugar and base derived amino acid and thiyl radicals. These radical in presence of oxygen are converted to peroxyl radical that induce chain reaction. The consequence of lipid peroxidation by ROS are cross linking of protein, change in membrane fluidity and formation of lipid-protein, lipid-DNA adduct which may be detrimental to the functioning of the cell (Beckman et al, 1997).Extracellular release of low levels of these potent mediators play an important role in the increased release of chemokines (IL-8), cytokines and endothelial leukocyte adhesion molecules and resulting in the amplification of inflammation process of various degrees. Their role in stress and inflammation:

a. Endothelial cell damage with increased vascular permeability. Adherent neutrophills when activated, not only produce their own toxic species but also stimulate the production of ROS in the endothelial cells
b. Injury to other cell types (parenchymal cell, red blood cells)
c. Inactivation of antiproteases like α-antitrypsin. These events lead to unopposed protease activity associated with increased destruction of extracellular matrix. In the case of lung, such events can contribute to the destruction of elastic tissues which happens in the case of emphysema.

Another potent mediator of oxidative stress is nitric oxide synthesized from l-arginine by the enzyme nitric oxide synthase (NOS). They are of three different types of Nitric oxide: endothelial (eNOS), neuronal (nNOS0 and inducible (iNOS). They play dual role in terms of oxidative stress by relaxing smooth muscle and promoting vasodilatation, thus contributing to the vascular reactions and also inhibits cellular components of inflammatory responses (Larox et al, 2001; Cirino et al, 2006).

In the case of DNA, ROS can interact with it and can potentially cause several types of damage such as modification of DNA bases, single and double strand DNA breaks, loss of purine (apurininc sites), damage to the deoxyribose sugar, DNA-protein cross linkage and damage to the DNA repair system. Protein can also undergo direct and indirect damage following interaction with ROS resulting in to peroxidation, changes in tertiary structure, proteolytic degradation, protein-protein cross linkage and fragmentation (Beckman et al, 1997).

Oxidative stress has been implicated in number of diseases like cancer, atherosclerosis, diabetes, neurological diseases such as Alzheimer's disease, Parkinson's disease etc. as well as in the ageing process. (Kunwar et al, 2011)

6.2. Antioxidant and their role in oxidative stress:

In order to protect the cells from the harmful of these harmful oxygen-derived free radical and reactive oxygen species that causes oxidative stress, there are molecules that cause antioxidant mechanism helping in the removal of these harmful mediators by the host serum, tissues fluids and cells. Antioxidants acts in the probable following mechanism:

a. The enzyme superoxide dismutase, which is found in or can be activated in a variety of cell types
b. The enzyme catalase, which detoxifies H_2O_2
c. Glutathione peroxidise, another powerful detoxifier of H_2O_2
d. The copper containing serum protein ceruloplasmin
e. The iron-free fraction of serum transferrin.

A number of synthetic antioxidant agents like butyl hydroxyanisol and butyl hydroxytoluene, have been developed to mitigate oxidative stress. These agents have become necessary as the normal antioxidant defence mechanism of the body become ineffective due to the present situation of emergence of different environmental pathogens However, different factors such as mutagenecity, cost and availability have limited their use in combating oxidative stress (Chihoung et al, 1992). Thus, natural antioxidants have assumed the pole of greater prominence owing to the advantage of mostly free from side effects, less expensive and abundant in many plant sources (Cai et al, 2004). Plant based antioxidant provide an advantage by playing defensive role by the prevention of free radical and thus they are extremely beneficial to alleviate oxidative stress (Akinmoladun et al, 2010; Ozen et al, 2010).

Polyphenols are effective ROS scavenger owing to the presence of multiple hydroxyl groups. Examples of natural antioxidant derived from polyphenols obtained from plant sources includes vitamin E, flavonoids, cinnamic acid derivatives, curcumin, caffeine, catechins, gallic acid derivatives, saliycylic acid derivatives, chlorogenic acid, resveratol, folate, anthocyanins and tannins (Bors et al, 1996). There are also some plant derived secondary metabolite having antioxidant potentiality such as melatonin, carotenoids, retinal, thiols, jasmonic acid, eicosapentaenoic acid, ascopyrones and allicin (Bendich, 1989 Olson, 1989; Goldman, 1995).

6.3. Principle:

Peroxidation of cell membrane lipids and release of toxic substances like free radicals can be mediated by stresses, physical damage, viral infection, cytotoxic or carcinogenic compounds which can culminate chemical or biological aggregation (Auroma,1998). Antioxidants are the substances that have the potentiality to interfere with the oxidation process by reacting with the free radicals, chelating catalytic metals and also by acting as oxygen scavenger (Shahidi and Wansundara, 1992). The interest of natural antioxidant obtained from plant sources have grown tremendously in recent times. DPPH (2,2-diphenyl-1-picrylhydrazyl) Free Radical Scavenging method is a method used in vitro in order to determine the free radical scavenging activity of the natural plant extract with the help of spectrophotometrical measurement of samples. DPPH is widely used to test the ability of compounds to act as free radical scavenger or hydrogen donors, and to evaluate antioxidant potential of plant extracts. The advantage of this method is to measure the overall antioxidant potential quantitatively of the sample and it can be used for either solid or liquid samples.

Sl. No.	Name of the sample	Amounts measured
1.	*Murdannia Nudiflora*	3.2mg
2.	*Tradescantia pallida*	3.2mg
3.	Ascorbic Acid	1.6mg

Table 6.1: Amount of sample and standard for the study

6.4.1. Preparation of the sample solution

3.2 mg of the crude plant extract was taken in a test tube was dissolved in 0.8ml methanol in such a way so that the concentration of the sample solution is 4mg/ml which is the mother solution. 200µl was separated from the stock solution so that the initial concentration can be adjusted to 200µg/ml then serial dilution was completed in the subsequent test tube by dissolving in equivalent amount of methanol. Thus, after completing serial dilution, the final concentrations are 200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.125µg/ml, 1.5625µg/ml and 0.78125µg/ml

6.4.2. Preparation of the standard & control solution

For this study, ascorbic acid was chosen as the standard of comparison for free radical scavenging activities of the crude plant extracts. For standard solution, 1.6mg of ascorbic acid was taken and dissolved in 0.4ml of methanol to have a concentration of 4mg/ml similar to that of sample solution. 0.2ml was separated and added to the first tube while the remaining solution undergoes serial dilution with the addition of equivalent of methanol in the similar manner. Thus, the final concentrations of the standard solution are 200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml,

12.5µg/ml, 6.25µg/ml, 3.125µg/ml, 1.5625µg/ml and 0.78125µg/ml. The blank solution was prepared by mixing 2ml of methanol with 2ml of DPPH solution to observe and omit the interference shown by the reagents.

6.4.3. Preparation of DPPH solution

4.4mg of DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical was added to 220ml of methanol to obtain a concentration of 20µg/ml to prepare the stock DPPH solution

Fig 6.2: Analysis using UV-Vis Spectrophotometer

6.5. Procedure & Method of Analysis:

1.8ml of methanol was added to each of the pre-marked test tube of both samples and standard solution so that the final volume obtained in 2ml and they were mixed well for the proper dissolution in methanol. 2ml of DPPH radical was added to each of the test tube and shaken well. Then, the standard and sample solutions were kept in dark places for 30 minutes. Finally, they were analysed spectrophotometrically by UV spectrophotometer (Hitachi Corporation) at 517nm. The absorbances were used to obtain the percentage of DPPH radical scavenging activity using the following equation:

DPPH Radical Scavenging Activity (%)=$[(A_0-A_1)/A_0 x100]$

Where, A_0= Absorbance of the control or blank

A_{1-9}=Absorbance of the sample/standard

The equivalent value was determined by this equation: % of $FRS_{(sample)}$/% of $FRS_{(AA)}$x100

IC_{50} values were determined using linear regression and probit analyses in Microsoft excel 2007.

Chapter-7

Analgesic evaluation by Acetic Acid Writhing Method

7.1. Mechanism of pain and inflammation

7.3. Mediator of inflammation

7.4. Principle

7.5.1. Experimental Animals

7.5.2. Experimental Design

7.5.3. Method of Identification of Animals

7.5.4. Preparation of the sample

7.5.6 Procedure

7.5.7. Counting of Writhing

7.5.8. Method of Analysis:

7. Analgesic evaluation by Acetic Acid Writhing Method

Inflammation is a complex localized response to foreign substances like bacteria or in some cases internally produced substances (Laupattarakasem et al, 2003; Schmid-Schnobein, 2006) with fever usually presenting as one of its consequences (Okumura et al ,2006). It underlies almost all disease conditions (Erlinger et al, 2004; Lucas et al, 2006; Schmid-Schnobein, 2006). Fundamentally, inflammation is a protective response and the ultimate goal of which is to rid the organism of both the initial cause of cell injury like microbes and toxins as well as the consequences of such injuries for example necrotic cells and tissues (Serhan 2004; Schmid-Schnobein, 2006). The process of inflammation is necessary for healing of wound, however if not controlled, lead to onset of diseases like vasomotor rhinnorhoea, rheumatoid arthritis and arthrosclerosis (Henson and Murphy, 1989). Inflammation is characterized by classical signs like edema, erythrema, pain, heat and subsequently loss of function.

Inflammatory are of two types, acute and chronic inflammatory conditions. Acute inflammation are designed to test drugs that modulate erythrema, changes in vascular permeability, leukocyte migration and chemotaxis, phagocytosis poly-morpho nuclear leucocytes and other phagocytic cells, measurement of local pain, antipyretic activity and local analgesic action (Barbosa-Filho et al, 2006). Chronic inflammatory conditions are designed to find drugs that may modulate the disease process and these include sponge and pellet implants and granuloma pouches that deposite granulation tissue, adjuvant induced arthritis and mononuclear arthritis that have immune etiology (Lewis, 1989).

7.1. Mechanism of pain and inflammation

The mechanism of pain and inflammation is not yet discovered specifically as there are lot of unknown territories to be discovered and the specific group of chemicals involved due to chemotaxis in the mediation of pain and inflammation and how they behave during pain and inflammation. The generalized mechanism is explained as below

1. Arising from inflamed or injured tissues, the pain may be spontaneous without the presence of an external trigger different to normal signs and symptoms that arise from tissue under exposure to stimuli of high intensity which gives a fair reflection of intensity, localization and timing of the stimuli that initiates the pain.
2. Noxious stimuli responses may be enhanced in the case of hyperalgesia or normally may produce pain (allodynmia) in the case of normally innocuous stimuli.
3. These events are non-specific and recognition of the distinct pathophysiological mechanism could not be explained. For example, symptoms related to the movement in

the case of osteoarthirits and pain occurred due to touch in case of herpetic neuralgia both are being the same example of mechanical pain completely distinct from each other in terms of their mechanism of action (Kidd and Urban, 2001).

4. Somatic tissues in the cutaneous and deep areas are often innervated by primary afferent neurons synapses with second-order neuron in the dorsal horn of the spinal cord. Primary affected neuron have three distinct function in nociception which are transduction dealing with the detection of noxious or damaging stimuli, another terminology called conduction explaining the pathway of the resulting sensory input from peripherial terminals to the spinal chord and thirdly the synaptic transfer of the input to neuron in the specific laminae of dorsal horn which is explained by the term transmission (Kidd and Urban, 2001).
5. Arachidonic acid, a 20-carbon polyunsaturated fatty acid derived from dietary source or by conversion of the essential fatty acid linoleic acid have particular role in pain and inflammation. It doesn't occur free in the cell, however, are normally esterified in membrane phospholids which is released upon the mediator of mechanical, chemical or other forms of stimuli from the membrane phospholipids through the action of cellular lipase enzyme, phospholipase A_2. The mediator of Arachidonic acid are often termed as eicosanoids, synthesized by the action of two distinct enzyme named cyclogenase for the generation of prostaglandin and lipoxygenase for the generation of leukotriene.
6. Prostaglandins are produced by mast cell, macrophages, endothelial cell and many other cells involved in the vascular and systemic reaction of inflammation. Produced upon the action of the enzyme cyclogenase, COX-1 and COX-2, these compounds are effective vasodilator, a potent inhibitor of platelet aggregation and marked potentiator of the permeability and chemotactic effect of other mediators. The most important compounds contributing to inflammation are PGE_2, PGD_2, PGF_{20}, PGI_2 (prostacyclin) and TxA_2 (thromboxane).
7. Leukotrienes, produced upon the action of the enzyme lipoxygenae, are secreted by leukocytes and facilitate the chemotaxis for leukocytes and also have vascular effect. LTB_4, a leukotriene is a potent chemotactic agent, activator of neutrophills, causing platelet aggregation and adhesion of the cells to venular endothelium, ROS generation and release of lysosomal enzyme. Some of them, notably LTC_4,LTD_4, LTE_4, can cause intense vasoconstriction, bronchospasm and increased vascular permeability.
8. Vanilloid receptor, a group of protein that facilitates the detection of noxious heat is another inducer of pain (Caterina et al, 2000). VR1 protein is a heat transducer due to their ability to convert thermal energy into electrical signal named action potential which is sent to the central nervous system, enabling the detection of stimuli as pain produced by heat.

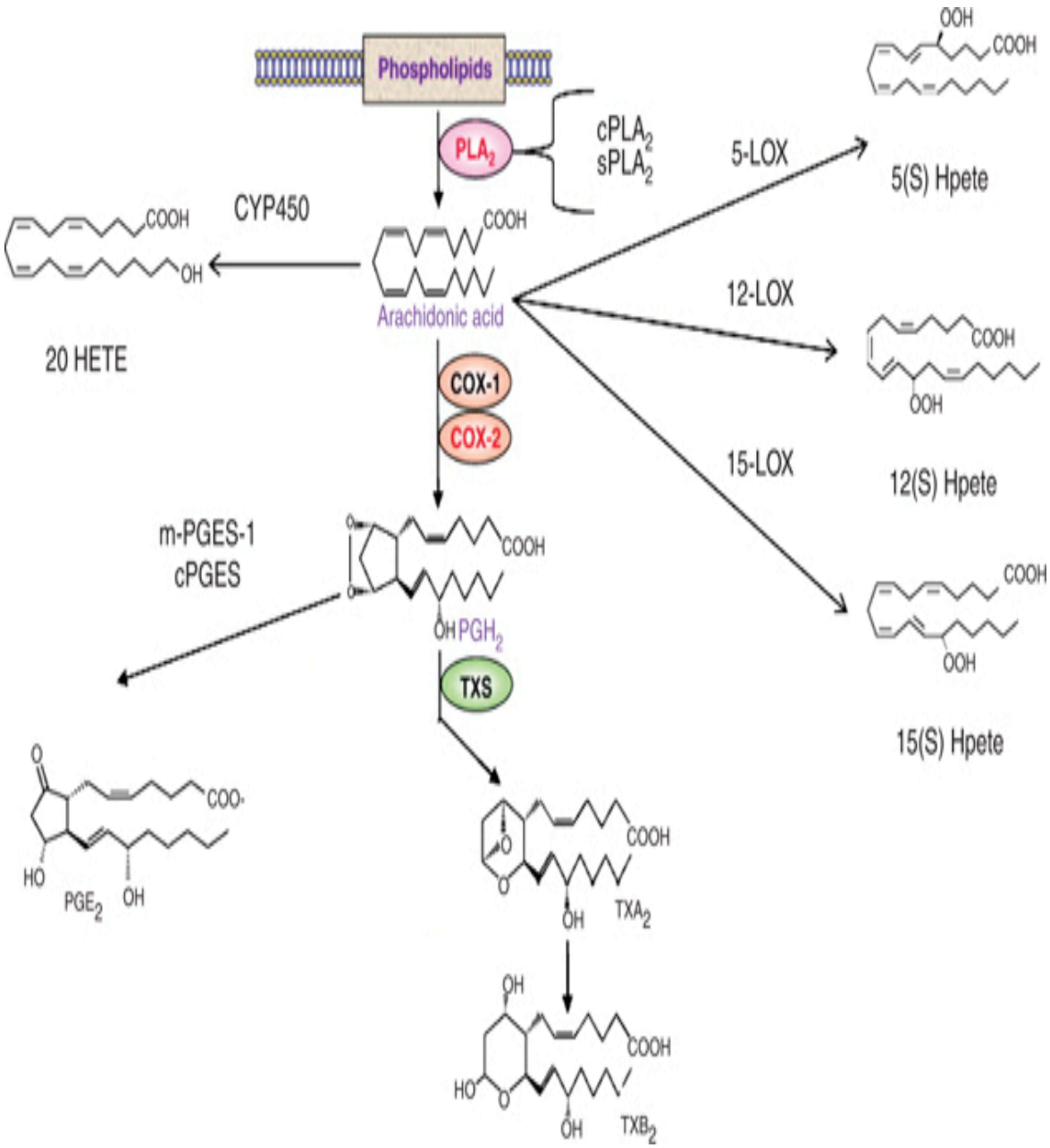


Fig 7.1: Arachidonic acid metabolism (source: nature.com)

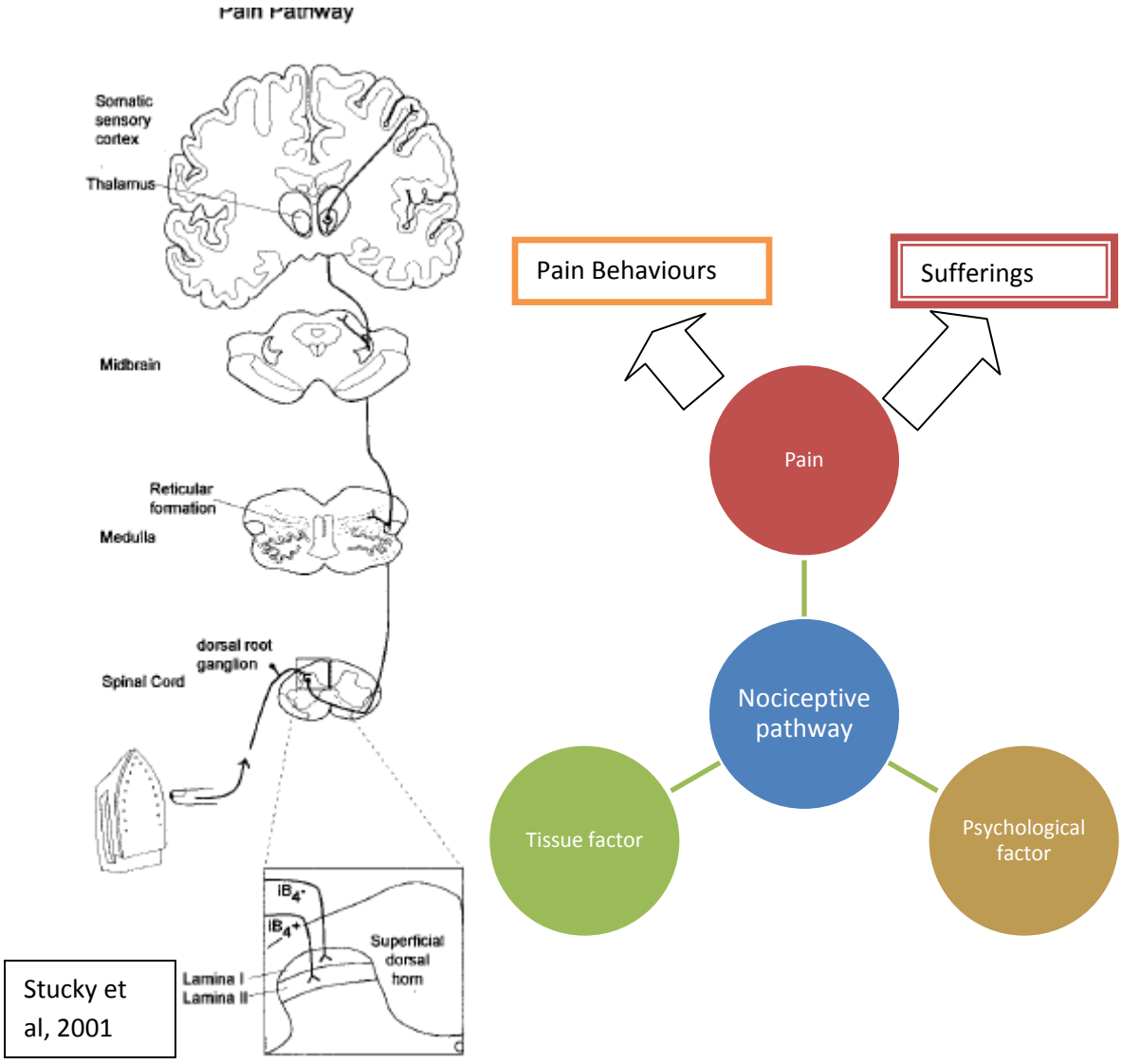


Fig 7.2: Mechanism of Pain

7.3. Mediator of inflammation

Inflammation can be mediated by various chemical that can trigger the reaction. These mediators can be of cell derived or plasma proteins. The cell-derived mediators are normally sequestered in intracellular and can be rapidly secreted by granules exocytosis (e.g. histamine in mast cell) or are synthesized de novo (e.g. prostaglandins, cytokines) in response to a stimulus. The major cell-derived mediators are platelets, neutrophills, monocytes/macrophages, mast cells, in addition to mesenchymal cells (endothelium, smooth muscle, fibroblasts). Plasma-derived mediators (e.g. complement proteins, kinin) are produced in the liver and present in the circulatory system as inactive precursors which must be activated, usually by a series of proteolytic cleavages, in order to acquire their biologic properties. The major stimuli include microbial products, substances

released from necrotic cells and the proteins of the complement, kinin and coagulation system activated by the microbes and damaged tissue. In some of the cases, one mediator can stimulate the release of other mediators. For example, the cytokine TNF acts on endothelial cells to stimulate the production of another cytokine IL-1 and many chemokines. After activation and release from cell, most of these mediators have a short life. They are either decayed quickly (e.g. kinase inactivates bradykinin) or are otherwise scavenged (e.g. antioxidant scavenge toxic oxygen metabolism) or are inhibited (e.g. complement regulatory protein break up and degrade activated complement components).

Mediator	Principal sources	Actions
Cell-Derived		
Histamine	Mast cells, basophiles, platelets	Vasodilatation, increased vascular permeability, endothelial activation
Serotonin	Platelets	Vasodilatation, increased vascular permeability
Prostaglandin	Mast cells, leukocytes	Vasodilatation, pain, fever
Leukotrienes	Mast cell, leukocytes	Increased vascular permeability, chemotaxis, leukocyte adhesion and activation
Reactive oxygen species	Leukocytes	Killing of microbes, tissue damage
Nitric oxide	Endothelium, macrophages	Vascular smooth muscle relaxation, killing of microbes
Cytokines(TNF, IL-1)	Macrophages, endothelial cells, mast cells	Local endothelial activation (expression of adhesion molecules), fever/pain/anorexia/hypotension, decreased vascular resistance (shock)
Chemokines	Leukocytes, activated macrophages	Chemotaxis, leukocyte activation
Plasma Protein-Derived		
Complement product (C5a, C3a, C4a)	Plasma (produced in liver)	Leukocyte chemotaxis and activation, vasodilatation (mast cell stimulation)
Kinins	Plasma (produced in liver)	Increased vascular permeability, smooth muscle contraction, vasodilatation, pain
Proteases activated during coagulation	Plasma (produced in liver)	Endothelial activation, leukocyte recruitment

Table 7.1: Mediators of pain

7.4. Principle

Analgesic activity of the crude plant extract was evaluated using acetic acid induced writhing method. (Koster et al, 1959; Whittle 1964; Vogel & Vogel, 1997; Ahmed et al, 2001,Mind et al ,2012). Intra-peritoneal administration of 0.7g of acetic acid is carried out to the experimental animal to create sensation of pain. Consequently, the animal squirm their body at regular interval in response to the pain, which is termed as writhing. The animals will continue to give writhing as long as they feel pain. Each of the writhing was counted and was taken as an indication of pain sensation. Any substances that can lessen the number of writhing of animal related to the decreased amount of pain sensation in a given time frame and with respect to the control group is an indication of analgesic activity. The writhing inhibition of positive control was taken as standard (in this case, Dicholefac sodium) and compared with the test sample for the evaluation of analgesic activity.

7.5.1. Experimental Animals

Swiss albino mice of either sex, aged 4-5 weeks having a mass of 20-30 gm on an average was obtained from the animal house of the department of pharmacy, Jahangirnagar University was used for the study. They were kept in standard environmental condition and fed with standard formulated mice pellets and water.

7.5.2. Experimental Design

The experimental animals were randomly selected and divided into six distinct group each consisting of 6 mice. The first two groups, Group-I and Group-II was used for the evaluation of activity of two different doses of *Murdannia Nudiflora* while the second two groups, Group-III and Group-IV was used for the evaluation of activity of two different doses of *Tradescantia pallida*. Group-V was used as standard and Group-VI was used for control group. Each of the mice were weighed properly before the feeding of the dosage and administration of the pain inducer and the dose of the sample and control materials were adjusted accordingly

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Fig 7.3: Oral feeding of extract

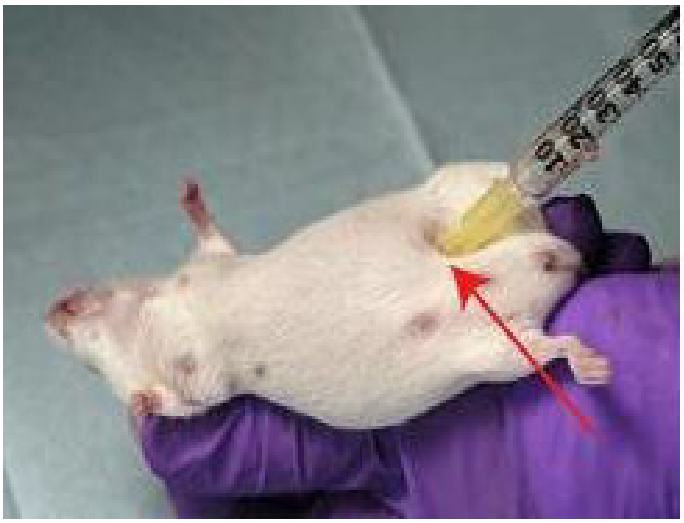


Fig 7.4: Intra-peritoneal administration of acetic acid

7.5.3. Method of Identification of Animals

Each group consisted of six mice. It is difficult to observe the biologic response of the six mices at a time receiving the same treatment, thus it is more than just necessary to identify individual animal to have accurate results during the treatment. The animals were marked in the following way

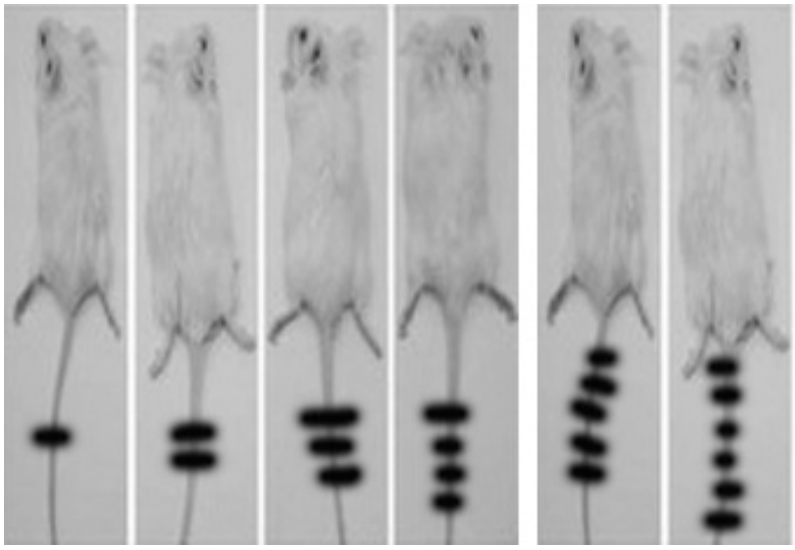


Fig 7.5: Process of Identification of Mice

7.5.4. Preparation of the sample

a. ***Preparation of the test material***

50mg of the crude extract of both the sample were accurately measured using electronic balance (AMD, USA) and dissolved in normal saline in such a way that the dosage of the crude extract received by the Group-I and Group-III would be 100mg/kg of body weight. Similarly, 100mg of the crude extract was dissolved in normal saline in order to adjust the dose as 200mg/kg of body weight. For the facilitation of dissolution of the crude extract with distilled water, unidirectional tituration with the small addition of Tween 80 or DMSO is used. The final volume of the sample was made 3ml for each of the sample group.

b. ***Preparation of standard sample***

For the preparation of standard sample, Dicholefac Na was prepared in such a way that the dosage was adjusted to 25mg/kg of body weight. Thus, 1ml of Dicholefac Na was added to 9ml of saline to prepare 10ml of suspension.

c. ***Preparation of Control sample***

Tween-80 (1%) and DMSO were mixed properly in normal saline and the volume was made upto 6ml.

Test Samples	Type	Group	Purpose	Dose (mg/kg)	Route of Administration
MN-I	Whole plant	I	Test sample	100	Oral
MN-II	Whole plant	II	Test sample	200	Oral
TP-I	Whole plant	III	Test sample	100	Oral
TP-II	Whole plant	IV	Test Sample	200	Oral
Dicholefac-NA	Chemical	V	Standard Group	25	Oral
1% Tween 80 in normal saline	Chemical	VI	Control Group	0.1ml/10gm of body weight	Oral
Glacial acetic acid (0.7%)	Chemical	-	Pain Inducer	0.1ml/10gm of body weight	Intreperitonial

MN=*Murdannia Nudiflora*, TP= *Tradescantia pallida*.

Table 7.2: Test materials used for the evaluation of analgesic activity of extracts of *Murdannia Nudiflora* and *Tradescantia pallida* using acetic acid induced writhing method

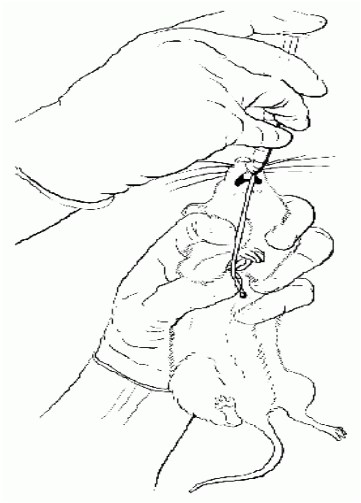
7.5.6 Procedure

a. Firstly, the test materials were administered orally by means of a long needle with a ball shaped end

b. After 30 minutes, acetic acid (0.7%0 was administered intra-peritoneal to each of the animals of the test sample group. Thirty minutes is required for the administration of test material and intra-peritoneal administration of acetic acid to ensure proper absorption of the administered sample

c. After 5 minutes, number of writhing for each mouse is counted till the completion of 15 minutes allowing a time space of 10 minutes.

d. Similarly, the standard and control group is carried out and the number of writhing was counted.



(a)

(b)

Fig 7.6: (a) Schematic diagram of oral administration of extract (b) Writhing after acetic acid induction

7.5.7. Counting of Writhing

Each of the mouse of every single group were observed individually using visual observation for counting the number of writhing they made in 15 minutes of intra-peritoneal administration of 0.7% acetic acid, commencing just after 5minutes of the administration. Full writhing is desired, however, it is not always accomplished, as for some of the cases of pain, it is not sufficient enough to cause full writhing. The incomplete writhing was counted as half writhing where two half-writhing completes one full writhing.

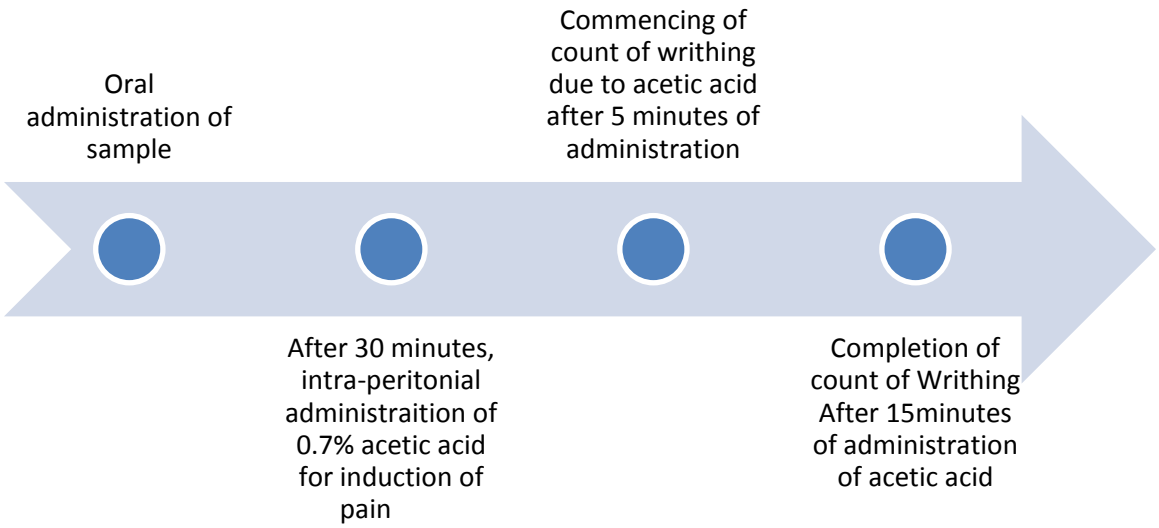


Fig 7.7: Schematic diagram of Induction and counting of writhing

7.5.8. Method of Analysis:

The significance analysis were completed using one-way ANOVA followed by Bonferoni and Dunnett-t test ($p<0.001$) in SPSS 17.0, the mean with standard error and standard deviation was determined using Microsoft excel and the percentage of inhibition of writhing was determined using the equation

% of inhibition of writhing= (1-(Mean writhing $_{(sample)}$/Mean writhing $_{(control)}$)) x100

Chapter-8

Observation and Result

8.1. Preliminary phytochemical evaluation:

8.2. In vitro cytotoxicity study by brine shrimp lethality assay:

8.3. In vitro antioxidant evaluation by DPPH Free Radical Scavanging Method

8.4. In vivo analgesic evaluation by acetic acid induced writhing Method

8. Observation and Result:

8.1. Preliminary phytochemical evaluation:

After completing wide range of chemical test for the identification of major classes of therapeutically important compounds, alkaloid, tannins, flavonoids, saponins were found in both plant. Reducing sugars were found in moderate amount and after filtration with activated charcoal with trace amount in *Tradescantia pallida*. The following table will give us a broad idea about phytochemicals present in these plants

Sl. No.	**Name of the Test**	***Murdannia nudiflora***	***Tradescantia pallida***
1.	Carbohydrate		
	Molisch Test	-	-
	Fehling's Test	-	+
2.	Tannins		
	10% Potassium Dichromate	++	++
	5% Ferric Chloride	+++	++
	1% Lead Acetate	++	+++
3.	Alkaloids		
	Mayer's Test	+++	++
	Dragendroff's Test	++	+++
	Hager's Test	++	++
4.	Test for Saponins	++++	++++
5.	Test for Proteins	-	-
6.	Test for Flavonoids	++	++
7.	Test for Steroids	-	-

++++=Present in high amount, ++=Present in moderate amount, +=Present in Trace Amount

-=Absent,

Table 8.1: List of Phytochemicals found in *Murdannia nudiflora* and *Tradescantia pallida*

8.2. In vitro cytotoxicity study by brine shrimp lethality assay:

The cytotoxic potential of the plants was obtained using brine shrimp lethality assay. After the nauplii were exposed to 24 hours of the study, it was found out at the maximum concentration for this study that is, at a concentration of 320µg/ml, *Murdannia nudiflora* showed about 55% mortality indicating the number of survived is nauplii is around 5 in an average. On the other hand, in the case of *Tradescantia pallida*, it was found at maximum concentration i.e. at 320µg/ml, the mortality rate is 45% which indicate the survival of more than 5 nauplii. For toxicological studies of the crude extract of the plant obtained, it was found out that *Murdannia nudiflora* showed at mortality of 60% and 80% at a concentration 640µg/ml and 1280µg/ml which in the case of *Tradescantia pallida*, the mortality rate is 50% and 70% respectively. For, the positive control, group 100% mortality was shown at a concentration of 8µg/ml and above which decrease to 30% at a concentration of 0.125µg/ml. The LC_{50} value of *Murdannia nudiflora* is 158.638 µg/ml and that of *Tradescantia pallida* is 833.85µg/ml.

Sample Concentration (µg/ml)	**Number of Nauplii Given**	**No. Of nauplii alive**				**Mean Percentage of Mortality**		**Vincristine Sulphate**			**Negative Control**
		MN		TP				Concentration	No. Of Nauplii Dead	Percentage of mortality	Number of Nauplii survived
		A	B	A	B	MN	TP				
320	10	4	5	6	5	55%	45%	40	10	100	10
160	10	7	7	7	8	30%	25%	20	10	100	10
80	10	8	7	8	9	25%	15%	10	10	100	10
40	10	9	8	9	9	15%	10%	5	9	90	10
20	10	9	9	9	9	10%	10%	2.5	8	80	10
10	10	10	9	10	9	5%	5%	1.25	6	60	10
5	10	10	10	10	10	0%	0%	0.625	5	50	10
2.5	10	10		10		0%	0%	0.3125	4	40	10
1.25	10	10		10		0%	0%	0.15625	3	30	10
640 (T)	10		4		5	60%	50%	80	10	100	10
1280(T)	10		2		3	80%	70%	160	10	100	10

(T)=For Toxicological studies, MN= *Murdannia nudiflora*, TP=*Tradescantia pallida*

Table 8.2: Percentage of morality with the survival nauplii by the crude extract of the plant after 24 hours of exposure in comparison to Vincristine sulphate

Sl. No.	Name of the plant	Concentration for LC_{50}	Regression Equation	R^2
1.	*Murdannia nudiflora*	158.638µg/ml	y=0.2939x-0.1467	0.9149
2.	*Tradescantia pallida*	833.85µg/ml	y=0.2205x-0.1441	0.8513
3.	Vincristine sulphate	1.021µg/ml	y=11.739x	0.5458

Table 8.3: Comparison LC_{50} of the given plant extracts

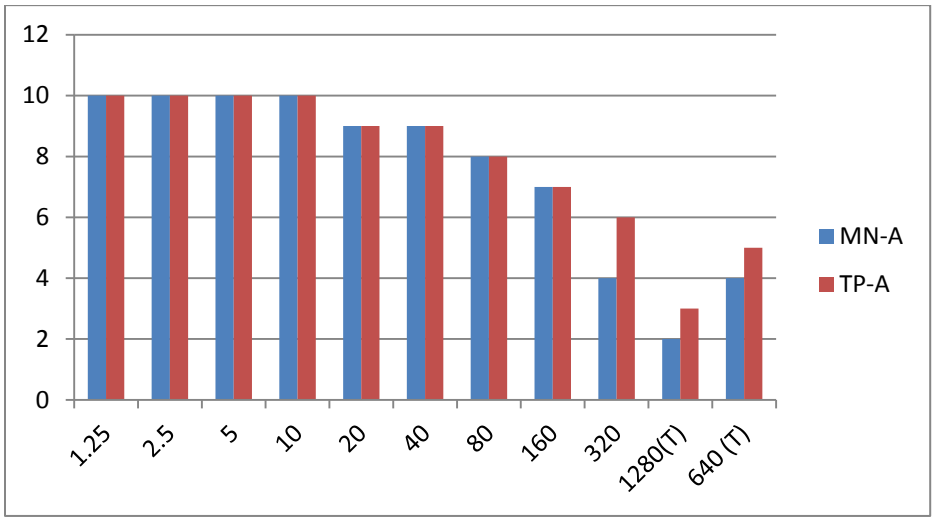


Fig 8.1: Survival of Nauplii in exposure to plant extract after 24 hours (minimum) (MN=*Murdannia Nudiflora*, TP=*Tradescantia pallida*)

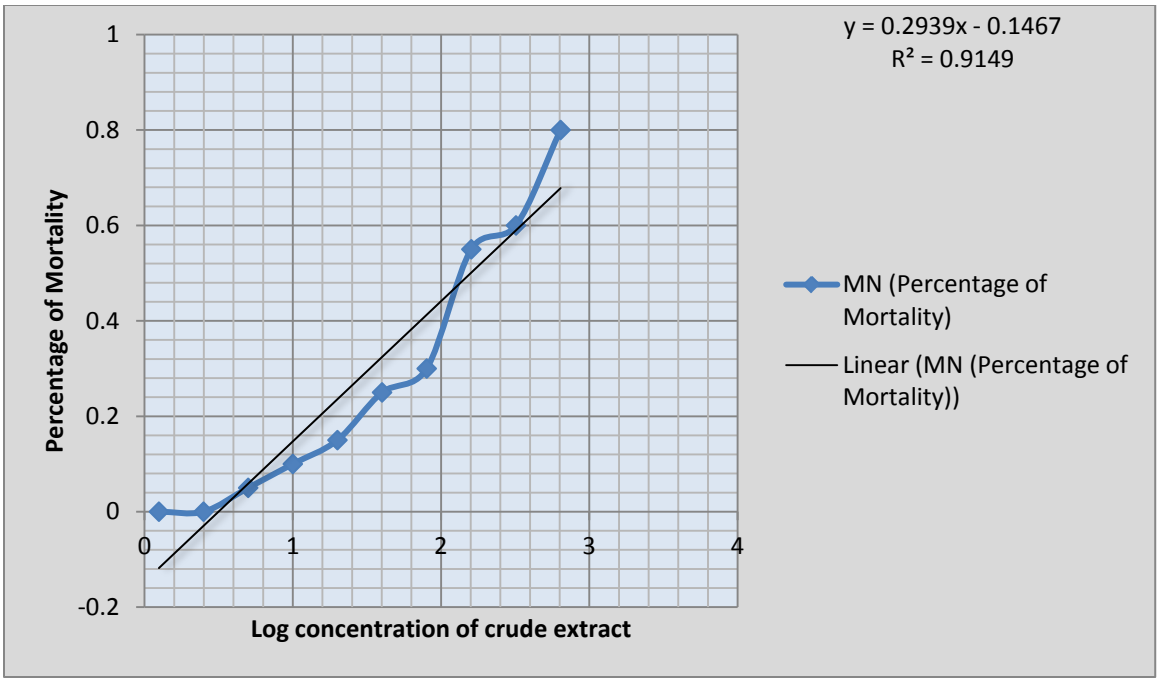


Fig 8.2: Percentage of mortality of *Murdannia Nudiflora*

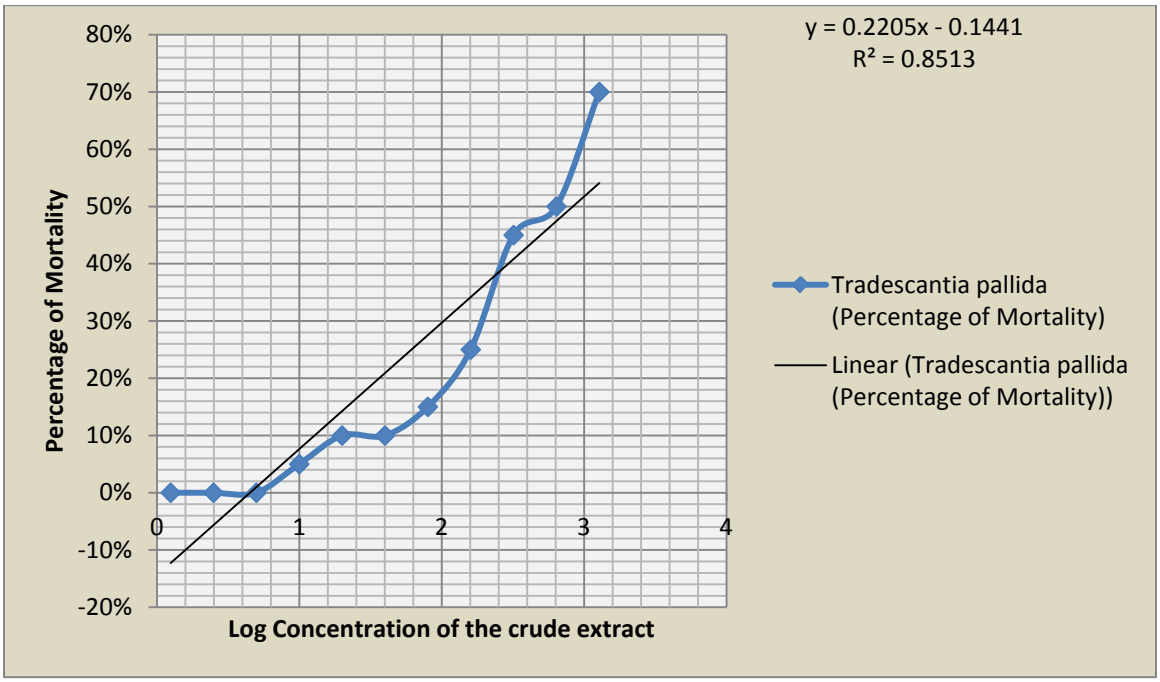


Fig 8.3: Percentage of Mortality of *Tradescantia pallida*

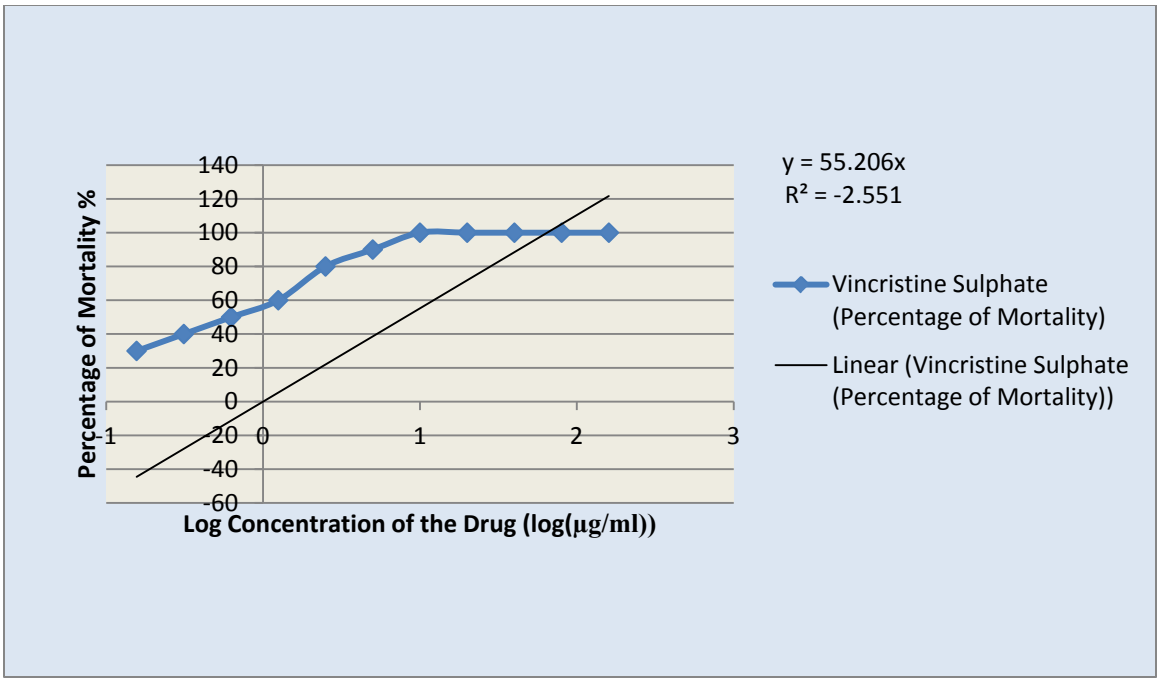


Fig 8.4: Percentage of Mortality of Vincristine Sulphate

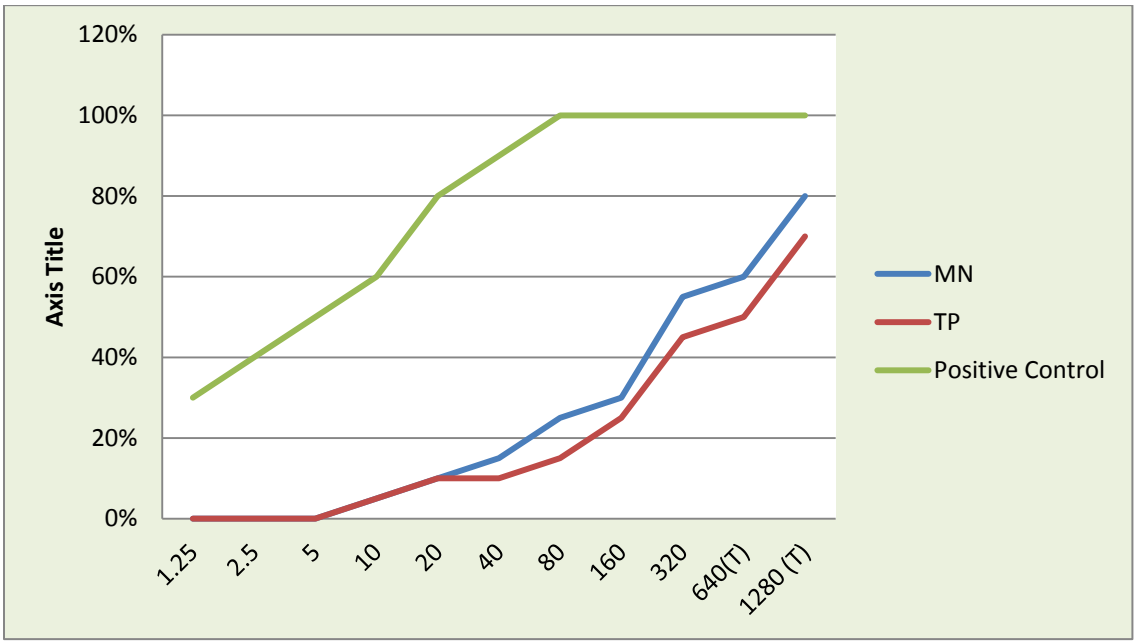


Fig 8.5: Comparison of Cytotoxicity with respect to standard (MN=*Murdannia nudiflora*, TP=*Tradescantia pallida*)

8.3. In vitro antioxidant evaluation by DPPH Free Radical Scavenging Method

The antioxidant activity was measured using DPPH Free Radical Scavenging method in order to exhibit their ability to inhibit oxidation of free radicals in the body. The percentage of free radical scavenging in the case of *Murdannia nudiflora* rages from 96.825% to 82.539%, while that of *Tradescantia pallida* ranges from 95.238% to 84.127% and that of ascorbic acid range from 96.825% to 87.302%. The amount of Free Radical Scavengers present in per 100mg of Ascorbic Acid was 95.76mg for *Murdannia Nudiflora* and 97.15mg for *Tradescantia pallida*. The IC_{50} value for Murdannia nudiflora was $1.51x10^{-10}$µg/ml and that of *Tradescantia pallida* was $5.48x10^{-10}$µg/ml.

Concentration (µg/ml)	**Absorbance of Blank (Control)**	**M.N.**		**T.P**		**A.A**		**FRS (mg /100mg of Ascorbic Acid)**			
		Absorbance	% of FRS	Absorbance	%of FRS	Absorbance	% of FRS	MN		TP	
								Individual	Mean	Individual	Mean
200		-0.008	87.302	-0.003	95.238	-0.003	95.238	91.667		100	
100		-0.004	93.651	-0.004	93.650	-0.002	96.825	96.721		96.721	
50		-0.002	96.825	-0.005	92.063	-0.004	93.651	103.389		98.305	
25		-0.006	90.476	-0.006	90.476	-0.005	92.063	98.275		96.551	
12.5	-0.063	-0.005	92.063	-0.007	88.889	-0.004	93.651	98.305	95.76	96.610	97.15
6.25		-0.007	88.889	-0.008	87.301	-0.006	90.476	98.245		96.491	
3.125		-0.008	87.302	-0.009	85.714	-0.007	88.889	98.214		96.429	
1.5625		-0.011	82.539	-0.010	84.127	-0.005	92.063	89.655		91.379	
0.78125		-0.010	84.127	-0.007	88.889	-0.008	87.302	96.363		101.818	

MN=*Murdannia nudiflora*, TP=*Tradescantia pallida*, AA=Ascorbic acid, FRS=Free Radical Scavenging

Table 8.4: Percentage of Free Radical Scavenging of plant extract in comparison to control group and the amount of free radical scavenger in comparison to 100mg of ascorbic acid

Sl. No.	Name of the Sample	IC_{50}	Regression equation	R^2
1.	*Murdannia nudiflora*	$1.51x10^{-10}$µg/ml	y=0.036x+0.8536	0.4446
2	*Tradescantia pallida*	$5.48x10^{-10}$µg/ml	y=0.0383x+0.8547	0.7797
3.	Ascorbic acid	$4.209x10^{-13}$µg/ml	y=0.0314x+0.8886	0.7654

Table 8.5: Determination of IC_{50} of the samples and standards

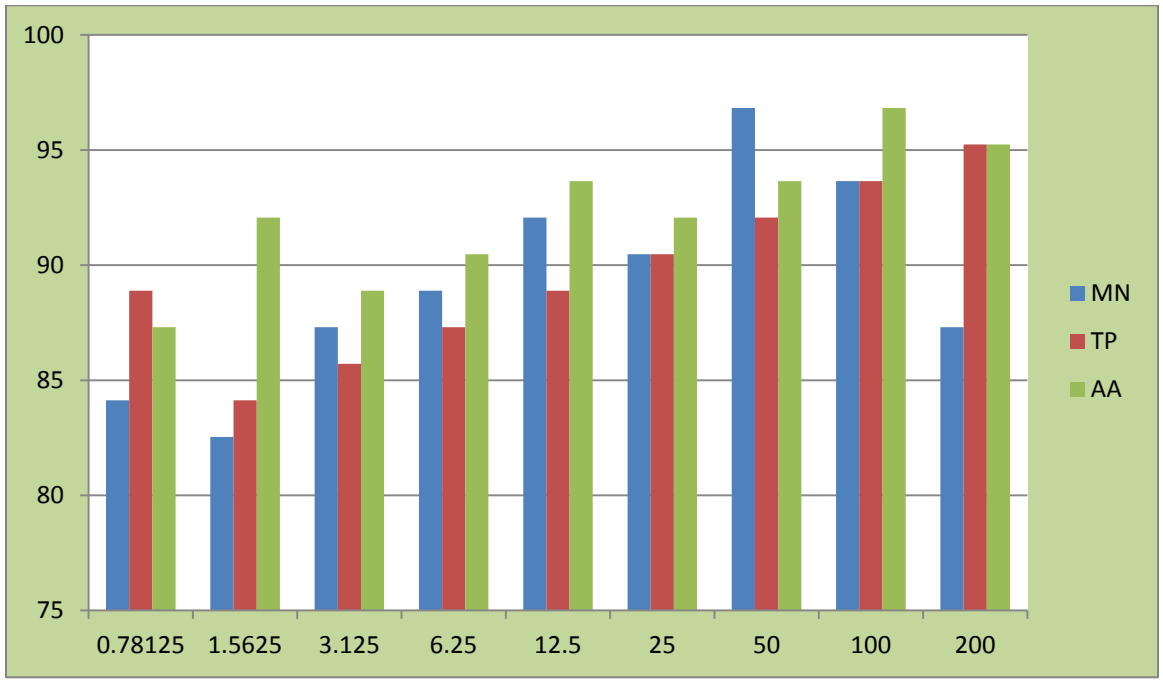


Fig 8.6: Percentage of Free Radical Scavenging by *Murdannia nudiflora* (MN), *Tradescantia pallida* (TP) and Ascorbic Acid

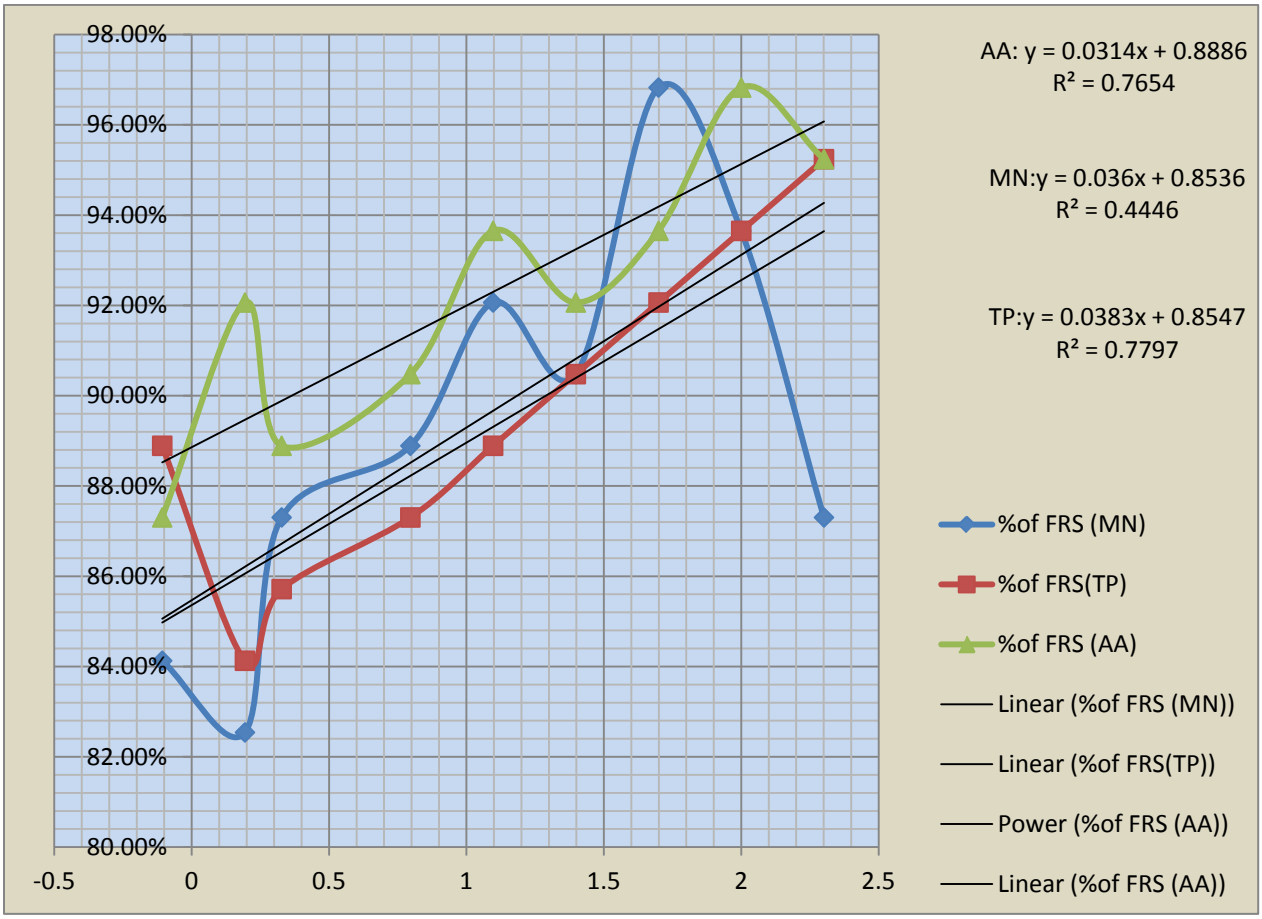


Fig 8.7: Evaluation of DPPH Free Scavenging Power of plant extract (MN=*Murdannia Nudiflora*, TP= *Tradescantia pallida,* AA=Ascorbic Acid)

8.4. In vivo analgesic evaluation by acetic acid induced writhing Method

The analgesic activity evaluation was completed using in vivo acetic acid induced writhing method in order to evaluate the central peripheral inhibition of pain sensation counted by the number of writhing given by the test animal. The result obtained showed that the extracts of both *Murdannia nudiflora* and *Tradescantia pallida* at all doses produced significant ($p<0.05$, $p<0.001$) inhibition of writhing. After the completion of counting of writhing by the end of 15 minutes of administration of 0.7% acetic acid by intra-peritoneal route, it was found out that mean amount of writhing count for the control group (Group VI) was 50.583, for Standard Group (Group V) was 9.75, for *Tradescantia pallida* at 200mg/kg of body weight (Group-IV) was 23.5 and that of 100mg/kg of body weight (Group-III) was 32.17 and lastly the significant reduction of number of writhing was found in *Murdannia nudiflora* extract, for a dose of 200mg/kg of body weight, the number of writhing was counted to 7.25 (Group-II) and that of 100mg/kg of body weight was 20.5 (Group-I). Thus, the maximum percentage of inhibition of writhing was showed by *Murdannia nudiflora* of 85.67% at 200mg/kg, while for the case of *Tradescantia pallida*, the percentage decreased to 54.55%. In the case of 100mg/kg of body weight, the percentage of inhibition of writhing for *Murdannia nudiflora* was 59.47% and that of Tradescantia pallida is 36.41%.

Animal Group	**Dose**	**Writhing Count**						**Mean with STD Error**	**Standard Deviation**	**% of Writhing**	**% of Inhibition of writhing**
		1	2	3	4	5	6				
Control		40	49	44.5	68	57	45	50.583 ±4.192	10.268	100	--
Standard	25mg/kg	11	6.5	10	12	10	9	9.75± 0.772	1.891	19.26	80.74
M.N.	100mg/kg	12.5	23	18.5	22	24	23	20.5± 1.779	4.359	40.53	59.47
M.N.	200mg/kg	4.5	3.5	6.5	12	6	11	7.25± 1.419	3.474	14.33	85.67
T.P.	100mg/kg	28	21	38	40	32	34	32.17± 2.83	6.94	63.59	36.41
T.P.	200mg/kg	24	23.5	27.5	22	23	21	23.5± 0.913	2.236	46.45	54.55

*Dicholefac sodium (25mg/kg) was used as standard. Test groups were treated with 100mg/kg and 200mg/kg of MN and TP. Data expressed as Mean±SEM, n=6. Statistical analysis was done by one way ANOVA followed by Bonferoni test and Dunnett test *p<0.5, **p<0.001

MN=*Murdannia nudiflora*, TP=*Tradescantia pallida*

Table 8.6: Evaluation of in vivo analgesic potential using acetic acid induced writhing method

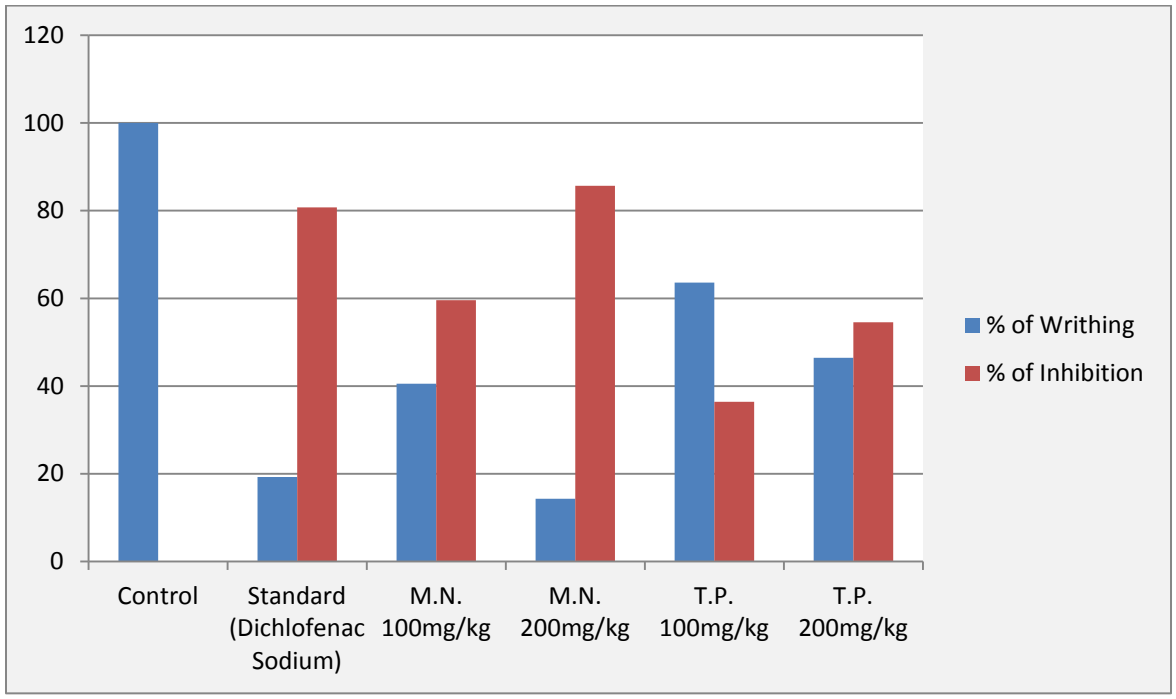


Fig 8.8: Percentage of inhibition of writhing by different groups exposed to acetic acid induced writhing

Chapter-9

Discussion

9.1. Phytochemical Evaluation

9.2. In vitro cytotoxic activity using brine shrimp lethality assay

9.3. In vitro antioxidant potential by DPPH Free Radical Scavanging Method

9.4. In vivo analgesic activity evaluation by acetic acid induced writhing method

9. Discussion

9.1. Phytochemical Evaluation

Phytochemical evaluation of both plants shows presence of alkaloids, particularly important in the inhibition of pain and inflammation by showing positive result in Mayer's test, Dragendroff's test and Hager's test; tannins, notable antimicrobial and antitumor agent by showing conformational result in 10% Potassium Dichromate in trace amount while showing positive test in 5% Ferric chloride test and 1% Lead Acetate test; saponins in notable amount, therapeutically important compounds having the potentiality of antihepatoxic, antiulcerogenic, anticoagulant, anticarcinogenic agent as well as notable inhibitor of dental caries and platelet aggregation confirmed by the presence of persistence frothing and finally the established notable flavonoids, the important group of polyphenols playing important anticancer property, anti-inflammatory activity and antibacterial activity, hepatoprotective property and most importantly the group of natural antioxidant that play significant role in free radical scavenging, metal ion chelation and inhibition of lipid peroxidation.

No protein and steroid molecule were found, not even in the traces of amount. Carbohydrate test shows negative for both plants in the case of Molisch test. However, presence of reducing sugar was found in *Tradescantia pallida* due to identification by Fehling's test both the cases of purification of activated charcoal and without purification by activated charcoal.

9.2. In vitro cytotoxic activity using brine shrimp lethality assay

After 24 hours of exposure, the plant extract showed moderate lethality at higher dosage. At 320µg/ml *Murdannia nudiflora* showed a lethality of 55% while that of *Tradescantia pallida* showed a moderate lethality of 45%. The rate of mortality decrease quite significantly and the extract becomes ineffective cytotoxic agent at 20µg/ml and complete inhibition of cytotoxic activities of both of the plants takes place at a concentration of 10µg/ml. When compared with the standard drug, Vincristine sulphate, where there is 100% mortality rate upto a concentration range 8µg/ml and higher and the presence of 50% lethal dose ranges in 0.5µg/ml, the cytotoxic potentiality is significantly low. These results indicate they are poor degree of immunosuppressant and might have moderate anti-tumorgenic potential at a significantly higher dosage.

From the toxicokinetic study of these plant extract, a moderately higher degree of lethality. For *Murdannia nudiflora*, the rate of mortality at 640µg/ml and 1280µg/ml is of 60% and 80% respectively and on the other hand, for a similar range of concentration, the mortality rate of *Tradescantia pallida* drops down to 50% and 70 % respectively. These studies indicate two dimensions. The first is they can be used in dietary supplement as they are not that highly lethal in small amount and secondarily they have the potentiality of causing serious adverse effect is taken accidentally at a larger amount.

While studying lethal concentration for 50% of the population, it was found out both required a concentration of 320µg/ml, specifically for *Murdannia nudiflora* it is 158.638µg/ml and for *Tradescantia pallida it is* 833.85µg/ml.While comparing it the standard drug Vincristine sulphate, whose LC_{50} value is 1.021µg/ml, the LC_{50} value of crude extract of the plant is exceedingly high which is an indicative low toxicity potential so they are safe to use in lower concentration.

However, there might be a complication that can enter during the study and interfere with the result. The complication is brine shrimp are moving living being and it is not always possible to have the exact number of nauplii in each of the test tube even though this is the ultimate goal of evaluation of cytotoxicity study. In addition to that, visual inspection under light it is sometimes may be difficult to identify the nauplii quantitatively especially for persons with weaker eye sight. Further investigation with other method should continue as the plant contain compound that playing notable roles in immunosupression

9.3. In vitro antioxidant potential by DPPH Free Radical Scavenging Method

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging method is used to visualize the total antioxidant potential of crude plant extract without the use of any test. DPPH radical has the ability to donate hydrogen and electron in order to remove reactive oxygen species and oxidative stress and to inhibit lipid peroxidation of the body. The plants are evaluated on the merit and potentiality of scavenging DPPH free radical and compared in respect to significant natural antioxidant named ascorbic acid to discover the effectiveness of the crude plant extract.

After UV-Spectrophotometer analysis, it was found out that both the plant have a higher percentage of Free Radical Scavenging activity which indicate the presence higher percentage of chemical having antioxidant properties. In the case of *Murdannia nudiflora*, the percentage of radical scavenging activity ranged between 96.825% and 82.539% at a concentration range started from 200µg/ml to the lowest concentration of 0.78125 µg/ml. For similar set of concentration, the percentage of radical scavenging potentials of *Tradescantia pallida* ranges between 95.238% and 84.127% and while the potentiality of ascorbic acid ranged from 96.825% to 87.302%. When the Free radical scavenging activity is compared with the ascorbic acid, the plant *Murdannia nudiflora* have a mean amount of 95.76mg of free radical scavenger per 100mg of ascorbic acid while that of *Tradescantia pallida* is 97.15mg per 100mg of ascorbic acid. The IC_{50} values for ascorbic acid was $4.209x10^{-13}$µg/ml, for *Murdannia nudiflora was* $1.51x10^{-10}$µg/ml and for *Tradescantia pallida was* $5.48x10^{-10}$µg/ml

The impressive results with the previous result of the presence of flavonoids and tannins shows the high presence of antioxidant compound of both of these plant extract. These antioxidant play not only important role in the body by anti-inflammatory, anti-microbial, free radical scavenging potentiality, these plants upon exposure to environment have huge role in removing volatile organic compounds which are potentially harmful as they can be inhaled through the human body unknowingly and their potentially to induce oxidative stress by producing reactive oxygen and nitrogen species resulting in lipid peroxidation and removing these compound can help in minimization of the adverse health impact over the human at a large scale.

9.4. In vivo analgesic activity evaluation by acetic acid induced writhing method

Analgesic activity was evaluated by using acetic acid induced writhing method. This method is useful in the evaluation of total pain sensation of the body in response to induced stimuli of pain. The extracts were given in two different doses of 100mg/kg of body weight and 200mg/kg of body weight for each group containing six mice and another group of mice was given standard Dicholefac sodium of 25mg/kg of body weight while the control group received normal saline. 0.7% of acetic acid was administered intra-peritoneal to induce pain and the analgesic activity was evaluated for a period of 10 minutes commencing at 5 minutes after administration of pain inducer and after the completion of 15 minutes of pain induction.

The analgesic potential of these plants are of notable statistical significance ($p<0.05$, $p<0.001$). The analgesic potential of *Murdannia nudiflora* is highly significant. They have shown maximum inhibition of writhing at a concentration of 200mg/kg of body weight of 85.67% higher than the inhibition of writhing of the standard drug which 80.74% while at 100mg/kg of body weight the analgesic activity showed a 59.47% inhibition of writhing. *Tradescantia pallida* showed moderate analgesic activity, a 54.55% inhibition of writhing at a dose of 200mg/kg of body weight while a mere 36.41% inhibition of writhing at a dose of 100mg/kg of body weight. The presence of alkaloids in the samples and the evaluation of inhibition of writhing by these crude plant extract shows a great deal of promise of new generation of analgesics in the future to come. However, owing to the evidence of use of *Tradescantia pallida* as an anti-inflammatory medicine the result has not been significant in terms to its use in traditional medicine.

Pain is mainly induced mainly due to inflamed or injured tissues and in the presence of pain inducing stimuli that can cause the breakdown of prostaglandin and leukotriene using the cycloxygenase and lipoxygenase pathway causing pain and inflammation. This method has been utilized using 0.7% of acetic acid to evaluate the inhibition of pain and inflammation by the mechanism causing inhibition of the synthesis of prostaglandin derivatives and leukotriene derivatives resulting in returning homeostasis and natural product would come into a great deal of value owing to the greater risk and side effects involved in the case of synthetic NSAIDs.

Chapter-10

Conclusion

10. Conclusion:

The project was intended and aimed to find out the first steps of discovery of phytochemicals which showed promising result of various kinds of important pharmacologically active secondary compound and the evaluation of different important pharmacological activities that has provided with the foundation of entering into the unknown territories of discoveries of the therapeutically important compound present in the plants and their assessment of potential to justify the scientific claim.

Phytochemical analysis of crude methanolic extract of both the plant showed the presence of alkaloids, tannins, saponins, flavonoids and the absence of protein and steroid. It gives us direction for guiding biological activities to discover their potential. Cytotoxicity assay completed by brime shrimp lethal assay showed the plants are poor immunosuppressant having LC_{50} value for *Murdannia nudiflora* of 562.636µg/ml and for *Tradescantia pallida it is* 833.85µg/ml in comparison with the standard drug, Vincristine sulphate whose LC_{50} is 4.259µg/ml, and it also gives the indication of being poor antitumor agent however also justifies the safety of these plant if accidentally ingested at small concentration. Antioxidant potential shows great promise for both the crude methanolic extract of the plant material. The percentage of Free radical scavenging for *Murdannia nudiflora* ranges from 96.825% and 82.539% at a concentration range started from 200µg/ml to the lowest concentration of 0.78125 µg/ml while for *Tradescantia pallida* it ranges between 95.238% and 84.127% for the similar set of concentrations. The IC_{50} values for *Murdannia nudiflora* was $1.51x10^{-10}$µg/ml and for *Tradescantia pallida* was $5.48x10^{-10}$µg/ml *Murdannia nudiflora* extracts shows a great deal of analgesic potential as it is found to inhibit 85.67% writhing at 200mg/kg of body weight of mice induced by 0.7% acetic acid while at 100mg/kg of body weight the percentage of inhibition of writhing was 59.47%. For a similar set of conditions, *Tradescantia pallida* has a low potential in comparison to both *Murdannia nudiflora* and standard drug, Dicholefac sodium, the inhibition of writhing in this case is 54.55% at 200mg/kg of body weight and 36.41% at 100mg/kg of body weight.

A lot of important assessment of these plants needed to be for further works for example anti-microbial potential, anti-viral potential as well as hepatoprotective, anti-carcinogenic potentials. Furthermore, the extraction of smaller group should also given priority on the basis of phytochemicals identified and the evaluation of the activities would give these initiatives more perfection in the sphere of change and global healthcare

Chapter-11

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