STUDIES OF PHYTOCHEMICAL AND ANTIOXIDANT PROPERTIES OF SELECTED LIVERWORTS OF DARJEELING HIMALAYA

A Thesis submitted to the University of North Bengal for the Award of Doctor of Philosophy in Botany

By

SUMIRA MUKHIA

Supervisor
Dr. Palash Mandal
Assistant Professor
University of North Bengal

Co-supervisor
Dr. D.K. Singh
Formerly Scientist G
Botanical Survey of India

Department of Botany
University of North Bengal
October, 2018

DECLARATION

I declare that the thesis entitled **"STUDIES OF PHYTOCHEMICAL AND ANTIOXIDANT PROPERTIES OF SELECTED LIVERWORTS OF DARJEELING HIMALAYA"** has been prepared by me under the supervision of Dr. Palash Mandal, Assistant Professor of Department of Botany and co-supervision of Dr. D.K Singh, Formerly Scientist G, Botanical Survey of India. No part of thesis has formed the basis for the award of any degree or fellowship previously.

Date: 19.11.2018

Sumira Mukhia

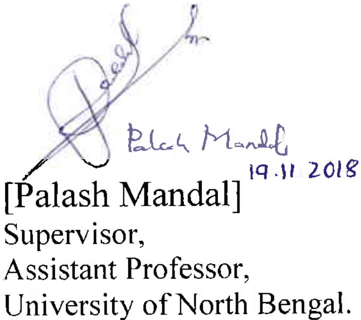
(SUMIRA MUKHIA)
Department of Botany,
University of North Bengal,
Raja Rammohunpur,
Siliguri, West Bengal, India-734013

UNIVERSITY OF NORTH BENGAL
ACCREDITED BY NAAC WITH GRADE A
Department of Botany
RajaRammohunpur Siliguri - 734013 West Bengal INDIA Phone: +91 353 2776337 FAX: +91 353 2699001

Dr. Palash Mandal
Asst. Professor
University of North Bengal

CERTIFICATE

This is to certify that the thesis entitled "**STUDIES OF PHYTOCHEMICAL AND ANTIOXIDANT PROPERTIES OF SELECTED LIVERWORTS OF DARJEELING HIMALAYA**", submitted by **Ms. Sumira Mukhia** for the award of the degree of Doctor of Philosophy in Botany is based on the results of experiments carried out by her. She has worked under my supervision at Department of Botany, University of North Bengal and Co-supervision of Dr. D.K Singh, Botanical Survey of India. I am forwarding her thesis for the Ph.D. Degree (Science) of the University of North Bengal. She has fulfilled all requirements according to the rules of the University of North Bengal regarding the works embodied in her thesis.



[Palash Mandal]
Supervisor,
Assistant Professor,
University of North Bengal.

DR. PALASH MANDAL
Assistant Professor,
Department of Botany
University of North Bengal

Dr. D.K. Singh
Scientist G (Retd.)
Botanical Survey of India
Member, Programme Advisory Committee, EcRP, &
Expert Committee on Scheme ABG
Ministry of Environment, Forests & Climate Change (GoI)

Flat No. 305, Block D
Saraswati Apartments, River View Enclave
Sector 4, Gomti Nagar Extension
Lucknow – 226 010 (U.P.)
M: +917080480720, 9433300177
E-mail: dksingh1954@gmail.com
singh_drdk@rediffmail.com

Dated: 19.11.2018

CERTIFICATE

This is to certify that the thesis, entitled "**Studies of Phytochemical and Antioxidant Properties of Selected Liverworts of Darjeeling Himalaya**" embodies the results of the work conducted by Ms. Sumira Mukhia, M.Sc., for the award of Ph.D. degree of the University of North Bengal under my co-supervision. She has carried out the work at the Department of Botany, University of North Bengal.

I am forwarding herewith her thesis for the award of Ph.D. degree in Science (Botany) of the University of North Bengal.

Dr. D.K. Singh
Scientist G (Retd.)
Botanical Survey of India

(URKUND

Urkund Analysis Result

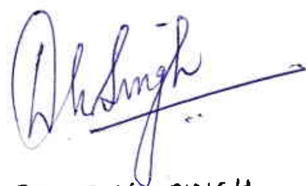
Analysed Document: Sumira Mukhia_Botany.pdf (D42359996)
Submitted: 10/10/2018 10:47:00 AM
Submitted By: nbuplg@gmail.com
Significance: 1 %

Sources included in the report:

http://14.139.13.47:8080/jspui/handle/10603/198141

Instances where selected sources appear:

2



19.11.2018

Palash Mandal

Dr. D.K. SINGH
CO-SUPERVISOR
SCIENTIST G (Retd.)
BOTANICAL SURVEY OF INDIA.

Dr. PALASH MANDAL
SUPERVISOR
DEPARTMENT OF BOTANY
UNIVERSITY OF NORTH BENGAL

DR. PALASH MANDAL
Assistant Professor,
Department of Botany
University of North Bengal

Sumira Mukhia
SUMIRA MUKHIA
(CANDIDATE)
DEPARTMENT OF BOTANY
UNIVERSITY OF NORTH BENGAL

ABSTRACT

For long time bryophytes have been neglected as a source of biologically active substances but the situation has changed now as many new biologically active compounds have been found in this group. Bryophytes can serve as the source of new therapeutically active phytochemicals. Till date, only a negligible number of species has been studied in detail. Therefore, present work aims to explore the phytochemical and pharmacological properties of some selected liverworts of Darjeeling. Accumulation of free radicals generates oxidative stress that plays major part in development of chronic and degenerative illness. Several reports document the use of antioxidants in reducing the level of oxidative stress. The antioxidant activity in present work was assessed by DPPH$^{\cdot}$, $ABTS^{+}$, superoxide, nitric oxide scavenging, metal chelating and reducing power assay. All the studied liverworts showed a promising antioxidant activity.

Diabetes mellitus is a chronic syndrome characterized by increase in the hepatic glucose production (hyperglycemia). α-amylase and α-glucosidase are the main enzymes involved in the digestion of carbohydrates. Thus by inhibiting the activity of these enzymes postprandial hyperglycemia can be controlled. In present study, highest α-amylase and α-glucosidase inhibitory activity was shown by *Marchantia subintegra*.

Anti-cancer activity was screened by studying the anti-proliferative activity against Human renal cancer cell line (ACHN). Anti-proliferative activity of studied liverworts against ACHN ranged between 69.15 and 308.98 µg/ml (IC_{50}). Among studied liverworts *Plagiochasma cordatum* showed highest cytotoxic effect against ACHN.

The phytochemicals present in the liverworts were screened by quantitative, qualitative phytochemical analysis and Thin Layer Chromatography (TLC). In *Marchantia emarginata* highest amount of phenol was recorded and the highest content of flavonoids was recorded in *Plagiochasma cordatum*. Estimation of ortho-dihydric phenol content using Arnow's reagent showed highest ortho-dihydric phenol content in *Dumortiera hirsuta* while, tannin content was highest in *Asterella wallichiana*. Qualitative phytochemical analysis confirmed the presence of steroid, tannin, triterpenoid, amino acid, resin, cardiac glycoside, alkaloid, flavonoid, reducing sugar, anthraglycoside and glycoside in the studied liverworts.

The correlation between the phytochemicals and pharmacological activities was studied by Pearson Correlation Coefficient (PCC) and Principal Component Analysis (PCA).

Result illustrated positive correlation between the pharmacological activity and phytochemical content. Phenolic content of liverworts was directly correlated with their DPPH˙, superoxide scavenging activity and reducing power. Similarly orthodihydric phenol content of liverworts was found to be positively correlated with their α-amylase inhibitory activity.

Bryophytes are successful colonizers whose life strategies are system of co-evolved adaptive qualities. An unfavourable growth condition leads to a stage when there occurs a little intracellular water content and reduced metabolic activity resulting in irrevocable damage to lipids, protein and nucleic acids by the production of Reactive species. To cope up with this condition an important strategies adapted by bryophytes is the production of strong antioxidant defence system. Study was done to evaluate the changes in antioxidant potential and phytochemical content of liverworts during unfavourable growth condition. The result showed significant increase in antioxidant activity during an unfavourable period of growth *i.e.* dry season (October –April). The phenol and flavonoid content also increased significantly during dry season. However, orthodihydric phenol content of studied liverwort was higher during rainy season. This result can be helpful in finding appropriate growth conditions for harvesting compounds with pharmacological value.

Purification and characterization of the phytochemicals with anti-diabetic potential from liverworts was done. *Marchantia paleacea* was selected for the purpose for its high bio prospective value and abundance in Darjeeling. Bioassay guided purification of *M. paleacea* yielded two bioactive fractions, FF1 and FF3. GC-MS analysis confirmed the presence of 19 phyto-compounds in these fractions. Network pharmacology is an important approach for drug discovery and development process. Through Network analysis, five phytochemicals was selected from 19 isolated compounds that specifically targeted important proteins associated with type 2 diabetes mellitus. This result highlighted the potential of *M. paleacea* to act as a source of phytochemicals having therapeutic potential against diabetes.

Chemical analysis on liverworts is restricted mainly due to their inadequate availability in nature, seasonal dependency and habitat specificity. These impediments, however, can be successfully addressed by *in-vitro* propagation of desired species. An attempt was made to initiate *in-vitro* culture of *Lunularia cruciata*. Culture was initiated from gemmae by giving continuous illumination of 4000-5000 lux at 21° ± 2°C. Half strength Murashige and Skoog media was most suitable medium for the propagation. Benzyl

aminopurine and naphthalene acetic acid (2: 0.5 mg/L) was the successful hormonal combinations. After proper *in vitro* growth of *L. cruciata*, a study was done to find out the difference in the pharmacological activities and phytochemical content of *in-vitro* and naturally grown *L. crucita*. Antioxidant potential was similar in *in-vitro* and naturally grown plants. Alpha-glycosidase inhibitory activity was high in *in-vitro* grown plants while alpha-amylase inhibitory activity was better in naturally grown plants. GC-MS analysis showed the presence of similar phytochemicals in both *in-vitro* and naturally grown plants which validated the use tissue cultured plants as a substitute for naturally grown plants to overcome the shortcomings restricting the use of liverworts for analytical purposes.

Nowadays increasing anthropogenic activities is posing serious threats to natural habitat of liverworts. Thus, study of factors that limits the growth of liverworts is highly essential form the conservational point of view. Survey related with ecology of epiphytic liverworts was done on fifty *Cryptomeria japonica* trees in Senchal Forest, Darjeeling. Factors like moisture, light intensity, age and the diameter of tree at breast height showed profuse influence on the abundance of epiphytic liverworts while biochemical properties of bark like phenol, orthodihydric phenol, flavonoid, tannin, sugar content and pH didn't show any remarkable effects; however the enhanced terpenoid content of the tree bark restricted the abundance of epiphytic liverworts. Similarly, study of the sites of growth of soil liverwort illustrated moisture content of the soil and light intensity to be the most important factors influencing the growth of liverworts. This work highlights the importance of old trees, canopy cover, moisture etc for maintenance of microclimate suitable for liverwort growth and suggests the responsible government authority to look at the activities resulting in habitat destruction of liverworts.

PREFACE

I am grateful to numerous peers who have contributed towards shaping this thesis. I would like to express my sincere gratitude to all of them. At the outset, I would like to express my appreciation and sincere gratitude to my guide Dr. Palash Mandal for his advice during my doctoral research endeavor for the past few years. As my supervisor, he has constantly inspired me to remain focused on achieving my goal. His observations and comments helped me to establish the overall direction of the research and to move forward with investigation in depth. I thank him for providing me with the opportunity to work with a talented team of researchers. It was indeed a rewarding experience.

At this juncture, I would like to thank my co- supervisor, Prof. D.K Singh Sir for his continuous support, patience and motivation throughout my Ph.D tenure. He has helped me in all possible ways and this shall always remain incised somewhere deep within me for the rest of my life. He himself being stationed far away from my research laboratory and study area was a constant source of inspiration during all these years and of course his insides time and again would open up new dimensions in my research.

Here again, I would like to show my gratitude to the Heads of the Department of Botany who looked after the department during my tenure of work for extending all the required facilities.

I am also thankful to all the teachers of Department of Botany, namely Prof. A.P. Das, Prof. B.N. Chakraborty, Prof. P. K. Sarkar, Prof. (Mrs) U. Chakraborty, Dr. A. Saha, Dr. A Sen, Dr. S.C. Roy and Dr. M. Chowdhury for their continuous support, help and encouragement. I would also like to thank the faculty in charge of SAP, Dr. U Chakraborty and Dr. A Saha for letting me utilize the facility and carryon my work smoothly.

It is indeed important to extent my gratitude to non-teaching staffs of the Department. They have all been cooperative and helpful in different ways.

I extent my special gratitude to Mr. Subhrojyoti Bagchi, Asst. Professor of Computer Sc. Malda College for assisting me while performing network analysis. Also, I would like to express

my heartiest gratitude to Dr. Anoop Kumar, Asst. Professor, Department of Biotechnology, and Dr. Pranab Ghosh, Professor, Department of Chemistry, University of North Bengal for their help during the course of my research work. I also want to extent my gratitude to Dr. Devendra Singh, Scientist D, Botanical Survey of India for helping me with the identification of plant samples.

I would also like to extend my gratitude to all my lab mates some old and new. People who come to this list are Ms. Arunika Subba, Mr. Sujoy Sen, Mr. Priyankar Roy, Mr. Dipayan Das, Mr. Salman Haider, Dr. Tarun Kumar Mishra, Ms. Suchisree Jha, Mr. Saran kumar Gupta, Dr. Mitali Ghosal and Dr. Dibakar Chowdhury, they all helped me in some way or other during my Ph.D tenure. Their support and co-operation was indispensable. Especially Miss Arunika Subba, my all season friend. She gave me mental and emotional support besides the academic help. She has helped me cheer up and get going whenever I felt low or needing in this span of my Ph.D tenure.

I am thankful to SAIF, IIT Madras and SAIF, IIT Mumbai for mass spectroscopic analysis of bioactive fractions. I am also grateful to Botanical survey of India for allowing the identifications of Plant samples.

Special thanks to University Grants Commission (UGC) for funding this work under the scheme of "Maulana Azad National fellowship".

Last but not the least, I want to express heartfelt gratitude to my parents, Mr. Mukesh Mukhia and Mrs. Asha Mukhia. They have been instrumental in building who I am today. They have been the source of constant support, strength and inspiration not only in my Ph.D but also in my entire academic endeavor. Their sacrifices are immense, not that I can enumerate. This acknowledgment would remain incomplete without mentioning Late. Chandra Prasad Mukhia, my Bajey (Grandfather). I know wherever he is, he would be proud of me today.

Sumira Mukhia

Place: **University of North Bengal** **[Sumira Mukhia]**

Date: 19. 11. 2018

TABLE OF CONTENTS

DECLARATION		ii
CERTIFICATES		iii-iv
ABSTRACT		v-vii
ACKNOWLEDGEMENT		viii-ix
TABLE OF CONTENTS		x-xvi
LIST OF TABLES		xvii-xviii
LIST OF FIGURES		xix-xxviii
LIST APPENDICES		xxix
Chapter I		
1	**INTRODUCTION AND BACKGROUND OF THE WORK**	1-6
Chapter II		
2	**REVIEW OF LITERATURE**	7-27
2.1	Status of Indian Hepaticae	7
2.2	Liverworts in traditional medicines	8-9
2.3	Chemical diversity of bryophytes	9-10
2.4	Status of study of phytochemicals of bryophytes in India	10
2.5	Biological activities	12-13
2.6	Free radicals and their effect in living organism	13-15
2.7	Antioxidants and their mechanism of action	15-16
2.8	Synthetic antioxidant	16
2.9	Free radical scavenging activity of liverworts	17-18
2.10	Diabetes	18-20
2.11	Cytotoxic activity of bryophytes	20-21
2.12	An overview of threats and conservation of Indian liverworts	22-23
2.13	*In vitro* culture	24-25

2.14	Effect of different physico-chemical factors on diversity, distribution and abundance of liverworts	25-27
Chapter III		
3	**STUDY AREA**	28-37
3.1	Rainfall	28
3.2	Temperature	28
3.3	Rainfall days	28
3.4	Sunshine hours	31
3.5	Enumeration of taxa	35-37
Chapter IV		
4	**MATERIALS AND METHODS**	38-56
4.1	Preparation of plant extract	38
4.2	**Antioxidant assays**	38-40
4.2.1	Determination of DPPH radical scavenging activity	38
4.2.2	Determination of ABTS radical scavenging activity	38
4.2.3	Determination of superoxide scavenging activity	39
4.2.4	Determination of nitric oxide scavenging activity	39
4.2.5	Determination of metal chelating activity	39
4.2.6	Determination of ferric ion reducing activity	40
4.3	**Anti-diabetic activity**	40
4.3.1	Determination of α-glucosidase inhibitory activity	40
4.3.2	Determination of α-amylase inhibitory activity	40
4.4	Anti- cancer activity	41
4.4.1	MTT assay for Mammalian Cell Viability	41
4.5	**Phytochemical analysis**	41-47
4.5.1	Quantitative phytochemical test	41-42
4.5.1a	Total phenol estimation	41
4.5.1b	Total flavonoid estimation	41-42
4.5.1c	Total orthodihydric phenol estimation	42
4.5.1d	Total Tannin estimation	42
4.5.1e	Total steroid estimation	42

4.5.2	Qualitative phytochemical analysis	42-44
4.5.2a	Test for amino acid	42
4.5.2b	Test for anthraquinones	43
4.5.2c	Test for phytosterol	43
4.5.2d	Test for tannin	43
4.5.2e	Test for triterpenoids	43
4.5.2f	Test for resins	43
4.5.2g	Test for cardiac glycosides	43
4.5.2h	Test for alkaloids	44
4.5.2i	Test for glycosides	44
4.5.2j	Test for reducing sugar	44
4.5.2	Test for flavonoids	44
4.5.3	**Phytochemical screening by Thin Layer Chromatography (TLC) analysis**	44-47
4.5.3a	Test for anthraglycosides	44-45
4.5.3b	Test for bitter principle	45
4.5.3c	Test for flavonoids	45
4.5.3d	Test for arbutin	45
4.5.3e	Test for alkaloids	46
4.5.3f	Test for cardiac glycosides	46
4.5.3g	Test for coumarins	46
4.5.3h	Test for phenol	46-47
4.6	**Seasonal changes in antioxidant activity and phenolic content in liverworts**	47
4.6.1	Sample preparation	47
4.6.2	Antioxidant assays	47
4.6.3	Quantitative phytochemical estimation	47
4.7	**Bioassay guided purification**	47-51
4.7.1	Extraction of plant sample	47-48
4.7.2	Bioassay guided screening of the fractions	48

4.7.3	Column chromatography	48-49
4.7.4	Analytical thin layer chromatography (TLC) and merging of fractions	49-50
4.7.5	Gas Chromatography – Mass Spectrometry analysis	50
4.7.6	Network pharmacology	50-51
4.7.6a	Data collection for network analysis	50-51
4.7.6b	Target-Target interactions	51
4.7.6c	Network construction and analysis	51
4.7.6d	Enrichment analysis	51
4.7.6e	Drug-likeliness prediction	51
4.8	***In vitro* propagation and comparison of bioactivities of axenically and naturally grown liverwort *Lunularia cruciata***	52-53
4.8.1	Plant material collection and method of *in vitro* culture	52-53
4.8.2	Screening of phytochemicals and biological activities	52
4.8.2a	Sample preparation	52
4.8.2b	Antioxidant activities	52
4.8.2c	Anti-diabetic activity	52
4.8.3	Phytochemical analysis through Gas Chromatography – Mass Spectrometry analysis	53
4.9	**Variation in the abundance of epiphytic liverworts in relation physico-chemical attributes**	53-55
4.9.1	Study area	53
4.9.2	pH measurement of the bark	53
4.9.3	Total phenol estimation	53
4.9.4	Total flavonoid estimation	54
4.9.5	Terpenoid estimation	54
4.9.6	Total soluble sugar estimation	54
4.9.7	Moisture content	54
4.9.8	Light intensity	54

4.9.9	Data analysis	55
4.10	**Variation in the abundance of liverworts growing on soil in relation to physical factor and soil properties**	55-56
4.10.1	Study area	55
4.10.2	Soil sampling and determination of physicochemical properties	55
4.10.3	Moisture content	56
4.10.4	Light intensity	56
4.11	Statistical analysis	56
Chapter V		
5	**RESULTS**	57-203
5.1	Free radical scavenging Activity	57-58
5.2	Anti-diabetic Activity	65
5.3	*In vitro* cytotoxicity screening	68
5.4	Phytochemical analysis	70-74
5.4.1	Quantitative estimation of phytochemicals	70
5.4.2	Qualitative phytochemical analysis	74
5.5.	Correlation between phytochemical content and pharmacological activities	74-93
5.5.1	Pearson Correlation Analysis	74
5.5.2	Principal Component Analysis	83
5.5.3	Heatmap Analysis	86-93
5.6	Seasonal changes in antioxidant activity and phenolic content in liverworts	96-97
5.7	Bioassay guided partial purification of liverwort *Marchantia paleacea* from Darjeeling Himalaya	100-122
5.7.1	Network pharmacology	137-163
5.7.1a	Identification of phytochemical compound	137
5.7.1b	Protein target compilation	137
5.7.1c	Phytochemical, Protein target and targeted disease	143
5.7.1d	Network robustness Study	150

5.7.1e	Network parameters and their relation	150
5.7.1f	Enrichment (KEGG) Analysis of Centralized Target Proteins	156-158
5.7.1g	Druggability Analysis and ADMET Study	162-163
5.8	*In vitro* propagation and comparison of bioactivity and phytochemical constituents of axenically cultured and naturally grown *Lunularia cruciata*	169
5.8.1	Comparison of free radical scavenging potential of *in-vitro* and naturally grown *Lunularia cruciata*	173-174
5.8.1a	$DPPH^{\bullet}$ scavenging activity	173
5.8.1b	$ABTS^{+}$ scavenging activity	173
5.8.1c	Metal chelating assay	173-174
5.8.2	Comparison of anti-diabetic activity of *in-vitro* and naturally grown *Lunularia cruciata*	174
5.8.2a	α-Glucosidase inhibitory activity	174
5.8.2b	α-amylase inhibitory activity	174
5.8.3	Phytochemical analysis of *in-vitro* and naturally grown *Lunularia cruciata*	176
5.9.1	Variation in the abundance of epiphytic liverworts in relation to physical and physico-chemical attributes	192-194
5.9.2	Variation in the abundance of liverworts growing on soil in relation to physical factor and soil properties	194
Chapter VI		
6	**DISCUSSION**	204-236
6.1	Free radical scavenging activity	204-208
6.2	Anti-diabetic activity	208-210
6.3	Cytotoxic activity	210-211
6.4	Phytochemical estimation	211-213
6.5	Correlation between phytochemical content and pharmacological activities	213-215
6.6	Seasonal changes in antioxidant activity and phenolic content in liverworts	215-217
6.7	Bioassay guided purification	217-227
6.8	*In vitro* propagation and comparison of	

	pharmacological activities and phytochemical constituents of *in vitro* and naturally grown liverwort *Lunularia cruciata*	228-231
6.9.1	Variation in the abundance of epiphytic liverworts in relation to physical and physico-chemical attributes	232-234
6.9.2	Variation in the abundance of liverworts growing on soil in relation to physical factor and soil properties	234-236
	CONCLUSION	237-238
	SUMMARY	239-243
	BIBLIOGRAPHY	244-266
	INDEX	267-268
	APPENDIX A	
	Chemical used	269-270
	APPENDIX B	
	Abbreviation used	271-274
	APPENDIX C	275
	List of publications	
	APPENDIX D	276
	Published articles [Based on the Thesis works]	

`

LIST OF TABLES

Table No.	*Table*	*Page No*
Table 2.1	List of some important phytochemicals present in liverworts of Indian Bryoflora	11
Table 3.1	Altitudinal details of the site of collection of studied eleven liverwort species	31
Table 5.1.1	DPPH˙ scavenging activity of studied liverworts	62
Table 5.1.2	$ABTS^+$ scavenging activity of studied liverworts	62
Table 5.1.3	Super oxide scavenging activity of studied liverworts	63
Table 5.1.4	Nitric oxide scavenging activity of studied liverworts (IC50 mg/ml)	63
Table 5.1.5	Metal Chelating activity of studied eleven liverworts	64
Table 5.1.6	Reducing potential of studied eleven liverworts	64
Table 5.2.1	Phytochemical profile of *Plagiochasma cordatum*	75
Table 5.2.2	Phytochemical profile of *Plagiochila nepalensis*	75
Table 5.2.3	Phytochemical profile of *Marchantia polymorpha*	75
Table 5.2.4	Phytochemical profile of *Asterella wallichiana*	76
Table 5.2.5	Phytochemical profile of *Pellia epiphylla*	76
Table 5.2.6	Phytochemical profile of *Marchantia subintegra*	76
Table 5.2.7	Phytochemical profile of *Dumortiera hirsuta*	77
Table 5.2.8	Phytochemical profile of *Lunularia cruciata*	77
Table 5.2.9	Phytochemical profile of *Conocephalum japonicum*	77
Table 5.2.10	Phytochemical profile of *Marchantia emarginata*	78
Table 5.2.11	Phytochemical profile of *Marchantia paleacea*	78
Table 5.3	Retardation factors of different analytes present in bioactive fractions from first column chromatograph of *Marcantia paleacea*	107-110
Table 5.4	Retardation factors of different analytes present in bioactive fractions from second column chromatograph of *Marcantia paleacea*	119-120
Table 5.5	List of phytochemicals present in fraction FF1 and their activities	129

Table No.	*Table*	*Page No*
Table 5.6	List of phytochemicals present in fraction FF3 and their activities	136
Table 5.7	List of phytochemicals present in FF1and their canonical smile and Pubchem ID	138
Table 5.8	List of phytochemicals present FF3 and their canonical smile and Pubchem ID	139
Table 5.9	List of proteins targeted by the phytochemicals in human	140-141
Table 5.10	List of diseases associated by target proteins	144-147
Table 5.11	Network list of first 30% of total targets analyzed through tripartite network	154
Table 5.12.1	Druggability analysis of phytochemicals for Absorption ability	164
Table 5.12.2	Druggability analysis of phytochemicals for distribution	165
Table 5.12.3	Druggability of phytochemicals for metabolism	166
Table 5.12.4	Druggability analysis of phytochemicals for Toxicity	167
Table 5.12.5	Druggability analysis of phytochemicals for excretion	168
Table 5.12.6	Druggability analysis according to Lipinski's criterion	168
Table 5.13	Effect of BAP/IAA on gemmae germination	170
Table 5.14	Phytochemicals present in *in-vitro* grown *Lunularia cruciata* methanol extract and their biological activities	177
Table 5.15	Phytochemicals present in *in-vitro* grown *Lunularia cruciata* methanol extract and their biological activities	184
Table 5.16	Comparison of phytochemicals present in *in-vitro* and *in-vivo* grown plant extract	191
Table 5.17	Correlation between epiphytic liverwort abundance and the physiological factors like moisture content of bark, pH of the bark and light intensity.	198
Table 5.18	Correlation between epiphytic liverwort abundance, terpenoid content of the tree barks and tree age.	198
Table 5.19	Correlation between epiphytic liverwort abundance and phenol, flavonoid, orthodihydric phenol, tannin and sugar content of the studied tree bark.	199
Table 5.20	Co-efficient of determination (R^2)	199

LIST OF FIGURES

Figure No.	*Figure*	*Page No.*
Figure 2.1	Free radicals and their harmful effects	14
Figure 2.2	Diagrammatic presentation of pathway of oxidative stress induced diabetes complications	19
Figure 3.1	Map of India showing the Study site i.e. Darjeeling area in West Bengal	29
Figure 3.2	Map of surveyed area of Darjeeling for liverwort sample collection (Senchal forest, Ghoom forest, Dhooteria forest, Kalej valley, Singmari, Lebong)	29
Figure 3.3	Average rainfall of Darjeeling throughout the year	30
Figure 3.4	Average temperature of Darjeeling throughout the year	30
Figure 3.5	Average rainfall of Darjeeling throughout the year	30
Figure 3.6	Average sunshine hours of Darjeeling throughout the year	30
Figure 3.7.1	Photographs of *Lunularia cruciata*	32
Figure 3.7.2	Photographs of *Dumortiera hirsuta*	32
Figure 3.7.3	Photographs of *Pellia epiphylla*	32
Figure 3.7.4	Photographs of *Marchantia paleacea*	32
Figure 3.7.5	Photographs of *Marchantia polymorpha*	33
Figure 3.7.6	Photographs of *Conocephalum japonicum*	33
Figure 3.7.7	Photographs of *Marchantia subintegra*	33
Figure 3.7.8	Photographs of *Plagiochasma cordatum*	33
Figure 3.7.9	Photographs of *Asterella wallichiana*	34
Figure 3.7.10	Photographs of *Marchantia emarginata*	34
Figure 3.7.11	Photographs of *Plagiochila nepalensis*	34
Figure 5.1.1	DPPH radical scavenging activity of studied eleven liverworts	59
Figure 5.1.2	$ABTS^+$ scavenging activity of studied eleven liverworts	59
Figure 5.1.3	Super oxide scavenging activity of studied eleven liverworts	60
Figure 5.1.4	Nitric oxide scavenging activity of studied eleven liverworts	60
Figure 5.1.5	Metal chelating activity of studied eleven liverworts	61
Figure 5.1.6	Reducing power of studied eleven liverworts	61
Figure 5.2.1	Anti-diabetic activity of *Asterella wallichiana*	66

Figure No.	***Figure***	***Page No.***
Figure 5.2.2	Anti-diabetic activity of *Conocephalum japonicum*	66
Figure 5.2.3	Anti-diabetic activity of *Dumortiera hirsuta*	66
Figure 5.2.4	Anti-diabetic activity of *Pellia epiphylla*	66
Figure 5.2.5	Anti-diabetic activity of *Lunularia cruciata*	66
Figure 5.2.6	Anti-diabetic activity of *Marchantia emarginata*	66
Figure 5.2.7	Anti-diabetic activity of *Marchantia polymorpha*	67
Figure 5.2.8	Anti-diabetic activity of *Marchantia subintegra*	67
Figure 5.2.9	Anti-diabetic activity of *Plagiochasma cordatum*	67
Figure 5.2.10	Anti-diabetic activity of *Plagiochila nepalensis*	67
Figure 5.3.1	Inhibitory activity of *Plagiochasma cordatum* on ACHN cell proliferation	69
Figure 5.3.2	Inhibitory activity of *Asterella wallichiana* on ACHN cell proliferation	69
Figure 5.3.3	Inhibitory activity of *Plagiochila nepalensis* on ACHN cell proliferation	69
Figure 5.3.4	Inhibitory activity of *Lunularia cruciata* on ACHN cell proliferation	69
Figure 5.3.5	Inhibitory activity of *Marchantia paleacea* on ACHN cell proliferation	
Figure 5.3.6	IC 50 values of anti-proliferative activity studied liverworts against ACHN cells	69
Figure 5.4.1	Phenol content of studied eleven liverwort species in different solvent extracts	71
Figure 5.4.2	Flavonoid content of studied eleven liverwort species in different solvent extracts	71
Figure 5.4.3	Ortho-dihydric phenol content of studied eleven liverwort species in different solvent extracts	71
Figure 5.4.4	Steroid content of studied eleven liverwort species in different solvent extracts	71
Figure 5.4.5	Tannin content of studied eleven liverwort species in different solvent extracts	71
Figure 5.4.6.1	Detection of phytochemicals in *Marchantia emarginata* by TLC analysis	79

Figure No.	*Figure*	*Page No.*
Figure 5.4.6.2	Detection of phytochemicals in *Marchantia subintegra* by TLC analysis	79
Figure 5.4.6.3	Detection of phytochemicals in *Plagiochasma cordatum* by TLC analysis	79
Figure 5.4.6.4	Detection of phytochemicals in *Asterella wallichiana* by TLC analysis	80
Figure 5.4.6.5	Detection of phytochemicals in *Conocephalum japonicum* by TLC analysis	80
Figure 5.4.6.6	Detection of phytochemicals in *Dumortiera hirsuta* by TLC analysis	80
Figure 5.4.6.7	Detection of phytochemicals in *Pellia epiphylla* by TLC analysis	81
Figure 5.4.6.8	Detection of phytochemicals in *Lunularia cruciata* by TLC analysis	81
Figure 5.4.6.9	Detection of phytochemicals in *Marchantia polymorpha* by TLC analysis	81
Figure 5.4.6.10	Detection of phytochemicals in *Marchantia paleacea* by TLC analysis	82
Figure 5.4.6.11	Detection of phytochemicals in *Plagiochila nepalensis* by TLC analysis	82
Figure 5.5.1	Correlation between phytochemical content and pharmacological activities	84
Figure 5.5.2	Principal component analysis of phytochemical content and antioxidant, anti-diabetic activities of studied liverworts	85
Figure 5.5.3.1	Heatmap analysis of role different solvents on extraction of bio-active phytochemicals from *Asterella wallichiana*	87
Figure 5.5.3.2	Heatmap analysis of role different solvents on extraction of bio-active phytochemicals from *Conocephalum japonicum*	87
Figure 5.5.3.3	Heatmap analysis of role different solvents on extraction of bio-active phytochemicals from *Dumortiera hirsuta*	89
Figure 5.5.3.4	Heatmap analysis of role different solvents on extraction of bio-active phytochemicals from *Lunularia cruciata*	89
Figure 5.5.3.5	Heatmap analysis of role different solvents on extraction of bio-active phytochemicals from *Pellia epiphylla*	90
Figure 5.5.3.6	Heatmap analysis of role different solvents on extraction of bio-active phytochemicals from *Marchantia paleacea*	90

Figure No.	*Figure*	*Page No.*
Figure 5.5.3.7	Heatmap analysis of role different solvents on extraction of bio-active phytochemicals from *Marchantia polymorpha*	92
Figure 5.5.3.8	Heatmap analysis of role different solvents on extraction of bio-active phytochemicals from *Marchantia emarginata*	92
Figure 5.5.3.9	Heatmap analysis of role different solvents on extraction of bio-active phytochemicals from *M.archantia subintegra*	94
Figure 5.5.3.10	Heatmap analysis of role different solvents on extraction of bio-active phytochemicals from *Plagiochasma cordatum*	94
Figure 5.5.3.11	Heatmap analysis of role different solvents on extraction of bio-active phytochemicals from *Plagiochila nepalensis*	95
Figure 5.6.1	comparison of $DPPH^{\cdot}$ scavenging activity of *Marchantia paleacea* during rainy and dry season	98
Figure 5.6.2	comparison of $ABTS^{\cdot}$ scavenging activity of *Marchantia paleacea* during rainy and dry season	98
Figure 5.6.3	Comparison of SO scavenging activity of *Marchantia. paleacea* during rainy and dry season	98
Figure 5.6.4	Comparison of NO scavenging potential of *Marchantia paleacea* during rainy and dry season	98
Figure 5.6.5	Comparison metal chelating activity of *Marchantia paleacea* during rainy and dry season	98
Figure 5.6.6	Comparison of reducing power of *Marchantia paleacea* during rainy and dry season	98
Figure 5.6.7	Comparison of phenolic content of *M. paleacea* during rainy and dry season	99
Figure 5.6.8	Comparison of flavonoid content of *M. paleacea* during rainy and dry season	99
Figure 5.6.9	Comparison Orthodihydric phenol content of *M. paleacea* during rainy and dry season	99
Figure 5.7	Schemes of fraction preparation of *Marchantia paleacea*	101
Figure 5.7.1	$DPPH^{\cdot}$ and $ABTS^{+}$ radical scavenging activities of main fractions of *Marchantia paleacea* obtained from successive extraction	102
Figure 5.7.2	Flavonoid content of main fractions of *Marchantia paleacea* obtained from successive extraction	102
Figure 5.7.3	$DPPH^{\cdot}$ scavenging activity of fractions of *Marchantia paleacea* obtained from first column chromatography	103

Figure No.	***Figure***	***Page No.***
Figure 5.7.4.	TLC profiling of bioactive sub-fractions of *Marchantia paleacea* obtained from first column chromatograph	104-106
Figure 5.7.5	DPPH$^{\cdot}$ scavenging activity of merged fractions of *Marchantia paleacea* obtained from first column chromatograph	111
Figure 5.7.6	ABTS^{+} scavenging activity of merged fractions of *Marchantia paleacea* obtained from first column chromatograph	111
Figure 5.7.7	Anti-diabetic activity (α-glucosidase inhibitory) of merged fractions of *Marchantia paleacea* obtained from first column chromatograph	112
Figure 5.7.8	Extractive yield of the merged fractions of *Marchantia paleacea* obtained from first column chromatograph	112
Figure 5.7.9.	DPPH$^{\cdot}$scavenging activity of fractions of *Marchantia paleacea* obtained from second column chromatograph	113
Figure 5.7.10	DPPH$^{\cdot}$ scavenging activity and flavonoid content of fractions of *Marchantia paleacea* obtained after second column chromatography	113
Figure 5.7.11	TLC profiling of bioactive fractions of *Marchantia paleacea* obtained from second column chromatograph	114-118
Figure 5.7.12	DPPH$^{\cdot}$scavenging activity of merged fractions of *Marchantia paleacea* obtained from second column chromatograph	121
Figure 5.7.13	Anti-diabetic activity of merged fractions of *Marchantia paleacea* obtained from second column chromatograph	121
Figure 5.7.14	HR- GCMS scanning chromatogram of fraction FF-1 of *Marchantia paleacea*	123
Figure 5.7.15	Structure and MS spectrum of (17á), Estra- 1,3,5 (10), 6- tetraene- 3,17- diol diacetate compared with NIST Library Spectral Database	124
Figure 5.7.16	Structure and MS spectrum of 4-methyl -1- (1-methylethyl) 3- Cyclohexen -1 ol compared with NIST Library Spectral Database	124
Figure 5.7.17	Structure and MS spectrum of 7-hydroxy-2- phenyl 4H-1- Benzopyran-4- one compared with NIST Library Spectral Database	125
Figure 5.7.18	Structure and MS spectrum of Phytol compared with NIST Library Spectral Database	125
Figure 5.7.19	Structure and MS spectrum of 4’, 5, 7- Trihydroxy isoflavone compared with NIST Library Spectral Database	126

Figure No.	*Figure*	*Page No.*
Figure 5.7.20	Structure and MS spectrum of 5- hydroxy-7- methoxy-2-(4-methoxyphenyl) 4H-1- benzopyran-4- one compared with NIST Library Spectral Database	126
Figure 5.7.21	Structure and MS spectrum of Pimaric acid compared with NIST Library Spectral Database	127
Figure 5.7.22	Structure and MS spectrum of Retinol compared with NIST Library Spectral Database	127
Figure 5.7.23	Structure and MS spectrum of Palustric acid compared with NIST Library Spectral Database	128
Figure 5.7.24	Structure and MS spectrum of 9-thiocyanato Androstan-4-en-3,17- diol- 11-one compared with NIST Library Spectral Database	128
Figure 5.7.25	HR- GCMS scanning chromatogram of fraction FF-3 of *Marchantia paleacea*	130
Figure 5.7.26	Structure and MS spectrum of 2,6-bis (1,1-dimethylethyl)-4-ethyl Phenol compared with NIST Library Spectral Database	131
Figure 5.7.27	Structure and MS spectrum of 2-tetradecanoyl-2-Cyclohexen-3,6-diol-1-one compared with NIST Library Spectral Database	131
Figure 5.7.28	Structure and MS spectrum of 1-Docosene compared with NIST Library Spectral Database	132
Figure 5.7.29	Structure and MS spectrum of Caryophyllene compared with NIST Library Spectral Database	132
Figure 5.7.30	Structure and MS spectrum of 7-(diethylamino) -2H-1-Benzopyran-2-one compared with NIST Library Spectral Database	133
Figure 5.7.31	Structure and MS spectrum of α-Myrcene compared with NIST Library Spectral Database	133
Figure 5.7.32	Structure and MS spectrum of 1- Octadecene compared with NIST Library Spectral Database	134
Figure 5.7.33	Structure and MS spectrum of 4H-1-Benzopyran-4-one, 5-hydroxy-7-methoxy-2-phenyl compared with NIST Library Spectral Database	134
Figure 5.7.34	Structure and MS spectrum of (9E), 3-Eicosene compared with NIST Library Spectral Database	135
Figure 5.7.35	Structure and MS spectrum of Methyl 2-(4-hydroxy-3-isopropyl) benzoate compared with NIST Library Spectral Database	135

Figure No.	*Figure*	*Page No.*
Figure 5.7.36	Interaction between target proteins of bioactive phytochemicals by using STRING Interactome Database	142
Figure 5.7.37	Pie chart representing the disease classes of different diseases associated with target protein.	148
Figure 5.7.38	Whole tripartite network of phytochemical, target protein and associated disease classes	149
Figure 5.7.39	Protein elimination by Network Perturbation	151-152
Figure 5.7.40	Network parameters versus percentage of network perturbation	153
Figure 5.7.41	Degree × betweenness × clossness analysis of 20 protein targets	155
Figure 5.7.42	Degree analysis of 20 protein targets	155
Figure 5.7.43	Betweenness centrality analysis of 20 protein targets	155
Figure 5.7.44	Function of eight protein target having high betweenness centrality value and percentage of heat	157
Figure 5.7.45	Insulin signalling pathway (hsa04931) obtained from KEGG database. Genes present in tripartite network is marked red	157
Figure 5.7.46	Insulin resistance pathway (hsa04931) obtained from KEGG database. Genes present tripartite network are marked in red	159
Figure 5.7.47	Insulin signalling pathway obtained from KEGG database. Genes present tripartite network are marked in red	160
Figure 5.7.48	Final tripartite network of five most important protein targets participating in diabetes pathway	161
Figure 5.8.1	Effect of different growth media on gemmae germination	170
Figure 5.8.2	*In-vitro* growth of *Lunularia cruciata*	171-172
Figure 5.8.3	DPPH$^{\cdot}$ scavenging activityof *in-vitro* and *in-vivo* (naturally grown) *Lunularia cruciata*	175
Figure 5.8.4	ABTS$^{\cdot}$ scavenging activity of *in-vitro* and *in-vivo* (naturally grown) *Lunularia cruciata*	175
Figure 5.8.5	Metal chelating activity of *in-vitro* and *in-vivo* (naturally grown) *Lunularia cruciata*	175
Figure 5.8.6	α-glucosidase inhibitory activity of *in-vitro* and *in-vivo* (naturally grown) *Lunularia cruciata*	175
Figure 5.8.7	α-amylase inhibitory activity of *in-vitro* and *in-vivo* (naturally grown) *Lunularia cruciata*	175
Figure 5.8.8	Gas chromatogram with mass-spectrometric detection of the methanolic extract of *in-vitro* grown plants	178

Figure No.	*Figure*	*Page No.*
Figure 5.8.9	Structure and MS spectrum of 1-butyl- Piperidine compared with NIST Library Spectral Database	179
Figure 5.8.10	Structure and MS spectrum of 3-Hydroxy-2-[(5-oxo-1-cyclopenten-1-yl)methyl]-2-cyclohexen-1-one compared with NIST Library Spectral Database	179
Figure 5.8.11	Structure and MS spectrum of flavone compared with NIST Library Spectral Database	180
Figure 5.8.12	Structure and MS spectrum of 4H-1-Benzopyren-4-one, 3-hydroxy-2-phenyl compared with NIST Library Spectral Database	180
Figure 5.8.13	Structure and MS spectrum of Tetradecanoic acid, ethyl ester compared with NIST Library Spectral Database	181
Figure 5.8.14	Structure and MS spectrum of Phytol compared with NIST Library Spectral Database	181
Figure 5.8.15	Structure and MS spectrum of 2-13-octadecen-1-yl acetate compared with NIST Library Spectral Database	182
Figure 5.8.16	Structure and MS spectrum of Isopropyl stearate compared with NIST Library Spectral Database	182
Figure 5.8.17	Structure and MS spectrum of Tricosane -2,4- dione compared with NIST Library Spectral Database	183
Figure 5.8.18	Gas chromatogram with mass-spectrometric detection of the methanolic extract of naturally grown plants	185
Figure 5.8.19	Structure and MS spectrum of Thujopsene-(12) compared with NIST Library Spectral Database	186
Figure 5.8.20	Structure and MS spectrum of Flavone compared with NIST Library Spectral Database	186
Figure 5.8.21	Structure and MS spectrum of Cyclopentaneudecanoic acid, methyl ester compared with NIST Library Spectral Database	187
Figure 5.8.22	Structure and MS spectrum of Cyclopentaneudecanoic acid compared with NIST Library Spectral Database	187
Figure 5.8.23	Structure and MS spectrum of 13,16-Octadecadienoic acid, methyl ester compared with NIST Library Spectral Database	188
Figure 5.8.24	Structure and MS spectrum of Phytol compared with NIST Library Spectral Database	188
Figure 5.8.25	Structure and MS spectrum of deoxy-Aspidodispermine compared with NIST Library Spectral Database	189

Figure No.	*Figure*	*Page No.*
Figure 5.8.26	Structure and MS spectrum of 13-Docosenoic acid, methyl ester compared with NIST Library Spectral Database	189
Figure 5.8.27	Structure and MS spectrum of 1-acetyl-5-ethyl-2-[3-[2-hydroxyethy]-1H-indol-2-yl]-α-methyl 4-Piperidineacetic acid, methyl ester compared with NIST Library Spectral Database	190
Figure 5.9.1	Variation in abundance % according to moisture content of bark	195
Figure 5.9.2	Normal probability curve of distribution of liverworts related with moisture content of the bark	195
Figure 5.9.3	Variation in abundance % according to light intensity	195
Figure 5.9.4	Normal probability curve of distribution of liverworts related with light intensity	195
Figure 5.9.5	Variation in abundance % according to pH of the bark	195
Figure 5.9.6	Normal probability curve of distribution of liverworts related with pH bark	195
Figure 5.9.7	Variation in abundance % according to girth of the tree	196
Figure 5.9.8	Normal probability curve of distribution of liverworts related with girth of the tree	196
Figure 5.9.9	Variation in abundance % according to phenol content of bark	196
Figure 5.9.10	Normal probability curve of distribution of liverworts related with phenol content of the bark	196
Figure 5.9.11	Variation in abundance % according to flavonoid content of bark	196
Figure 5.9.12	Normal probability curve of distribution of liverworts related with flavonoid content of bark	196
Figure 5.9.13	Variation in abundance % according to ortho-dihydric phenol content	197
Figure 5.9.14	Normal probability curve of distribution of liverworts related with ortho-dihydric phenol content of bark	197
Figure 5.9.15	Variation in abundance % according to terpenoid content of bark	197
Figure 5.9.16	Normal probability curve of distribution of liverworts related with terpenoid content of bark	197
Figure 5.9.17	Variation in abundance % according to total sugar content of bark	197
Figure 5.9.18	Normal probability curve of distribution of liverworts related with sugar content of bark	197

Figure No.	*Figure*	*Page No.*
Figure 5.9.19	Variation in abundance % according to tannin content of bark	198
Figure 5.9.20	Normal probability curve of distribution of liverworts related with tannin content of bark	198
Figure 5.9.21	Principle component analysis of epiphytic liverwort abundance and different physical factors (light, moisture) and biochemical characteristics of plants	200
Figure 5.9.22	Z-score heatmap of the epiphytic liverwort density and different physical and biochemical factors.	201
Figure 5.9.23	Influence of light intensity on abundance % of liverworts	202
Figure 5.9.24	Influence of soil moisture % on abundance % of liverworts	202
Figure 5.9.25	Influence of soil pH on abundance % of liverworts	202
Figure 5.9.26	Influence of organic matter on abundance % of liverworts	202
Figure 5.9.27	Influence of organic carbon on abundance % of liverworts	202
Figure 5.9.28	Influence of total nitrogen on abundance % of liverworts	202
Figure 5.9.29	Influence of avg. nitrogen on abundance % of liverworts	203
Figure 5.9.30	Influence of avg. P_2O_5 on abundance % of liverworts	203
Figure 5.9.31	Influence of C:N ratio on abundance % of liverworts	203
Figure 5.9.32	Influence of avg. K_2O on abundance % of liverworts	203
Figure 5.9.33	Influence of avg. sulphur on abundance % of liverworts	203
Figure 6.1	Assumed cellular effects of PPARG activation by 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one; 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one	223
Figure 6.2	Assumed cellular effects of PTPN1inactivation by Palustric and Pimaric acid; GSK3 by 4',5,7-Trihydroxy isoflavone and 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one	225
Figure 6.3	Assumed cellular effects of ADORA1 inactivation by 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one; 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one	227

LIST OF APPENDIX

APPENDIX-A List of Chemicals used

APPENDIX-B Abbreviation and symbols used

APPENDIX-C List of publication from this thesis

APPENDIX-D Published articles [Based on the Thesis works]

Chapter 1

INTRODUCTION

1. INTRODUCTION AND BACKGROUND OF THE WORK

Darjeeling hill is the part of Eastern Himalayan region of India, lying between 87º59′ to 88º53′ E and 28º31′ to 27º13′ N. It covers an area of about 2320 km^2 with versatile altitude ranging between 130 m to 3660 m (Saha *et al*., 2011). Due to its geographic uniqueness, variability in altitude and climatic condition, this region favours the luxuriant growth of diversified vegetation. Bryophytes, the second largest group of green land plants after angiosperms, are one of the major constituents of this ecologically rich biodiversity. Bryophytes, also known as the "amphibians" of the plant kingdom, are small plants characterized by their simple organization and little or no organized vascular tissue. Their non-vascular nature is due to poorly developed conducting tissue that lack xylem. The bryophytes consist of three subgroups: Bryophyta (mosses), Marchantiophyta (liverworts or hepatics) and Anthocerotophyta (hornworts) and occupy an intermediate position in between algae and pteridophytes. This primitive group of plants play significant role in soil formation, maintaining soil moisture, recycling nutrients, preventing soil erosion, bio-monitoring pollutants, bio-accumulation of toxic minerals, pH indication and also as medicine.

For thousands of years nature has been a source of many therapeutic drugs. Traditional practices had provided knowledge about the useful plants for the development of many important drugs (Rado *et al*., 1989). Since time immemorial, different group of plants are used in different culture for the treatment of different ailments, and many scientific reports are also available indicating the application of bryophytes, especially liverworts in traditional medicine. But, in comparison to vascular plants their utilization was rather rare in ancient traditional medicine. However, they have been utilized in ancient Chinese, European, North American and Indian medicine to treat illness of cardiovascular system, tonsillitis, bronchitis, tympanitis in skin diseases and burns, etc. (Khanam *et al*., 2011). About 40 bryophyte species in China are reported to be used for the treatment of ulcer, cardiovascular disorders, bronchitis, tympanitis, cystitis, skin diseases and burns as well as antifungal and antibacterial agents. Recent studies have established cytotoxic, anticancer, anti-tumour (Shi *et al*., 2008a; Shen *et al*., 2010), antifungal (Niu *et al*., 2006; Veljic *et al*., 2010; Ivanova *et al*., 2007), and anti-inflammatory responses (Ivanova *et al*., 2007) properties of liverworts and mosses.

Nowadays, the increasing activities of mankind are directly leading to the alteration of distribution patterns of bryophytes and subsequently influencing the functional and structural roles of species in the ecosystem. Bryophytes are not well known to common people, even among some conservationists. They are thought to be quite useless members of the plant kingdom by the people and are being disturbed without even being noticed. However, several ethno medicinal studies and recent pharmacological studies have confirmed the unique medicinal properties of bryophytes, but still much is left to unearth.

For a long time the phytochemistry of bryophytes has been neglected and the information on biological activities of this second biggest group of land plants remained neglected and unknown (Sabovljevic and Sabovljevic, 2008). However, over the past two decades, biologists, chemists and pharmacologist have shown interest in this group of plants, and recent studies on liverworts and mosses have revealed that they produce numerous distinct active substances (Dulger *et al*., 2005; Ilhan *et al*., 2006; Ojo *et al*., 2007). Nowadays, bryophytes are being considered as a remarkable reservoir of new and distinct natural secondary compounds. Studies have revealed the presence of wide array of secondary metabolites in bryophytes (Manoj *et al*., 2012a). Many new compounds were described from this comparatively unexplored group of plants, mainly from liverworts (Asakawa, 2008; Sabovljevic and Sabovljevic, 2008). In spite of being treasure house for diverse, naturally occurring phytochemicals, the investigations on its phytochemicals are still in nascent stage only. In this context, assessment of bioactive components present in this group of plant is highly essential considering their therapeutic and clinical utility.

Secondary metabolites discovered from bryophytes have shown various important biological activities (Asakawa, 2008). Studies have revealed that very few reports on antioxidative activity of bryophytes have been detailed till now in comparison to other biological activities. In this aspect generation of appropriate knowledge base about antioxidant phytochemistry are essential. Antioxidative activity shown by plants is considered to be due to the phenolic compounds present in them. Antioxidants are substance that detoxifies reactive oxygen species (ROS) generated usually from exogenous chemicals and other common endogenous metabolic processes in human body (Nandy *et al*., 2012). Reactive oxygen species, or free radicals, are molecules having one or more unpaired electrons in the outer shell which makes them extremely reactive. The production of reactive oxygen species like hydrogen peroxide (H_2O_2), superoxide ($O^{\cdot}{}_2{}^{-}$) and hydroxyl radicals is an

usual physiological phenomenon in the human body (Nordberg and Arner, 2001). However excess production of free radicals is considered harmful to health. These ROS are short lived but have the potential to initiate a chain reaction that results in creation of more free radical by destabilizing other molecules (Vats and Alam, 2013). Excessive production of free radicals imposes oxidative stress that causes hypertension, atherosclerosis, diabetes, emphysema, inflammation, cancer and many degenerative diseases including premature aging, neurodegenerative diseases like Parkinson's and Alzheimer's and cardiovascular diseases (Halliwell and Gutteridge, 1990; Kris-Etherton *et al.*, 2002; Pejin and Bogdanovic-Pristov, 2012). In addition to this, free radicals (ROS) also attack unsaturated fatty acids of cell membranes, resulting in lipid peroxidation, reduction in membrane fluidity, membrane protein damage and failure of enzyme and receptor activities (Dean and Davies, 1993). Thus, for the survival all living system is equipped with antioxidant molecules that detoxify the harmful effect of free radicals. However, under prolonged stress, tiredness, or diminished immunity inner antioxidant system may be insufficient. Thus, there is a need of exogenous supplimentation of antioxidants. Antioxidant can reduce the oxidative damage by chelating catalytic metals, neutralizing ROS and by acting as oxygen scavengers (Gulcin *et al.*, 2003). Antioxidant can be either natural or synthetic. The most commonly used synthetic antioxidants are butylatedhydroxyanisole (BHA), butylatedhydroxytoluene (BHT), propylgalate and *tert*-butylhydroquinone (Sherwin, 1990). However, synthetic antioxidants are considered harmful for health due to its carcinogenicity (Jayaprakasha *et al.*, 2003). To meet the growing demand of natural antioxidants in pharmaceutical, cosmetic and food industries, the discovery of new natural sources with potential pharmaceutical and antioxidant capabilities are of great interest (Cowan, 1999). Several reports highlight scientific investigations regarding different antioxidant rich sources like vegetables, fruits, and other angiospermic, gymnospermic plant parts, but only little work has been done on other group of plants, especially bryophytes (Chobot *et al.*, 2008). Recent pharmacological investigations have proved that active principles present in bryophytes are quite unique having potential therapeutic activity (Gokbulut *et al.*, 2012). As this group can cope up with extreme climates and stresses, they are considered to possess strong antioxidative enzymatic machinery (Dey and De, 2012). Thus, investigation of antioxidative activity of this group of plants can prove to be beneficial in future for pharmaceutical and cosmetic industries.

Although liverworts have shown different biological activities, literature reviews have revealed that reports dealing with the antioxidant activity of bryophytes are rather few. In the

same way very little is known about the role of Eastern Himalayan bryophytes as natural sources of antioxidant. They are not as well explored as angiosperms in terms of antioxidant potential. Till now mosses like *Octoblepharum albidum, Hyophila involuta, Hyophila perannulata* (*Trichostomum criotum*), *Syrrhopodon subconfertus* (*Syrrhopodon confertus*), *Erythrodontium julaceum and Sematophyllum subhumile* (Mukhopadhyay *et al*., 2013) and the liverwort *Pellia endivifolia* (Dey *et al*., 2013) from Darjeeling Himalaya were studied to analyze their antioxidant activity, but there are still many more to unearth.

Liverworts have also shown some cytotoxic effect against cancer cells. Terpenoids and other aromatic compounds present in liverworts are reported to be responsible for the cytotoxicity shown by the plants. Liverworts like *Pellia endiviifolia*, *P. perrotetiana, Lophocolea heterophylla* and *Radula perrotetiana* showed cytotoxicity against P-388 cells (Toyota *et al*., 1990). Terpenoids from *Plagiochila pulcherrima* are cytotoxic against Hela cells (Wang *et al*., 2013). Metabolites, such as germacronolids, eudesmanolides, guaianolids, lunularin, plagiochiline A, (-)-ent-Arbusculin B (94b) and (-)-ent-costunolide, costunolide are reported to be responsible for cytotoxic activity of liverworts (Asakawa, 1995; Asakawa, 1990). However, studies have shown that no work has been done till date to explore the cytotoxic activity of liverworts of Darjeeling hills. Inhibitory activity against α-glucosidase was also displayed by marchantin C isolated from liverworts (Harinantenaina and Asakawa, 2007). But, only few reports are available on the anti-diabetic activity of liverworts worldwide. Thus, studies of α-glucosidase and α-amylase inhibitory activities can be of importance from pharmaceutical point of view.

Recently, there are increasing public demand for herbal medicines due to their low or no side-effects on human health. To meet this requirement there is a need to search for more natural resources with potential pharmacological activity. Till date, angiosperms have been mostly investigated for the drug development and no doubt secondary metabolites from these plants have lead to the development of a wide variety of therapeutic drugs. But, as plants belonging to same group are known to have similar phytochemical constituents, researchers are looking for novel resources of new therapeutic substances in unexplored group of plants. In comparison to other plant groups, bryophytes remain comparatively untouched in the course of drug discovery. At present, only a small percentage of liverworts and mosses are chemically studied worldwide. Similarly, in India only recently studies have been initiated to screen the phytochemicals and also to determine antioxidant activities of bryophytes (Alam,

2012). Eastern Himalayan Biodiversity hotspot is a huge repository of bryophytes with rich generic and species diversity, but they have not been explored chemically till date (Singh, 1997, 2001; Mukhopadhyay *et al.*, 2013). Further, pharmacological and chemical analysis on the secondary metabolites of bryophytes may give us a number of different new compounds which could be useful for pharmaceutical, cosmetic or agricultural fields. Therefore, more research on these aspects is required.

Liverworts despite being the storehouse of many phytochemicals unique to plant kingdom, very little is known about the phytochemistry of these plants. Various factors restricting the chemical analysis and isolation of bioactive compounds of bryophytes are (i) their seasonal availability, (ii) niche specificity restricting them to grow only in selected geographic area, and (iii) difficulty in collection of pure sample in sufficient quantity. These impediments, however, can be successfully addressed by *in-vitro* propagation of desired species (Vujcic *et al.*, 2017). The standardization of appropriate growth medium for liverwort culture will, therefore, be of great help to overcome the difficulties in the study of chemical constituents of liverworts.

Liverworts are significant component of biodiversity; still these plants are neglected by the conservationists as useless weeds. Nowadays increasing human population leading to various anthropogenic activities, like deforestation, urbanization, tourism, etc. are posing serious threat to natural habitats of fauna and flora including these plants. In India nearly 10% of the liverworts and hornworts are considered as rare, endangered and threatened due to various biotic factors (Singh, 2008). India has developed an elaborate Protected Area (PA) network comprising Wildlife Sanctuaries, National Parks, Community Reserves and Conservation Reserves for *in situ* conservation of its biodiversity. While, liverworts occurring in these PAs are conserved *in situ*, there is a need to conserve populations / habitats outside these areas by establishing 'Species Specific Sites' (Singh, 1999). Besides, to conserve small isolated populations of bryophytes *ex situ*, there is a need to establish 'moss houses' in different eco-climatic zones of the country. Lack of both proper knowledge on this group and appreciation by the masses, including general botanists, may cause serious threats leading to extinction of these plants. Through facilities like 'Bryophyte Gardens', 'Moss Houses' and 'Species Specific Sites', liverworts can be familiarized to students, researchers, scientists and the common people, which may inculcate interest among them and help to reduce threats to this unique group of plants. Thus more effort in establishment of suitable *ex situ* conservation

facilities is essential to save this fascinating yet less understood wealth of nature. Apart from this, study of habitat requirements of liverworts are also important to consider for their conservation. Factors such as light, moisture, temperature, substrate, etc. highly effect the distribution and density of liverworts. Several works focusing on the factors affecting species diversity and abundance have been carried out worldwide; however no such studies have been done in Darjeeling area. So, the record of habitat requirement can be of immense significance for proper conservation of this unique plant group.

Following are the objectives of this work:

1. Characterization of free radical scavenging properties of liverworts

2. To determine the anti-diabetic activity of bio-active molecules *in-vitro*

3. Quantitative evaluation of primary phytochemicals present in the plant sample

4. Detection and profiling of different phytochemicals by using TLC analysis

5. Bioassay guided isolation and purification of bioactive components

6. Spectral characterization and detection of bioactive components isolated through purification

7. To establish the influence of seasonal variation on the antioxidant property

8. To establish suitable conservation strategies for selected liverwort species

Chapter 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 STATUS OF INDIAN HEPATICAE

Hepaticae, also known as liverworts, are less known group of plant biodiversity. India harbors about 13% of total world population of liverwort flora (Singh, 1999). Recent checklist reports that in India there are 930 species and infra-specific taxa under 140 genera and 59 families of liverworts and hornworts (Singh *et al*., 2016). India has extreme diversity of climatic and geographical zones contributing to rich vegetation diversity. This vast landform varies from hot deserts of Rajasthan to ice caped cold mountains of Himalayas, humid tropical Western Ghats to moderate climatic zone of central India, warm coast of Peninsular India to moist Eastern Himalayas. India represents about 11% of the total floristic resources of world and ranks fourth in Asia and eleventh in the world among the 17 mega biodiverse countries (Mittermeier *et al*., 1997; Williams *et al*., 2001). Owing to diverse geomorphological and climatic variation, eight bryogeographical regions have been recognized in India by Pande (1958) and Singh (1992, 1997). India has four biodiversity hotspots, viz. the Himalayas, Indo-Burma (including six North-eastern states of India south of River Brahmaputra and Andaman Islands), Western Ghats-Sri Lanka (including Western Ghats) and the Sundaland (including Nicobar Islands). Bryophytes are important component of vegetation in these regions as these plants largely occur in the regions having cool and moist climates. Status survey has revealed many liverworts and hornworts are rare in Indian bryoflora (Udar and Srivastava, 1983; Pant *et al*., 1992, 1994; Singh 1997). Singh (1999) reported that about 83 species of Indian liverworts and hornworts are rare. Recently, Singh *et al.* (2016) have listed 181 endemic liverwort species in Indian bryoflora. Liverworts are moisture loving plants and thus show their maximum diversity in moist cool areas of Eastern Himalaya, Western Himalaya and Western Ghats in India. Darjeeling, a part of Eastern Himalaya, located at the elevation of 2043.5 meters home to large variety of liverworts. This area experiences mean annual precipitation of ca 2981.8 mm and the mean annual temperature ranges from 8.9^{o} to 15.98^{o}C favoring luxuriant growth of liverworts. Many rare species of liverworts have been reported from this area. Some species are even reported to be confined to this particular area only. Species such as, *Haplomitrium gibbsiae* (Steph.) R.M. Schust. (*Colobryum dentatum* Kumar *et* Udar, *C. indicum* Udar *et* Chandra, *Haplomitrium grollei* Kumar *et* Udar, *H. hashyapii* Udar *et* Kumar), *Frullania hattoriantha* Udar *et* Nath, *F. sharpantha* Udar *et* A. Kumar, *Folioceros physocladus* (Schiff & Pande) are rare and are

confined only to Darjeeling and Sikkim in India (Singh, 1999; Singh *et al*., 2016). It is reported that another rare species, *Haplonutrium hookeri* (Sm.) Nees was initially reported in Indian bryoflora from Darjeeling, but couldn't be collected again from the same area (Singh, 1999).

2.2 LIVERWORTS IN TRADITIONAL MEDICINES

Liverworts are plants with unique biological characteristics. They differ from tracheophytes by two important characters. First, their ecologically persistent, photosynthetic phase of life cycle is the haploid gametophyte generation rather than the diploid sporophyte, which is very short-lived and attached to and nutritionally dependent on their gametophytes and are monosporangiate. Second, xylem tissue is absent in bryophytes, a lignified water-conducting tissue present in the sporophytes of all vascular plants. They are an integral part of ecosystem and have important functional role in many ecosystem processes (Frego, 2007). Apart from ecological roles, studies also report the use of bryophytes, including liverworts in traditional practices and ethno medicine. Because of no side effect, currently world's 80% population depends on herbal medicine as the first choice of primary health care (Hasan *et al*., 2009). Chinese, Europeans, North American and Indian medicine reports traditional use of bryophytes to treat illness of skin, cardiovascular system, bronchitis, tosilities, burns etc. (Khanam *et al*., 2011). Liverworts are popular among the tribal people in different parts of the world as natural remedy of many illnesses. Tribal community of Melghat area, India use liverworts in ethnomedicinal health care system. *Marchantia polymorpha* is used against inflammation; *Plagiochasma appendiculatum* for skin disease; *Polytrichum* sp. for hair growth and *Riccia* sp. against ringworm in children. Gaddi tribes of Kangra valley, Himachal Pradesh of India use *Plagiochasma appendiculatum* for treating burns, boils and other skin diseases (Kumar *et al*., 2000). Singh (2011) reported use of liverworts like *Conocephalum conicum*, *P. appendiculatum* by traditional healers to cure burn infection. *Riccia* spp. is used as external application to treat ringworm in the villages of Pithoragarh in Western Himalayan region (Pant and Tewari, 1989). In Melghat region of India, *Plagiochila beddomei*is reported to be used for treatment of skin diseases (Manoj and Murugan, 2012). Information is also available detailing the use of fresh thalli of *Marchantia palmata* to cure heat or hot water induced inflammation (Tag *et al*., 2007). *Conocephalum conicum* is used for treatment of cut, burn, against snake bite and gallstones. *Frullania tamarisci*is reported to have antiseptic activity. Other liverworts, like *Marchantia polymorpha* is used as antidotal, diuretic and also to cure burns, fractures, snake bites and open wounds. *Reboulia hemisphaerica* is used for

blotches, external wounds and bruises (Ding 1982). In china and Bolivia, *Conocephalum conicum* and *Polytrichum commune* are boiled together to make syrup to treat common cold. These liverworts are also reported to dissolve kidney and gall bladder stones (Glime, 2007). In Western Ghats and Kerala of India, paste of *Targionia hypophylla*, a liverwort is used to cure itches, scabies and other skin diseases (Ramesh and Manju, 2009).

2.3 CHEMICAL DIVERSITY OF BRYOPHYTES

Bryophytes are the second largest taxonomic group in plant kingdom (Asakawa, 2013a). However, the study of phytochemistry of bryophytes has long been neglected. Large number of biologically active compounds has been reported recently from this less explored group of plants (Fu *et al*., 2012; Cheng *et al*., 2012). Many new compounds, that have never been isolated from other plant groups, have been reported from bryophytes (Sabovljevic and Sabovljevic, 2008). Thus, bryophytes are being considered as a remarkable reservoir of new and active phytochemicals. Presence of phenolics, terpenoids, lipids, glycosidic group and some rare aromatic compounds has been recorded (Savaroglu *et al*., 2011). Liverworts are mainly composed of acetogenins (which constitutes oil bodies), lipophilic monoterpenoids, diterpenoids, sesqui- aromatic compounds like benzoates, bibenzyles, bis-bibenzyls, long chain alkyl phenols, cinnamates, isocoumarins, napthalenes, pthalides, etc. (Asakawa 1993; 1995). In bryophytes, terpenoids are the largest class of secondary metabolites (Lu *et al*., 2005). The most important characteristics feature of sesqui and diterpenoids present in liverworts are they are enantiomers of those present in higher group of plants e.g. pinguisanes, ventricosanes and myltaylanes. Few exceptional forms that are similar to higher plants are drimane, guaianes and germacrane. Hydrophobic terpenoids is detected very rarely in liverworts (Asakawa, 2011). More than 220 aromatic compound and 700 terpenoids isolated from liverworts are studied for their biological activities. Steroids such as sitosterol, brassicasterol, stigmasterolin are present (Ando, 1983). Large number of flavonoids glycosides is also found to be present in liverworts (Asakawa, 2011). In addition, fatty acidderivatives and sulphur and nitrogen containing compounds are also produced by bryophytes (Xie and Lou, 2009).

Bibenzyls or dihydrostelbene are the phenolic compounds present in bryophytes. Flavonoids are common components of bryophytes, (Asakawa, 2013b). Of the mosses, liverworts and hornworts, liverworts are studied in much detail because of the presence of

cellular oil bodies that contain phytochemicals having biological activities. These oil bodies are composed of lipophilic terpenoids and aromatic compounds (Askawa, 2001).

2.4 STATUS OF STUDY OF PHYTOCHEMICALS OF BRYOPHYTES IN INDIA

Literature review showed that very limited efforts have been made till date to explore the phyto constituents of bryophytes from India. In an experiment performed by Kishnan and Murugan (2013) to study the phytochemicals present in liverwort *Marchantia linearis* and *M. polymorpha* collected from Kerala, revealed the presence of components such as Phenol 2,4-bis (1,1-dimethyl ethyl), Hexadecanoic acid, 1-heptacosanol, Octadecanoic acid, Eicosylheptaflurobutyrate, Isochiapin B, 1,2-benzene dicarboxylic acid, 9-octadecanoic acid, Dichloro acetic acid 1-adamantyl methyl ester, Ethyl adamantine. Presence of amino acids, carbohydrate, fats, flavonoids, anthraquinones, cardiac glycosides, glycosides, tannins, proteins, steroids, terpenoids, alkaloids was recorded in mosses *Hyophilainvoluta* and *Entodonplicatus* from Rajasthan by Singh *et al* (2016). Saponin was found to be absent in the studied plants. Kadam (2017) reported the presence of alkaloids, coumarins, flavonoids, phenols, steroids and sugar in bryophytes collected from Lonavla. Eastern Himalayan region being the storehouse of large number of bryophytes harbors many rare and endemic liverworts of Indian bryoflora. Still, liverworts of this area remain untouched for chemical studies. Till date not a single report detailing the phytochemical constituents of liverworts from this area has been reported.

Table 2.1: List of some important phytochemicals present in liverworts of Indian bryoflora

Sl no	Name	Distribution pattern in India	Important phytochemical	Bioactivity
1	*Conocephalum conicum*	Eastern and Western Himalaya	2α, 5β-Dihydroxybornane-2-cinnamate	Cytotoxic (Lu *et al*., 2006)
2	*Dumortiera hirsuta*	All bryo-geographical region of India	Lunularin	Cytotoxic (Lu *et al*., 2006); anti-microbial (Lu *et al*., 2006)
3	*Marchantia polymorpha*	Eastern Himalaya, Western Himalaya and Western ghat	Marchantin A	Cytotoxic (Asakawa, 1990b); antibacterial (Asakawa, 1990a), anti-HIV-1acivity (Asakawa*et al*., 2008); 5- lipoxygenase, calmodulin activity (Asakawa 1990b)
4	*Mastigophora dicladose*	Eastern Himalaya, Nicobar Island, western Ghat	Herbertene-1,2-diol	Antioxidant (Komala*et al*., 2010a) Antimicrobial (Komala*et al*., 2010a)
5	*Plagiochila ovalifolia*	Eastern Himalaya	3,5-dihydroxy-2-(3-methyl-2-butenyl)-bibenzyl	Antioxidant (Sadamori, 2009)
6	*Jungermannias ubulata*	Eastern Himalaya	Subulatin	Antioxidant (Tazaki*et al*., 2002)
7	*Marchantia paleacea*	Western Himalaya, Eastern Himalaya, Gangetic Plains and Central India	Marchantin A	Cytotoxic (Asakawa, 1990b); antibacterial (Asakawa, 1990a), anti-HIV-1acivity (Asakawa*et al*., 2008); 5- lipoxygenase, calmodulin activity (Asakawa 1990b)
8	*Marchantia palmata*	Western Himalaya, Eastern Himalaya, Gangetic Plains and Central India	Isomarchantin C	Cathepsin L and cathepsin B inhibitor (Asakawa, 2008a)

2.5 BIOLOGICAL ACTIVITIES

Various novel and rare phytochemicals have been found to be present in bryophytes. These phytocompounds are responsible for some interesting biological activities shown by the bryophytes. Cytotoxic, antimicrobial, α and β glucosidase inhibitory, superoxide radical releasing, NO production inhibitory, insecticidal, muscle relaxing, neurotrophic, insect anti-feedant, antioxidant, 5-lipoxygenase, calmodulin, hyaluronidse, cyclooxygenase activities are shown by bryophytes. Jian-bo *et al.* (2006) have found that flavonoids from *Marchantia convoluta* have shown anti-hepatitis B virus activity. Flavonoids from *Marchantia convoluta* have showed protective effect against acute hepatic injury caused by CCl_4 in mice. It has also shown anti-infammatory, diuretic activity in mice and antibacterial effects on *Bacillus enteritidis, Salmonella typhi, Streptococcus pyogenes, Escherichia coli, Staphylococcus aureus* and *Pneumococcus* (Xiao *et al*., 2005). Marchantins present in liverworts have been reported to have many biological activities. Marchantins and related compounds were tested for their inhibitory activity against key enzyme of arachidonic acid cascade, 5-lipooxygenase 5- (LOX) and cycloxygenase (COX). Tested marchantins and related compounds showed significant inhibitory activity against 5-LOX and COX (Schwartner *et al*., 1995). Isoflavonids present in bryophyte extract is proven to have the antimicrobial activity (Seeger, 1993).Aqueous, acetonic and methanolic extract of *Riccia gangetica* has showed significant reduction in germination of spores and hyphal elongation of *Curvularia lunata* (Deora and Suhalka, 2017). Many other reports are available documenting antifungal activities of bryophytes. *Homalia trichomanoides* has antifungal activity against *Candida albicans* (Wang *et al*., 2004); *Dumortiera hirsuta*, *Sphagnum portarecense* are active against *Candida albicans* (Madsen and Pates, 1952). Bryophytes have strong antibiotic activity too. Phytochemicals such as 4-hydro-3-methoxybibenzyl and a-and b-pininealloromadendrine from *Plagiochila stevensoniana*, Norpiguisone from *Conocephalum conicum*, polygodial from *Porella*, Lunularin from *Lunularia cruciata* have shown significant antimicrobial activities (Lorimeres *et al*., 1993; Harborne, 1998). Harborne 1998, reports *Plagiochila fasciculate* has *Trichophyton mentagrophytes, Herpes Simplex type 1, Candida albicans, Polio type 1, Bacillus subtilis*, *Cladosporium resinae* inhibitory activity. Liverwort *Marchantia palmate* has shown the ability to inhibit the growth of *Klebsiella pneumonia*, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Proteus mirabilis* (Khanam *et al*., 2011). Excessive production of superoxide in living organism causes angiopathies like cardiac infraction and arterial sclerosis. Plagiochilide from *Plagiochila fruticosa*, *P.*

yokogurensis, *P. ovalifolia* and infuscaic acid from *Solenostoma infuscum* (*Jungermannia infusca*) prevents the release of superoxide from rabbit polymorphonuclear leukocytes and from formyl methionylleucyl phenylalanine guinea pig peritoneal macrophage (Asakawa, 1990). Compounds like norpinguisone methyl ether, cyclomyltaylyl-10-caffeate, sesquiterpenoids, bicycleogermacrenal, herbentene-1,2-diol, diterpenoids, infuscaside A, B, perrottetianal A isolated from different bryophytes inhibit release of superoxide from guinea pig peritoneal macrophage (Asakawa, 1995).

Anti DNA polymerase β activity is one of the several activities shown by bryophytes, especially liverworts. Bis-bibenzyl dimmers, pusilatins B isolated from *Blasia pusilla* possess DNA polymerase β inhibitory activity (Yoshida *et al*., 1996). Anti-diabetic activity shown by liverworts is reported to be due to a phytochemical marchantic C. Harinantenaina and Asakwa (2007) reported for the first time the α-glucosidase inhibitory activity of this macrocyclic bis-bibenzyls. Marchantins are one of the active compounds found in liverworts responsible for many important biological activities. Cyclic bis-bibenzyls specifically Marchantin A and its methyl ester displayed muscle relaxing activity (Taira *et al*., 1994).

Various compounds obtained from liverworts have shown the cytotoxic activity. Marchantin A isolated from *Marchantia emarginata* subsp. *Tosana* induced apoptosis in MCF-7 cells. Extracts of *Bazzania novae-zelandiae* were found to be cytotoxic against tumor cell lines (Burgess *et al*., 2000). In A172 and HeLa cells, marchantin C obtained from liverworts induced cell arrest at G_2/M phase (Shi *et al*., 2009). Bryophytes are also reported to have strong antioxidant activity. Herbertene-1,2-diol and tigophorene isolated from *Mastigophora diclados* showed strong antioxidant activity which was better than vitamin C and equal to quercetin (Komala *et al*., 2010). Compounds like marchantin A (Huang *et al*., 2010), marchantin H (Hsiao *et al*., 1996), Perrottetin D (Schwartner *et al*., 1996), 3,5-dihydroxy-2-(3-methyl-2-butenyl)-bibenzyl and plagiochin D (Sadamori, 2009) isolated from liverworts showed strong antioxidant activity.

2.6 FREE RADICALS AND THEIR EFFECT IN LIVING ORGANISM

Free radicals are molecules or fragment of molecules containing one or more unpaired electrons in its orbit (Halliwell and Gutteridge, 1999). This unpaired electron(s) are responsible for reactivity of free radicals. Free radicals are generated *in situ* as a consequence of normal cell metabolism or from external sources like radiation, pollution,

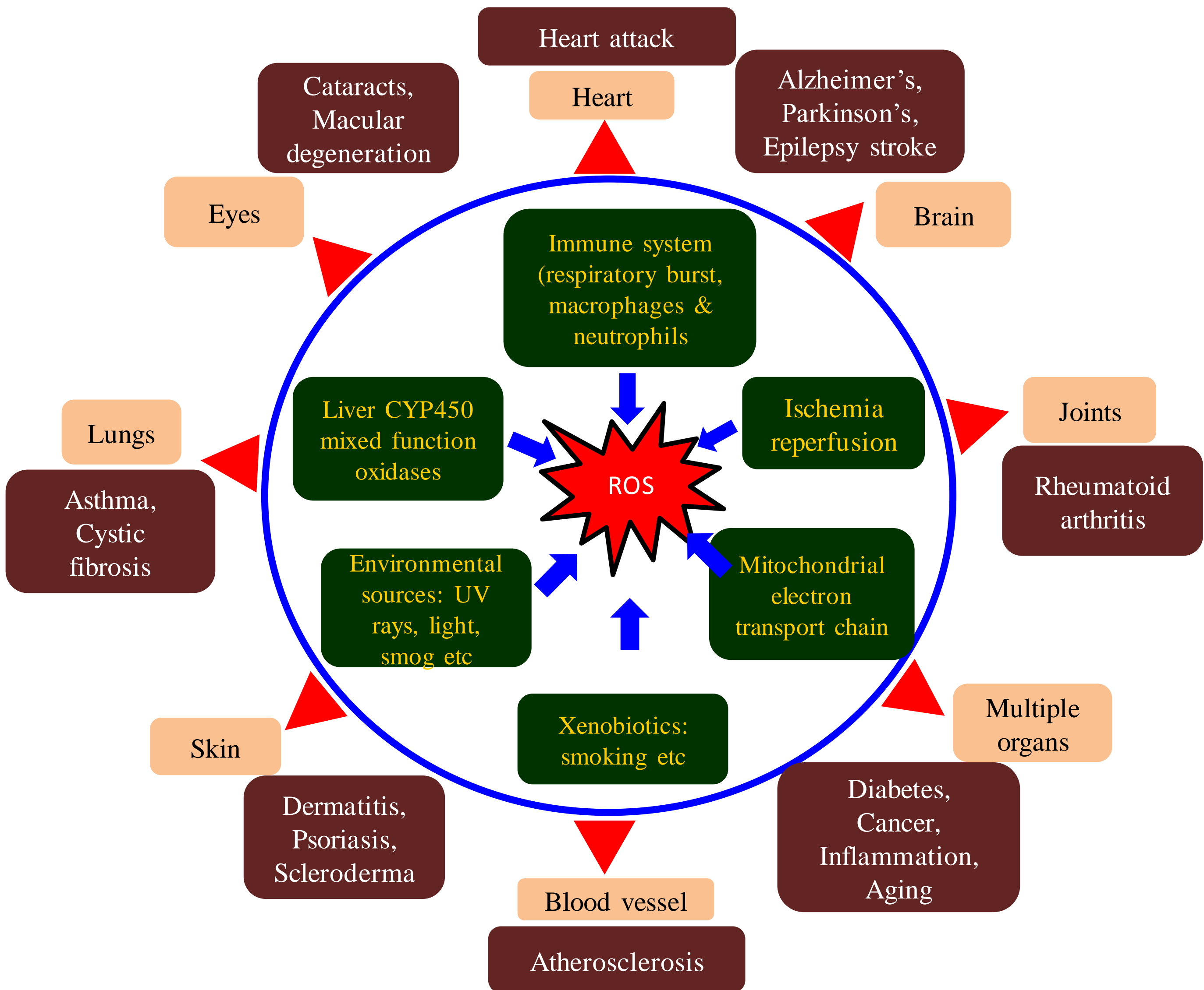


Fig 2.1. : Free radicals and their harmful effects

medication, etc. (Pham-Huy *et al.*, 2008). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the by-products that result from the cellular redox processes. ROS are produced within mitochondria as a consequence of ATP production by electron transport chain (Cadenas and Sies, 1998). In living system radicals generated from oxygen are the most reactive free radicals (Miller *et al.*, 1990). Superoxide anion generated from metabolic processes or through oxygen "activation" by physical irradiation further reacts with other molecules leading to the production of secondary ROS (Valko *et al.*, 2005). RNS are generated by nitric oxide synthase during the metabolism of arginine to citrulline (Ghafourifar and Cadenas, 2005). ROS and RNS have both deleterious and beneficial effects on living organism. The balance between these two antagonistic effects are essential for sustenance of life. ROS and RNS have beneficial effects on immune function and cellular responses at moderate or low levels. Over production of ROS results in oxidative stress that leads to the damage of lipid, proteins and DNA. Nitric oxide ($NO^{\bullet}$) is an important biological signaling molecule involved in large variety of physiological processes (Bergendi *et al.*, 1999). However, overproduction of reactive nitrogen species, known as nitrosative stress, is harmful. Nitrosative stress causes nitrosylation reactions which leads to the alteration in protein structure and disturb their normal function. Oxidative stress finally leads to chronic and degenerative diseases such as cancer, aging, arthritis, autoimmune disorders, diabetes, neurodegenerative diseases and cardiovascular diseases.

2.7 ANTIOXIDANTS AND THEIR MECHANISM OF ACTION

Antioxidants are first line of defence against the oxidative damage in living organism and are important for maintaining health. Humans have developed complex antioxidant protection system, that function interactively and synergistically to combat the free radicals (Jacob, 1995). Antioxidants can be both endogenous and exogenous in origin. Endogenous antioxidative system includes enzymatic and non-enzymatic systems. Body has antioxidant enzymes- glutathione peroxidise, superoxide dismutase (SOD), catalase (CAT) as endogenous antioxidant defence system. SOD are present in the cytosol and mitochondria, they convert $O_2^{\bullet-}$ into oxygen and hydrogen peroxide (Gough and Cotter, 2011). CAT enzyme that is present in the peroxisomes converts hydrogen peroxide into oxygen and water (Stone and Yang, 2006). Glutathione are water-soluble antioxidants synthesized from amino acids glycine, cysteine and glutamate. Glutathione quenches reactive oxygen species like lipid peroxides and play a role in xenobiotic metabolism. Studies suggest that glutathione and

vitamin C functions interactively to neutralize free radicals (Jacob 1995). The non-enzymatic system includes carotene, tocopherol, ascorbic acid, lipoic acid etc. Lipoic acid is sulphur containing molecule capable of scavenging free radicals in lipid and aqueous domains as well. Lipoic acid serves as antioxidants by chelating with pro-oxidant metals (Packer and Witt, 1995).

Antioxidants can also be obtained by dietary uptake. Vitamin E, Vitamin C, beta carotene are the most common dietary antioxidants. Vitamin C is the water soluble antioxidant capable of neutralizing ROS by donating reducing equivalents (Oh *et al.*, 2010). Vitamin E is a lipid soluble antioxidant. It breaks free radical chain within a cell membrane and protects membrane fatty acids from lipid peroxidation (Sies *et al.*, 1992). Beside these traditional vitamins, many other dietary antioxidant substances exist. Plant phytochemicals are increasingly being considered for their antioxidant activity nowadays. Food rich in naturally occurring antioxidants are widely recommended by health organizations. Plant phytochemicals like flavonol (myricetin, quercetin), flavonols (taxifolin), flavone (apigenin, luteolin), catechin, Carotenoids (lycopene), anthocyanidin, isoflavine are found to be potent antioxidants. Bioflavonoids have protective effect on H_2O_2 induced DNA damage (Russo *et al.*, 2000). Protective effect of flavonoids on DNA is reported to be due to chelating effect of metal ions such as copper or iron complexed with flavonoids (de Souza and De Giovani, 2004). Anthocynidins terminates the damaging oxidative chain reaction by donating an electron to a free radical from –OH group attached to its phenolic rings (Castaneda-Ovando *et al.*, 2009). Another natural antioxidant carotenoids, scavenge the peroxyl radicals generated in the process of lipid peroxidation and provides protection against cellular membrane and lipoprotein damage (Stahl and Sies, 2004). Lycopene and β-carotene demonstrate potential antioxidant property due to its ability to quench singlet oxygen (Erhardt *et al.*, 2003; Di Mascio *et al.*, 1989).

2.8 SYNTHETIC ANTIOXIDANT

Synthetic antioxidants are added to fats and oil to extend their shelf life. They are added in different food products as preservatives to prevent the peroxidation of lipid. The most frequently used synthetic antoxidants are butylatedhydroxyanisole (BHA), butylatedhydroxytoluene (BHT), gallates and tertiary butyl hydroquinone (TBHQ).

2.9 FREE RADICAL SCAVENGING ACTIVITY OF LIVERWORTS

Demands for herbal medicines are high due to its low or no side effects. Till date, large number of drugs has been developed from secondary metabolites obtained from plants. However, to meet the ever increasing demand of herbal medicine there is a need to look for novel sources of new therapeutic substances. Bryophytes are known to contain many rare and novel phytochemicals (Nagashima *et al*., 2002). More specifically, liverworts contain unique compounds like pinguisane (sesquiterpene), sacculatane (diterpenoids) and bis (bi-benzyl) aromatic compounds that are not found in higher plant groups (Asakawa, 1999). Still, bryophytes are less explored for its pharmacological activities.

Higher plants cannot withstand dryness, they die when their relative content of water drops below 20-25 %. Only few can dry up to 4-13% without injury (Gaff, 1997) and they are known as "dessication tolerant" plant. Desiccation tolerance is also found in bryophytes (Oliver *et al*., 2000). During extreme dryness there occurs irreversible damage to lipids, proteins and nucleic acid due to the production of ROS. ROS production increases under dry condition and solar radiation (Kranner and Lutzoni, 1999). Desiccation tolerant plants limit the dryness caused damage by extensive use of free radical scavenging systems (Kranner and Lutzoni, 1999). This fact suggests existence of strong antioxidant system in bryophytes.

Many experiments have been performed to study free radical scavenging activity of liverworts. Gokbulut *et al*. (2012) tested antioxidant activity of the ethyl acetate and methanol extracts of *Marchantia polymorpha*. $DPPH^{\bullet}$ and $ABTS^{+}$ assay was used to evaluate the antioxidant activity of the ethyl acetate and methanolic extracts of *M. polymorpha*. Luteolin present in the plant are considered to be responsible for such activity. Phenolic compound present in *Plagiochila beddomei* also displayed the potential to scavenge the DPPH and hydroxyl radical (Manoj *et al*., 2012). *P. beddomei* showed significant potential to inhibit DPPH radical and peroxidation. It similarly displayed high FRAP value (496 µmol $FeSO_4$/g DW). Manoj and Murugan (2012a) also reported antioxidant activity in *Plagiochila beddomei.* Hsiao *et al.* (1996) reported inhibition of Fe^{2+} induced lipid peroxidation in rat brain homogenates by marchantin H isolated from *Marchantia diptera.* This anti lipid peroxidation activity of marchantin H is more potent than desferrioxamineor other classical antioxidants. Free radical scavenging potential of marchantin was also reported by Schwartner *et al.* (1996) using pulse radiolysis and EPR techniques. Subulatin isolated from *Jungermannia subulata* showed free radical scavenging property which was equal to α-tocopherol (Tazaki *et al*., 2002). Strong antioxidant activity was reported for phytochemicals

isolated from herbertene-1,2-diol, mastigophorene C and mastigophorene D from *Mastigophora diclados*. Their activities were similar to quercetin and stronger than vitamin C (*Komala et al*., 2010).

Dey *et al.* (2013) studied the antioxidant activity of *Pellia endiviifolia* collected from Darjeeling by studying its potential to scavenge DPPH radical. They found that this liverwort has significant potential to scavenge DPPH radical. More work focusing on the pharmacological efficacy may lead to the clinical trials and identification of phyto-constituents of diverse therapeutic application.

2.10 DIABETES:

Diabetes is associated with severe long term health complications. Disturbance in glucose metabolism leads to diabetes. Insulin secreted from pancreatic β cells is responsible for glucose homeostasis (Sesti, 2006). It suppresses the output of glucose from liver and stimulates hepatocytes, myocytes and adipocytes to uptake glucose from the circulatory system. Inappropriate use of insulin leads to a condition where cells fail to respond to normal levels of circulating insulin, a condition known as insulin resistance (Berg *et al*., 2002). This condition leads to chronic hyperglycemia which is a characteristic of diabetes. As T2DM progresses, insulin secreting β cell deteriorates in function and normal level of insulin in the body gets disturbed. Thus, there will be the need of therapeutic approach to treat postprandial hyperglycemia (Ross *et al*., 2004). Two enzymes, α-amylase and α-glucosidase are involved in the digestion of carbohydrates. α-amylase catalyzes the hydrolysis of starch into mixture of maltose, maltotriose and oligoglucans. α-glucosidase further breaks these compounds to glucose and are absorbed into the bloodstream (Gomathi *et al*., 2012). Postprandial hyperglycemia can be controlled by inhibiting these two hydrolyzing enzymes.

Under conditions of sustained hyperglycemia, oxidative stress is largely increased (Araki and Nishikawa, 2010). Oxidative stress in diabetes arises from different mechanisms such as auto-oxidation of glucose, increased glycation and alteration in polyol pathway (Araki and Nishikawa, 2010). These free radicals damages β cells impairing its function and finally leads to the pathogenesis and development of other complications from diabetes (Niedowicz and Daleke, 2005) like accelerated coronary artery disease, neuropathy, nephropathy and retinopathy (Philips *et al*., 2004). Thus, antioxidant therapies

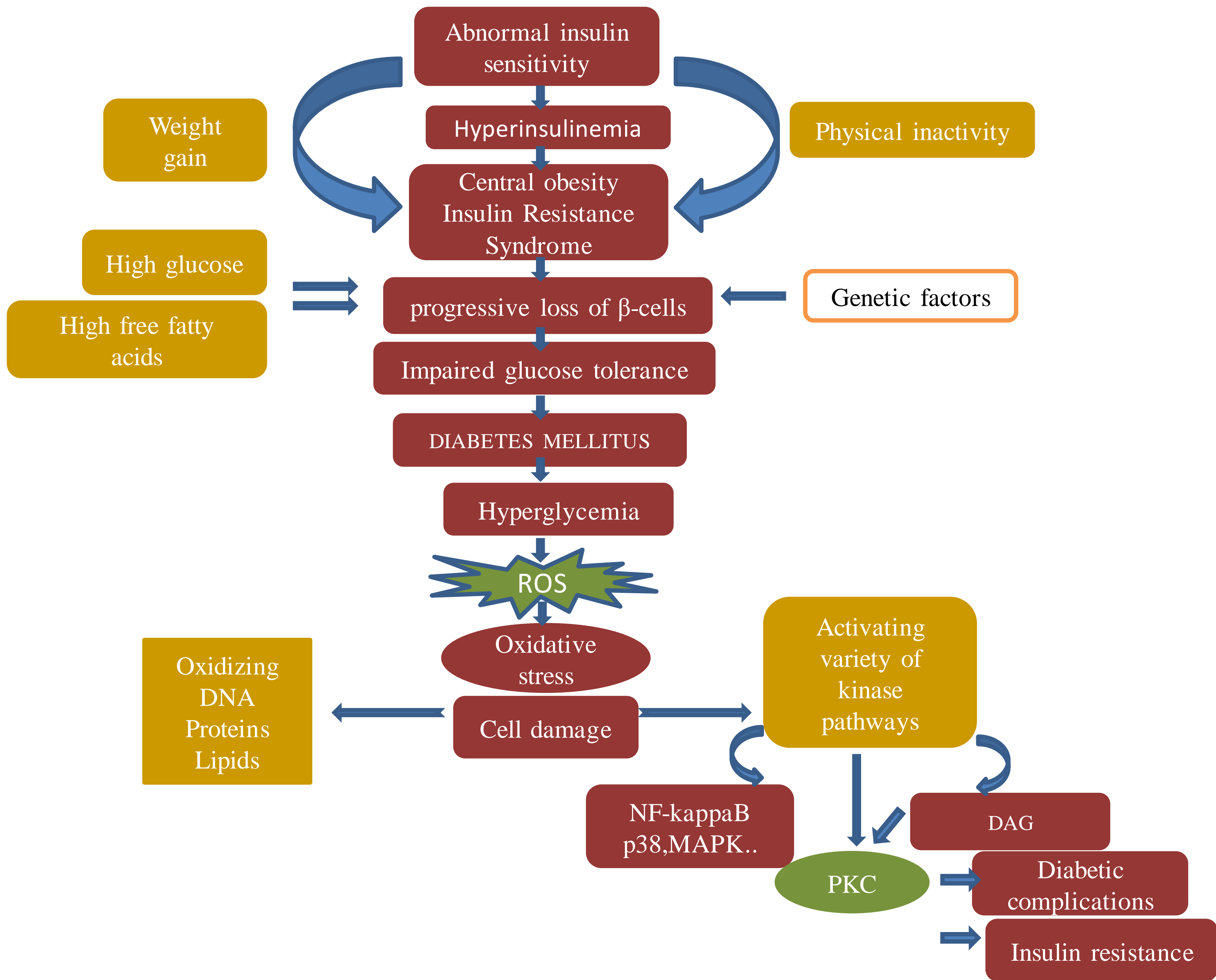


Fig 2.2: Diagrammatic presentation of pathway of oxidative stress induced diabetes complications

targeting diabetes-induced oxidative stress can be proved to be useful for preventing downstream diabetic complications (Niedowicz and Daleke, 2005).

Natural compounds isolated from some plants are reported to efficiently inhibit the α-amylase and α-glucosidase activities. Studies have shown that bioactivity of polyphenols present in plants are responsible for such activities (Pant, 2011). In liverworts, marchantin C has shown α-glucosidase inhibitory activity (Harinantenaina and Asakawa, 2007). Shilajit is a herbo-mineral drug which has been widely used in Ayurveda for diverse clinical conditions. People living in Himalaya and adjoining regions are reported to use shilajit to combat the effect of diabetes (Tiwari *et al*., 1973). Medical researchers (Gupta, 1966; Bhattacharya, 1995) have supported the anti-diabetic actions of Shilajit by their findings. There are different hypothesis regarding the origin of shilajit. *Euphorbia royleana* and *Trifolium repens* are latex bearing plants that are found at the vicinity of shilajit bearing rocks. These plants are thought to be the likely source of shilajit (Ghosal *et al*., 1988). Moreover, recent research found that the liverwort species such as *Asterella*, *Plagiochasma*, *Dumortiera*, *Pellia*, *Marchantia*, and mosses like *Barbula, Fissidens, Mnium, Thuidium* to be present in the vicinity of shilajit exuding rocks and are considered to be active constituent of shilajit (Joshi *et al*., 1994). Furthermore, minerals like zinc, copper, iron, silver, lead, etc., that are present in the shilajit, are also present in the tissues of bryophytes, thereby supporting that bryophytes contribute to the formation of shilajit (Mirza *et al*., 2010).

2.11 CYTOTOXIC ACTIVITY OF BRYOPHYTES

Bryophytes are devoid of vascular tissue. They are small plants that grow closely on rocks, soil or as epiphytes on the tree trunks and leaves. While growing, they produce diverse secondary metabolites to cope up with a large number of biotic and abiotic stresses like ultraviolet radiation, microbial decomposition and predation (Xie and Lou, 2009). Among the three groups of bryophytes, liverworts mainly contain lipophilic monoterpenoids, sesquiterpenoids, diterpenoids and aromatic compounds like benzoates, naphthalenes, bibenzyls, bis-bibenzyls, cinnamates, isocoumarins, long-chain alkyl phenolsphthalides and acetogenins (which constitute the oil bodies). In liverworts, bibenzyls or dihydrostelbene are characteristic phenolic compounds. These compounds are occasionally found in some higher plants and are completely absent in hornworts and mosses (Banerjee, 2001).These metabolites exhibit a variety of biological activities including cytotoxic activity (Asakawa, 2008).

Triterpenoids derived from plants had shown significant potential as anticancer agent against various cancer cell lines (Setzer and Setzer, 2003). Monoterpene ester; 2-α,5-β-dihydroxybornane-2-cinnamate isolated from *Conocephalum conicum* and lunularin isolated from *Dumortiera hirsuta* has been found to be cytotoxic against human HepG2 cells (Lu *et al*., 2006). Potential anticancer activity was shown by the sesquiterpenes isolated from *Porella cordaeana*, *Chiloscysphus polyanthos* var. *rivularis* and *Frullania nisquallensis*. These sesquiterpenes are categorized as DNA damaging natural products (Gunatilakaa and Kingston, 1997). Cytotoxic diterpenes are also isolated from several liverworts. Diterpene like 8,9-secokaurane, isolated from liverworts *Lepidolaena taylorii* and *Lepidolaena palpebrifolia* are found to have cytotoxic activity against human tumor cell lines (Perry *et al*., 1996; Perry *et al*., 1999). Ent-kaurene type diterpenoids isolated from *Jungermannia* sp. was cytotoxic against a human leukemia cell line (Nagashima *et al*., 2003). Ent-11 alpha-hydroxy-16-kauren-15-one, a diterpene obtained from *Solenostoma truncatum* (=*Jungermannia trucata*) was found to have apoptosis property against different human cancer cells like HL-60 (Suzuki *et al*., 2004a), human leukemia cells (Suzuki *et al*., 2004b). The apoptotic activity of this compound could be mediated by caspase dependent pathway or by p38 mediated mitogen –activated protein kinase (Suzuki *et al*., 2004a).

Marchantin, an important phytochemical found in liverworts has shown strong cytotoxic activity. Marchantin A has shown cytotoxicity against KB cells (Asakawa, 1982); marchantin C reported to promote apoptosis in human glioma A172 cells (Shi *et* al., 2008) and also to be cytotoxic against P388 leukemia cells (Scher *et al*., 2002). Strong antimicrotubule activities of marchantin A and C isolated from *Reboulia hemisphaerica* was reported by examining their effect on human tumor cell line Hela. Many other phytochemicals present in liverworts, like neomarchantins, lunularin, plagiochin, perrottetin, riccardin C, pakyonol are reported to have cytotoxicity against many human cancer cells (Scher *et al*., 2002; Lu *et al*., 2006; Asakawa 1982).

Positive result of many experiments relating anti-cancerous efficacy of liverworts indicated that liverworts can serve as an attractive candidate as a source of valuable phytochemical having anti-cancerous property. With the help of modern instruments like liquid chromatography (LC-MS), high performance liquid chromatography (HPLC), X-ray crystallography, IR, NMR, etc. it has become possible to isolate the bioactive compounds and study their activities. Isolation, characterization, pharmacological evaluation and clinical trial of phyto-compounds present in liverworts might open a pathway for future anti-cancer drug development Programmme.

2.12 AN OVERVIEW OF THREATS AND CONSERVATION OF INDIAN LIVERWORTS

Bryophytes are integral part of forest ecosystem and are the second largest taxonomic group of plant kingdom (Asakawa *et al*, 2013),but due to small size and problem in identification they have been neglected for long and considered useless as a source of biologically important substances. Recent studies, however, have found the presence of many active compounds in these plants (Dulger *et al*., 2005). Liverworts, despite of being the storehouse of many phytochemicals unique to plant kingdom, are still not the preferred experimental material for phytochemical studies. Such studies are particularly constrained by often restricted geographical distribution of taxa, seasonality, limited availability in nature, and more importantly lack of basic floristic and taxonomic knowledge on these plants. *In vitro* culture is one the important technique that helps to overcome the problems restricting the study of pharmacological bioactivities and isolation of bioactive compound from liverworts growing in their natural habitat (Vujcic *et al*., 2017). Therefore, standardization of appropriate axenic culture technique might help to overcome the various exertions that restrict their utilization for research purposes.

Bryophytes are significant part of diversity but they are neglected even by conservationists. Study of distribution pattern of liverworts have showed that Eastern Himalayan region of India harbors over 70%, Western Ghats harbours 41% and Western Himalaya harbours 33% of their total taxa found in India (Singh *et al*., 2016). Moist and cool weather of Eastern Himalaya favours the luxuriant growth of liverworts in this area. Liverworts are found to grow up to the altitude of 5000 m in the Himalayas (Bhattacharya, 2011). Varied topography ranging from tropical-subtropical to alpine forest supported by moist, cool climate of this region facilitate the growth of large variety of terrestrial, corticolous (growing on bark) and foliicolous (growing on leaf) species of liverworts in this region. However, liverworts have got less attention as a source of valuable natural products as compared to higher plants. As a consequence of which liverworts are being threatened in their habitat and even some has become rare in this area. With the increase in human population leading to increased anthropogenic activities such as deforestation, tourism, urbanization, etc. diversity of liverworts is declining rapidly in this area. Many species of liverworts have been marked as rare. The main threat to liverwort diversity by far is the habitat destruction. Use of forest products like timber is destroying the habitat for epiphytic

liverworts. Hilly region of Himalaya is mainly the home to Indian liverworts which is the popular tourist destination also. Thus liverworts growing in this region are stressed due to anthropogenic activities. Apart from habitat destruction, another important aspect is the lack of knowledge about this plant group among common people and also among many conservationists. Because of the small size of these plants they usually escape the attention. As a result, the habitat of many liverworts is being destroyed unknowingly. In India very few people are working to study the biochemical aspect as well as the ecological aspect of liverworts. Due to lack of intensive and extensive survey of these plants many species are known from a single locality only, while some have not even been collected after their first report. About 10% of Indian liverworts are considered rare, endangered and threatened (Singh, 2008). About 20.3% of Indian liverworts are endemic to the country (Singh *et al.,* 2016). India has an elaborate network of 771 protected areas comprising of Wildlife Sanctuaries, National Parks, Conservation Reserves and Community Reserves. Interestingly, about 541 species of liverworts present in these protected areas are conserved *in situ* (Singh *et al.*, 2016). However, to conserve liverworts and their habitat outside these protected areas 'Species Specific Sites' are needed to be established (Singh, 1999). The interest of among the students, scientists, researchers and the common people can be generated by creating facilities like 'Moss Houses', 'Bryophyte Gardens' and 'Species Specific Sites' which will help to reduce threats to this plant. To conserve these plants in *ex situ* conservation sites, it is highly essential to study the habitat requirements of this fascinating group of plants. Liverworts grow in particular eco-climatic zones so it is important to study light intensity, moisture content of the environment, temperature and substrate requirement of these plants effecting their distribution and density. Studies focusing on the factors affecting the abundance and species diversity in particular area has been done worldwide for the important diversity sites of bryophytes, but no such work has been done in Darjeeling Himalayan region though it is the richest site of liverwort diversity of India. So the study of habitat preferentiality of liverworts growing in this region could be of enormous significance for conservation by *ex-situ* means.

2.13 *IN VITRO* CULTURE

Liverworts have been receiving only limited attention as a source of new bio active phytochemicals. Liverworts have restricted geographical distribution pattern. In India, Eastern and Western Himalaya and Western Ghats are the main areas where liverworts grow

and show their maximum diversity. So, there is the major problem of material availability for researchers working on areas of less sample availability. One solution to all the factors constraining the chemical analysis is to establish *in vitro* culture, to find out the appropriate conditions to propagate and multiply the sample. *In vitro* propagation will help to overcome the problem of seasonal dependency of researchers working on bryophytes so that they do not have to wait for long time for sample collection. Most importantly it will help to overcome the major problem of sample collection in large and pure amount for different chemical analysis required for the validation of their application in pharmacological, clinical and cosmetic industrial use. Apart from solving the problem of clinical study, *in vitro* culture will also help in the conservation of many rare, endangered and red listed species. Spores of threatened rare Indian liverwort *Cryptomitrium himalayense* Kashyap has been propagated successfully by *in-vitro* culture method (Awasthi *et al.*, 2013). Awasthi *et al.* (2012) have reported the successful regeneration of liverworts *Conocephalum conicum*, *Reboulia hemisphaerica* and *Marchantia paleacea* from the vegetative parts. *Plagiochasma appendiculatum* has been reported to be used by many tribes. Mature thalli have been developed successfully from the spores of this ethno medicinally important liverwort by *in vitro* method. The thalli developed were reported to be acclimatized and then transferred into the soil where they have also been reported to produce gametangia (Awasthi *et al.*, 2012). Krishnan and Murugan (2014a) reported successful regeneration of entire plant from the spores of liverwort *Marchantia linearis*. There are many reports of successful regeneration of several other rare and entho medicinally important liverworts through *in vitro* culture method. *In vitro* culture is not only utilized for the conservation of the species but it has also been used for the isolation of biocompounds having an important biological activities. Krishnan and Murugan (2013) established cell suspension culture for maximum production of flavonoids in *Marchantia linearis*. Isolated flavonoids have shown significant anti-cancer activities.

In vitro propagation is the rapid means of multiplication of the plant material. However, there is a chance of alteration of composition of bioactive phytochemicals of *in vitro* grown plants because of the alteration of biochemical pathway as a result of artificial culture system (Senarath *et al.*, 2017). Many works has been done to check whether there exists any difference in the phytochemical composition of *in-vitro* and naturally grown plants. In this context Sabovljevic *et al.* (2011) compared the bio-activities of some axenically and naturally grown bryophytes. From the result of this work they have reported

the enhancement in the antifungal activity of the *in vitro* grown plant extract than naturally grown plants. Along with the attempt to see the changes in the biological activity of *in vitro* grown plants, some researchers have also studied the phytochemical profiles of *in vitro* grown plants. In this context Neves *et al.* (2001) studied the phytochemical profile of liverwort *Targionia lorbeeriana*. They have compared the terpenoid constituent of *in vitro* and *in vivo* grown plants and found that sesquiterpene lactones was produced by *in vitro* grown plants in the same relative amount as that of wild plants. Diversity of essential oil obtained from wild growing *Targionia lorbeeriana* was higher than *in vitro* grown, and some new compounds were noticed to be accumulated under *in vitro* conditions that are not found in wild grown *Targionia lorbeeriana*.

Studies on higher plant group have also supported the synthesis of similar kind of bio active molecules by *in vitro* and *in vivo* grown plants. When *in vivo* and *in vitro* grown *Centaurea ragusina* was compared, it was found that callus tissue of the plant produced higher levels of chlorogenic acid and naringenin than *in vivo* grown plants (Vujcic *et al.*, 2017). Similar kinds of phytochemicals were found to be produced by both *in vitro* and naturally grown *Kaempferia galanga* plant (Senarath *et al.*, 2017). All these reports supported the possibility of *in vitro* grown plants to be used as substitute for naturally grown plants for research purposes. Thus, *in vitro* method of propagation is worth considering for overcoming different factors that impede the use liverworts for researches.

2.14 EFFECT OF DIFFERENT PHYSICO-CHEMICAL FACTORS ON DIVERSITY, DISTRIBUTION AND ABUNDANCE OF LIVERWORTS

Bryophytes are important part of biodiversity having significant functional relationship with different ecological processes (Frego, 2007). They influence nutrient cycling and the moisture retention of forest ecosystem. Apart from this they play significant role in soil formation, bio accumulation of toxic minerals, prevention of soil erosion and pH indication. This important plant group needs major attention for conservation due to increasing deterioration of their habitat and changing environmental condition. Due to phyto geographic location and diverse climatic regiments, India has rich liverwort diversity. In recent year the rich bryophyte flora have shown considerable decline (Singh, 1999). Being the moisture loving plants, in India the maximum diversity of liverworts are recorded in the hilly regions like Eastern and Western Himalaya and Western Ghats. As these areas are famous tourist destinations, increasing anthropogenic activities is one of the main threats to

the diversity of liverworts in these areas. Increasing human population and other anthropogenic activities are also equally important. Moreover, change in climatic condition due to global warming also leads to loss of liverwort diversity (Bhattacharya, 2011). This plant group usually prefers to grow in cool areas with moist climate. Their growth is entirely dependent on the availability of water that is why they are also known as the 'amphibians of plant kingdom'. Global warming is leading to change in microclimatic condition of earth. This changed climatic condition is directly affecting the habitat of liverworts by altering the precipitation amount and temperature of that area. Thus, to have a good knowledge about the factors required for the growth of these plants is highly important.

Due to their small body structure, these plants are mostly ignored as weeds and their habitats are being destroyed unnoticed. By facilitating the concepts such as creation of 'Bryophyte Gardens', 'Liverwort Houses', 'Moss Houses' and 'Species Specific Sites' liverworts can be familiarized among the common people. This will help reduce the threats to these plants. Thus, for sustainable conservation it is important to have good knowledge regarding ecological factors that controls the species diversity, distribution and abundance of liverworts.

Lukaszkiewicz and Kosmala (2008) studied the effect of factors such as canopy effect, diameter at breast height, number of growth anomalies and forest density. They concluded that with the increase in the canopy cover, diameter at breast height (DBH), growth anomalies and tree density the probability of occurrence of indicator bryophyte species also increased. In a study related to distribution of epiphytic bryophytes in natural old forest of Latviana, it was found that DBH has a significant positive correlation with the epiphytic species richness. pH and bark crevice depth did not affect the richness of the epiphytic bryophytes (Mezaka, 2008). In another such study it was reported that the size of the tree log is positively related to the occurrence of bryophytes. Furthermore, it was found that different types of bryophytes preferred logs of different decay stages for their growth and proliferation. Common epixylic species were found to have greater tolerance to decay stage of wood (Odor and Van Hees, 2004).Whitelaw and Burton (2015) studied the distribution and diversity of epiphytic bryophytes on apple orchards. They found that pH of the tree has no correlation with the diversity or the bryophyte cover. Age of the tree is considered to be an important factor that influences the distribution of epiphytic bryophytes, but in this study they have found no correlation between tree age and bryophyte distribution. They justified that in orchards epiphytes are continuously removed as a measure to protect tree health and this practice might have affected the distribution pattern and density. Werner *et al.* (2003) in their

study have found that soil moisture and pH have a great effect on the species richness of ground vegetation.

Liverworts grow in areas with maximum moisture and low temperature which restricts its distribution in particular climatic zones and similar eco-climatic conditions are required for *ex situ* conservation. Study of effects of physico-chemical factors on the distribution, abundance and diversity of liverworts can be helpful for keeping the record of optimum condition of environmental factors required for their growth and proliferation.

Chapter 3

STUDY AREA

3. STUDY AREA

Darjeeling is a town in the state of West Bengal of India. It is a part of Eastern Himalaya which has an elevation of 2042 meter and stretches between 27° 2′ 9.6253″ N and 88° 15′ 6192″ E. It experiences a temperate climatic condition with wet summers caused by cool moisture laden monsoon rains. Cool and humid climatic condition of this place makes it a perfect habitat for bryophytic growth. Maximum diversity of Indian Bryofolra is seen in Eastern Himalaya. In the present study, Darjeeling is taken as the study area for collection of liverworts. Places surveyed for the collecting liverwort species are presented in figure 3.2 and the details of their location is given in table 3.1.

3.1 RAINFALL

The rainfall in Darjeeling occurs under the influence of Southwest Monsoon. The average annual precipitation is 309.2 (cm). This area experiences an average of 126 days of rain per year. Rainy season prevails from late May to early October. Maximum rainfall occurs in July. December is the driest month of the year with minimum rainfall. March receives maximum amount of winter rain.

3.2 TEMPERATURE

The annual average maximum temperature of Darjeeling is 14.9°C while annual average minimum temperature is 8.9°C. During the coldest months of December and January the mean maximum temperature remains 10.5°C and minimum mean temperature remains 2.5°C. Rapid rise in temperature occurs from March to May and remains almost constant till September resulting in maximum mean temperature of 18.2°C and minimum mean temperature of 11.8°C during this time of the year.

3.3 RAINFALL DAYS

Maximum number of rainy days is observed in the months of May, June, July, August and September. November and December are the driest months of the year with almost no rainfall. Months of January February and March sometimes receives few winter rain.

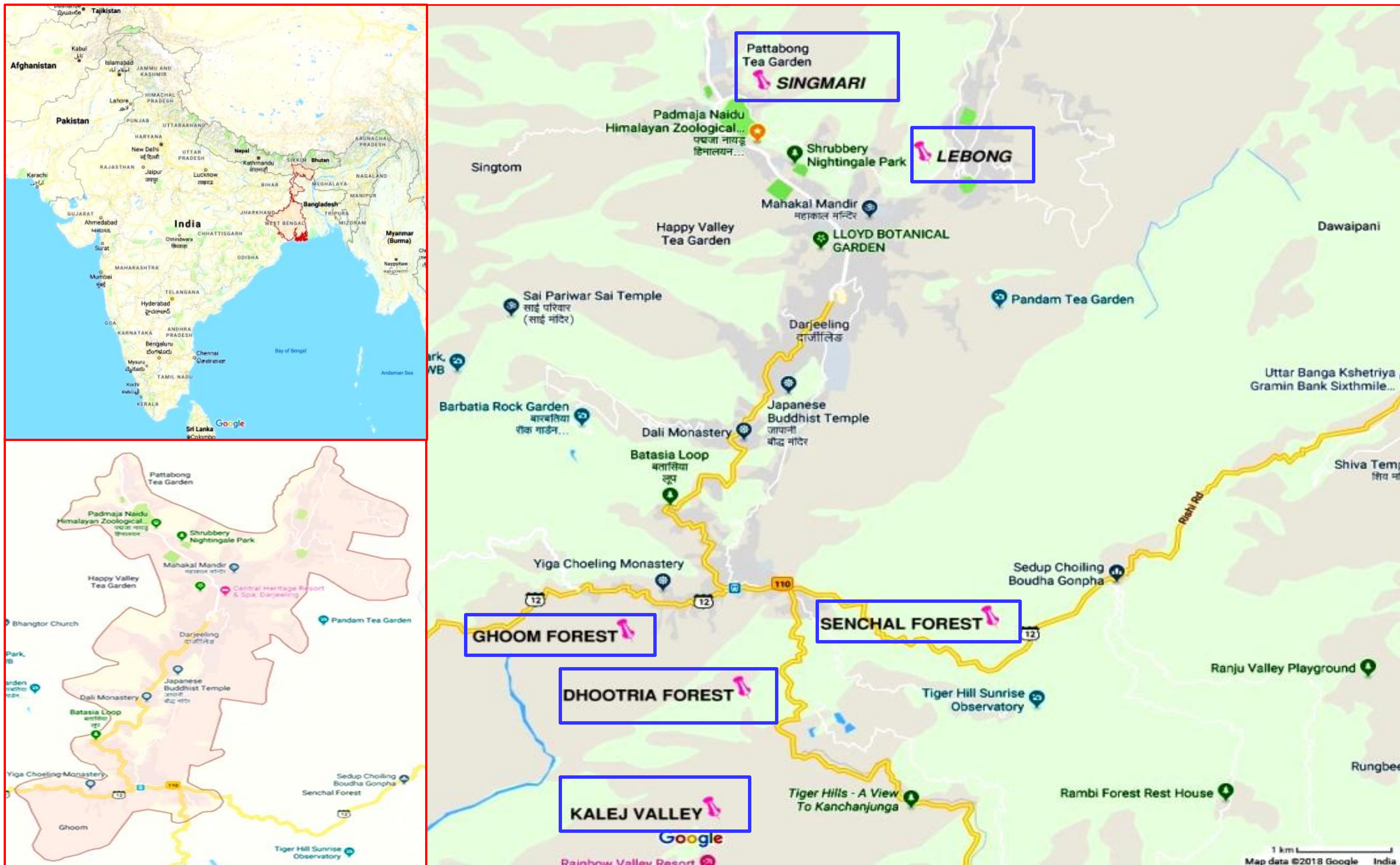


Fig 3.2: Map of surveyed areas of Darjeeling for liverwort sample collection (Senchal forest, Ghoom forest, Dhooteria forest, Kalej valley, Singmari, Lebong)

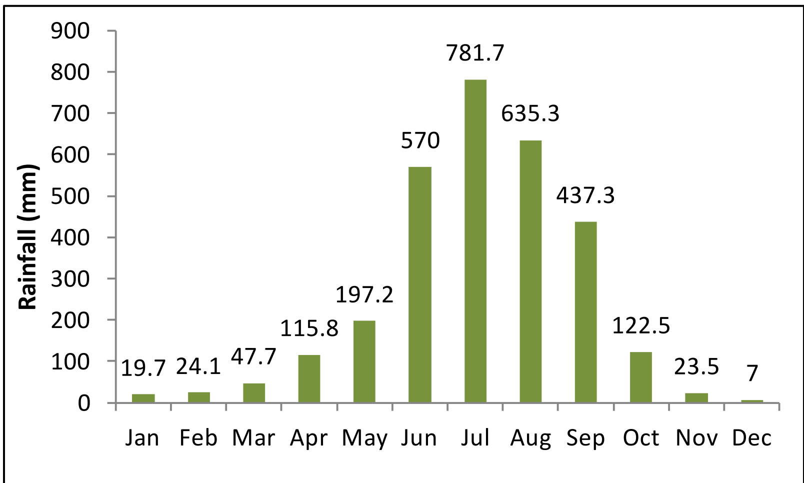


Fig 3.3: Average rainfall of Darjeeling throughout the year

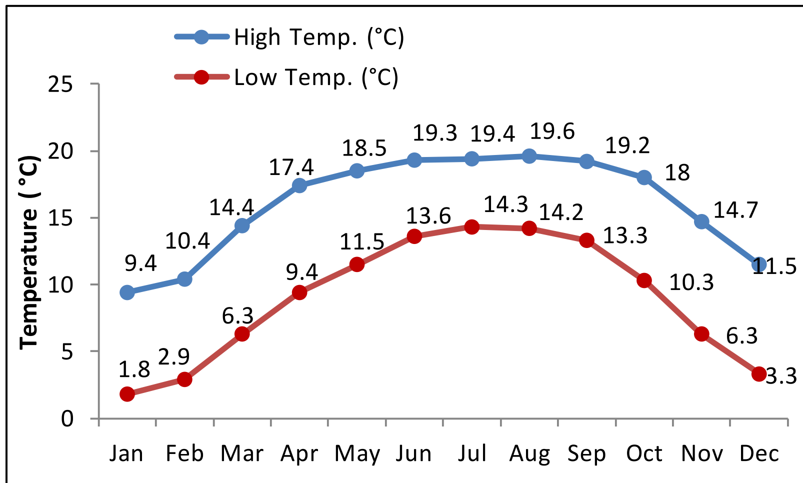


Fig 3.4: Average temperature of Darjeeling throughout the year

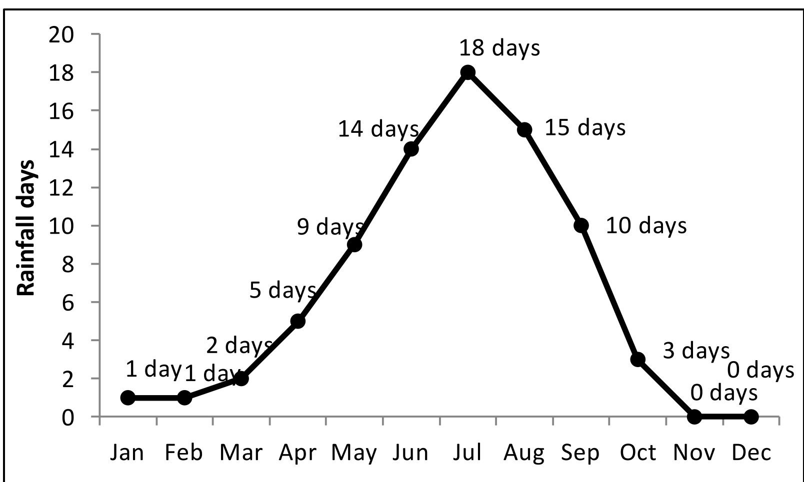


Fig 3.5: Average rainfall of Darjeeling throughout the year

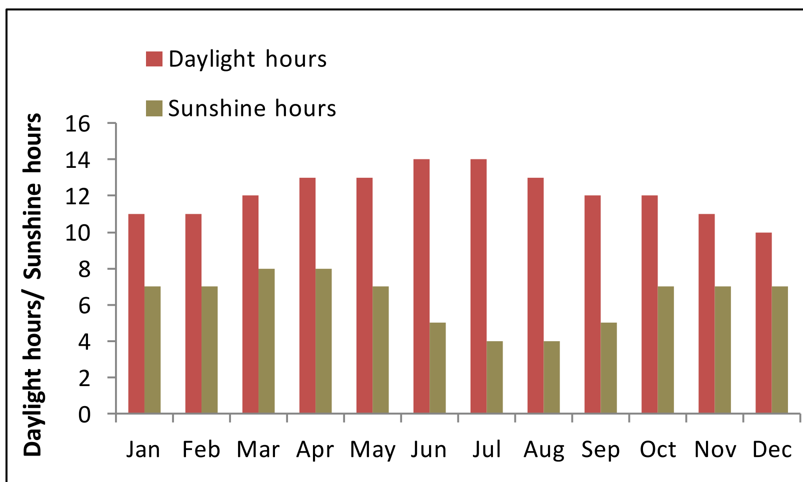


Fig 3.6: Average sunshine hours of Darjeeling throughout the year

[Source: Indian Meteorological Department]

3.4 SUNSHINE HOURS

Average daylight hours are shorter during the months of November, December, January, February and March but the average sunshine hours in these months is higher than rest of the months of a year. From April daylight light hours increases, reach the highest during the month of June and July and gradually start to decrease from the month of August. However the average sunlight hours stars decreasing from the month of April and reaches minimum in July and August. Average sunshine starts increasing again from the month of October.

Table 3.1: Altitudinal details of the sites of collection of studied eleven liverwort species

Locality	***Altitude***	***Latitude***	***Longitude***	***Species***	***Abundance at the site of collection***
Senchal Forest, Darjeeling	2471 m	27.0072° N	88.2826°E	*Pellia epiphylla* (L.) Corda	+++
Ghoom, Darjeeling	2196 m	27.0008° N,	88.2437° E	*Conocephalum japonicum* (Thunb.) Grolle	++
Singmari, Darjeeling	2137 m	27.0606° N	88.2565° E	*Lunularia cruciata*(L.) Dumort. Ex Lindb	+
Kalej Valley, Daejeeling	1698 m	26.9816ºN	88.2524° E	*Dumortiera hirsute* (Sw.) Nees	+++
Dhootria, Darjeeling	1789 m	26.9953 ºN	88.2525° E	*Marchantia emarginata* Reinw., Blume & Nees subsp. *emarginata*	++
Dhootria, Darjeeling	1789 m	26.9954 ºN	88.2542° E	*Marchantia subintegra* Mitt.	+
Singmari, Darjeeling	2133 m	27.0594 ºN	88.2491° E	*Marchantia polymorpha* L. subsp. *ruderalis* Bischl. & Boisselier-Dubayle	++
Dhootria, Darjeeling	1824 m	26.9953 ºN	88.2525° E	*Plagiochasma cordatum* Lehm. & Lindenb	++
Lebong, Darjeeling	1257 m	27.0528 ºN	88.2745° E	*Asterella wallichiana* (Lehm.) Grolle	++
Kalej Valley, Darjeeling	1745 m	26.9812 ºN	88.2535° E	*Plagiochila nepalensis* Lindenb.	+
Singmari, Darjeeling	2135 m	27.0593 ºN	88.2485° E	*Marchantia paleacea* Bertol.	+++

NB: += moderate, ++= high, +++= very high

Fig 3.7.1: Photograph of *Lunularia cruciata*

Fig 3.7.2: Photograph of *Dumortiera hirsuta*

Fig 3.7.3: Photograph of *Pellia epiphylla*

Fig 3.7.4: Photograph of *Marchantia paleacea*

Fig 3.7.5: Photograph of *Marchantia polymorpha*

Fig 3.7.6: Photograph of *Conocephalum japonicum*

Fig 3.7.7: Photograph of *Marchantia subintegra*

Fig 3.7.8: Photograph of *Plagiochasma cordatum*

Fig 3.7.9: Photograph of *Asterella wallichiana*

Fig 3.7.10: Photograph of *Marchantia emarginata*

Fig 3.7.11: Photograph of *Plagiochila nepalensis*

3.5 ENUMERATION OF TAXA

1. *Marchantia paleacea* Bertol. subsp. *paleacea*
Family: Marchantiaceae

Marchantia paleacea Bertol., Opusc. Sci. 1: 242. 1817; Bischl., Bryophyt. Biblioth. 38: 93. 1989. D.Singh & D.K.Singh, Proc. Natl. Acad. Sci., India, Sect. B Biol. Sci. 83(1): 23. 2013. *Marchantia nepalensis* Lehm. & Lindenb. in Lehm., Nov. Strip. pug. 4: 10. 1832; V.B. Singh, Natl. Bot. Gard. Bull. 125: 15. 1966. *Marchantia nitida* Lehm. & Lindenb. in Lehm., Nov. Strip. pug. 4: 11. 1832; Mitt., J. Proc. Linn. Soc., Bot. 5: 125. 1861. subsp. *paleacea*

Voucher specimens: India, Eastern Himalaya, Darjeeling, Singmari, 2133 m, 15/6/2012, *Sumira Mukhia* 51876/C (CAL)

2. *Pellia epiphylla* (L.) Corda.
Family: Pelliaceae

Pellia epiphylla (L.) Corda in Opiz (ed.) Beitr. Zur Naturg. 12 (1829) 654; R.S. Chopra in J. Indian Bot. Soc. 22 (1943) 241. *Jungermannia epiphylla* L., Sp. pl. 2 (1753) 1135.

Voucher specimens: India, Eastern Himalaya, Darjeeling, Senchal, 2471 m, 06.03.2013, *Sumira Mukhia* 51871/6 (CAL)

3. *Dumortiera hirsuta* (Sw.) Nees
Family: Dumortieraceae

Dumortiera hirsuta (Sw.) Nees, Fl. Bras. Enum. Pl. 1: 307. 1833 subsp. hirsuta; Kashyap, Liverw. W.Himal. 1: 42. 1929; Sushil K.Singh & D.K.Singh, Hepat. Anthoc. Gr. Him. Nat. Park 377. 2009.. *Marchantia hirsuta* Sw., Prodr. 145. 1788.

Voucher specimens: India, Eastern Himalaya, Darjeeling, Kalej Valley, 1698 m, 09.08.2015, *Sumira Mukhia* 51892/16 (CAL)

4. *Marchantia polymorpha* L. subsp. *polymorpha*
Family: Marchantiaceae

Marchantia polymorpha L., Sp. Pl. 1137. 1753; Mitt., J. Proc. Linn. Soc., Bot. 5: 125. 1861; V.B. Singh, Natl. Bot. Gard. Bull.125: 20. 1966; Bischl., Bryophyt. Biblioth. 38: 74. 1989; D.Singh & D.K.Singh, Proc. Natl. Acad. Sci., India, Sect. B Biol. Sci. 83(1): 24. 2013. subsp. *polymorpha*

Voucher specimens: India, Eastern Himalaya, Darjeeling, Singmari, 1698 m, 12.08.2015, *Sumira Mukhia* 51879/1 (CAL)

5. *Conocephalum japonicum* (Thunb.) Grolle
Family: Conocephalaceae

Conocephalum japonicum (Thunb.) Grolle, J. Hattori Bot. Lab. 68: 423. 1990; D.K.Singh & Sushil K.Singh, Indian J. Forest. 26(4): 442. 2003. *Marchantia japonica* Thunb., Fl. Jap. 344. 1784.

Voucher specimens: India, Eastern Himalaya, Darjeeling, Ghoom, 1698 m, 07.08.2015, *Sumira Mukhia* 51882/4 (CAL)

6. *Asterella wallichiana* (Lehm. & Lindenb.) Pande & al. ex Grolle
Family: Aytoniaceae

Pande et al. ex Grolle, Ergenisse der Forschungs-Unternehmens Nepal Himalaya 1 (1966) 262; D.G. Long in Bryophyt. Biblioth. 63 (2006) 144. Fimbriaria wallichiana Lehm. & Lindenb., Nov. Strip. pug. 4 (1832) 4. Asterella angusta (Steph.) Mahab. & Bhate in J. Univ. Bombay 13 (5) (1945) 5; R.S. Chopra in J. Indian Bot. Soc. 22 (1943) 238.

Voucher specimens: India, Eastern Himalaya, Darjeeling, Lebong, 1257 m, 06.08.2015, *Sumira Mukhia* 51881/3 (CAL)

7. *Plagiochila nepalensis* Lindenb.
Family: *Plagiochilaceae*

Plagiochila nepalensis Lindenb., Sp. Hepat. (*Plagiochila* fasc. 2 – 4): 93. 1840; M.L.So, Syst. Bot. Monog. 60: 156. 2001; K.K.Rawat & S.C.Srivast., Genus *Plagiochila* in E. Himal. 176. 2007.

Voucher specimens: India, Eastern Himalaya, Darjeeling, Kalej Valley, 1745 m, 11.08.2015, *Sumira Mukhia* 51889/11 (CAL)

8. *Marchantia subintegra* Mitt.
Family: Marchantiaceae

Marchantia subintegra Mitt., J. Proc. Linn. Soc., Bot. 5: 125. 1861; V.B. Singh, Natl. Bot. Gard. Bull. 125: 23. 1966; Bischl., Bryophyt. Biblioth. 38: 74. 1989; D. Singh & D.K.Singh, Proc. Natl. Acad. Sci., India, Sect. B Biol. Sci. 83: 25. 2013. *Marchantia papulosa* Amakawa in Hara, Fl E. Himal. 535. 1966.

Voucher specimens: India, Eastern Himalaya, Darjeeling, Dhootria, 1745 m, 09.08.2015, *Sumira Mukhia* 51884/6 (CAL)

9. *Lunularia cruciata* (L.) Dumort. ex Lindenb.
Family: Lunulariaceae

Lunularia cruciata (L.) Dumort. ex Lindenb., Not. Sällsk. Fauna Fl. Fenn. Förh. 9: 298. 1868. M*archantia cruciata* L., Sp. Pl. 1137. 1753. subsp. *cruciate*

Voucher specimens: India, Eastern Himalaya, Darjeeling, Singmari, 1928 m, 09.08.2015, *Sumira Mukhia* 51891/17 (CAL)

10. ***Marchantia emarginata*** **Reinw. Blume & Nees**
Family: Marchantiaceae

Marchantia emarginata Reinw. Blume & Nees, Nova Acta Phys.-Med. Acad. Caes. Leop.-Carol. Nat. Cur. 12: 192. 1824; Bischl., Bryophyt. Biblioth. 38: 183. 1989. *Marchantia palmata* Reinw. Blume & Nees, Nova Acta Phys.-Med. Acad. Caes. Leop.-Carol. Nat. Cur. 12: 193. 1824. subsp. emarginata

Voucher specimens: India, Eastern Himalaya, Darjeeling, Dhootria, 1789 m, 09.08.2015, *Sumira Mukhia* 51887/9 (CAL)

11. ***Plagiochasma cordatum*** **Lehm. & Lindenb.**
Family: Aytoniaceae

Plagiochasma cordatum Lehm. & Lindenb. in Lehm., Nov. Stirp. Pug. 4: 13. 1832; Kashyap, Liverw. W. Himal. Pl. 1: 81. 1929. *Plagiochasma paradoxum* Griff., Not. Pl. Asiat. 2: 330. 1849

Voucher specimens: India, Eastern Himalaya, Darjeeling, Dhootria, 1824 m, 09.08.2015, *Sumira Mukhia* 51883/5 (CAL)

Chapter 4

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 PREPARATION OF PLANT EXTRACTS

Liverwort samples were first carefully examined for the separation of specimen of interest and cleaned cautiously to remove contaminants such as soil and other plant specimens. Samples were then air dried and crushed into powder. Powdered sample of each plant was extracted successively by soxhletion using solvents having different polarity (from non polar to polar). 10g sample was extracted successively with heptane, diethyl ether, ethyl acetate, acetone, butanol and methanol. Solvents were completely evaporated and the crude extract obtained was reconstituted in methanol and stored for further use.

4.2 ANTIOXIDANT ASSAYS

4.2.1 Determination of DPPH$^{\bullet}$ scavenging activity

DPPH$^{\bullet}$ scavenging potential of plants was determined by following the method of Sidduraju *et al.* (2002). Plant extract (200 µl) was added to 2 ml 0.2mM DPPH$^{\bullet}$ solution and the change in color of DPPH$^{\bullet}$ from violet to light yellow was measured spectrophotometrically at 517 nm against reagent blank. The inhibition percentage was calculated by using following equation:

Inhibition percent (%) = $[(A_{control} - A_{sample}) / A_{control} \times 100]$

Where, $A_{control}$ is the absorbance of control and A_{sample} is the absorbance of sample. IC_{50} value was used to express the antioxidant activity. IC_{50} value is defined as concentration of antioxidant required for decreasing the free radical concentration by 50% (Gaikwad *et al.*, 2011).

4.2.2 Determination of ABTS^{+} scavenging activity

Method described by Li *et al.* (2009), was followed for estimating ABTS^{+} scavenging activity. ABTS^{+} stock was prepared by dissolving ABTS^{+} to 7 mM concentration. ABTS^{+} stock solution was mixed with 24mM potassium persulfate to prepare ABTS^{+} cation. The solution was kept in dark for 12- 16 h at room temperature before use. Before using solution was diluted with distilled water to give an absorbance of 0.700 (±0.02) at 734 nm. 2ml ABTS^{+} solution was then added to 20 µl plant extract and incubated for 10 minutes.

Absorbance was measured at 734 nm. Inhibition percentage was calculated by using similar chemical equation as mentioned above.

4.2.3 Determination of superoxide scavenging activity

Superoxide scavenging activity was determined by the method described by Fu *et al.* (2010), 1 ml extract and 1ml nitroblue tetrazolium chloride (312µM in phosphate buffer, pH 7.4) were mixed, to this 1ml nicotinamide adenine dinucleotide (936µM in phosphate buffer, pH 7.4) was added after 5 minutes. The mixture was centrifuged to remove precipitate. 10 µl phenazine methosulphate was added to the mixture and was incubated for 30 minutes with exposure to fluorescent light. Absorbance was measured at 560 nm. Formula used for calculating inhibition percentage:

Inhibition percent (%) = $[(A_{control} - A_{sample}) / A_{control} \times 100]$

4.2.4 Determination of nitric oxide scavenging activity

Method of Marcocci *et al.* (1994), was used for estimating nitric oxide scavenging activity. Nitric oxide was generated from sodium nitroprusside. 2 ml 20mM sodium nitroprusside was mixed with 0.5 ml phosphate buffer and 0.5 ml phosphate buffer. The mixture was incubated for 150 minutes at 25°C. 3 ml Griess reagent was added after the completion of incubation time and again allowed to stand for 30 minutes at room temperature. Absorbance was measured spectrophotometrically at 540 nm. Formula used for calculating inhibition percentage:

Inhibition percent (%) = $[(A_{control} - A_{sample}) / A_{control} \times 100]$

4.2.5 Determination of metal chelating activity

Ferric ion chelating activity was determined using the method described by Dinis *et al* (1994). Plant extract (400 µl), 40 µl 2 mM $FeCl_2$ and 80 µl 5mM Ferrozine was mixed and allowed to equilibrate for 10 minutes. Metal chelating activity was measured by decrease in the absorbance of iron (II)-ferrozine complex at 562 nm. Formula used for calculating inhibition percentage:

Inhibition percent (%) = $[(A_{control} - A_{sample}) / A_{control} \times 100]$

4.2.6 Determination of ferric ion reducing activity

Previously described method of Gulcin (2009), was used for estimating iron reducing ability. At first 1 ml extract and 2.5 ml phosphate buffer (0.2M) was mixed. To this mixture 2.5 ml 1% potassium ferricyanide was added and incubated for 20 minutes at 50°C. After completion of incubation period, 2.5 ml 10% trichloroacetic acid was added. Mixture was then cooled and centrifuged for 10 minutes. 2.5 ml supernatant was collected; to this 2.5 ml distilled water and 250 µl 0.1 % $FeCl_3$ was added. Iron reducing potential was determined by plotting absorbance value against different concentrations. Absorbance was measured at 700 nm.

4.3 ANTI-DIABETIC ACTIVITY

4.3.1 Determination of α-glucosidase inhibitory activity

The α-glucosidase enzyme inhibitory activity was determined by the method of Kim *et al.* (2005). For the assay, 2.5 ml 0.2mM phosphate buffer, 0.1 α-glucosidase enzyme and 0.1 ml 3 mM reduced glutathione was mixed and incubated for 15 minutes at 37°C. Later 0.5 ml sample and 0.25 ml 3 mM ρ-NPG was added and incubated for 15 minutes. 0.1 M Na_2CO_3 was added after 15 minutes to stop the reaction. Absorbance measured at 450 nm. Formula used for calculating inhibition percentage:

Inhibition percentage (%) = $[1-(A_s - A_b) / A_c] \times 100$

Where, A_s = absorbance of sample, A_b = absorbance of blank, A_c = absorbance of control

4.3.2 Determination of α-amylase inhibitory activity

α-amylase inhibitory activity was estimated by the method of Kim *et al.* (2005) mixture of 0.1 ml extract, 0.1 ml α-amylase enzyme and 0.3 ml 0.02 M sodium phosphate buffer was incubated for 10 minutes. 500 µl starch solution was added after 10 minutes. 1 ml dinitrosalicylic acid added at the end to terminate the reaction. The reaction mixture was heated at 100ºC for 15 minutes. Absorbance measured at 540 nm. Formula used for calculating inhibition percentage:

Inhibition percentage (%) = $(A_{Control} - A_{Sample}) / A_{Control} \times 100$

4.4 ANTI- CANCER ACTIVITY

4.4.1 MTT assay for Mammalian Cell Viability

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide by mitochondrial succinate dehydrogenase of living cell to a blue formazan product. The effect of liverworts *P. cordatum*, *A. wallichiana*, *P. nepalensis*, *L. cruciata* and *M. paleacea* on 'Kidney cancer cell line (ACHN) was measured by following the MTT assay method described by Denizot and Lang (1986) with few modifications. The cancerous cell line ACHN was seeded in 96 well plates at a density of 5×10^3 cells/well in 100 µl culture medium and incubated for 24 hrs at 37ºC in a humified incubator with 5% CO_2. After incubation and attachment of cells on well, the cells were washed with 1X PBS (Phosphate-buffered saline) twice and treated with different aliquots of studied liverwort extract and kept for 24 hrs. The media was replaced with MTT solution prepared in PBS (10 µl of 5mg/ml/well) after 24 hrs of treatment and again incubated for 3 hours at 37ºC. 50 µl isopropanol was added to solubilize the formazan crystals. The plates were then gently shaken for 1 min and the absorbance was measures at 620 nm by microtiter plate reader (BMG LABTECH SPECTRO star^{Nano} Germany).

4.5 PHYTOCHEMICAL ANALYSIS

4.5.1 Quantitative phytochemical test

4.5.1a Total phenol estimation

Phenol content estimated following the method of Kadam *et al*. (2013) using Folin-Ciocaolteu reagent. For this purpose 1 ml ethanol, 1 ml extract, 0.5 ml 50 % Folin Ciocalteu reagent and 5 ml distilled water were mixed. 1 ml 5% sodium carbonate added after 5 minutes and the mixture was incubated for 1 hour. Absorbance was measured at 725 nm. Gallic acid was used as standard.

4.5.1b Total flavonoid estimation

Method described by Atanassova *et al*. (2011) was followed for the estimation of flavonoid. 0.5 ml extract, 0.3 ml 5% sodium nitrite and 4 ml distilled water was mixed and allowed to stand for 5 minutes. After 5 minutes 0.3 ml 10% aluminium chloride was added and the mixture was left for 6 minutes. After completion of incubation time 2 ml 1 M sodium

hydroxide and 2.4 ml distilled water were added subsequently. Absorbance was measured at 510 nm. Quercetin was used as standard.

4.5.1c Total orthodihydric phenol estimation

Orthodihydric phenol content was estimated by following the method of Mahadevan and Sridhar (1982). Arnow's reagent was prepared by dissolving 10g sodium nitrite and 10g sodium molybdate in 100 ml water and stored in brown bottle. 0.5 ml Arnow's reagent was added to 0.5 ml plant extract. To this mixture 5 ml water and 1 ml of 1 (N) NaOH was added. Absorbance was measured at 515 nm. Catechol was used as standard.

4.5.1d Total Tannin estimation

Tannin content was estimated by following the method described by Thimmaiah (1999). Reaction was initiated by adding 5 ml water and 1 ml sodium carbonate to 0.1 ml plant extract. After 30 minutes of incubation, absorbance was measured at 700 nm. Tannic acid was used as standard.

4.5.1e Total steroid estimation

Total steroid content was estimated according to the method described by Rai *et al* (2013). At first 1 ml extract and 4 ml chloroform was mixed and shaken vigorously. From this mixture, 1 ml chloroform layer was taken and evaporated. The extract remaining on the test tube after evaporation of chloroform was then dissolved in 2 ml Liebermann Burchard's Reagent which is prepared by adding 0.5 ml H_2SO_4 in 10 ml acetic anhydride. Absorbance was measured at 640 nm. Solasodine was used as standard.

4.5.2 Qualitative phytochemical analysis

4.5.2a Test for amino acid

0.5 ml methanolic plant extract was reacted with few drops of ninhydrin reagent (30 mg ninhydrin dissolved in 10 ml n-butanol and mixed with 0.3 ml 98% acetic acid). The mixture was heated in a water bath. Purple colour of the mixture indicated the occurrence of amino acid (Kumar *et al*., 2009).

4.5.2b Test for anthraquinones

1 ml plant extract was evaporated and dissolved in 2 ml chloroform. To this, 2 ml ammonia wad added. Mixture turning into red or orange colour confirms the presence of anthraquinones (Kumar *et al.*, 2009).

4.5.2c Test for phytosterol

0.5 ml methanolic plant extract was evaporated and reconstituted in 2 ml chloroform. 2 ml concentrated H_2SO_4 was added. Formation of red colour ring indicated the presence of steroid (Kumar *et al.*, 2009)

4.5.2d Test for tannin

0.5 ml methanolic plants extract and 1% lead acetate was mixed. Appearance of brownish to yellowish precipitation indicated the presence of tannin (Kumar *et al.*, 2009).

4.5.2e Test for triterpenoids

0.5 ml extract was evaporated and reconstituted in 2 ml chloroform. 1 ml acetic anhydride was added to this mixture and chilled. After cooling 2 ml H_2SO_4 was added. Turning of the mixture into reddish violet colour confirms the presence of triterpenoids (Kumar *et al.*, 2009)

4.5.2f Test for resins

From 0.5 ml methanolic extract, the entire methanol was evaporated in a water bath and the remaining plant extract was dissolved in 1 ml petroleum ether. 2 ml 2% cupper acetate was added, shaken vigorously and allowed to separate. Appearance of green colour on the lower layer confirms the presence of resins (Trease and Evans, 1983).

4.5.2g Test for cardiac glycosides

Methanolic extract (0.5 ml) was evaporated and the remaining plant extract was dissolved in 1 ml glacial acetic acid. To this mixture, 1 drop of 10% ferric chloride was added. After the addition of ferric chloride, 1 ml concentrated H_2SO_4 added by the side of the test tube. Appearance of brown ring at interface and green colour of upper layer confirms the presence of cardiac glycoside (Ngbede *et al.*, 2008).

4.5.2h Test for alkaloids

Reaction was initiated by mixing 0.5 ml methanolic extract and 0.2 ml 36.5% HCl. To this mixture 0.2 ml Dragendroff's reagent was added. Formation of orange or red precipitation indicated the presence of alkaloids (Kumar *et al*., 2009).

4.5.2i Test for glycosides

For the detection of glycosides 0.5 ml methanolic extract was mixed with 2 ml of 50% HCl and hydrolyzed for 2 hours on a water bath. After 2 hours 1 ml pyridine, few drops of 1% sodium nitropruside and 5% sodium hydroxide were added. Appearance of red to pink colour confirms the presence of glycosides (Kumar *et al.*, 2009)

4.5.2j Test for reducing sugar

Reaction initiated by mixing 0.5 ml extract and 2 ml (1:1) mixture of Fehling's solution A and Fehling's solution B. The mixture was then heated in water bath for 5 minutes. Formation of brick red precipitate confirms the presence of reducing sugar (Ibrahim, 2009).

4.5.2k Test for flavonoids

Few drops of 10% ferric chloride were added to 1 ml methanolic extract. Turning of the reacting mixture to blue or green colour confirms the presence of flavonoids (Ibrahim, 2009).

4.5.3 Phytochemical screening by Thin Layer Chromatography (TLC) analysis

To screen the presence of secondary metabolites, TLC analysis was performed following the standard method of Wagner and Bladt, 1996. Silica gel 60 F_{254} pre-coated plates (Merck, Darmstadt Germany) were used in this method.

4.5.3a Test for anthraglycosides

Sample preparation: 1 g powdered sample was extracted with 5 ml methanol by heating on a water bath for 10 minutes. 20 µl filtrate was for the analysis.

Separation and identification: Running solvent used for the separation of anthraglycosides was ethylacetate: methanol: water (100: 13.5: 10). For detection, plate was sprayed with 10%

ethanolic potassium hydroxide and evaluated under UV- 365 nm. Red band confirms the presence of anthraquinones.

4.5.3b Test for bitter principle

Sample preparation: 1g powdered sample was extracted by heating on a water bath for 10 minutes with 5 ml methanol. 20 µl filtrate was used for the analysis.

Separation and identification: Running solvent used for separation was ethylacetate: methanol: water (100: 13.5: 10). For detection, plate was sprayed with 10 ml 1% ethanolic vanillin solution followed immediately by 10 ml 10% ethanolic sulphuric acid. Plate was then heated at 110ºC for 10 minutes. Plate was evaluated visually. Appearance of Red/ yellow/ brown or blue-green coloured band confirms the presence of bitter principle.

4.5.3c Test for flavonoids

Sample preparation: 1 g powdered sample was extracted with 5 ml methanol by heating on a water bath for 10 minutes. 20 µl filtrate was used for the analysis.

Separation and identification: Running solvent used for flavonoid separation was ethyl acetate: formic acid: glacial acetic acid: water (100: 11: 11 diphenylboric acid-β-ethylamino ester: 26). For detection, plate was sprayed with 10 ml 1% methanolic followed by 8 ml 5% etahnolic polyethylene glycol-4000. Plate was evaluated in UV-365 nm. Yellow/ green/orange coloured band indicates the presence of flavonoids.

4.5.3d Test for arbutin

Sample preparation: 1g powdered sample was extracted with 5 ml methanol by heating on a water bath for 10 minutes. 20 µl filtrate was used for the analysis.

Separation and identification: Solvent combination ethylacetate: methanol: water in the proportion of 100: 13.5: 10 was used as a running solvent. For detection, plate was sprayed with 10g ferric chloride and 0.5g potassium hexacyanoferrate in 100 ml water. Plate was evaluated visually. Blue coloured band indicated the presence of arbutin.

4.5.3e Test for alkaloids

Sample preparation: Powdered sample was moistened with 1 ml 10% ammonia solution. 5 ml methanol was added to the sample and extracted for 10 minutes in a water bath. 20 µl of the filtrate was used for analysis.

Separation and detection: Running solvent used for separation of alkaloids was ethylacetate: methanol: water (100: 13.5: 10). For detection, plate was sprayed with 1 ml Dragendroff reagent mixed with 2 ml glacial acetic acid and 10 ml water. Plate was evaluated visually. Orange- brown band confirms the presence of alkaloids.

4.5.3f Test for cardiac glycosides

Sample preparation: 1 g powdered sample was mixed with 5 ml 50% methanol and 10 ml 10% lead (II) acetate solution and in a water bath for 10 minutes. The filtrate was then cooled to room temperature and extracted with 10 ml dichloromethane for two times. The extract was then evaporated and the residual extract was dissolved in dichloromethane- methanol (1:1) solution. 100 µl of the filtrate was used for the analysis.

Separation and detection: Running solvent used for separation of cardiac glycoside was ethylacetate: methanol: water (100: 13.5: 10). For detection, plate was sprayed with 15-20 ml 20% solution of antimony-II-chloride in chloroform and heated for 5-6 minutes at 110ºC. Plate was evaluated visually. Appearance of blue coloured band indicates the presence of cardiac glycosides.

4.5.3g Test for coumarins

Sample preparation: Sample was prepared by heating 1g powdered sample with 10 ml dichloromethane for 15 minutes under reflux. Filtrate was evaporated and the residue was dissolved in 0.5 ml toluene. 20-40 µl filtrates was used for analysis.

Separation and detection: Running solvent used for separation of coumarins was toluene: ethyl acetate (93:7). For detection, plate was sprayed with 10 ml 10% ethanolic potassium hydroxide and evaluated in UV 365 nm. Light blue or brown coloured band confirms the presence of coumarins.

4.5.3h Test for phenol

Sample preparation: Sample was prepared in the same way as for coumarins.

Separation and detection: Solvent combination Ethyl acetate: methanol: water in the ratio 100: 13.5: 10 were used as running solvent for separation of phenols. For detection, plate was sprayed with 8 ml Fast blue reagent prepared by dissolving 0.5g fast blue salt B in 100 ml water. Evaluation of plate was done visually. Formation of reddish brown band confirms the presence of phenols.

4.6 SEASONAL CHANGES IN ANTIOXIDANT ACTIVITY AND PHENOLIC CONTENT IN LIVERWORTS

4.6.1 Sample preparation

Liverwort *Marchantia paleacea* was collected during two seasons: one during unfavorable growth condition *i.e.* when there is scarcity of water during the month of October to March and the other during favorable environmental condition *i.e.* when water is present in sufficient amount in the environment. Collected samples were then air dried and crushed into powder. 10g powdered sample was then extracted successively with heptane, diethyl ether, ethyl acetate, acetone, butanol and methanol. Solvents were completely evaporated and the crude extract obtained was reconstituted in methanol and stored for further use.

4.6.2 Antioxidant assays

$DPPH^{\cdot}$, $ABTS^{+}$, super oxide, nitric oxide scavenging, metal chelating and reducing power assays were performed following the methods of Sidduraju *et al*. (2002) Li *et al*. (2009), Fu *et al*.(2010), Marcocci *et al*. (1994), Dinis *et al*.(1994) and Gulcin (2009) described earlier in the methodology section for antioxidant assays.

4.6.3 Quantitative phytochemical estimation

Phenol, flavonoid and ortho-dihydric phenol content was estimated following the methods of Kadam *et al*. (2013) Atanassova *et al*. (2011) Mahadevan and Sridhar (1986) described earlier in this methodology section for antioxidant assays.

4.7 BIOASSAY GUIDED PURIFICATION

4.7.1 Extraction of plant sample

1 kg plant sample (*Marchantia paleacea)* was cleaned initially under tap water, dried and ground into fine powder. It was then finely crushed into powder and extracted successively by soxlation method with hexane, toluene, diethyl ether, ethyl acetate,

chloroform, acetone, acetonitryl, ethanol, methanol and water according to the increasing polarity of solvents. The fraction showing the best antioxidant, anti-diabetic activities and flavonoid content was subjected to column chromatography separation method for isolation of active constituents.

4.7.2 Bioassay guided screening of the fractions

DPPH$^{\bullet}$ scavenging assay, anti-diabetic assay and flavonoid content estimation was done following the method of Sidduraju *et al*. (2002), Kim *et al*. (2005) and Atanassova *et al*. (2011) as described earlier.

4.7.3 Column chromatography

Powdered and dried plant sample (1Kg) was sequentially extracted with hexane, toluene, diethyl ether, ethyl acetate, chloroform, acetone, acetonitryl, ethanol, methanol and water. Each fraction was analyzed for antioxidant, antidiabetic activity and flavonoid content. The diethyl ether showed the highest antioxidant, anti-diabetic activities as well as flavonoid content. The diethyl ether fraction was then chromatographed on silica gel (75g) column. 10g extract was subjected to column chromatography for separating the extracts into its successive fractions. Silica gel 200-400 M was utilized as the stationary phase and different solvent combinations with increasing polarity were utilized as the mobile phase for the analysis. For the preparation of column, at first a small piece of cotton was stocked at the bottom of the glass column with the help of glass rod to prevent the straining out of the silica gel. Thin layer of sea sand was deposited above the cotton wool for the uniform deposition of silica. The slurry of 75g silica gel and 150 ml benzene was poured carefully into the column. While pouring the slurry, the tap of the column was kept open for free flow of solvent. The solvent was drained freely until a clear solvent carrying no silica gel or cotton wool was obtained. The tap was closed and allowed to stand for 24 hours for stabilization. After 24 hours, the solvent on top of silica gel was drained to the meniscus of silica gel. The sample was prepared by absorbing 10g extract on 20g silica gel by applying a gentle heat. Low heat was given until the sample completely gets attached to the silica gel firmly and dry powder was obtained. Powder was gently layered on the top of silica gel packed in the column. Benzene was then poured gently into the column. Tap of the column was opened and the eluent was allowed to flow at the rate of 30 drops per minute. Solvent systems of increasing polarity were used for the elution of the extract. Solvent systems used were: Benzene: diethyl ether 100: 0, 75: 25, 50: 50, 25: 75; 0: 100; diethyl ether: chloroform 75: 25, 50: 50, 25: 75, 0:

100; chloroform: ethyl acetate 75: 25, 50: 50, 25: 75; 0: 100; ethyl acetate: methanol 75: 25, 50: 50, 25: 75; 0: 100; methanol: water 75: 25, 50: 50, 25: 75; 0: 100. 500 ml of each solvent combination was added gradually by the side of the mouth of the column. This was done carefully as the distortion of the silica gel layer results in non-uniform draining. The eluted sample was collected in the test tube in aliquots of 10 ml. A total of 208 sub fractions were collected and combined on the basis of their antioxidant activity and TLC profiles. Antioxidant property was screened through DPPH radical scavenging activity. Sub fractions with good DPPH radical scavenging activity were subjected to analytical thin layer chromatography.

4.7.4 Analytical thin layer chromatography (TLC) and merging of fractions

The content of the selected sub fractions were spotted on TLC plates pre-coated with silica gel 60 F_{254}; Merck, Darmstadt, Germany. TLC plate was cut into small strips (2cm × 8cm) and 20 µl of concentrated sample (sub fraction of interest) was loaded on the plate with micro pipette above 0.5 cm from the edge. The sample spot was dried by applying hot air. The plate was then gently lowered into a chromatographic jar that contained running solvent system (ethyl acetate: methanol: water:: 100: 13.5: 10). The jar was closed with a lid. The solvent was allowed to run on the plate. The plate was removed and heated in an oven for few minutes to remove the solvents. Dried plate was then viewed under UV lamp at 365nm. On the basis of TLC profiles, sub fractions were merged into eleven main fractions as follows: F1A, F1B, F1C, F1D, F1E, F1F, F1G, F1H, F1I, F1J, F1K. These main fractions were used for antioxidant and anti-diabetic assays. $ABTS^{+}$ and $DPPH^{\cdot}$ scavenging assays were done to evaluate the free radical scavenging activity of the merged fractions. Earlier described method was followed for screening $ABTS^{+}$ and $DPPH^{\cdot}$ scavenging activity. Anti-diabetic activity were screened by measuring their potential to inhibit the activity of α- glucosidase enzyme. Earlier described method was followed. Fraction F1F showed best antioxidant and antidiabetic activity and was subjected to additional fractionation. F1F fraction was chromatographed again on a silica gel column. Column was prepared in the similar method as described earlier. The solvent combinations used for elution process were petroleum ether: benzene 100: 0, 70:30, 50:50, 30:70, 0:100; benzene: diethyl ether 100:0, 70:30, 50:50, 30:70, 0:100; diethyl ether: chloroform 100: 0, 70:30, 50:50, 30:70, 0:100; chloroform: ethyl acetate 100: 0, 70:30, 50:50, 30:70, 0:100; ethyl acetate: methanol 100: 0, 70:30, 50:50, 30:70, 0:100. The eluents were collected at the rate of 30 drops per minute as aliquots of 5 ml. A total of 546 sub fractions were collected and screened for antioxidant anti-diabetic

activities (DPPH˙ scavenging assay) and phytochemical content (flavonoid content). Sub fractions showing good antioxidant activity and high flavonoid content were combined on the basis of their TLC (ethyl acetate: methanol: water:: 10: 1.3: 1) profiles into two main fractions and were subjected to Gas chromatography-mass spectrometry for the identification of active compounds present in the fraction.

4.7.5 Gas Chromatography – Mass Spectrometry analysis

For the analysis, Perkin-Elmer GC Clarus 500 system with Gas chromatograph interfaced to a Mass Spectrometer and AOC-20i auto sampler was utilized. For separations Elite-5MS (5% diphenyl/ 95% dimethyl poly siloxane) and capillary tube (30 × 0.25 μm ID × 0.25 μm film thickness) was used. Carrier gas helium (99.9%) was used at a constant flow rate of 1 ml for 1 minute with a 10.0 mL/ min split flow rate. Mass spectrometer was operated at 70ev ionization energy and a scan interval of 0.5 s scanning from 45 to 450 m/z. Temperature of the column was maintained at 250ºC and temperature 200ºC was maintained for ion source. Column was programmed at temperature 110ºC for 2 minutes with an increase of 10ºC/minute to 200ºC, 5ºC/ minute to 280º C and finally held for 9 minutes at 280º C. The total runtime was 36 minutes. 2 μl samples were loaded in a mode injector. TurboMass Gold^{TM} PerkinElmer was used as the mass detector and Turbo-Mass ver-5.2 was used as software for mass spectral analysis and chromatogram. NIST Library database was used for comparing MS fragmentation patterns and retention time of the compound. Compounds relative percentage was estimated by comparing average peak to total area.

4.7.6 Network pharmacology

4.7.6a Data collection for network analysis

Active phytochemicals present in *M. paleacea* was identified by performing Gas Chromatography –Mass Spectrometry analysis of bio-active fractions obtained from column chromatography. Chemical information like Canonical SMILES, PubChemID etc were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov). Human proteins targeted by the active phytochemicals present in the liverwort were predicted from Binding Data Base (BindingDB) [https://www.bindingdb.org]. To access the high confidence human protein targets, phytochemical-protein interactions having similarity search value ≥ 0.75 was screened. BindingDB is an open access database having binding affinities of approximately 20,000 protein-ligand complexes. It works on the basic principle that similar compounds most likely bind identical proteins. BindingDB aims to support drug discovery through

literature awareness and validation of computational chemistry, development of structure activity relations and molecular modeling approaches. Gene ID of the human protein targets were obtained from UniprotKB (http://www.uniport.org).

Target associated Diseases of all target proteins was determined from Therapeutic Target Database (TTD) [http://bidd.nus.edu.sg]. The diseases were classified according to ICD 10 codes (https://www.icd10data.com). TTD provides information of known, earlier explored therapeutic drugs, pathway information, targeted disease, nucleic acid targets and corresponding drugs directed at targets.

4.7.6b Target-Target interactions

First-degree interaction of the target proteins was identified using STRING (https://string-db.org). STRING (Search Tool for the Retrieval of Interacting Genes/ Proteins) is a biological database of known and predicted protein- protein interactions.

4.7.6c Network construction and analysis

Pharmacological properties of the phyto-compounds were studied by constructing tripartite network through interactions between phytochemical-target protein-associated diseases using Cytoscape (http://www.cytoscape.org). Cytoscape is a platform that analyzes complex molecular networks and integrates these with gene expression profiles.

4.7.6d Enrichment analysis

The functional enrichments were highlighted in list of isolated centralized proteins using KEGG (Kyoto Encyclopedia of Genes and genomes) pathway (https://www.genome.jp/keg/pathway).

4.7.6e Drug-likeliness prediction:

Pharmacokinetic and toxicity properties of phytochemicals were studied by using pKCSM that utilizes graph based signatures using predictive models of ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) properties for drug development.

4.8 *IN VITRO* PROPAGATION AND COMPARISON OF BIOACTIVITIES OF AXENICALLY AND NATURALLY GROWN LIVERWORT *LUNULARIA CRUCIATA*

4.8.1 Plant material collection and method of *in vitro* culture

Liverwort *Lunularia cruciata* was collected from Darjeeling and the voucher specimen was deposited in the herbarium of Botanical Survey of India, Kolkata. Axenic culture was initiated from the gemmae as sporophytes were not found in proper developmental stage. Gemmae present in the gemma cup were carefully taken out and rinsed carefully with distilled water. Sterilization of the gemmae was done in 1, 2, 3 and 4% solution of sodium hypochlorite for different time intervals like 30 seconds, 1 minute, 2 minutes and 4 minutes. Gammae was then immediately washed with sterilized double distilled water. Different culture mediums like half and full strength of Murashige and Skoog medium, Knop's medium and Gamborg G5 medium were used at first to standardize the suitable media for micropropagation. Media was gelled using 0.8% agar and the pH was maintained at 5.8. All the glasswares and media were sterilized by autoclaving them at 15 lb/sq inch for 15 minutes. In the Laminar Air Flow cabinet, the sterilized gammae was inoculated into the media. The culture was carefully maintained under controlled and aseptic condition under illumination of alternating 4000-5000 lux light and dark period of 14 and 10 hours respectively at 21±2°C. Germinated gemmae were transferred into another media supplemented with growth regulators like 1-Naphthaleneacetic and 6-Benzylaminopurine.

4.8.2 Screening of phytochemicals and biological activities

4.8.2a Sample preparation

Naturally grown as well as *in vitro* grown *L. cruciata* were dried carefully and ground into fine powder. Powder was then refluxed separately in methanol for 4 hours. The extracts were concentrated and stored for future use.

4.8.2b Antioxidant activities

DPPH radical scavenging assay, ABTS radical scavenging assay and metal chelating assay were performed to evaluate the free radical scavenging activity of *in vitro* and *in vivo* grown plants. Earlier described methods for $DPPH^{\bullet}$, $ABTS^{+}$ and metal chelating assay were followed.

4.8.2c Anti-diabetic activity

α- glucosidase and α- amylase enzyme inhibitory assays were performed to evaluate anti-diabetic potential of *in-vitro* grown and naturally grown plants. Earlier described methods for α- glucosidase and α- amylase inhibitory assays were followed.

4.8.3 Phytochemical analysis through Gas Chromatography – Mass Spectrometry analysis

Phytochemicals present in axenically and naturally grown liverwort *L. cruciata* were studied by Gas Chromatography – Mass Spectrometry analysis. Previously described method for GC-MS was followed.

4.9 VARIATION IN THE ABUNDANCE OF EPIPHYTIC LIVERWORTS IN RELATION TO PHYSICO-CHEMICAL ATTRIBUTES

4.9.1 Study area

Senchal Wildlife Sanctuary, Darjeeling, West Bengal, India was studied for habitat study. It is one of the oldest wildlife sanctuaries of India situated at an elevation of 1500 to 2600 m and covers an area of 38.88 km^2. The mean annual temperature ranges from 8.9℃ to 15.98℃ and the annual precipitation in the area is 29.81.8 mm. Studied sample plot was selected randomly. In this area, *Cryptomeria japonica* is the dominant tree species and it also favours the luxuriant growth of epiphytic liverworts. Thus, the study of abundance of liverworts was conducted on a total of fifty *C. japonica* trees of different age groups. Density, presence or absence of the epiphytic liverworts was recorded up to diameter at breast height (dbh). Five quadrats of 5× 5cm size were placed randomly on each tree for the sampling of bryophytes along the underlying bark.

4.9.2 pH measurement of the bark

Bark has been cleaned carefully by removing all the epiphytic species growing on it. 0.5g bark was cut into small pieces. 20 ml 1M KCl solution was added and shaken vigorously. After 1 hour pH was measured using pH meter.

4.9.3 Total phenol estimation

3g bark was powdered and refluxed with methanol for two hours. The filtered extract was used for phenol estimation. Method of Kadam *et al*. (2013) was followed. One ml

sample, 1 ml 95% ethanol, 5 ml distilled water and 0.5 ml 50% Folin Ciocalteau reagent were mixed, followed by addition of 1 ml 5% Na_2CO_3 after 5 minutes. The absorbance was measured at 725 nm. Standard curve was calibrated using different concentrations of gallic acid.

4.9.4 Total flavonoid estimation

Flavonoids were estimated following the method of Atanassova *et al.* (2011). 0.5 ml extract, 4 ml distilled water and 0.3 ml 5% $NaNO_2$ was mixed. To this, 0.3 ml 10% $AlCl_3$ and 2 ml 1.0 M NaOH was added after 5 minutes and 6 minutes respectively. The whole mixture was diluted by adding 2.4 ml of distilled water. Absorbance was measured at 510 nm. Quercetin was used as standard.

4.9.5 Terpenoid estimation

Method of Theng and Korpenwar (2013) was followed. Two gram bark powder was soaked in 50 ml 95% ethanol for 24 hours. It was then filtered. The filtrate was extracted with petroleum ether (60°C – 80°C). After extraction, petroleum ether fraction was kept and dried. Content of total terpenoids were determined from extractive weight of the petroleum ether fraction.

4.9.6 Total soluble sugar estimation

One hundred milligram (100 mg) bark powder was boiled for 3 hours with 5 ml 2.5N HCl in water bath. Extract was neutralized by adding sodium carbonate and the volume was made up to 100 ml. The extract was then centrifuged and supernatant collected was used for estimation of total soluble sugar following the earlier described method of Thimmaiah (1999). One milliliter (1 ml) aliquote was mixed with 4 ml anthrone reagent and heated for 8 minutes in a water bath. The reactant was cooled rapidly and the absorbance as measured at 630 nm.

4.9.7 Moisture content

Three gram bark was dried at 50°C for 1 day until it lost all the moisture content and its weight was stabilized. Bark was weighed again. Moisture content was measured using following formula

$$Moisture\ \% = \frac{initial\ weight - weight\ after\ drying}{initial\ weight} \times 100$$

4.9.8 Light intensity

Intensity of light falling on a particular area was measured using Lutron lux meter LX-101.

4.9.9 Data analysis

Effects of physical and biochemical parameters on epiphytic distribution pattern were studied by Box plot and Histogram test using XLSTAT 2014 software. Correlation co-efficient matrix was prepared using SPSS (Version 12.00) for determining the relation of liverwort density with environmental and biochemical factors. Regression value was calculated using XLSTAT 2014 software. Relationship between epiphytic liverwort density, environmental factors and chemical attributes of trees were studied by Principle component analysis (PCA) and Heatmap using Multivariate Statistical Package (MSVP 3.1) and R software (version 3.4.0) respectively.

4.10 VARIATION IN THE ABUNDANCE OF LIVERWORTS GROWING ON SOIL IN RELATION TO PHYSICAL FACTOR AND SOIL PROPERTIES

4.10.1 Study area

The study of abundance of liverworts was conducted on a total six different locations. Five quadrats of 10 × 10 cm size were set at an interval of 10 cm along the studied sites. A screen with 25 grids (2 cm x 2cm) was placed on each quadrate. The percentage cover of the bryophytes was calculated on the basis of space and number of grids occupied by bryophytes (Fernando *et al*., 2008).

4.10.2 Soil sampling and determination of physicochemical properties

Soil samples are collected from five different sites and processed for physicochemical analysis viz. moisture content, pH, carbon nitrogen ratio of soil, organic carbon, available form of nitrogen, phosphorous and potash content (Jackson, 1973).

4.10.3 Moisture content

Soil sample was dried at room temperature until it lost all the moisture content and its weight was stabilized. Soil was weighed again. Moisture content was measured using following formula

$$Moisture\,\% = \frac{initial\,weight - weight\,after\,drying}{initial\,weight} \times 100$$

4.10.4 Light intensity

Intensity of light falling on a particular area was measured using Lutron lux meter

4.11 STATISTICAL ANALYSIS

The data were collected in triplicate. IC_{50} values and standard error of estimates was determined using MS Excel 2007 (Microsoft, Redmond, WA, USA). Duncan's Multiple Range Test (DMRT) through DSAASTAT software (version 1.002; DSAASTAT, Peruglia, Italy) was used for comparing means; $p < 0.05$ was considered to be statistically significant for differences in the mean level of components. Correlation co-efficient matrix using SPSS (Version 12.00, SPSS Inc., Chicago, IL, USA) and a principal component analysis (PCA) using Multivariate Statistical Package (MVSP 3.1) was used for drawing relation between pharmacological attributes and the phytochemical constituents of liverworts. Heatmap by Multivariate Statistical Package (MSVP 3.1) and R software (version 3.4.0) used for analyzing the function of different biological solvents on extraction of phytochemicals from studied liverworts.

Chapter 5

RESULTS

5. RESULT

5.1 FREE RADICAL SCAVENGING ACTIVITY

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are beneficial for normal physiological functions when they occur at low or moderate concentrations. Overproduction of reactive ROS/RNS results in damage of cell structure, protein and DNA (Valko *et al.*, 2006). In the present work free radical scavenging potential of eleven liverworts namely, *Pellia epiphylla*, *Conocephalum japonicum*, *Lunularia cruciata*, *Dumortiera hirsuta*, *Marchantia emarginata*, *M subintegra*, *M polymorpha*, *Plagiochasma cordatum*, *Asterella wallichiana*, and *Plagiochila nepalensis* and *Marchantia paleacea* were studied by their ability to scavenge Superoxide (SO), Nitric oxide (NO), $DPPH^{\cdot}$, $ABTS^{+}$ radicals and metal ion chelating Activity (MC). $DPPH^{\bullet}$ is mostly used for investing the free radical scavenging properties of natural compounds. Studied eleven liverworts showed DPPH radical scavenging activity ranging between 0.1 mg/ml and 34.7 mg/ml (IC_{50} value). Among the studied liverworts *Marchantia paleaea*, *Plagiochasma cordatum* and *Plagiochila nepalensis* showed the lowest IC_{50} value which signifies the highest $DPPH^{\cdot}$ scavenging activity of these liverworts (Fig 5.1.1). Another important finding of this assay was determination of suitable solvent for the extraction of phytochemicals with high $DPPH^{\cdot}$ scavenging potential. Table 5.1 lists the $DPPH^{\bullet}$ scavenging activity of studied liverworts. In all the studied liverworts, diethyl ether and ethyl acetate extracts have shown the highest $DPPH^{+}$ scavenging activity. $ABTS^{+}$ is another stable free radicals used to study the antioxidant activity of plants. In the present study, $ABTS^{+}$ scavenging activity ranged between 0.02 mg/ml and 10.19 mg/ml (IC_{50} value). *Marchantia polymorpha*, *Marchantia paleacea*, *Plagiochasma cordatum* and *Plagiochila nepalensis* showed highest $ABTS^{+}$ scavenging activity (Fig 5.1.2). $ABTS^{+}$ was scavenged with maximum strength by the diethyl ether and ethyl acetate extracts in studied liverworts except in case of *Dumortiera hirsuta* where acetone extract showed the highest $ABTS^{+}$ scavenging activity (Table 5.2).

In living organism superoxide generated by the auto-oxidation reaction is the most harmful free radical. It induces oxidation of lipid by singlet oxygen generation (Halliwell *et al.*, 1978). Table 5.3 represents the SO scavenging activity of studied liverwort species. SO scavenging activity ranged between 0.11 mg/ ml and 15.57 mg/ml (IC_{50} value). Liverworts

like *Marchantia polymorpha*, *Marchantia paleacea*, *Marchantia subintegra*, *Marchantia emerginata* and *Asterella wallichiana* showed the highest superoxide scavenging activity (Fig 5.1.3). Nitric oxide is an important signalling molecule; however its sustained level in the body is very harmful. Liverworts showed large variation in the NO scavenging Activity (Fig 5.1.4). The highest NO scavenging activity was shown by heptane extract of *Dumortiera hirsuta.* Other liverworts like *Lunularia cruciata*, *Dumortiera hirsuta*, *Conocephalum japonicum* and *Marchantia paleacea* also showed significant NO scavenging activity. Lowest NO scavenging activity was shown by methanolic extract of *Marchantia subintegra* (Table 5.4). Suitable solvent for extracting active nitric oxide scavenging phytochemicals varied among studied liverworts. In *Marchantia paleacea*, *M. subintegra*, *Pellia epiphylla*, *Plagiochasma cordatum* and *Astetrella wallichiana* heptane extract showed highest NO scavenging activity. Whereas, in *Marchantia polymorpha* and *Dumortiera hirsuta*, butanol extract showed the NO scavenging activity. In *Marchantia emarginata* and *Lunularia cruciata* ethyl acetate extract and in *Conocephalum japonicum* and *Plagiochila nepalensis* acetone extract showed highest NO scavenging activity (Fig 5.1.4).

Metal chelating assay is another important antioxidant assay. Complexes of ferrozine and Fe^{2+} together with samples have metal chelating activity, and thus probability for the production of complexes to yield hydroxyl radical will be decreased. Studied liverworts have showed metal chelating activity ranging between 0.09 mg/ml and 25.48 mg/ml (IC_{50}value) (Table 5.5). Liverwort species like *Marchantia paleacea*, *M. subintegra*, *M. emarginata*, *Plagiochasma cordatum*, *Plagiochila nepalensis*, *Dumortiera hirsuta* and *Lunularia cruciata* displayed a strong metal chelating activity among studied liverworts.

Reducing power of bioactive compounds provides direct assessment to their antioxidative property. Figure 5.1.6 represents the ferrous ion reducing potential of studied eleven liverworts. Diethyl ether extract of *Plagiochasma cordatum* showed the highest metal ion reducing potential. Liverworts like *Marchantia paleacea*, *M. emerginta, Plagiochila nepalensis*, *Pellia epiphylla* also displayed an impressive potential to reduce ferrous ion. Result also revealed that in *Marchantia paleacea*, *M. subintegra*, *Conocephalum japonicum*, *Pellia epiphylla*, *Plagiochasma cordatum*, *Plagiochila nepalensis* and *Dumortiera hirsuta* diethyl ether showed highest ferrous ion reducing activity. Whereas in *Marchantia polymorpha*, *M. emarginata*, *Lunularia crucita* and *Asterella wallichiana* ethyl acetate extract showed highest ferrous ion reducing actcivity (Table 5.6

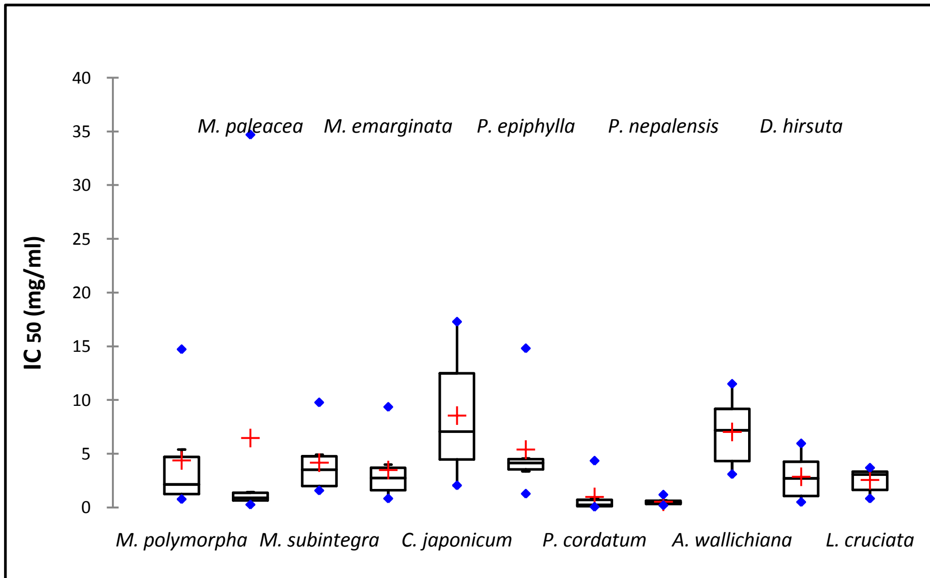


Fig 5.1.1: DPPH radical scavenging activity of studied eleven liverworts. For each sample, mean, standard deviation, maximum and minimum values, outliers are shown.

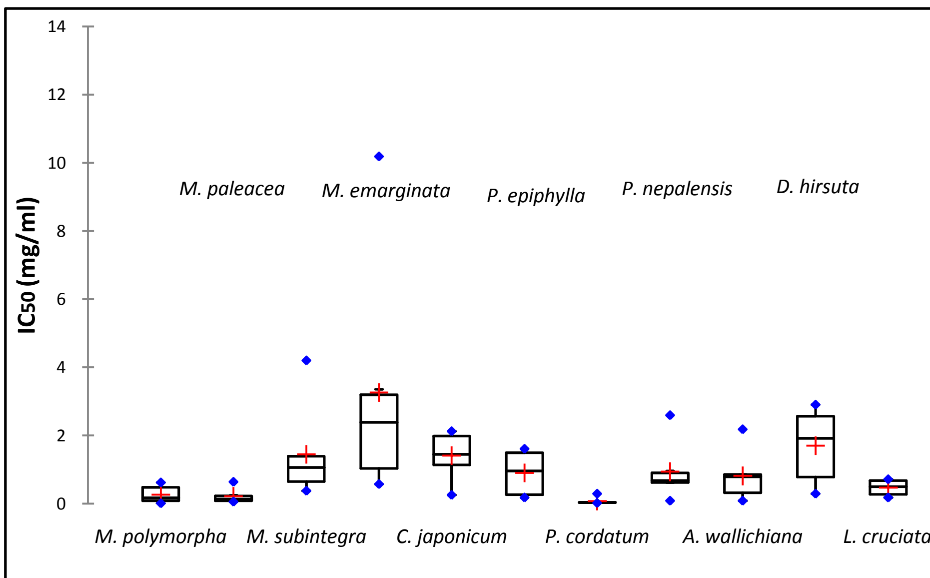


Fig 5.1.2 ABTS radical scavenging activity of studied eleven liverworts. For each sample, mean, standard deviation, maximum and minimum values, outliers are shown.

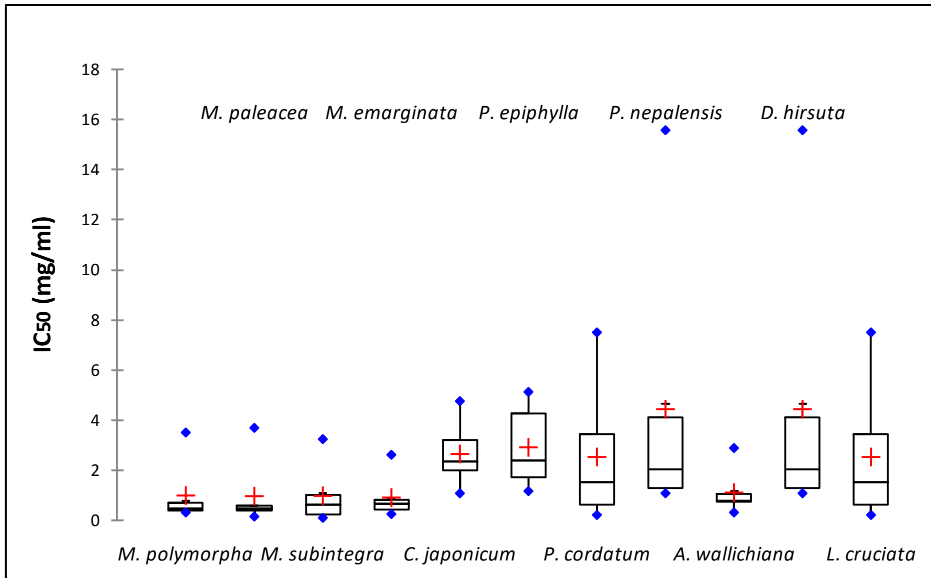


Fig 5.1.3: Super oxide scavenging activity of studied eleven liverworts. For each sample, mean, standard deviation, maximum and minimum values, outliers are shown.

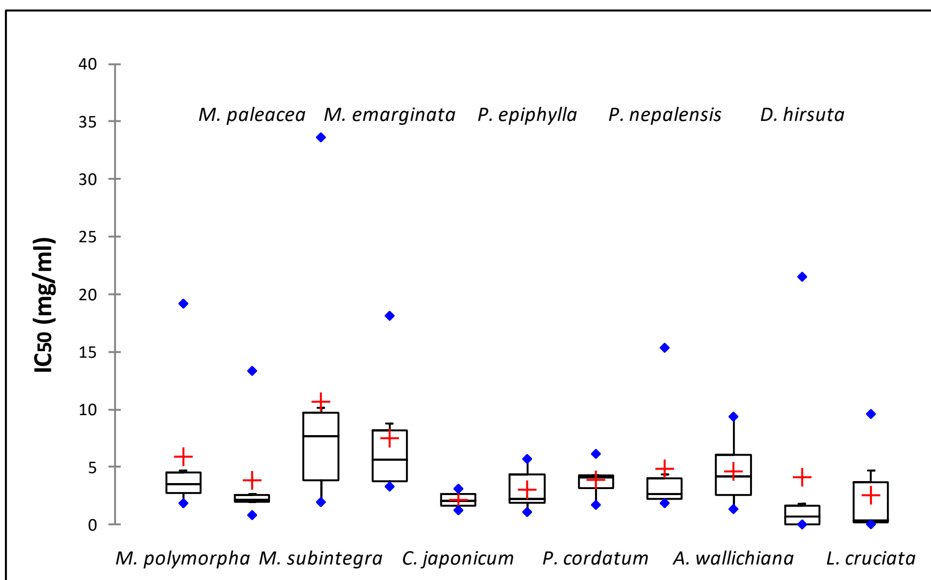


Fig 5.1.4: Nitric oxide scavenging activity of studied eleven liverworts. For each sample, mean, standard deviation, maximum and minimum values, outliers are shown.

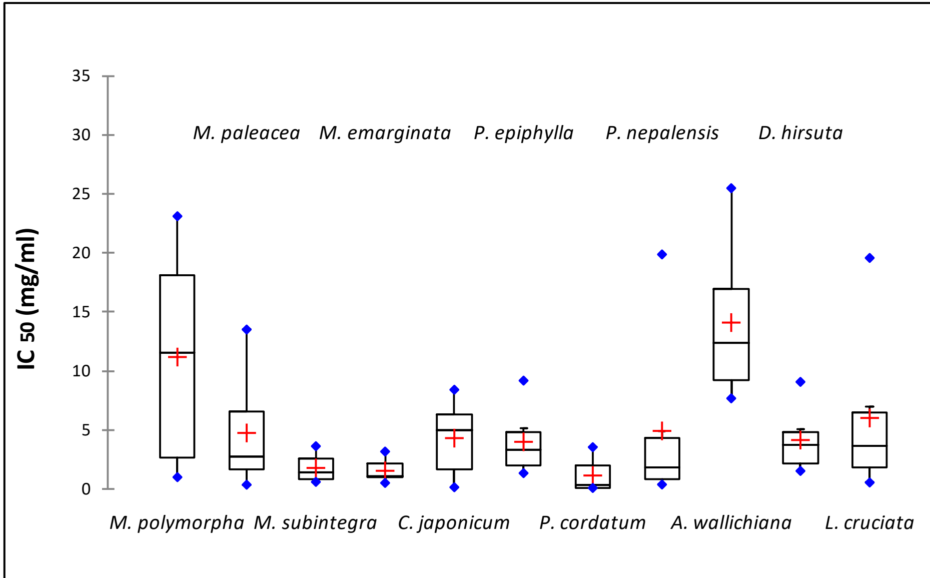


Fig 5.1.5: Metal chelating activity of studied eleven liverworts. For each sample, mean, standard deviation, maximum and minimum values, outliers are shown.

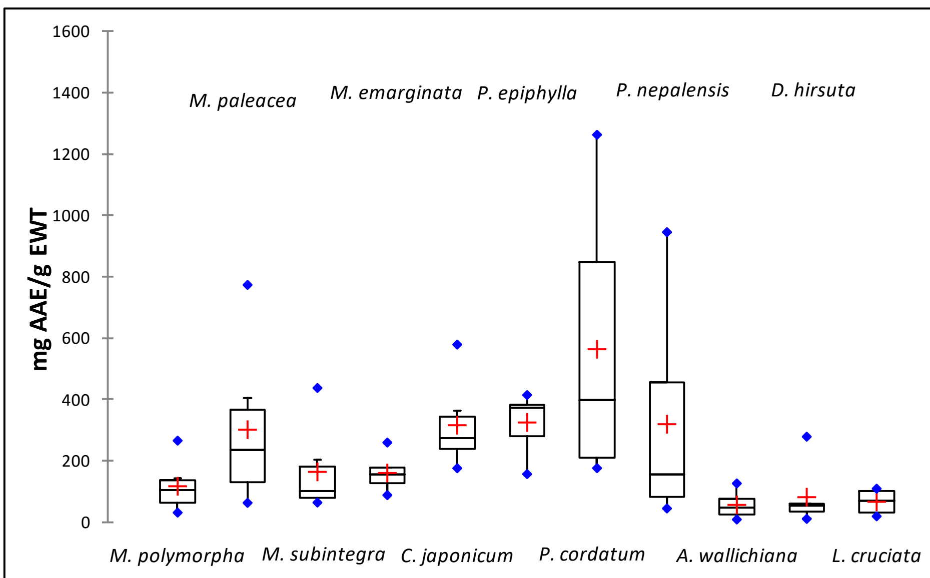


Fig 5.1.6: Reducing power of studied eleven liverworts. For each sample, mean, standard deviation, maximum and minimum values, outliers are shown.

Table 5.1.1: $DPPH^{\cdot}$ scavenging activity of studied liverworts (IC_{50} mg/ml)

Solvents used	***M. polymorpha***	***M. paleacea***	***M. subintegra***	***M. emarginata***	***C. japonicum***	***P. epiphylla***	***P. cordatum***	***P. nepalensis***	***A. wallichiana***	***D. hirsuta***	***L. cruciata***
Heptane	5.38±0.19	34.7±2.08	4.29±0.44	9.35±0.23	17.3±0.32	14.8±0.45	0.87±0.01	0.66±0.01	7.20±0.06	1.60±0.1	2.89±0.23
Diethyl ether	1.13±1.45	0.27±0.01	2.74±0.23	1.24±0.04	2.04±1.52	3.37±0.38	0.10±0.01	0.19±0.03	3.37±0.16	0.90±0.12	1.22±0.09
Ethyl acetate	0.76±0.67	0.62±0.37	1.58±0.04	0.84±0.07	4.30±0.58	1.27±0.07	0.18±0.02	0.27±0.01	3.10±0.36	3.78±0.59	0.84±0.38
acetone	1.62±0.21	1.43±0.07	9.77±0.32	3.98±0.13	9.19±0.88	4.55±0.11	0.06±0.05	0.41±0.13	11.5±0.67	4.41±0.38	3.25±0.36
Butanol	2.63±0.46	0.67±0.03	1.74±0.01	2.63±0.01	4.91±0.05	3.99±0.13	0.26±0.10	0.50±0.03	9.82±0.17	0.51±0.02	3.37±0.16
Methanol	14.73±3.66	1.11±0.05	4.92±0.44	2.82±0.10	13.6±0.7	4.29±0.59	4.34±0.10	1.18±0.11	7.16±0.25	5.96±1.15	3.70±0.58

Table 5. 1.2: $ABTS^{+}$ scavenging activity of studied liverworts (IC_{50} mg/ml)

Solvents used	***M. polymorpha***	***M. paleacea***	***M. subintegra***	***M. emarginata***	***C. japonicum***	***P. epiphylla***	***P. cordatum***	***P. nepalensis***	***A. wallichiana***	***D. hirsuta***	***L. cruciata***
Heptane	0.20±0.04	0.64±0.4	1.41±0.31	10.19±0.25	1.70±0.21	1.41±0.02	0.04±0.01	0.72±0.29	2.18± 0.04	1.40±0.10	0.70±0.08
Diethyl ether	0.57±0.10	0.06±0.01	0.37±0.14	0.69±0.02	0.26±0.03	0.19±0.01	0.02±0.01	0.08±0.02	0.19± 0.03	0.58±0.13	0.37±0.02
Ethyl acetate	0.07±0.04	0.16±0.01	0.59±0.07	0.57±0.03	2.12±0.39	1.61±0.26	0.03±0.01	0.96±0.27	0.09± 0.05	2.44±0.25	0.09±0.03
acetone	0.13±0.02	0.24±0.04	4.2±0.22	3.36±0.26	1.12±0.04	0.50±0.1	0.04±0.02	2.60±0.20	0.72± 0.01	2.61±0.29	0.71±0.01
Butanol	0.01±0.02	0.08±0.01	0.80±0.41	2.72±0.02	1.20±0.01	1.52±0.44	0.02±0.01	0.62±0.38	0.86± 0.04	0.29±0.09	0.24±0.1
Methanol	0.62±0.43	0.10±0.01	1.34±0.77	2.06±1.08	2.08±0.07	0.18±0.03	0.29±0.10	0.62±0.34	0.85± 0.03	2.90±0.21	0.62±0.16

Table5.1.3: Super oxide scavenging activity of studied liverworts (IC_{50} mg/ml)

Solvents used	*M. polymorpha*	*M. paleacea*	*M. subintegra*	*M. emarginata*	*C. japonicum*	*P. epiphylla*	*P. cordatum*	*P. nepalensis*	*A. wallichiana*	*D. hirsuta*	*L. cruciata*
Heptane	0.46±0.16	3.7±0.28	1.12±0.23	0.88±0.09	4.76±0.76	2.55±0.33	7.51±0.05	1.64±0.07	0.72±0.28	1.64±0.07	7.51±0.05
Diethyl ether	0.80±0.18	0.16±0.01	0.11±0.03	0.26±0.17	1.09±0.59	1.18±0.98	0.62±0.04	1.2±0.05	0.82±0.32	1.20±0.05	0.62±0.04
Ethyl acetate	0.32±0.03	0.39±0.26	0.15±0.07	0.38±0.17	1.91±1.09	1.57±0.92	0.64±0.09	2.47±0.02	0.32±0.07	2.47±0.02	0.64±0.09
acetone	0.51±0.33	0.61±0.05	0.81±0.12	0.71±0.09	2.26±0.31	2.24±0.55	0.22±0.06	4.67±0.07	1.16±0.40	4.67±0.07	0.22±0.06
Butanol	0.39±0.13	0.52±0.03	0.48±0.12	0.66±0.03	2.46±0.36	5.13±1.08	2.42±0.06	1.09±0.01	0.78±0.20	1.09±0.01	2.42±0.16
Methanol	3.51±0.17	0.47±0.01	3.25±0.53	2.63±0.33	3.46±0.7	4.86±0.91	3.82±0.28	15.5±0.12	2.90±0.66	15.6±0.12	3.82±0.28

Table 5.1.4: Nitric oxide scavenging activity of studied liverworts (IC_{50} mg/ml)

Solvents used	*M. polymorpha*	*M. paleacea*	*M. subintegra*	*M. emarginata*	*C. japonicum*	*P. epiphylla*	*P. cordatum*	*P. nepalensis*	*A. wallichiana*	*D. hirsuta*	*L. cruciata*
Heptane	1.85±0.6	13.35±1.4	8.62±2.33	6.42±0.3	2.08±0.69	1.89±0.46	3.97±0.73	1.86±0.64	6.09±2.7	0.02±0.01	0.03±0.02
Diethyl ether	4.73±1.6	0.82±0.3	1.95±3.52	4.92±0.1	1.25±0.42	1.09±075	2.87±0.72	2.21±0.21	1.34±0.4	0.03±0.02	0.15±0.69
Ethyl acetate	3.93±0.9	2.68±0.1	2.90±1.78	3.30±0.1	1.48±0.78	1.89±0.19	6.14±0.91	4.31±0.24	2.57±0.9	0.16±0.05	0.37±0.86
acetone	2.65±0.4	2.31±0.8	6.78±2.02	3.43±1.5	2.06±0.45	2.59±0.76	1.70±0.47	2.99±0.44	2.53±0.6	1.78±0.84	0.41±0.09
Butanol	3.03±0.2	1.98±0.6	10.1±0.42	8.79±0.3	3.11±0.16	5.7±0.95	4.31±0.50	2.33±0.12	5.80±1.8	1.18±0.19	9.6±083
Methanol	19.2±1.5	1.93±0.1	33.6±0.16	18.1±0.04	2.88±0.80	5±1.11	4.30±0.38	15.4±1.56	9.37±1.6	21.5±1.42	4.72±0.12

Table 5.1.5: Metal Chelating activity of studied eleven liverworts (IC_{50} mg/ml)

Solvents used	*M. Polymorpha*	*M. paleacea*	*M. subintegra*	*M. emarginata*	*C. japonicum*	*P. epiphylla*	*P. cordatum*	*P. nepalensis*	*A. wallichiana*	*D. hirsuta*	*L. cruciata*
Heptane	1.3±0.06	0.37±0.15	0.61±0.26	0.96±0.1	5.57±1.32	1.35±0.32	0.09±0.02	4.94±0.29	8.42±0.38	1.4±0.02	0.7±0.08
Diethyl ether	23.1±0.82	13.5±0.03	0.94±0.27	1.07±0.06	4.42±0.25	1.7±0.13	0.10±0.02	0.74±0.38	18.2±0.18	0.58±0.03	0.37±0.02
Ethyl acetate	18.6±0.64	2.95±0.44	0.78±0.01	0.52±0.04	8.41±0.11	2.85±0.36	0.16±0.09	1.09±0.45	11.7±0.52	2.44±0.16	0.18±0.04
acetone	16.5±0.65	7.74±0.11	2.83±0.43	2.48±0.06	0.15±0.31	9.19±0.53	0.44±0.01	0.38±0.37	25.5±0.55	2.61±0.18	0.71±0.01
Butanol	1.01±0.01	2.61±0.21	1.95±0.05	1.09±0.04	6.55±0.29	3.74±0.47	2.57±0.13	2.52±0.13	13.2±0.44	0.29±0.1	0.24±0.10
Methanol	6.59±1.3	1.34±0.24	3.63±1.16	3.18±0.30	0.79±0.43	5.18±0.64	3.55±0.43	19.8±0.45	7.68±0.03	2.9±0.02	0.62±0.16

Table 5.1.6: Reducing potential of studied eleven liverworts (mg ascorbic acid equivalent/ g extractive weight)

Solvents used	*M. polymorpha*	*M. paleacea*	*M. subintegra*	*M. emarginata*	*C. japonicum*	*P. epiphylla*	*P. cordatum*	*P. nepalensis*	*A. wallichiana*	*D. hirsuta*	*L. cruciata*
Heptane	98.9±17.1	63.2±17.2	92.3±3.03	175±30	176±18.6	383±22.7	215±0.08	169±0.87	54.2±15.9	61.3±9.2	88.9±14.5
Diethyl ether	144±16.5	773±8.3	437±93.1	134±29.9	578±65.6	414±20.5	1261±0.54	944±30.8	85.5±15.3	278±19.8	104±5.7
Ethyl acetate	265±23.3	242±27.1	203±7.13	259±10.3	233±27.7	251±40.1	210±0.07	550±47.1	126±25.3	55.6±14.5	109±16.4
acetone	113±0.48	226±7.45	64±15.8	125±15.7	363±15.7	382±33.4	936±0.02	44.9±15.6	42.5±21.0	28.9±10.1	53.5±5.8
Butanol	50.5±0.1	406±16.7	109±14.1	178±11.1	258±2.56	156±7.11	581±0.04	142±3.67	8.97±4.79	54.6±0.08	19.4±4.13
Methanol	31.6±7.9	98.6±7.57	77.4±10.3	88.4±7.5	287±21.4	360±39.3	176±0.07	64.9±12.5	21.7±16.2	10.9±0.82	24.8±3.13

5.2 ANTI-DIABETIC ACTIVITY

The α-glucosidase and α-amylase enzymes inhibitory activity was investigated and the results were presented in Figure 5.2.1 to 5.2.11. Studied liverworts inhibited the activity of α-glucosidase enzyme significantly; their IC_{50} value ranged between 0.09 mg/ml and 64.82 mg/ml and α- amylase inhibitory activity ranged between 0.058 mg/ml to 30.07 mg/ml (IC_{50} value). Diethyl ether extract of *Marchantia subintegra* showed the highest α-glucosidase enzyme inhibitory activity (IC_{50} value 0.09 mg/ml), the activity was almost similar to and α-amylase inhibitory activity of standard Metformin (0.17 mg/ml). Similarly, diethyl ether extract of *Marchantia subintegra* also showed highest α-amylase enzyme inhibitory activity (IC_{50} value 0.058 mg/mg). Variations in the α- amylase and α- glucosidase inhibitory activity was seen within different solvent extracts of a same species. Among six solvents used for extraction, diethyl ether and ethyl acetate extracts of *Marchantia polymorpha*, *M. paleacea*, *M. subintegra*, *M. Subintegra, Pellia epiphylla*, *Plagiochasma cordatum*, *Asteralla wallichiana* and *Dumortiera hirsuta* showed the highest α. -glucosidase and α- amylase inhibitory activities. In *Conocephalum japonicum* and *Lunularia cruciata*, acetone extract and in *Plagiochila nepalensis* heptane extract showed the highest α-glucosidase and α-amylase inhibitory activity.

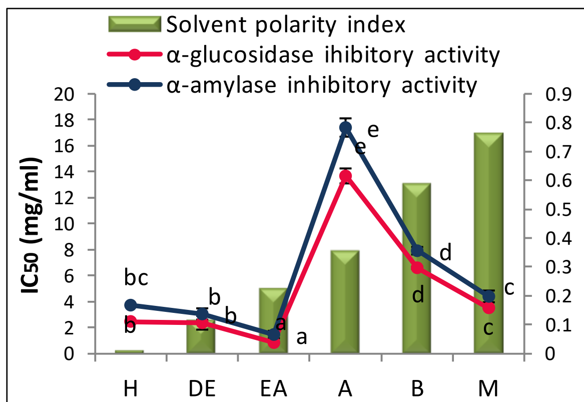


Fig 5.2.1: Anti-diabetic activity of *Asterella wallichiana*

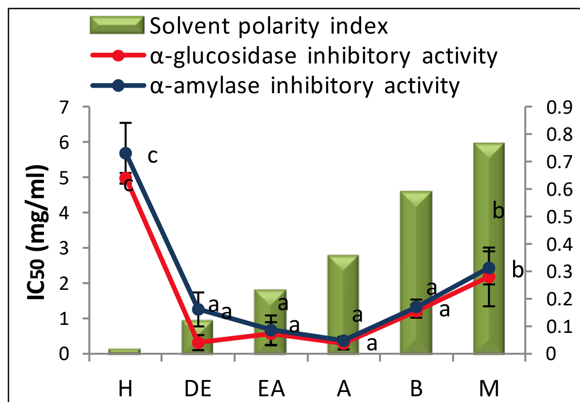


Fig 5.2.2: Anti-diabetic activity of *Conocephalum japonicum*

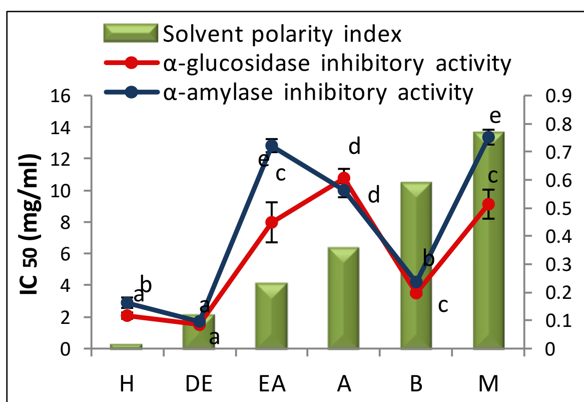


Fig 5.2.3: Anti-diabetic activity of *Dumortiera hirsuta*

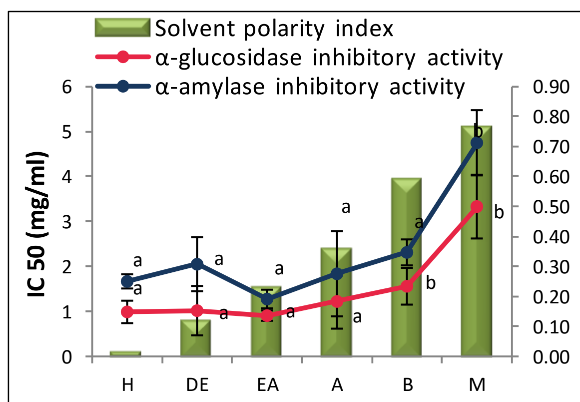


Fig 5.2.4: Anti-diabetic activity of *Pellia epiphylla*

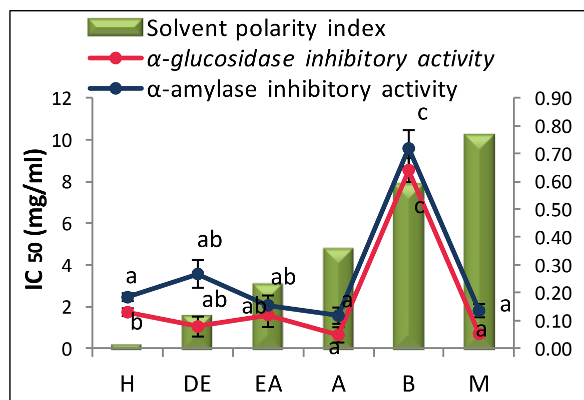


Fig 5.2.5: Anti-diabetic activity of *Lunularia cruciata*

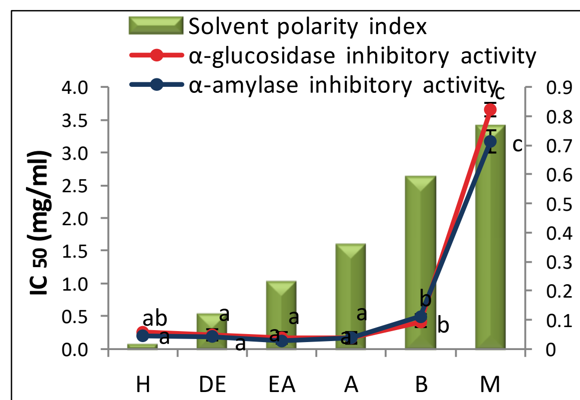


Fig 5.2.6: Anti-diabetic activity of *Marchantia emarginata*

NB: H= heptane, DE=diethyl ether, EA= ethyl acetate, A= acetone, B=butanol, M=methanol

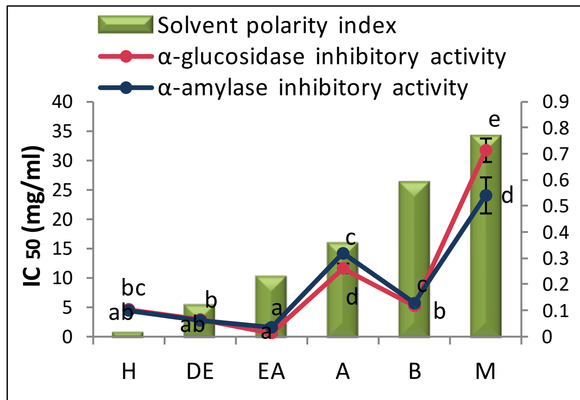


Fig 5.2.7: Anti-diabetic activity of *Marchantia polymorpha*

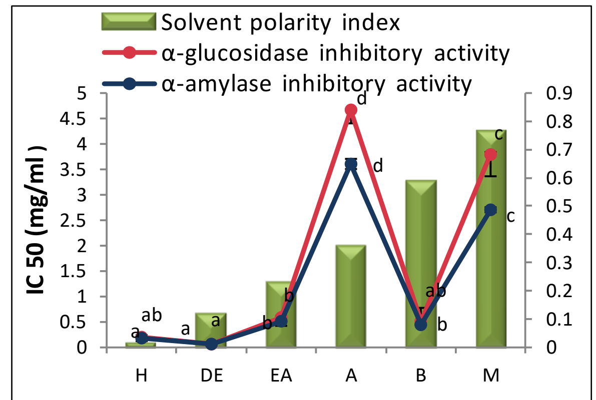


Fig 5.2.8: Anti-diabetic activity of *Marchantia subintegra*

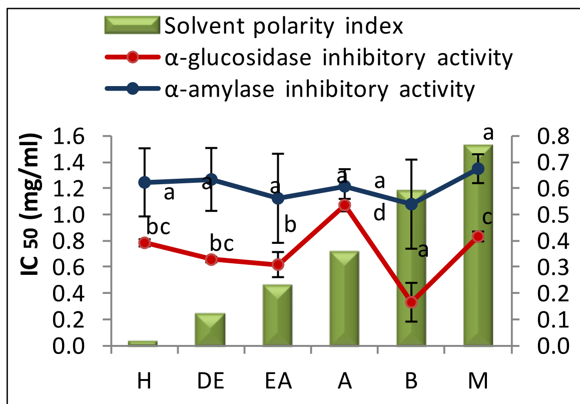


Fig 5.2.9: Anti-diabetic activity of *Plagiochasma cordatum*

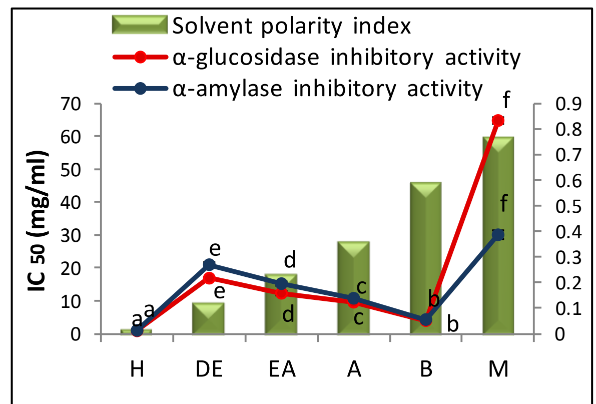


Fig 5.2.10: Anti-diabetic activity of *Plagiochila nepalensis*

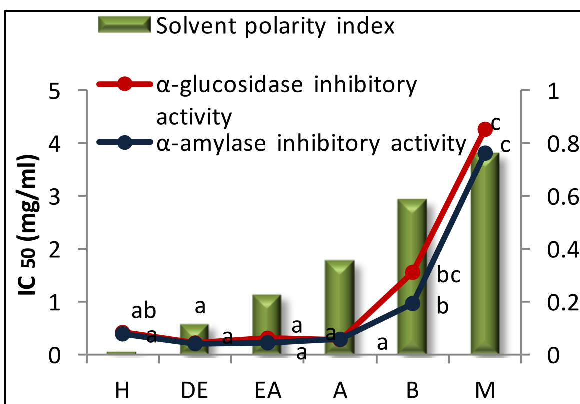


Fig 5.2.11: Anti-diabetic activity of *Marchantia paleacea*

NB: H= heptane, DE=diethyl ether, EA= ethyl acetate, A= acetone, B=butanol, M=methanol

5.3 *IN VITRO* CYTOTOXICITY SCREENING

In this study, we screened the extracts of five species of liverworts: *Plagiochasma cordatum, Asterella wallichiana*, *Lunularia cruciata Marchantia paleacea* and *Plagiochila nepalensis* against human kidney cancer cell line (ACHN). Perusal of literature revealed that liverwort under study has not been included previously in a bio prospecting program for anti-cancer activity. The cytotoxic effect of extracts on human kidney cancer cell line was studied by MTT assay. The effect of plant extract on viability of cell was studied by calculating the percentage of MTT reduction on incubation of ACHN cells with increasing concentration of extract. Extracts caused a dose dependent reduction in cell viability. Extract concentration range 100 µg/ ml to 500 µg/ ml were used. The calculation of cell viability percentage showed that in present work *Plagiochasma cordatum* had the highest potential to induce maximum cell death at a minimum concentration of 100 µg/ ml. Dose response studies of studied liverwort extracts are presented in figures 5.3.1 to 5.3.5. Studied liverworts showed up to 73 % cell death of human kidney cancer cell line. The IC_{50} value of anti-proliferative activity of liverwort under analysis ranged between 69.15 µg/ml to 308.98 µg/ml. Atjanasuppat *et al*. (2009) categorized the anti-proliferative activities of the extracts into four groups: ≤ 20 µg/mL, active; >20–100 µg/mL, moderately active; >100–1000 µg/mL, weakly active; and >1000 µg/mL, inactive. Based on the report, IC_{50} values of the studied liverworts falls under range of extracts having anti-proliferative activity. The lowest IC_{50} value was shown by *Plagiochasma cordatum* which indicated that this liverwort is most active in inducing death of human kidney cancer cell line than other studied liverworts (Fig 5.3.6).

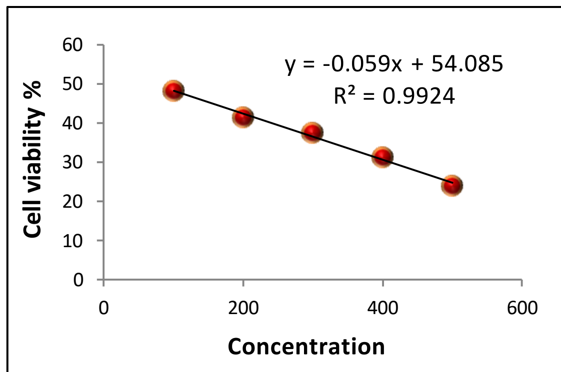


Fig 5.3.1: Inhibitory activity of *Plagiochasma cordatum* on ACHN cell proliferation.

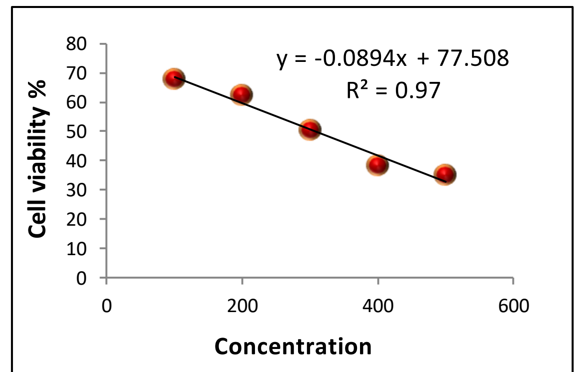


Fig 5.3.2: Inhibitory activity of *Asterella wallichiana* on ACHN cell proliferation.

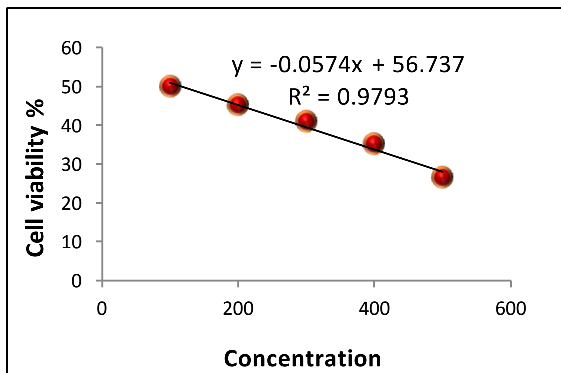


Fig 5.3.3: Inhibitory activity of *Plagiochila nepalensis* on ACHN cell proliferation.

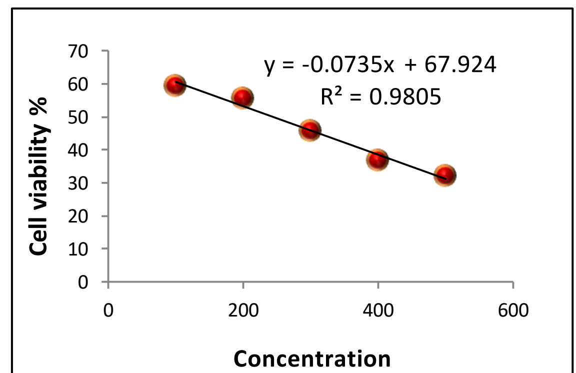


Fig 5.3.4: Inhibitory activity of *Lunularia cruciata* on ACHN cell proliferation.

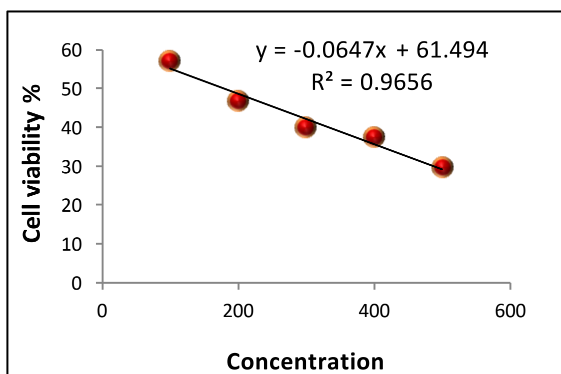


Fig 5.3.5: Inhibitory activity of *Marchantia paleacea* on ACHN cell proliferation.

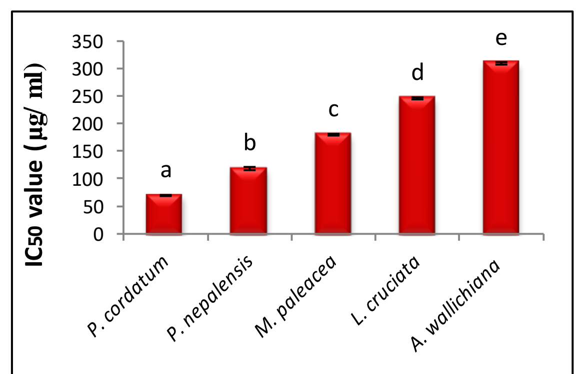


Fig 5.3.6: IC_{50} values of anti-proliferative activity studied liverworts against ACHN cells. Value with different letters (a, b, c, d etc.) differ significantly ($p < 0.05$) from each other by Duncan's Multiple Test (DMRT)

5.4 PHYTOCHEMICAL ANALYSIS

5.4.1 Quantitative estimation of phytochemicals

Quantitative estimation of phenols, flavonoids, orthodihydric phenol, steroid and tannin content was done. Studied liverworts showed large variation in the content of phenol which ranged between 4.8 mg Gallic acid equivalent / g extractive weight to 377.9 mg GAE/g EW (Fig 5.4.1). Highest phenol content was recorded in diethyl ether extract of *Marchantia emarginata* and lowest content of phenol was recorded in methanolic extract of *Dumortiera hirsuta*. Significant effects of solvent's polarity were observed on extraction of phenolic compounds. It was found that diethyl ether extract of all studied liverworts except *Marchantia polymorpha* and *Asterella wallichiana* contained the highest amount of phenols. Whereas in case of *Marchantia polymorpha* butanolic extract and in *Asterella wallichiana*, heptane extract showed highest phenol content. Flavonoid content of the studied plants ranged between 0.3 mg Quercetin equivalent/ g extractive weight and 94.2 mg Quercetin equivalent/ g extractive weight. *Plagiochasma cordatum* showed highest flavonoid content among studied liverworts (Fig 5.4.2).

Orthodihydric phenol content in the liverworts ranged between 0.34 mg catechol equivalent / g extractive weight and 47.20 mg catechol equivalent / g extractive weight (Fig 5.4.3). In *Dumortiera hirsuta* the presence of highest amount of orthdihydric phenol content was found. Presence of steroid was also detected in the studied liverworts which ranged between 0.002 mg solasodine equivalent/ g extractive weight and 1.64 mg solasodine equivalent/ g extractive weight (Fig 5.4.4). Tannin content of the plant ranged between 0.02 mg Tannic acid equivalent / g extractive weight and 2.56 mg Tannic acid equivalent / g extractive weight. Highest tannin content was recorded in methanolic extract of *Asterella wallichiana* (Fig 5.4.5).

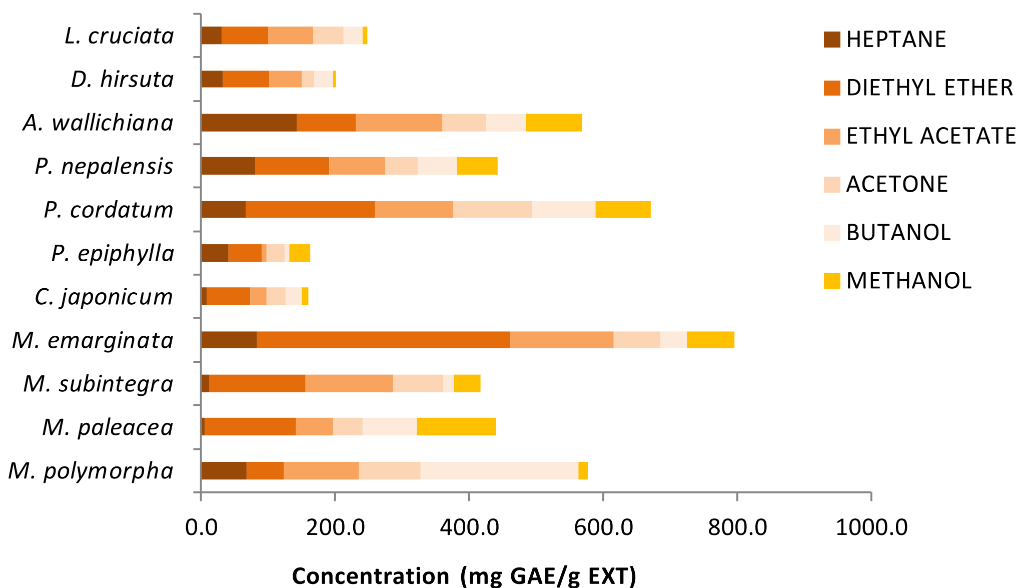


Fig 5.4.1: Phenol content of studied eleven liverwort species in different solvent extracts

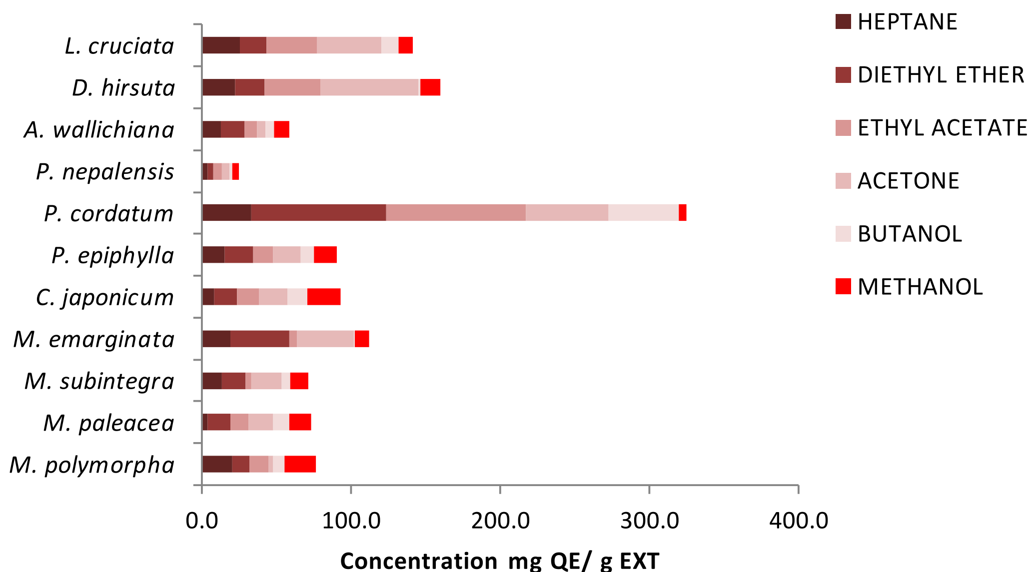


Fig 5.4.2: Flavonoid content of studied eleven liverwort species in different solvent extracts

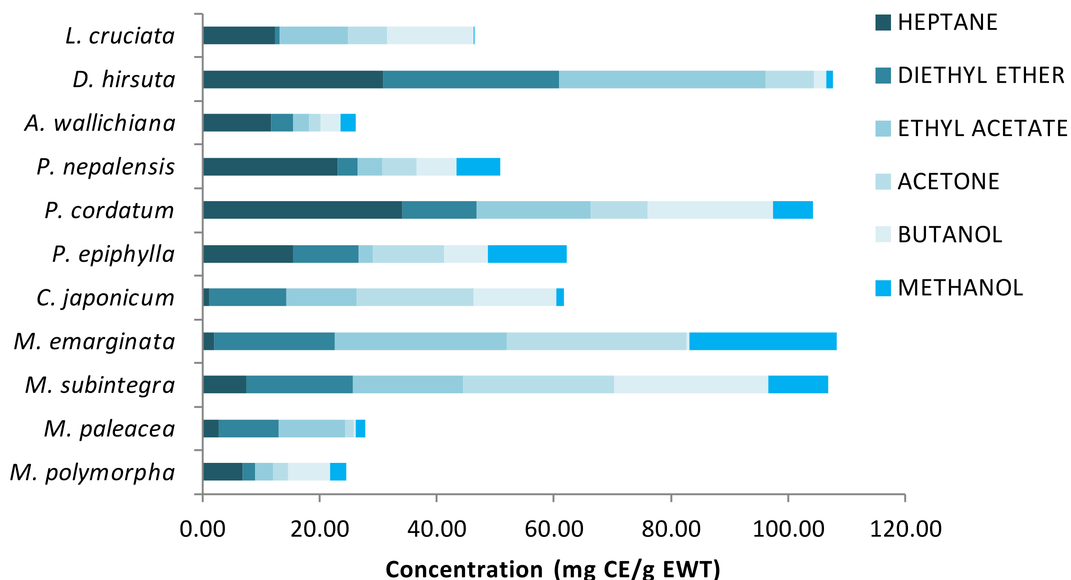


Fig 5.4.3: Ortho-dihydric phenol content of studied eleven liverwort species in different solvent extracts

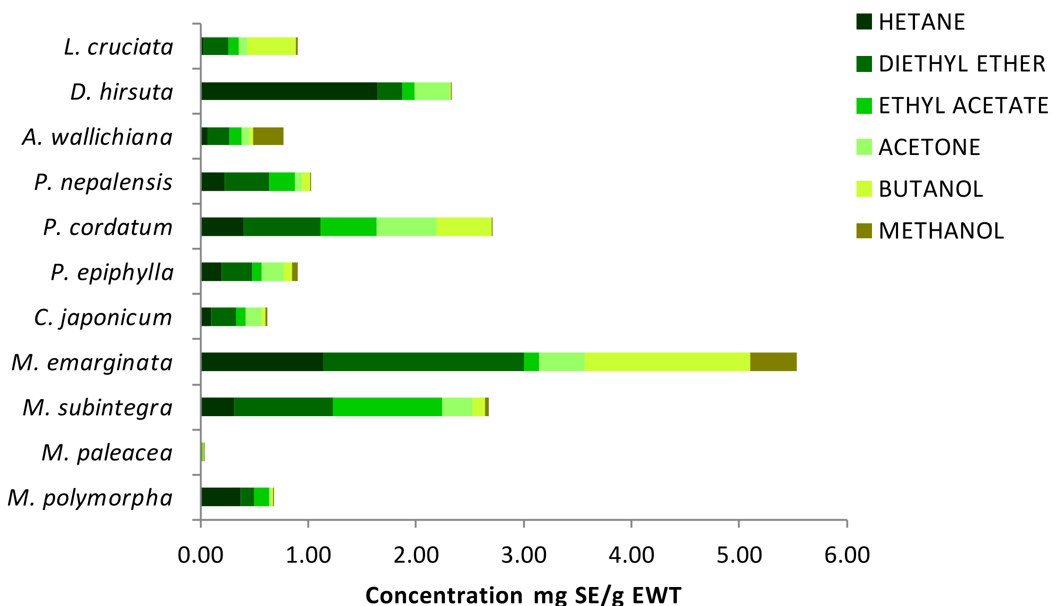


Fig 5.4.4: Steroid content of studied eleven liverwort species in different solvent extracts

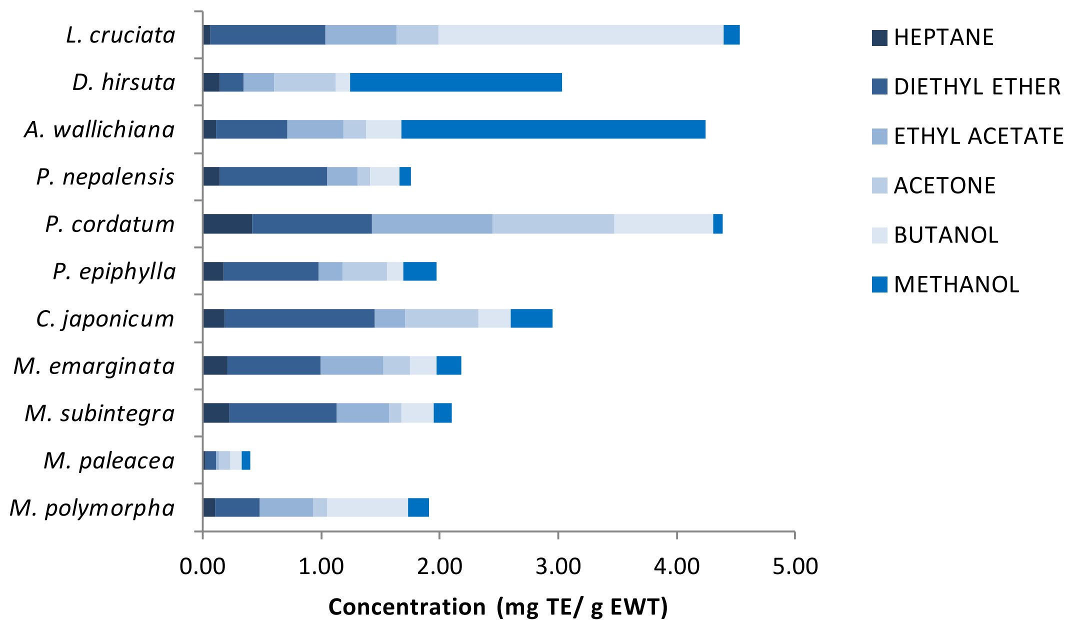


Fig 5.4.5: Tannin content of studied eleven liverwort species in different solvent extracts

5.4.2 Qualitative phytochemical analysis

Presence of phytochemicals like steroid (ST), tannin (TAN), triterpenoid (TT), amino acid (AA), resin (RES), cardiac glycoside (CG), alkaloid (ALK), flavonoid (FLA), reducing sugar (RS), anthraglycoside (ANT) and glycoside (GLY) were detected by qualitative phytochemical analysis (Table 5.2.1 -5.2.11).

5.4.3 Thin layer Chromatography (TLC)

Thin layer chromatography analysis has shown the presence of phytochemicals like steroid, tannin, triterpenoids, cardiac glycoside, flavonoids, resin, reducing sugar, amino acid, glycoside, anthraglycoside, arbutin, phenol, bitter principle and coumarin in studied liverworts.

5.5 CORRELATION BETWEEN PHYTOCHEMICAL CONTENT AND PHARMACOLOGICAL ACTIVITIES

5.5.1 Pearson Correlation Analysis

Pearson Correlation analysis was performed to evaluate how the pharmacological activities and phytochemical content of studied liverworts were correlated with each other (Fig 5.5.1). Result showed a negative correlation between total phenol content of the plant and the IC_{50} value of $DPPH^{\cdot}$ and superoxide (SO) scavenging assay. This indicated that the phenolic compounds that are present in the plants actively participated in the reduction of DPPH radical and SO ions. Reducing power (RP) of the plant was positively correlated with total phenol and flavonoids content. Ferric reducing activity is considered equivalent to free radical scavenging potential of plant. Positive correlation between ferric reducing ability and phenolic content suggests that phenolic compounds are main free radical scavengers that are produced by plants as secondary metabolites. In Pearson Correlation analysis, another important class of phenolic compound, the orthodihydric phenol was found to be negatively correlated with IC_{50} value of metal chelating activity and the α-amylase (α-A) inhibitory activity. Thus, it can be interpreted that orthodihydric phenol was main phytochemical that are responsible for metal chelating activity displayed by liverworts. Data also suggested that anti-diabetic activity of the plants was mainly contributed by the orthodihydric phenol present in the liverworts.

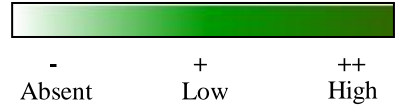


Table 5.2.1: Phytochemical profile of *Plagiochasma cordatum*

	ST	TAN	TT	AA	RES	CG	ALK	FLA	RS	ANT	GLY
H	+ +	+	+ +	-	-	+ +	+	-	+ +	-	+
DE	+ +	+	+ +	-	+	+ +	+	-	+ +	-	+
EA	+ +	+	+ +	-	-	+ +	+	+	+ +	-	+
A	+	+	-	-	-	+ +	+	-	+ +	-	+
B	+ +	+	+ +	-	+	+ +	+	+	+ +	-	+
M	+ +	+ +	+	-	+	-	-	-	-	-	+ +

Table 5.2.2: Phytochemical profile of *Plagiochila nepalensis*

	ST	TAN	TT	AA	RES	CG	ALK	FLA	RS	ANT	GLY
H	+ +	+	+	-	+	+	-	-	-	-	+
DE	+ +	+	+	-	+	+	-	-	+	-	+
EA	+ +	+	-	-	-	+ +	-	-	+	-	+
A	+ +	+	+	-	-	+	-	-	+	-	+
B	+	+	+	-	-	-	-	-	+	-	+
M	+ +	+ +	+	+	+	-	-	-	+	-	+ +

Table 5.2.3: Phytochemical profile of *Marchantia polymorpha*

	ST	TAN	TT	AA	RES	CG	ALK	FLA	RS	ANT	GLY
H	+ +	+	+	-	-	+ +	-	-	+	-	+
DE	+ +	+ +	+ +	-	-	+ +	-	-	v+	-	+
EA	+ +	+ +	+ +	-	+	+ +	+	+	+	-	+
A	+	+ +	-	-	+	-	-	-	+	-	+
B	+	+ +	+ +	-	-	-	-	-	+	-	+
M	+ +	+ +	+	-	-	-	-	-	+	-	+ +

NB: ST= steroid, Tan= tannin, TT= triterpenoid, AA=amino acid, RES=resine, CG= cardiac glycoside, ALK=alkaloid, FLA= flavonoid, RS= reducing sugar, ANT=anthraglycoside, GLY= glycoside

-	+	++
Absent	Low	High

Table 5.2.4: Phytochemical profile of *Asterella wallichiana*

	ST	TAN	TT	AA	RES	CG	ALK	FLA	RS	ANT	GLY
H	+ +	+	+ +	-	-	+ +	-	-	+	-	+
DE	+ +	+	+	-	+	+ +	-	-	+	-	+
EA	+ +	+	+ +	+	+	+ +	+	-	+	-	+
A	+ +	+	+	+	-	+	-	-	+	+	+
B	+	+	+	-	-	-	-	-	+	-	+
M	+ +	+ +	+ +		-	-	-	-	+	-	+ +

Table 5.2.5: Phytochemical profile of *Pellia epiphylla*

	ST	TAN	TT	AA	RES	CG	ALK	FLA	RS	ANT	GLY
H	+ +	+	+	-	-	+ +	-	-	-	-	+
DE	+	+	+	-	+	+ +	-	+	+ +	-	+
EA	+ +	+	+	-	-	+	-	+	+	-	+
A	+	+	+	-	+	+	-	+	+	-	+
B	+ +	+	-	-	+	-	-	-	+	-	+
M	+ +	+ +	+	-	+	+	-	+	+	-	+ +

Table 5.2.6: Phytochemical profile of *Marchantia subintegra*

	ST	TAN	TT	AA	RES	CG	ALK	FLA	RS	ANT	GLY
H	+	+	+	-	-	+	-	-	+ +	-	+
DE	+	+	+	-	+	+	-	+	+	-	+
EA	+	+	+	+	-	-	-	-	+	-	+
A	+	+	-	+	-	-	-	+	+	-	+
B	+	+ +	+	-	+	-	-	-	+	-	+
M	+	+ +	+	+	+	+	-	+	+ +	-	+ +

NB: ST= steroid, Tan= tannin, TT= triterpenoid, AA=amino acid, RES=resine, CG= cardiac glycoside, ALK=alkaloid, FLA= flavonoid, RS= reducing sugar, ANT=anthraglycoside, GLY= glycoside

- Absent | + Low | ++ High

Table 5.2.7: Phytochemical profile of *Dumortiera hirsuta*

	ST	TAN	TT	AA	RES	CG	ALK	FLA	RS	ANT	GLY
H	++	+	+	-	+	++	-	-	+	-	++
DE	++	+	+	-	++	++	-	+	+	-	++
EA	++	+	+	+	++	++	-	-	+	-	+
A	++	+	-	+	-	-	-	-	+	-	+
B	+	+	-	-	+	-	-	-	+	-	+
M	++	++	-	+	-	-	-	-	+	+	++

Table 5.2.8: Phytochemical profile of *Lunularia cruciata*

	ST	TAN	TT	AA	RES	CG	ALK	FLA	RS	ANT	GLY
H	+	+	-	-	-	+	-	-	+	-	+
DE	+	+	+	-	+	++	-	+	+	-	+
EA	++	++	++	+	+	++	+	+	+	-	+
A	++	+	+	-	-	++	-	+	+	-	+
B	+	+	-	-	+	++	+	-	+	-	+
M	++	++	-	-	-	-	-	-	+	-	++

Table 5.2.9: Phytochemical profile of *Conocephalum japonicum*

	ST	TAN	TT	AA	RES	CG	ALK	FLA	RS	ANT	GLY
H	++	+	+	-	-	++	-	-	-	-	+
DE	++	+	+	-	+	++	-	-	-	-	+
EA	++	+	+	-	+	++	-	-	-	-	+
A	+	+	-	-	-	-	-	-	-	-	+
B	+	+	-	-	-	-	-	-	-	-	+
M	+	+	-	-	-	-	-	-	-	-	++

NB: ST= steroid, Tan= tannin, TT= triterpenoid, AA=amino acid, RES=resine, CG= cardiac glycoside, ALK=alkaloid, FLA= flavonoid, RS= reducing sugar, ANT=anthraglycoside, GLY= glycoside

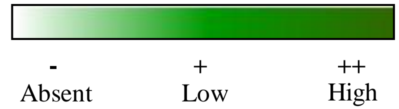


Table 5.2.10: Phytochemical profile of *Marchantia emarginata*

	ST	TAN	+TT	AA	RES	CG	ALK	FLA	RS	ANT	GLY
H	+	+	+	-	-	+	-	-	-	-	+
DE	+	+	+	-	-	+	-	+	-	-	+
EA	+ +	+	+	-	-	+	-	-	-	-	+
A	+	+	-	-	-	-	-	-	-	-	+
B	+	+	-	-	-	-	-	-	-	-	+
M	+	+ +	-	-	+	-	+	-	-	-	+ +

Table 5.2.11: Phytochemical profile of *Marchantia paleacea*

	ST	TAN	TT	AA	RES	CG	ALK	FLA	RS	ANT	GLY
H	-	+	+ +	+	-	+ +	-	-	+ +	-	+
DE	-	+	+ +	+	+	+ +	-	+	+ +	-	+ +
EA	+	+ +	+ +	+	+ +	+ +	-	+ +	+ +	-	+ +
A	+ +	+ +	+ +	+	+	+ +	-	+ +	+ +	-	-
B	+ +	+ +	+	+	+ +	+ +	-	+ +	+ +	-	+ +
M	+ +	+ +	+	+	+ +	+ +	-	+ +	+ +	-	+ +

NB: ST= steroid, Tan= tannin, TT= triterpenoid, AA=amino acid, RES=resine, CG= cardiac glycoside, ALK=alkaloid, FLA= flavonoid, RS= reducing sugar, ANT=anthraglycoside, GLY= glycoside

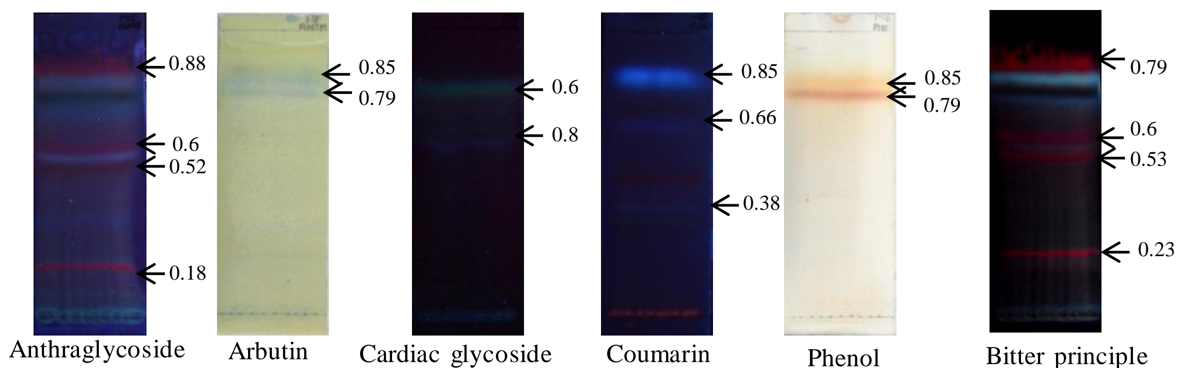


Fig 5.4.6.1: Detection of phytochemicals in *Marchantia emarginata* by TLC analysis

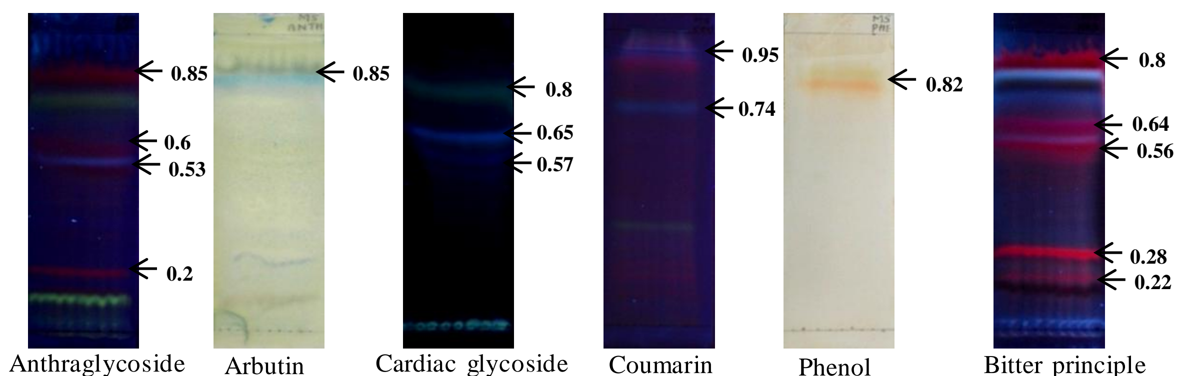


Fig 5.4.6.2 : Detection of phytochemicals in *Marchantia subintegra* by TLC analysis

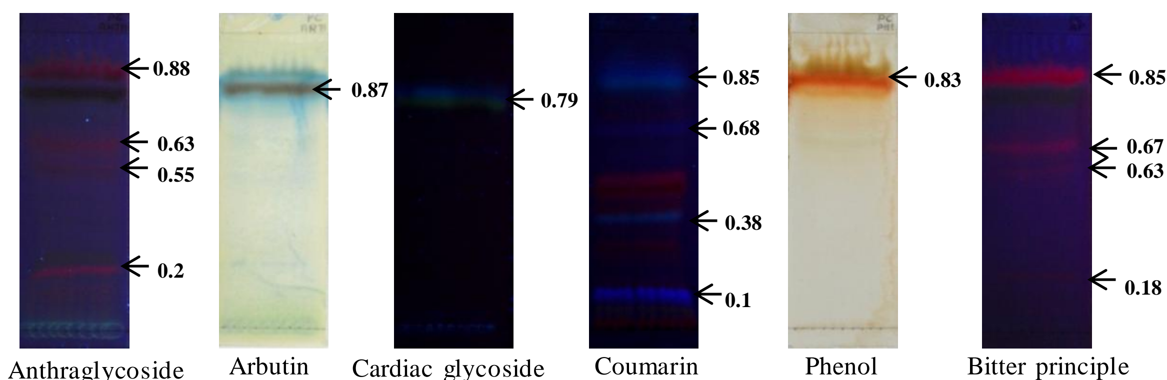
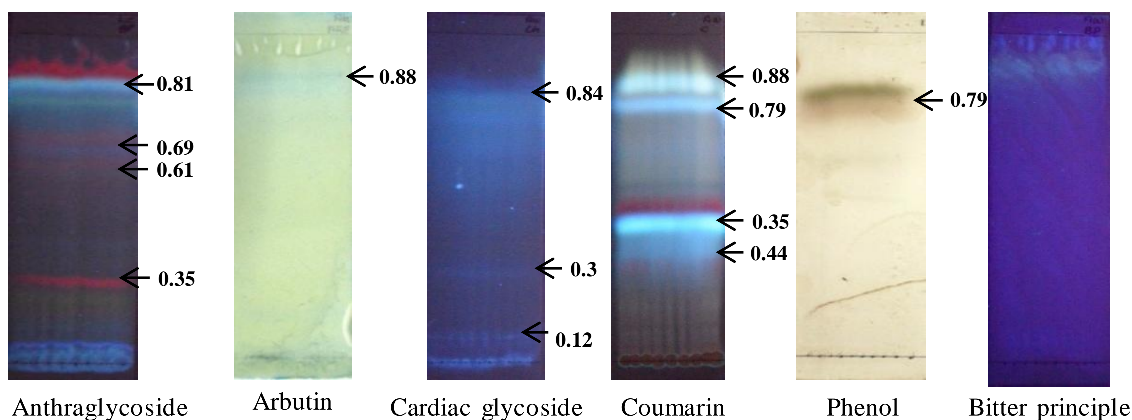
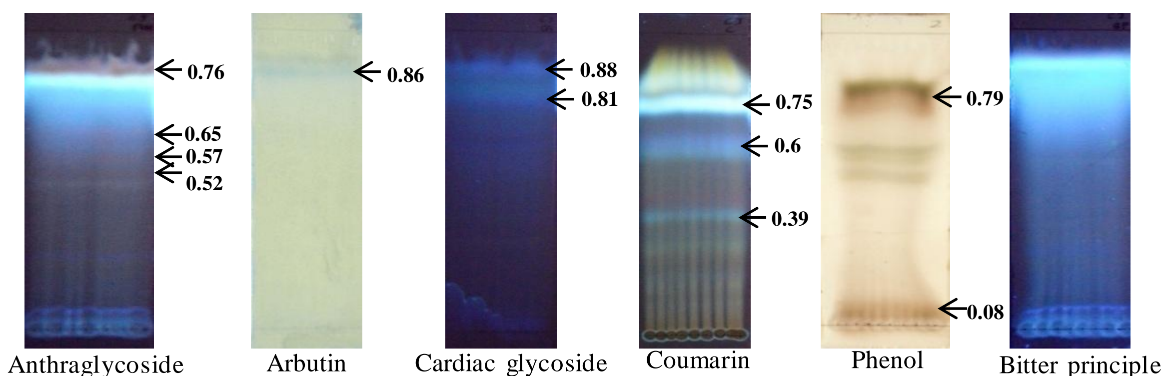


Fig 5.4.6.3 : Detection of phytochemicals in *Plagiochasma cordatum* by TLC analysis

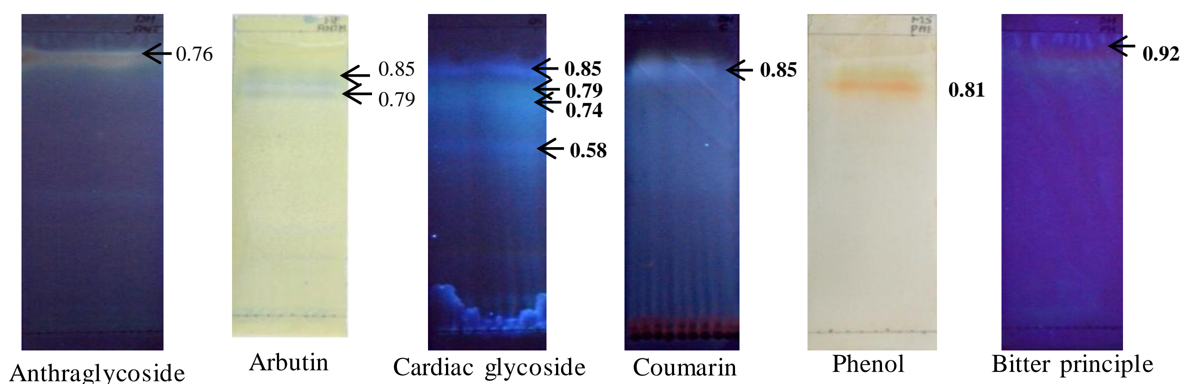
Phytochemical	*Identifying band colour*
Anthraglycoside	red band (UV-365)
Arbutin	blue band (Visible)
Cardiac glycoside	blue band(UV-365)
Coumarin	light blue band (UV-365)
Phenol	brown band (visible)
Bitter principle	red band (UV-365)



5.4.6.4 : Detection of phytochemicals in *Asterella wallichiana* by TLC analysis

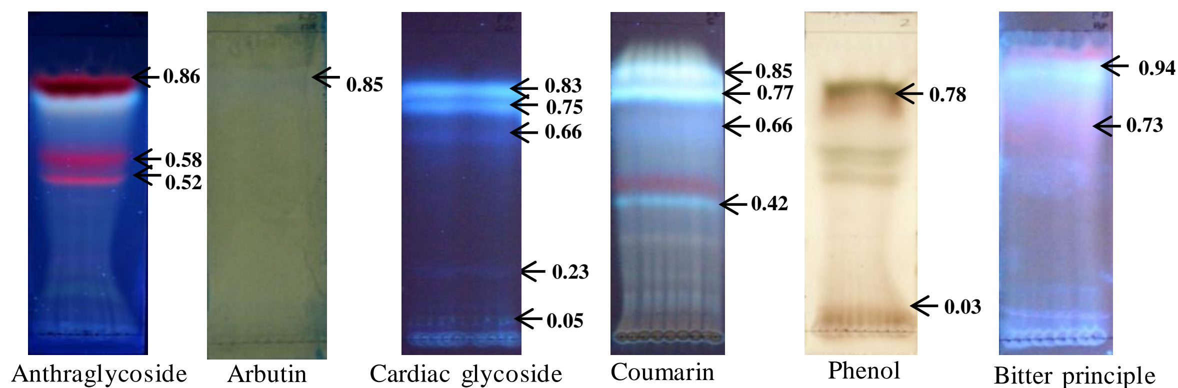


5.4.6.5 : Detection of phytochemicals in *Conocephalum japonicum* by TLC analysis

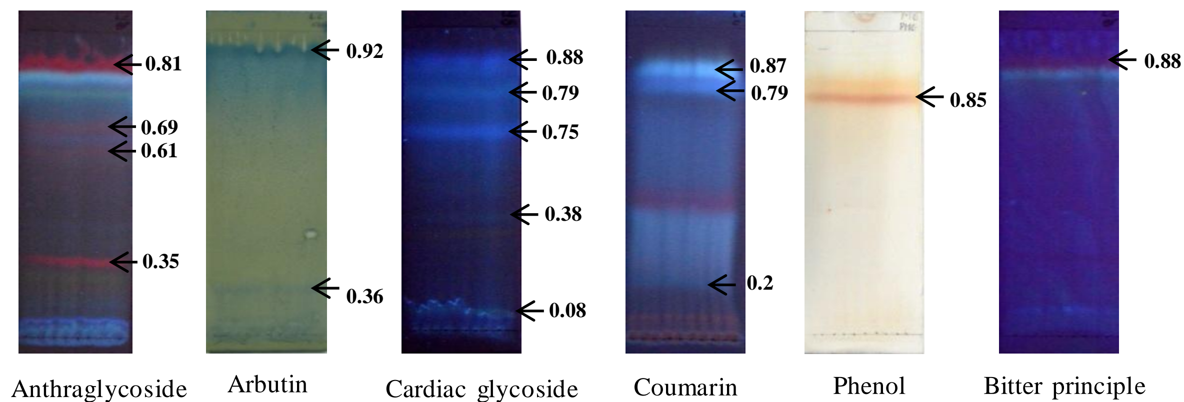


5.4.6.6 : Detection of phytochemicals in *Dumortiera hirsuta* by TLC analysis

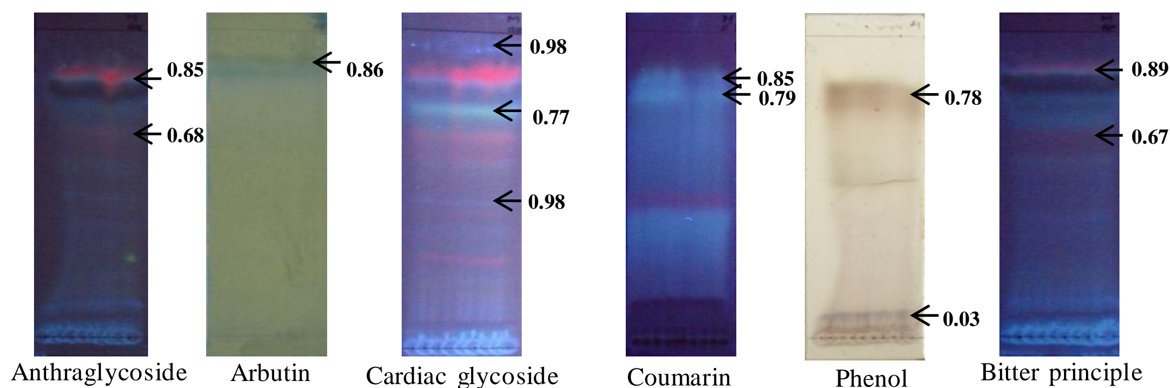
Phytochemical	*Identifying band colour*
Anthraglycoside	red band (UV-365)
Arbutin	blue band (Visible)
Cardiac glycoside	blue band(UV-365)
Coumarin	light blue band(UV-365)
Phenol	brown band(UV-365)
Bitter principle	red band(UV-365)



5.4.6.7: Detection of phytochemicals in *Pellia epiphylla* by TLC analysis

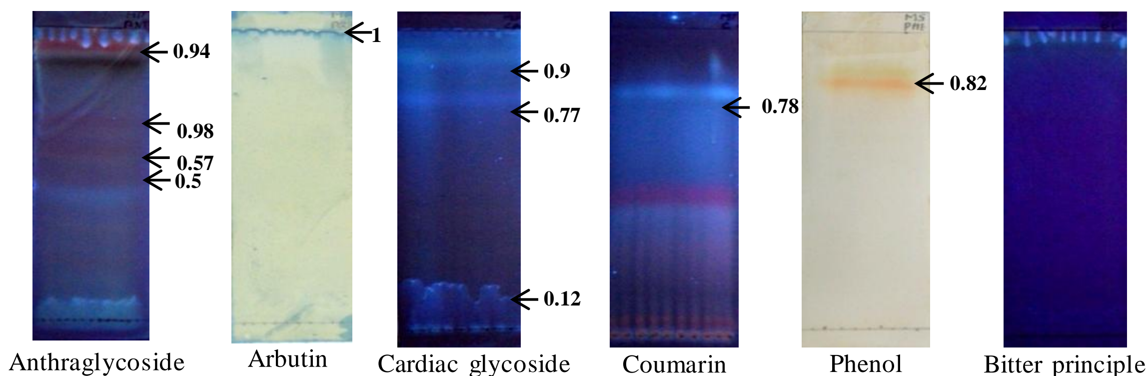


5.4.6.8: Detection of phytochemicals in *Lunularia cruciata* by TLC analysis

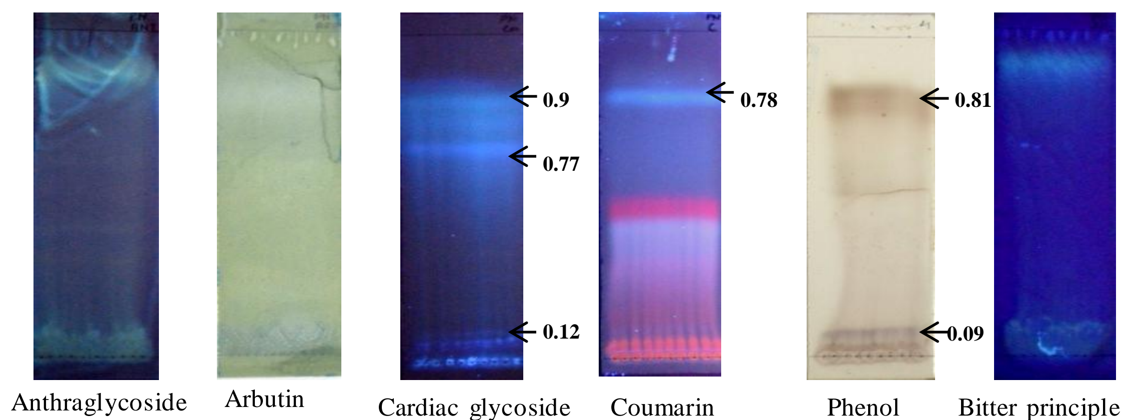


5.4.6.9: Detection of phytochemicals in *Marchantia polymorpha* by TLC analysis

Phytochemical	***Identifying band colour***
Anthraglycoside	red band (UV-365)
Arbutin	blue band (Visible)
Cardiac glycoside	blue band(UV-365)
Coumarin	light blue band(UV-365)
Phenol	brown band(UV-365)
Bitter principle	red band(UV-365)



5.4.6.10: Detection of phytochemicals in *Marchantia paleacea* by TLC analysis



5.4.6.11: Detection of phytochemicals in *Plagiochila nepalensis* by TLC analysis

Phytochemical	***Identifying band colour***
Anthraglycoside	red band (UV-365)
Arbutin	blue band (Visible)
Cardiac glycoside	blue band(UV-365)
Coumarin	light blue band(UV-365)
Phenol	brown band(UV-365)
Bitter principle	red band(UV-365)

Steroid content of the liverworts also found to be involved in metal chelating activity as suggested by the negative correlation between metal chelating activity and steroid content in Pearson Correlation analysis.

5.5.2 Principal Component Analysis

Multivariate analysis like principal component analysis helps to summarize the variability of complex set of data and translates it into most interpretable form. The PCA analysis of phytochemical content and pharmacological activities of the studied liverworts were carried out. The loading of the first principal component (PC1) accounted for 19.84% of the variance and second principal components (PC2) accounted for 13.59% of the variance (Fig 5.5.2). Analyzed parameters were grouped into three clusters (A, B and C). By the loadings of PC1, phytochemicals like phenol, flavonoids, alkaloid, cardiac glycoside were clustered together in group A. Ferric ion reducing activity was also clustered along with other phytochemicals in group A. By PC1, phytochemicals and reducing power (Group A) of the plants were separated from the antioxidant and anti-diabetic activities of plants (Group C). This indicated negative correlation between the phytochemicals of Group A and IC_{50} value of different antioxidant and anti-diabetic assays suggesting phytochemicals like phenols, flavonoids, alkaloid, cardiac glycoside present in the studied plants to be responsible for free radical scavenging and α- amylase, α- glucosidase enzyme inhibiting activities. Negative correlation between FRAP assay and biological activities suggested antioxidant and anti-diabetic activities increased with the increase of metal ion reducing potential of plant. On the basis of loading of PC2, phytochemicals such as tannin, resin and amino acid were included in group B and $ABTS^+$ scavenging activity in group D. PC2 separated the phytochemicals of group B from the IC_{50} value of $ABTS^+$ scavenging activity. This result suggested that tannin like compounds present in liverworts were involved in $ABTS^+$ scavenging activity.

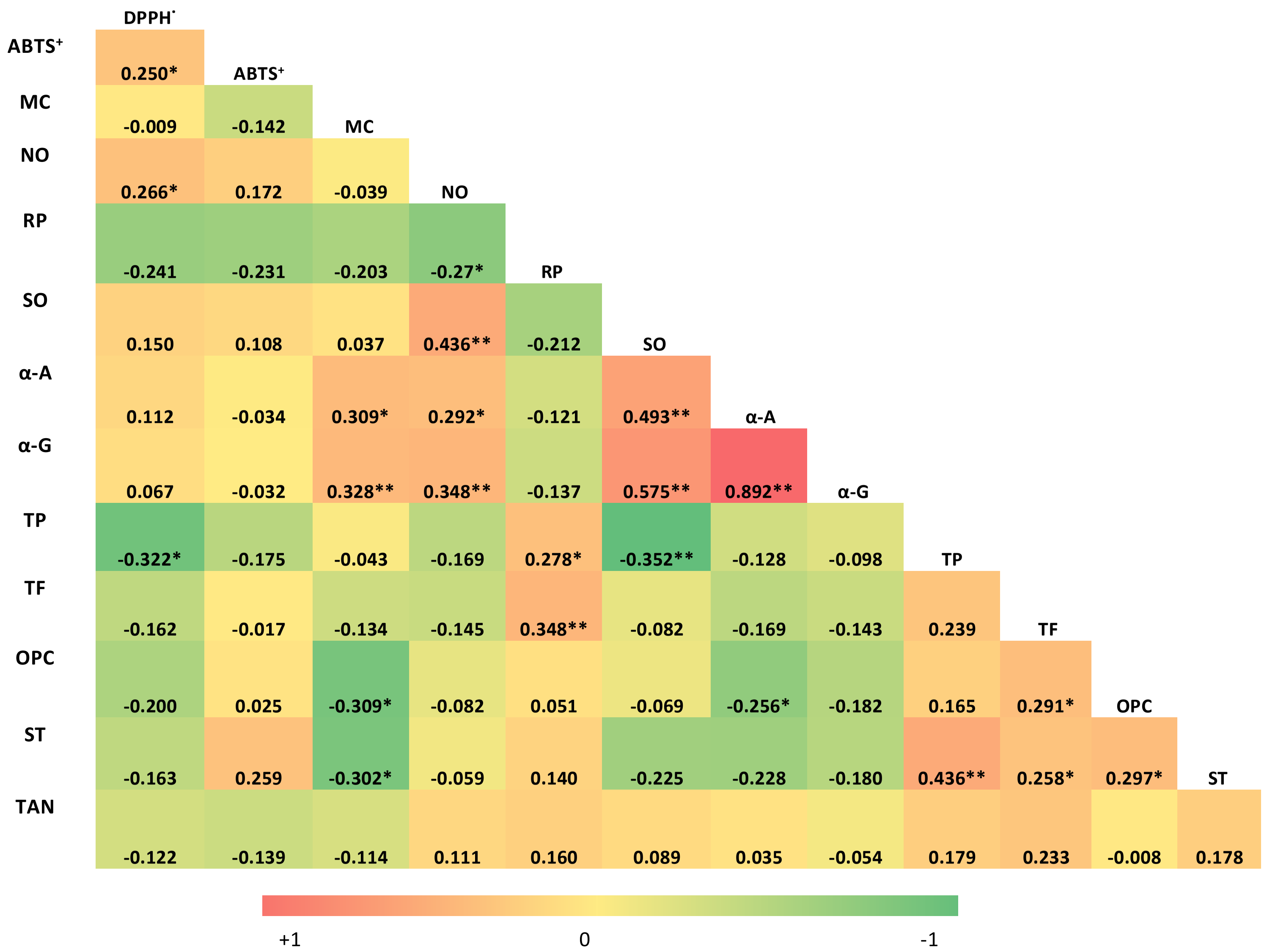


Fig 5.5.1: Correlation between the pharmacological activities and phytochemical content of the studied eleven liverwors. *. Correlation is significant at the 0.05 level (2 tailed) **. Correlation is significant at the 0.01 level (2 tailed)

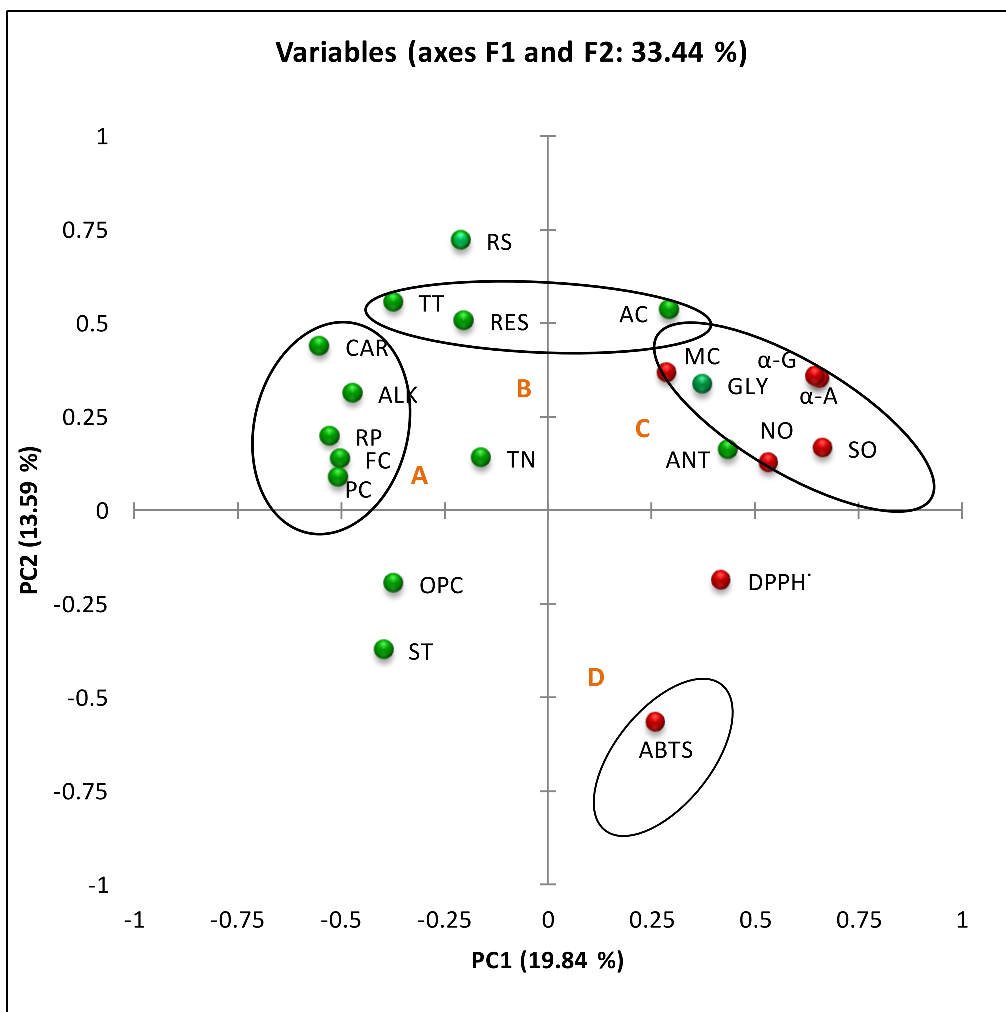


Fig 5.5.2: Principal component analysis of phytochemical content and antioxidant, anti-diabetic activities of studied liverworts

NB: CAR= cardiac glycoside, ALK= alkaloid, RP=reducing power, FC= flavonoid content, PC= phenol content, OPC= orthodihydric phenol content, ST= steroid, RS= reducing sugar, TT= triterpenoid, RES= resine, TN=tannin, AC= amino acid, ANT= anthraglycoside, GLY= glycoside, MC= metal chelating activity, NO= nitric oxide scavenging activity, SO= superoxide scavenging activity, DPPH˙= DPPH˙ scavenging activity, ABTS= ABTS scavenging activity, α-G= α-glucosidase inhibitory activity, α-A= α-amylase inhibitory activity

= antioxidant and anti-diabetic assays **= phytochemical content**

5.5.3 Heat map Analysis

Heat map analysis was performed specifically to find the effect of the polarity of solvents on the extraction of phytochemicals having high antioxidant and anti-diabetic activities. Solvents used for extraction were placed on the vertical axis and the phytochemical content, antioxidant and anti-diabetic activities of the studied plants were placed on the horizontal axis. In case of *Asterella wallichiana* (Fig 5.5.3.1) diethyl ether, ethyl acetate and heptane were clustered together (Group A) indicating that extracts of these solvents have similar pharmacological activities and phytochemical content. Colour key in graphical analysis signified the presence of high amount of plant phytochemicals having high free radical scavenging activity and anti-diabetic enzyme inhibition activity in diethyl ether, ethyl acetate and heptane extracts. Another cluster (Group B) on the vertical axis consisted of solvents acetone and butanol. These solvent extracts showed much lesser pharmacological activities and phytochemical content. Methanol was placed alone in group C. In methanolic extract tannin was found in higher concentration than other phytochemicals. Methanolic extract was noticed to be effective in scavenging selective free radicals only. Thus for *Asterella wallichiana,* non-polar (heptane, diethyl ether) to moderately polar (ethyl acetate) solvents were found to be most appropriate for extraction of phytochemicals having high pharmacological activities.

In case of *Conocephalum japonicum* butanol, ethyl acetate, acetone and diethyl ether were clustered in one group (A) while heptane and methanol extracts were clustered together in another group (Group B). Solvents falling under one group shows similar pattern of biological activities and phytochemical content. Solvents of group A displayed significant pharmacological activities but the content of phytochemicals under study was low. Graphical analysis interpreted that diethyl ether extract had the highest antioxidant, anti-diabetic activities and phytochemical contents. Heptane and methanol extracts showed low pharmacological activities and phytochemicals as well. Thus for *Conocephalum japonicum* moderately polar and non-polar solvents were better for extraction of plant metabolites with significant pharmacological activities (Fig 5.5.3.2).

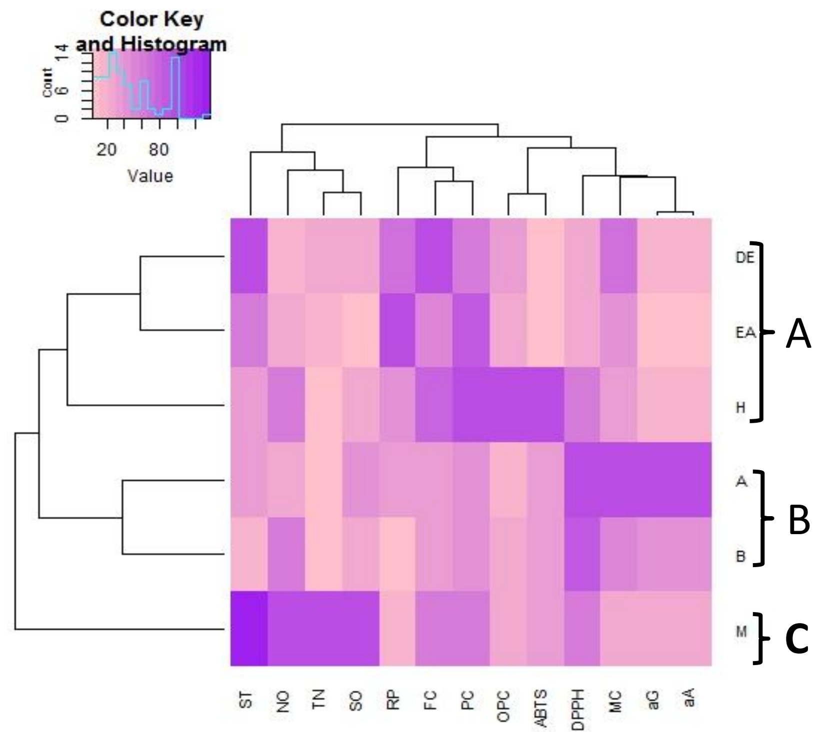


Fig 5.5.3.1: Heat map test for determining the role of different solvents on bioactive phytochemical extraction from *Asterella wallichiana*

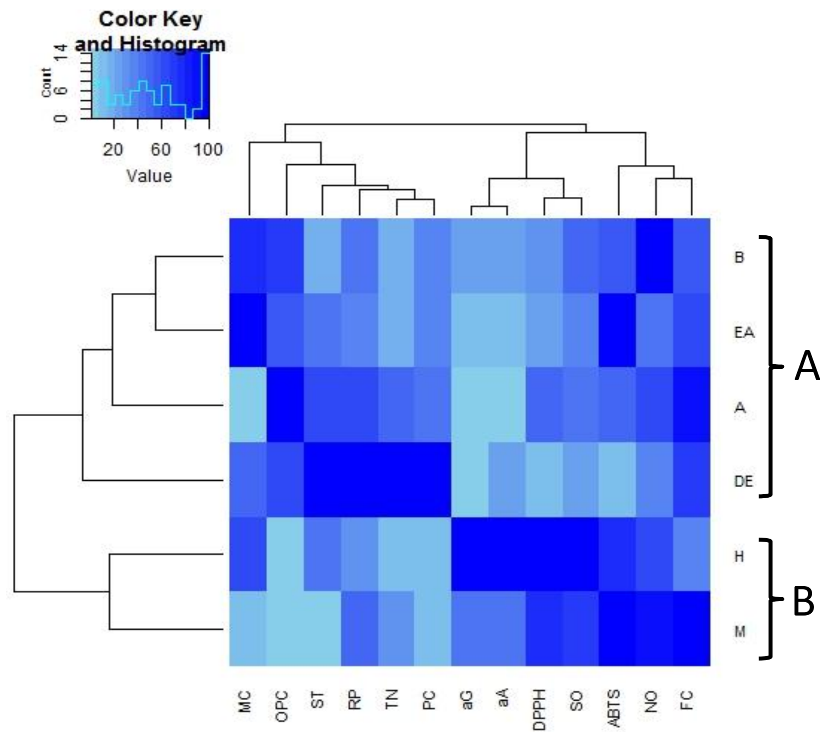


Fig 5.5.3..2 : Heat map test for determining the role of different solvents on bioactive phytochemical extraction from *Conocephalum japonicum*

Heat map of *Dumortiera hirsuta* clusters the solvent extracts into two major groups - A and B (Fig 5.5.3.3). In group A acetone, ethyl acetate and methanol were included and in group B diethyl ether, heptane and butanol were clustered. Graphical analysis signified that diethyl ether and heptane extracts had high antioxidant and anti-diabetic activities. Phytochemical content was also high in these solvent extracts. Apart from these, butanol extract had also showed high pharmacological activities but the content of phytochemicals was low indicating that plant metabolites other than those belonging to polyphenolic group are responsible of the pharmacological activities displayed by butanol extract. However, pharmacological activities and phytochemical content was insignificant in acetone, ethyl acetate and methanol extract.

Different solvents used for extraction of *Lunularia cruciata* were grouped into three major groups (Fig 5.5.3.4). Group A included ethyl acetate, diethyl ether and acetone. Group B included heptane and methanol, and group C included butanol. Colour key indicated that diethyl ether and ethyl acetate have extracted the phytochemicals that have high free radical scavenging potential. Acetone, heptane and methanol extracts were found to be active in scavenging only selective free radicals and also the content of polyphenols were low in these extracts. While in butanol both the pharmacological property and content of phytochemicals was found to be insignificant. Thus, it can be inferred that, moderately non-polar and moderately polar solvents like diethyl ether and ethyl acetate were most suitable for extraction of active phytochemical from *Lunularia cruciata*.

In case of *Pellia epiphylla*, acetone, diethyl ether, heptane and methanol extracts have showed high antioxidant, anti-diabetic activities and phytochemical content (Fig 5.5.3.5). While, butanol and ethyl acetate extracts (Group A) had high pharmacological activities but low phytochemicals content. Colour key in graphical analysis showed highest antioxidant and anti-diabetic activity in diethyl ether and ethyl acetate extracts. Thus in *Pellia epiphylla*, for extraction of biologically active substances non-polar to moderately polar solvents were found to be suitable.

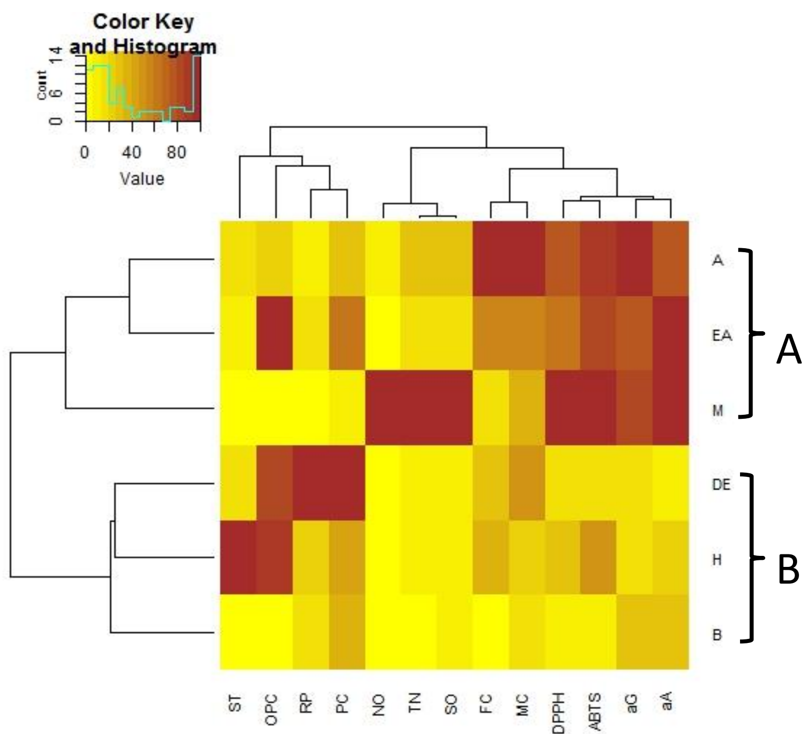


Fig 5.5.3.3: Heatmap test for determining the role of different solvents on bioactive phytochemical extraction from *Dumortiera hirsuta*

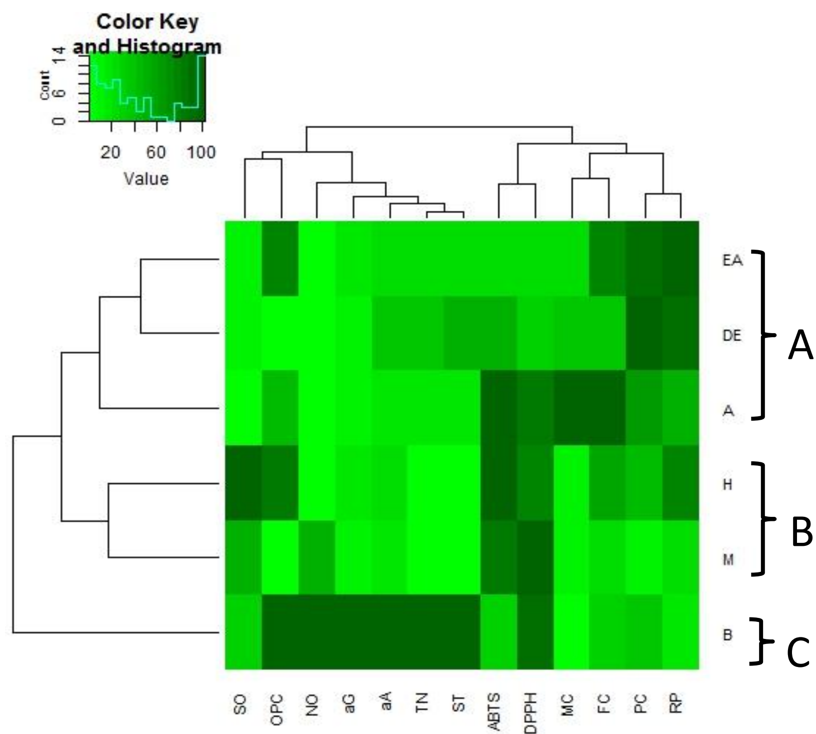


Fig 5.5.3.4: Heatmap test for determining the role of different solvents on bioactive phytochemical extraction from *Lunularia cruciata*

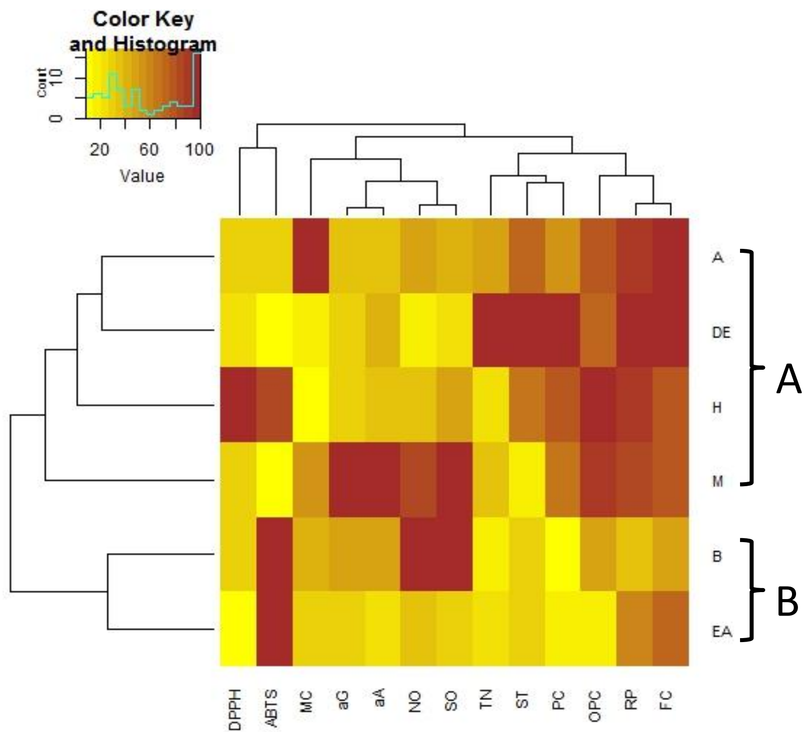


Fig 5.5.3.5: Heatmap test for determining the role of different solvents on bioactive phytochemical extraction from *Pellia epiphylla*

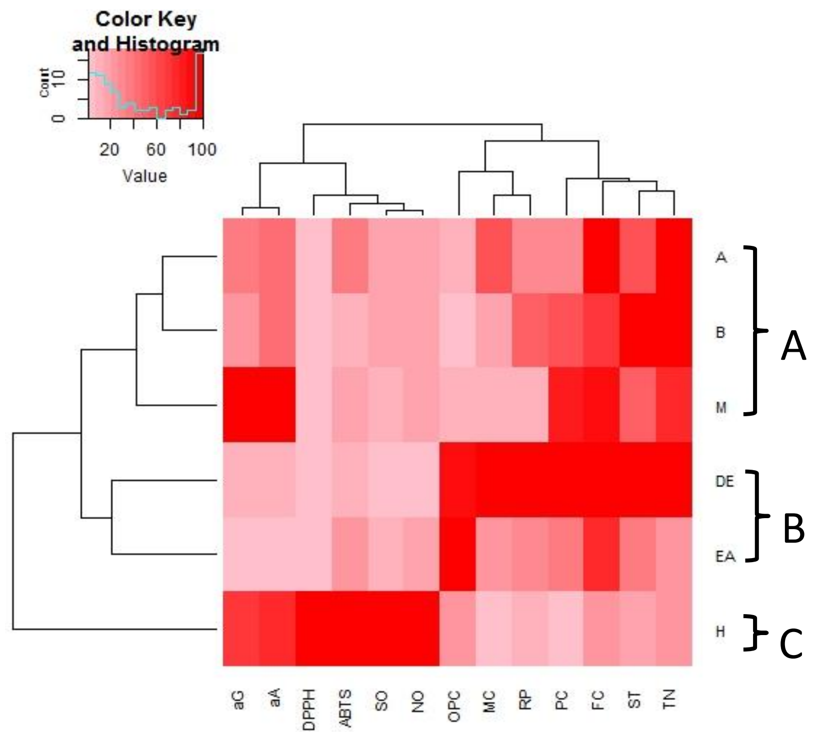


Fig 5.5.3.6: Heatmap test for determining the role of different solvents on bioactive phytochemical extraction from *Marchantia paleacea*

In *Marchantia paleacea*, hierarchical clustering placed the solvent extracts under study in three groups A, B and C (Fig 5.5.3.6). Graphical analysis suggested solvents included in group A (acetone, butanol and methanol) and B (diethyl ether and ethyl acetate) have extracted phytochemicals from *M. paleacea* that are active in scavenging free radicals. However, solvents of group A were less active in inhibiting the activity of α-amylase and α-glucosidase enzymes than solvents of group B. In heptane extract both phytochemicals and pharmacological activities were low. Thus, for *M. paleacea* too ethyl acetate and diethyl ether were best solvents for extracting phytochemicals having high pharmacological activities.

In *Marchantia polymorpha* except methanolic extract all the other extracts showed a significant potential to scavenge free radical and to inhibit α-amylase and α-glucosidase enzymes. Solvents of Group A (diethyl ether, ethyl acetate and acetone) extracted the studied phytochemicals in high amount and the extracted bio-active phytochemicals also displayed a strong antioxidant and anti-diabetic activities. In butanol and heptane extracts (Group B) steroid content was high whereas other phytochemicals like phenol, flavonoid, tannin etc were present in lower amount. These solvent extracts scavenged the free radicals and inhibited the activity of α-amylase and α-glucosidase efficiently. However methanol extract have shown low pharmacological activities and phytochemical content. Thus, for *M. polymorpha* non-polar and moderately polar solvents were found to be suitable for bio-active phytochemical extraction (Fig 5.5.3.7).

In *Marchantia emarginata*, six solvents used for extracting bioactive phytochemicals were grouped into three major groups by heatmap hierarchical clustering (Fig 5.5.3.8). Acetone, ethyl acetate and diethyl ether were clustered together in group A, heptane and butanol were clustered together in Group B and methanol was placed separately in group C on the vertical axis. Solvent extracts of group A showed good antioxidant, anti-diabetic activity and high phytochemical content. Butanol and heptane extracts showed good free radical scavenging and anti-diabetic activities suggesting that steroids present in these extracts are responsible for mentioned activities. In group B solvent extracts, amount of steroid and iron reducing potential were high whereas polyphenol content was low. Phytochemical content, antioxidant and anti-diabetic activities were insignificant in methanolic extract. Result indicated that, in *M. emarginata* too ethyl acetate and diethyl ether were best solvents for bioactive phytochemicals extraction. Acetone though included in

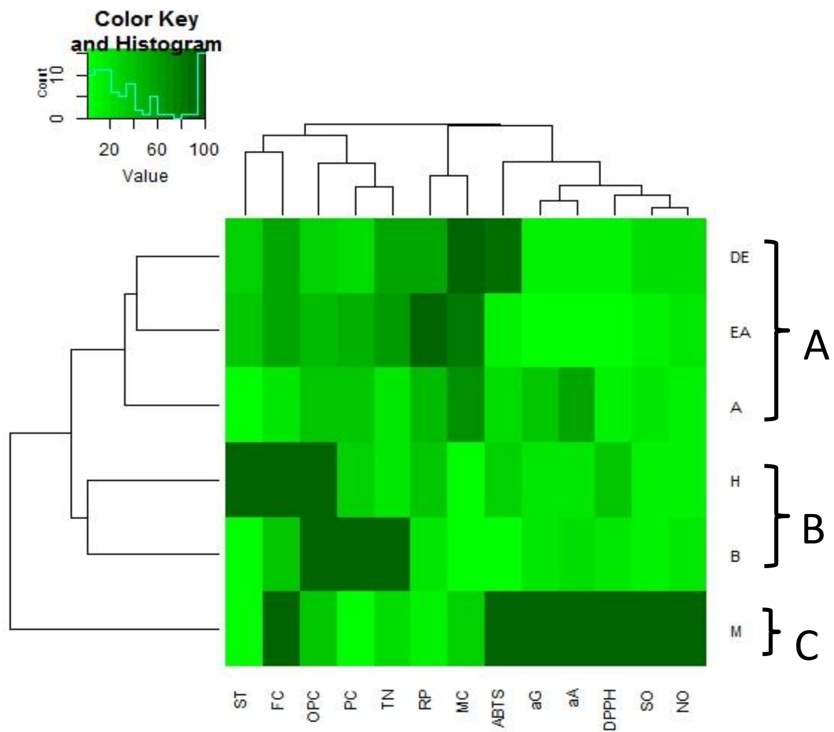


Fig 5.5.3.7. : Heat map test for determining the role of different solvents on bioactive phytochemical extraction from *Marchantia polymorpha*

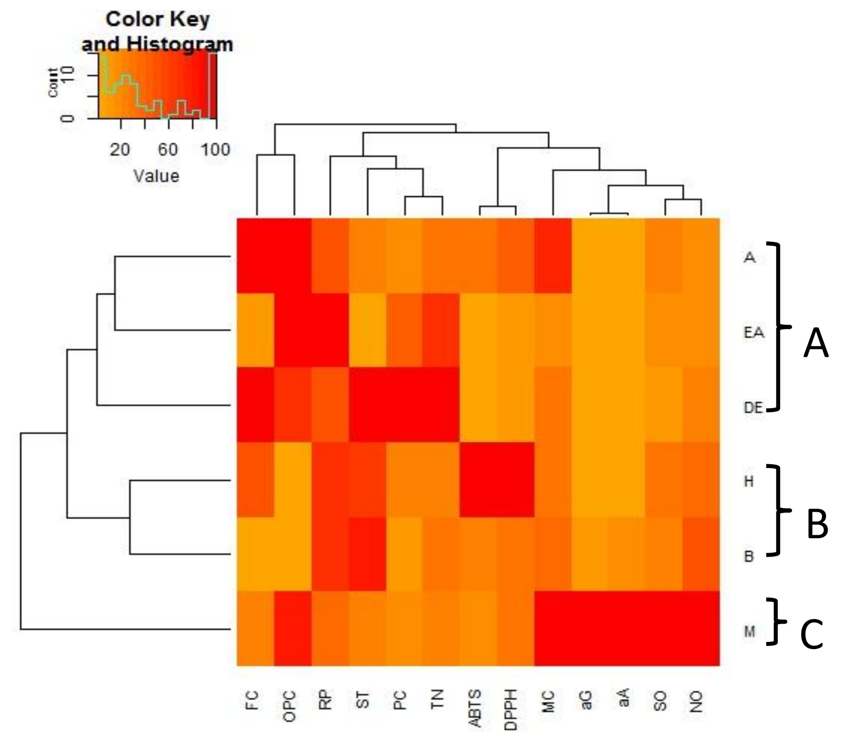


Fig 5.5.3.8 : Heat map test for determining the role of different solvents on bioactive phytochemical extraction from *Marchantia emarginata*

group A showed weaker metal chelating and DPPH$^{\cdot}$ scavenging activities and thus was not considered as good solvent for active phytochemical extraction.

Heat map analysis for *Marchantia subintegra* placed the solvent extracts under study in three discrete groups namely A, B and C (Fig 5.5.3.9). The extracts of group C (diethyl ether and ethyl acetate extracts) showed high antioxidant, anti-daibetic activities as well as high bioactive phytochemical content. Butanol and heptane extracts placed under group B showed significant free radical scavenging and anti-diabetic activities but low polyphenol content. In methanol and acetone extracts (group A) both lower phytochemical content and pharmacological activities were noticed. Thus in *M. subintergra* non-polar and moderately polar solvents were found to be suitable for extracting bio-active phytochemicals while polar solvents were less effective in extraction of bioactive phytochemicals.

In *Plagiochasma cordatum*, hierarchical clustering clustered the studied solvents under three groups (Fig 5.5.3.10). Solvents included under group A (diethyl ether and acetone) and B (ethyl acetate and butanol) extracted phytochemicals having significant biological activities in high amount. While group C solvents extracts (methanol and heptane) showed low content of studied phytochemicals as well as low free radical scavenging activity. Thus for *Plagiochasma cordatum,* solvents with moderate polarity like diethyl ether, ethyl acetate, acetone and butanol were more suitable for bioactive phytochemical extraction.

In *Plagiochila nepalensis*, hierarchical clustering placed solvent extracts into four groups (Fig 5.5.3.11). In extracts of Group A (ethyl acetate and acetone) and B (heptane and butanol) significant antioxidant and anti-diabetic activities were recorded but the content of studied phytochemicals were low. While, diethyl ether extract showed high pharmacological activities and phytochemical content. However, methanolic extract showed very low antioxidant and anti-diabetic activities. Thus for *P. nepalensis* non polar and moderately polar solvents were found to be suitable for extraction of bioactive phytochemicals.

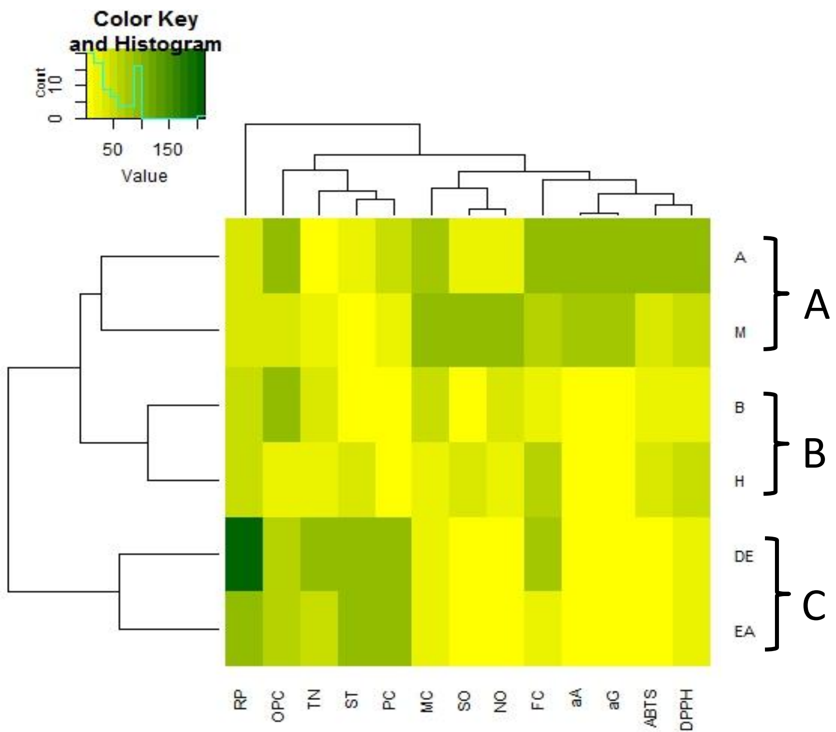


Fig 5.5.3.9 : Heat map test for determining the role of different solvents on bioactive phytochemical extraction from *M.archantia subintegra*

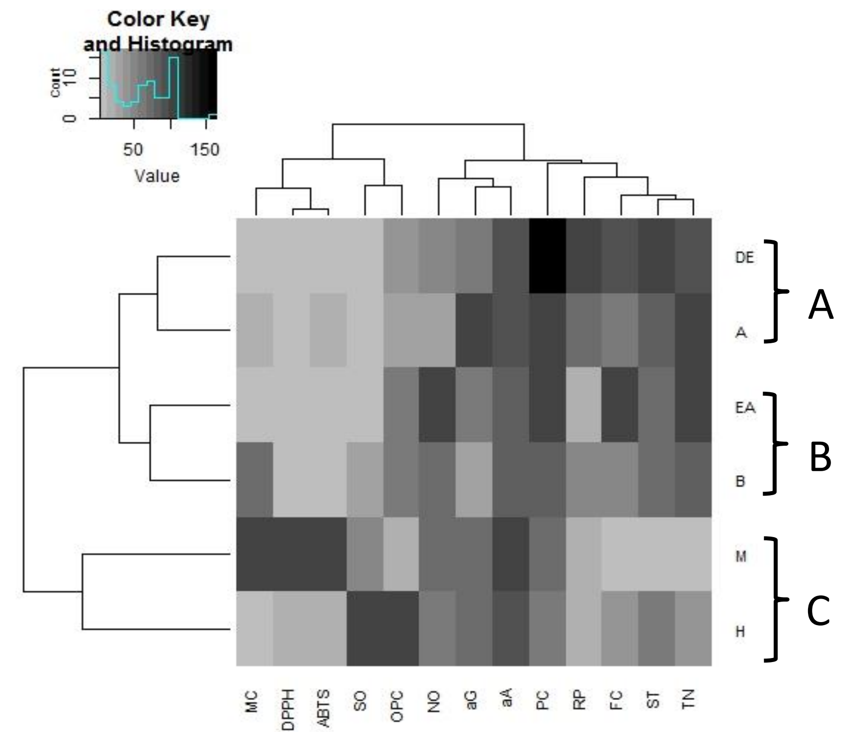


Fig 5.5.3.10 : Heat map test for determining the role of different solvents on bioactive phytochemical extraction from *Plagiochasma cordatum*

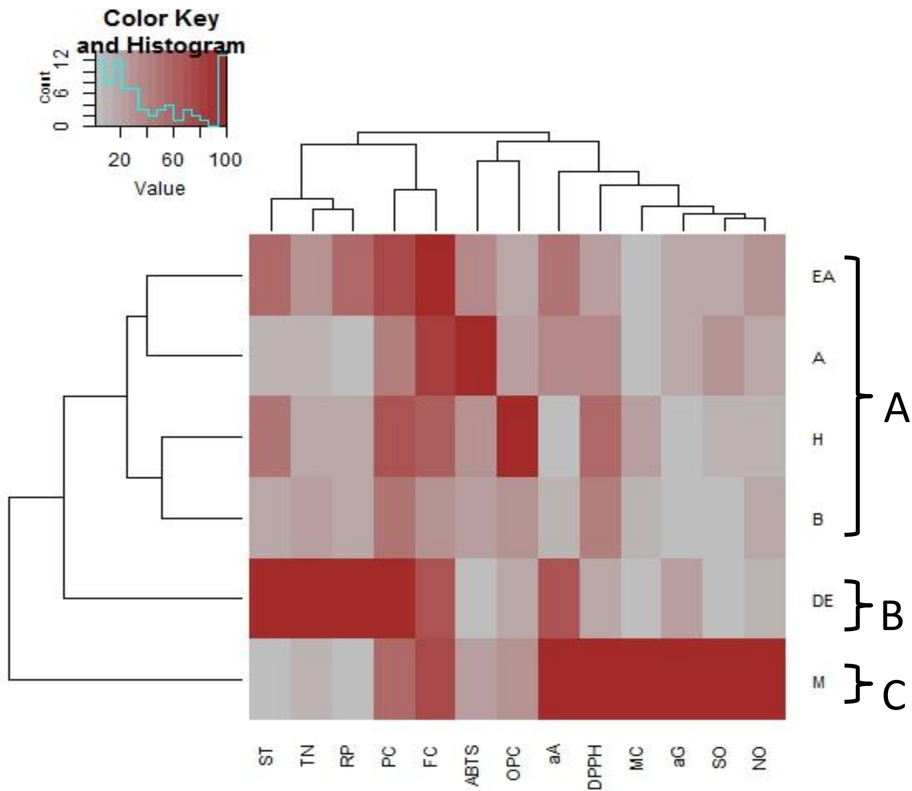


Fig 5.5.3.11: Heat map test for determining the role of different solvents on bioactive phytochemical extraction from *Plagiochila nepalensis*

5.6 SEASONAL CHANGES IN ANTIOXIDANT ACTIVITY AND PHENOLIC CONTENT IN LIVERWORTS

Seasonal changes in free radical scavenging activity and phenolic content were studied in liverwort *Marchantia paleacea* collected during two seasons *i.e.* rainy season prevailing from May till October and dry season prevailing from October till March of a year. Six different solvents, heptane, diethyl ether, ethyl acetate, acetone, butanol and methanol were used for the extraction of phytochemicals. Different kinds of free radicals are generated in the body that are responsible for inducing oxidative stress. We have selected $DPPH^{\cdot}$, $ABTS^{+}$, nitric oxide (NO), super oxide (SO) radicals to evaluate the variation in antioxidant activity of *M. paleacea* during rainy and dry season. $DPPH^{\cdot}$ scavenging activity of *M. paleacea* increased significantly during dry season. The $DPPH^{\cdot}$ scavenging activity (IC_{50}) ranged between 0.27 mg/ml and 34.75 mg/ml during rainy season and in dry season activity ranged between 0.22 mg/ml and 2.63 mg/ml in different solvent extracts. Significant increase in $DPPH^{\cdot}$ activity was observed in heptane, diethyl ether, ethyl acetate, acetone and butanol extracts during dry season while methanolic extract's potential to scavenge this free radical decreased during dry season as compared to rainy season (Fig 5.6.1).

$ABTS^{+}$ scavenging activity varied from 0.06 mg/ ml to 0.64 mg/ml (IC_{50} values) during rainy season in six solvent extracts. While in dry season, IC_{50} value ranged between 0.06 mg/ml and 0.40 mg/ml. $ABTS^{+}$ scavenging activity of heptane, ethyl acetate and acetone extracts increased during dry season. The activity decreased in methanol, butanol and very slightly in diethyl ether extract during dry season (Fig 5.6.2). In superoxide scavenging assay also similar result was obtained. Superoxide scavenging activity of *M. paleacea* increased significantly during dry season in heptane, ethyl acetate and acetone extracts, while in methanol, butanol and diethyl ether extracts, potential to scavenge SO was found to be better during rainy season. IC_{50} value of SO scavenging activity ranged from 0.16 mg/ml to 3.7 mg/ml during rainy season and from 0.19 mg/ml to 3.7 mg/ml during dry season (Fig 5.6.3). However when all other studied free radicals scavenging activity was increased during drought condition in selected solvent extracts, the result of nitric oxide scavenging assay were rather contradictory. *M. paleacea* showed increased potential to scavenge nitric oxide during rainy season than dry season (Fig 5.6.4). During rainy season the NO scavenging activity ranged between 0.82 mg/ml and 13.35 mg/ml (IC_{50}), while during dry season the said activity ranged between 2.56 mg/ml and 21.8 mg/ ml (IC_{50}).

Free ferrous ions are the most powerful pro-oxidants and thus its reduction is important for protection against oxidative damage and lipid peroxidation by Fenton reaction. Result of the present study showed that metal chelating potential of *M. paleacea* increased significantly during dry season in diethyl ether, ethyl acetate and acetone extract. While heptane, methanol and butanol extracts showed better ferrous iron chelating activity during rainy season. Metal chelating activity of *M. paleacea* varied from 0.37 mg/ml to 13.51 (IC_{50} value) during rainy season and 0.96 mg/ml to 4.72 mg/ml (IC_{50} value) during dry season (Fig 5.6.5).

As plant polyphenols are mainly reported of having a role as antioxidants, we have studied the change in pattern of polyphenolic compounds content during rainy and dry season to check if there exist any correlation between the phenolic content and the free radical scavenging activities. Content of phenols in studied extracts during two seasons explained the variation in the antioxidant activity to some extent. With the increase in antioxidant activity of heptane, diethyl ether, ethyl acetate and acetone extracts during dry season the increase in the phenolic compound was also recorded. Similarly, the increase in antioxidant activity of methanolic and butanolic extracts was accompanied with the increase in phenolic compound in these extracts. Phenol content ranged between 4.81 mg gallic acid equivalent/g extractive weight (mg GAE/g EW) and 113.9 mg GAE/g EW in rainy season and between 24.1 mg GAE/g EW and 117.4 mg GAE/g EW (Fig 5.6.7). Similarly, flavonoid content of the plant was also found to increase during dry season. Concentration of flavonoid increased significantly in butanol, ethyl acetate, diethyl ether and heptane extracts during dry season. Flavonoid content varied from 3.8 mg quercetin equivalent/ g extractive weight (mg QE/ g EW) to 16.3 mg QE/ g EW during rainy season and from 9.1 mg QE/ g EW to 32.1 mg QE/ g EW during dry season (Fig 5.6.8). However, orthodihydric phenol content of the plant was found to decrease during unfavorable condition where there is scarcity of water (Fig 5.6.7). During rainy season content of orthodihydric phenol ranged between 0.34 mg Catechol/ g extractive weight (mg CE/ g EW) to 11.45 mg CE/ g EW. In dry season 0.11 mg CE/ g EW to 7.11 mg CE/ g EW content of orthodihydric phenol was recorded in six solvent extracts.

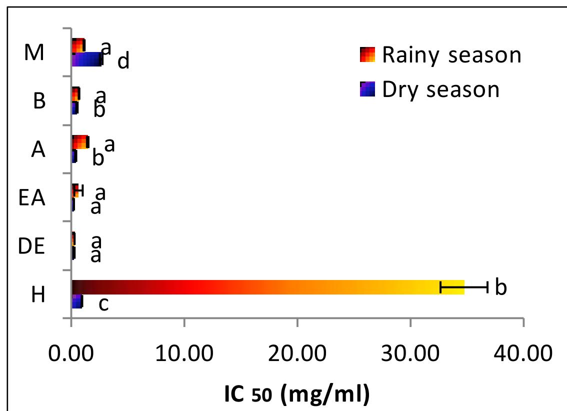


Fig 5.6.1: Comparison of DPPH˙ scavenging activity of *M. paleacea* during rainy and dry season

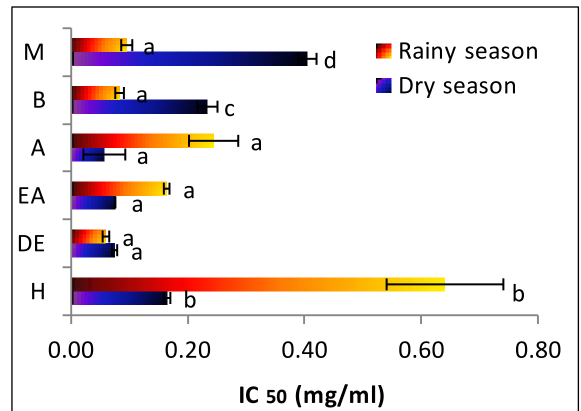


Fig 5.6.2: Comparison of ABTS˙ scavenging activity of *M. paleacea* during rainy and dry season

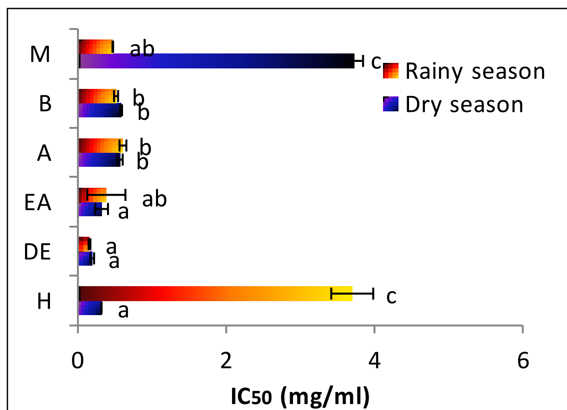


Fig 5.6.3: Comparison of SO scavenging activity of *M. paleacea* during rainy and dry season

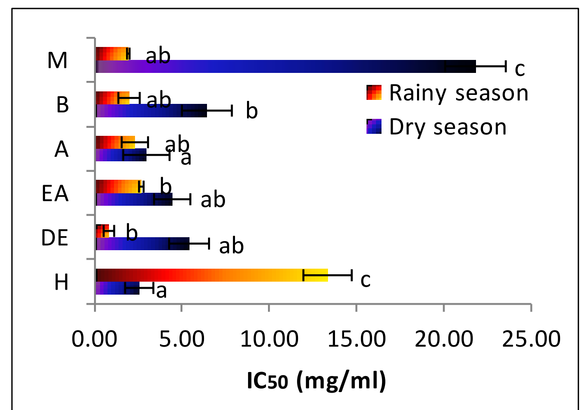


Fig 5.6.4: Comparison of NO scavenging potential of *M. paleacea* during rainy and dry season

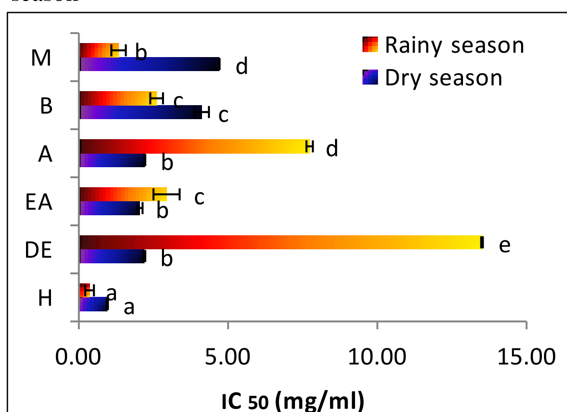


Fig 5.6.5: Comparison metal chelating activity of *M. paleacea* during rainy and dry season

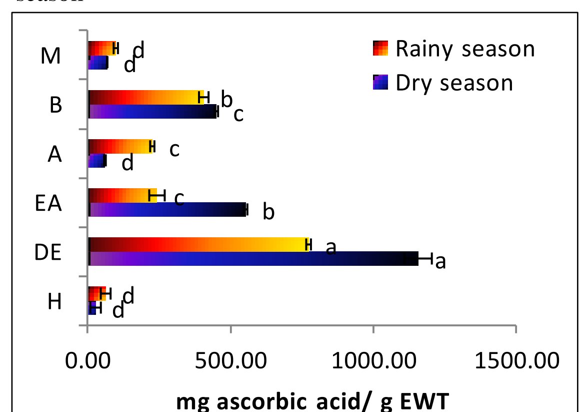


Fig 5.6.6: Comparison of reducing power of *M. paleacea* during rainy and dry season

NB: H= heptane, DE=diethyl ether, EA= ethyl acetate, A= acetone, B=butanol, M=methanol Value with different letters (a, b, c, d etc.) differ significantly ($p < 0.05$) from each other by Duncan's Multiple Test (DMRT).

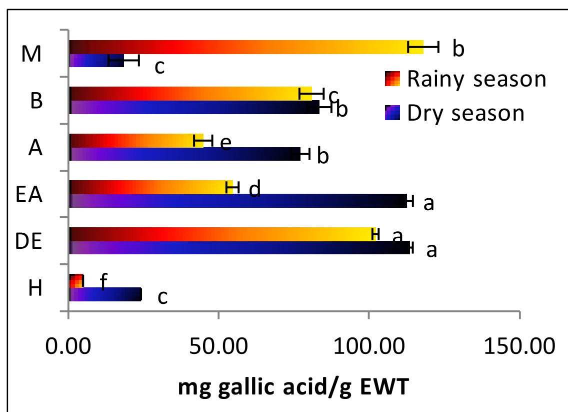


Fig 5.6.7: Comparison of phenolic content of *M. paleacea* during rainy and dry season

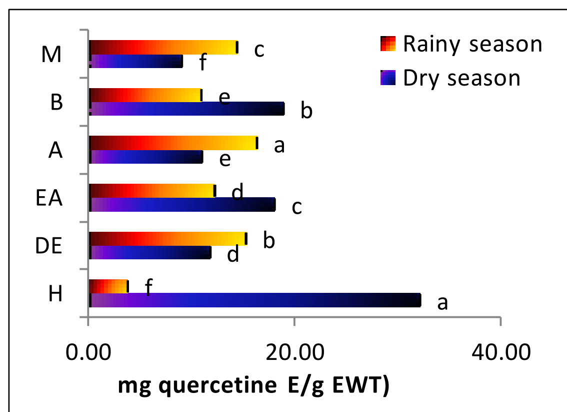


Fig 5.6.7: Comparison of flavonoid content of *M. paleacea* during rainy and dry season

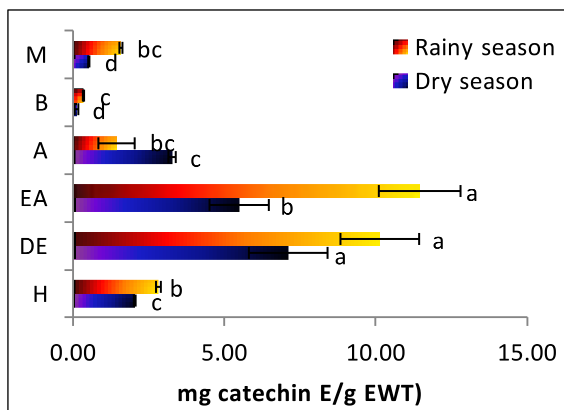


Fig 5.6.7: Comparison Orthodihydric phenol content of *M. paleacea* during rainy and dry season

NB: H= heptane, DE=diethyl ether, EA= ethyl acetate, A= acetone, B=butanol, M=methanol Value with different letters (a, b, c, d etc.) differ significantly (p <0.05) from each other by Duncan's Multiple Test (DMRT)

5.7 BIOASSAY GUIDED PARTIAL PURIFICATION OF LIVERWORT *MARCHANTIA PALEACEA*

Bryophytes were used as medicines in ancient Chinese, European and North American traditional medicine. One important class of bryophyte, the liverworts, has been used as ethnomedicine worldwide and few reports are also available recording their use in Indian culture. *Marchantia paleacea*, has been reported to be used in traditional medicines; it is used in skin tumefaction, hepatitis and also as an antipyretic (Chandra *et al*., 2017). Moreover, result of assays that were performed earlier in this work showed that *M. paleacea* has a significant free radical scavenging and anti-diabetic potential along with moderate anti-proliferation activity. In Darjeeling, *M. paleacea* grows abundantly, so, on the basis of medicinal properties and abundance, *M. paleacea* was selected for bioassay guided purification of phytochemicals. Phytochemicals present in *M. paleacea* was extracted successively with solvents of increasing polarity (hexane, toluene, diethyl ether, ethyl acetate, chloroform, acetone, acetonitrile, ethanol, methanol, water). All ten extracts obtained after successive extraction were screened for the phytochemical content and antioxidant activity. DPPH˙ assay is the most common assay for screening free radical scavenging potential (Brand-Williams *et al*., 2005).

On screening the preliminary extracts for its DPPH˙ scavenging activity, diethyl-ether extract was found to exhibit considerably stronger antioxidant property than other solvent extracts (Fig 5.7.1). Similarly Fig 5.7.2 represents the quantitative profile of flavonoid which shows that diethyl ether extract also contained higher amount of flavonoid compounds. On the basis of the antioxidant activity, anti-diabetic potential and content of flavonoid compounds, diethyl ether fraction was chromatographed on silica gel column. A total of 208 sub-fractions (20 ml each) were collected and screened for DPPH˙ scavenging activity (Fig 5.7.3). Sub-fractions with high antioxidant activity were then combined on the basis of their TLC profiles (Fig 5.7.4). Active sub-fractions were grouped into eleven main fractions (F1-A, F1-B, F1-C, F1-D, F1-E, F1-F, F1-G, F1-H, F1-I, F1-J, F1-K). These main fractions were further screened by antioxidant and anti-diabetic assays (Fig 5.7.5 – 5.7.7). Fraction F1-F showed best antioxidant and anti-diabetic activity. Therefore, F1-F was subjected to additional fractionation by second column chromatograph. In total, 546 sub fractions (10 ml each) were collected and screened for their antioxidant (DPPH˙ scavenging activity) property and flavonoid content (Fig 5.7.10). Less active fractions were discarded

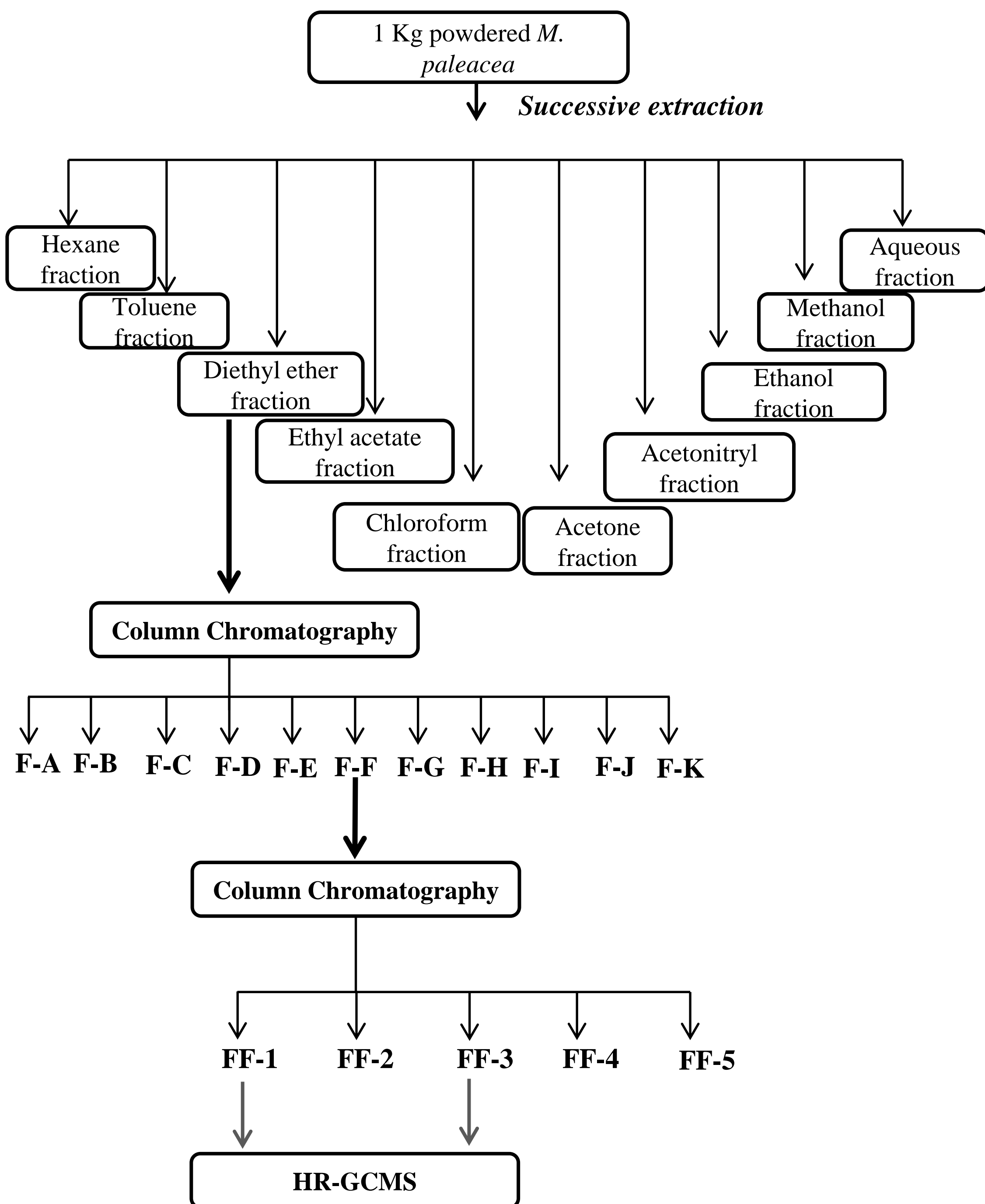


Fig 5.7: Schemes of fraction preparation of *Marchantia paleacea.* F= fractions obtained from first column chromatography, FF= fraction obtained from second column chromatography

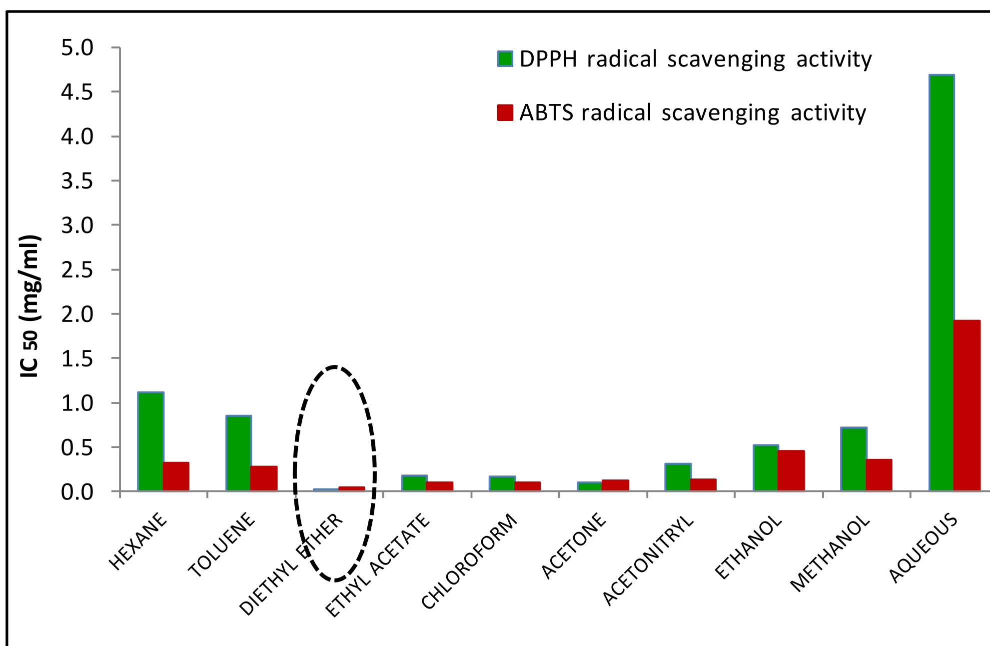


Fig 5.7.1: DPPH and ABTS radical scavenging activities of main fractions obtained from successive extraction

NB: Bar within the ellipse indicates highest DPPH˙ and ABTS⁺ activity.

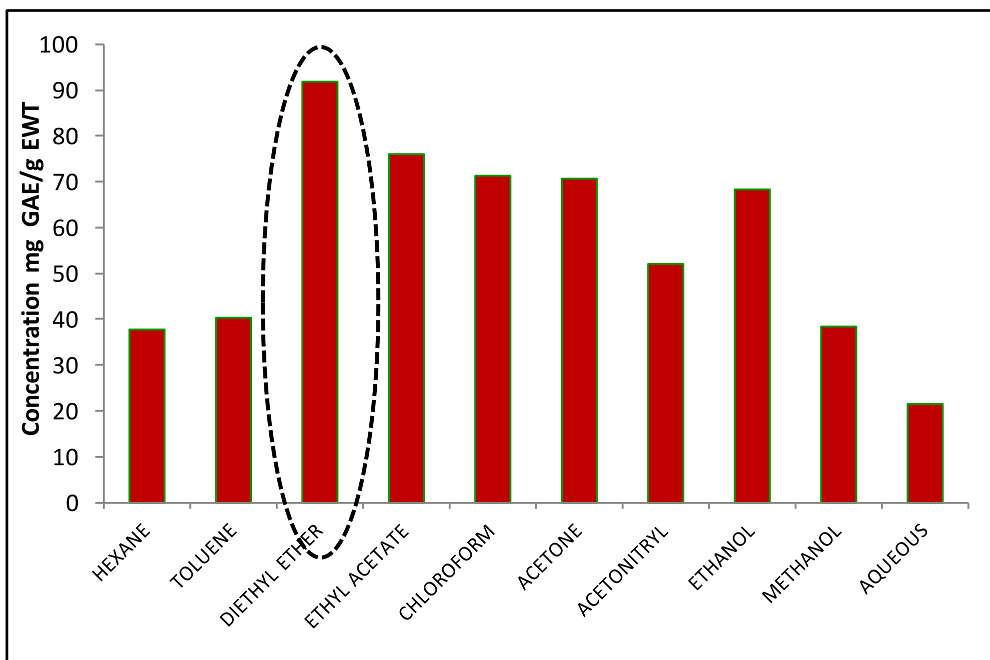


Fig 5.7.2: Flavonoid content of main fractions obtained from successive extraction

NB: Bar within the ellipse indicates highest flavonoid content.

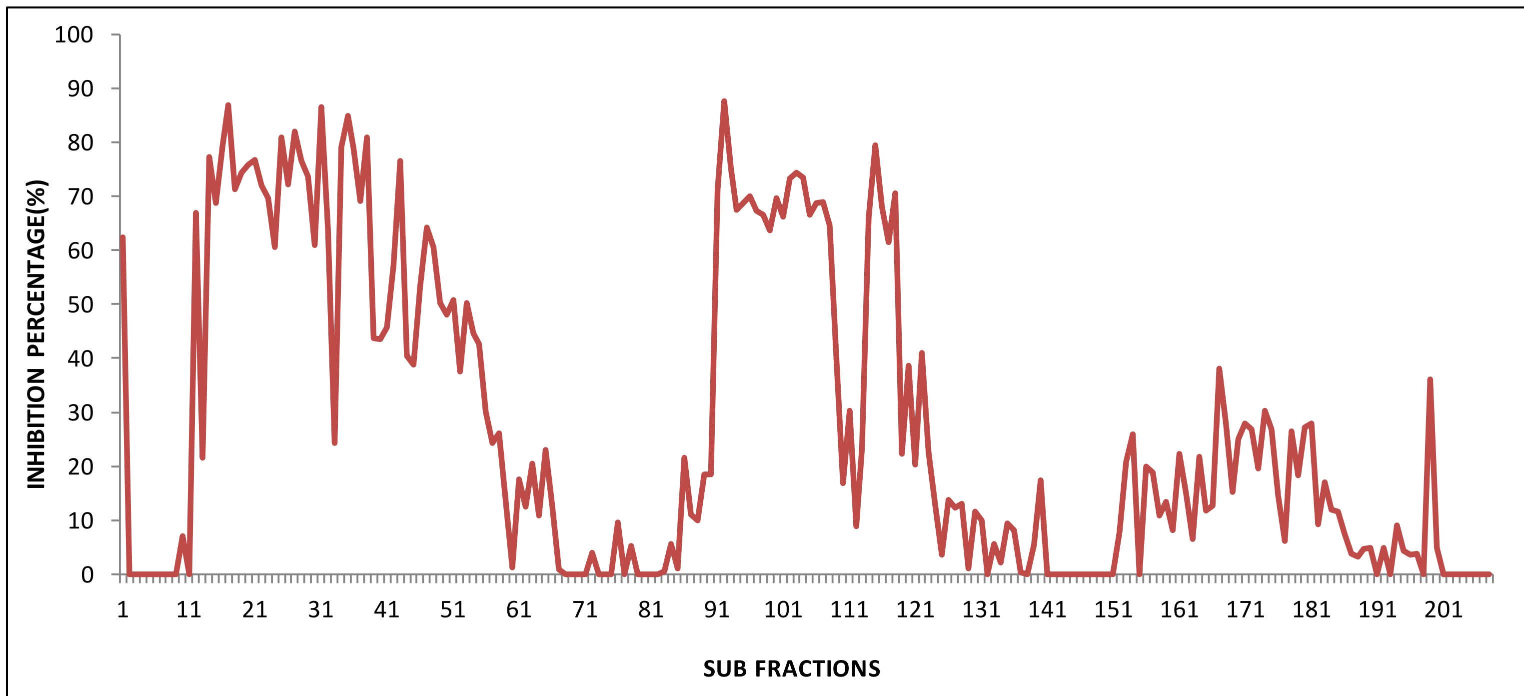


Fig 5.7.3: DPPH˙ scavenging activity of fractions of *M. paleacea* obtained from first column chromatography

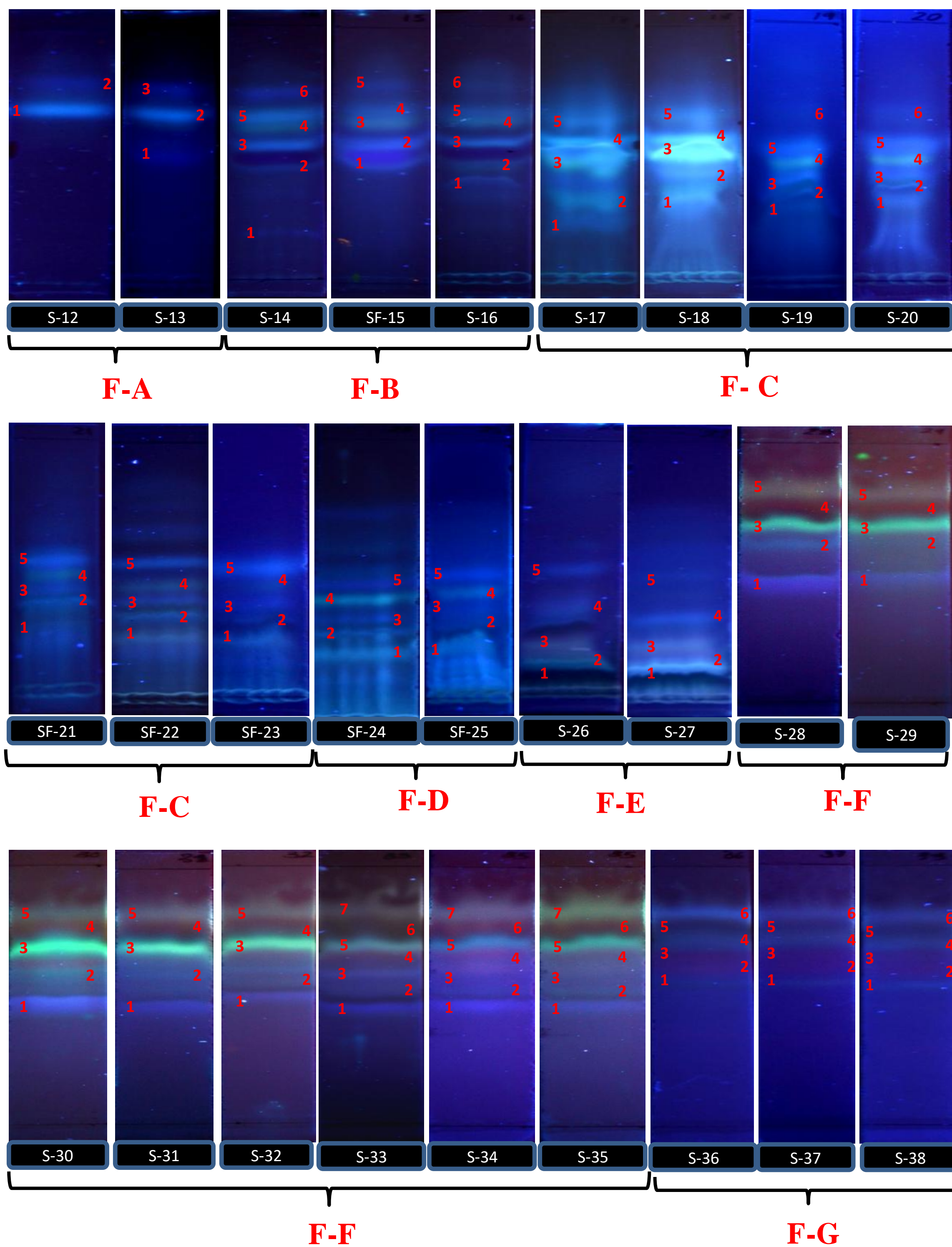
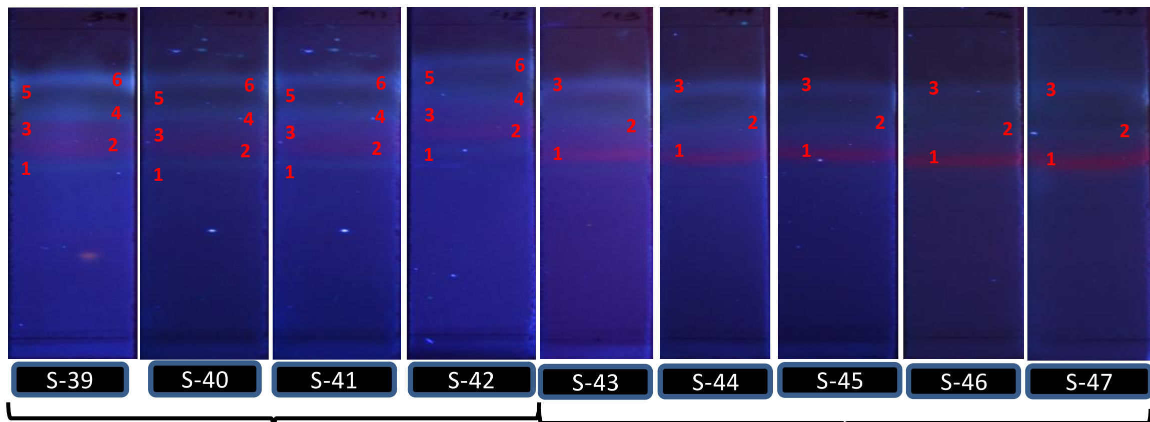
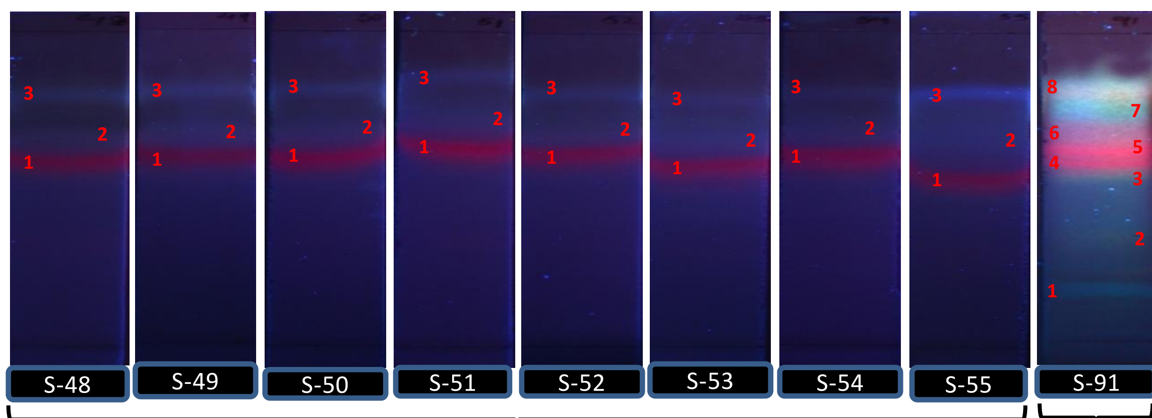


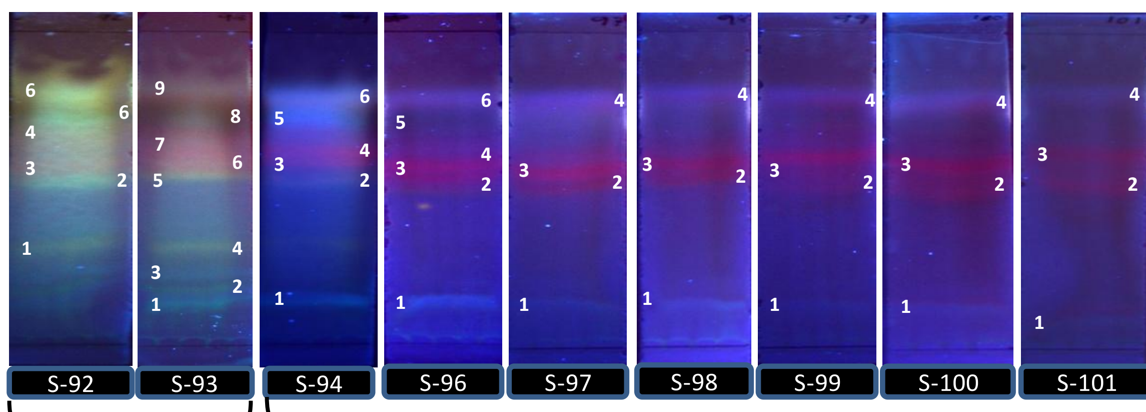
Fig 5.7.4 : TLC profiling of bioactive sub-fractions of *M. paleacea* obtained from first column chromatograph



F-G **F-H**

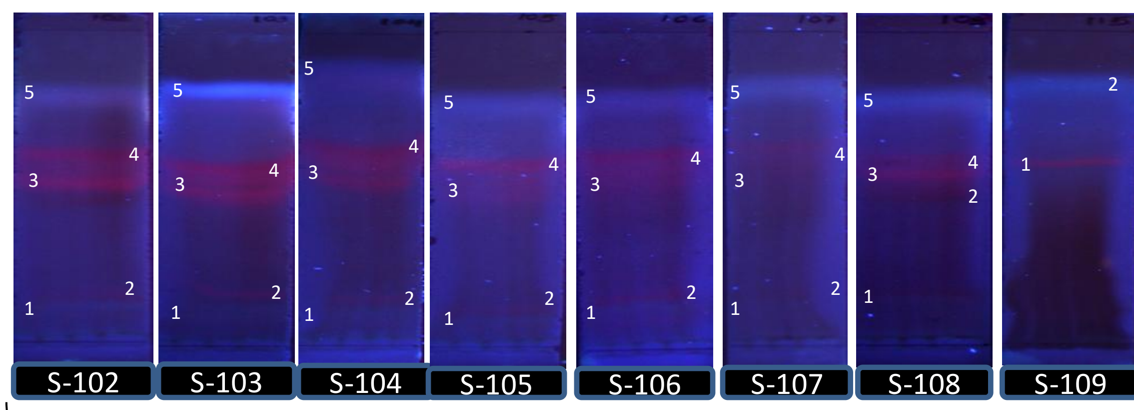


F-H **F-I**



F-I **F-J**

Fig 5.7.4 : TLC profiling of bioactive sub-fractions of *M. paleacea* obtained from first column chromatograph (Continuation)



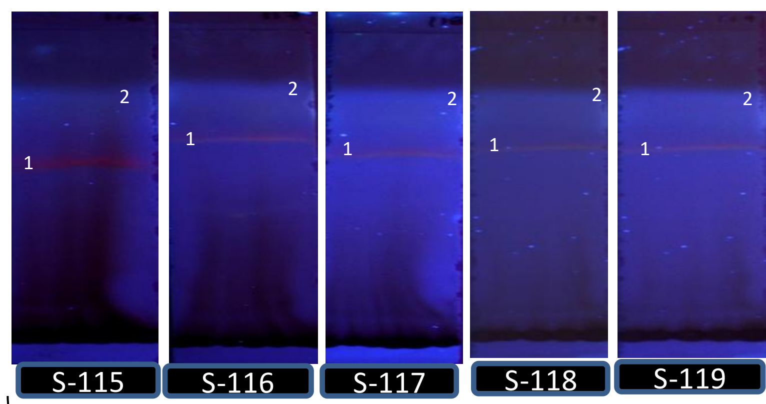


Fig 5.7.4: TLC profiling of bioactive sub-fractions of *M. paleacea* obtained from first column chromatograph (Continuation)

Table 5.3: Retardation factors of different analytes present in bioactive fractions of *M. paleacea* obtained from first column chromatograph of *M. paleacea*

Sub fractions	***Identity of bands***	**Rf**	***Sub fractions***	***Identity of bands***	**Rf**
12	1	0.65	22	1	0.25
	2	0.77		2	0.34
				3	0.37
				4	0.42
				5	0.51
13	1	0.48	23	1	0.23
	2	0.65		2	0.31
	3	0.77		3	0.41
				4	0.47
				5	0.51
14	1	0.17	24	1	0.25
	2	0.34		2	0.34
	3	0.43		3	0.37
	4	0.6		4	0.45
	5	0.51		5	0.51
	6	0.65			
	7	0.75			
15	1	0.48	25	1	0.25
	2	0.5		2	0.34
	3	0.53		3	0.37
	4	0.67		4	0.42
	5	0.77		5	0.51
17	1	0.15	26	1	0.15
	2	0.34		2	0.17
	3	0.48		3	0.24
	4	0.56		4	0.27
	5	0.65		5	0.51
18	1	0.15	27	1	0.15
	2	0.34		2	0.17
	3	0.48		3	0.24
	4	0.56		4	0.27
	5	0.65		5	0.50
19	1	0.3	28	1	0.46
	2	0.37		2	0.51
	3	0.41		3	0.68
	4	0.43		4	0.74
	5	0.48		5	0.81
	6	0.52			
20	1	0.3	29	1	0.46
	2	0.37		2	0.51
	3	0.41		3	0.68
	4	0.43		4	0.74
	5	0.48		5	0.81
	6	0.52			
21	1	0.29	30	1	0.46
	2	0.36		2	0.51
	3	0.41		3	0.68
	4	0.45		4	0.74
	5	0.51		5	0.81

Table 5.3: Retardation factors of different analytes present in bioactive fractions of *M. paleacea* obtained from first column chromatograph of *M. paleacea* (continuation)

Sub fractions	***Identity of bands***	***Rf***	***Sub fractions***	***Identity of bands***	***Rf***
31	1	0.48	40	1	0.51
	2	0.53		2	0.55
	3	0.58		3	0.60
	4	0.68		4	0.65
	5	0.74		5	0.71
	6	0.81		6	0.81
32	1	0.48	41	1	0.51
	2	0.53		2	0.55
	3	0.58		3	0.60
	4	0.68		4	0.65
	5	0.74		5	0.71
	6	0.81		6	0.81
33	1	0.46	42	1	0.51
	2	0.56		2	0.55
	3	0.60		3	0.60
	4	0.65		4	0.65
	5	0.68		5	0.71
	6	0.75		6	0.81
	7	0.81			
34	1	0.46	43	1	0.59
	2	0.56		2	0.72
	3	0.60		3	0.82
	4	0.65			
	5	0.68			
	6	0.75			
	7	0.81			
35	1	0.46	44	1	0.59
	2	0.56		2	0.72
	3	0.60		3	0.82
	4	0.65			
	5	0.68			
	6	0.75			
	7	0.81			
36	1	0.51	45	1	0.59
	2	0.55		2	0.72
	3	0.60		3	0.82
	4	0.65			
	5	0.70			
	6	0.81			
37	1	0.51	46	1	0.59
	2	0.55		2	0.72
	3	0.60		3	0.82
	4	0.65			
	5	0.71			
	6	0.81			
38	1	0.51	47	1	0.58
	2	0.55		2	0.64
	3	0.60		3	0.82
	4	0.65			
	5	0.71			
	6	0.81			
39	1	0.51	48	1	0.58
	2	0.55		2	0.64
	3	0.60		3	0.82
	4	0.65			
	5	0.71			
	6	0.81			

Table 5.3: Retardation factors of different analytes present in bioactive fractions of *M. paleacea* obtained from first column chromatograph of *M. paleacea* (continuation)

Sub fractions	***Identity of bands***	***Rf***	***Sub fractions***	***Identity of bands***	***Rf***
49	1	0.58		1	7.3
	2	0.64		2	0.52
	3	0.82	94	3	0.64
				4	0.68
				5	0.70
				6	0.78
50	1	0.58		1	7.3
	2	0.64		2	0.52
	3	0.82	95	3	0.64
				4	0.68
				5	0.70
				6	0.78
				1	7.3
	1	0.58		2	0.52
51	2	0.64	96	3	0.64
	3	0.82		4	0.68
				5	0.70
				6	0.78
	1	0.58		1	0.15
52	2	0.64	97	2	0.5
	3	0.82		3	0.6
				4	0.82
	1	0.58		1	0.15
53	2	0.64	98	2	0.5
	3	0.82		3	0.6
				4	0.82
	1	0.17			
	2	0.19			
	3	0.32		1	0.15
	4	0.52		2	0.5
91	5	0.56	99	3	0.6
	6	0.59		4	0.82
	7	0.73			
	8	0.78			
	1	0.17			
	2	0.19		1	0.15
92	3	0.32	100	2	0.5
	4	0.51		3	0.6
	5	0.70		4	0.82
	6	0.78			
	1	0.14	101	1	0.15
	2	0.19		2	0.5
	3	0.21		3	0.6
	4	0.32		4	0.82
93	5	0.52			
	6	0.56			
	7	0.59			
	8	0.73			
	9	0.78			

Table 5.3: Retardation factors of different analytes present in bioactive fractions of *M. paleacea* obtained from first column chromatograph of *M. paleacea* (continuation)

Sub fractions	*Identity of bands*	*Rf*	*Sub fractions*	*Identity of bands*	*Rf*
102	1	0.15	108	1	0.31
	2	0.5		2	0.48
	3	0.6		3	0.55
	4	0.82		4	0.58
				5	0.60
103	1	0.15	109	1	0.58
	2	0.5		2	0.79
	3	0.6			
	4	0.82			
104	1	0.31	115	1	0.60
	2	0.51		2	0.79
	3	0.56			
	4	0.6			
	5	0.77			
105	1	0.12	116	1	0.60
	2	0.15		2	0.79
	3	0.56			
	4	0.6			
	5	0.77			
106	1	0.12	117	1	0.60
	2	0.15		2	0.79
	3	0.56			
	4	0.6			
	5	0.77			
107	1	0.12	118	1	0.60
	2	0.15		2	0.79
	3	0.56			
	4	0.6			
	5	0.77			

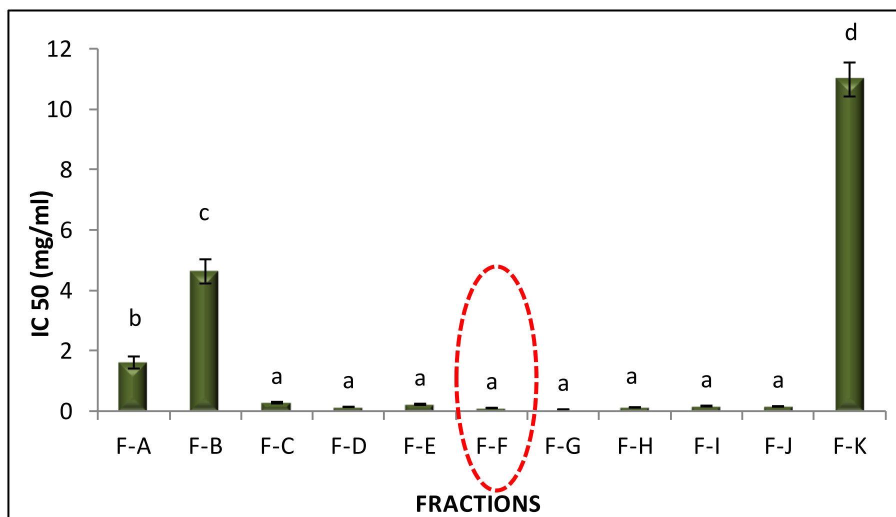


Fig 5.7.5: DPPH˙ scavenging activity of merged fractions of *M. paleacea* obtained from first column chromatograph. NB: Value with different letters (a, b, c, d etc) differ significantly ($p < 0.05$) from each other by Duncan's Multiple Test (DMRT).

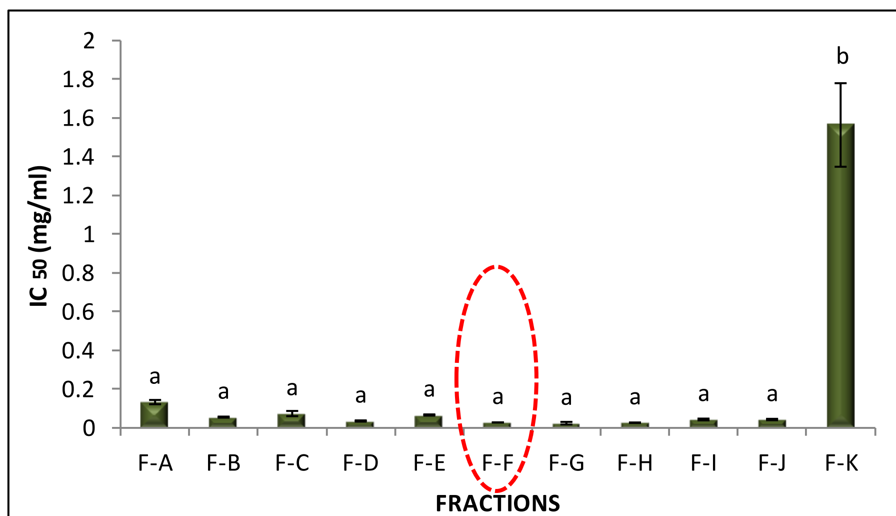


Fig 5.7.6: $ABTS^+$ scavenging activity of merged fractions of *M. paleacea* obtained from first column chromatograph. NB: Value with different letters (a, b, c, d etc) differ significantly ($p < 0.05$) from each other by Duncan's Multiple Test (DMRT).

NB: Bar within the ellipse indicates highest DPPH˙ and $ABTS^+$ activity.

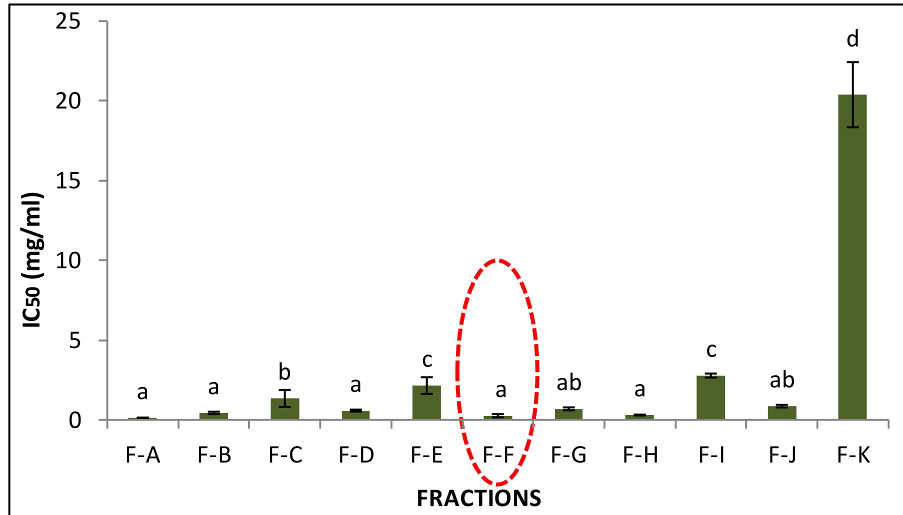


Fig 5.7.7: Anti-diabetic activity (α-glucosidase inhibitory) of merged fractions of *M. paleacea* obtained from first column chromatograph. NB: Value with different letters (a, b, c, d etc) differ significantly ($p < 0.05$) from each other by Duncan's Multiple Test (DMRT).

NB: Bar within the ellipse indicates highest anti-diabetic activity

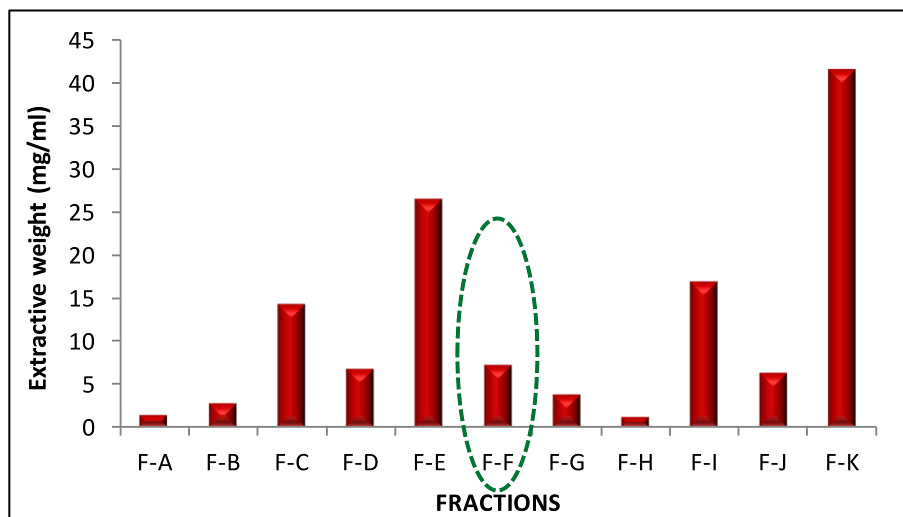


Fig 5.7.8: Extractive yield of the merged fractions of *M. paleacea* obtained from first column chromatograph

NB: Bar within the ellipse indicates highest extractive yield

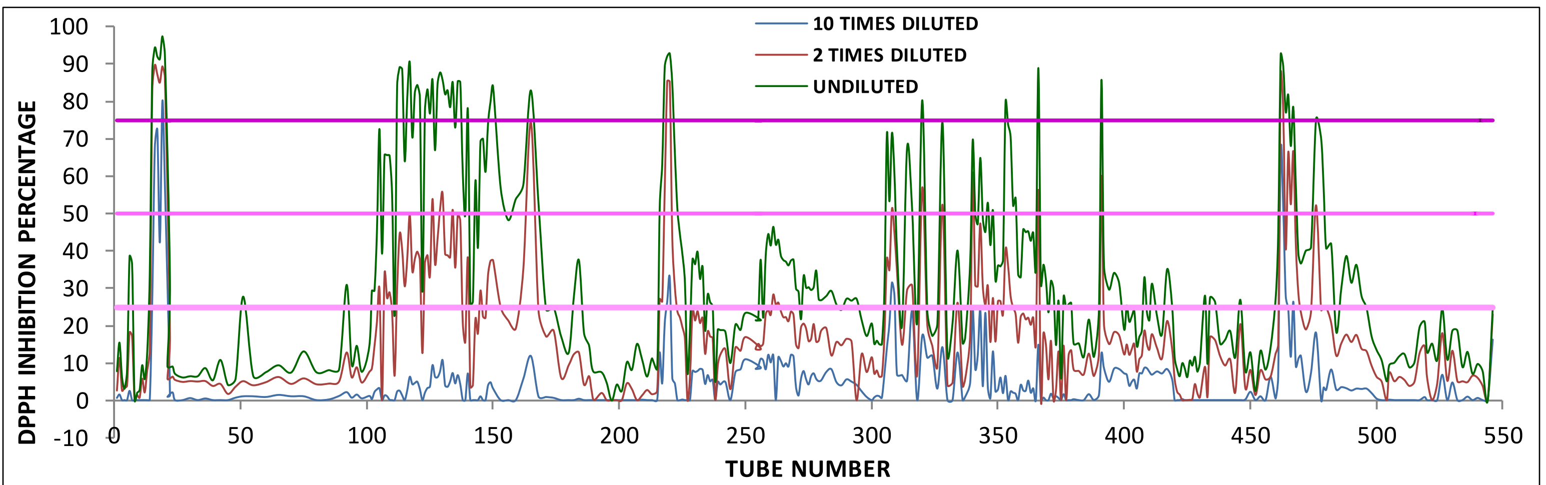


Fig 5.7.9 : DPPH˙ scavenging activity of fractions of *M. paleacea* obtained after second column chromatography

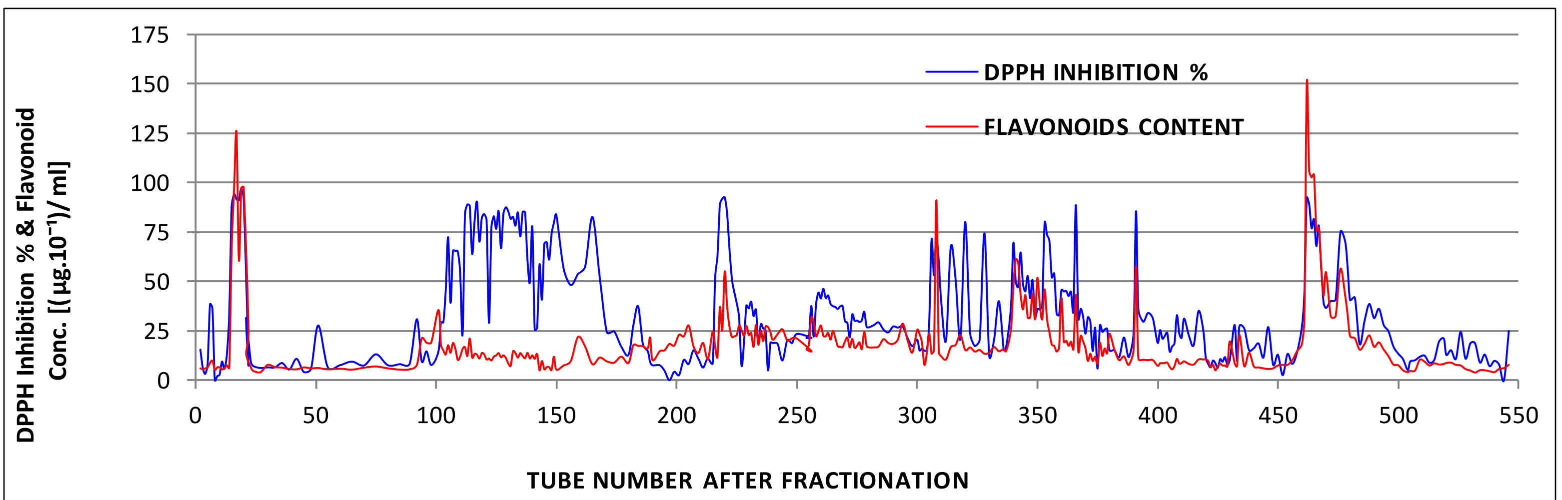


Fig 5.7.10: DPPH˙ scavenging activity and flavonoid content of fractions of *M. paleacea* obtained after second column chromatography

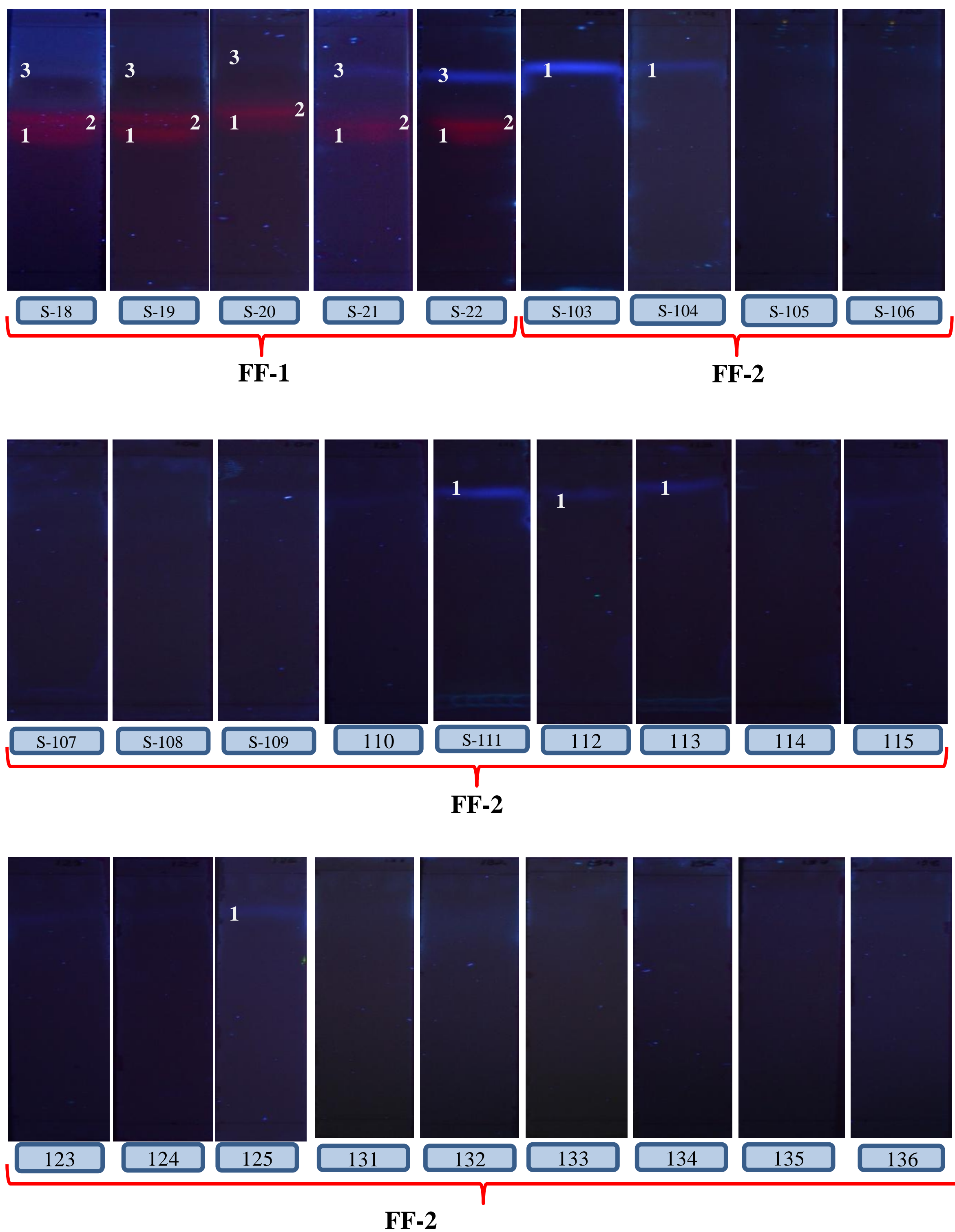


Fig 5.7.11: TLC profiling of bioactive fractions of *M. paleacea* obtained from second column chromatograph

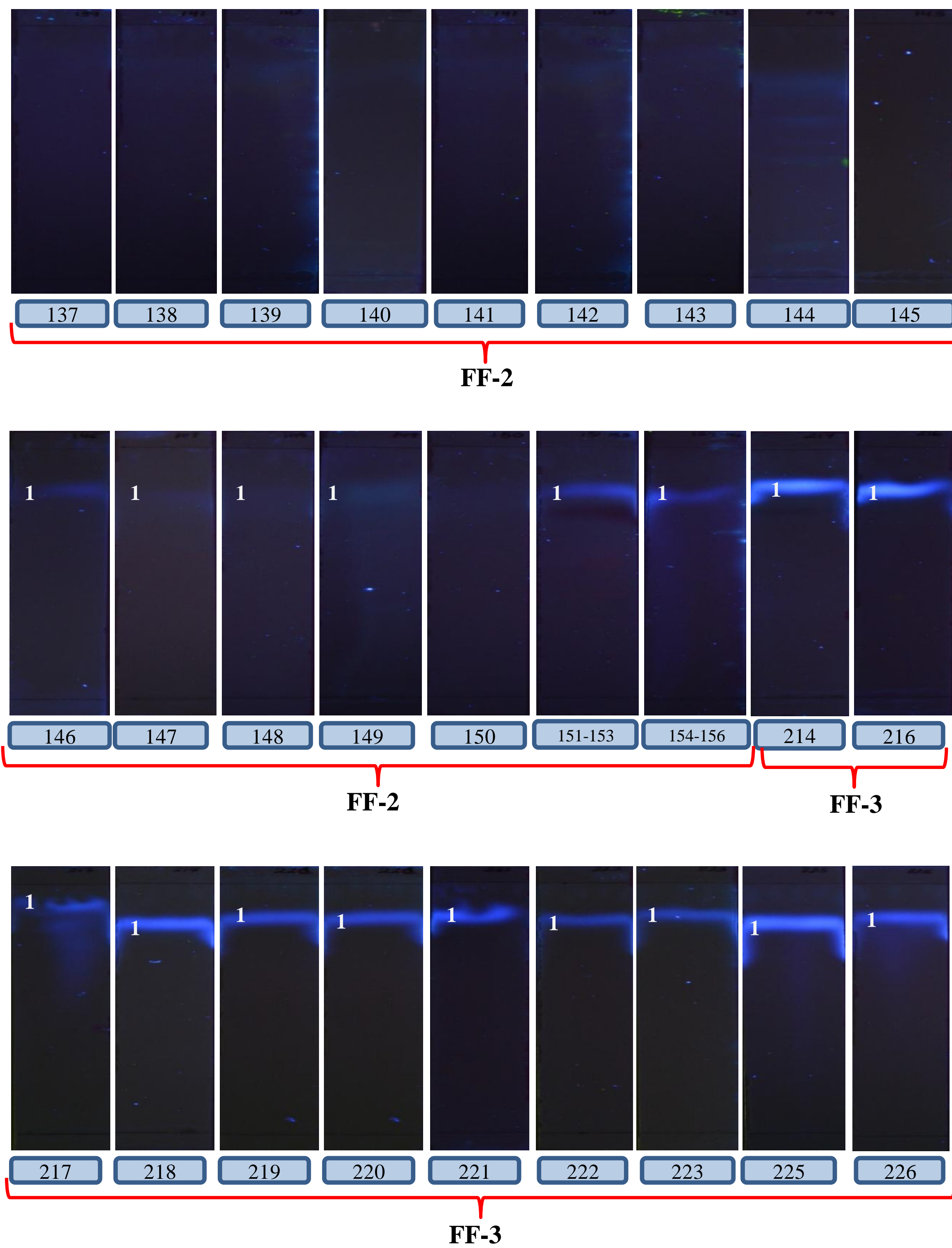


Fig 5.7.11: TLC profiling of bioactive fractions of *M. paleacea* obtained from second column chromatograph (Continuation)

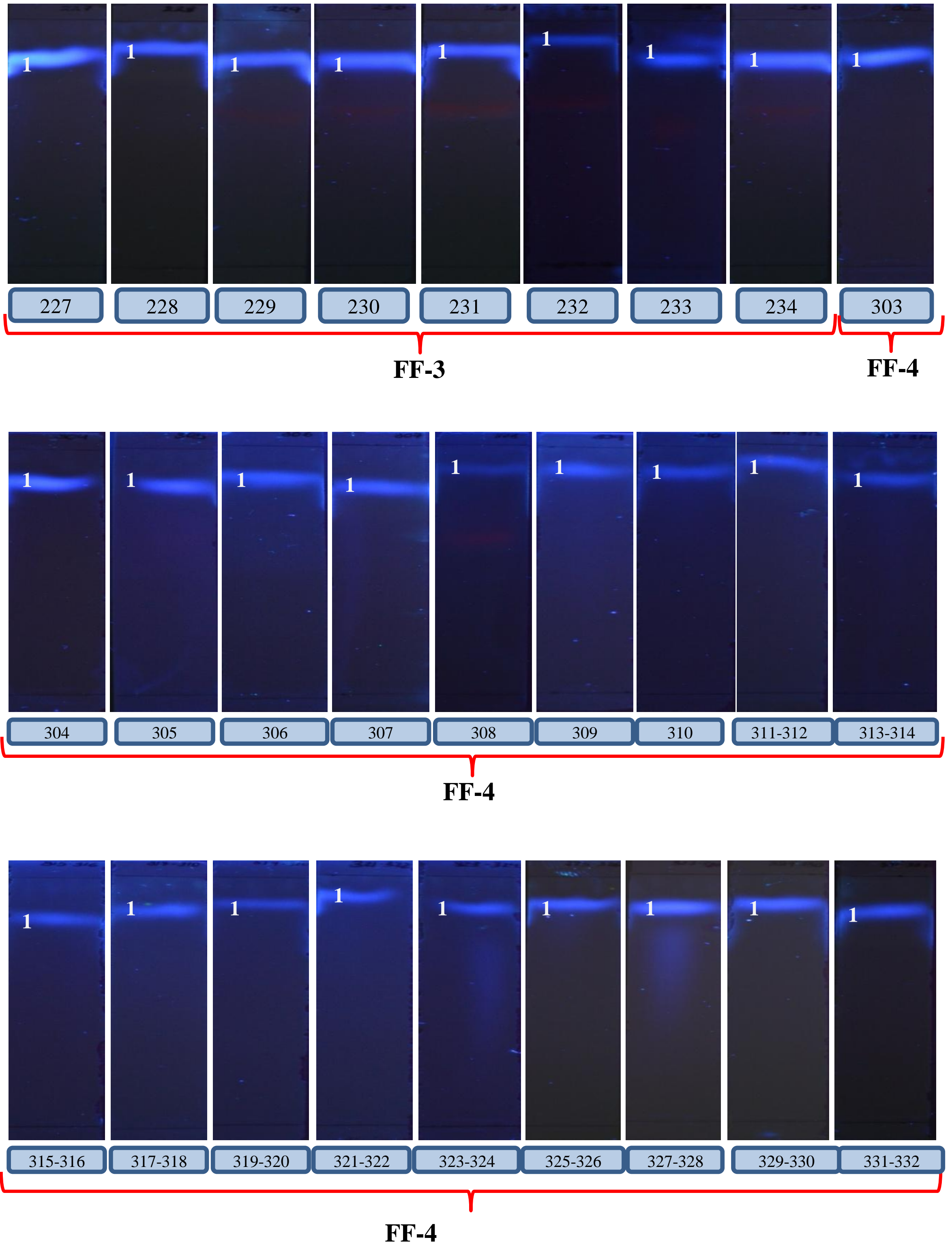
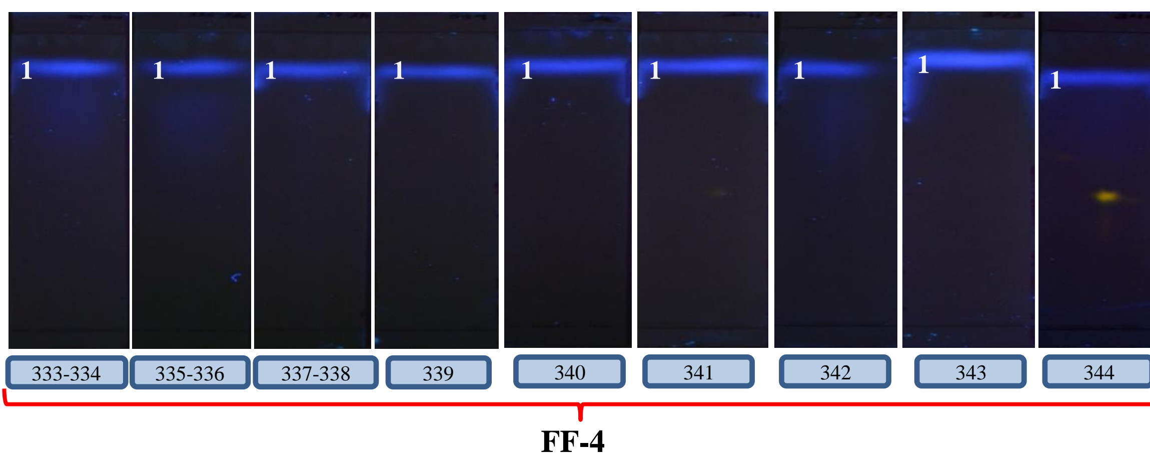
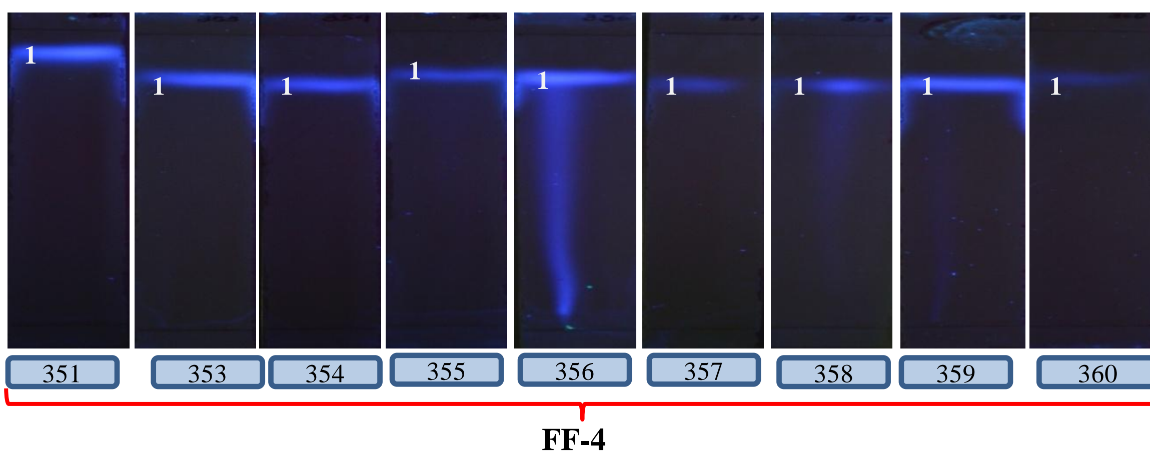


Fig 5.7.11: TLC profiling of bioactive fractions of *M. paleacea* obtained from second column chromatograph (Continuation).





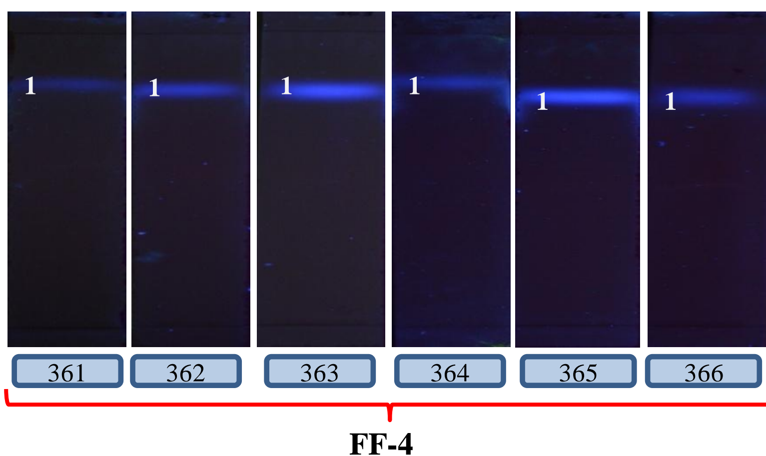
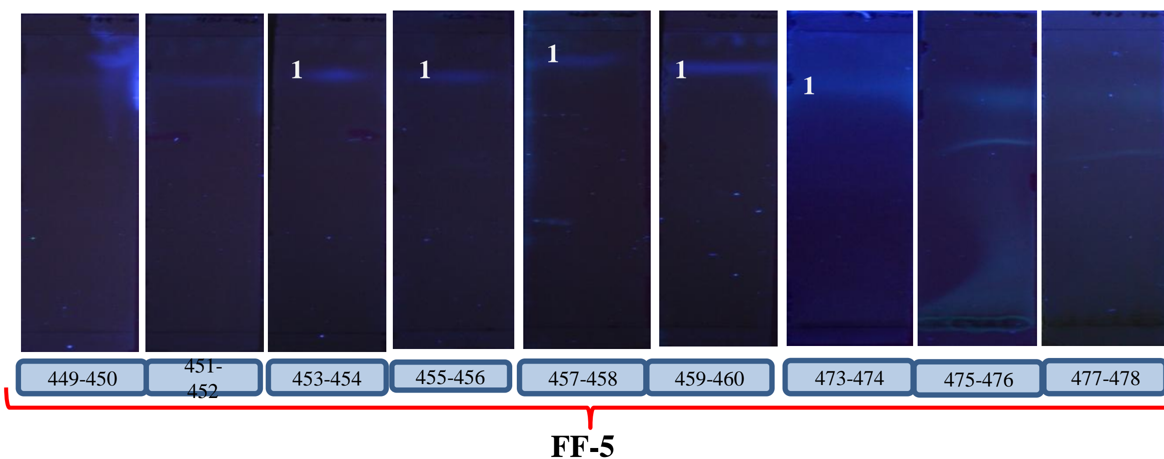


Fig 5.7.11: TLC profiling of bioactive fractions of *M. paleacea* obtained from second column chromatograph (Continuation).



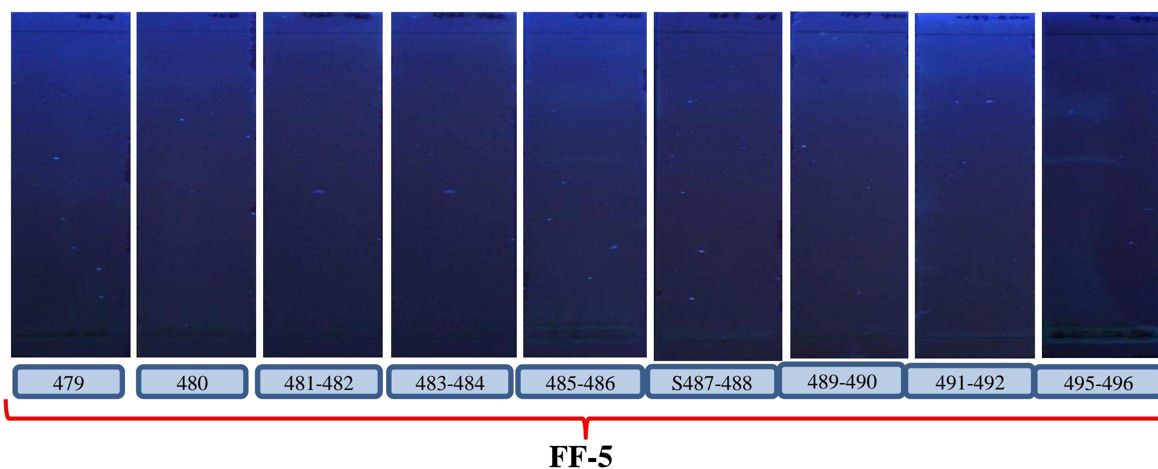


Fig 5.7.11: TLC profiling of bioactive fractions of *M. paleacea* obtained from second column chromatograph (Continuation).

Table 5.4: Retardation factors of different analytes present in bioactive fractions of *M. paleacea* obtained from second column chromatograph

Sub fractions	***Identity of bands***	***Rf***	***Sub fractions***	***Identity of bank***	***Rf***
18	1	0.54	144	1	0.13
	2	0.59		2	0.46
	3	0.79		3	0.63
				4	0.76
19	1	0.54	145	0	No bands
	2	0.59			
	3	0.79			
20	1	0.54	146	1	0.83
	2	0.59			
	3	0.79			
21	1	0.54	147	1	0.83
	2	0.59			
	3	0.79			
22	1	0.54	148	1	0.83
	2	0.59			
	3	0.79			
103	1	0.83	149	1	0.83
104	1	0.83	150	1	0.83
105	0	No bands	151-153	1	0.83
106	0	No bands	154-156	1	0.83
107	0	No bands	214	1	0.83
108	0	No bands	216	1	0.83
109	0	No bands	217	1	0.83
110	0	No bands	218	1	0.83
111	1	0.83	219	1	0.83
112	1	0.83	220	1	0.83
113	0	No bands	221	1	0.83
114	0	No bands	222	1	0.83
115	0	No bands	223	1	0.83
123	0	No bands	224	1	0.83
124	0	No bands	225	1	0.83
125	0	No bands	226	1	0.83
131	0	No bands	228	1	0.83
132	0	No bands	229	1	0.83
133	0	No bands	230	1	0.83
134	0	No bands	231	1	0.83
135	0	No bands	232	1	0.83
136	0	No bands	233	1	0.83
137	0	No bands	234	1	0.83
138	0	No bands	303	1	0.87
139	0	No bands	304	1	0.87
140	0	No bands	305	1	0.87
141	0	No bands	306	1	0.87
142	0	No bands	307	1	0.87
143	0	No bands	308	1	0.87

Table 5.4: Retardation factors of different analytes present in bioactive fractions of *M. paleacea* obtained from second column chromatograph (continuation)

Sub fractions	***Identity of bands***	***Rf***	***Sub fractions***	***Identity of bank***	***Rf***
309	1	0.87	477-478	0	No bands
310	1	0.87	479	0	No bands
311-312	1	0.87	480	0	No bands
313-314	1	0.87	481-482	0	No bands
315-316	1	0.87	483-484	0	No bands
317-318	1	0.87	485-486	0	No bands
319-320	1	0.87	487-488	0	No bands
321-322	1	0.87	489-490	0	No bands
323-324	1	0.87	491-492	0	No bands
325-326	1	0.87	495-496	0	No bands
327-328	1	0.87	497-498	0	No bands
329-330	1	0.87	499-500	0	No bands
331-332	1	0.87			
333-334	1	0.87			
335-336	1	0.87			
337-338	1	0.87			
339	1	0.87			
340	1	0.87			
341	1	0.87			
342	1	0.87			
343	1	0.87			
344	1	0.87			
351	1	0.87			
353	1	0.87			
354	1	0.87			
355	1	0.87			
356	1	0.87			
357	1	0.87			
358	1	0.87			
359	1	0.87			
360	1	0.87			
361	1	0.87			
362	1	0.87			
363	1	0.87			
364	1	0.87			
365	1	0.87			
366	1	0.87			
449-450	0	No bands			
451-452	0	No bands			
453-454	1	0.87			
455-456	1	0.87			
457-458	1	0.87			
459-460	1	0.87			
475-476	0	No bands			

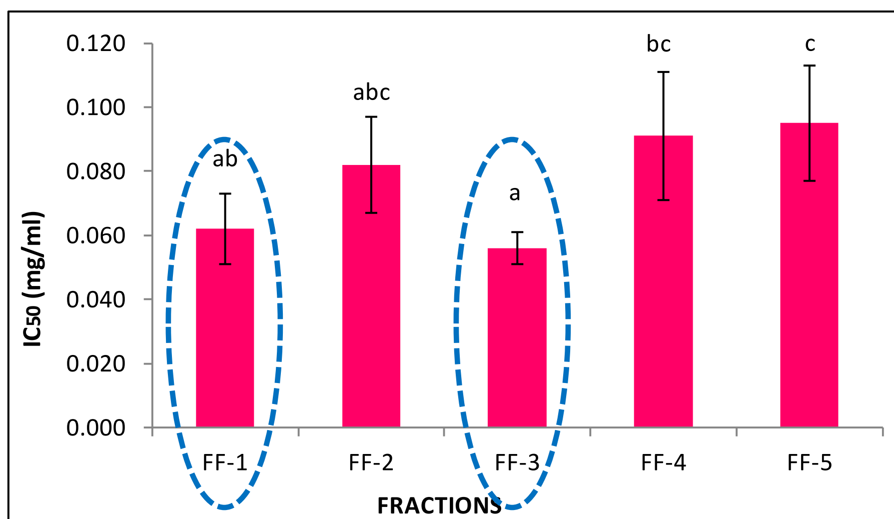


Fig 5.7.12: DPPH˙ scavenging activity of merged fractions of *M. paleacea* obtained from second column chromatograph . NB: Value with different letters (a, b, c, d etc) differ significantly ($p < 0.05$) from each other by Duncan's Multiple Test (DMRT).

NB: Bar within the ellipse indicates highest DPPH˙ scavenging activity

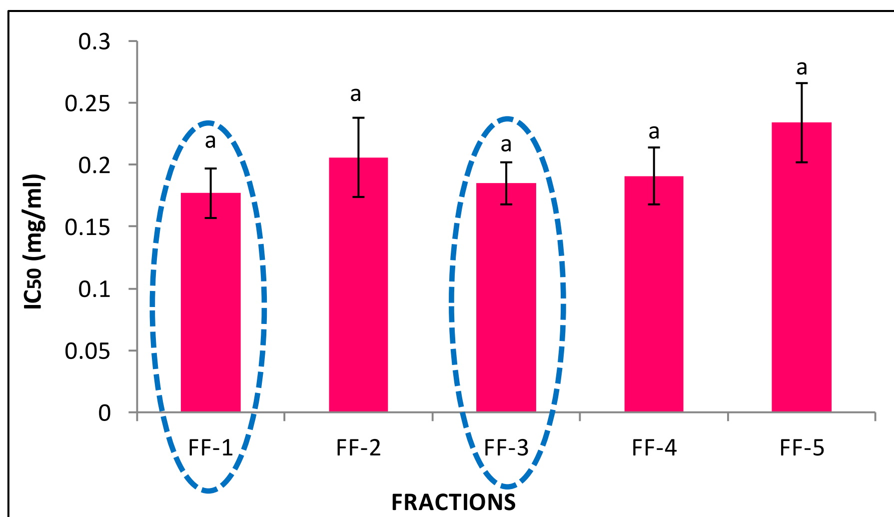


Fig 5.7.13: α-glucosidase inhibitory activity of merged fractions of *M. paleacea* obtained from second column chromatograph . NB: Value with different letters (a, b, c, d etc) differ significantly ($p < 0.05$) from each other by Duncan's Multiple Test (DMRT).

NB: Bar within the ellipse indicates highest DPPH˙ scavenging activity

and active sub fractions were merged together on the basis of their TLC profiles into five main fractions as follows: FF-1, FF-2, FF-3, FF-4 and FF-5 (Fig 5.7.11) Among five main fractions, FF-1 and FF-3 were selected on the basis of their high antioxidant and anti-diabetic activities. After that fractions FF-1 and FF-3 were processed through Gas chromatography mass spectrometry (GC-MS); the chromatogram of the bioactive fractions are presented in Fig 5.7.12 to Fig 5.7.23. The library matching (NIST Library) of mass peaks of fraction FF-1 showed the presence of nine compounds and that of fraction FF-3 showed the presence of nine phyto compounds. Presence of phytochemicals such as phenols, flavonoids, terpenes, alkanes and flavones were found in FF-1 and FF-3 which might be responsible for the antioxidant and anti-diabetic activities displayed by *M. paleacea* (Table 5.5 and Table 5.6). Network pharmacology is an important approach in drug development and discovery process. After the identification of bioactive compound through GC-MS analysis and NIST Library matching, the underlying mechanism of action of these compounds at cellular level was studied by network pharmacology followed by KEGG Metabolic Pathway analysis.

Phenol, 2, 6 bis (1,1-dimethylethyl)-4-ethyl present in fraction FF-3 is a phenylpropanes that are active antioxidants and anti-fungal agents (Varsha *et al.*, 2015). Different forms of terpenes (mono, di, tri, sesqui) were also found to be present in the analyzed fractions. β-Caryophyllene, α-Myrcene, 3-Cyclohexen-1-ol,4-methyl-1-(1-methylethyl), Phytol, Pimaric acid, Palustric acid were detected in the analyzed fractions. Phyto-compounds like 4-H-Benzopyran-4-one,5-hydroxyl-7-methoxy-2-phenyl; 4H-1-Benzopyran-4-one,5-hydroxy-2-(-4 methoxypheny) and 4H-1-Benzopyran-4-one, 7-hydroxy-2 phenyl were also detected which belongs to the class of compound flavonoids.. Apart from this, hydrocarbons like 1-docosene and 1-octadecene were found to be present in the active fractions FF-1 and FF-3. 1-docosene and 1-octadecene detected in the extracts are reported to have important pharmacological activities (Kuppuswamy *et al.*, 2013). Other phytochemical that were present in the fractions FF-1 and FF-3 were 2-Cyclohexen-3,6-diol-1-one, 2-tetradecanoyl; Methyl 2-(4-hydroxy-3-isopropylbenzoyl) benzoate; 2H-1-benzopyran-2-one,7 (diethylamino); Estra-1,3,5 (10), 6-tetraene-3,17-diol, diacetate and Androstan-4-en,3,17-diol-11-one, 9-thiocyanato. No biological activities have been reported for these compounds till date. For drug discovery and development process network pharmacology plays a very important role. After the identification of bioactive compound through GC-MS analysis and NIST Library matching, the underlying mechanism of action of

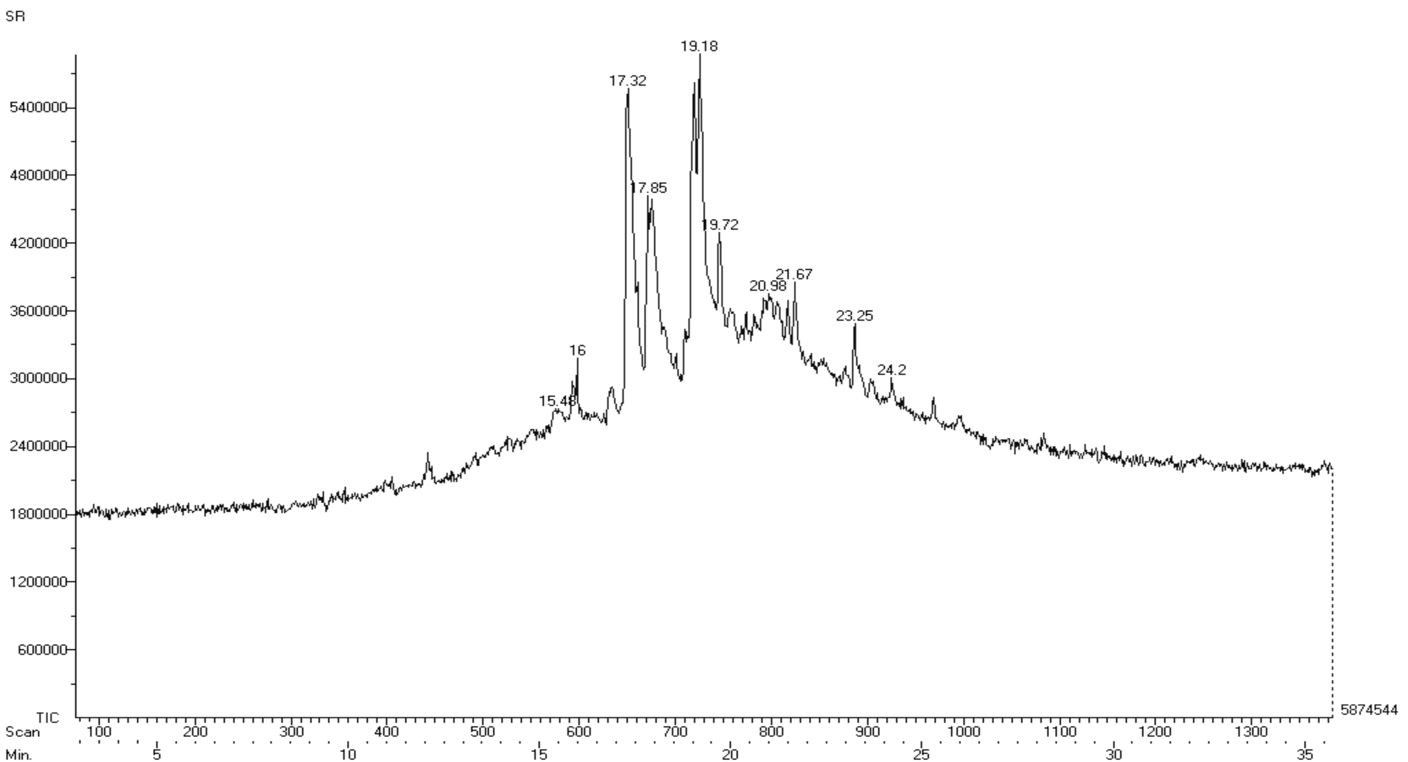


Fig 5.7.14: HR- GCMS scanning chromatogram of fraction FF-1 of *M. paleacea*

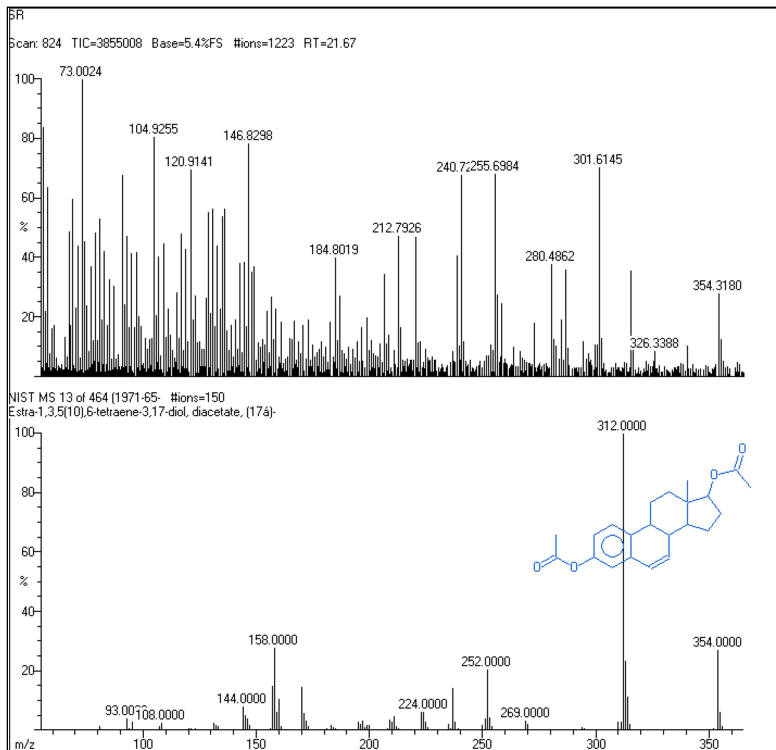


Fig 5.7.15: Structure and MS spectrum of (17á), Estra-1,3,5 (10), 6- tetraene- 3,17- diol diacetate compared with NIST Library Spectral Database

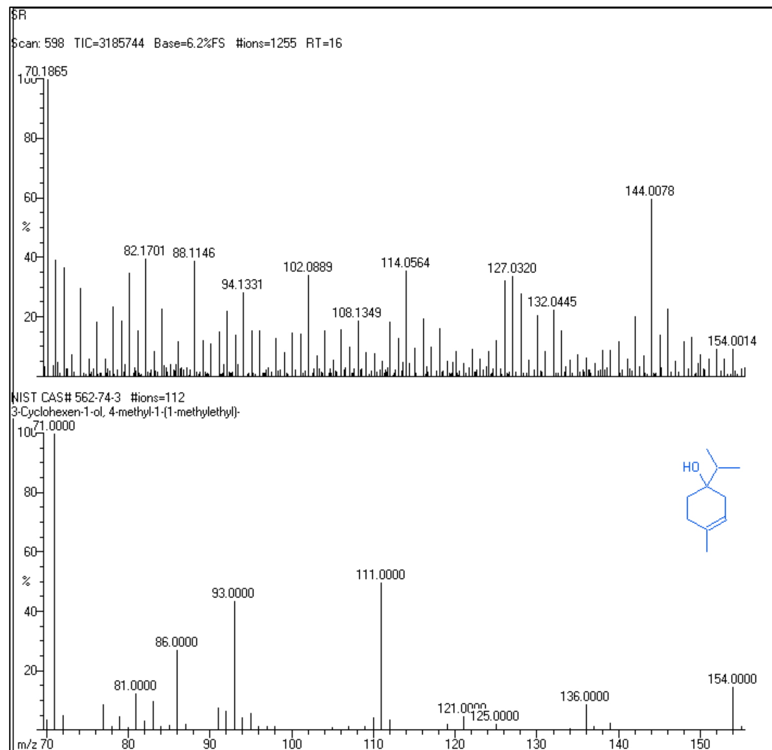


Fig 5.7. 16: Structure and MS spectrum of 4-methyl -1-(1-methylethyl) 3- Cyclohexen -1 ol compared with NIST Library Spectral Database

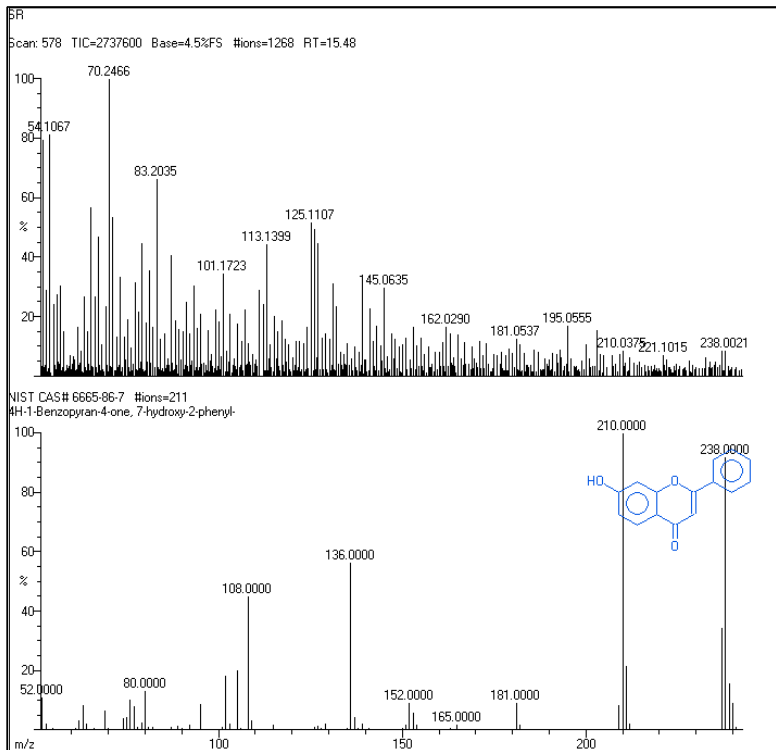


Fig 5.7.17: Structure and MS spectrum of 7-hydroxy-2-phenyl 4H-1- Benzopyran-4- one compared with NIST Library Spectral Database

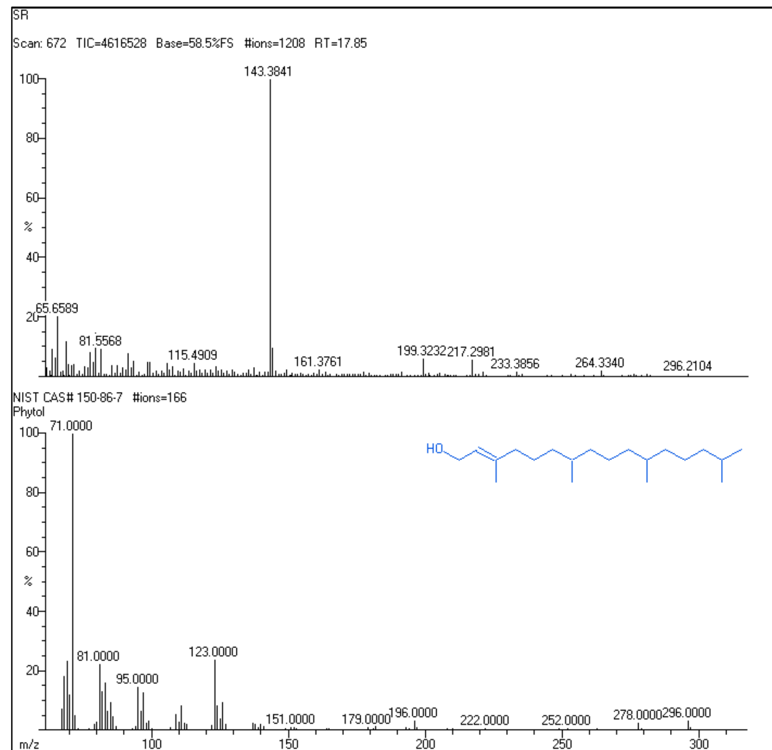


Fig 5.7.18: Structure and MS spectrum of Phytol compared with NIST Library Spectral Database

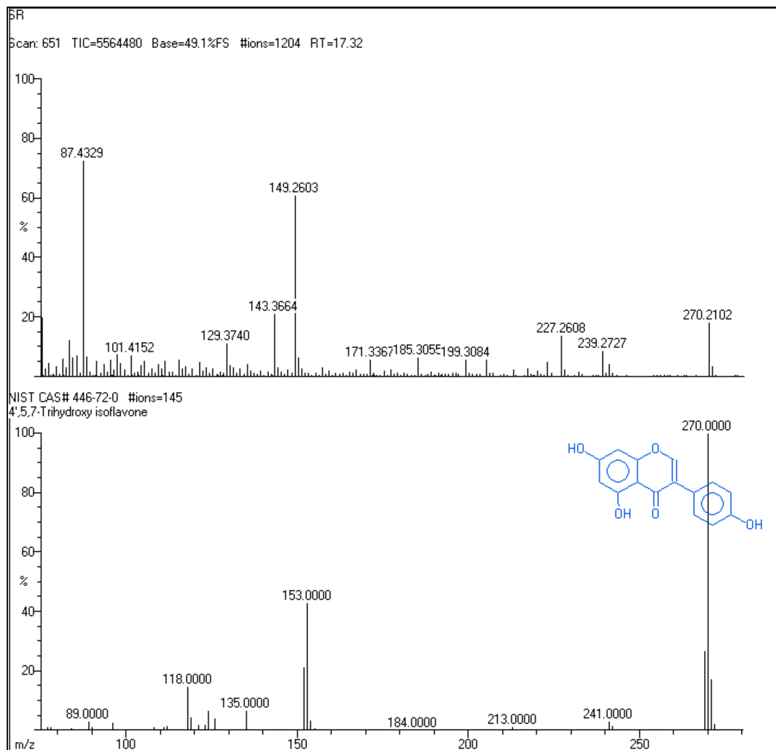


Fig 5.7.19 : Structure and MS spectrum of 4’, 5, 7-Trihydroxy isoflavone compared with NIST Library Spectral Database

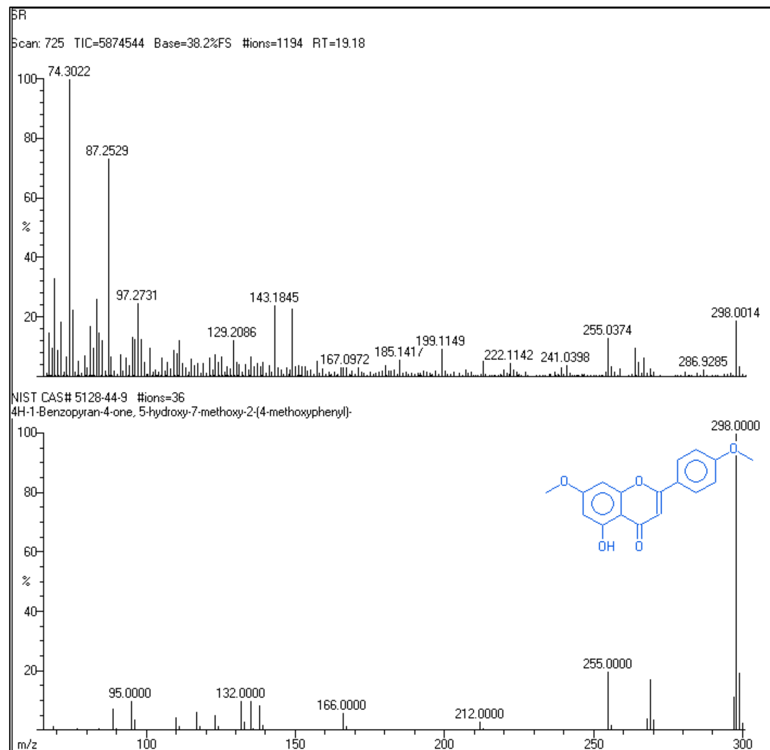


Fig 5.7.20: Structure and MS spectrum of 5- hydroxy-7-methoxy-2-(4-methoxyphenyl) 4H-1- benzopyran-4-one compared with NIST Library Spectral Database

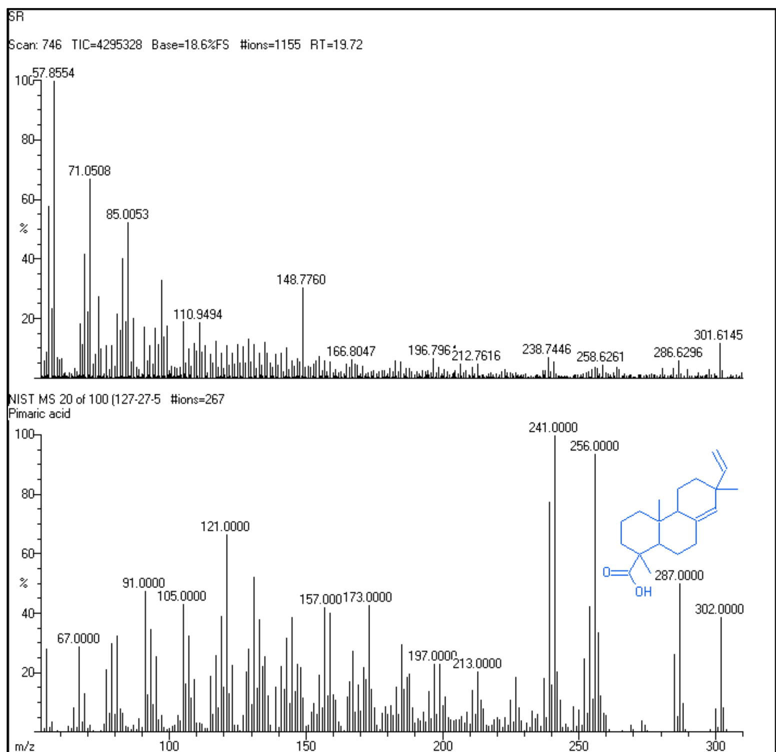


Fig 5.7.21: Structure and MS spectrum of Pimaric acid compared with NIST Library Spectral Database

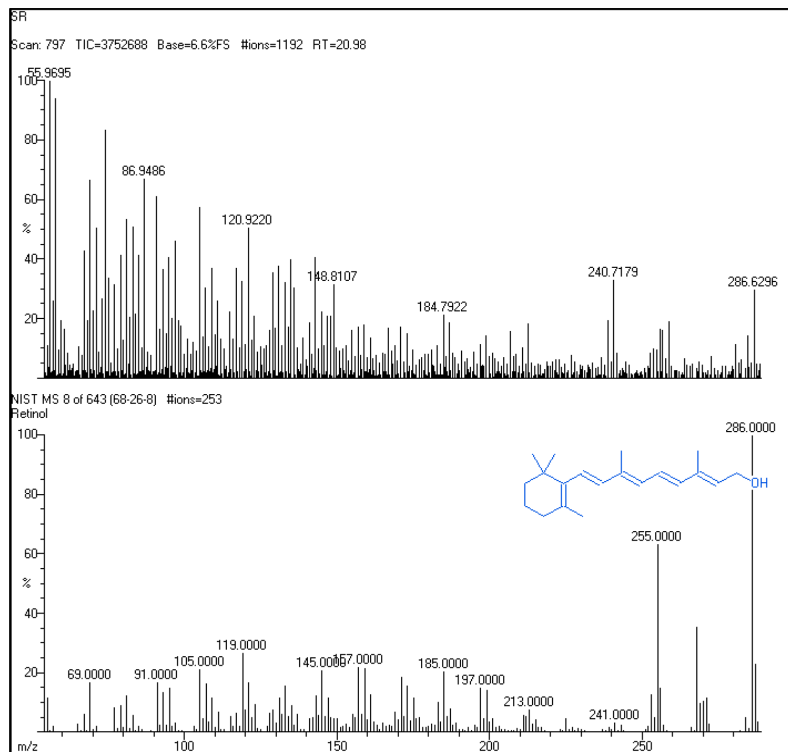


Fig 5.7.22: Structure and MS spectrum of Retinol compared with NIST Library Spectral Database

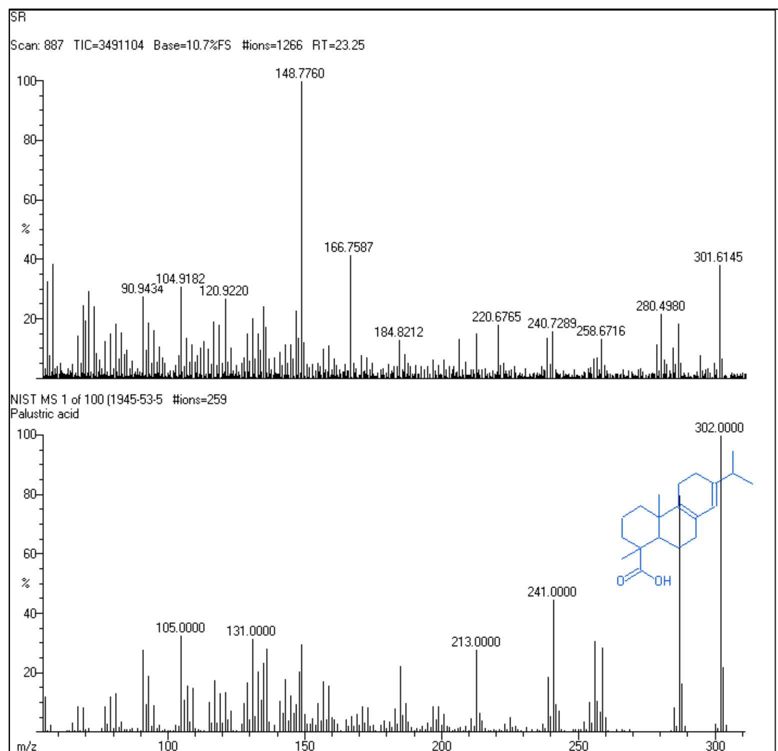


Fig 5.7.23: Structure and MS spectrum of Palustric acid compared with NIST Library Spectral Database

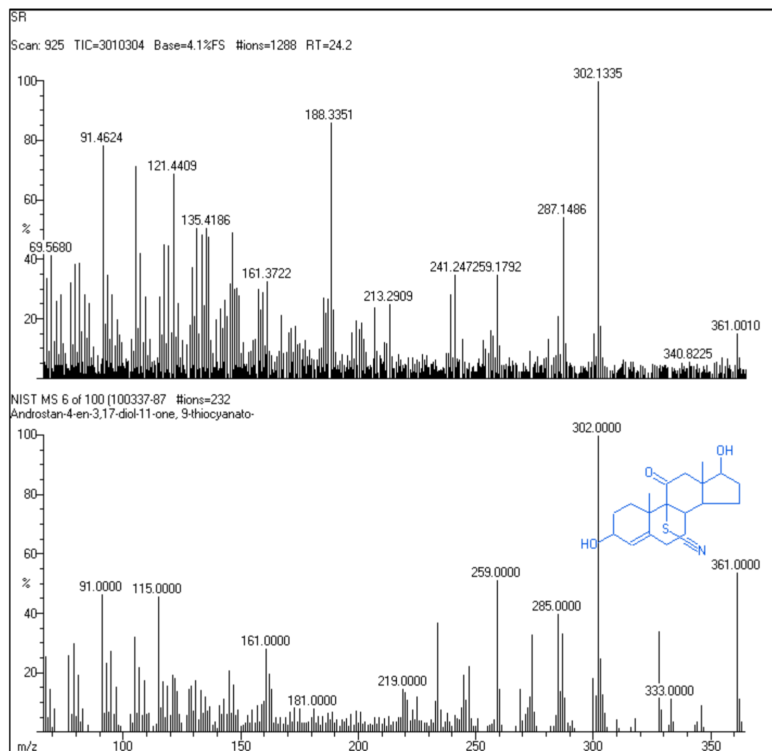


Fig 5.7.24: Structure and MS spectrum of 9-thiocyanato Androstan-4-en-3,17- diol- 11-one compared with NIST Library Spectral Database

Table 5.5: List of phytochemicals present in fraction FF1 of *M. paleacea* and their bioactivities

Sl. no	***Compound name***	***RT***	***Molecular formula***	***Molecular weight***	***Percentage amount***	***Compound nature***	***Bioactivity***
1	(17á), Estra-1,3,5 (10), 6-tetraene-3,17-diol,diacetate	21.67	$C_{22}H_{26}O_4$	354.446	11%	Not found	No activities reported
2	4-methyl-1 -(1-methylethyl) 3-cyclohexen-1-ol	16	$C_{10}H_{18}O$	154.249	9%	Monoterpenoids.	antioxidant (Chung IM, 2011)
3	7-hydroxy-2 phenyl 4H-1-Benzopyran-4-one	15.48	$C_{15}H_{10}O_3$	238.242	7%	Isoflavone	estrogenic activity (Vitale DC, 2013)
4	Phytol	17.85	$C_{20}H_{40}O$	296.539	13%	Diterpene	antinociceptive (Santos *et* al., 2013) antioxidant (Santos *et* al., 2013
5	4',5,7 trihydroxy isoflavone	17.32	$C_{15}H_{10}O_5$	270.24	15%	Isoflavone	antioxidant (Dowling *et al.*, 2010)
6	5-hydroxy-7-methoxy-2-(4-methoxyphenyl)-4H-1-Benzopyran-4-one	19.18	$C_{17}H_{14}O_5$	298.290	16%	flavonoid	estrogenic activity (Vitale DC, 2013); antioxidant (Dowling *et al.*, 2010)
7	Pimaric acid	19.72	$C_{20}H_{30}O_2$	302.458	12%	Diterpene	antimicrobial (Ali *et al.*, 2012)
8	Palustric acid	23.25	$C_{20}H_{30}O_2$	302.458	10%	Diterpene	anti-microbial activity (Savluchinske-Feio *et al.*, 2006)
9	9-thiocyanato Androstan-4-en-3,17-diol-11-one	24.2	$C_{20}H_{27}NO_3S$	361.498	8%	Not found	No activities reported

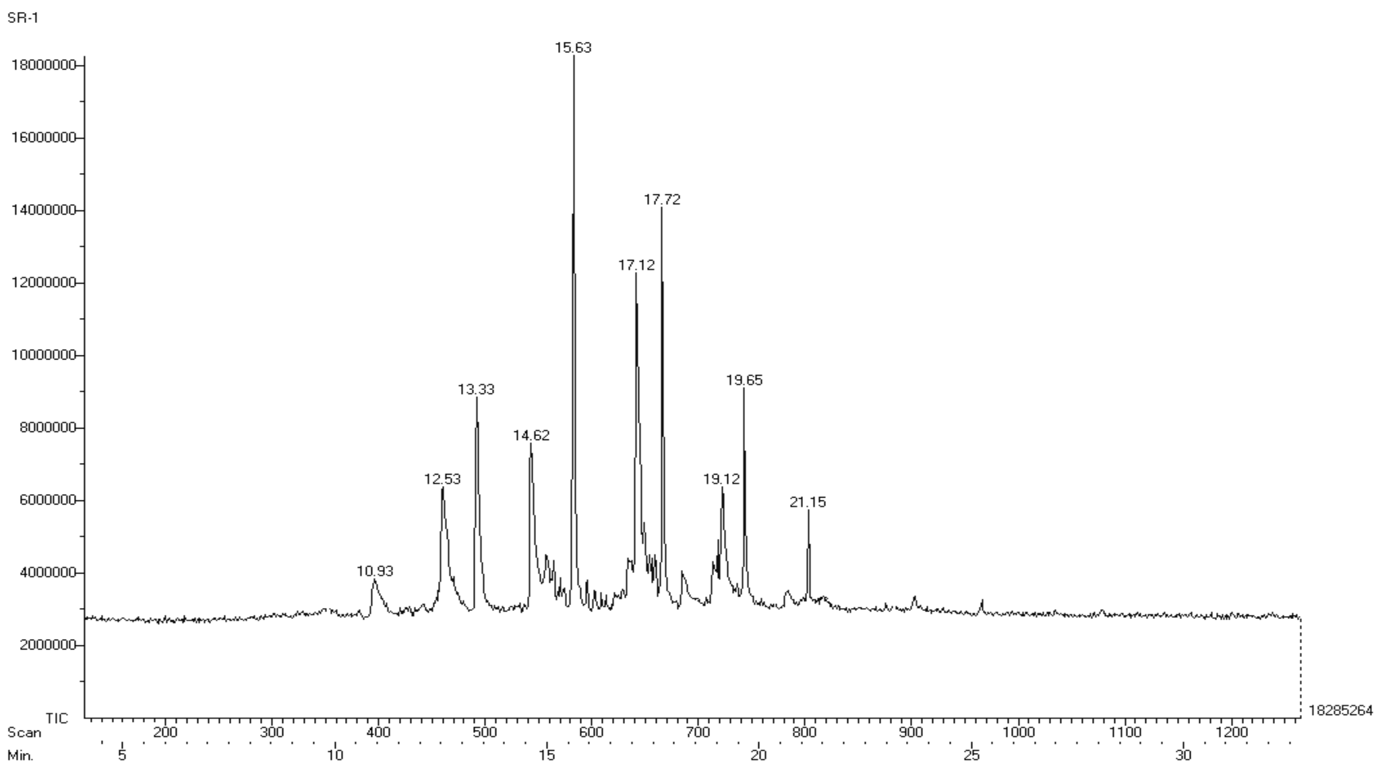


Fig 5.7.25: HR- GCMS scanning chromatogram of fraction FF-3 of *M. paleacea*

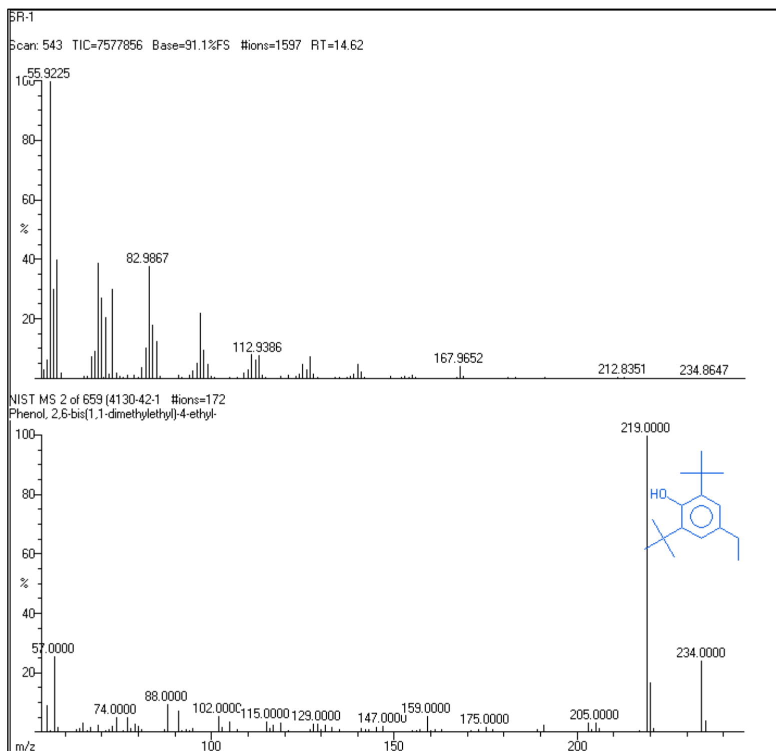


Fig 5.7.26: Structure and MS spectrum of 2,6-bis (1,1-dimethylethyl)-4- ethyl Phenol compared with NIST Library Spectral Database

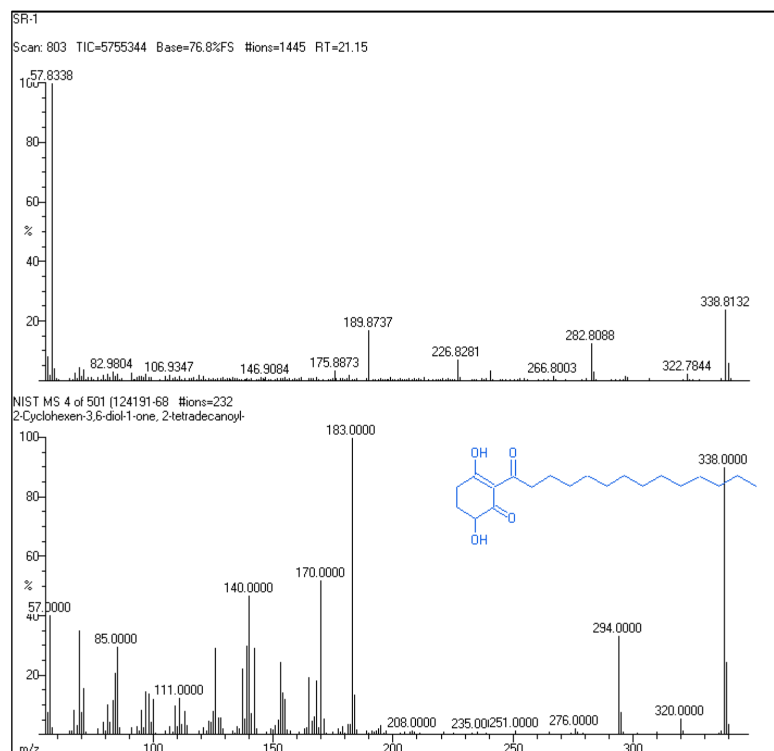


Fig 5.7.27: Structure and MS spectrum of 2-tetradecanoyl-2-Cyclohexen-3,6-diol-1-one compared with NIST Library Spectral Database

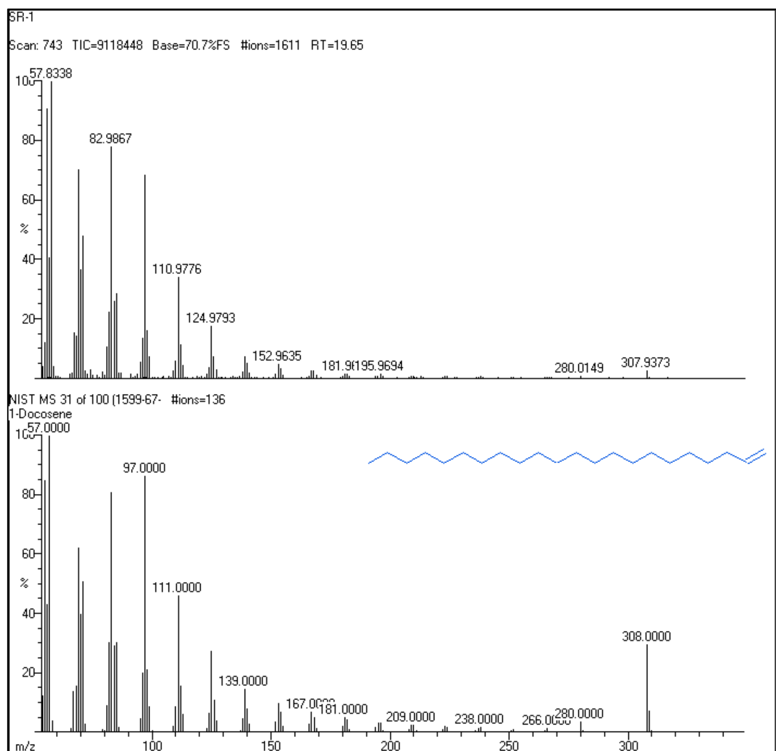


Fig 5.7.28: Structure and MS spectrum of 1-Docosene compared with NIST Library Spectral Database

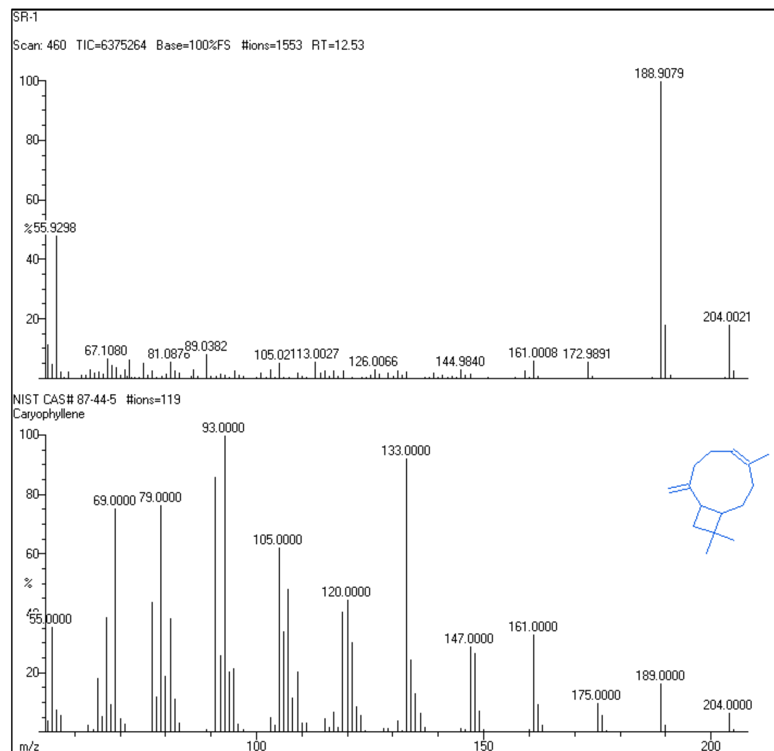


Fig 5.7.29: Structure and MS spectrum of Caryophyllene compared with NIST Library Spectral Database

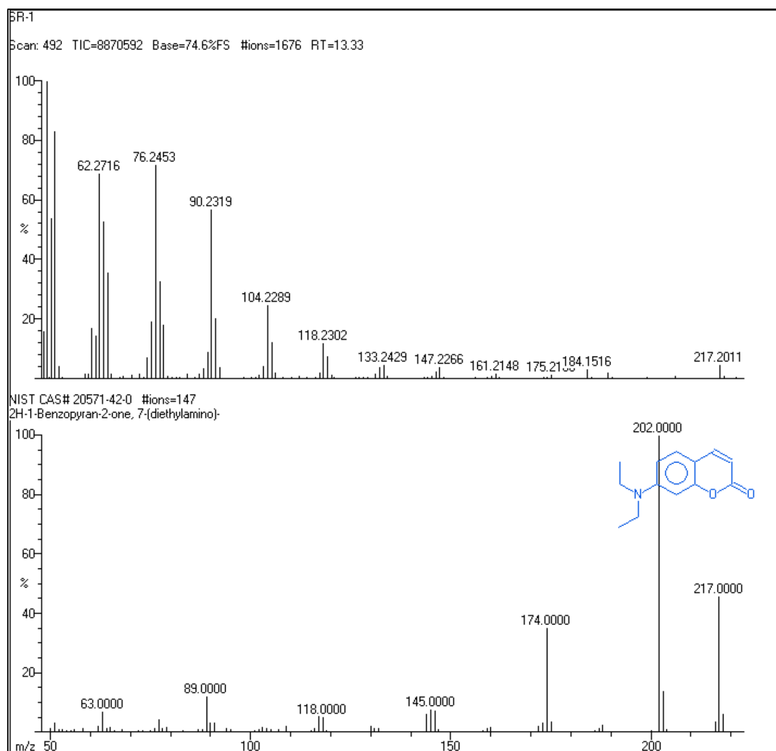


Fig 5.7.30: Structure and MS spectrum of 7-(diethylamino) -2H-1-Benzopyran-2-one compared with NIST Library Spectral Database

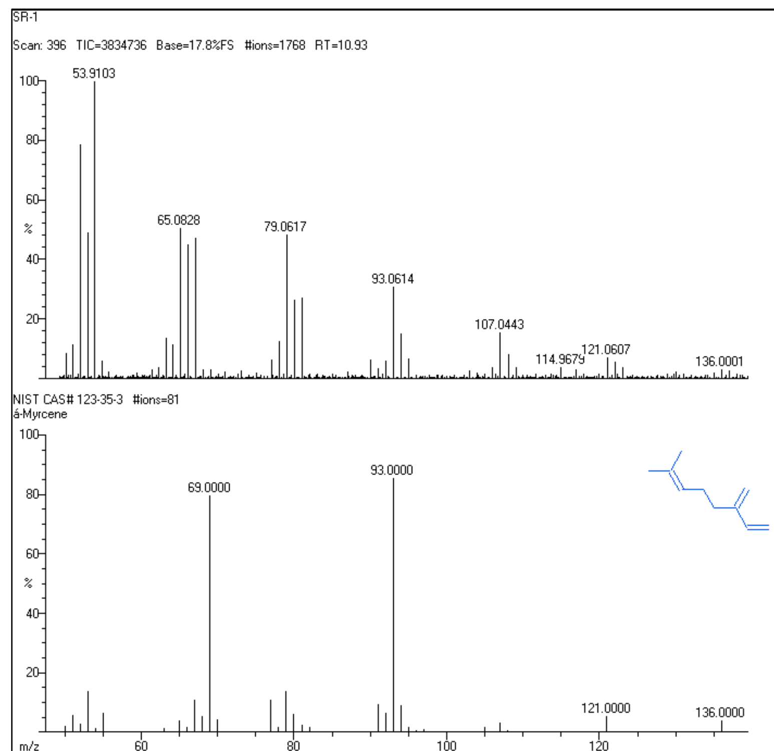


Fig 5.7.31: Structure and MS spectrum of α-Myrcene compared with NIST Library Spectral Database

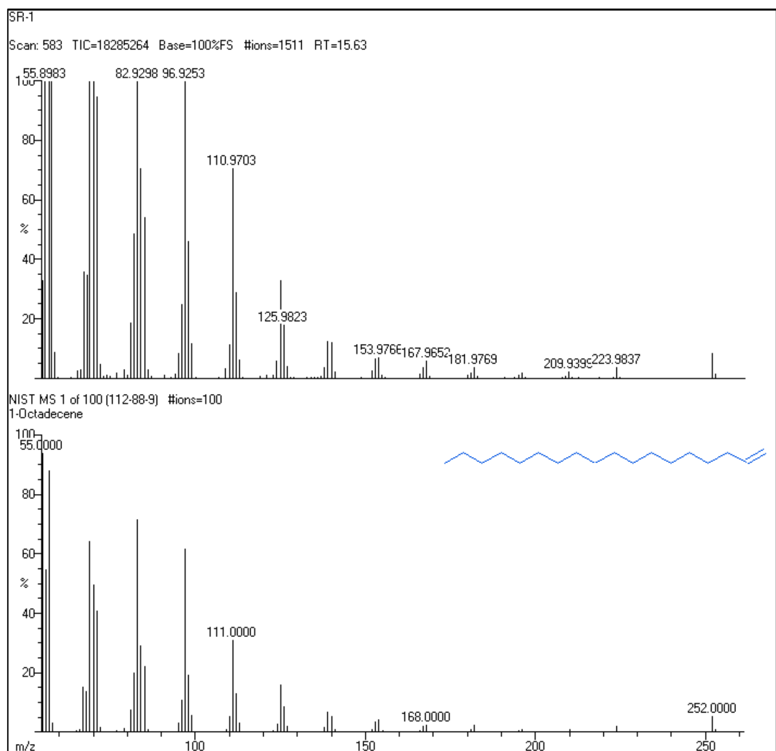


Fig 5.7.32: Structure and MS spectrum of 1-Octadecene compared with NIST Library Spectral Database

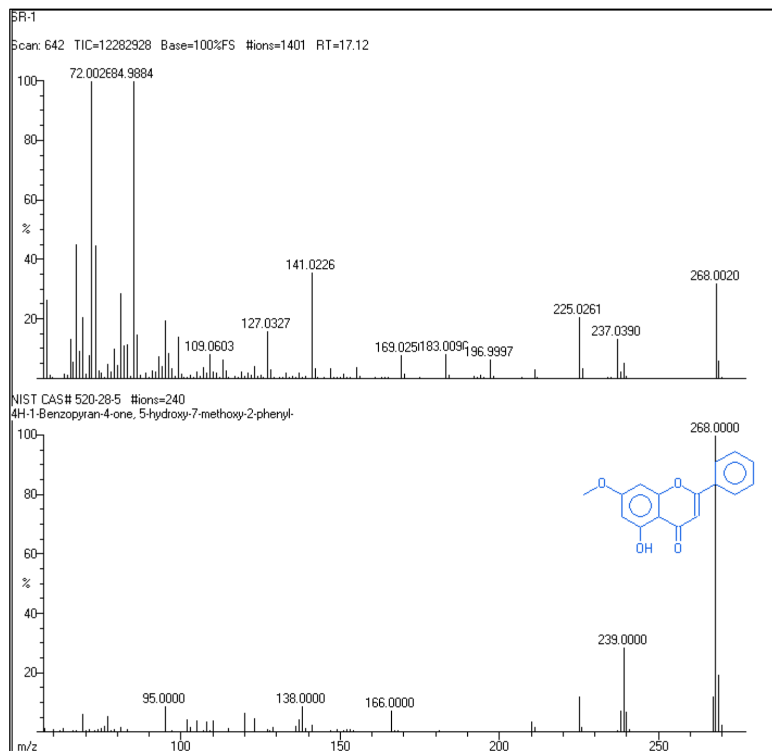


Fig 5.7.33: Structure and MS spectrum of 4H-1-Benzopyran-4-one, 5-hydroxy-7-methoxy-2-phenyl compared with NIST Library Spectral Database

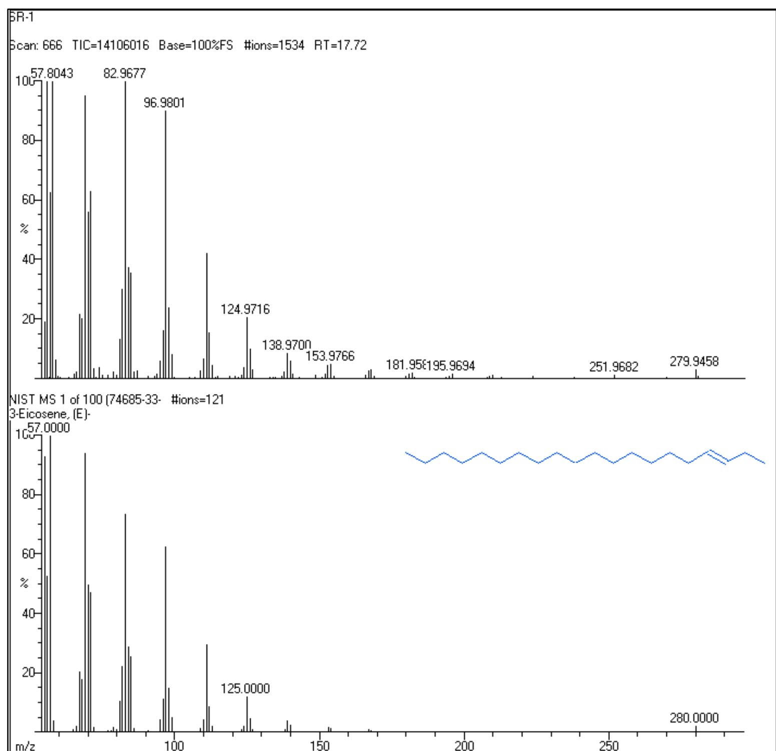


Fig 5.7.34: Structure and MS spectrum of (9E), 3-Eicosene compared with NIST Library Spectral Database

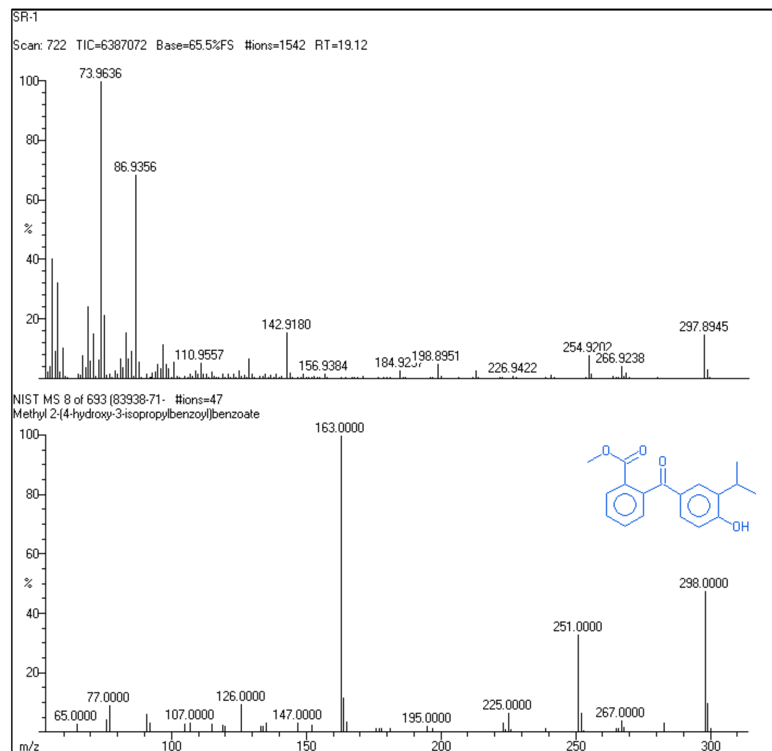


Fig 5.7.35: Structure and MS spectrum of Methyl 2-(4-hydroxy-3-isopropyl) benzoate compared with NIST Library Spectral Database

Table 5.6: List of phytochemicals present in fraction FF3 of *M. paleacea* and their bioactivities

Sl no	*Compound name*	*RT*	*Molecular formula*	*Molecular weight*	*Percentage amount*	*Compound nature*	*Bioactivity*
1	2,6-bis (1,1-dimethylethyl)-4-ethyl phehol	14.62	$C_{16}H_{26}O$	234.377	8%	Phenylpropanes	Antioxidant (KKet al., 2015
2	2-tetradecanoyl-2-Cyclohexen-3,6-diol-1-one	21.15	Not found	Not found	6%	Not found	No activities reported
3	1-docosene	19.65	$C_{22}H_{44}$	308.594	10%	aliphatic hydrocarbons.	No activities reported
4	beta-Caryophyllene	12.53	$C_{15}H_{24}$	204.357	7%	sesquiterpene	anticancer activities
5	7-(diethylamino) 2H-1-Benzopyran-2-one	13.33	$C_{15}H_{17}O_3$	259.305	10%	Not found	No activities reported
6	Myrecene	10.93	$C_{10}H_{16}$	136.238	4%	Myrcene	anti-inflammatory properties [22] and has been widely used in pharmaceutical and cosmetic product12)
7	1-octadecene	15.63	$C_{18}H_{36}$	252.486	20%	alkane	anti-bacterial (20,21)
8	5-hydroxy-7-methoxy-2-phenyl 4H-1-benzopyran 4 one	17.12	$C_{18}H_{12}O_4$	268.264	13%	flavonoids	anti-mitotic (Hadjeri et al., 2004), anti-bacterial (Tracanna et al., 2010)
9	3-Eicosene	17.72	$C_{20}H_{40}$	280.531	15%	aliphatic hydrocarbons	anti-microbial (Kuppuswamy et al., 2013)
10	Methyl 2-(4-hydroxy-3-isopropyllbenzoyl) benzoate	19.12	$C_8H_7NO_5$	197.146	7%	Not found	No activities reported

these compounds at cellular level was studied by network pharmacology followed by KEGG Metabolic Pathway analysis.

5.7.1 Network pharmacology

5.7.1a Identification of phytochemical compound

Active phytochemicals present on *M. paleacea* were identified by Gas Chromatography-Mass Spectrometry (GC-MS) of bioactive fractions (FF-1 and FF-3) obtained from column chromatography. In total 19 phyto-compounds have been identified from the two bioactive fractions of *M. paleacea*. Their name, unique ID and canonical smiles are presented in table 5.7 and table 5.8.

5.7.1b Protein target compilation

The therapeutic potential of any phyto-compound depends on the number of proteins that the particular compound targets. To determine the therapeutic potential of identified compounds a network analysis of phytochemicals and their protein targets was performed. In total 81 unique human proteins was targeted by the phytochemicals. Among ninteen phytochemicals under study, human protein was potentially targeted by fifteen phytochemicals. All the protein targets of studied phytochemicals were represented in table 5.9 along with their gene ID. Interaction between target proteins of bioactive phytochemicalas by suing STRING Interactome Database is presented figure 5.7.3

Table 5.7: List of phytochemicals present in FF1 of *M. paleacea* and their canonical smile and Pubchem ID

Sl. No	*Phytochemical*	*Canonical smile*	*PubChem CID*	*Compound Class*
1	(17α)-estra-1,3,5(10),6-tetraene-3,17-diol, diacetate	[H]C12CCC3C(C=CC4=C3C=CC(OC(C)=O)=C4)C1CCC2OC(C)=O	Not found	Not found
2	4-methyl-1 -(1-methylethyl), 3-cyclohexen-1-ol	CC1=CCC(CC1)(C(C)C)O	11230	Monoterpenoids
3	7-hydroxy-2-phenyl, 4H-1-Benzopyran-4-one	C1=CC=C(C=C1)C2=CC(=O)C3=C(O2)C=C(C=C3)O	5281894	Isoflavone
4	Phytol	CC(C)CCCC(C)CCCC(C)CCCC(=CCO)C	5280435	Diterpene
5	4',5,7-Trihydroxy isoflavone	C1=CC(=CC=C1C2=COC3=CC(=CC(=C3C2=O)O)O)O	5280961	Isoflavone
6	5-hydroxy-2-(4-methoxyphenyl) 4-H-1 Benzopyran-4-one	COC1=CC=C(C=C1)C2=CC(=O)C3=C(C=C(C=C3O2)OC)O	5281601	Flavonoid
7	Pimaric acid	CC1(CCC2C(=C1)CCC3C2(CCCC3(C)C(=O)O)C)C=C	220338	Diterpene
8	Retinol	CC1=C(C(CCC1)(C)C)C=CC(=CC=CC(=CCO)C)C	445354	Not found
9	Palustric Acid	CC(C)C1=CC2=C(CC1)C3(CCCC(C3CC2)(C)C(=O)O)C	443613	Diterpene
10	9-thiocyanato, androstan-4-en 3,17-diol-11-one	Not found	Not found	Not found

Table 5.8: List of phytochemicals present FF3 of *M. paleacea* and their canonical smile and Pubchem ID

Sl. no	***Phytochemical***	***Canonical smile***	***PubChem CID***	***Compound Class***
1	2,6-bis(1,1-dimethylethyl)-4-ethyl-Phenol	CCC1=CC(=C(C(=C1)C(C)(C)C)O)C(C)(C)C	20087	Phenylpropanes
2	2-tetradecanoyl-2-Cyclohexen-3,6-diol-1-one	Not found	Not found	Not found
3	1-Docosene	CCCCCCCCCCCCCCCCCCCCC=C	Not found	Aliphatic hydrocarbons
4	beta-Caryophyllene	CC1=CCCC(=C)C2CC(C2CC1)(C)C	5281515	Sesquiterpene
5	7-diethylamino,2H-1-Benzopyran-2-one	CCN(CC)C1=CC2=C(C=C1)C=CC(=O)O2	88598	Not found
6	a-Myrcene	CC(=CCCC(=C)C=C)C	Not found	Myrcene
7	1-Octadecene	CCCCCCCCCCCCCCCCC=C	Not found	Alkane
8	5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one	COC1=CC(=C2C(=C1)OC(=CC2=O)C3=CC=CC=C3)O	5281954	Flavonoids
9	3-Eicosene	Not found	Not found	Aliphatic hydrocarbons
10	Methyl 2-(4-hydroxy-3-isopropyllbenzoyl) benzoate	Not found	Not found	Not found

Table 5.9: List of human proteins targeted by the phytochemicals

Sl.no.	*Target proteins (human)*	*Gene ID*
1	3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA)	HMGCR
2	Androgen Receptor	AR
3	Estrogen receptor	ESR1
4	Liver X receptor beta (LXRB)	NR1H2
5	LXR-alpha	NR1H3
6	12-Lipoxygenase (12-LOX)	ALOX12
7	5-lipoxygenase/FLAP	ALOX5
8	Acetylcholinesterase	ACHE
9	Adenosine receptor	ADORA1
10	Adenosine Receptor A3	CA7
11	Aldose reductase	AKR1B1
13	Arachidonate 15-lipoxygenase	ALOX15
14	Arachidonate 5-lipoxygenase	ALOX5
15	Aromatase (CYP19)	CYP19A1
16	Aryl hydrocarbon receptor	AHR
17	AXL receptor tyrosine kinase	AXL
18	Beta amyloid A4 protein	APP
19	Butyrylcholine esterase	BCHE
20	Carbonic anhydrase	CA12
21	Carbonic anhydrase 2	CA2
22	Carbonic Anhydrase IV	CA4
23	Casein kinase II alpha/beta	CSNK2A1
24	Cyclin-dependent kinase	CDK1
25	Cyclooxygenase	PTGS2
26	Cystic fibrosis transmembrane conductance regulator	CFTR
27	D(2) dopamine receptor	DRD2
28	Delta-type opioid receptor	OPRD1
29	DNA topoisomerase 1	TOP1
30	Estradiol 17-beta-dehydrogenase 1 (17beta-HSD1)	HSD17B1
31	Estradiol 17-beta-dehydrogenase 2	HSD17B2
33	Glycogen Synthase Kinase-3, beta	GSK3B
34	Glyoxalase I	GLO1
35	Insulin-like growth factor 1 receptor (IGF1R)	IGF1R
36	Matrix metalloproteinase-12 (MMP12)	MMP12
37	Monoamine oxidase A (MAO-A)	MAOA
38	Monoamine oxidase B (MAO-B)	MAOB
39	Multidrug resistance protein 1 (MRP1)	ABCC1
40	Insulin-like growth factor 1 receptor (IGF1R)	IGF1R
41	Lck	LCK
42	Sucrase-isomaltase	SI

Table 5.9: List of human proteins targeted by the phytochemicals (continuation)

Sl.no.	*Target proteins (human)*	*Gene ID*
42	NADPH oxidase 4	NOX4
43	Neurotrophic tyrosine kinase receptor type 2	NTRK2
44	Placenta growth factor	PGF
45	Protein E6	E6
46	RXR alpha/PPAR gamma	PPARG
47	Serine/threonine-protein kinase Aurora-B	AURKB
48	Sigma opioid receptor	SIGMAR1
49	Telomerase reverse transcriptase	TERT
50	Transthyretin (TTR)	TTR
51	Vascular endothelial growth factor A	VEGFA
52	Xanthine oxidase	XDH
53	Aldehyde dehydrogenase (ALDH2)	ALDH2
54	Aldehyde oxidase	AOX1
55	Epidermal growth factor receptor (EGFR)	EGFR
56	Estrogen-related receptor alpha	ESRRA
57	Macrophage migration inhibitory factor (MIF)	MIF
58	MER intracellular domain/EGFR extracellular domain chimera	EGFR
59	Glucocorticoid receptor	NR3C1
60	Testis-specific androgen-binding protein	SHBG
61	11-beta-hydroxysteroid dehydrogenase 1	HSD11B1
62	11-beta-hydroxysteroid dehydrogenase 2	HSD11B2
63	Dual specificity phosphatase Cdc25B	CDC25B
64	Prostaglandin E synthase	PTGES
65	Protein-tyrosine phosphatase 1B (PTP1B)	PTPN1
66	RXR alpha/PPAR gamma	RXRA
67	Carbonic Anhydrase IX	CA9
68	D(4) dopamine receptor	DRD4
69	Progesterone receptor	PGR
70	Adenosine Receptor A3	ADORA3
71	Adenosine Receptors A2a (A2a)	ADORA2A
72	Arachidonate 12-lipoxygenase	ALOX12
73	ATP-binding cassette sub-family G member 2	ABCG2
74	Beta-secretase 1	BACE1
75	Cyclin-dependent kinase	CDK1
76	Cyclooxygenase-1 (COX-1)	PTGS1
77	Cytochrome P450 1B1	CYP1B1
78	Tyrosine-protein kinase Met	MET
79	Cannabinoid receptor	CNR2
80	GABA receptor alpha-1/beta-2/gamma-2 subunit	GABRA1
81	Dopamine Transporter (DAT)	SLCA3

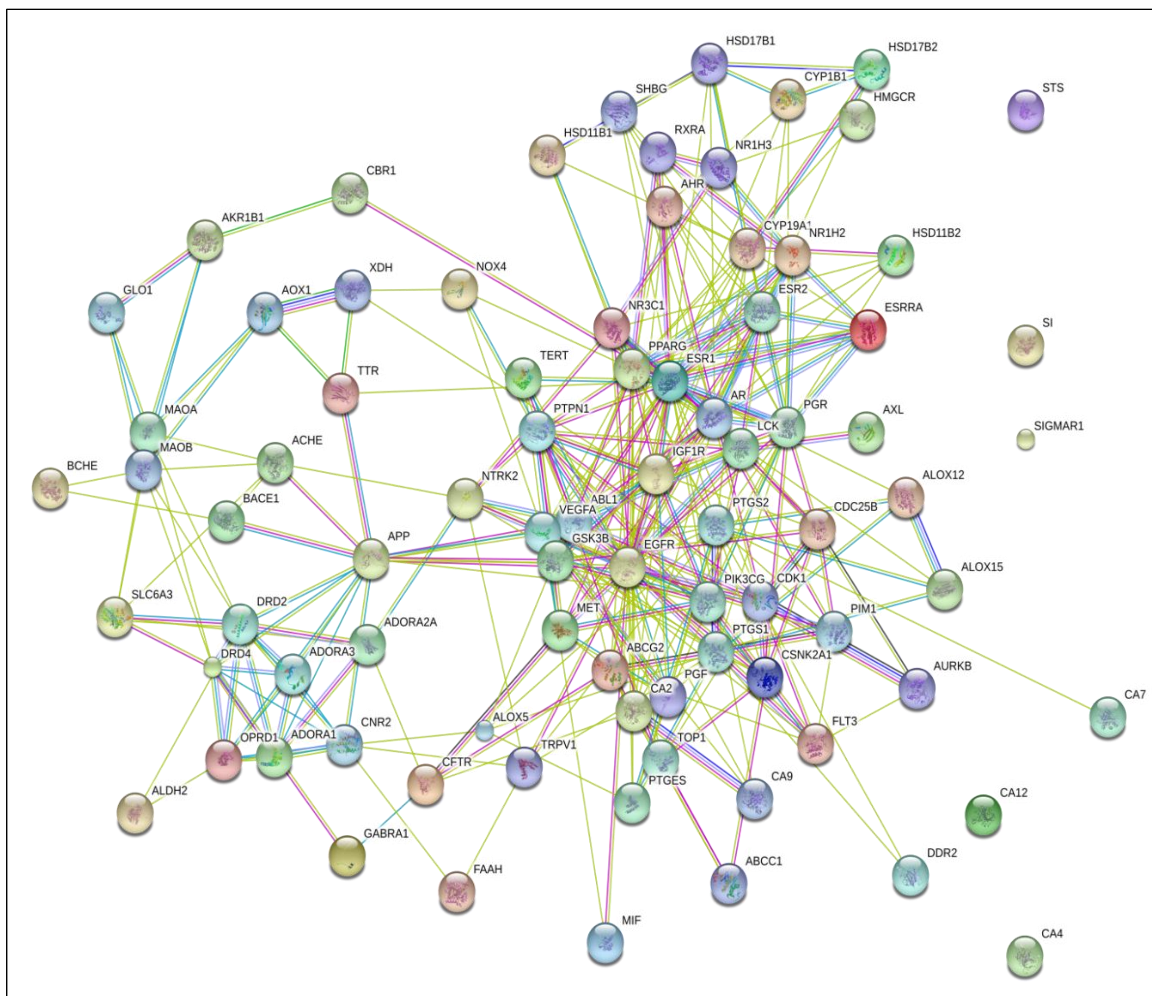


Fig 5.7.36: Interaction between target proteins of bioactive phytochemicals by using STRING Interactome Database

5.7.1c Phytochemical, Protein target and targeted disease

To determine the diseases targeted by *M. paleacea,* a tripartite network was constructed using its phytochemicals, their protein targets and associated diseases via Cytoscape. A total of 180 disease targets (Table 5.10) were identified and were classified into 22 broad categories (Fig 5.7.35). Figure 5.7.36 represents the interaction between phytochemicals, proteins and disease targets.. Among the diseases targeted by the proteins, 26% percent of the total diseases belonged to the class malignant neoplasm, 13% belonged to endocrine, nutritional and metabolic diseases, 9% belonged to disease of circulatory system and 8% belonged to certain infectious and parasitic diseases. Each of the remaining disease classes constituted 1-5% of all the disease targets. In this work phytochemicals obtained by *in-vitro* bioassay guided purification. The assays used for extraction of phytochemicals were *in-vitro* antioxidant and anti-diabetic assays. Network analysis of phytochemicals-protein targets-associated diseases revealed that the endocrine, nutritional and metabolic diseases were associated with the protein target of studied phytochemicals which proved that phytochemicals extracted using *in-vitro* anti-diabetic activity efficiently targets the proteins involved in endocrine nutritional and metabolic pathways. Second highest number of disease associated with the target proteins was disease of endocrine, nutritional and metabolic diseases (13%), highest being the diseases belonging to the disease class malignant neoplasm.

The aim of this work was to isolate phytochemicals having anti-diabetic activity and to validate the therapeutic relevance of these compounds through network analysis. So, the further network analysis was mainly focused on disease class endocrine, nutritional and metabolic diseases.

Table 5.10: List of diseases associated with target proteins

Disease	Disease ID	Class	Disease	Disease ID	Class
Diabetic complications	ICD10: E08-E13	ENDOCRINE, NUTRITIONAL AND METABOLIC DISEASES	Alzheimer's disease	ICD10: G30, G31.84	DISEASE OF NERVOUS SYSTEM
Diabetic neuropathy	ICD10: E08-E13		Motor neurone disease	ICD10: G12.2	
Diabetic retinopathy	ICD10: H36		Parkinson's disease		
Non-insulin-dependent diabetes mellitus	ICD10: E08-E13		Analgesics	ICD10: R52	
Cystic fibrosis	ICD10: E84, L90.5		Vascular diseases	ICD10: G45-G46	
Endocrine disease	ICD10: E00-E35		Neurodegenerative diseases	ICD10: G30-G32	
Hormone deficiency	ICD10: E00-E90		Cardiovascular disease	ICD10: G45-G46, I00-I99, I10, I11, I12	
Hypogonadism	ICD10: E23.0, E28.3, E29.1		Disabling peak-dose dyskinesias	ICD10: G24.9	
Male hormonal deficiencies	ICD10: E20-E35		Neurological diseases	ICD10: G00-G99	
Testosterone deficiency	ICD10: E29.1		Lateral sclerosis	ICD10: G12.2	
Peripheral precocious puberty	ICD10: E22.8, E30.1		Motor neurone disease	ICD10: G12.2	
Obesity	ICD10: E66		Dyskinesia	ICD10: G24.9	
Amyloidosis	ICD10: E85		Neuropathic pain	ICD10: G64, G90.0	
TTR amyloidosis	ICD10: E85				
Diabetic angiopathy	ICD10: E10.5, E11.5, E13.5		Insomnia	ICD10: F51.0, G47.0	
Diabetes mellitus	ICD10: E23.2		Melanoma	ICD10: C43	MELIGNANT MELANOMA OF SKIN
Inflammation	ICD10: E08-E13, E10.2		Diarrhea	ICD10: A09, K59.1	CERTAIN INFECTIOUS AND PARASITIC DISEASE
Hyperlipidemia	ICD10: E78				
Metabolic disease	ICD10: E70-E89		HIV-associated diarrhoea	ICD10: A09	
Diabetes mellitus type 2	ICD10: E11		Streptococcus pneumoniae infections	ICD10: A09	
Dyslipidemia	ICD10: E78		HIV infections	ICD10: A00-B99	
Hypercholesterolemia	ICD10: E78.1		HCV infection	ICD10: B17.1, B18.2	
Hypertriglyceridemia	ICD10: E78.1		Septic shock	ICD10: A41.9	
Metabolic disorders	ICD10: E70-E89				

Table 5.10: List of diseases associated with target proteins (continuation)

Disease	Disease ID	Class	Disease	Disease ID	Class
Adult respiratory distress syndrome	ICD10: J80	DISEASE OF RESPIRATORY SYSTEM	Tumors	ICD10: C00-C75, C7A, C7B, D10-D36, D3A	MALIGNANT NEOPLASM
Allergic rhinitis	ICD10: J00, J30, J31.0, T78.4		Cancer	ICD10: C00-C96	
Asthma	ICD10: J45		Bladder cancer	ICD10: C00-C96, C67	
Pulmonary fibrosis	ICD10: J84.1		Hormone refractory prostate cancer	ICD10: C00-C96, C61	
Emphysema	ICD10: J43		Metastasis	CD10: C00-C97	
Respiratory tract inflammation	ICD10: J00-J99		Salivary glands cancer	ICD10: C00-C96	
Allergic diseases	ICD10: T78.4	INJURY, POISONING AND CERTAIN OTHER CONSEQUENCES OF EXTERNAL CAUSES	Breast cancer	ICD10: C50	
Allergic rhinitis	ICD10: J00, J30, J31.0, T78.4		Multiple myeloma	ICD10: C90.0	
Antigen-induced decrease in coronary flow and cardiac anaphylaxis	ICD10: T78.2		Myeloma	ICD10: C90.0	
Brain injury	ICD10: S09.90		Solid tumours	ICD10: C00-C75	
Sepsis	ICD10: A40, A41, T78		Inflammatory diseases	ICD10: K75.9	
			Colorectal cancer	ICD10: C18-C21	
Bronchiolar carcinoma	ICD10: C34	MALIGNANT NEOPLASM	Lung cancer	ICD10: C33-C34	
Cervical cancer	ICD10: C00-C96		Hormone-sensitive breast cancer	ICD10: C00-C96	
Chronic myeloid leukemia	ICD10: C92.1		Endocrine independent cancer	ICD9: 140-229 ICD10: C00-C96	
Gastrointestinal cancers	ICD10: C15-C26		Osteosarcoma	ICD10: C40-C41	
Pancreatic cancer	ICD10: C00-C96		Ewing's sarcoma	ICD10: C41	
Urological cancers	ICD10: C00-C96		Medulloblastoma	ICD10: C71	
Peripheral neuroectodermal tumor	ICD10: C00-C75		Renal cell carcinoma	ICD10: C64	
Non-small cell lung cancer	ICD10: C00-C96		Brain cancer	ICD10: C71, D33	

Table 5.10: List of diseases associated with target proteins (continuation)

Disease	*Disease ID*	*Class*	*Disease*	*Disease ID*	*Class*
Ovarian cancer	ICD10: C00-C96	**MALIGNANT NEOPLASM**	Myocardial infarction	ICD10: I21	**DISEASES OF THE CIRCULATORY SYSTEM**
Acute myeloid leukemia	ICD10: C92.0		Cardiomyopathy	ICD10: I42.0	
Leukemia	ICD10: C91-C95		Angiogenesis disorder	ICD10: I00-I99	
MLL-rearranged acute lymphoblastic leukemias	ICD10: C91-C95		Coronary artery disease	ICD10: I20-I25	
Metastatic colorectal cancer	ICD10: C18-C21		Ischemia reperfusion injuries	ICD10: I99.8	
Metastatic prostate cancer	ICD10: C00-C96		Stroke	ICD10: I61-I63	
Melanoma	ICD10: C43		Coronary heart disease	ICD10: I25.1	
Chronic myelogenous leukemia	ICD10: C91-C95, C92.1		Essential hypertension	ICD10: I10, I11, I12, I13, I15	
Colon adenocarcinoma	ICD10: C00-C75		Pulmonary hypertension	ICD10: I27.0, I27.2	
Philadelphia-positive leukemia	ICD9: 208.9 ICD10: C91-C95		Subarachnoid hemorrhage	ICD10: I60, P10.3, S06.6	
Acute leukaemia	ICD10: C91-C95		Prostate hyperplasia	ICD10: N40	**DISEASES OF THE GENITOURINARY SYSTEM**
Glioblastoma multiforme	ICD10: C71		Glomerulonephritis	ICD10: N00, N01	
Head and neck tumors	ICD10: C00-C75		Endometriosis	ICD10: N80	
Squamous cell carcinoma	ICD10: C44		Male infertility	ICD10: N46	
ER beta-positive prostate tumors	ICD10: C00-C75		Renal failure	ICD10: N17, N18, N19	
Head and neck squamous cell carcinomas	ICD10: C44		Stroke	ICD10: I61-I63, I80-I82	
Cerebral vasospasm	ICD10: I73.9	**DISEASES OF THE CIRCULATORY SYSTEM**	Postmenopausal symptoms	ICD10: N95.0	
Myocardial ischemia	ICD10: I99.8		Renal diseases	ICD10: N00-N39	
Cardiovascular disease	ICD10: I00-I99		Menopause	ICD10: N95.0	
Heart failure	ICD10: I21, I22, I50		Inflammatory bowel disease	ICD10: K50, K51	**DISEASES OF THE DIGESTIVE SYSTEM**
Atherosclerosis	ICD10: I70		Chronic ileitis	ICD10: K50-K52	
Ischemia	ICD10: I99.8		Ulcerative colitis	ICD10: K51	
Coronary atherosclerosis	ICD10: I70		Crohn's disease	ICD10: K50	

Table 5.10: List of diseases associated with target proteins (continuation)

Disease	Disease ID	Class	Disease	Disease ID	Class
Inflammatory diseases	ICD10: C00-C96	DISEASES OF THE DIGESTIVE SYSTEM	Delusional disorder	ICD10: F22.0	
Gastro-intestinal ulcers	ICD10: K25		Erectile dysfunction	ICD10: F52.2	
Inflammatory response in alcoholic liver disease	ICD10: K70-K77		Parkinson's disease	[ICD10: F02.3	
Magnesium deficiency dermatitis	ICD10: L20-L30	DISEASES OF THE SKIN AND SUBCUTANEOUS TISSUE	Bipolar affective disorder	ICD10: F31	
Psoriasis	ICD10: L40		Social phobias	ICD10: F40.1	
Acne vulgaris	ICD10: L70.0		Major depressive disorder	ICD10: F32, F33, M32	
Dermatological disease	ICD10: L00-L99		Chronic alcoholism	ICD10: F10.2]	
Fibrosis	ICD10: L90.5		Drug dependence	ICD10: F10-F19	
Alopecia	ICD10: L65.9		Alopecia	ICD10: L65.9	NONSCARRING HAIR LOSS
Systemic lupus erythematosus	ICD10: L51-L54		Contraception	ICD10: Z30	FACTORS INFLUENCING HEALTH STATUS AND CONTACT WITH HEALTH SERVICES
Osteoarthritis	ICD10: M15-M19, M47	DISEASES OF THE MUSCULOSKELETAL SYSTEM AND CONNECTIVE TISSUE	Pain	ICD10: G64	PAIN
Muscle atrophy	ICD10: M62.5		Analgesics	ICD10: R52	
Osteoporosis	ICD10: M80-M81		Glaucoma	ICD10: H40-H42	DISEASES OF THE EYE AND ADNEXA
Spinal and bulbar muscular atrophy	ICD10: M62.5		Ocular hypertension	ICD10: H40.0	
Gout	ICD10: M10		Ocular inflammation	ICD10: H00-H59	
Rheumatoid arthritis	ICD10: M05-M06		Macular degeneration	ICD10: H35.3	
Sjogren-Larsson syndrome	ICD10: Q87.1	CONGENITAL MALFORMATIONS, DEFORMATIONS AND CHROMOSOMAL ABNORMALITIES	Oxygen-induced retinopathy	ICD10: H35-H35.2	
Ulcerative colitis	ICD10: K51	NON-INFECTIVE ENTERITIS AND COLITIS	Nausea and vomiting	ICD10: R11	SYMPTOMS AND SIGNS INVOLVING THE DIGESTIVE SYSTEM AND ABDOMEN
Cognitive deficits	ICD10: F01-F07, F04, R41.3	MENTAL, BEHAVIOURAL AND NEURO-DEVELOPMENTAL DISORDERS	Vomiting	ICD10: R11	
Depression	ICD10: F32		Cough	ICD10: R05	SYMPTOMS AND SIGNS INVOLVING THE CIRCULATORY AND RESPIRATORY SYSTEMS
Dementia	ICD10: F01-F07, G30		Dyspnea	ICD10: R06.0	
Schizophrenia	ICD10: F20, M32		Immunodeficiency	ICD10: D84.9	DISEASES OF THE BLOOD AND BLOOD-FORMING ORGANS AND CERTAIN DISORDERS INVOLVING THE IMMUNE MECHANISM
Anxiety disorders	ICD10: F40-F42		Angioedema	ICD10: D84.1	
Attention deficit hyperactivity disorder	ICD10: F90		Autoimmune diseases	ICD10: D84.9	
Cocaine dependence	ICD10: F14.2, F32		Male infertility	ICD10: N46	DISEASES OF MALE GENITAL ORGANS

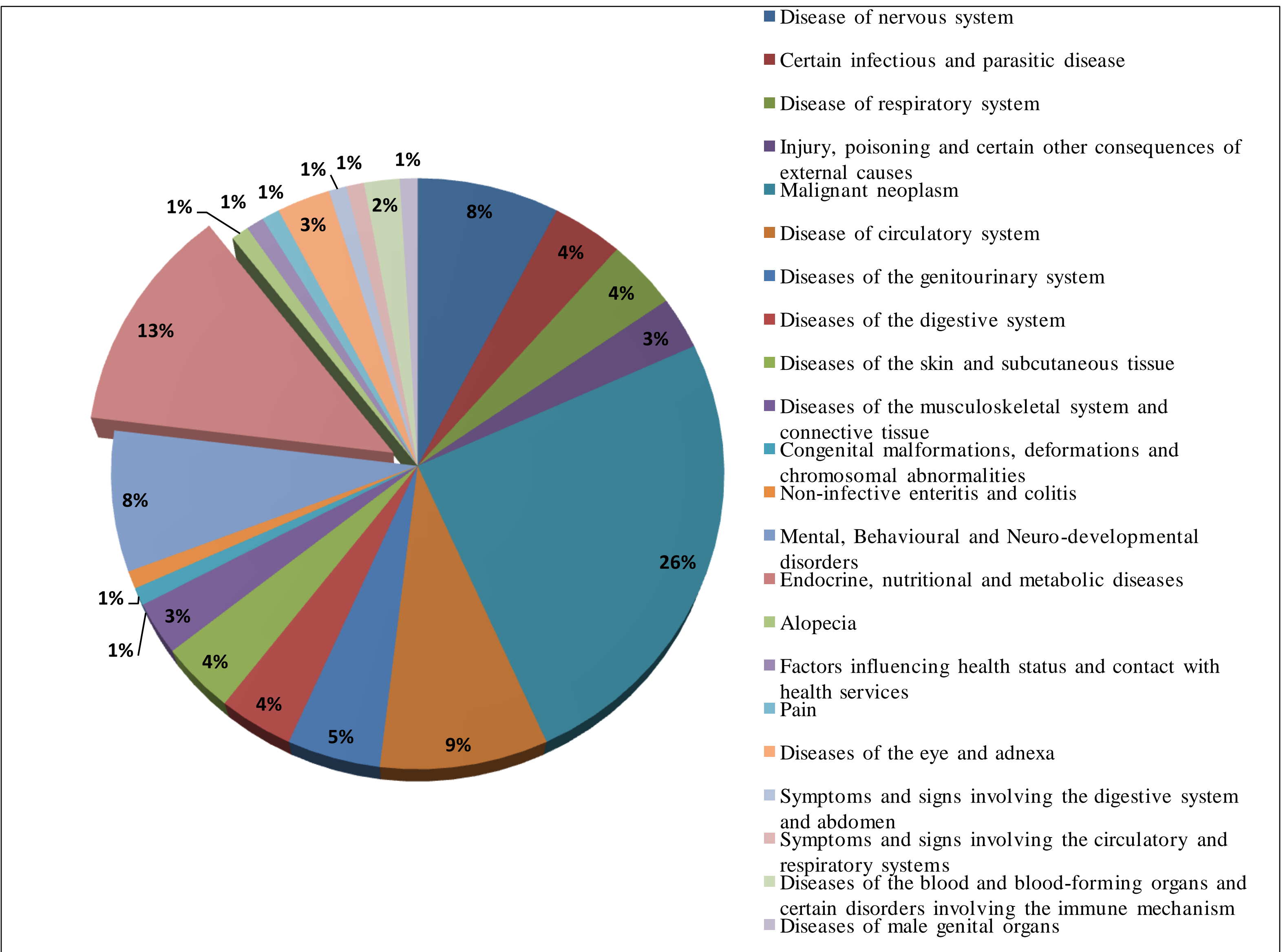


Fig 5.7.37: Pie chart representing the disease classes associated with target protein.

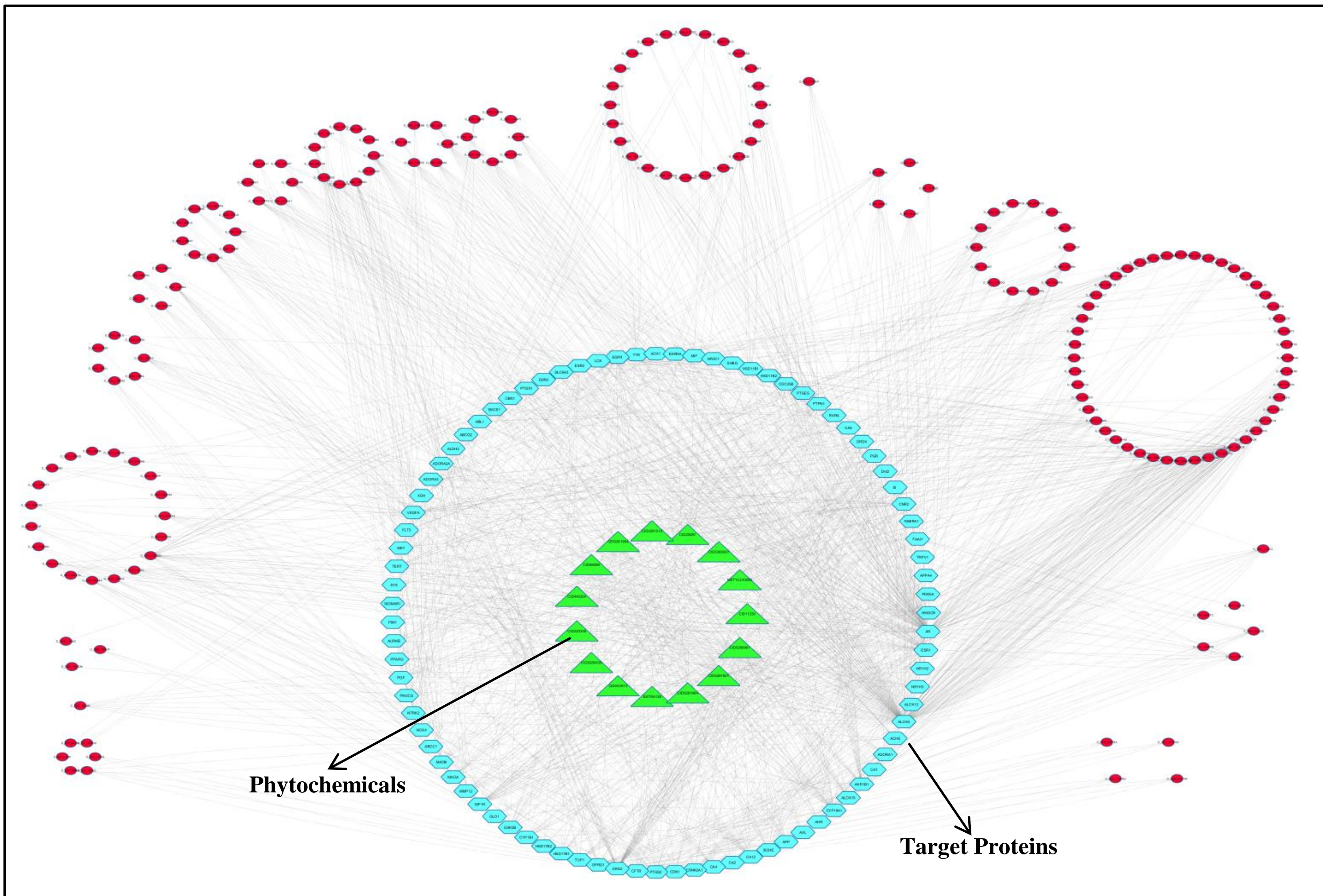


Fig 5.7.38: Whole tripartite network of phytochemicals, target proteins and associated disease classes.

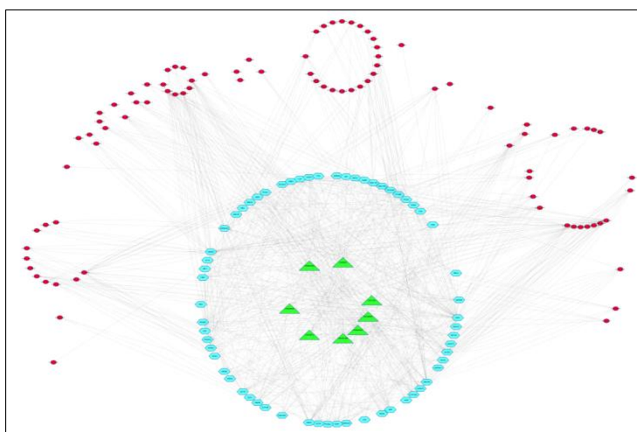
5.7.1d Network robustness Study

Network robustness was analyzed by the elimination of proteins from the sub network on the basis of Degree × Betweenness × Closeness value. For breaking the robust structure of network, about 30% of targeted proteins were deleted from network through gradual elimination of 5% proteins for 6 times (Fig 5.7.37). Degree centrality is the number of links incident upon a node (Freeman, 1979). The average length of the shortest path between a node and all other nodes in a network is Closeness centrality of a node. So if the node is at the centre, closer it will be to all the other nodes (Bavelas, 1950). The total number of times a node bridges the shortest path between other two nodes of a network is its Betweenness centrality. It is a centrality measure of a vertex within a network (Freeman, 1977).

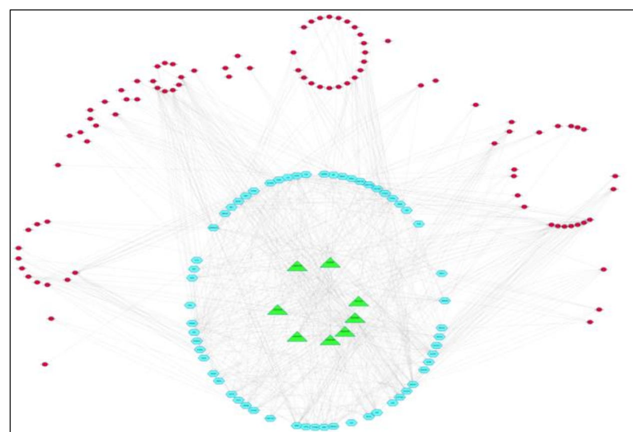
Degree × Betweenness × Closeness (indicators of centrality) identifies the most important vertices in the network. In this work, vertices represent the target proteins. Thus the Network Robustness helps to resolve the complexness of network by removing the centralized targets. Network robustness analysis found twenty protein targets of phytochemicals isolated from *M. paleacea* to be involved in endocrine, nutritional and metabolic diseases. List of the protein targets is presented in table 5.11

5.7.1e Network parameters and their relation

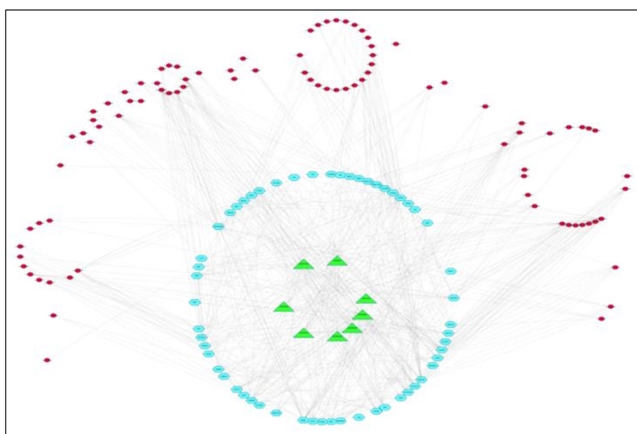
Different parameters like isolated nodes, characteristic path length, clustering coefficient and network diameter were studied for network analysis and are presented in Fig 5.7.38. Parameters like isolated nodes and characteristic path length was found to increase, while decrease in clustering co-efficient was noticed. Diameter of the network first increased and then remained constant.



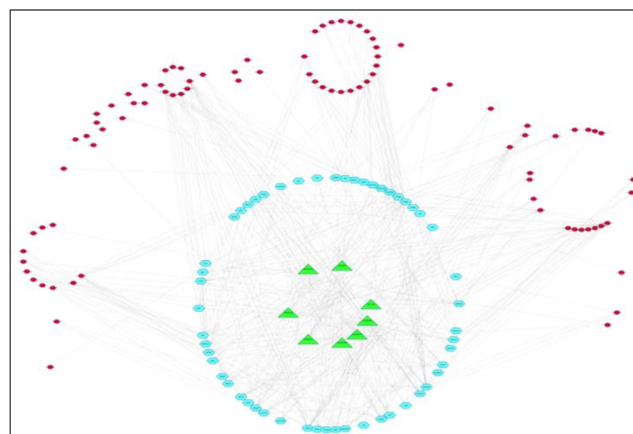
1.5 % protein elimination by Network Perturbation



5 % protein elimination by Network Perturbation

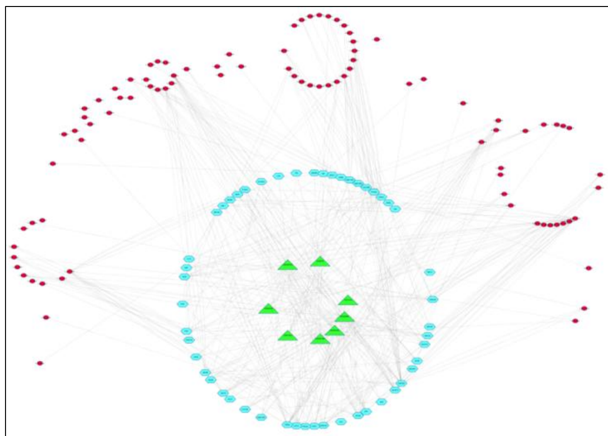


10% protein elimination by Network Perturbation

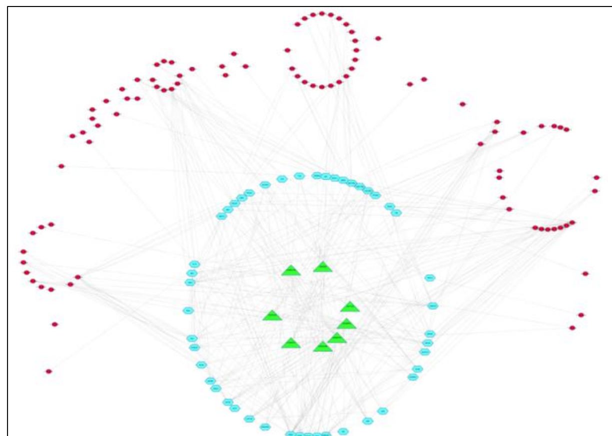


15% protein elimination by Network Perturbation

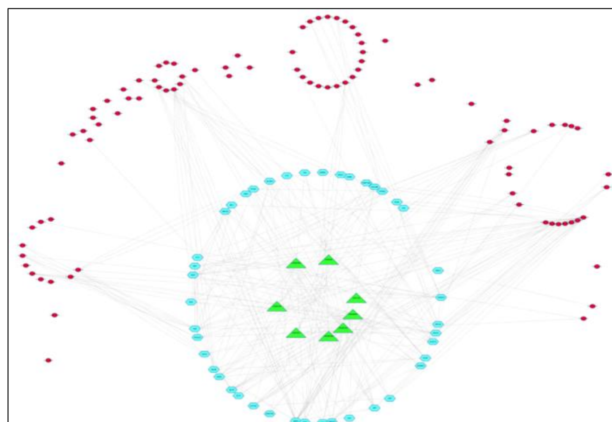
Fig 5.7.39: Protein elimination by Network Perturbation



20% protein elimination by Network Perturbation

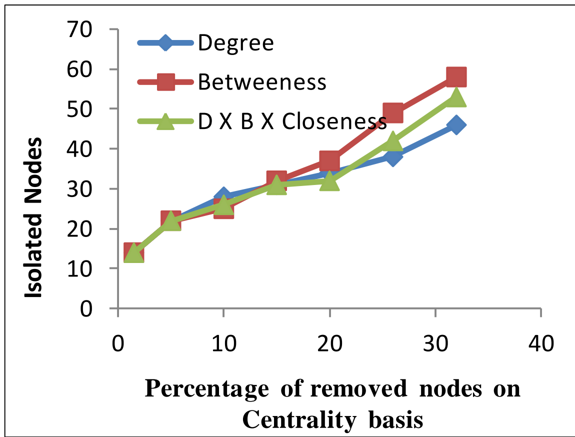


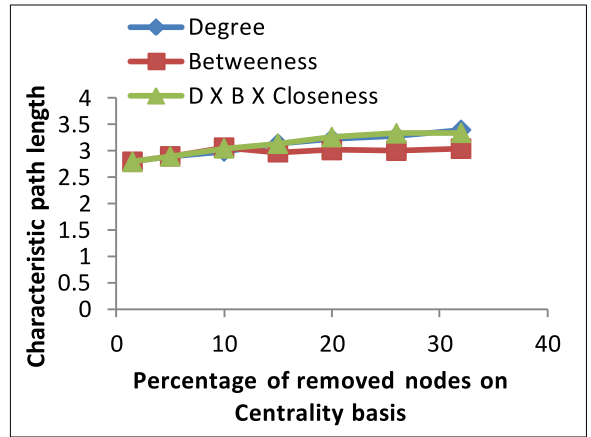
26% protein elimination by Network Perturbation

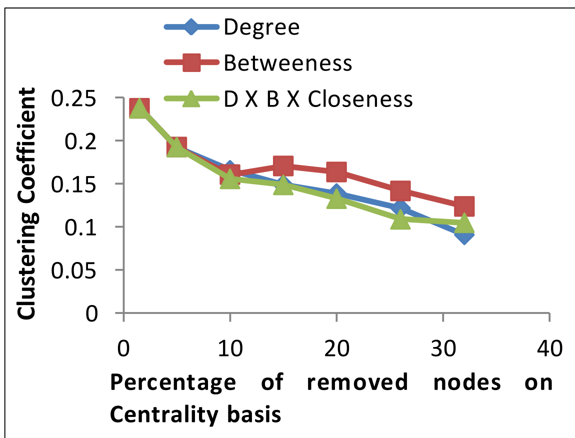


32% protein elimination by Network Perturbation

Fig 5.7.39: Protein elimination by Network Perturbation (continuation)







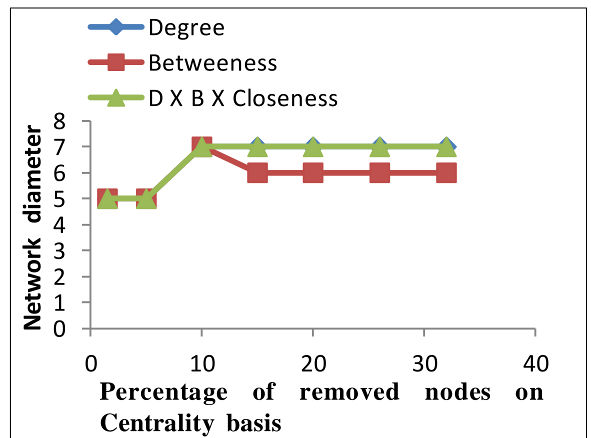


Fig 5.7.40: Network parameters versus percentage of network perturbation

Table 5.11: Network list of first 30% of total targets analyzed through tripartite network

Gene	*Betweenness Centrality*	*Closeness Centrality*	*Clustering Coefficient*	*Degree*	*D × B × Close*
PPARG	0.072	0.518	0.309	30	1.122
VEGFA	0.125	0.501	0.195	59	3.704
AKR1B1	0.042	0.381	0.153	25	0.409
PTPN1	0.043	0.435	0.2	24	0.450
HMGCR	0.048	0.367	0.030	12	0.214
EGFR	0.070	0.519	0.258	38	1.392
HSD11B1	0.026	0.380	0.075	17	0.172
MIF	0.063	0.380	0.060	12	0.290
BCHE	0.022	0.386	0.076	19	0.166
GSK3B	0.049	0.456	0.187	26	0.593
ESR1	0.152	0.523	0.122	70	5.578
CYP19A1	0.017	0.473	0.411	30	0.246
AR	0.242	0.523	0.105	142	18.03
ALOX15	0.033	0.397	0.219	19	0.253
BACE1	0.017	0.396	0.145	17	0.118
DRD2	0.018	0.382	0.269	23	0.163
NTRK2	0.040	0.431	0.166	26	0.451
ESR2	0.023	0.459	0.307	43	0.458
ADORA1	0.068	0.405	0.098	29	0.801
ALOX5	0.028	0.407	0.179	38	0.438

Fig 5.7.41: Degree × betweenness × clossness analysis of 20 protein targets

Fig 5.7.42: Degree analysis of 20 protein targets

Fig 5.7.43: Betweenness centrality analysis of 20 protein targets

5.7.1f. Enrichment (KEGG) Analysis of Centralized Target Proteins

Betweenness Centrality analysis and percentage of heat from CooLGen data mining had identified eight targets having high betweenness centrality and heat percentage from earlier identified twenty targets by robustness analysis (Fig 5.7.39 to Fig 5.7.41). These targets were PTPN1, AKR1B1, PPARG, ADORA1, MIF, GSK3B, HMGCR and VEGFA (Fig 5.7.43). The functional enrichments of isolated centralized proteins were done through KEGG pathway to find out the role of these targets in initiating endocrine, nutritional and metabolic diseases. Biological pathways in which these targets are involved are described sequentially in the following section.

VEGFR: associated with MAP kinase pathway in cancer.

HSDII: involved in steroid hormone metabolism pathway.

MIF: involved in tyrosin metabolism pathway

BCHE: Butylcholineesterase inhibitor

AKR1B1: mainly associated with steroid hormone synthesis pathway. Insulin when binds with the insulin receptor of ovary it triggers the biosynthesis of AKR1B1. It then utilizes cholesterol inside ovary and produces hormone like testesteron and projesterone etc.

ESR1: in ovarian system it acts as estrogen receptor when leuteinising hormone is received by the leutonising hormone receptor.

CYP1: when insulin binds with insulin receptor in ovary during ovarian steroidogenesis, CYP1 converts cholesterol present in ovary into testerone and projesterone hormones and insulin signalling pathway particularly enhances the expression of CYP1.

AR: in protrate epithelial cell testerone binds with androgen receptor and subsequently genes required for cell proliferation are going to be expressed, which might create prostate cancer.

ALOX15: associated with linolic acid metabolism.

BACE1: In Alzemier disease, BACE1 inhibits acetylcholine.

Fig 5.7.44: Function of eight protein target having high betweenness centrality value and percentage of heat

Fig 5.7. 45: Insulin signaling pathway (hsa04931) obtained from KEGG database. Genes present in tripartite network is marked red

ALOX5: in ovarian tissue ALOX is activated by arachidonic acid which is produced through phospholipase A by stimulatory G_{α} and cyclic AMP. ALOX is a type of lypooxygenase which produce different arachidonic metabolites and express the genes of steroidal sex hormone biosynthesis like testerone and projesterone.

PPARG: responsible for insulin resistance

PTPN1: responsible for insulin receptor insensitivity

GSK3: inhibits glycogen synthase

ADORA1: inhibits the process of fatty acid degradation and lipolysis.

From Enrichment analysis of centralized target by KEGG pathway, it was found that four protein targets were mainly involved in the pathways associated with diabetic diseases. Entire pathway and involved genes of these targets are presented in the following section.

PPARG: Insulin resistance pathway

PPAR nuclear receptors and winged-helix-forkhead box class O (FOXO) are most important transcription factors that regulate GLUT4 gene expression. In absence of ligand, PPARG forms a heterodimer with retinoid X receptor (RXR) and binds to PPAR response elements (PPREs) of target gene (GLUT4). The PPARG-RXR heterodimer then binds with co-repressor and represses the expression of GLUT4 gene. However, in presence of ligand the corepressor will be replaced from PPARG by a coactivator complex and leads to transcriptional activation of GLUT4.

PTPN1: Insulin signalling pathway

PTPN1 is responsible for insulin receptor insensitivity as it blocks insulin receptor and insulin receptor substrate 1 which results in the inhibition of the process of glycogenesis.

ADORA1: Insulin signalling pathway

ADORA1 induces production of inhibitory G_i protein and inhibits adenyl cyclase. Adenyl cyclase when present in the active form is able to activate the protein Kinase A

Fig 5.7.46: Insulin resistance pathway (hsa04931) obtained from KEGG database. Genes present in tripartite network are marked in red

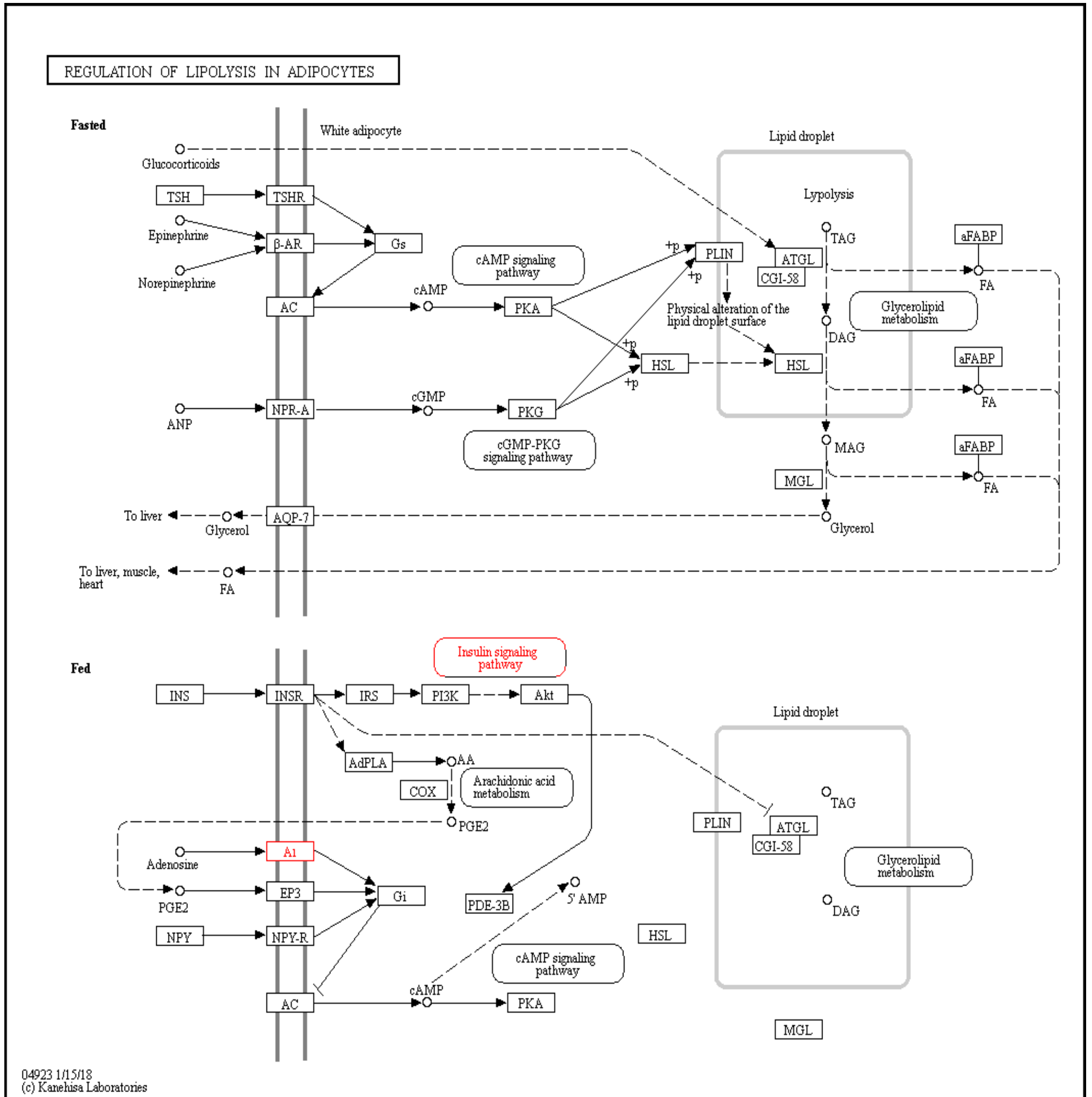


Fig 5.7.47: Insulin signaling pathway (has 04923) obtained from KEGG database. Genes present in tripartite network are marked in red

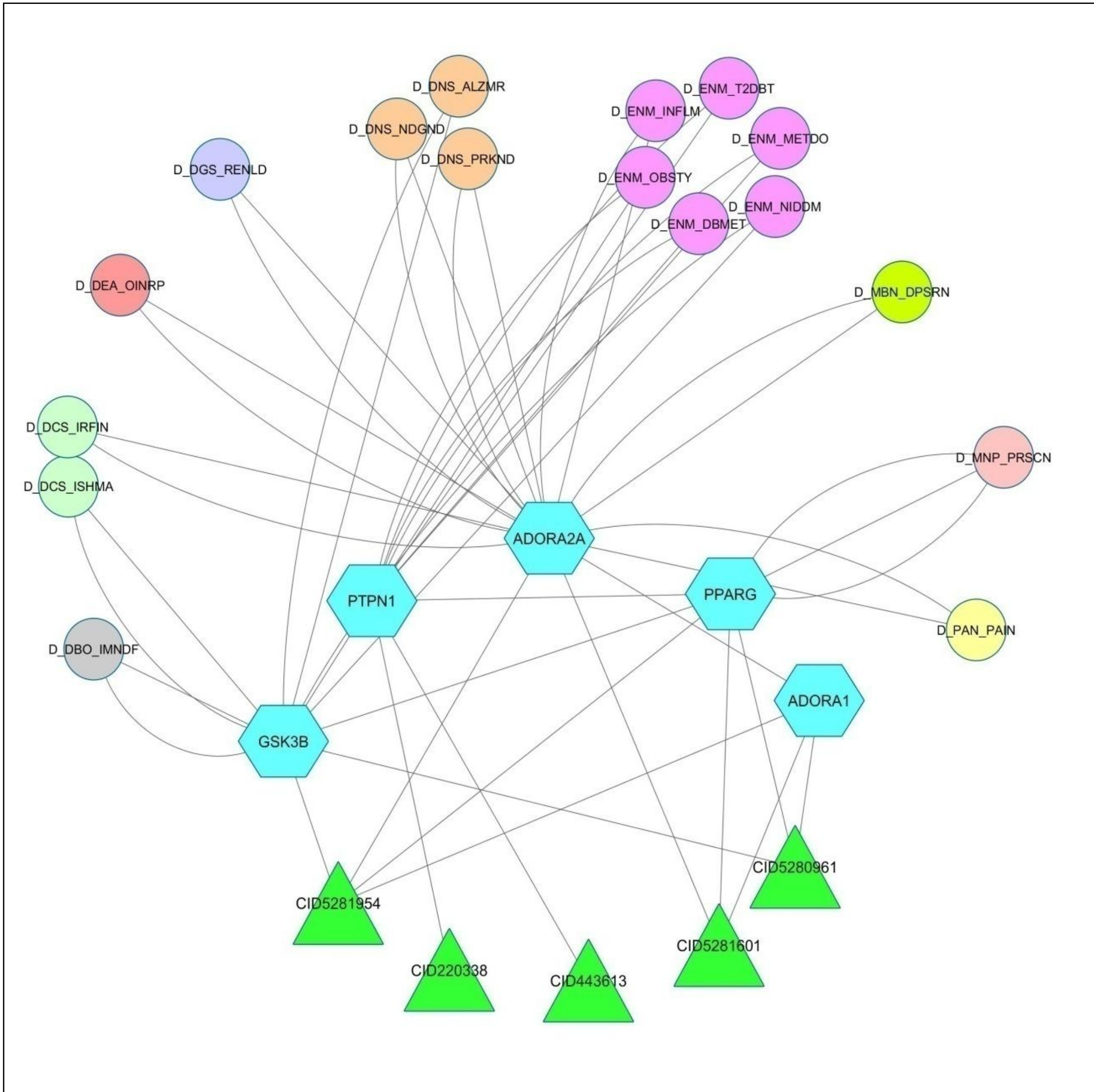


Fig 5.7.48: Final tripartite network of five most important protein targets participating in diabetes pathway

(PKA) through generation of secondary messenger cAMP. Activated PKA then degrades fatty acid and causes lipolysis in adipose tissue. Thus ADORA1 inhibits the process of fatty acid degradation and lipolysis by inhibiting the PKA activation.

5.7.1g Druggability Analysis and ADMET Study

Druaggability analysis is important for drug discovery process. Estimation of ADMET properties is highly important for considering a drug candidate for further clinical trials (Van De Waterbeemd and Gifford *et al*., 2003). Analysis of ADMET properties of five phytochemicals of *M. paleacea* that are found to be good candidate for preventing diabetes was studied. *In silico* test showed that the intestinal absorption percentage of studied five phytochemicals was more than 90%. $Caco_2$ cell permeability was also high i.e. more than 0.9 of the concerned phytochemicals. It is important to consider the capability of a drug to cross blood brain barrier (BBB) for reducing side effects and toxicities or improve efficiency drug acting within brain. Studied phytochemicals showed good BBB permeability (logBB values were more than -1) [Table 5.12.1]. Similarly, these phytochemicals also showed the Central Nervous System (CNS) penetrating potential, the CNS penetration values were less than -3 (Table 5.12.2). Another important parameter to be considered in the drug discovery is their clearance from the body. Organic Cation Transporter 2 is a transporter (OCT2) that helps in renal clearance of drugs and other compounds. Drugs capable of being transported by OCT2 provide information about its clearance. Phytochemicals 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one and Palustric acid found to be capable of acting as a substrate of OCT2 (Table 5.12.5).

The study of toxic potency of a drug is highly important. Ames test is used to access the mutagenic potential of a drug. *In silico* Ames test showed that the analyzed phytochemicals have no mutagenic effect. Estimation of maximum recommended tolerated dose (MRTD) estimates the threshold toxic dose of chemicals. Studied phytochemicals found to have low maximum recommended tolerated dose. Human ether-a-go-go gene (hERG) codes for the potassium channels. Blocking of these channel are the main cause of development of fatal arrhythmia. Studied phytochemicals was found to have hERG I and II inhibitory activity. Drug induced liver damage is of high concern in drug development and is one of the main cause of drug attrition. 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-

4-one, 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one and 4’,5,7-Trihydroxy isoflavone showed no hepatotoxicity in *in silico* test while pimaric acid and palustric acid showed hepatoxicity. Evaluating whether a drug can induce allergic contact dermatitis and cause skin sensitisation is highly important. No skin sensitization is induced by the studied five phytochemicals (Table 5.12.4).

Lipinski’s “rule of five” criterion measures the likeliness of the compounds to act as a drug (Lipinski *et al.*, 1997). Criteria for Lipinski’s rule are: (i.) molecular weight less than 500 Dalton, (ii.) Number of hydrogen bond donors less than 5, (iii.) Number of hydrogen bond acceptors less than 10, (iv.) logP value (octanal-water partition coefficient) less than 5. All tested phytochemicals found to maintain the entire criteria (Table 5.12.6).

Table 5.12 .1: Druggability analysis of phytochemicals for absorption ability

Property	***unit***	***Predicted value***					
		Data prediction	***4',5,7-Trihydroxy isoflavone***	***5-hydroxy-2-(4-methoxyphenyl),4-H-1 Benzopyran-4-one***	***5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one***	***Palustric acid***	***pimaric acid***
Water solubility	Numeric (log mol/L)	The higher the better	-3.595	-3.714	-3.641	-3.88	-3.683
Caco2 permeability	Numeric (log Papp in 10^{-6} cm/s)	> 0.90 = high Caco2 permeability	0.9	1.106	1.248	1.621	1.742
Intestinal absorption (human)	Numeric (% Absorbed)	Absorbence of less than 30 % is considered to be poorly absorbed	93.387	95.453	95.229	97.612	98.196
Skin Permeability	Numeric (log Kp)	> -2.5 = Low skin permeability	-2.735	-2.666	-2.758	-2.715	-2.727
P-glycoprotein substrate	Categorical (Yes/No)		Yes	No	Yes	No	No
P-glycoprotein I inhibitor	Categorical (Yes/No)		No	No	No	No	No
P-glycoprotein II inhibitor	Categorical (Yes/No)		No	Yes	Yes	No	No

Table 5.12 .2: Druggability analysis of phytochemicals for distribution

Property	***unit***	***Predicted value***					
		Prediction	***4',5,7-Trihydroxy isoflavone***	***5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one***	***5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one***	***Palustric acid***	***pimaric acid***
VDss (human)	Numeric (log L/kg)	Low below 0.71 and high above 2.81	0.094	-0.1	-0.047	-0.546	-0.645
Fraction unbound (human)	Numeric (Fu)	For a given compound the predicted fraction that would be unbound in plasma will be calculated.	0.087	0.156	0.119	0.073	0.081
BBB permeability	Numeric (log BB)	> 0.3 = readealy cross the blood brain barrier, < -1 = poorly distributed in brain	-0.71	-0.458	0.003	0.324	0.273
CNS permeability	Numeric (log PS)	> -2 = penetrate the central nervous system, <-3= unable to penetrate the CNS	-2.048	-2.144	-1.992	-2.022	-2

Table 5.12.3: Druggability of phytochemicals for metabolism

Property	***unit***	***Predicted value***				
		4',5,7-Trihydroxy isoflavone	***5-hydroxy-2-(4-methoxyphenyl),4-H-1 Benzopyran-4-one***	***5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one***	***Palustric acid***	***pimaric acid***
CYP2D6 substrate	Categorical (Yes/No)	No	No	No	No	No
CYP3A4 substrate	Categorical (Yes/No)	No	Yes	Yes	Yes	Yes
CYP1A2 inhibitior	Categorical (Yes/No)	Yes	Yes	Yes	No	No
CYP2C19 inhibitior	Categorical (Yes/No)	Yes	Yes	Yes	Yes	No
CYP2C9 inhibitior	Categorical (Yes/No)	No	Yes	Yes	Yes	Yes
CYP2D6 inhibitior	Categorical (Yes/No)	No	No	No	No	No
CYP3A4 inhibitior	Categorical (Yes/No)	No	Yes	Yes	No	No

Table 5.12.4: Druggability analysis of phytochemicals for toxicity

Property	***unit***	***Predicted value***					
		Prediction	***4',5,7-Trihydroxy isoflavone***	***5-hydroxy-2-(4-methoxyphenyl),4-H-1 Benzopyran-4-one***	***5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one***	***Palustric acid***	***pimaric acid***
AMES toxicity	Categorical (Yes/No)		No	No	No	No	No
Max. tolerated dose (human)	Numeric (log mg/kg/day)	Low = Less than and equal to 0.477, high = greater than 0.477	0.478	0.193	-0.105	-0.086	0.008
hERG I inhibitor	Categorical (Yes/No)		No	No	No	No	No
hERG II inhibitor	Categorical (Yes/No)		No	No	No	No	No
Oral Rat Acute Toxicity (LD50)	Numeric (mol/kg)	The Lower the better	2.268	2.085	2.042	1.869	1.838
Oral Rat Chronic Toxicity (LOAEL)	Numeric (log mg/kg_bw/day)	The higher the better	2.189	1.432	0.744	2.344	2.207
Hepatotoxicity	Categorical (Yes/No)		No	No	No	Yes	Yes
Skin Sensitisation	Categorical (Yes/No)		No	No	No	No	No
T.Pyriformis toxicity	Numeric (log ug/L)	> -0.5 = toxic	0.377	0.567	0.638	0.388	0.31
Minnow toxicity	Numeric (log mM)	Below (<) 0.5 =high acute toxicity	1.941	0.609	-0.04	-0.491	-0.35

Table 5.12.5: Druggability analysis of phytochemicals for excretion

Property	***unit***	***Predicted value***					
		Prediction	***4',5,7-Trihydroxy isoflavone***	***5-hydroxy-2-(4-methoxyphenyl),4-H-1 Benzopyran-4-one***	***5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one***	***Palustric acid***	***pimaric acid***
Total Clearance	Numeric (log ml/min/kg)	The higher the better	0.151	0.737	0.457	0.917	0.941
Renal OCT2 substrate	Categorical (Yes/No)		No	Yes	No	Yes	No

Table 5.12.6: Druggability analysis according to Lipinski's criterion

Property	***Values***				
	4',5,7-Trihydroxy isoflavone	***5-hydroxy-2-(4-methoxyphenyl),4-H-1 Benzopyran-4-one***	***5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one***	***Palustric acid***	***pimaric acid***
Molecular weight	270.24	298.29	268.27	302.46	302.46
Log P	2.58	3.18	3.17	5.35	5.21
Number of hydrogen bond donors	3	1	1	1	1
Number of hydrogen bond acceptor	5	5	4	1	1

5.8 *IN VITRO* PROPAGATION AND COMPARISON OF BIOACTIVITIES OF AXENICALLY AND NATURALLY GROWN LIVERWORT *LUNULARIA CRUCIATA*

Result of antioxidant activities revealed that liverworts *Plagiochasma cordatum, Asterella wallichiana*, *Lunularia cruciata*, *Marchantia paleacea* and *Plagichila nepalensis* have high potential to scavenge free radicals. Considering the bio prospective value of the plant and their lower availability in nature, axenic culture was started with gemmae of liverwort *Lunularia cruciata*. As sporophytes were not found in proper stage of development, an attempt was made to initiate axenic culture from the gemmae. Germination of gemmae started after 7-8 days. Gemmae increased in size absorbing moisture and the viable gemmae were turned green in colour. Half strength Murashige and Skoog (MS) medium in alternate light (L) and dark condition (D) of 14hrs L/ 12hrs D was found suitable, supporting maximum gemmae germination. Germination of few gemmae was noticed in Knop's micronutrient medium also, but no further growth and development was observed. No germination was noticed in half and full strength Gamborg B-5 medium. Continuous illumination of 4000-5000 lux and temperature 21^0C $\pm 2^0$C was found to be favourable. Among different concentration of sterilants used either 1% sodium hypochlorite with exposure time of 4 minutes or 4% sodium hypochlorite with exposure time of 1 minute proved effective as gemmae remained alive and maximum microbes were killed. Exposure to higher concentration of sterilant solution even for few seconds was found to be toxic to plant material. Similarly, treatment of the explants in lower concentration given for longer time was also found to be lethal.

The early sign of germination was the change in the colour of gemmae from brown to green. Highest percentage (90%) of germination was observed in half strength MS media followed by half strength Knop's medium (20%), while no germination was noticed in full strength MS media, Knop's medium, Gamborg B5 medium and half strength Gamborg G5 medium (figure 5.8.1). Gemmae then started to increase in size and green colour became more prominent. Young thalli started developing from gemma after 10-12 days. After 2 weeks of development of thalli, gemmae turned into dark blackish green coloured undifferentiated tissue called callus. At this stage profound effect of growth regulators like Benzylaminopurine and Indole acetic acid was noticed. Effect of different combination of growth regulator concentrations was studied which confirmed 2 mg/L BAP and 0.5 mg/L

	NAA (0.5 mg/L)	NAA (1 mg/L)	NAA (2 mg/L)
BAP (1 mg)	No germination	No germination	No germination
BAP (2 mg)	Germination	No germination	No germination
BAP (3 mg)	No germination	No germination	No germination

Table 5.13: Effect of BAP/NAA on gemmae germination

Fig 5.8.1: Effect of different growth medium on gemmae germination

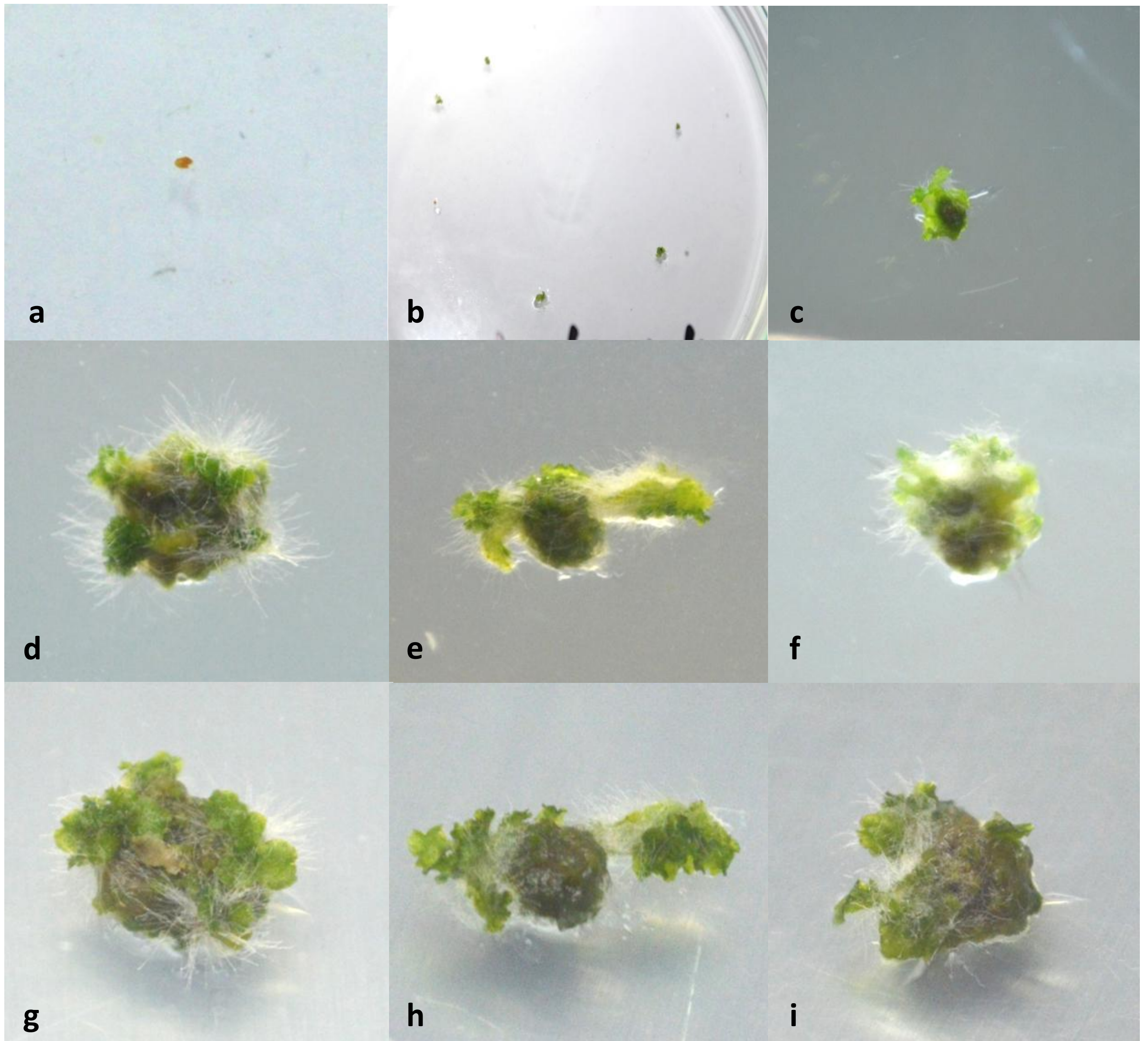


Fig 5.8.2.: **In-vitro growth of *Lunularia cruciata*.** **a.** Gemmae placed on MS media **b-c.** germinating gemmae after 16-17 days **d-f.** thallus and rhizoids developed from gemmae **g-i.** well developed thalli after repeated sub culturing **j-l.** control of excessive growth of rhizoids by alternating hormone ratio and growth of dichotomously branched thalli

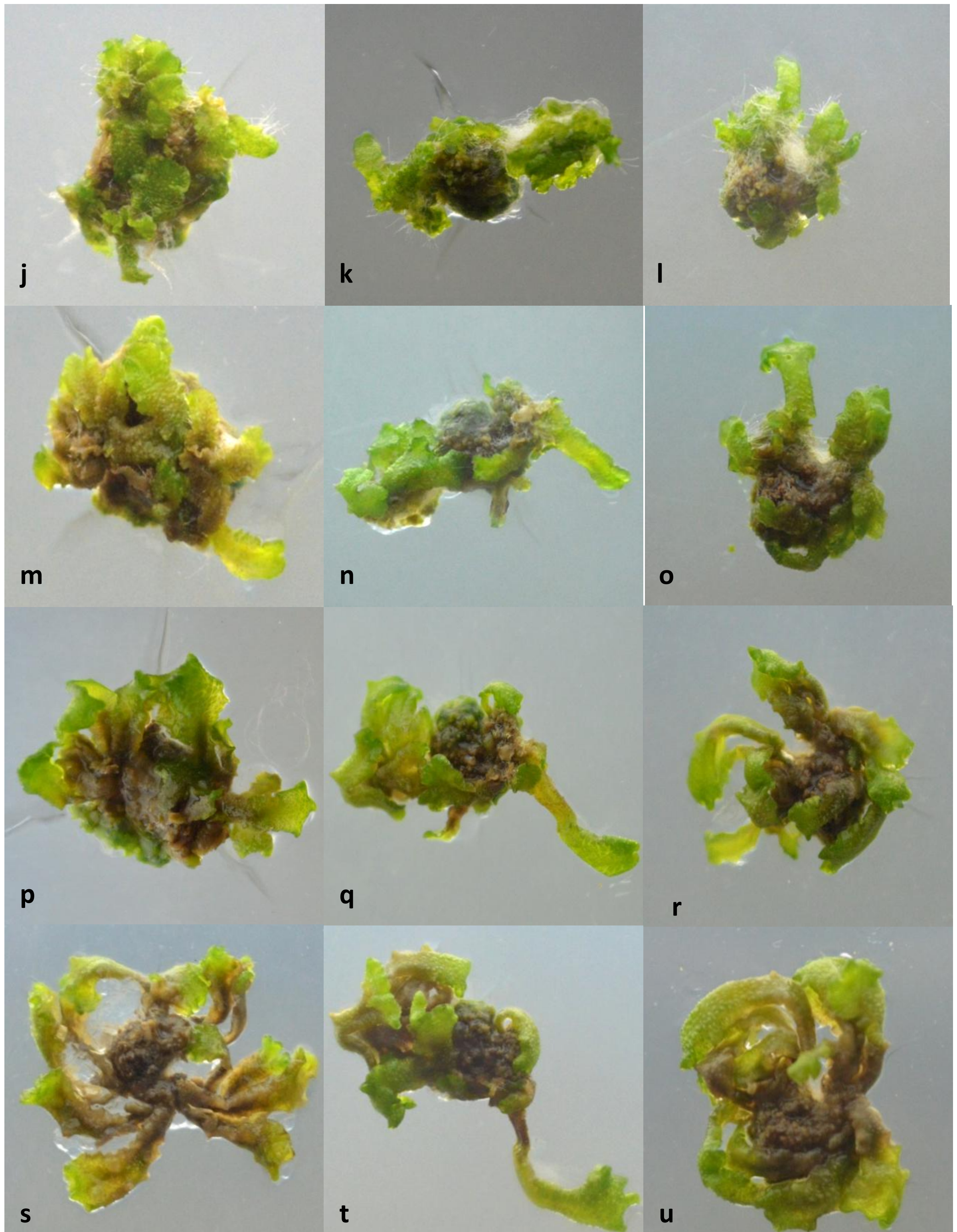


Fig 5.8.2.: ***In-vitro* growth of *Lunularia cruciata*** . **j-l** control of excessive growth of rhizoids by alternating hormone ratio and growth of dichotomously branched thalli **m-u** growth and maturation of thalli (continuation).

NAA to be most efficient for differentiation (Table 5.13). Higher concentration of growth regulators had shown detrimental effect on callus. Large number of rhizoids growing from all part of callus was noticed in the media containing 0.5 mg/L NAA hindering further differentiation of tissues. The transfer of callus in the media containing 4 mg/L BAP and 0.2 mg/L IAA lead to the development of thallus from undifferentiated tissue with much reduced number of rhizoids on the dorsal side of the thallus. With illumination of 4000-5000 lux under alternate light and dark condition of 14 hrs and 10 hrs and temperature maintained at $21^0C \pm 2^0C$ the thallus further continued to branch and grow (Fig 5.8.2). After the maturation of thallus an *in-vitro* antioxidant and anti-diabetic activities of this *in-vitro* grown liverwort was compared with its naturally growing counterparts to check if there occurs any change in the biological activities when liverworts are grown in artificial culture medium.

5.8.1 Comparison of free radical scavenging potential of *in-vitro* and naturally grown *Lunularia cruciata*

5.8.1a *DPPH*$^{\bullet}$ *scavenging activity*

The result of DPPH scavenging assay revealed substantial ability of both the extracts to scavenge DPPH radical (Fig 5.8.3). *In-vitro* and *in-vivo* plant extract showed only slight variation in the magnitude of radical scavenging activity. *In-vivo* plant extract showed 73%, 40% and 24% inhibition at 650 µg, 350 µg and 250 µg concentrations respectively. Extract from *in vitro* source showed 48%, 35% and 22% inhibition of DPPH at 650 µg, 350 µg and 250 µg concentrations respectively.

5.8.1b *ABTS*$^{+}$ *scavenging activity*

This assay is widely used in *in-vitro* studies to assess the antioxidant property. Free radical scavenging property of extracts was further confirmed by $ABTS^+$ scavenging assay (Fig 5.8.4). 98% and 88% inhibition of ABTS radical was shown by *in-vitro* and *in-vivo* extracts respectively at 2 mg/ml concentration. Data highlighted slightly better $ABTS^+$ scavenging property of *in-vitro* plant extract than that of *in-vivo* plant extract.

5.8.1c *Metal chelating assay*

Variation between *in-vitro* and *in-vivo* grown plant extracts was recorded for metal chelating activity. A reduction of 85% in free ferrous ion concentration was shown by 4.5 mg

in-vivo plant extract while *in-vitro* plant extracts showed 46% reduction at 4.5 mg concentration (Fig 5.8.5).

5.8.2 Comparison of anti-diabetic activity of *in-vitro* and naturally grown *Lunularia cruciata*

5.8.2a *α-Glucosidase inhibitory activity*

Result of α-glucosidase inhibitory assay revealed the property of *in-vitro* and *in-vivo* plant extracts to inhibit the α-glucosidase enzyme activity to an impressive level. *In-vitro* plant extract showed IC_{50} value 0.24 mg/ml and *in-vivo* plant showed IC_{50} value 0.51 mg/ml (Fig 5.8.6). Lower IC_{50} value of *in-vitro* plant extract indicates its higher α-glucosidase inhibitory activity than the *in-vivo* grown plants.

5.8.2b *α-amylase inhibitory activity*

In-vitro and *in-vivo* grown plants both showed α-amylase enzyme inhibitory activity. IC_{50} value of 0.48 mg/ml and 0.40mg/ml was recorded for *in-vitro* and *in-vivo* grown plant extract respectively (Fig 5.8.7).

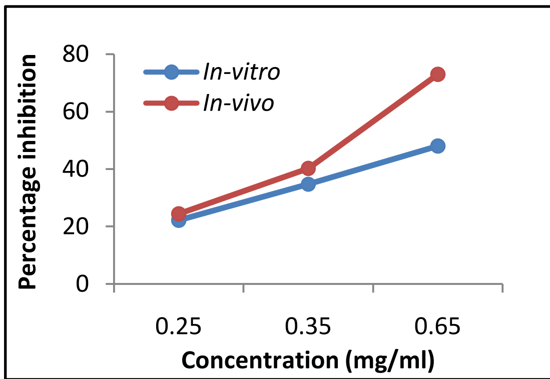


Fig 5.8.3: DPPH˙ scavenging activity of *in-vitro* and naturally grown *L. cruciata*

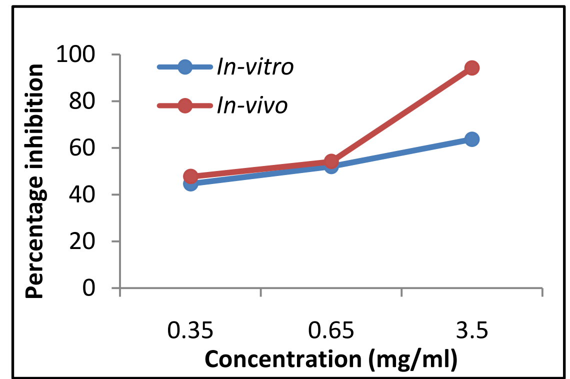


Fig 5.8.4: ABTS˙ scavenging activity of *in-vitro* and naturally grown *L. cruciata*

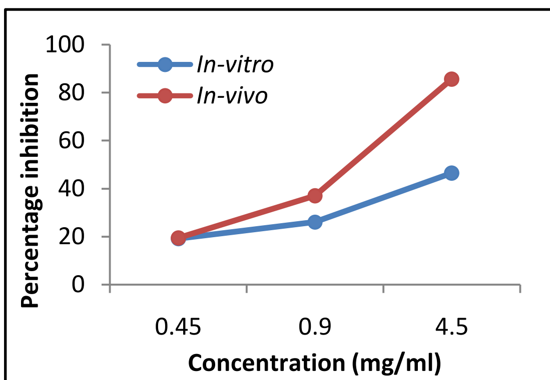


Fig 5.8.5: Metal chelating activity of *in-vitro* and naturally grown *L. cruciata*

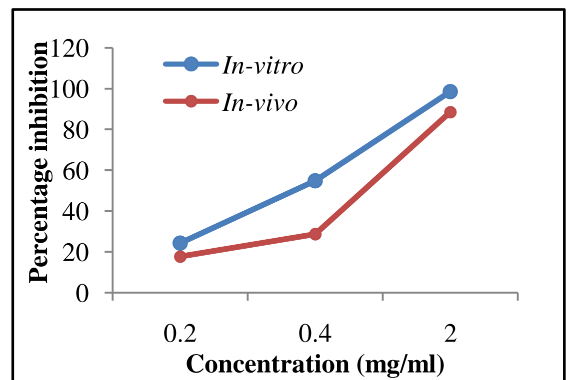


Fig 5.8.6: α-glucosidase inhibitory activity of *in-vitro* and naturally grown *L. cruciata*

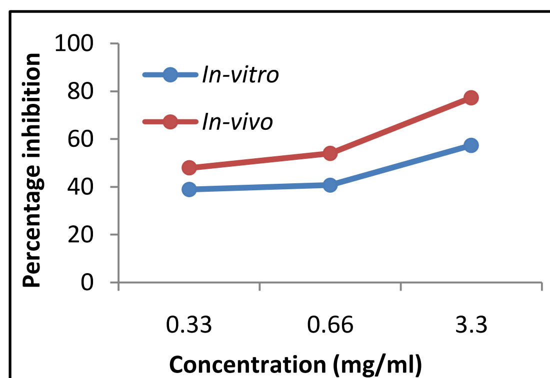


Fig 5.8.7: α-amylase inhibitory activity of *in-vitro* and naturally grown *L. cruciata*

5.8.3 Phytochemical analysis of *in-vitro* and naturally grown *Lunularia cruciata*

GC-MS analysis was performed to study the variation in chemical profile of *in-vitro* and naturally grown plants and also to characterize the possible sources of liverwort extracts for biological activity. Methanolic extract of *in-vitro* and naturally grown plants showed the presence of different types of alkaloids, flavonoids, terpenes, fatty acid, aliphatic hydrocarbon and acyclic alkanes on analyses. Total of 9 compounds have been identified from naturally grown plant methanolic extract, this includes thujopsene; flavones; cyclopentaneudecanoic acid, methyl ester; cyclopentaneundecanoic acid; 13,16-octadecadienoic acid; methyl ester; phytol; deoxyaspidodispermine; 13-docosenoic acid; methyl ester and 4-piperidieacetic acid,1-acetyl-5-ethyl-2-[3(2-hydroxyethyl)-1H-indol-2-yl]-a-methyl and methyl ester (Table 5.14). Methanolic extract of *in-vitro* grown plant also showed the presence of 9 compounds *namely*, piperidine, 1-butyl; 2-cyclohexen-1-one, 3hydroxy-2-[(5-oxo-1-cyclopenten-1-yl) methyl]; flavones; 4H-1-benzopyran-4-one, 3 hydroxy-2-phenyl, tetradecanoic acid, ethyl ester; phytol; Z-13-octadecen-1-yl acetate; Isopropyl stearate and tricosane-2,4-dione (Table 5.15).

GC-MS analysis has showed the present of phytol and flavones in plants grown in both natural and artificial growth condition. These compounds showed similar retention time as well. Alkaloid group was present in both naturally and artificially grown *L. cruciata* but in different forms. 1-butyl Piperidine was found in *in-vitro* grown plants while slightly modified form, 4-piperdineacetic acid, 1 acetyl 5 ethyl-2[3-(2 hydroxy ethyl)-1H-indol-2-yl]-a methyl, methyl ester was found in grown grown plants. Fatty acid and their esters were also detected in plants grown in both the habitats. Ethyl tetradecanoate, Isopropyl stearate were fatty acids present in *in-vitro* grown plants and Cyclopentaneudecanoic acid, Cyclopentaneudecanoic acid, methyl ester, 13-Decosenoic acid, methyl ester, 13,16-Octadecanoic acid, methyl ester are form of fatty acids that were present in naturally grown plants. However, presence of aliphatic hydrocarbon and alkanes were found only in *in-vivo* grown plants (Table 5.16).

Table 5.14 : Phytochemicals present in *in-vitro* grown *Lunularia cruciata* methanolic extract and their biological activities

Name	***RT***	***TIC***	***Compound nature***	***Biological activities***
1-1-butyl Piperidine	12.08	481975	alkaloid	Anti-microbial (Kumar *et al*., 2009); cytotoxic (Rinaldi *et al*., 2017); Antimalarial (Wirasathien *et al*., 2006)
3-Hydroxy-2-[(5-oxo-1-cyclopenten-1-yl)methyl]-2-cyclohexen-1-one	14.17			No activities reported
Flavone	16.5	596325	Flavonoid	Antioxidant (Pietta, 2000), Hepatoprotective (Akachi *et al*., 2010), Antibacterial (Cushnie and Lamb, 2005), Anti-Inflammatory (Serafini *et al*., 2010), Anticancer (Zhang *et al*., 2008), Antiviral Activity (Lani *et al*., 2016)
3-hydroxy-2-phenyl 4H-1-Benzopyran-4-one	17.27	1412798	Flavonols	Antioxidant (Pietta, 2000), Hepatoprotective (Akachi *et al*., 2010), Antibacterial (Cushnie and Lamb, 2005), Anti-Inflammatory (Serafini *et al*., 2010), Anticancer (Zhang *et al*., 2008), Antiviral Activity (Lani *et al*., 2016)
Tetradecanoic acid, ethyl ester	18.03	632812	Fatty acid	No activities reported
Phytol	18.95	2122402	Diterpene alcohol	Antimicrobial (Cardoso *et al*., 2012), Anti-inflammatory (Khiev *et al*., 2011), Anti cancer (Islam, 2017), Cardiovascular and diuretic activity (Somova *et al*., 2001)
Z-13-octadecen-1yl acetate	20.43	1229732	Aliphatic hydrocarbon	No activities reported
Isopropyl stearate	22	833849	Fatty acid ester	No activities reported
Tricosane 2,4-dione	23.17	607218	acyclic alkanes	No activities reported

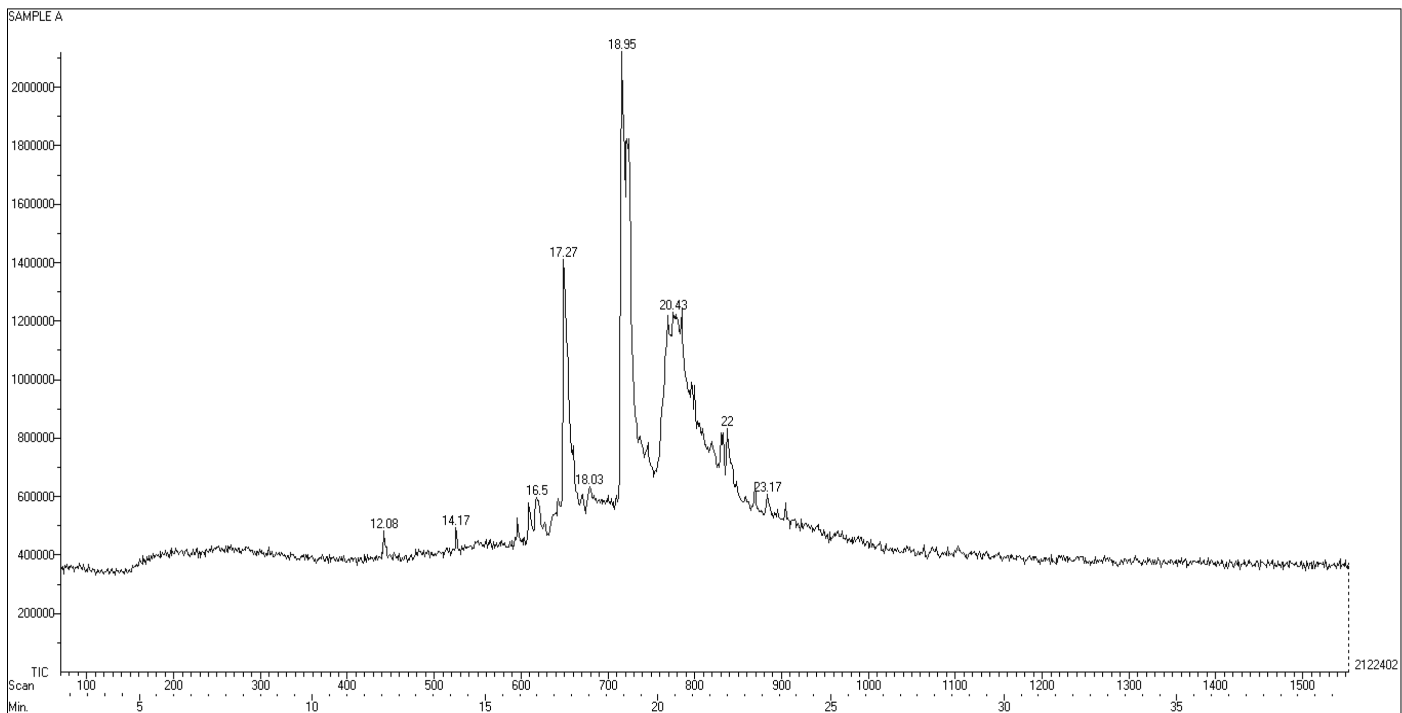


Fig 5.8.8: Gas chromatogram with mass-spectrometric detection of the methanolic extract of *in-vitro* grown *Lunularia cruciata*

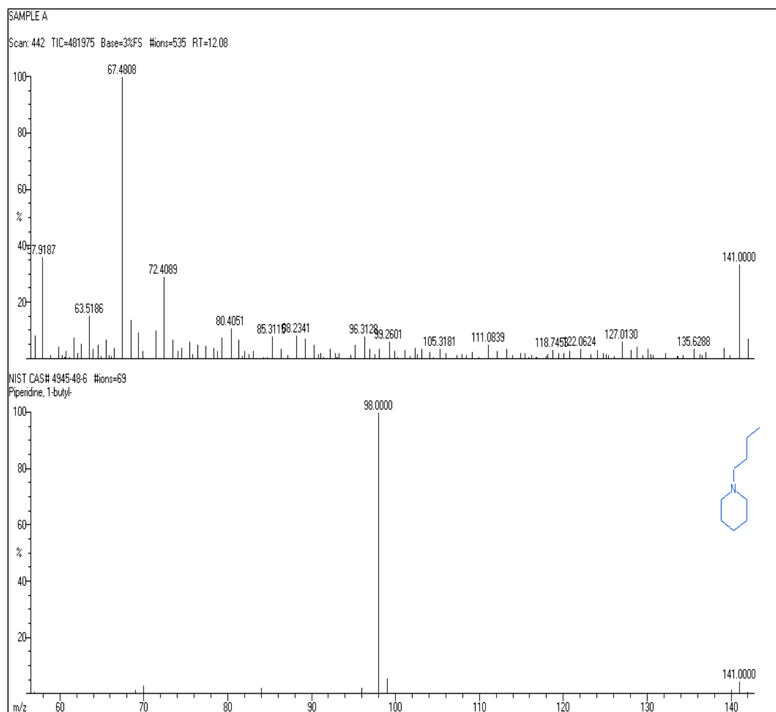


Fig 5.8.9: Structure and MS spectrum of 1-butyl-Piperidine compared with NIST Library Spectral Database

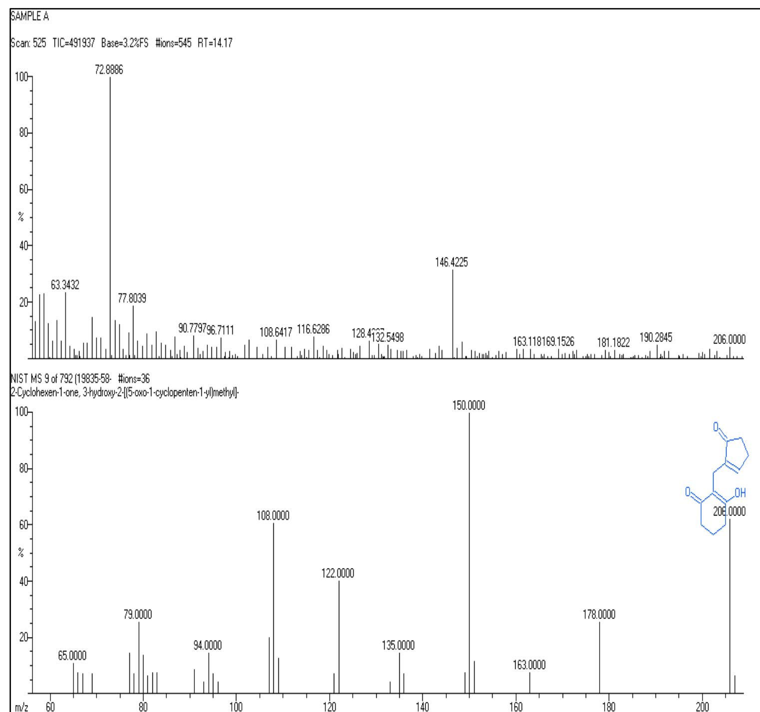


Fig 5.8.10: Structure and MS spectrum of 3-Hydroxy-2-[(5-oxo-1-cyclopenten-1-yl)methyl]-2-cyclohexen-1-one compared with NIST Library Spectral Database

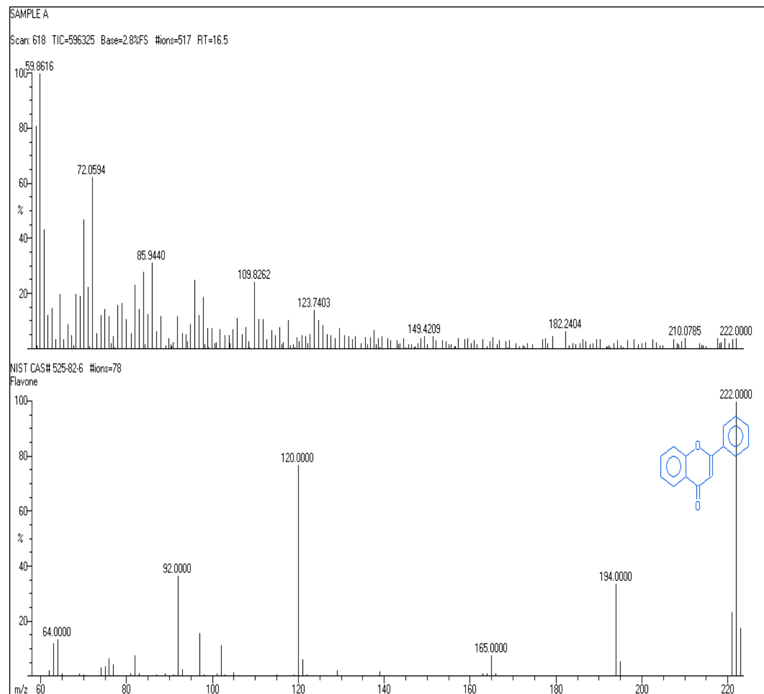


Fig 5.8.11: Structure and MS spectrum of flavone compared with NIST Library Spectral Database

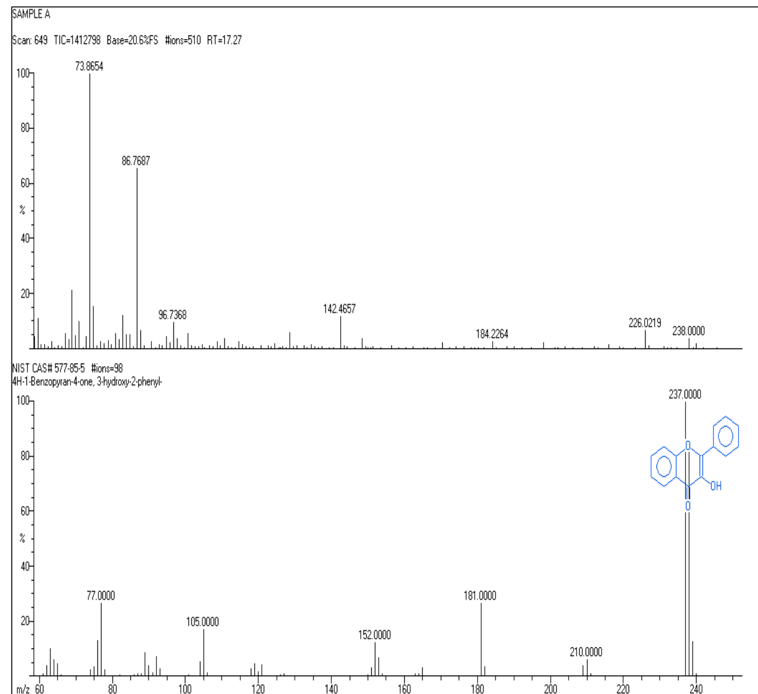


Fig 5.8.12: Structure and MS spectrum of 4H-1-Benzopyren-4-one, 3-hydroxy-2-phenyl compared with NIST Library Spectral Database

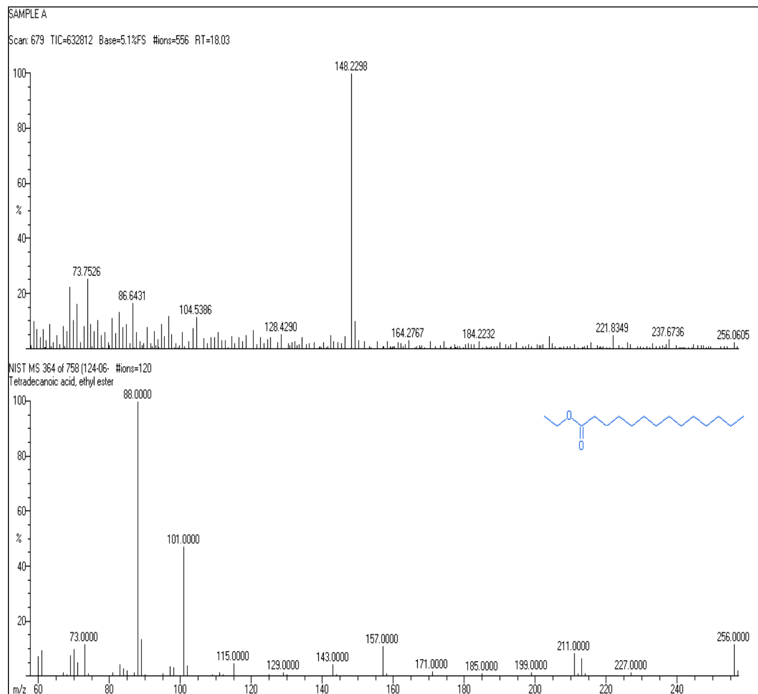


Fig 5.7.13: Structure and MS spectrum of Tetradecanoic acid, ethyl ester compared with NIST Library Spectral Database

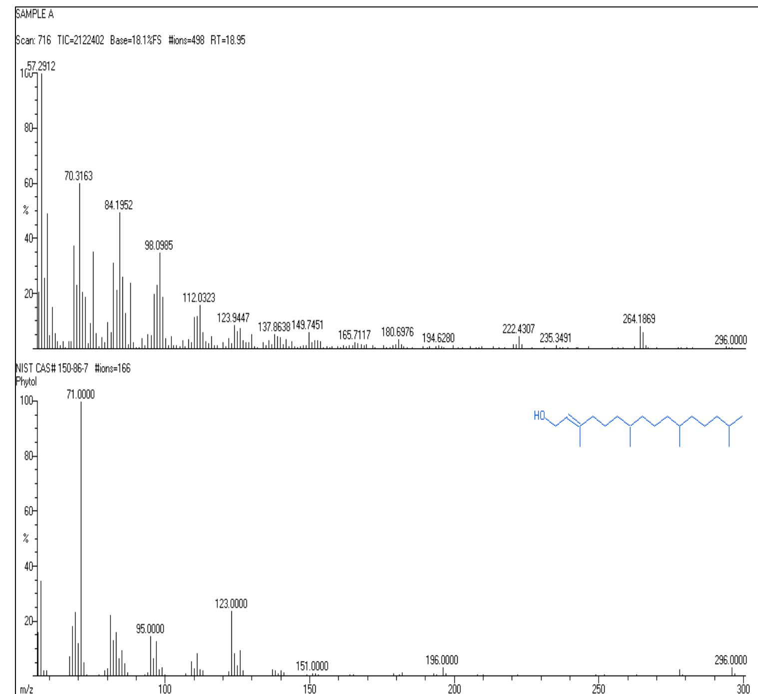


Fig 5.7.14: Structure and MS spectrum of Phytol compared with NIST Library Spectral Database

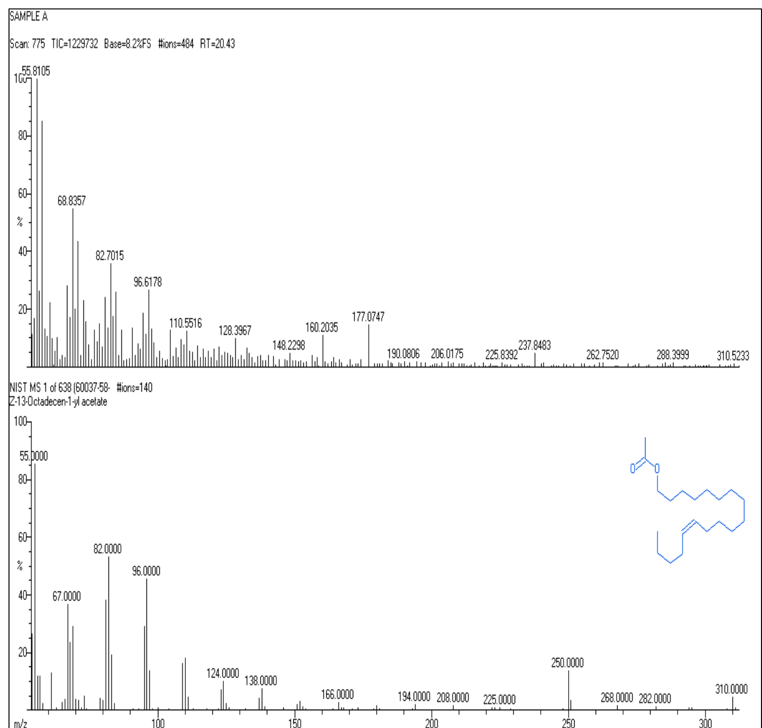


Fig 5.7.15: Structure and MS spectrum of 2-13-octadecen-1-yl acetate compared with NIST Library Spectral Database

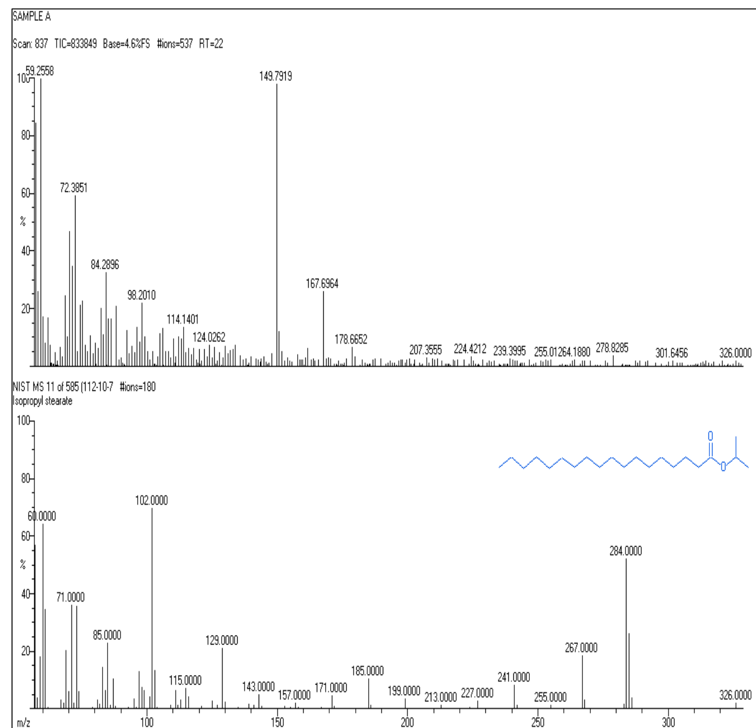


Fig 5.7.16: Structure and MS spectrum of Isopropyl stearate compared with NIST Library Spectral Database

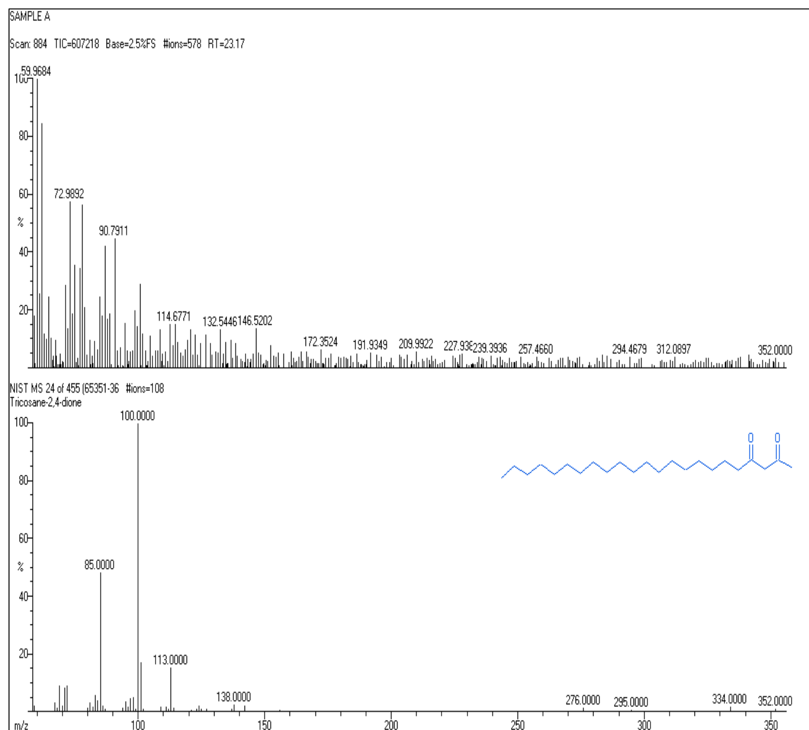


Fig 5.8.17: Structure and MS spectrum of Tricosane -2,4- dione compared with NIST Library Spectral Database

Table 5.15 : Phytochemicals present in naturally grown *Lunularia cruciata* methanolic extract and their biological activities

Name	***RT***	***TIC***	***Compound nature***	***Biological activities***
Thujopsene-[12]	14.03	873686	sesquiterpene	Antimicrobial (Cardoso *et al*., 2012), Anti-inflammatory (Khiev *et al*., 2011), Anti cancer (Islam 2017), Cardiovascular and diuretic activity (Somova *et al*., 2001)
Flavone	15.75	1138027	Flavonoid	Antioxidant (Pietta, 2000), Hepatoprotective (Akachi *et al*., 2010), Antibacterial (Cushnie and Lamb, 2005), Anti-Inflammatory (Serafini *et al*., 2010), Anticancer (Zhang *et al*., 2008), Antiviral Activity (Lani *et al*., 2016)
Cyclopentaneudecanoic acid, methyl ester	17.17	1822103	Fatty acid ester	No activities found
Cyclopentaneudecanoic acid	18.08	1274201	Fatty acid	No activities found
13,16-Octadecanoic acid, methyl ester	18.85	2536991	Fatty acid ester	No activities found
Phytol	19.03	2799556	Diterpene alcohol	Antimicrobial (Cardoso *et al*., 2012), Anti-inflammatory (Khiev *et al*., 2011), Anti cancer (Islam 2017), Cardiovascular and diuretic activity (Somova *et al*., 2001)
Deoxy aspidodispermine	20.43	2148295	alkaloid	No activities found
13-Decosenoic acid, methyl ester	21.77	1202092	Fatty acid ester	No activities found
1 acetyl 5 ethyl-2[3-(2 hydroxy ethyl)-1H-indol-2-yl]-a methyl, 4-piperdineacetic acid, methyl ester	21.95	1105543	Alkaloid	Anti-microbial (Kumar *et al*., 2009); cytotoxic (Rinaldi *et al*.,2017); Antimalarial (Wirasathien *et al*., 2006)

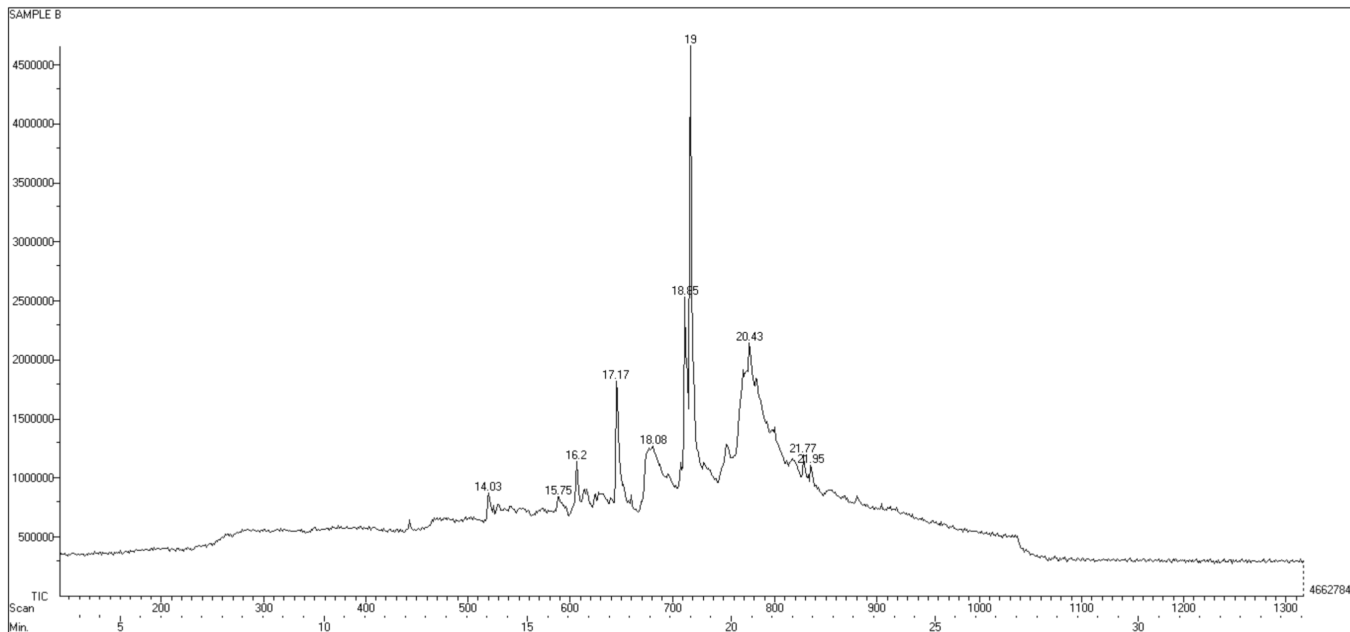


Fig 5.8.18: Gas chromatogram with mass-spectrometric detection of the methanolic extract of naturally grown *Lunularia cruciata*

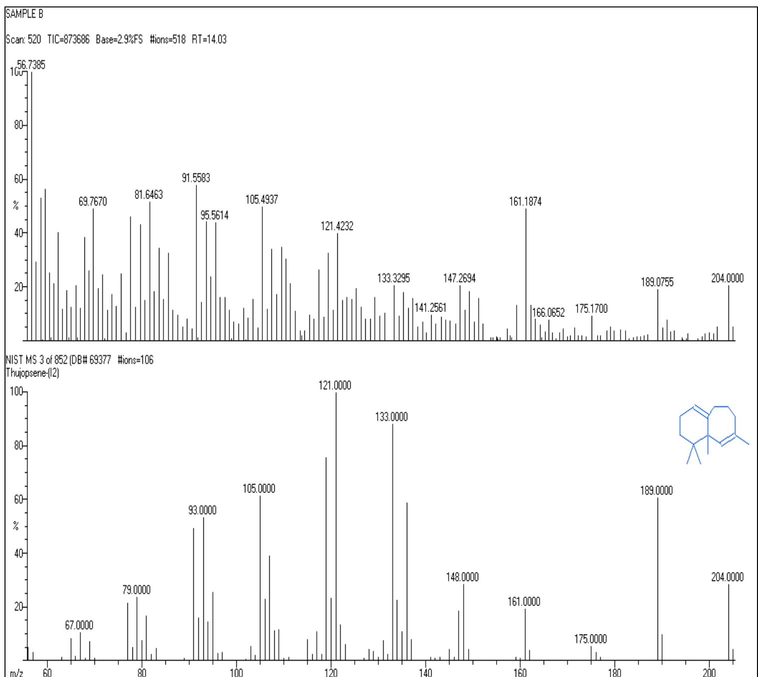


Fig 5.8.19: Structure and MS spectrum of Thujopsene-(12) compared with NIST Library Spectral Database

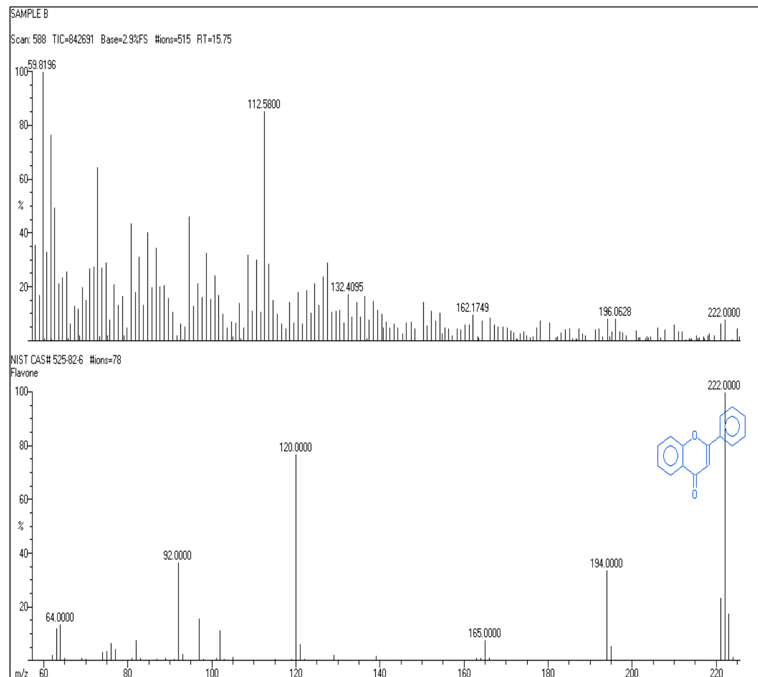


Fig 5.8.20 : Structure and MS spectrum of Flavone compared with NIST Library Spectral Database

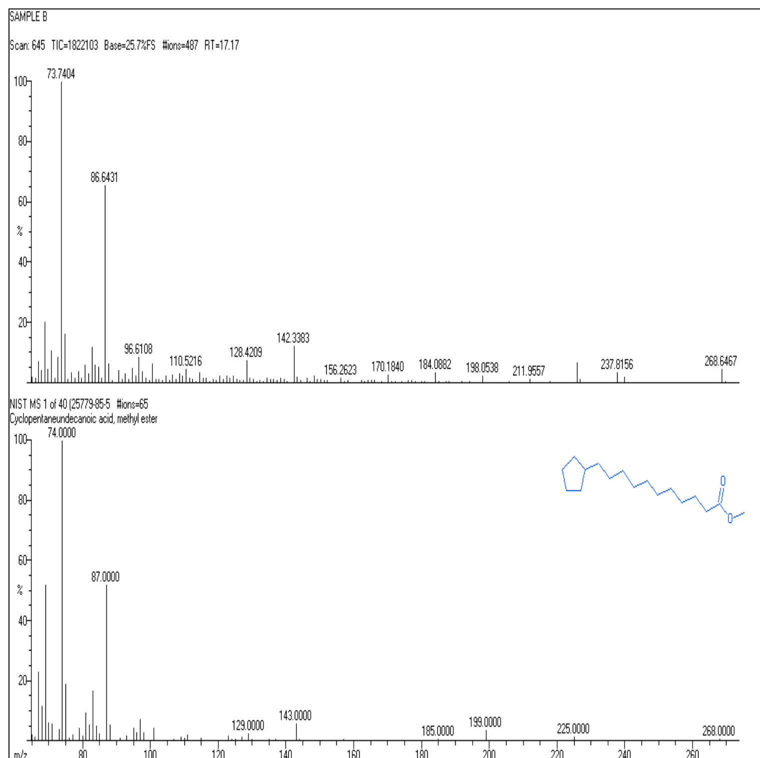


Fig 5.8.21: Structure and MS spectrum of Cyclopentaneudecanoic acid, methyl ester compared with NIST Library Spectral Database

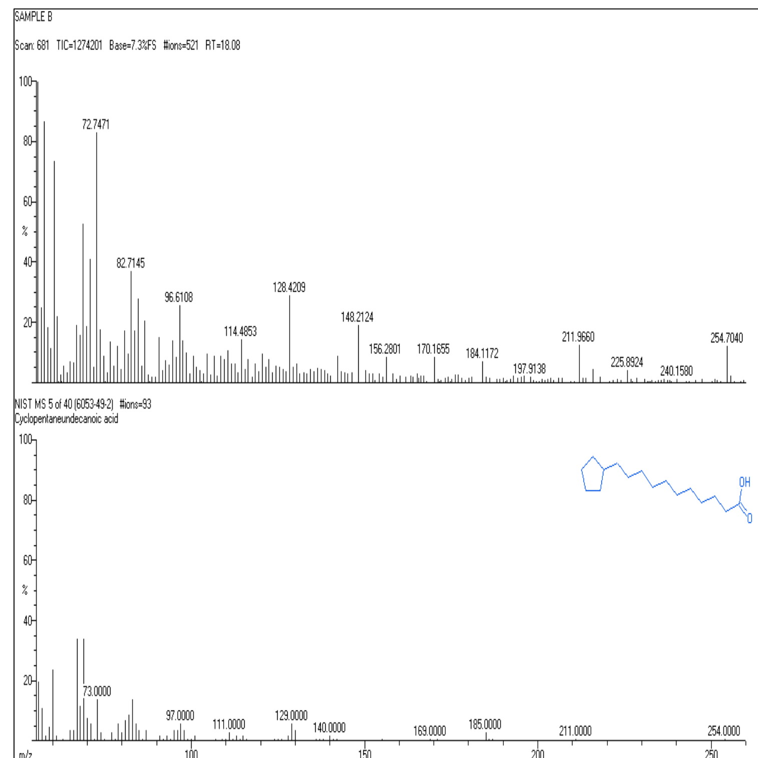


Fig 5.8.22: Structure and MS spectrum of Cyclopentaneudecanoic acid compared with NIST Library Spectral Database

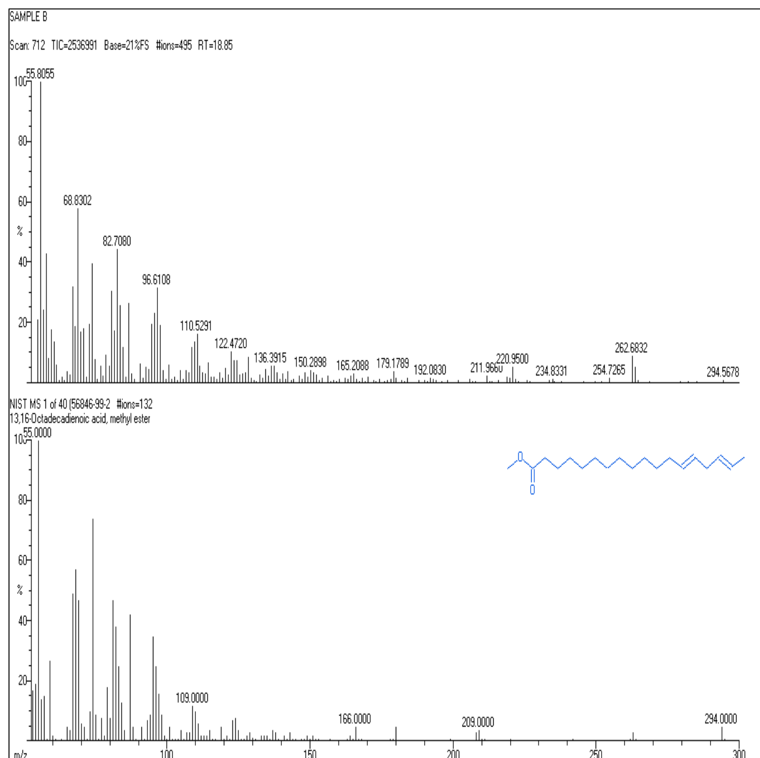


Fig 5.8.23: Structure and MS spectrum of 13,16-Octadecadienoic acid, methyl ester compared with NIST Library Spectral Database

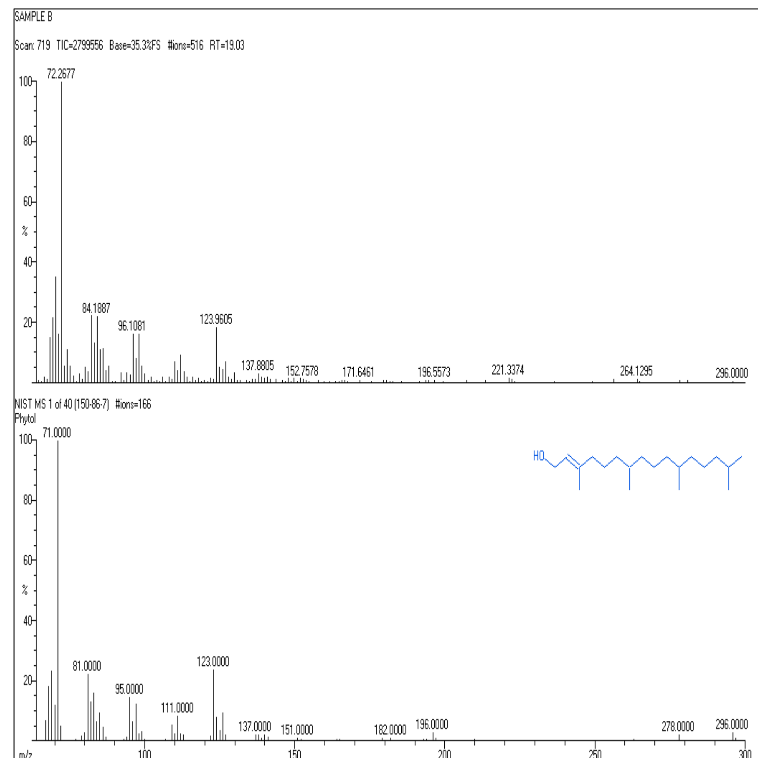


Fig 5.8.24 : Structure and MS spectrum of Phytol compared with NIST Library Spectral Database

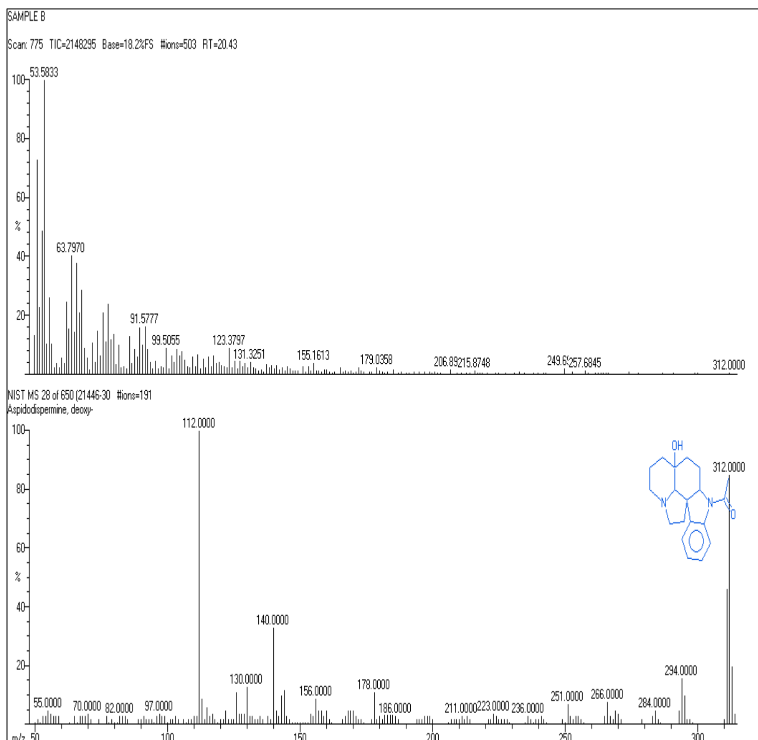


Fig 5.8.25: Structure and MS spectrum of deoxy-Aspidodispermine compared with NIST Library Spectral Database

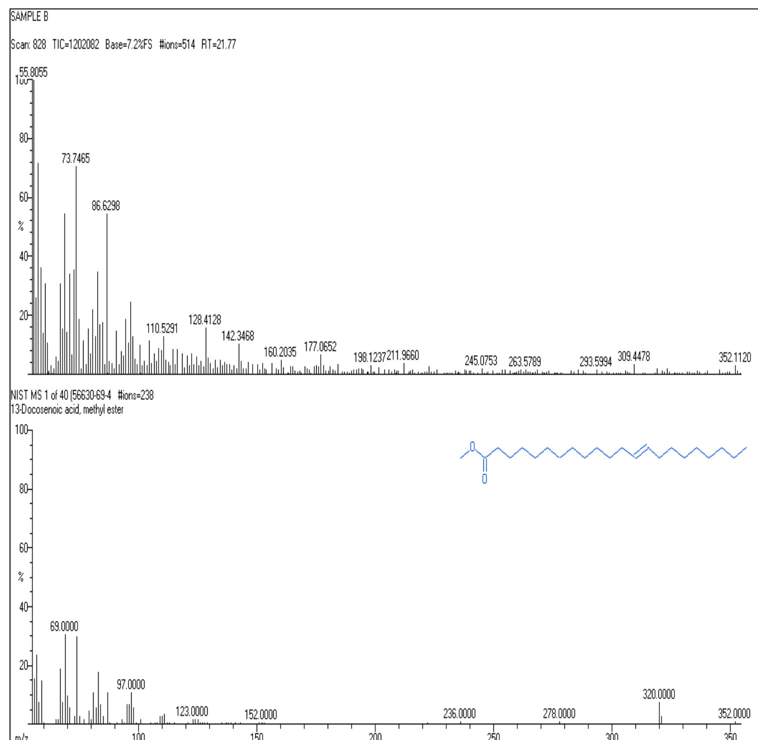


Fig 5.8.26: Structure and MS spectrum of 13-Docosenoic acid, methyl ester compared with NIST Library Spectral Database

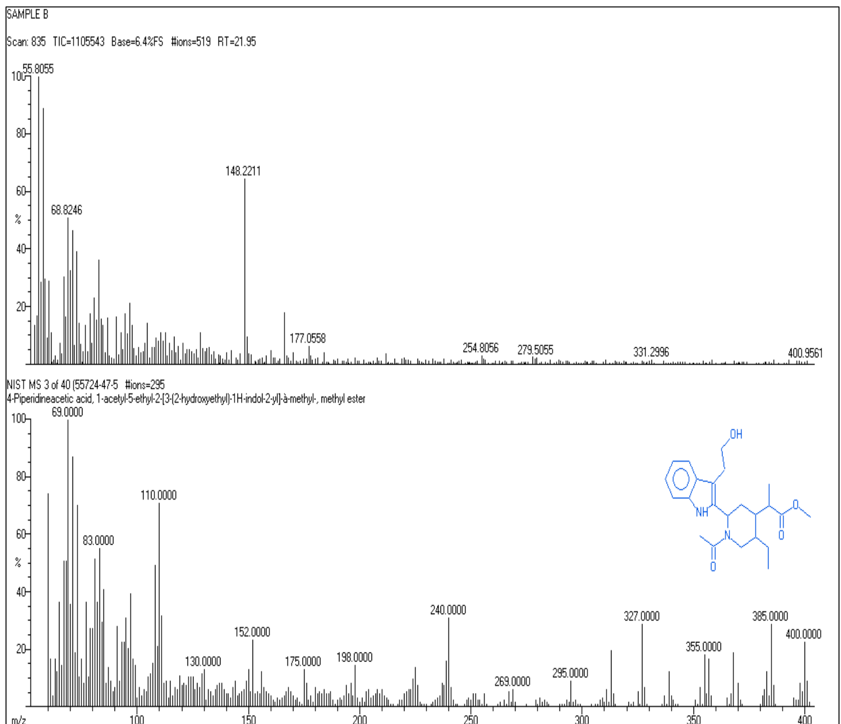


Fig 5.8.27: Structure and MS spectrum of 1-acetyl-5-ethyl-2-[3-[2-hydroxyethy]-1H-indol-2-yl]-α-methyl 4-Piperidineacetic acid, methyl ester compared with NIST Library Spectral Database

Table 5.16: Comparison of phytochemicals present in *in-vitro* and *in-vivo* grown Lunularia cruciata

Sl. No.	*Compound class*	*Plant growth condition*			*Retention time*	*Relative abundance*
		In-vitro	*Relative abundance*	*Naturally grown*		
1	**Alkaloid**	1-butyl Piperidine	23%	-	12.08	
		-		4-piperdineacetic acid,; 1 acetyl 5 ethyl-2[3-(2 hydroxy ethyl)-1H-indol-2-yl]-a methyl, methyl ester	21.95	40%
2	**Terpenes**	Phytol	100%	Phytol	18.95 (*in-vitro*), 19.03 (*in-vivo*)	100%,
		-		Thujopsene-[12]	14.03	31%
3	**Flavonoid**	Flavone ;	28%	Flavone	16.5	71%
		3-hydroxy-2-phenyl 4H-1-Benzopyran-4-one,	67%	-	16.80	
4	**Fatty acid**	Ethyl tetradecanoate	30%	-	18.03	
				Cyclopentaneudecanoic acid	18.08	46%
5	**Fatty acid ester**	Isopropyl stearate	39%		22	
		-		Cyclopentaneudecanoic acid, methyl ester ;	17.17	65%
		-		13,16-Octadecanoic acid, methyl ester ;	18.85	91%
		-		13-Decosenoic acid, methyl ester	21.77	43%
6	**Aliphatic hydrocarbon**	Z-13-octadecen-1yl acetate	58%	-	20.43	
7	**Alkanes**	Tricosane 2,4-dione	29%	-	23.17	

5.9.1 VARIATION IN THE ABUNDANCE OF EPIPHYTIC LIVERWORTS IN RELATION PHYSICO-CHEMICAL ATTRIBUTES

This study demonstrates the effects of different environmental factors and biochemical characteristics of *Cryptomeria japonica* tree bark on the relative abundance of the epiphytic liverworts. Liverwort flora on *C. japonica* bark consisted mainly of three species *viz*, *Bazzania oshimensis*, *Ptycanthus striatus* and *Pellia epiphylla* belonging to families Lepidoziaceae, Lejeuneaceae and Peliaceae respectively. According to abundance percentage, *B. oshimensis* was the most dominant species while *Pellia epiphylla* was found scarcely.

The study on epiphytic liverworts' abundance in Senchal forest showed moisture content of the substrate (bark) to be best correlated with the epiphytic cover in positive manner showing coefficient of correlation value of 0.648 with $R^2 = 0.420$. Barks with moisture content ranging between 52.24% and 84.48% favours luxurious growth of epiphytic liverworts while, their frequency of occurrence declined drastically below 50% moisture level (Fig 5.9.1). Among analyzed trees, maximum trees showed 65–71% moisture content in their bark (Fig 5.9.2). Light intensity on the studied sites ranged between 1620 lux and 15200 lux. Data suggested that light intensity in the canopy and epiphytic liverwort cover were inversely related with each other. Pearson correlation matrix value of -0.77 signifies inverse relationship between two variables. In the studied sites, maximum trees favouring epiphytic liverwort cover experienced 2800–3700 lux light intensity (Fig 5.9.4). High epiphytic liverwort abundance was recorded in light intensity range between 1900 to 4600 lux (Fig 5.9.3).

The pH of bark of *C. japonica* was slightly acidic (pH = 3.0 – 5.1). In our study, pH of the bark didn't affect the abundance of the epiphytic liverwort on the tree trunk [$R^2 = 0.0043$] (Table 5.20). Maximum trees had pH ranging between 3.2–3.4 (Fig 5.9.6). Presence of epiphytic liverworts was noticed on moderate to old aged trees, while they were completely absent on the young trees (Fig 5.9.5). Coefficients of determination (R^2) value of $R^2 = 0.602$ between abundance and dbh indicates positive correlation between the studied variables (Pearson Correlation matrix = 0.776). Epiphytic liverwort cover was noticed on trees having girth above 100 cm, while no growth were observed on trees having girth size below this level (Fig 5.9.7).

In studied trees, phenol content ranged between 36–844 μg gallic acid equivalent (eq.) / mg fresh weight tissue (fwt). Maximum trees under study showed phenol content in a range of 85–170 μg gallic acid eq. / mg fwt (Fig 5.9.10). Epiphytic liverwort cover was noticed on trees with phenol content ranging between 36–510 μg gallic acid eq./ mg fwt. Higher content of phenol showed inhibitory activity against liverwort proliferation (Fig 5.9.9) with few exceptions. However, Pearson correlation test didn't indicate any direct correlation between phenol content and bryophyte cover (Table 5.19).

Flavonoid content of the tree also didn't affect the liverwort proliferation. Co-efficient of determination and Pearson correlation value indicated no correlation between two variables. Epiphytic liverwort cover was noticed on trees having flavonoid content above 5.7 μg Quercetin equivalent/ mg fwt (Fig 5.9.11). Maximum epiphytic liverwort proliferation was noticed on trees containing flavonoid in the range 11.3–14.2 μg quercetin eqv. / mg fwt (Fig 5.9.12). Similar to flavonoids, no correlation was found between ortho-dihydric phenol content and liverwort cover by Coefficient of determination and Pearson correlation analysis (Table 5.19). Epiphytic liverwort abundance didn't increase or decrease with the content of orthodihydric phenol (Fig 5.9.13). Maximum percentage of epiphytic liverwort abundance was noticed on trees having 3.8–4.5 μg catechol eqv./ mg fwt ortho-dihydric phenol (Fig 5.9.14). Phenol, flavonoid and ortho-dihydric phenol content of the studied trees varied irrespective of tree age, suggesting no effect of age on phenolic compound content of plants. Another important class of secondary metabolite synthesized by plants is terpenoids.

Terpenoids are the primary constituents of pine oleoresin (Rodrigues-Correa *et al.*, 2013). Co-efficient of determination value, R^2= 0.938 suggests direct correlation between terpenoid content and liverwort cover (Table 5.20); Pearson correlation value of -0.959 (Table 5.18) suggested correlation to be negative between two variables. Coefficient of determination (Table 5.20); R^2= 0.654 (Pearson correlation matrix = - 0.851) suggested that terpenoid content in the studied trees decreased with the increase in tree age (Table 5.18). Maximum epiphytic growth was observed on the tree having terpenoid content in a range between 1.32 mg to 13.2 mg fwt. However, increase in the concentration of terpenoid affected the proliferation of liverworts in a negative manner.

Two more biochemical variables, total sugar and tannin content of the bark were studied to understand their possible role in epiphytic liverwort distribution. Optimal

proliferation of epiphytic liverworts was noticed on trees containing sugar in a range between 0.19 - 1.16 µg glucose eqv. /mg fwt (Fig: 5.9.17). Coefficient of determination test value R^2= 0.05 (Table 5.20) and Pearson correlation test value -0.087 (Table 5.18) indicates no direct or indirect correlation between sugar content of the bark and epiphytic cover. Similarly tannin also didn't affect the epiphytic distribution on the tree bark (R^2= 0.07). Maximum growth of epiphytic liverworts was noticed on trees having 0.3–0.15 µg tannic acid eqv. / mg fwt tannic acid (Fig 5.9.20).

5.9.2 VARIATION IN THE ABUNDANCE OF LIVERWORTS GROWING ON SOIL IN RELATION TO PHYSICAL FACTOR AND SOIL PROPERTIES

There was significant influence of light on the abundance of liverworts growing on soil. Coefficient of determination test value R^2= 0.877 indicated direct correlation between the two attributes (Fig 5.9.23). It was noticed that light and bryophyte abundance were correlated negatively each other. Moisture content of the soil also significantly influenced the bryophyte abundance (Coefficient of determination test value R^2=0.983). Liverwort abundance was positively correlated with the moisture content of the soil (Fig: 5.9.24). No influence of soil pH was found on the abundance of liverworts in this work (0.006). Similarly no correlation between the liverwort abundance and other physico chemical properties of soil like organic carbon (R^2=0.130) [Fig 5.9.27], nitrogen (R^2=0.203) [Fig 5.9.28], phosphorous (R^2=0.014) [Fig 5.9.30], potassium (R^2=0.001) [Fig 5.9.32] and sulphur content (R^2=0.006) [Fig 5.9.33] was found in this work. While, carbon and nitrogen ratio of the soil was found to be positively correlated with the liverwort abundance, although weakly so (R^2=0.579) [Fig 5.9.31].

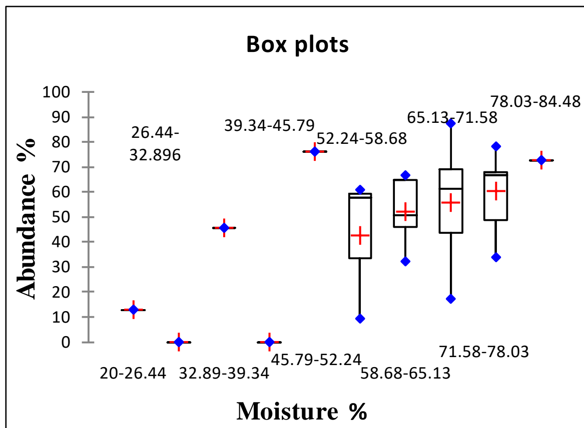


Fig 5.9.1: Variation in abundance % according to moisture content of bark

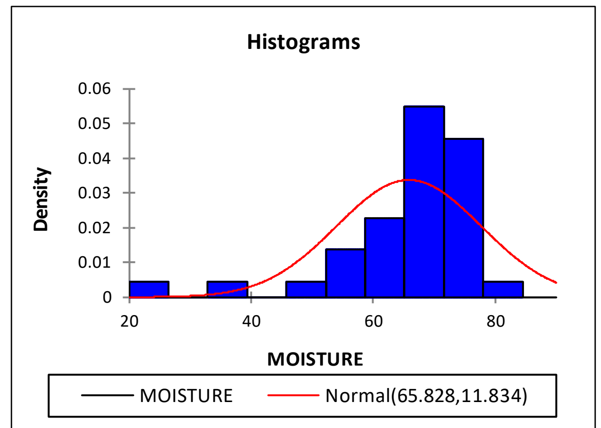


Fig 5.9.2: Normal probability curve of distribution of liverworts related with moisture content of the bark

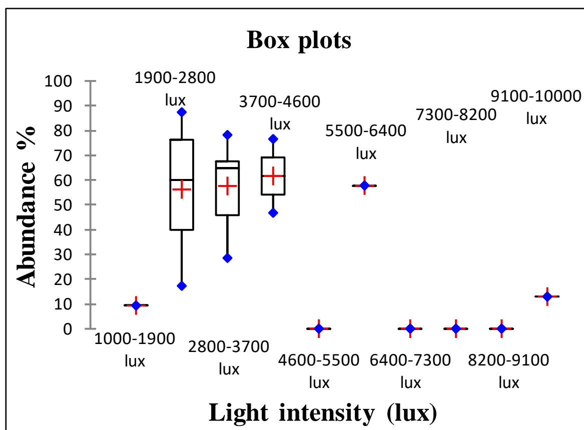


Fig 5.9.3: Variation in abundance % according to light intensity

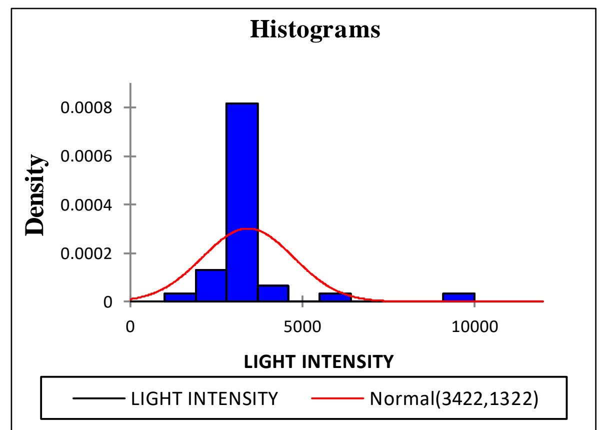


Fig 5.9.4: Normal probability curve of distribution of liverworts related with light intensity

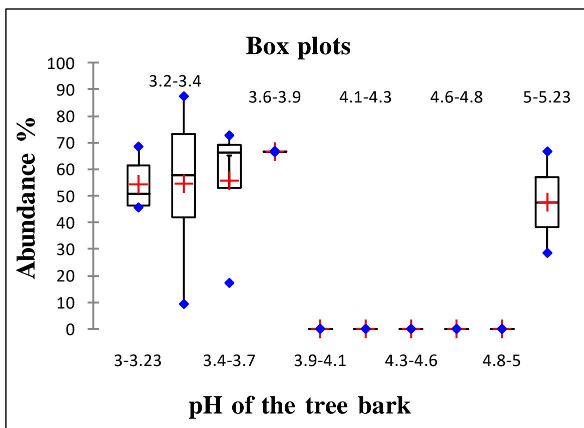


Fig 5.9. 5: Variation in abundance % according to pH of the bark

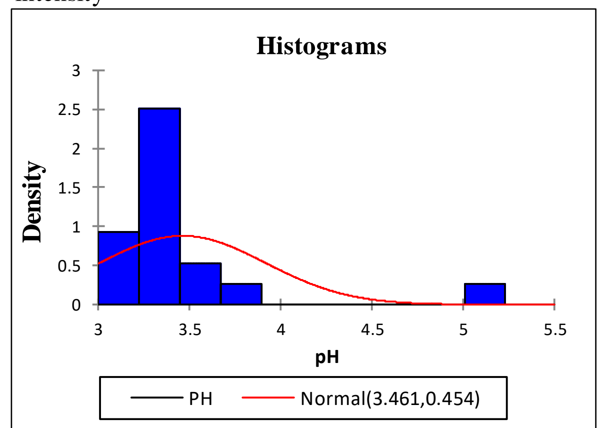
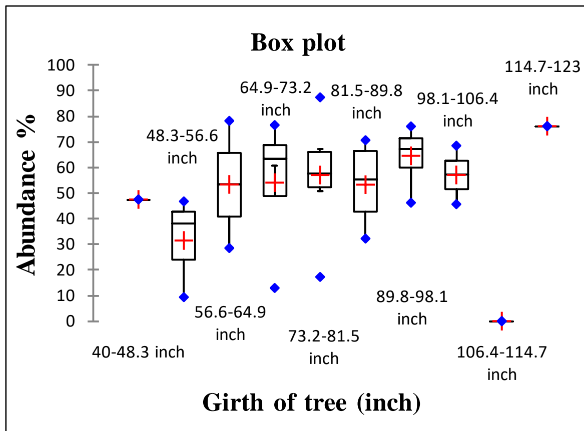


Fig 5.9.6: Normal probability curve of distribution of liverworts related with pH bark



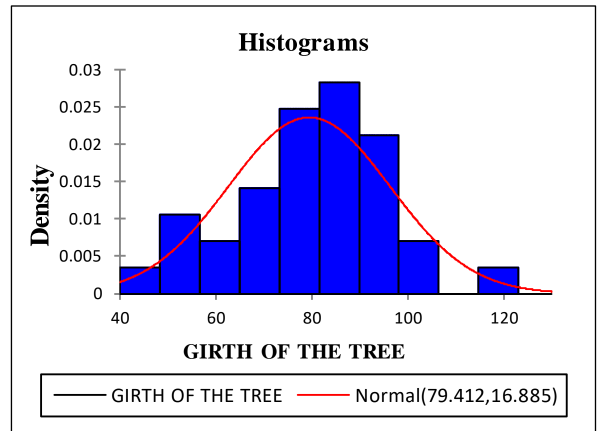
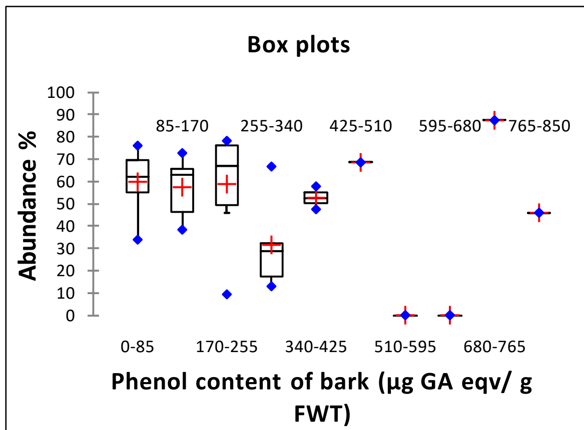


Fig 5.9 7: Variation in abundance % according to girth of the tree

Fig 5.9.8: Normal probability curve of distribution of liverworts related with girth of the tree



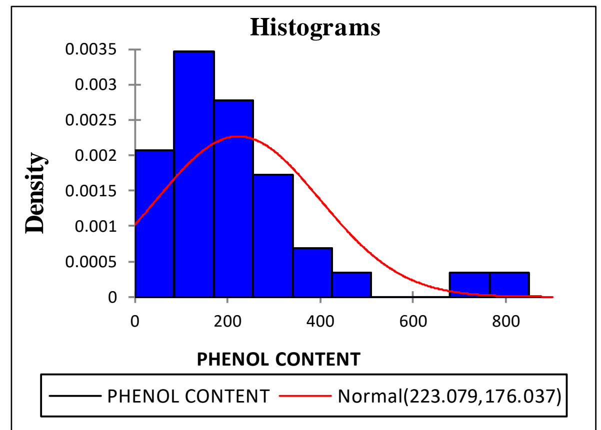
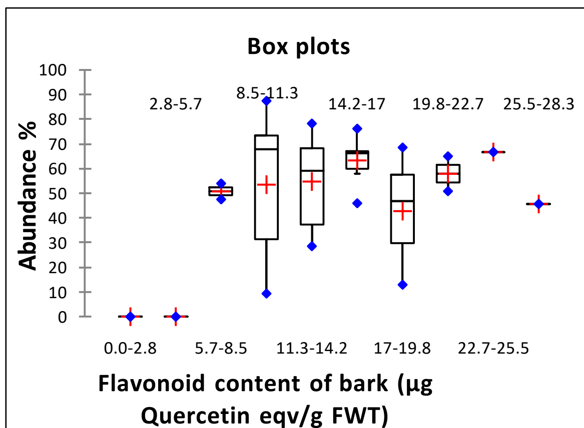


Fig 5.9 .9: Variation in abundance % according to phenol content of bark

Fig 5.9.10: Normal probability curve of distribution of liverworts related with phenol content of the bark



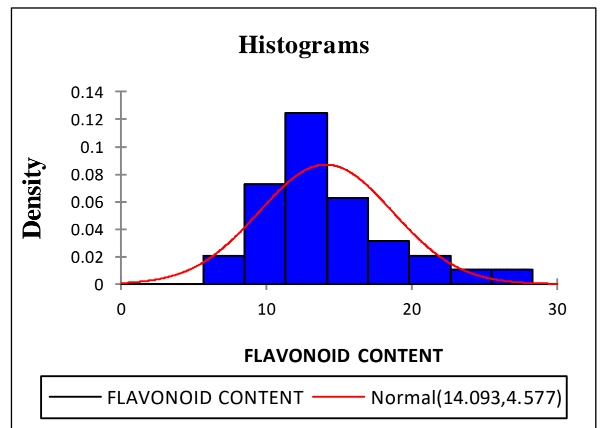
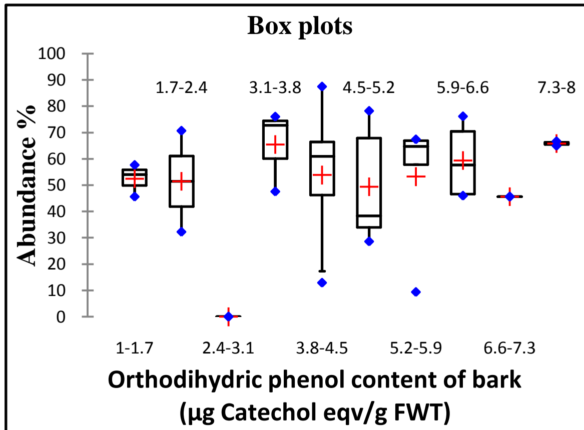


Fig 5.9.11: Variation in abundance % according to flavonoid content of bark

Fig 5.9.12: Normal probability curve of distribution of liverworts related with flavonoid content of bark



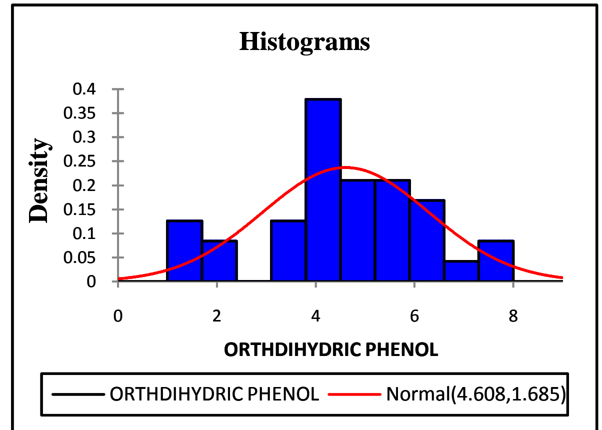
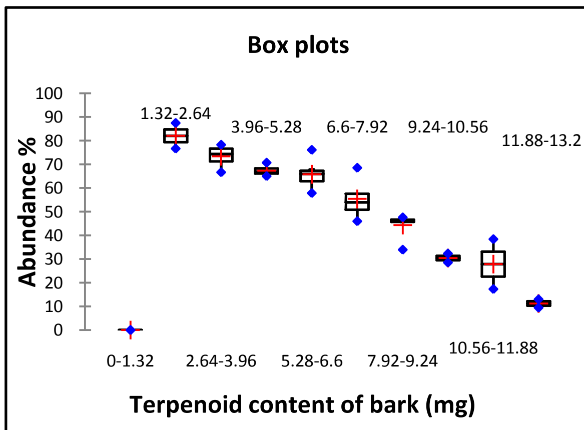


Fig 5.9.13: variation in abundance % according to ortho-dihydric phenol content

Fig 5.9.14: Normal probability curve of distribution of liverworts related with ortho-dihydric phenol content of bark



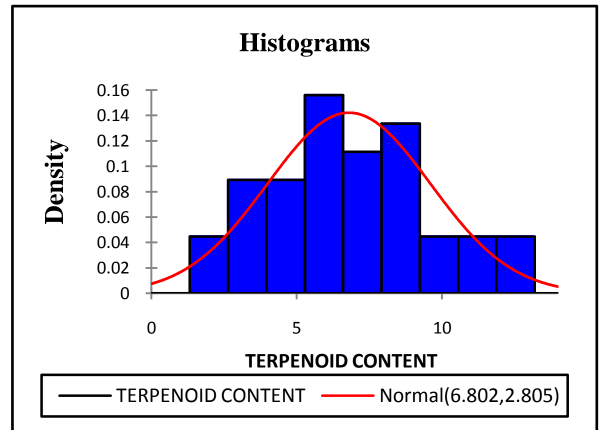
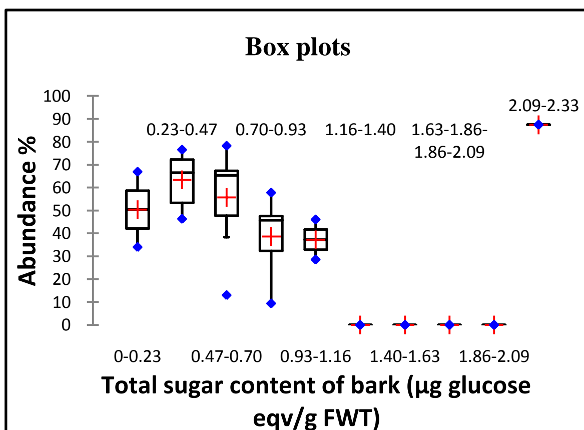


Fig 5.9.15: Variation in abundance % according to terpenoid content of bark

Fig 5.9.16: Normal probability curve of distribution of liverworts related with terpenoid content of bark



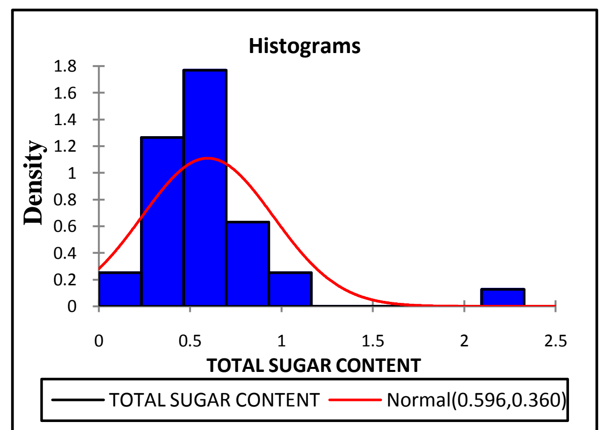


Fig 5.9.17: Variation in abundance % according to total sugar content of bark

Fig 5.9.18: Normal probability curve of distribution of liverworts related with sugar content of bark

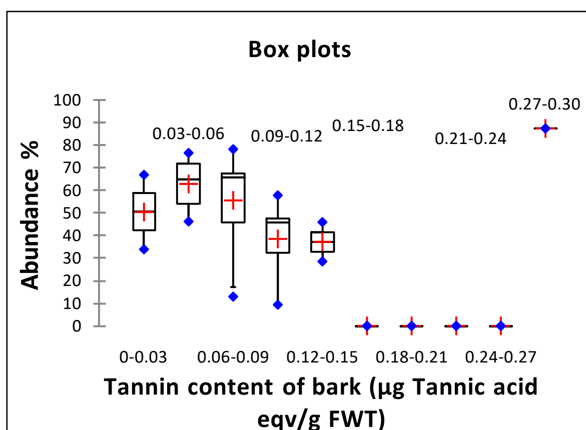


Fig 5.9.19: Variation in abundance % according to tannin content of bark

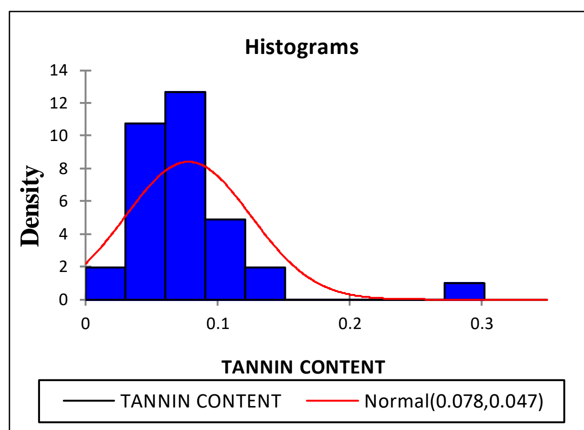


Fig 5.9.20: Normal probability curve of distribution of liverworts related with tannin content of bark

Table 5.17: Correlation between epiphytic liverwort abundance and the physiological factors like moisture content of bark, pH of the bark and light intensity. **. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).

	Abundance	***Moisture***	***Ph***	***Light Intensity***
Abundance	1	**0.648****	0.065	**-0.769****
Moist ure	**0.648****	1	0.220	**-0.606****
Ph	0.065	0.220	1	-0.123
Light intensity	**-0.769****	**-0.606****	-0.123	1

Table 5.18: Correlation between epiphytic liverwort abundance, terpenoid content of the tree barks and tree age. **. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).

	Abundance	***Terpenoid content***	***Tree age***
Abundance	1	**-0.959****	**0.937****
Terpenoid content	**-0.959****	1	**-0.836****
Tree age	**0.937****	**-0.836****	1

Table 5.19: Correlation between epiphytic liverwort abundance and phenol, flavonoid, orthodihydric phenol, tannin and sugar content of the studied tree bark. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed)

	Abun	*Gir*	*Phen*	*Flav*	*Odp*	*Tann*	*Sug*
Abun	1	**0.776****	-0.137	0.007	-0.066	-0.084	-0.087
Gir	**0.776****	1	-0.100	0.037	-0.101	0.005	-0.005
Phen	-0.137	-0.100	1	**0.675****	**0.679****	-0.213	-0.227
Flav	0.007	0.037	**0.675****	1	**0.647****	**-0.335***	**-0.347***
Odp	-0.066	-0.101	**0.679****	**0.647****	1	-0.145	-0.176
Tann	-0.084	0.005	-0.213	**-0.335***	-0.145	1	**0.996****
Sug	-0.087	-0.005	-0.227	**-0.347***	-0.176	**0.996****	1

NB: Abun= abundance, Gir= girth, Phen= phenol, Flav= flavonoid, Odp= orthodihydric phenol, Tann= tannin, Sug= total sugar

Table 5.20: Co-efficient of determination (R^2)

	Abun	*Gir*	*Phen*	*Flav*	*Odp*	*Moist*	*Phen*	*Lig*	*Sug*	*Tann*
Gir	0.602									
Phen	0.006	0.000								
Flav	0.000	0.001	0.008							
Odp	0.004	0.010	0.017	0.419						
Moist	0.420	0.246	0.034	0.017	0.012					
Ph	0.004	0.003	0.004	0.027	0.023	0.048				
Lig	0.591	0.523	0.003	0.017	0.019	0.368	0.015			
Sug	0.005	0.000	0.603	0.115	0.030	0.007	0.004	0.000		
Tann	0.007	0.000	0.619	0.114	0.026	0.008	0.004	0.001	0.996	
Terpen	0.938	0.654	0.001	0.000	0.005	0.422	0.015	0.684	0.001	0.002

NB: Abun= abundance, Gir= girth, Phen= phenol, Flav= flavonoid, Odp= orthodihydric phenol, Moist= moisture, Lig= light, Sug= total sugar, Tann= tannin, Terpen= terpenoid

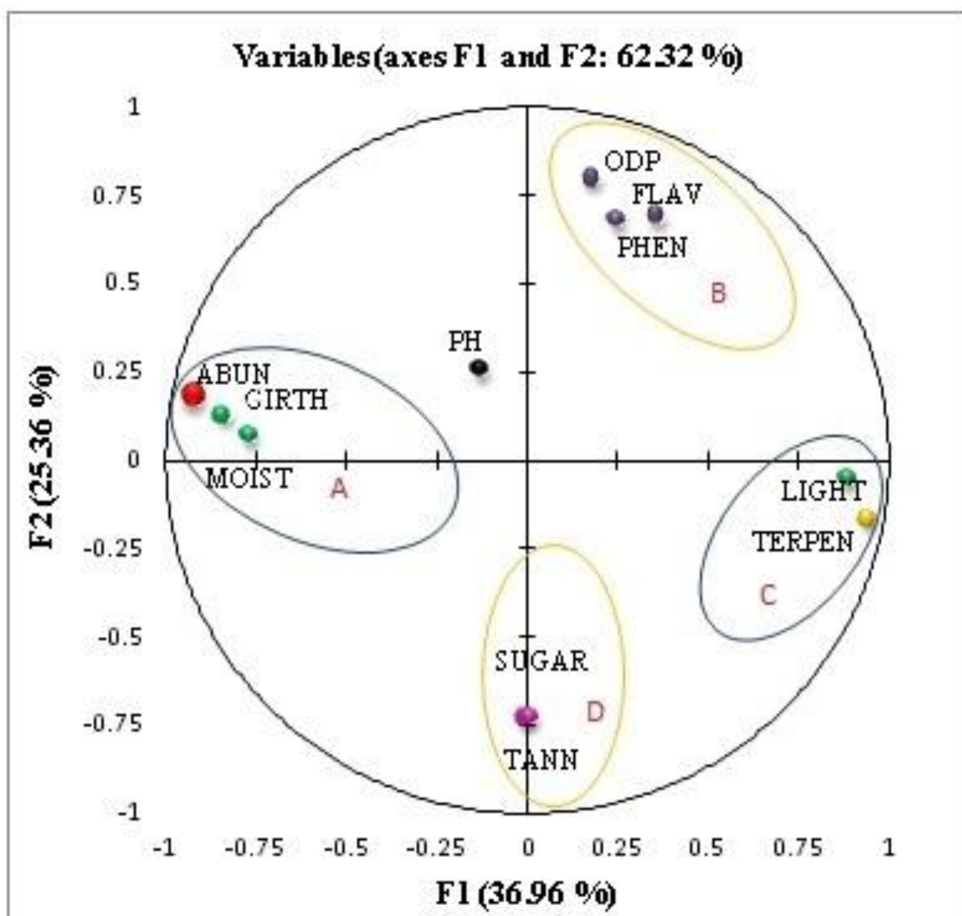


Fig 5.9.21: Principle component analysis of epiphytic liverwort abundance and different physical factors (light, moisture) and biochemical characteristics of plants (pH, girth, phenol, flavonoid, orthodihydric phenol, sugar, tannin and terpenoid conent).

NB: **ABUN= abundance, GIR= girth, PHEN= phenol, FLAV= flavonoid, ODP= orthodihydric phenol, MOIST= moisture, LIG= light, SUG= total sugar, TAN= tannin, TERPEN= terpenoid**

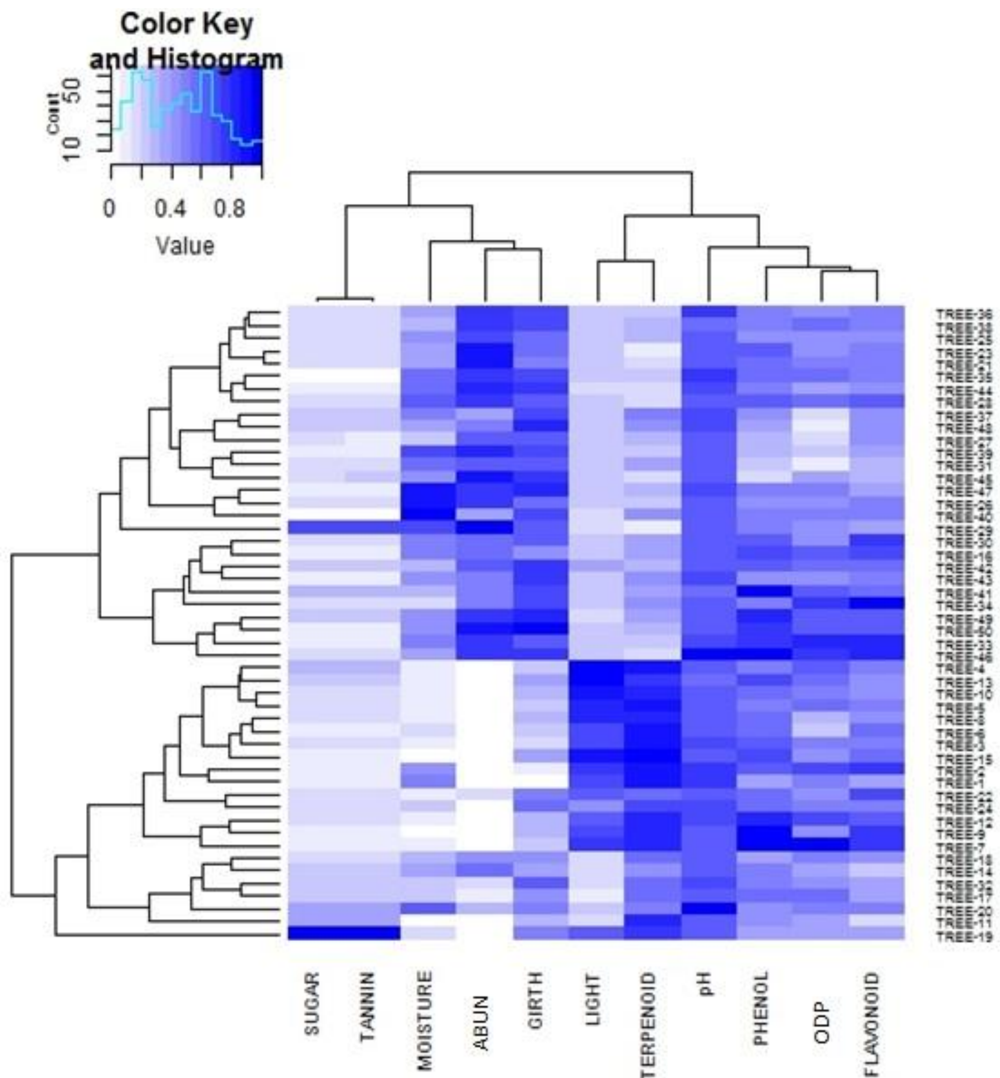


Fig 5.9.22: Z-score heatmap of the epiphytic liverwort density and different physical and biochemical factors.

NB: ABUN= abundance, ODP= orthodihydric phenol, TREE= Trees on which the abundance of epiphytic liverwort was studied

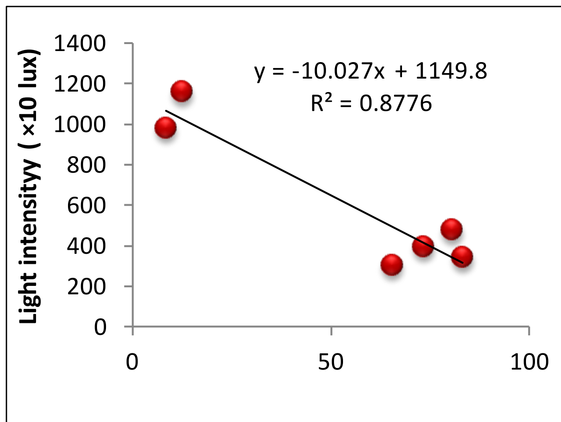


Fig 5.9.23: Influence of light intensity on abundance % of liverworts

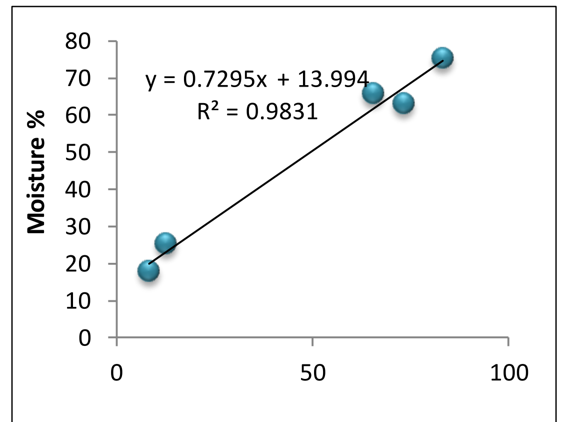


Fig 5.9.24 : Influence of soil moisture % on abundance % of liverworts

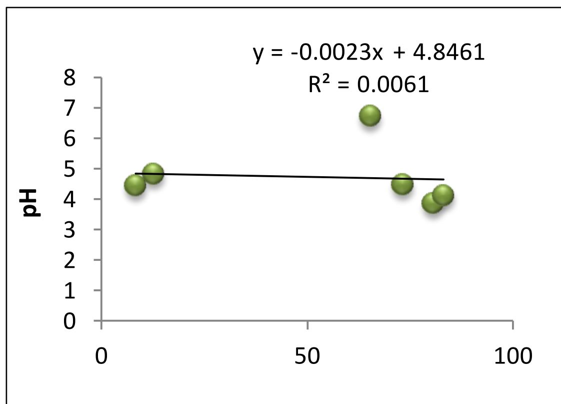


Fig 5.9.25 : Influence of soil pH on abundance % of liverworts

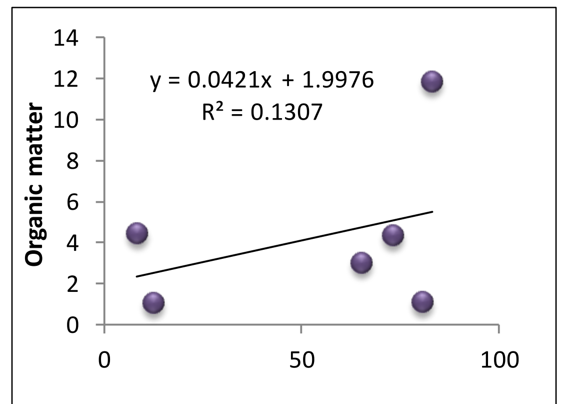


Fig 5.9.26: Influence of organic matter on abundance % of liverworts

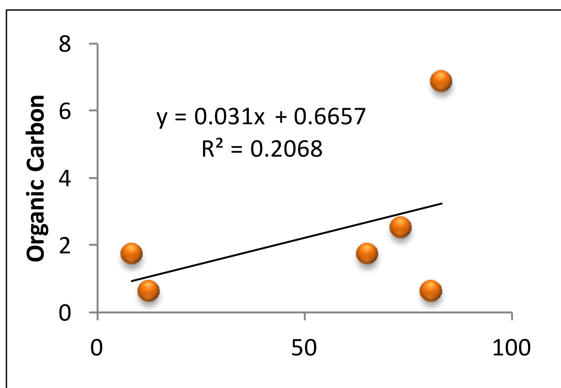


Fig 5.9.27: Influence of organic carbon on abundance % of liverworts

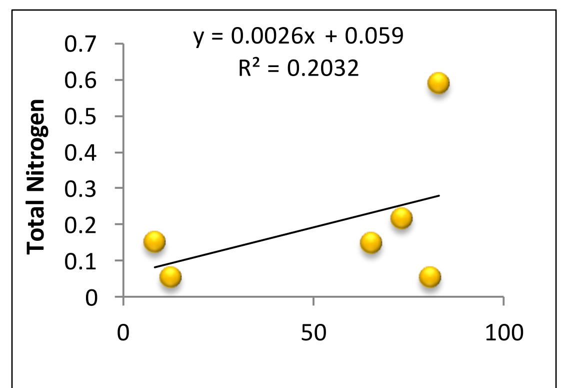


Fig 5.9.28: Influence of total nitrogen on abundance % of liverworts

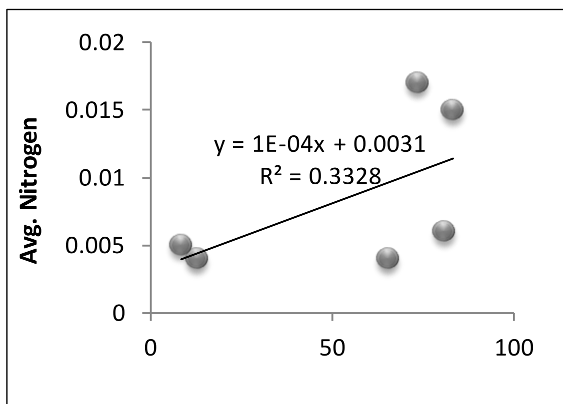


Fig 5.9.29: Influence of avg. nitrogen on abundance % of liverworts

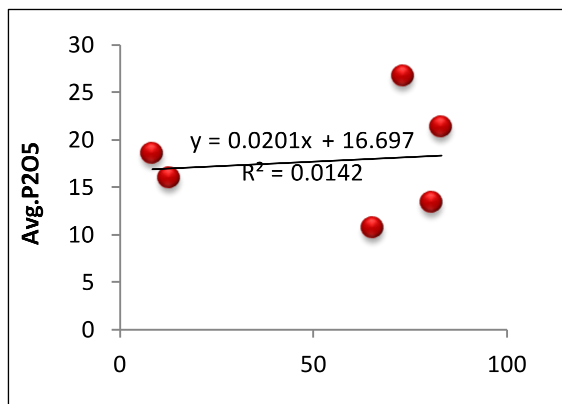


Fig 5.9.30: Influence of avg. P_2O_5 on abundance % of liverworts

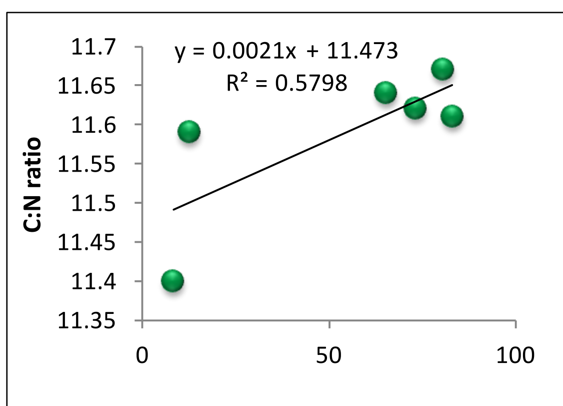


Fig 5.9.31: Influence of C:N ratio on abundance % of liverworts

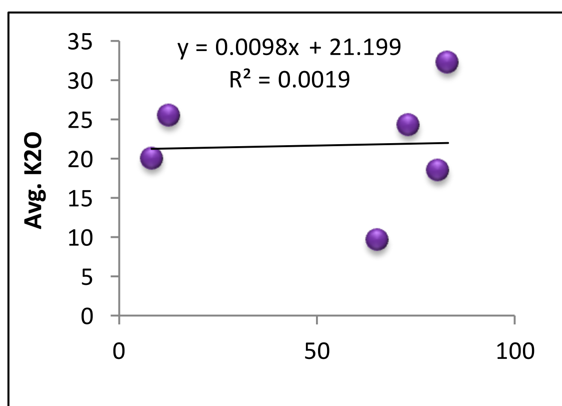


Fig 5.9.32: Influence of avg. K_2O on abundance % of liverworts

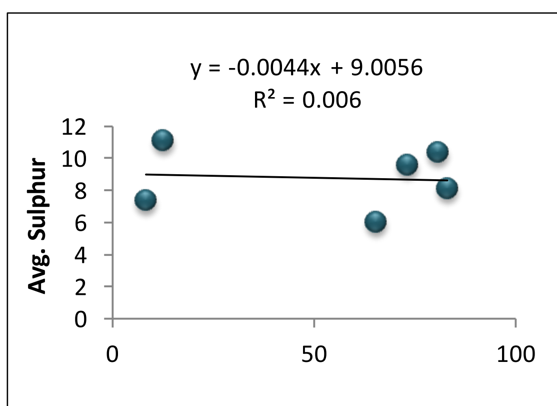


Fig 5.9.33: Influence of avg. sulphur on abundance % of liverworts

Chapter 6

DISCUSSION

6. DISCUSSION

6.1 FREE RADICAL SCAVENGING ACTIVITY

Free radicals are constantly generated in the body as a result of aerobic metabolism in cells. $O_2^{\bullet}$ are generated slowly by the leakage of electrons from electron carriers of the electron transport chain of mitochondria to O_2. Beside this other endogenous and exogenous factors such as infection, exercise, aging, radiations, pollutants, etc. contribute to the generation of free radicals which ultimately leads to the development of oxidative stress (Zima *et al.*, 2001). Different types of ROS includes singlet oxygen, nitric oxide ($NO^{\bullet}$), superoxide anion ($O_2^{\bullet}$), hypochlorite radical and hydrogen peroxide (H_2O_2). For protection against free radicals, human system has developed a complex and sophisticated antioxidant protection system. Antioxidants are endogenous and exogenous in origin. Endogenous antioxidants are different antioxidant enzymes like glutathione peroxidase, superoxide dismutase (SOD), and catalase. Apart from this, non enzymatic molecules including thiols, thioredoxin and disulfide bonding also constitute antioxidant defense system. Exogenous antioxidants are obtained from food in the form of vitamin C, vitamin E and beta carotene. In addition, a number of plant derived substances were also proved to contain antioxidant activity. Phytochemicals such as flavonoids, tannins and phenolic compounds are considered as the main antioxidant contributing compounds of plants (Wong *et al.*, 2006). Bryophytes, a second largest group of plant kingdom have demonstrated potent antifungal, antimicrobial, cytotoxic and other biological activities however documentation of information regarding their biological activities is rather less as compared to higher groups of plants. So the main aim of this study was to explore the pharmacological properties and phytochemical constituents of liverworts.

Eastern Himalaya is a home to large number of bryophyte species (Liverwort, hornwort and moss). Eleven liverwort species were selected from this area based on their uses in traditional medicine and abundance; they are *Pellia epiphylla* (L.) Corda, *Conocephalum japonicum* (Thunb.) Grolle, *Lunularia cruciata* (L.) Dumort. Ex Lindb, *Dumortiera hirsuta* (Sw.) Nees, *Marchantia emarginata* Reinw., Blume & Nees subsp. *emarginata*, *M subintegra* Mitt., *M polymorpha* L. subsp. *ruderalis* Bischl. & Boisselier-Dubayle, *Plagiochasma cordatum* Lehm. & Lindenb, *Asterella wallichiana* (Lehm.) Grolle, and *Plagiochila nepalensis* Lindenb. and *Marchantia paleacea* Bertol.

Extraction is an important step for pulling out desired phyto-compounds from plants for further analysis. Depending on the nature of bioactive compounds, solvent system for their extraction was selected. For extraction of hydrophilic compounds, polar solvents like methanol, ethanol or ethyl acetate is used (Cosa *et al.*, 2006). Akinmoladun *et al.* (2007), working on *Ocimum gratissimum* found the presence of anthraquinones only in aqueous extract while alkaloids were detected in methanolic extract only. Bryophytes are composed of different types of polysaccharides, lipids, polyphenols and other secondary metabolites (Klavina, 2014). In the present work both non polar and polar substances were extracted almost in equal amount indicating the presence of lower and higher polarity phytocomponds in equal amount. Klavina *et al.* (2015) reported the presence of polar substances in lower amount in case of mosses than the non polar compounds. While in case of higher group of plant the presence of polar substances was found in higher amount than non polar substances (Saeed *et al.*, 2012). Thus, the result obtained in the present work is in contradiction with those found in higher group of plants. This highlights the uniqueness of phytochemicals in this group of plants; so, further chemical analysis on the secondary metabolites of liverworts can identify a number of different new compounds which could be useful in pharmaceutical, cosmetic or agricultural fields.

In recent years wide ranges of spectrophotometric assays were used to measure the antioxidant properties of plants (Thaipong *et al.*, 2006). These assays are based on the principle of generation of coloured synthetic radicals, their chelation by antioxidants present in the studied plant and monitoring the free radical scavenging activity spectrophotometrically. These assays are categorised into two groups. One approach involves transfer of electron and reduction of coloured free radicals, like the case of $DPPH^{\bullet}$ and $ABTS^{+}$. In another approach antioxidants and substrate compete for thermally generated peroxyl radicals such as the case of oxygen radical absorbance ability and ferric reducing ability (Rodriguez- Amaya, 2010). $DPPH^{\bullet}$ and $ABTS^{+}$ are stable free radical method which is a rapid, easy and sensitive way to study the antioxidant activity of plant extracts (Koleva *et al.*, 2002). $DPPH^{\bullet}$ is a well known stable organic nitrogen free lipophilic radical which has strong absorption band at about 520 nm. $DPPH^{\bullet}$ radical in solution has a deep violet colour and becomes pale yellow when it is neutralized. Antioxidants neutralize $DPPH^{\bullet}$ free radical either by transferring an electron or hydrogen atom. Studied liverworts demonstrated $DPPH^{\bullet}$ radical scavenging activity in a concentration dependent manner. Liverworts like *Marchantia paleacea*, *Plagiochasma cordatum*, *Plagiochila nepalensis, Dumortiera hirusta* (IC_{50} value 60

µg/ml -510 µg/ml) showed significant potential to scavenge free radicals which are as good as DPPH radical scavenging potential of Algerian medicinal plants (4.30 µg/ml - 486.6 µg/ml) (Soumia *et al.*, 2014). $DPPH^{\bullet}$ scavenging activity of studied liverworts was also found to be more or less similar to wetland medicinal plants (Ho *et al.*, 2012). Significant $DPPH^{\bullet}$ radical scavenging activity can be attributed to hydrogen or electron donating ability of the phytochemicals present in these liverworts.

$ABTS^{+}$ is another commonly used assay to assess the antioxidant property of the plant. $ABTS^{+}$ scavenging assay is based on the generation of green $ABTS^{+}$ by reacting $ABTS^{+}$ and potassium persulfate. Addition of samples under study to $ABTS^{+}$ solution reduces it depending on the antioxidative potential of the samples. In the present work diethyl ether extract of *Plagiochasma cordatum* showed highest $ABTS^{+}$ scavenging activity. *Marchantia polymorpha*, *M. paleacea*, *M. subintegra*, *Pellia epiphylla*, *Plagiochila nepalensis* and *Asterella wallichiana* also showed strong $ABTS^{+}$ scavenging activity. Diethyl ether and ethyl acetate extract of all the liverworts have shown the highest activity than other solvent extracts. Methanolic fraction after diethyl ether and ethyl acetate extract showed good $ABTS^{+}$ activity. This result showed that for liverworts, diethyl ether and ethyl acetate are best solvents for extracting phytochemicals with significant $ABTS^{+}$ scavenging activity.

Superoxide anion is one among the most powerful reactive species generated by the auto-oxidation reactions, enzymatic processes and nonenzymatic electron transfer reactions (Michelson *et al.*, 1977). They are mostly produced within the mitochondria and are usually produced by enzymes xanthine oxidase (Kuppusamy *et al.*, 1989), cyclooxygenase (McIntyre *et al.*, 1999), lipooxygenase and NADPH dependent oxidase. Superoxide is considered more harmful because it leads to the production of other harmful radicals such as hydroxyl ion. Superoxide ion exists in two forms, O_2^- or hydroperoxyl radical (HO_2) at low pH (Bielski and Cabelli, 1996). Under physiological pH superoxide exists in O_2^- form and reduces iron complexes such as cytochrome c and ferric-thylene diaminetetra acetic acid reducing Fe^{+3} to Fe^{+2}. Hydroperoxyl radical formed can easily enter the phospholipid bilayer causing its oxidation. Studied liverworts have shown significant potential in scavenging SO radicals. Liverworts like *Marchantia subintegra* and *M. paleacea* showed SO scavenging activity (IC_{50} value of 109 µg/ml and 160 µg/ml respectively) that was similar to the SO scavenging activity of medicinal plants like *Prunella vulgaris*, *Saurauia oldhamii*, *Rubus parvifolius*, *Jassiaea repens* showing (IC_{50} value 113-159 µg/ml) (Shyur *et al.*, 2005). SO scavenging activity of *Marchantia subintegra* (IC_{50} value 109 µg/ml) was comparable to that of the

aqueous extract of medicinal plant *Acorus calamus* (IC_{50} value 101 μg/ml) which is used as a central nervous system relaxant, an appetite stimulant, an anthelmintic, a sedative and an antibacterial agent (Barua *et al.*, 2014). In the present case, moderate polarity solvent extracts scavenged SO more efficiently than extreme polar and non polar solvent extracts.

Free radicals are grouped into two group namely reactive oxygen species and reactive nitrogen species. Most important reactive nitrogen species is nitric oxide. However nitric oxide is an important signalling molecule in living organisms. Enzyme nitric oxide synthase that converts L-arginine to L-citrulline generates nitric oxide (Andrew and Mayer, 1999). It is soluble in both lipid and water thus can diffuse easily through cytoplasm and plasma membrane and act as an important intracellular second messenger (Chiuch, 1999). It helps in muscle relaxation in blood vessel by stimulating guanylate cyclase and protein kinase. It is also an important redox regulator of the cells (Wink and Mitchell, 1998). Other biological activities like blood pressure regulation, neurotransmission and smooth muscle relaxation also requires $NO^{\bullet}$, however its sustained level in the biological system is harmful. Result of this work showed the presence of NO scavenging property in the studied liverworts. Among studied liverworts *Marchantia paleacea, Dumortiera hirsuta* and *Lunularia cruciata* (IC_{50} 20-82 μg/ml), scavenged the *in vitro* originated $NO^{\bullet}$ more efficiently than medicinal plants *Phyllunthus fraternus, Triumfetta rhomboidea, Casuarina littorea* (IC_{50} 48.27-196.89 μg/ml) (Parul *et al.* (2013). Similarly, *Marchantia paleacea, Dumortiera hirsuta* and *Lunularia cruciata* displayed higher $NO^{\bullet}$ scavenging ability than medicinal plant *Guettarda speciosa* having IC_{50} value 77.22 μg/ml in its aqueous extract (Revathi *et al* 2015). Study of the effect of solvent on extraction of $NO^{\bullet}$ scavenging phytochemicals from liverworts revealed that non polar solvents to moderately polar solvents are best for the extraction of phytochemicals with potential NO scavenging activity. This result is in disparity with that of higher group of plants where better NO has been shown by the extracts of polar solvent.

The ferric reducing ability of plasma (FRAP) assay is different from all other antioxidant assays as no free radicals is generated here. Instead in this assay reduction of ferric ion (Fe^{+3}) to ferrous ion (Fe^{+2}) is monitored. Reducing potential is the measure of the ability of antioxidants to transfer electron (Meir *et al.*, 1995) and thus, are considered as the direct measure of antioxidant activity. In the present study, *Plagiochasma cordatum* showed the highest ferrous ion reducing potential.

Metal ions accelerate lipid peroxidation by decomposing hydrogen peroxide to form alkoxyl and peroxyl radicals. Transition metal also reacts with hydrogen peroxide to form hydroxyl ion. Metal chelating activity has high perceptibility in living system as it lowers the concentration of transition metals in the process like lipid peroxidation. Result showed that among studied liverworts *Marchantia paleacea*, *Conocephalum japonicum*, *Plagiochasma cordatum* and *Plagiochila nepalensis* have high metal chelating potential with IC_{50} value ranging between 90 µg/ml to 380 µg/ml. The metal chelating activity of medicinal plants (IC_{50} 80- 250 µg/ml) found in Jordan (Bilto, 2015) were somewhat similar to the result obtained in the present study.

Present work showed that different solvent extracts of a single plant have wide variation in the radical scavenging activity. Extraction of phytochemicals having different polarity might have resulted in the variation of the activity. It was noticed that in all studied liverworts diethyl ether and ethyl acetate extracts have showed best antioxidat activity. Butanol and acetone fractions have shown an average activity while least activity was recorded in heptane and methanol extracts. However, transition metal chelating activity was better in case of heptane extract. It has been reported that terpenoids and aromatic compounds present are responsible for different biological activities by the liverwort (Asakawa, 2007). Terpenoids are naturally occurring non polar compounds derived from isoprene unit that can be extracted by using non polar solvents (Harman-Ware *et al*., 2016). In the present study, better antioxidant activity of diethyl ether and ethyl acetate extracts can be assumed due to the presence of non-polar compounds like terpenoids in the extract which is similar to the findings of other workers (Asakawa, 2007).

6.2 ANTI-DIABETIC ACTIVITY

Diabetes mellitus (DM) is one amongst the major health problems worldwide. Reduction in insulin sensitivity and postprandial hyperglycemia are the characteristics of type 2 diabetes (Mousinho *et al.,* 2013). Lowering postprandial hyperglycemia can be an important measure to control diabetes. Postprandial hyperglycemia can be controlled by inhibiting the activity of enzymes α-amylase and α-glucosidase (carbohydrate hydrolyzing enzymes) (Ali *et al.,* 2006). Inhibition of these enzymes will delay carbohydrate digestion which will result in the reduction of rate of glucose absorption and consequently blunts the postprandial hyperglycemia. Liverworts like *Conocephalum japonicum*, *Pellia epiphylla*, *Marchantia emarginata*, *M. subintergra* and *Plagichasma cordatum* showed high α-amylase

and α-glucosidase enzyme inhibitory activity. Diethyl ether extract of *Marchantia subintegra* inhibited the α-glucosidase activity with greater efficiency than that of standard metformin. α-amylase and α-glucosidase inhibitory activity of medicinal plant *Solanum surratense* L (Manoj, 2012) was somewhat similar to the result obtained in the present study. Traditionally used plant *Calamus erectus* for treatment of diabetes have α -amylase and α-glucosidase inhibitory activity (IC_{50}- 1.69 and 2.00 mg/ml) similar to the result obtained in the present work. Hence, this result supports the potential of liverworts as an active source of phytochemicals having anti-diabetic activity. Bis(benzyls) namely marchantin C found in liverworts are reported to be responsible for α-glucosidase inhibitory activity (Harinantenaina and Asakawa, 2007).

In *Asterella wallichiana*, *Pellia epiphylla*, *Marchantia emarginata*, *M. polymorpha* and *Plagiochasma cordatum* ethyl acetate extract showed the highest α-glucosidase inhibitory activity. In *Dumortiera hirsuta* and *Marchantia subintegra* diethyl ether, in *Conocephalum japonicum* and *Lunularia cruciata* acetone and in *Plagiochila nepalensis* butanol extract showed the highest α-glucosidase inhibitory activity. It has been noticed that solvent having moderate polarity i.e. solvents having polarity between two extreme polarity values are more efficient in extraction of phytochemicals with significant α-glucosidase reducing potential in liverworts. While, many studies (Zarena and Sankar, 2009; Mohsen and Ammar, 2009) reports the greater efficiency of polar solvents in extraction of phytochemicals with better pharmacological activities. Extraction of phenolic group in polar solvents is responsible for the said biological activities. The result of present study is different from these findings suggesting that in liverworts non polar to moderately polar phytochemicals are pharmacologically more active. Many new compounds uncommon in higher plant groups are found to be present in bryophytes (Sabovljevic and Sabovljevic, 2008). Result of the present study is in conformity with the earlier findings suggesting the presence of unique phytochemicals in liverworts than those found in higher group of plants. Less polar solvents like acetone was also found to be more suitable for extraction of phenolics compounds from flowers (Liu *et al*., 2009). Phenolics varying from simple (anthocyanins, phenolic acids) to highly polymerized form (tannins) are present in plants. Higher α-glucosidase inhibitory activity of moderate polarity extracts of studied liverworts might be due to the presence of more active moderate polarity phytochemicals than highly polar one in liverworts.

In *Asterella wallichiana*, *Pellia epiphylla* , *Marchantia emarginata* and *M. polymorpha* ethyl acetate fraction showed the highest α-amylase inhibitory activity. In *Dumortiera hirsuta* and *Marchantia subintegra* diethyl ether extract, in *Conocephalum japonicum* and *Lunularia cruciata* acetone extract and in *Plagiochila nepalensis* butanol extract displayed the highest α-amylase inhibitory activity. Diethyl ether, ethyl acetate, acetone and butanol extracts also showed good α–amylase inhibitory activity suggesting moderate polarity phytochemicals to be more active against α–amylase. It has been reported that terpenoids and aromatic compounds present in the liverwort are responsible for different biological activities (Asakawa, 2007). Better anti-diabetic activity of diethyl ether and ethyl acetate extracts shown in the present study might be due to the presence of non-polar compounds like terpenoids in the extract which is similar with the findings of other workers like Asakawa (2007).

Oxidative stress increases largely under sustained hyperglycaemia. During hyperglycaemia, free radical generates from self-oxidation of glucose, increased glycation and alteration of polyol pathway. These free radicals cause damage to β-cells and leads to the development and progression of complications associated with diabetes such as memory impairment, neuropathy, nephropathy and retinopathy (Maritim *et al*., 2003). Therefore, antioxidant therapies targeting diabetes induced oxidative stress can be considered to prevent downstream diabetic complications (Araki and Nishikawa, 2010). Thus, considering the potential free radical scavenging activity, studied liverworts can be used for reducing downstream diabetic complications.

6.3 CYTOTOXIC ACTIVITY

Renal cell carcinoma (RCC) is the third most frequent malignancy of genitourinary organs (Cheng *et al*., 2017). In most of the cases, RCC is diagnosed at the later stages (Jemal *et al*., 2009). It is found to be usually resistant to chemotherapy and radiotherapy. Recently certain drugs have been used in targeted therapeutic approach (Escudier *et al*., 2012) but this approach also faced some limitations of development of drug resistance (Cheng *et al*., 2017). So, there is a need to find novel source of chemotherapeutic agents that can target renal cell carcinoma without acquiring drug resistance. Bryophytes have been investigated for active biomolecules having potential cytotoxicity against cancer cells. Both isolated compounds and crude extracts from bryophytes were found to have cytotoxic properties. Active biomolecules like terpenoids (Perry *et al*., 1996), bi- bisbenzyl (Asakawa *et al*., 1982), marchantin (Asakawa *et al*., 1982), plagiochn (Shi *et al*., 2008), perrottetin (Asakawa *et al*., 1982),

lunularin (Lu *et al.,* 2006) found in liverworts are reported to have potential cytotoxic activities. In our study, liverworts *P. cordatum, A. wallichiana*, *L. cruciata*, *M. paleacea* and *M. nepalensis* were studied for their anti-proliferative activity against human kidney cancer cell line (ACHN). Studied liverworts showed potent cytotoxic effects in ACHN human renal cancer cells. *P. cordatum* showed highest cytotoxic effect (IC_{50} 69.15 µg/ml) against ACHN among studied liverworts. The cytotoxic activity of *P. cordatum, P. nepalensis* and *M. paleacea* against ACHN was found to be better than that of Semen Euphorbiae (dried ripe seed of *Euphorbia lathyris*) extract (IC_{50} 185.2 µg/ml) studied by Cheng *et al*. (2017). Extracts having cytotoxicity value ranging between ≤ 20 and1000 µg/ml (IC_{50}) are considered active and extracts having cytotoxicity value more than 1000 µg/ml are considered inactive according to the reports of Atjanasuppat *et al*. (2009). Studied liverworts have anti-proliferative activity value ranging between 69.15 µg/ml and 308.98 µg/ml (IC_{50}). Extracts of studied liverworts exhibited promising cytotoxic activity. The result of this study suggested that liverworts also have the anti-proliferative potential and thus can act as the source of novel bioactive compounds with therapeutic and anti-cancer properties. Till date, liverworts are less explored for their bio-prospective values and were just ignored, but the result of this study provides the basis for further investigation on this group of plants as a source of potent bioactive compounds having anti-cancer property.

6.4 PHYTOCHEMICAL ESTIMATION

The phytochemicals present in plant are responsible for the biological activities displayed by plant. An insight into chemical nature can present rich data in understanding correlation between phytochemicals and their biological activities. Plant phenols are an important group of natural antioxidants having tremendous therapeutic potential. Variations in the structure of phenolic compounds lead to their solubility in solvents of different polarity. Thus, nature of extraction solvent has a significant impact on the extraction of polyphenols from plants. In this work, six solvents namely heptane, diethyl ether, ethyl acetate, acetone, butanol and methanol were used. Optimization of extraction conditions for polyphenolic compounds indicated that the most suitable solvent is usually different for different plant samples. Ngo *et al*. (2017) and Yang *et al*. (2017) reported acetone as the optimal extraction solvent for extraction of phenolic compounds from their studied plants. Addai *et al*. (2013) and Ferhat *et al*. (2017) found methanol to be optimal extraction solvent for phenols. Singh *et al*. (2014) found mixture of ethanol, diethyl ether, water (8:1:1) to be optimal solvent mixture for phenolic compound extraction. In this study, variation was

noticed in the phenol content among extracts obtained from liverworts using solvents of different polarity. In *Marchantia polymorpha*, *M. paleacea* and *Pellia epiphylla*, diethyl ether, ethyl acetate and methanol showed high phenol content. In *Marchantia subintegra*, *M. emarginata, Plagiochila nepalensis, Dumortiera hirsuta, Lunularia cruciata* and *Conocephalum japonicum* ethyl acetate and diethyl ether showed optimal extraction of phenolic compounds. Phenol content of studied liverworts varied from 4.8 mg Gallic acid equivalent / g extractive weight to 377.9 mg GAE/g EW. The phenol content of bryophytes such as *Pellia endiviifolia* (80.3 µg/mg) [Dey *et al*., 2013] was found to be lower than that of studied liverworts except *Conocephalum japonicum* (66.4 µg/mg) and *Pellia epiphylla* (49.5 µg/mg). Phenol content of *Barbula javanica* (30 µg/ ml) [Vats and Alam, 201] was lower than that of liverworts studied in this work. The phenol content of moss *Bryum moravicum* (356.4 µg/mg) was slightly lesser than the phenol content of *Marchantia emarginata* (377.9 µg/mg) obtained in present study. Similarly screening of phenol content of higher group of plants with medicinal property showed phenol content (69.89-382.57 mg GAE/g) somewhat similar to result obtained in present work (4.8 to 377.9 mg GAE/g).

Flavonoids are important group of phenolic compounds that are reported to have strong antioxidant activity (Pietta *et al*., 2000). In the present work presence of significant amount of flavonoids was recorded. Flavonoid content ranged between 0.3 to 94.2 mg quercetin equivalent/ g extractive weight. Flavonoid content in some liverworts like *Plagichasma cordatum*, *Dumortiera hirsuta*, *Lunularia cruciata*, *Marchantia emarginata* and *M. subintegra* was higher than that of Macedonian medicinal plants *Digitalis ferruginea* and *Digitalis lanata* having flavonoid content 2.86 and 11.19 mg CE/g DW, respectively (Tusevski *et al*., 2014). However, variation in flavonoid content was noticed with the polarity of solvent. Presence of high amount of flavonoids was recorded in non polar, moderately polar and polar solvent. This result differs from the findings of Yumrutas and Saygideger, (2012) and Ghasemzadeh *et al*. (2011) who reported higher flavonoid content in polar solvents only. Therefore, it can be inferred that in liverworts less polar flavonoid aglycones are present in high amount which results in its extraction in higher amount in non polar solvents. Polar flavonoids like flavonoid glycosides might have extracted in polar solvents. Ortho-dihydric phenol was also found to be present in studied liverworts. Presence of ortho-hydric phenol was recorded in almost equal amount in all the six extracts.

Steroids are group of secondary metabolites having many medicinal applications (Sultan and Raza, 2015). It has cardiotonic; anticancer, anti-viral, insecticidal and

antimicrobial activities (Kokpol *et al.*, 1984). Steroid content in studied liverworts ranged between 0.002 to 1.64 mg solasodine equivalent/ gram extractive weight. Madhu *et al.* (2016) studied the content of steroid in ten medicinal plants which ranged between 30.45 to 69.20 μg/ml. Steroid content of these medicinal plant was found to be lesser than that of liverworts studied in the present work. Steroid compounds are non polar in nature and are expected to be extracted in non polar solvents (Nuryanti and Puspitasari, 2017). Estimation of steroids using Libermann-Burchad reagent showed that steroids was extracted in higher amount in non polar solvents. Least amount of steroids has been detected in polar solvents.

Tannins are polyphenols having high molecular weight. It has many therapeutic potential like cardio-protective, anti-diabetic, anti-carcinogenic potential (Kumari and Jain, 2015). The presence of tannins in *Litsea cubeba* and *Zanthoxylum acanthopodium* supports the traditional medicinal use of these plants in the treatment of different diseases. Trease and Evans (1989) stated that tannins have potent antimicrobial, anticancer as well as antioxidants activities. Studied liverworts showed the presence tannins ranging between 0.02 to 2.56 mg Tannic acid equivalent/g extractive weights. Tannin content of *Halimium halimifolium* (Rebaya *et al.*, 2015) was lesser (0.05-2.20 g/100 g) than that of studied liverworts. While fifty three trees which are traditionally used in the treatment of diarrhoea contained tannin in higher amount (6-10 mg/ml) than studied liverwort (Wurger *et al.*, 2013). In *Marchantia subintegra*, *M. emarginata*, *Conocephalum japonicum*, *Pellia epiphylla* , *Plagiochasma cordatum*, *Plagiochila nepalensis* and *Lunularia cruciata* dietyl ether extract contained highest tannin content than other solvent. While in case of *Marchantia polymorpha*, *M. paleacea*, *Asterella wallichiana* and *Dumortiera hirsuta* moderately non polar solvents like methanol, butanol and acetone has extracted tannin in greater amount than the other solvents. Pansera *et al.*, 2004 and Yuliana *et al.*, 2014 have also reported polar solvents to be more suitable for extraction of tannins.

6.5 CORRELATION BETWEEN PHYTOCHEMICAL CONTENT AND PHARMACOLOGICAL ACTIVITIES

6.5.1 Pearson correlation test

Plants were used as medicines since prehistoric times until synthetic drugs were developed in 19th century. The health beneficial and health promoting effects of plants are due to the active principles they possess. Plant secondary metabolites are mainly responsible for displayed pharmacological properties. Phenolics are ubiquitous bioactive compounds present in plants (Robards *et al.*1999). Phenolic acids like chlorogenic, coumaric, caffeic and

ferulic acids are strong antioxidants (Larson, 1988). Phenolic compound β-carotene acts as antioxidants by quenching singlet oxygen. *p*- coumaric acids act by inhibiting the generation free radicals (Laranjinha *et al*., 1995). Flavonoids are other group of phenolic compounds that donate electrons and quench free radicals. However, data in the literature on the correlation between the phenolic compounds and their antioxidant activities are sometimes contradictory. Some researchers have noticed good correlation while some others have reported very weak or no correlation between antioxidant activity and phenolic compounds. Qusti *et al.* (2010) found good correlation between the phenolics and antioxidant activity of fruits. High correlation between the antioxidant activity and the phenolic compound was also observed in case of flax seeds and cereals (Velioglu *et al*., 1998). Similarly, phenolic compounds contributing to the antioxidant activity was also reported by Wong *et al*. (2005) in case of 30 Chinese medicinal plants.

Like higher group of plants, high correlation between antioxidant activity and total phenolic content was also noticed in liverworts studied in present work. Manoj *et al.* (2012) observed linear correlation between antioxidant activity and phenol content of bryophytes like *Plagiochila beddomei*, *Leucobryum bowringii* and *Octoblepharum albidum*. In this work, correlation between the antioxidant activities, anti-diabetic activity and phenolic content was studied using Pearson Correlation coefficient and Principle Component Analysis. Data showed correlation between studied pharmacological activity (antioxidant and anti-diabetic activity) and phenolic compounds present in plants (Fig 5.5.1). Correlation was analyzed by comparing IC_{50} value of the assays and phenolic content of the plants. Lower the IC_{50} value, higher is the antioxidant activity. Negative value of Pearson correlation test in our study indicates positive correlation between phenol content and $DPPH^{\cdot}$ scavenging activity. Value is negative because higher phenolic content results in lowering of IC_{50} value (i.e. $DPPH^{\cdot}$ scavenging activity increases) showing negative correlation between the two parameters. Phenols present in the studied liverworts were also found to have a strong correlation with the superoxide scavenging activity. Ferric reducing activity is considered equivalent to free radical scavenging potential of plant. The result of the present work also showed positive correlation between ferric reducing ability and phenolic content. It was suggested that phenolic compounds are main free radical scavengers that are produced by studied liverworts as secondary metabolites. Suganya Devi *et al.* (2012) also found strong positive correlation between total phenol and FRAP assay. Flavonoids having many hydroxyl group are very good scavengers of pyroxyl radicals (Robards *et al.*, 1999). Flavonoid content of the

liverworts was positively correlated with the reducing power suggesting flavonoids group of phenolic compounds to be another active free radical scavengers.

Furthermore orthodihydic phenol in the present study showed positive correlation with metal iron chelating activity and α-amylase inhibitory activity (anti-diabetic activity). The inhibition of the activity of carbohydrate hydrolyzing enzymes, α-amylase and α-glucosidase is an important strategy in treating diabetes. Result of the study showed that orthodihdric phenols were involved in inhibition of enzyme α- amylase thus contributing to the anti-diabetic activity displayed by the studied liverworts. Steroids a group of secondary metabolites are low molecular weight compounds having many medicinal applications. In our case, steroid present in the liverworts was positively correlated with the metal chelating activity.

6.5.2 Principal Component Analysis

Principal component analysis (PCA) was done to further confirm relationships between the studied variables from the eleven liverworts species. The loading of the first principal component (PC1) accounted for 19.84% of the variance and second principal components (PC2) accounted for 13.59% of the variance. In PC1, phytochemicals content and reducing power of the plants were separated from the antioxidant and anti-diabetic activities of plants. Loadings of PC1 were phenol, flavonoids, cardiac glycoside, alkaloid content, reducing power, metal chelating, nitric oxide scavenging, superoxide scavenging, α-amylase inhibitory and α-glucosidase inhibitory activities. Phytochemicals like phenol, flavonoids, alkaloid and cardiac glycoside showed negative correlation with the IC_{50} value of different antioxidant and anti-diabetic assays. This indicated that phytochemicals like phenol, flavonoids, alkaloid and cardiac glycoside present in the studied plants were responsible for free radical scavenging and α-amylase, α-glucosidase enzyme inhibiting activities. Ribeiro *et al.* (2013) also found a positive correlation between total phenolic content and antioxidant activity. Result of the PCA analysis showed positive correlation of phytochemicals with reducing power (FRAP assay). Kang *et al.* (2014) also found positive correlation between the flavonoids content and FRAP assay. Reducing power (FRAP) was negatively correlated with studied pharmacological activities suggesting that at higher metal ion concentration, reducing potential of plant results in better antioxidant and anti-diabetic activities. Loadings of PC2 were triterpene, resine, amino acid content and $ABTS^{+}$ scavenging activity as revealed from their squared cosine values. By PC2 triterpene, resine and amino acid were separated from

$ABTS^+$ scavenging activity suggesting the contribution of these phytochemicals on $ABTS^+$ scavenging property of liverworts.

6.6 SEASONAL CHANGES IN ANTIOXIDANT ACTIVITY AND PHENOLIC CONTENT IN LIVERWORTS

Life strategies of bryophytes are the system of co-evolved adaptive qualities. They can survive extreme environmental conditions and are considered as successful colonizers. Plants die when the relative water level becomes lesser than 20-50%. Selective plants, including bryophytes, dries up to 4-13% and can still survive and resurrects when favourable condition prevails. Hence they are referred as the desiccation tolerant plants. At the time of excessive dryness, there prevails a condition when there is little intracellular water content and reduction in metabolic activity resulting in irrevocable damage to lipids, protein and nucleic acids by the production of RS. Different physiological and chemical adaptations made by bryophytes help them to recover from water loss and resurrect under favourable environmental conditions. One of the important strategies for limiting damage in cellular membrane and organelles to a repairable level is the production of strong antioxidant defence system composed of enzymatic and non enzymatic mechanisms (Van Breusegem *et al*., 2001). Plant secondary metabolites are strong non enzymatic antioxidative machinery which helps them cope with different biotic and abiotic stresses (Dey and De, 2012).

Study of changes in antioxidant activity and phytochemical content of liverwort *M. paleacea* during rainy season (May to October) and dry season (October to March) showed that antioxidant activities increased significantly during dry season when the environmental conditions are not suitable for the bryophytic growth. This may be due to increase in enzymatic and non enzymatic defence mechanism against reactive oxygen species (ROS) produced under stressful conditions. The generation of reactive oxygen species during desiccation was also reported in vascular plant *Atrichum androgynum* by Mayaba *et al*., 2002 and in algae *Scytosiphon arbuscula* by Burritt *et al*., 2002. In drought tolerant moss *Otoblepharum albidum* too desiccation stress resulted in sharp increase of H_2O_2 as result of enhanced production of ROS (Lubiana *et al.,* 2013). These drought induced ROS acts as a signal to induce antioxidant mediated defence system (Weisany *et al*., 2012).

Result of the present work showed that the $DPPH^\cdot$ scavenging activity of *M. paleacea* increased significantly during dry season in all extracts (heptane, diethyl ether, ethyl acetate, butanol, acetone) except methanolic extract. $ABTS^+$ scavenging potential was also found to

increase in heptane, diethyl ether, ethyl acetate and acetone extracts during dry season; while methanol and butanol extracts of *M. paleacea* collected during rainy season showed better $ABTS^+$ scavenging activity. $ABTS^+$ scavenging potential of diethyl extract was similar during rainy and dry season.

Reductions of Free ferrous ions are important for protection against oxidative damage and lipid peroxidation through Fenton reaction. Result of present study showed that metal chelating potential of *M. paleacea* was increased significantly during dry season in diethyl ether, ethyl acetate and acetone extract. While heptane, methanol and butanol extracts showed better ferrous iron chelating activity during rainy season. Superoxide is a strong radical which leads to the generation of other RS like H_2O_2, $O_2^{\cdot-}$ and $OH^{\cdot}$ that are extremely reactive and capable of damaging bio-molecules of living system. Superoxide scavenging activity was also found to increase in heptane, diethyl ether, ethyl acetate and acetone during dry season. However all other studied free radicals scavenging activity increased during drought condition, the result of nitric oxide scavenging assay was rather contradictory. *M. paleacea* showed increased potential to scavenge nitric oxide during rainy season than dry season. Nitric oxide ($NO^{\bullet}$) is an important bio-molecule, but its sustained level is toxic to tissue and thus is required to scavenge from the body.

From the results of different antioxidant assays it was observed that among six solvents used for extraction of bioactive phytochemicals from *M. paleacea* during rainy and dry season, radical scavenging activities increased in all the extracts during dry season except in methanol and butanol. This result suggested that during drought stress there is an increase in the synthesis of phenolic compounds that provide crucial protection against oxidative damage caused by almost no metabolic activity due to water loss. This was evident from the result of phenolic compound screening where the phenolic content in all the extracts except methanolic extract increased during dry season. During rainy season higher phenol content in methanolic extract indicated the synthesis of polar phenolic compounds in higher concentration during rainy season imparting protection to the plants against reactive species. Flavonoid content was also found to increase during dry season. Flavonoid content increased significantly in heptane extract. This result indicated that flavonoids were synthesized in high amount by *M. paleacea* as a result of chemical adaptations during the unfavorable condition. Whereas, ortho-dihydric phenol content of plant was found to be present in higher concentration in *M. paleacea* during rainy season. This result suggests the less involvement

of orthodihydric phenol compounds in imparting protection against reactive species to plants under stress.

6.7 BIOASSAY GUIDED PURIFICATION

Assay guided purification are usually implemented for the extraction and identification of principal active components from the crude extract. A large number of bioactive substances have been isolated from crude extract following assay guided purification. Panichayupakarananta *et al*. (2010) by antioxidant assay guided purification have isolated ellagic acid that is responsible for antioxidant activity displayed by the pomegranate peel. Similarly, through bioassay guided purification method Ejele *et al.* (2012) isolated three phyto-compunds from *Garcinia kola* having significant antimicrobial activity. Phytochemicals having anti-ulcer activity was isolated from *Cassia singueana* by Ode *et al.* (2011). Similarly, four novel cyclic bisbenzyl dimmers were isolated from liverwort *Blasia pusilla* by Yoshida *et al,* (1996). In this work also, an attempt was made to perform an *in vitro* antioxidant and anti-diabetic assay guided purification of bioactive substances from liverwort *Marchantia paleacea*. Plant contains a wide array of phytochemicals varying from polar to non-polar compounds. For isolating major groups of phytochemicals a sequential elution method can be used for separating lipids initially and more polar substances afterwards. In the present work, solvent from lower to higher polarity were sequentially used in the elution process. Lower to higher polarity solvents was also used by Jassbi *et al.* (2016) for isolation of bioactive phytochemicals. Two bioactive fractions showing high pharmacological activity and phytochemical content were finally selected and the phytochemical present in the active fraction was detected by using Gas Chromatography Mass Spectrometry.

6.7.1 GC-MS analysis and identification of phytochemicals

GCMS result of bioactive fraction obtained from *M. paleacea* in this study showed the presence of phytochemicals like phenols, flavonoids, terpenoids and alkanes. Thus the promising antioxidant and anti-diabetic activity of this plant can be attributed to these phytochemicals. Through spectrum analysis 2,6-bis (1,1-dimethylethyl)-4-ethyl phenol was found to be present in the bioactive fraction. The identified compound 2,6-bis(1,1-dimethylethyl)-4-ethyl phenol is a strong antioxidant generally used for food, cosmetics and pharmaceuticals. Antioxidant and anti-diabetic activities of compound 2,6-bis(1,1-dimethylethyl)-4 -ethyl phenol was earlier reported by Unnikrishnan *et al*. (2015). Plant polyphenols are important group of metabolites that have a number of beneficial therapeutic

effects including their role as free radical scavengers. Researchers have found a positive correlation between the antioxidant activity and polyphenolic content of plants (Oki *et al*., 2002). Thus promising antioxidant activity shown by the purified fraction can be due the presence of compound 2,6-bis (1,1-dimethylethyl)-4-ethyl phenol in *M. paleacea*. Krishnan and Murugan (2014b) also reported the presence of 2,6-bis (1,1-dimethylethyl)-4-ethyl phenol in liverwort *Marchantia linearis*. This compound was also reported to be present in *Mesembryanthemum crystallinum* (Bouftira *et al*., 2007). Among identified phytochemicals, 7-hydroxy-2-phenyl 4H-1-benzopyran-4-one; 5-hydroxy-7-methoxy-2-phenyl 4H-1-benzopyran-4-one; 4',5,7 trihydroxy isoflavone and 5-hydroxy-2-(-4 methoxypheny) 4H-1-Benzopyran-4-one belonged to flavonoid group of compound which possess various activities such as anti-mitotic (Hadjeri *et al*., 2004), estrogenic (Vitale, 2013), antioxidant activities (Dowling *et al*., 2010) etc. Suba *et al*., 2004 from the result of their work suggested that flavonoids present in plants mainly contribute to its anti-diabetic activities. GC-MS analysis of *M. paleacea* confirms the presence of large amount of flavonoids which suggests that *M. paleacea* can be a promising source of pharmacologically active bio-molecules.

Terpenoids are most abundant and structurally diverse products of plants. This class of compounds have displayed a wide range of important pharmacological properties. Mass spectrometry of the most active purified fraction from *M. paleacea* had shown the presence of different kind of terpenoid compounds like Caryophyllene, Myrcene, Pimaric acid, Phytol and Palustric acid. Caryophyllene, also known as beta-caryophyllene is a natural bicyclic sesquiterpene which is a constituent of many essential oils of some aromatic plants. When assessed for its pharmacological properties caryophyllene was found to have an anticancer, analgesic properties (Fidyt *et al*., 2016), etc. Caryophyllene was also reported from liverwort *Marchantia convulata* (Xiao *et* al., 2006) which showed cytotoxicity against human liver and lung cancer cells. Presence of caryophyllene in bryophytes was also confirmed by Sorbo *et al*. (2004). Caryophyllene was also found to present in higher group of plants like *Polygonum minus* (Baharum *et al*, 2010).

Another constituent of purified fraction was myrcene which belongs to the class of organic compounds known as acyclic monoterpenoids and are reported to have anti-inflammatory properties (Jakovlev *et al*., 1979). Sonwa and Konig, (2002) also found the presence of myrcene in the essential oil of hornwort *Anthoceros caucasicus.* 4-methyl-1 -(1-methylethyl), 3-cyclohexen-1-ol, is a menthane monoterpenoids having a strong antioxidant activity (Chung, 2011). Phytol belongs to the class of organic compounds known as

acyclic diterpene alcohol used as a precursor in the manufacturing of synthetic forms of vitamin K1 and vitamin E[1]. Phytol is a biologically active compound which were reported to have many pharmacological activities such as anti-nociceptive; antioxidant (Santos *et* al., 2013) etc. Presence of phytol was also reported from other mosses such as *Rhytidiadelphus triquetrus* and *Polytrichum commune* (Klavina *et al*., 2015). Other compounds present belonging to terpene family were pimaric acid and palustric acid. Pimaric acid is a diterpene which was recorded to have an antimicrobial (Ali *et al*., 2012), anti-atherosclerotic activity (Suh *et al*., 2012). Pimaric acid among the bryophyte family has been found to be present in high concentration in *Ptilium crista-castrensis* (Klavina *et al*., 2015). Pimaric acid was also isolated from *Aralia cordata* which showed strong anti-atherosclerotic activity. Palustric acid belonging to the organic compounds class diterpenoids are generally reported of having an anti-microbial activity (Savluchinske-Feio *et al*., 2006).

Another important bioactive compound found was 3-Eicosene, an aliphatic hydrocarbon. 3-eicosene was reported to have a strong anti-antioxidant activity (Adeosum *et al.,* 2013). Other compounds like 2-tetradecanoyl 2-Cyclohexen-3,6-diol-1-one; Methyl 2-(4-hydroxy-3-isopropylbenzoyl) benzoate; 7 (diethylamino) 2H-1-benzopyran-2-one; (17α)-estra-1,3,5(10),6-tetraene-3,17-diol, diacetate and 9-thiocyanato androstan-4-en,3,17-diol-11-one were also present in the purified fractions. These compounds are not reported to have biological activities till date. Thus from the GC-MS analysis of purified fractions FF-1 and FF-3 it can be interpreted that promising antioxidant and anti-diabetic activities displayed by the fraction were mostly due to the presence of bioactive phytochemicals belonging to class phenols, flavonoids, terpenoids and alkanes. The result of the bioassay guided purification of *M. paleacea* confirms that this liverwort can serve as a potent source of phytochemicals having many pharmacological properties.

6.7.2 Network pharmacology

Marchantia paleacea, a liverwort belonging to family Marchantiaceae has been used in many traditional medicines. It is used in treatment of hepatitis, in skin tumefacation, antipyretic etc. liverworts has been used as ethnomedicine worldwide. Recent advancement in scientific research have proved that plant belonging to this group have many important pharmacological properties. Present work aims to study the role of *M. paleacea* as anti-diabetic agent. In Liverwort only Marchantin C has so far has shown anti-diabetic activity (Harinantenaina and Asakawa, 2007). In the present work, *in-vitro* anti-diabetic activity guided column chromatography has lead to the isolation of 19 phytochemicals from *M.*

paleacea having potential anti-diabetic activity. After identifying of phytochemicals, the underlying mechanism of action at cellular level of these compounds was studied by network pharmacology. Network pharmacology is an important approach for drug discovery and development process. This approach involves data base mining, phytochemical-protein target – target disease relationship study and drug likeliness study. The therapeutic effect of phytochemical is correlated with the binding of these compounds to particular protein or nucleic acid target. Therapeutic target database (TTD) allows the user to find protein and nucleic acid targets of concerned protein sequence or drug structure. In total 81 human proteins was targeted by the 15 out of 19 phyto-compounds. No human protein target was found for the other five phyto-compounds. Further the diseases associated with the target proteins have been identified and classified into 22 broad categories according to ICD 10 codes. The highest number of diseases found to be associated with target proteins was those belonging to class malignant neoplasm. While second highest number of disease found to be associated with target proteins was endocrine, nutritional and metabolic diseases i.e. disease related with diabetes. To explore the involvement of isolated phytochemicals from *M. paleacea* in diabetes disease a tripartite network was constructed with phytochemicals, their protein targets and target disease. In total 20 protein targets: PPARG, VEGFA, AKR1B1, PTPN1, HMGCR, EGFR, HSD11B1, MIF, BCHE, GSK3B, ESR1, CYP19A1, AR, ALOX15, BACE1, DRD2, NTRK2, ESR2, ADORA2A and ALOX5 were found to be involved in endocrine, nutritional and metabolic diseases. CooLGen data mining of all diabetes targets ultimately identified 8 targets having high percentage of Heat and Centrality Betweenness value. Protein targets PTPN1, AKR1B1, PPARG, ADORA1, MIF, GSK3B, HMGCR and VEGFA were identified as the main targets of phytochemicals to regulate the diabetes. Among them, PTPN1, PPARG and GSK3B directly affect the insulin signalling pathway (KEGG ID: has04910, hsa04931).

In adipose and muscle cells, PPAR nuclear receptors and winged-helix-forkhead box class O (FOXO) are main transcription factors that regulate GLUT4 gene expression (Fig 6.1). Impaired regulation of GLUT4 gene is directly associated with type 2 diabetes mellitus. PPAR is also associated with the regulation of pro-inflammatory factors and increased level of free fatty acid. Effect of PPARG depends on the presence of ligands, kinases, cofactors and growth factors. In unliganded state, PPARG forms a heterodimer with retinoid X receptor (RXR) and binds to PPAR response elements (PPREs) of target gene (GLUT4). The PPARG-RXR heterodimer then binds with co-repressor and represses the expression of GLUT4 gene.

However, when ligand binds with PPARG, the corepressor will be replaced by a coactivator complex and leads to transcriptional activation of GLUT4. Thus, PPARG ligands have strong role in insulin responsiveness. PPARG is also associated with the regulation of pro-inflammatory factors. In the anti-inflammatory action, PPAR interferes with nuclear factor (NF) κB pathway and causes trans-repression of the concerned gene.

FOXO1 is a negative regulator of insulin sensitivity (Nakae *et al.*, 2002). FOXO1 represses the transcription of PPAR-G1 or PPAR-G2 by repressing their promoter. Insulin completely or partially reverses the effect of FOXO1 by causing PKB (Protein kinase B) or AKT activation which leads to hierarchical phosphorylation of FOXO1 at three sites. FOXO1 cycles between the cytoplasm and the nucleus but mostly are localized in the nucleus. Phosphorylation by PKB causes its exclusion from the nucleus and de-repression of PPARG1 or PPARG2 promoter. PPARG are considered as an important target for anti-diabetic drug development. Therefore, PPARG agonists are clinically used as insulin sensitizers (Kletzien *et al.*, 1992). Synthetic drugs like thiazolidinediones (rosiglitazone and pioglitazone) or glitazones are used to activate PPARG and decrease serum glucose level by decreasing insulin resistance in patients suffering from type-2 diabetes mellitus. In the present study, Peroxisome proliferators activated receptor gamma (PPARG) is targeted by phytochemicals, 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one and 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one present in the studied liverwort. BindingDB (Binding batabase) has shown that these compounds have structural similarity with Bavachinin obtained from *Psoralea corylifolia*. Bavachinin has been reported to act as agonist to PPARG and improve glucose uptake by sensitizing insulin signalling pathway and AMPK activation (Lee *et al.*, 2016). Thus, it can be interpreted that, 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one and 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one present in *M. paleacea* might have therapeutic potential to activate insulin signalling pathways and cure type 2 diabetes. Thus, further detailed work can be focused to understand the series of events leading to interaction of these phytochemicals and PPARG receptor.

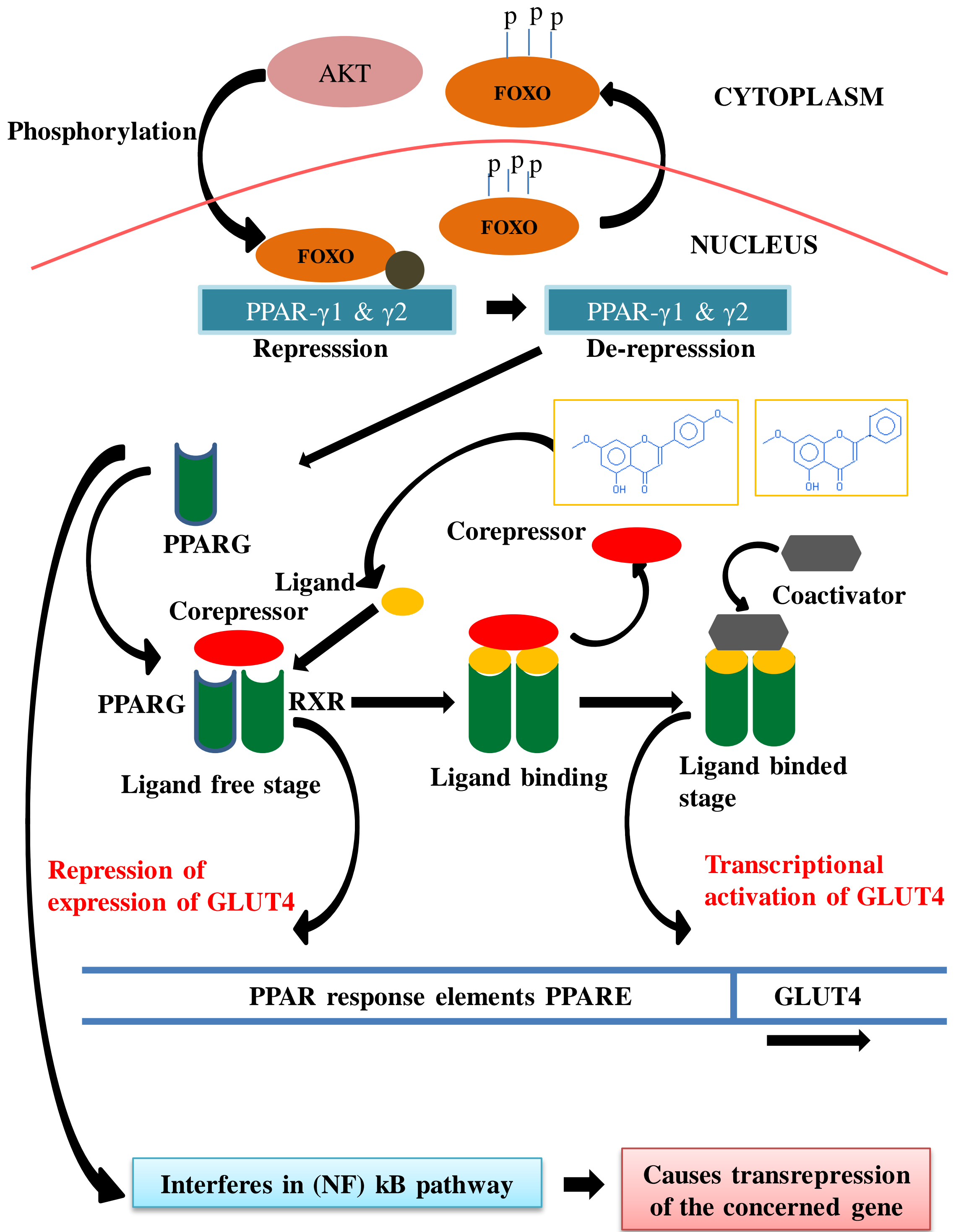


Fig 6.1: Assumed cellular effects of PPARG activation by 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one; 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one

Glycogen synthase kinase (GSK3) is regulated by 4′,5,7-Trihydroxy isoflavone and 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one. GSK plays a key role in insulin resistance by regulating glycogen synthesis. GSK3 was identified as the key regulator in glycogen synthesis by Woodgett and Cohen (1984). GSK3 is a serine/threonine protein kinase that acts as suppressor of many signalling pathway when present in the active form (Fig 6.2). Key target of insulin signalling, glycogen synthase and insulin receptor substrate 1 are inactivated by GSK. Thus, GSK3 becomes an important target for therapeutic interference for insulin resistance. Kaidanovich and Finkelman (2002) reported GSK3B as the main drug target for diabetes type 2. Lappas (2014) reported the increase of GSK3β in adipose tissue and skeletal muscle in gestational diabetes. Result of network analysis showed two phytochemicals extracted from *M. paleacea*, 4',5,7-Trihydroxy isoflavone and 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one targets GSK3B. Natural drug targeting GSK3B are of high significance thus, further study on the interaction of phytochemicals and GSK3B can be useful to explore role of these phytochemicals in insulin resistance.

Another important protein target of phytochemical isolated from *M. paleacea*, involved in insulin signalling pathway was PTPN1. In the present study, tyrosine protein phosphatase non-receptor type 1 (PTPN1) was targeted by palustric acid and pimaric acid. PTPN1 is an enzyme that acts as negative regulator of insulin signalling pathway (Fig 6.2). PTP1B (protein tyrosine phosphatase-1B) encoded by PTPN1 inactivates insulin receptor (INSR) and insulin receptor substrate 1 (IRS1) by dephosphorylation and leads to insulin resistance (type 2 diabetes mellitus). Mackawy *et al.* (2015) found PTPN1 promoter variant 1023> A to be associated with type 2 diabetes patient. PTPN1 is considered another very important therapeutic target for drug discovery. Further work to explore the role of palustric acid and pimaric acid on PTPN1 gene expression can be of great significance.

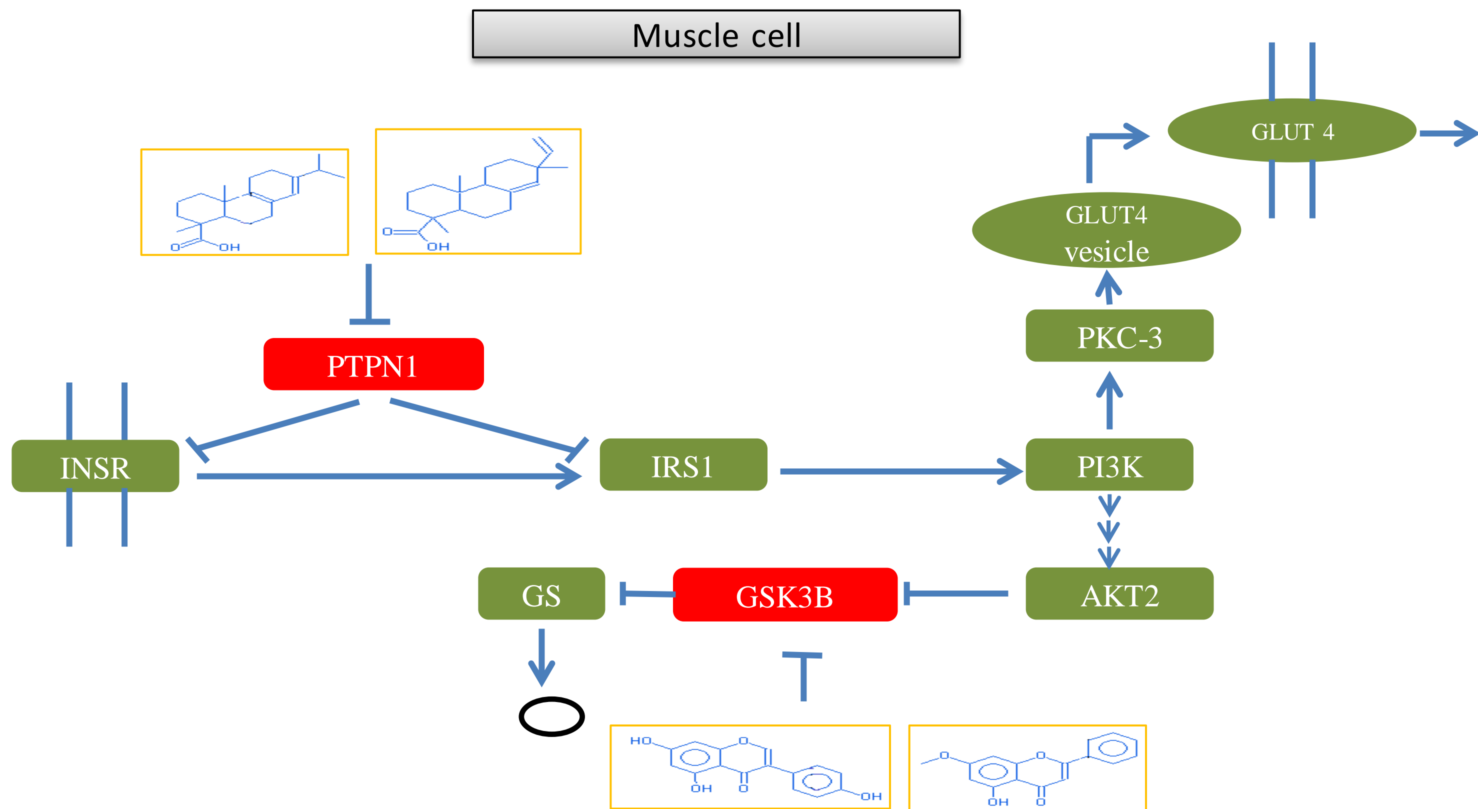


Fig 6.2: Assumed cellular effects of PTPN1inactivation by Palustric and Pimaric acid; GSK3 by 4’,5,7-Trihydroxy isoflavone, 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one

Adenosine receptor A1 (ADORA) causes diabetes retinopathy by blocking the activity of adenyl cyclase through regulatory Gi protein. Inactivated adenyl cyclase fails to activate protein kinase A (PKA) which has an important role in fatty acid degradation (Fig 6.3). Abrogation of ADORA1 might improve the metabolic disorder causing diabetes retinopathy. Attenuation of metabolic dysfunction by abrogating the adenosine mediated activation of ADORA1 was earlier reported by Yang *et al.* (2015). In the present study ADORA1 was found to be targeted by phytochemicals, 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one and 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one.

Insulin resistance pathway has lead to the identification of five active phytochemicals namely, 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one; 4',5,7-Trihydroxy isoflavone and 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one, palustric acid and pimaric acid present in *M. paleacea* that are important for curing insulin resistance in human. Druaggability analysis is important for drug discovery process. Estimation of ADMET properties is highly important for considering a drug candidate for further clinical trials (Waterbeemd and Gifford, 2003). Studied five phytochemical in *in silico* test showed high intestinal absorption percentage and Caco2 (human epithelial colorectal adenocarcinoma) cell permeability. Blood brain barrier permeability and Central Nervous System penetrating potential was also shown by the studied phytochemicals. *In silico* test for excretion found two phytochemical, 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one and Palustric acid are found to be capable of acting as a substrate of Organic Cation Transporter 2 (OCT2) that helps in renal clearance of drugs. Toxic potency of a drug is an important parameter to consider for a drug. Toxicity was measured by Ames test, maximum recommended tolerated dose (MRTD), hERG I and II inhibitory activity and skin sensitization. Phytochemicals studied cleared the entire toxicity test. 5-hydroxy-2-(4-methoxyphenyl), 5 hydroxy-7-methoxy-2-phenyl, 4-H-1 Benzopyran-4-one, 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one, and 4',5,7-Trihydroxy isoflavone showed no hepatotoxicity in *in silico* test while hepatoxicity was shown by pimaric acid and palustric acid. Druaggability of five phytochemicals was also analyzed by Lipinski's "rule of five" criterion. Studied drugs clearly passed Lipinski's "rule of five" criterion.

Information of drugs regarding diabetes was collected from DrugBank database and target of these drugs are collected from Therapeutic Database (TTD). On matching the synthetic drug targets from Drugbank and centralized protein targets, 1 taget i.e. PPARG was matched. So it can be concluded that PPARG is one of the main therapeutic target in diabetes.

Thus, as mentioned earlier agonist of PPARG can act as good candidate for increasing insulin sensitivity and curing type 2 diabetes. In this study PPARG was targeted by 5 hydroxy-7-methoxy-2-phenyl, 4-H-1 Benzopyran-4-one and 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one. Thus, these phytochemicals can be explored further to find the entire mechanism by which these drug like molecules can activate the PPARG.

Therefore, this network assisted work has paved a new way to find the potential of liverworts in curing diabetes. Thus, this study will prove important for understanding phytochemical-protein level interaction that will eventually help in the identification of novel natural drug compound from liverworts against diabetes mellitus.

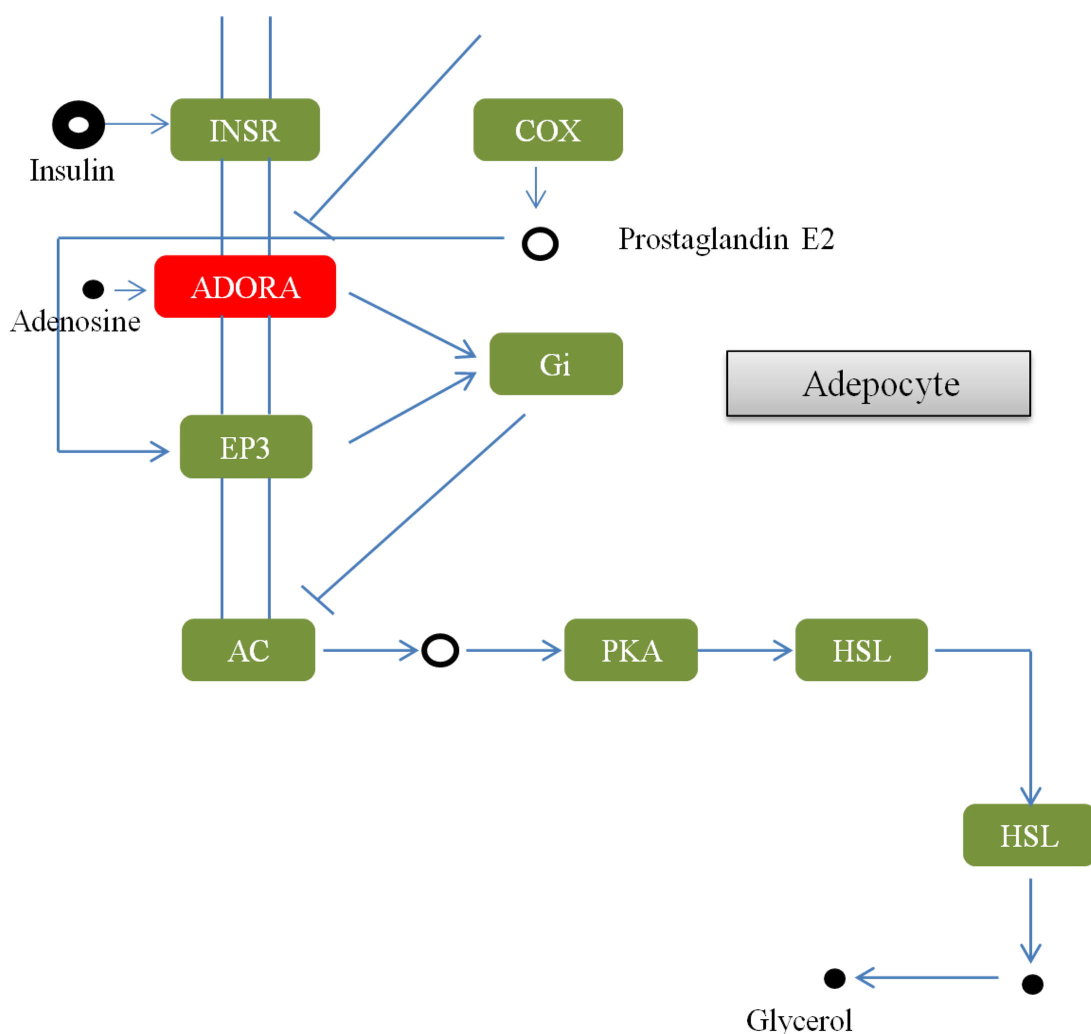


Fig 6.3: Assumed cellular effects of ADORA1 inactivation by 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one; 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one

6.8 *IN VITRO* PROPAGATION AND COMPARISON OF BIOACTIVITIES OF AXENICALLY AND NATURALLY GROWN LIVERWORT *LUNULARIA CRUCIATA*

Research on the study of phytochemicals and pharmacological activities of bryophytes has been neglected for long in spite of being treasure house for large number of photochemical. Restriction in their study is mainly due to i) habitat preference allowing them to grow only within a selected geographic area ii) seasonal dependence for sample collection and iii) less availability in natural habitat. So, the aim of this work is *in vitro* propagation and evaluation of phytochemical and pharmacological activity of artificially grown and naturally grown *Lunularia cruciata*.

In vitro propagation of *L. cruciata* was initiated from gemmae which started germinating after 8-12 days of inoculation. Spores of moss *Erythrodontium julaceum* and liverwort *Marchantia linearis* also found to require the similar time period for germination (Awasthi *et al.*, 2012; Krishnan and Murugan, 2014). Awasthi *et al.* (2013) found diluted nutrient medium to be most suitable for germination of the spores of endangered liverwort *Cryptomitrium himalayensis*. In present work different culture media such as Gamborg B-5, Knop, MS and diluted MS/2 were used for optimizing micro propagation of *L. cruciata*. Among the media used, germination of gemmae was successfully obtained in half strength Murashige and Skoog medium. Profound effect of growth regulators BAP and NAA was noticed on the growth and multiplication of thalli. Krishnan and Murugan (2014a) and Senarath *et al.* (2017) used 2 mg/L Benzylaminopurine (BAP) and 0.5 mg/L Naphthaleneacetic acid (NAA) for thallus and rhizoids differentiation from the spore of *Marchantia linearis*. In the present work, thallus and rhizoids differentiation from callus was initiated by supplementing basal salt media with 2 mg/L BAP and 0.5 mg/L NAA. Alteration of hormone concentration at later stage of growth and development was found to be helpful. Increased BAP (4 mg/L) and decreased NAA concentration (0.2 mg/L) restricted the excessive growth of rhizoids and restored normal growth and multiplication of the thallus in the present work. Alternation of concentration of growth regulators was also found to be helpful by other researchers in achieving proper growth and proliferation of bryophytes in *in vitro* condition. Krishnan and Murugan (2014a) achieved root induction by transferring the differentiating leafy thalli of *Marchatia linearis* in a rooting medium containing 2 mg/L Indolebutaric acid. This result highlighted the importance of plant hormones in controlling

the growth and proliferation of plants in *in-vitro*. Young thalli then grew continuously to develop dichotomously branched fully grown thalli after 12 weeks.

In-vitro propagation provides the means for rapid multiplication of plants which helps to overcome various exertions related to isolation of phytochemicals from wild varieties due to factors like seasonal dependence, lower availability in nature, low rates of growth and over exploitation of wild varieties. However, there is a possibility that there occurs alteration in the phytochemical composition of naturally grown plants due to change in biochemical pathways resulting from artificial growth system (Senarath *et al*., 2017). Thus, comparison of phytochemicals in *in-vitro* and naturally grown plants are essential if the axenically grown plants are to be used as alternative to naturally grown plants (Manivannan *et al*., 2015). GC-MS analysis of methanolic extract showed the presence of ten compounds in naturally grown plants and nine compounds in *in-vitro* grown plants. Presence of similar kind of phytochemicals was found in naturally and artificially grown plants. Synthesis of similar kind of phytochemicals in naturally and naturally grown plants was also reported by Nikolova *et al*. (2013); Seranath *et al*. (2017). Alkaloids, flavonoids, terpenes, fatty acid, aliphatic hydrocarbon and acyclic alkanes were the phyto-compounds that had been detected in the studied plant extracts. Compounds like flavone, phytol, piperidine were common in both the plant extracts. While many other compounds were detected that differed slightly in structure, but belonged to same metabolite class and had similar retention time.

Ethyl tetradecanoate and cyclopentaneudecanoic acid present in *in-vitro* and naturally grown plants respectively were fatty acids detected in similar retention time (18.03 and 18.08 respectively). Alkaloid group was detected in both the extracts, in *in-vitro* grown plant alkaloid was present in the form of 1-1-butyl piperidine, while slightly modified ester form, 4-piperdineacetic acid, 1 acetyl 5 ethyl-2[3-(2 hydroxy ethyl)-1H-indol-2-yl]-a methyl; methyl ester was detected in naturally grown plants. Studies suggest that alkaloids present in plants have many medicinal properties like anti-microbial (Kumar *et al*., 2009), cytotoxic (Rinaldi *et al*., 2017), antimalarial activities (Wirasathien *et al*., 2006), etc. Apart from alkaloids, fatty acid esters was also found to be present, tricosane 2,4-dione was detected in *in-vitro* grown plants and 13,16-octadecanoic acid, methyl ester; cyclopentaneudecanoic acid, methyl ester; 13-decasenoic acid, methyl ester were obtained in naturally grown plants. Another class of compounds detected were terpenes; phytol was present in both extracts while thujopsene –[12] was found to be present only in naturally grown plants. Researchers while exploring plants for their medicinal properties found terpenes to be responsible for

many important health beneficial activities like antimicrobial (Cardoso *et al.*, 2012), anti-inflammatory (Khiev *et al.*, 2011), anti cancer (Islam, 2017), cardiovascular and diuretic activity (Somova *et al.*, 2001).

Flavonoid group was also found to be present in *L. cruciata* extract. Flavonoids like flavones were present in both extracts while 4H-1-Benzopyran-4-one, 3-hydroxy-2-phenyl was detected in naturally grown plant extract only. Flavonoids are another important class of phytochemicals in plants having many important activities like antioxidant (Pietta, 2000), hepatoprotective (Akachi *et al.*, 2010), antibacterial (Cushnie and Lamb, 2005), anti-inflammatory (Serafini *et al.*, 2010), anticancer (Zhang *et al.*, 2008) and antiviral activity (Lani *et al.*, 2016). GC-MS analysis has showed the presence of alkane only in *in-vitro* grown plants. Result of this study showed that the remarkable substances like phenols, terpenes and alkaloids that are present in naturally grown plants were also been synthesized in plants grown under artificial condition. *In vitro* grown *Fossombronia pusilla* has produced the same terpenoid group as is produced by its natural counterparts (Sauerwein and Becker, 1990). Different fatty acid, fatty acid ester, fatty acid alcohol and alkane have also found to be synthesized in *in-vitro* grown plants like their natural counterparts. This suggests possible application of *in-vitro* cultured plants for clinical validation, bioprospection studies and commercial exploitation of novel compounds through bio farming without over harvesting plants from their natural habitats. The presence of similar phytochemicals and biological activities in *in-vitro* and naturally grown plants were also reported by Kumari (2013); Vujcic *et al.* (2017); Senarath *et al.* (2017).

Liverworts are moisture loving plants with a restricted pattern of distribution growing mostly during rainy season under natural condition (Singh, 1999; Bhatacharyya, 2011); these drawbacks are responsible for their least use in research purposes. Also their low availability in nature is the biggest challenge for identification and isolation of biologically active phytochemicals in sufficient amount from these plants (Klavina *et al.*, 2015). Our finding supports the potential of *in-vitro* grown plants to overcome the challenges of seasonal dependence and low availability of liverworts for structural elucidation of compounds and biological assay. Potential of *in-vitro* grown plants to be used in place of naturally grown plants has also been reported by other researchers, like two times increase in sesquiterpene was recorded by Otha *et al.* (1990) in cell culture of liverwort *Calypogeia granulata* and *Marchantia polymorpha*. Better production of effective phytochemical in artificial condition rather than natural habitat was also noticed by Sabovljevic *et al.* (2011).

Different health beneficiary properties shown by herbal medicines are due to bioactive phytochemicals present in the plant (Sahoo *et al*., 2013; Saravanam and Parimelazhagan, 2014). Presence of many phytochemicals in naturally and *in-vitro* grown plants were recorded through GC-MS analysis. In this work, antioxidative and anti-diabetic assays were performed to check if there are any biological activities of these phytochemicals, and if so, study was done to find out whether any change takes place in the activity of phytochemicals if grown in artificial habitat. DPPH is the stable organic nitrogen free lipophilic radical commonly used to investigate the free radical scavenging properties. Both naturally and *in-vitro* grown plant extracts has shown DPPH radical chelating property which might be due to the donation of electron or hydrogen atom to DPPH radical by phytochemicals (Shirwaikar, 2006) present in the studied plants. Naturally grown plants were recorded to have slightly better (IC_{50}= 0.45 mg/ml) DPPH radical scavenging activity than *in-vitro* grown plants (IC_{50}= 0.62 mg/ml). Slightly better DPPH activity of *in-vitro* grown plants was also reported by Mohan *et al*. (2011) in his work with *Bacopa monnieri*. In ABTS radical scavenging assay it was found that artificial condition of growth does not hinder the ABTS radical scavenging property, rather little improvement in the activity was noticed. Little improvement in free radical scavenging activity by *in-vitro* shoot extract was also reported by Manivannan *et al.* (2015). In living system, free ferrous ions are the powerful pro-oxidants which cause oxidative damage and lipid peroxidation by Fenton reaction (Saravanam and Parimelazhagen, 2014). It is important to scavenge such radicals from the body of a living organism. On testing the *in-vitro* and naturally grown *L. cruciata* extracts for metal chelating property we have found plant grown on both habitat have developed ability to scavenge ferrous ion. In this assay Fe^{2+} and ferrozine forms complexes to produce hydroxyl radical, chelating effect observed might be due to interference of plant phytochemicals in the complex formation. Free radical scavenging property might be attributed to the presence of phenolic compounds like flavone, 4H-1-Benzopyran-4-one, 3-hydroxy-2-phenyl present in the extracts. Phenolic compound acting as radical scavengers has also been reported by workers like Wong *et al*. (2006); Tusevski *et al*. (2014). Apart from phenolic group, other bioactive metabolites like alkaloids, terpenoids identified might have contributed to biological activities.

Reduction in insulin sensitivity and postprandial hyperglycemia are the characteristics of type 2 diabetes (Mousinho *et al*., 2013). Lowering postprandial hyperglycemia can be an important measure to control diabetes. Postprandial hyperglycemia can be controlled by inhibiting the activity of enzymes α-amylase and α-glucosidase (carbohydrate hydrolyzing

enzymes) (Ali *et al*., 2006). *In-vitro* and naturally grown plants inhibited the activity of α-amylase and α-glucosidase enzymes to an impressive level. Betterment of α-glucosidase inhibitory activity was recorded *in-vitro* grown (IC_{50} 0.24 mg/ml) plants in comparison to in-vivo grown (IC_{50} 0.51 mg/ml) while, α-amylase activity was better in case of naturally grown plants (naturally grown: IC_{50} 0.49 mg/ml; *in-vitro* grown: IC_{50} 0.59 mg/ml)

Here we report more or less similar biological activity and presence of similar phytochemicals in *in-vitro* and naturally grown plants. All these results showing similar biological activity of in-vitro grown plants supported the objective of our work to use tissue cultured plants as a substitute for naturally grown plants to overcome the shortcomings restricting the use of liverworts for analytical purposes, despite of being the storehouse of many unique phytochemicals.

6.9.1 VARIATION IN THE ABUNDANCE OF EPIPHYTIC LIVERWORTS IN RELATION PHYSICO-CHEMICAL ATTRIBUTES

Bryophytes are integral part of forest ecosystem having strong functional relationship with many ecosystem processes. Darjeeling is home to large variety of epiphytic bryophytes. Nowadays increasing anthropogenic activities are posing serious threats to natural habitats of liverworts. They are generally considered as weed and are being ignored. Lack of proper knowledge and appreciation by the masses, including general botanists are serious threats leading to extinction of these plants. However, through facilities like 'Bryophyte Gardens', 'Moss Houses and 'Species Specific Sites', liverworts can be familiarized to students, researchers and the common people, which may inculcate interest among them and help to reduce threats to this unique group of plants. Therefore study of habitat requirements of liverworts are important to consider for their conservation.

Study of growth and proliferation of epiphytes on trees occurring in more or less similar environment is a feasible method for analyzing the effect of physical and biochemical variables on proliferation of epiphytes. Several factors may account for epiphytic liverwort density on trees. The diameter at breast height (dbh) exerts a profound influence on distribution of epiphytic liverworts. Density of epiphytic liverwort increased with the increase in the diameter of the tree indicating positive correlation between two variables. Dbh is the indicator of habitat quality of epiphytes. Growth anomaly becomes more frequent and the bark texture becomes more fissured with the increase in dbh. Similar to the findings of present work, McGee and Kimmere (2002), Friedel *et al*. (2006) and Orjan *et al*. (2009) also

observed an increase in epiphytic species with the increase in tree diameter. Positive correlation was found between dbh and the age of the tree by Lukaszkiewicz and Kosmala (2008). So it can be stated that old trees are most favoured site by epiphytic liverworts. Suitable substrate formation with the increase in tree age might have favoured the increase in density of liverworts. Exceptional occurrence of epiphytic liverwort on tree with much small bark area (40 – 48.3 inch) suggested that abundance might not be solely dependent on tree girth. This highlights complex interplay of role of different factors on distribution pattern.

Moisture is an important physical factor affecting species composition and diversity (Hettenbergerova *et al.*, 2013). Most epiphytic species of bryophytes are stenoecious demanding shady and highly humid condition (Friedel *et al.*, 2006). Result of the present study also highlighted the importance of moisture in sustaining the diversity and density of epiphytic liverworts. Positive correlation between two variables shows significant impact of increasing moisture content on increasing density of epiphytes.

Most epiphytic liverworts suffer from abrupt exposure to radiations. Light condition exerted profound influence on the species density. Statistical analysis of data revealed an interesting fact about the correlation between light intensity and epiphytic liverwort cover. Liverwort abundance reduced dramatically above and below particular threshold limits of light. Growth of epiphytic liverworts was recorded in light intensity range 1900–4600 lux, above this range density of epiphytic liverworts decreased dramatically. It was also noticed that light of very low intensity also reduced liverwort abundance. Thus, it was inferred that shade to half shade condition was favoured by the studied epiphytic liverworts. Connection between richness of epiphytes and the pH of tree bark has been recorded by various authors (Mezaka and Znotina, 2006; Lobel *et al*., 2006). But in our study variation in epiphytic cover was not significantly characterized by the pH of the bark.

Apart from physical attributes, biochemical characteristics of tree bark might also affect the epiphytic richness and distribution. Probable role of plant secondary metabolites on the density of epiphytic liverwort cover was studied in present work. *C. japonica* exudes resin, the major components of which are terpenes. Resin protects plants from invading pathogens. So we studied possible effect of terpenes on epiphytic abundance. We have noticed an increase in the liverwort abundance with the decrease in terpenoid content of the trees, suggesting that the two attributes are correlated in a negative manner. Similarly, tree age and terpenoid content were found to be negatively correlated. Kim (2001) also noticed

negative correlation between terpenes and age of the tree. Unlike pathogen, bryophytes do not invade the sieve or blast tissues of the tree; they remain attached to cork layers through rhizoids. Nevertheless they are subjected to bark allelochemicals that are protective against plant pathogens. This study therefore confirmed our hypothesis that bark phytochemical might have some allelopathic effect on epiphytic liverworts and the resistance action of *Cryptomeria japonica* against these liverworts might be due to higher accumulation of terpenoid compounds particularly at young age.

Phenolic group of phytochemicals are mostly involved in plant defense. However, we found that phenolic group didn't exert allelopathic influence on liverwort cover. Epiphytic abundance varied irrespective of the total phenolics content of the bark. Other researchers have noticed decrease in phenol content with the increase in tree age. In our study, no correlation was observed between the tree age and phenolic compounds. Various biotic and abiotic factors affect the composition and concentration of secondary metabolites of a plant species. Sites selected for analysis being natural, trees growing within this area may experience wide variation in biotic and abiotic stresses. Different environmental stresses experienced by the trees might have contributed for variation in the secondary metabolite content irrespective of age of the plant.

For better understanding of complex interplay of the roles of different physical and biochemical factors, Principal Component Analysis (PCA) and Heatmap analysis was performed. In Principal component analysis, variables were clustered in four groups, *viz.* Cluster A, B, C and D. It was noticed that the variables of cluster A were negatively correlated with the variables of cluster C. Variables like moisture, abundance and girth of the tree were closely related. With increase in diameter of the trees, the physical and chemical characteristics of bark changes continuously (Barkman, 1958). With the increase in tree size and age, the bark of the tree becomes more porous, absorbent and resinous facilitating the settlement of epiphytic species (Schumacher, 2000) and thus abundance increases with the increase in the phorophyte diameter. With the increase in tree age, also the bark composition changes and becomes more absorbent favouring luxuriant growth of epiphytes. Furthermore, cover of the tree layer and tree density are also important parameters affecting the moisture and thus the abundance of the bryophytes.

Heatmap test result interprets that high moisture content in air as well as substrate are required to ensure the occurrence and luxuriance of epiphytes. PCA and heatmap focuses on

the significant impact of light condition on density of epiphytic cover. Abrupt exposure to light affects the species requiring consistent humidity and shady condition (Friedel *et al.*, 2006). Moreover, tree density as a substitute for light condition, also affects the density of liverworts, as thinning of the forest after logging may lead to the exposure of species, demanding shady and humid condition, to radiation. Old growth forest stands containing large old trees, dead logs and canopy cover preventing exposure to sunlight are important for epiphytic growth and conservation. Light of higher intensity are found to be detrimental for moisture loving epiphytic species. Heatmap and PCA suggested that among plant secondary metabolites terpenes content of the plant has a negative impact on epiphytic growth. It can be interpreted that not only the favourable substrate developed with the increase in tree age favours epiphyte abundance but also the reduced terpene content with the increasing plant age is highly important for maintenance the epiphytic cover.

Senchal Wildlife Sanctuary, is one among the important tourist places of Darjeeling. Nowadays increasing tourist activity is imposing serious threat to the biodiversity of this area and chances are there that many plant species might disappear from this area unnoticed due to increasing anthropogenic activity. Bryophytes have always been neglected form the ecological as well as biochemical point of view as compared to other plant group. So, the result of the present study reports about the habitat preference of epiphytic liverworts in Senchal Wildlife Sanctuary. Dbh, moisture of tree bark were the important factors that influenced the abundance of epiphytic liverworts. In addition light condition was of great importance for the proliferation of epiphytic liverworts. Terpenoid content of the tree on which the liverwort was found to grow influenced the epiphytic liverwort abundance immensely. The terpenoid content was found to be inversely related to the age of trees suggesting old trees to be the preferred habitat of epiphytic liverworts. Thus, forest management or the responsible authority therefore should ensure the presence of old, large and rough barked trees to sustain the epiphytic liverwort cover in this area. Liverworts are moisture loving plants so care should also to be taken to maintain the tree density and canopy cover as abrupt exposure to light may prove lethal for epiphytic liverwort diversity and density.

6.9.2 VARIATION IN THE ABUNDANCE OF LIVERWORTS GROWING ON SOIL IN RELATION TO PHYSICAL FACTOR AND SOIL PROPERTIES

Determination of factors that controls the relative abundance of liverworts is of great significance for understanding their habitat preference. Studies have showed that nutrient

supply rates can influence the abundance of species in communities. Much less is known about the role of physical factors and biochemical attributes of soil on liverwort species abundance. Thus in this study an effort was made to study role different environmental factors and biochemical attributes of soil on liverwort abundance. It was found that moisture content of the soil is very important for the sustenance and growth of liverworts. High moisture content is favourable for growth and proliferation of liverworts. Another important factor that was found to have significant influence on liverwort abundance was light intensity. It was observed that low light intensity was mostly preferred by the liverworts. Liverwort abundance decreased with the increase in the intensity of light. Among different biochemical properties of soil that has been analyzed, carbon to nitrogen ratio found to be positively correlated with the abundance of liverworts while organic carbon, organic nitrogen, available phosphorous, potassium and sulphur content of the soil didn't influence the abundance of liverworts in the present study. However, Virtanen *et al*. (2000) found positive correlation between the soil pH and the presence of liverworts but in the present study pH of the soil didn't influence the liverwort abundance. Thus, proper understanding of this plant group is necessary for its proper conservation.

CONCLUSION

Liverworts are largely neglected in the search for new biologically active compounds. Very few works has been dedicated to study the composition of its secondary metabolites. Till date, research is mainly focused on higher group of plants, mainly angiosperms and no doubt a large numbers of pharmacologically active compounds have been isolated from this group; but as all these plants belong to the same Angiosperm group which contain similar kind of phytochemicals, there's a need to look for novel natural resources from under-explored area for the discovery of new therapeutic agents. Bryophytes are the plant group which remain untouched in course of drug discovery. Considering this, in the present study, liverworts common in the natural environment of Darjeeling was selected and their pharmacological property as well as phytochemical composition was documented. Taking account of the need for conservation due to increasing human threats to liverworts, the information about the environmental conditions influencing their abundance was also investigated. Problem of adequate sample availability is mainly restricting the functional area of bio-prospection of liverworts, which could be resolved through establishing the techniques of *in vitro* culture. Comparing the biological activities as well as the phytochemical composition of *in vitro* grown plants with that of naturally growing individuals, present work recommends using *in vitro* technique as an alternative for solving the problem of low sample availability as no significant variation in biological activity and phyto-constituents between *in vitro* cultured and naturally grown liverworts has been recorded. From the result of the present study, following aspects are deserved to be considered important like:

i. Studied liverworts showed significant free radical scavenging activity similar to that of some medical plants belonging to higher group of plants. More *in vitro* and *in vivo* studies alongside detailed phyto-constituent analysis are suggested in this field for use of liverworts in disease preventing therapies.

ii. It was also known from the result that liverworts possess significant anti-diabetic activity too. *In vitro* bioassay guided purification by column chromatography has resulted in the identification of five phytochemicals namely, 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one; Pimaric acid; 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one; Palustric acid and 4′,5,7-Trihydroxy isoflavone from liverwort *Marchantia paleacea* that specifically targets the proteins that are involved in insulin resistance pathway. Network pharmacology technique identified the phytochemicals and their protein targets along with

the mechanism of action of these proteins in insulin signalling pathway. By acting either as the agonist or antagonist, these phytochemicals were found to improve the insulin sensitivity in insulin signalling pathway. The result obtained from pharmacological network highlights the potential of liverworts in curing diabetes. Thus, work is needed to understand the series of events leading to interaction of these phytochemicals with their targets

iii. Outcome of the experiment performed for screening the cytotoxic activity of liverworts provided groundwork to use liverworts as the active source of compounds with cytotoxic activity. Darjeeling, a part of Eastern Himalaya is a home to large number of liverworts but till date, no work related to cytotoxic activity of liverworts of this area has been reported. Thus more focused work in this field is required.

vi. Secondary metabolites present in plants are accountable for the biological activities of the plant. The results of the qualitative and quantitative test for phytochemicals done in this work, showed the presence of variety of phyto-compounds that could be isolated and further studied to understand the detailed mechanism on how they help to inhibit the activity of α-amylase and α-glucosidase enzyme in diabetes, reduce elevated free radicals during oxidative stress and decrease proliferation in cancer cell line.

v. Results showing similar or slightly changed biological activity of *in vitro* grown plants supported the objective of this work to use tissue cultured plants as a substitute for naturally grown plants to overcome the shortcomings restricting the use of liverworts for analytical purposes.

vi. Study of the effect of physical and biochemical factors on liverworts density found that light, moisture and age of tree exerts major influence on liverwort density. Therefore, forest management should ensure the presence of large, old trees as well as care should also be taken to maintain the forest canopy cover as abrupt exposure to light causes increased radiation leading to the changes in the microclimate resulting in habitat destruction of liverworts.

SUMMARY

Present work deals with the "Studies of phytochemical and antioxidant properties of selected liverworts of Darjeeling Himalaya". A brief introduction of the work and a review of literature are presented in the beginning of the thesis. The review starts with detailing of the status of liverworts in India and their uses in traditional medicines. Since time immemorial plants are used for the treatment of different ailments and scientific reports indicated the application of liverworts in traditional medicine. It has been proved that phytochemicals present are responsible for the biological activities showed by plant. In this context, work done for the evaluation of phytochemicals present in liverworts worldwide and in India was reviewed. Presence of many biologically active phytochemicals has been recorded and some among them are even reported to be unique to the plant kingdom. The conceptual background of free radicals and their harmful effects in human health was presented. Free radicals generated during different metabolic processes in the body at their optimized concentration have beneficial role in cell signaling and other cellular functions. However excess free radical accumulation in the body leads to severe deterioration of the human health. Antioxidants are the first line of defense to combat the harmful effect of free radicals generated during oxidative stress. When endogenous antioxidant protection system fails, intake of antioxidants from external sources is the only solution. So the source of dietary antioxidants and their mechanism of action against free radicals were schematically presented. However, high doses of dietary uptake of synthetic antioxidants have major side effects like carcinogenic effect. Plant secondary metabolites have a great potential to act as a free radical scavengers. Thus, in this study potential of liverworts to act as free radical scavengers was reviewed. Apart from the anti-oxidative activity liverworts are reported of having many other pharmacological activities. Besides the antioxidative role, the present work also aims to study their anti-diabetic potential and cytotoxic activity of liverworts against kidney cancer cell line. Therefore, literature on the anti diabetic and cytotoxic role of liverworts against specific cancer cell lines is also reviewed.

Liverworts are important component of biodiversity however; they are usually neglected due to their small size and less economic values. As a result of this many species are being threatened and some are even on the verge of extinction. So, the threats as well as the conservation technique undertaken for the protection of liverworts in India were reviewed. Apart

from conservation, liverworts are receiving limited attention in laboratory researches too. Restricted geographical distribution pattern, seasonal availability and problem of sample availability in sufficient amount for research are restricting researches on this group of plants. One of the main solutions to these problems is to propagate and multiply the plants *in vitro*. Thus, literatures on *in vitro* culture method of liverworts were reviewed. Optimum condition for *in vitro* growth, increase or decrease in pharmacological activity and phytochemical constituents of liverworts when grown under artificial growth condition were discussed under this topic. In natural habitat, different physical factors affect the species richness and abundance of liverworts. Due to small body structure and less economic importance, liverworts are usually considered as weeds and their habitats are being destroyed without even being noticed. Thus, facilitating the concepts of *ex-situ* conservation might help in the conservation and familiarization of liverworts to common people. Sustainable conservation requires good knowledge about ecological factors that affects the diversity, distribution and abundance of liverworts. Thus, literature on the study of physical and biochemical factors affecting the richness and abundance of liverworts was critically studied.

In the next chapters, results of the work performed for fulfilling the objectives was presented and the important outcome of the works has been discussed. Eleven liverworts were selected for the study in present work considering their abundance in nature and the use in traditional medicines. Free radical scavenging potential was screened through $DPPH^{\bullet}$, $ABTS^{+}$, nitric oxide, superoxide, metal chelation and ferric reducing method. Promising free radical scavenging activities were obtained from liverworts *Plagiochasma cordatum, Asterella wallichiana*, *Lunularia cruciata*, *Marchantia paleacea* and *Marchantia nepalensis* among the studied liverworts. Type 2 diabetes mellitus is caused by the reduction in insulin sensitivity which results to a condition called hyperglycemia. Postpandrial hyperglycemia can be lowered by inhibiting the activity of enzyme α-amylase and α-glucosidase. *Marchantia subintegra* showed highest α-amylase and α-glucosidase inhibitory activity which was almost similar to that of standard drug Metformin. Oxidative stress also increased under sustained hyperglycemia. Therefore antioxidant therapies that targets diabetes induced oxidative stress can be useful to prevent downstream complications of diabetes. Cytotoxic activity of liverworts was assessed by measuring their toxicity to human kidney cancer cell line (ACHN). Cytotoxicity value ranging between ≤ 20 and1000 µg/ml (IC_{50}) are considered active and extracts having cytotoxicity value

more than 1000 µg/ml are considered inactive. *Plagiochasma cordatum, Asterella wallichiana*, *Lunularia cruciata*, *Marchantia paleacea* and *Marchantia nepalensis* were found to have active in controlling the proliferation of human kidney cancer cell line cells.

As the phytochemicals present in plants are responsible for their pharmacological properties, qualitative and quantitative estimation of phytochemicals was done. Significant amount of polyphenolic components were recorded in studied liverworts. Apart from polyphenolic group, presence of other important group of secondary metabolites like terpenes was recorded. Data analysis by software programs such as Pearson Correlation test and Principal Component Analysis (PCA), showed a positive correlation of phyto-compounds with antioxidant and anti-diabetic activities.

Life strategies of bryophytes are system of co-evolved adaptive qualities. They are successful colonizers and can survive extreme environmental conditions. Survival of extreme environmental condition leads to different physical and chemical adaptations in the bryophytes. One of the important strategies adapted by plants is the generation of strong antioxidant defense system. Thus, the changes in antioxidant defense system under favorable (rainy season) and unfavorable growth conditions (season with scanty water in the environment) was studied. *Marchantia paleacea* was selected as the model plant for this work. Result of the study suggested that free radical scavenging activity increases in unfavorable growth condition. The polyphenol synthesis was also found to increase during unfavorable condition of growth and it was assumed that increased polyphenolic compounds might have contributed to enhanced free radical scavenging activity.

One of the main objectives of the present work was *in-vitro* bioassay guided isolation of bioactive phytochemicals. Considering medicinal value, abundance in nature and promising free radical scavenging, anti-diabetic and cytotoxic activity, *Marchantia paleacea* was selected for *in-vitro* bioassay guided purification of bioactive compounds having promising anti-diabetic activity. Purification of phyto-compounds by column chromatography identified nineteen phycto-compounds having potential antioxidant and anti-diabetic activity. Network pharmacology is an important approach in drug development and discovery process. After the identification of bioactive compound through GC-MS analysis and NIST Library matching, the underlying mechanism of action of these compounds at cellular level was studied by network pharmacology followed by KEGG Metabolic Pathway analysis. This approach finally lead to the

identification of phytochemicals namely, 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one; Pimaric acid; 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one; Palustric acid and 4′,5,7-Trihydroxy isoflavone which directly targets the main human proteins that are involved in the insulin signaling pathway. The protein target of these phytochemicals was PPARG, GSK3B, PTPN1 and ADORA1. By acting either as the agonist or the antagonist of these protein targets identified phytochemicals was found to increase the insulin sensitivity in insulin resistant pathway. Flavononolic compounds, 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one and 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one present in *Marchantia paleacea* were found by network analysis to increase insulin sensitivity by acting as the agonist of protein PPARG in the insulin signalling pathway. Similarly flavonoid 4′,5,7-Trihydroxy isoflavone along with 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one were predicted to prevent insulin resistant by acting as an antagonist to the GSK3B protein. Network analysis showed terpenes like Palustric acid and Pimaric acid present in the bioactive fraction of *Marchantia paleacea* by acting as agonist as to PTPN1 protein can reduce the insulin sensitivity. Phytochemical-protein-disease network showed that 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one and 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one also targets protein ADORA1 which is responsible for causing metabolic disorder.

Number of research works going on to study about pharmacological activity phytochemical constituents of bryophytes are considerably low. Main reason that constrains the research work on this group of plants is the problem of sample availability in sufficient amount to carry on the sophisticated analytical work. *In-virto* culture technique was tried to establish for liverwort *Lunularia cruciata* in this work as a contribution to resolve the problem of sample unavailability. On the basis of bio-prospective value and low availability in nature *in vitro* culture was established with *Lunularia cruciata*. *In vitro* propagation was initiated from gemmae. Continuous illumination of 4000-5000 lux at 21°±2°C initiated germination of gemmae after 8-12 days in a half strength Murashige and Skoog media. Profound effect of growth regulators benylaminopurine (BAP) and naphthelen acetic acid (NAA) was noticed on the growth and multiplication of thalli. Young thalli then grew continuously to develop dichotomously branched thalli after 12 weeks. Hormone combination (BAP 2 mg/L and IAA 0.5 mg/L) initiated differentiation of thallus and rhizoids from the gemmae. Further the phytochemicals composition and biological activities of naturally grown and *in vitro* grown

liverwort was compared to determine if there occurs any change in biological activity or phytochemical composition when *in vitro* condition was used for the propagation. Considering the bio prospective value and lower availability in natural condition, in the present work *in vitro* culture was initiated from liverwort *Lunularia cruciata.* No significant changes in the bioactivity were seen. Phytochemicals constituents of two differently grown plants also did not varied much.

Changing atmospheric scenario is posing serious threat to world's biodiversity. Area of study of the present work is one of the famous tourist places. Thus increasing tourism as well as urbanization in this area are causing serious anthropogenic threats to bryophytes as they are uncommon to people and are usually considered as weeds. So an attempt was made to demonstrate the effects of different physical and chemical factors on species richness and abundance of epiphytic liverworts and ground liverworts. Moisture content of the substrate was found to be best correlated with liverwort abundance in positive manner. Light intensity also influenced the abundance immensely. Liverwort abundance was found to reduce dramatically under abrupt exposure to light. Age of the tree on which the epiphytic liverworts grow also influences the density of the liverworts. With increase in the age of the tree increase in the density of liverworts was recorded. Biochemical nature of the tree bark such as phenol, flavonoid, tannin, steroid content, pH did not exert much effect on the liverwort abundance in the present work. However terpenoid content of the trees in case of epiphytic liverworts found to exert a negative effect of their abundance. In case of liverworts growing on the ground, moisture content of the substrate and light intensity were the most important factors influencing the abundance. Other factors like pH, carbon, nitrogen, phosphorus, potassium, sulphur content and carbon nitrogen ratio of the soil didn't influence the abundance of liverworts. By discussing the important outcomes of objectives of the present work, the result and discussion chapters were concluded. At the end thesis writing was ended with short conclusion of the framed work.

BIBLIOGRAPHY

Addai ZR, Abdullah A, Mutalib SA. 2013. Effect of extraction solvents on the phenolic content and antioxidant properties of two papaya cultivars. *Journal of Medicinal Plants Research*. 7: 3354-59.

Adeosun CB, Olaseinde S, Opeifa AO and Atolani O. 2013. Essential oil from the stem bark of *Cordia sebestena* scavenges free radicals. *Journal of Acute Medicine*. 3: 138–41.

Akachi T, Shiina Y, Ohishi Y, Kawaquchi T, Kawaqishi H, Morita T *et al.* 2010. Hepatoprotective effects of flavonoids from shekwasha (*Citrus depressa*) against D-galactosamine-induced liver injury in rats. *Journal of Nutritional Science Vitaminology (Tokyo)*. 56: 60-7.

Akinmoladun AC, Ibukun EO, Afor E, Obuotor EM. 2007. Phytochemical constituent and antioxidant ectract from the leaves of *Ocimum gratissimum*. *Scientific Research and Essays*. 7: 163-6.

Alam A. 2012. Some Indian bryophytes known for their biologically active compounds. *International Journal of Applied Biology and Pharmaceutical Technology*. 3: 239–46.

Ali H, Houghton PJ, Soumyanath A. 2006. α-Amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to *Phyllanthus amarus*. *Journal of Ethnopharmacology*. 107: 449–55.

Ando H. 1983. Use of bryophytes in China 2. Mosses indispensable to the production of Chinese gallnuts. *Proceedings of Bryological Society of Japan*. 3: 124 - 5.

Andrew PJ, Mayer B. 1999. Enzymatic function of nitric oxide synthases. *Cardiovascular Research*. 43: 521–31.

Araki E, Nishikawa T. 2010. Oxidative stress: a cause and therapeutic target of diabetic complications. *Journal of Diabetes Investigation*. 1: 90–6.

Asakawa Y, Ludwiczuk A, Nagashima F. 2013. Chemical constituents of bryophytes: bio- and cchemical diversity, biological activity, and chemosystematics (Progress in the Chemistry of Organic Natural Products); Springer: Vienna, Austria; 796.

Asakawa Y, Toyota M, Taira Z, Takemoto T. 1982. Biologically active cyclic bisbenzyls and terpenoids isolated from liverworts. In: *Proceeding of the 25th Symposium on Chemistry of Natural Products*. Tokyo; 337-44.

Asakawa Y. 1990a. Biologically Active Substances From Bryophytes. In: Chopra, R.N., Bhatla, S.C. (Eds.), *Bryophyte Development: Physiology and Biochemistry*. CRC Press: Boca Raton; 259–87.

Asakawa Y. 1990b. Terpenoids and Aromatic Compounds with Pharmacological Activity from Bryophytes. In: Zinsmeister, D.H., Mues, R. (Eds.), *Bryophytes: Their Chemistry and Chemical Taxonomy*. Oxford University Press: Oxford; 369–410.

Asakawa Y. 1993. Biologically active terpenoids and aromatic com- pounds from liverworts and the inedible mushroom *Cryptoporus volvatus*. In: Colgetae, S.M., Molyneux, R.J. (Eds.), *Bioactive Natural Products-Detection, Isolation, and Structural Determination*. 319-47.

Asakawa Y. 1995. Chemical Constituents of the Bryophytes. In: Herz, W, Kirby WB, Moore RE, Steglich W, Tamm Ch. (Eds.), *Progress in the Chemistry of Organic Natural Products*, Springer: Vienna; 65: 1–618.

Asakawa Y. 2001. Recent advances in phytochemistry of bryophytes acetogenins, terpenoids and bis(bibenzyl)s from selected Japanese, Taiwanese, New Zealand, Argentinean and European liverworts. *Phytochemistry.* 56: 297–312.

Asakawa Y. 2007. Biologically active compounds from bryophytes. *Pure and Applied Chemistry*. 79: 557-80.

Asakawa Y. 2008. Liverworts- potential source of medicinal compounds. *Current Pharmaceutical Design*. 14: 3067-88.

Asakawa Y. 2011. Recent advances in phytochemistry of bryophytes-acetogenins, terpenoids and bis(bibenzyl)s from selected Japanese, Taiwanese, New Zealand, Argentinean and European liverworts. *Phytochemistry*. 56: 297-312.

Asakawa YL. 1995. Chemical constituents of the bryophytes. In: Herz W, Kirby WB, Moore RE, Steglich W, Tamm Ch. (Eds.), *Progress in the Chemistry of Organic Natural products*. Springer-Verlag: Vienna; 65: 1-618.

Asakawa, Y, Ludwiczuk A. 2008. Bryophytes–chemical diversity, bioactivity and chemosystematics. Part I. Chemical diversity and bioactivity. *Ros ́liny Lecznicze w Polsce i na S ́wiecie* (Medical Plants in Poland and in the World). 2: 33–53.

Atanassova M, Georgieva S, Ivancheva K. 2011. Total phenolic and total flavonoid contents, antioxidant capacity and biological contaminants in medicinal herbs. *Journal of the University of Chemical Technology and Metallurgy*. 46: 81-8.

Atjanasuppat K, Wongkham W, Meepowpan P, Kittakoop P, Sobhon P, Bartlett A *et al* 2009. In vitro screening for anthelmintic and antitumour activity of ethnomedicinal plants from Thailand. *Journal of Ethnopharmacology*. 123: 475–82.

Awasthi V, Asthana AK, Nath V. 2013. In- vitro study on the reproductive behavior of the endemic and threatened Indian liverwort: *Cryptomitrium himalayense* Kashyap (Aytoniaceae). *Cryptogamie Brologie*. 34: 313-23.

Awasthi V, Nath V, Asthana AK. 2012. In vitro study and micropropagation of ethnomedicinally important bryophyte: *Plagiochasma appendiculatum* Lehm. & Lindenb. *Proceeding of the National Academy of Sciences, India, Section B: Biological sciences*. 82: 405-412.

Baharum SN, Bunawan H, Ghani MA, Mustapha WAW, Noor NM. 2010. Analysis of the chemical composition of the essential oil of *Polygonum minus* Huds. Using two dimensional gas chromatography-time-of-flight mass spectrometry (GC-TOF MS). *Molecules.* 15: 7006-15.

Banerjee RD. 2001. Recent advances in the chemistry of liverworts. In: Nath V, Asthana AK, editors, *Perspectives in Indian bryology* (Proceedings National Conference on Bryology): Bishen Singh Mahendra Pal Singh. Dehra Dun: India; 171-207.

Barkman JJ. 1958. Phytosociology and ecology of cryptogamic epiphytes, including a taxonomic survey and description of their vegetation units in Europe. Van Gorcum, Assen. 628.

Barua CC, Sen S, Das AS, Talukdar A, Hazarika NJ, Barua AG, Baruah AM, Barua I. 2014. A comparative study of the in-vitro antioxidant property of different extracts of *Acorus calamus* Linn. *Journal of Natural Product and Plant Resources*. 4: 8-18.

Bavelas A. 1950. Communication patterns in task orientated groups. *Journal of the Acoustical Society of America*. 22: 271–88.

Berg JM, Tymoczko JL, Styer L. 2002. Biochemistry, 5th edition, W.H. Freeman and Company, New York.

Bergendi L, Benes L, Durackova Z, Ferencik M. 1999. Chemistry, physiology and pathology of free radicals. *Life Sciences*. 65: 1865–74.

Bhattacharya SD. 2011. Threats and conservation of liverworts in India: an overview. *Biological and Environmental Sciences*. 7: 168-72

Bhattacharya SK. 1995. Shilajit attenuates streptozotocin induced DM and decrease in pancreatic islet superoxide dismutase activity in rats. *Phytotherapy Research*. 9: 41-4.

Bielski BHJ, Cabelli DE. 1996. Superoxide and hydroxyl radical chemistry in aqueous solution. *Active Oxygen in Chemistry*. 2: 66–104.

Bilto Y. 2015. Antioxidant properties of twelve selected medicinal plants commonly used in Jordan. *British Journal of Pharmaceutical Research*. 6: 121-30.

Bouftira I, Abdelly C, Sfar S. 2007. Identification of a naturally occurring 2, 6-bis (1.dimethylethyl)-4-methylphenol from purple leaves of the halophyte plant *Mesembryanthemum crystallinum*. *African Journal of Biotechnology*. 6: 1136-9.

Burgess EJ, Larsen L, Perry NB. 2000. A cytotoxic sesquiterpene caffeate from the liverwort Bazzania novae-zelandiae. *Journal of Natural Product*. 63: 537-9.

Burritt DJ, Larkindale J, Hurd K. 2002. Antioxidant metabolism in the intertidal red seaweed *Stictosiphonia arbuscula* following desiccation. *Planta*. 215: 829–38.

Cadenas E, Sies H. 1998. The lag phase. *Free Radical Research*. 28: 601–9.

Cardoso SAN, Cavalcante TT, Araujo AX, Dos Santos HS, Albuquerque MR, Bandeira PN, Da Cunha RM, Cavada BS, Teixeira EH. 2012. Antimicrobial and antibiofilm action of Casbane Diterpene from *Croton nepetaefolius* against oral bacteria. *Archives of Oral Biology.* 57: 550-5.

Castaneda-Ovando A, Pacheco-Hernandez ML, Paez-Hernandez ME, Rodrıguez JA, Vidal GC. 2009. Chemical studies of anthocyanins: review. *Food Chemistry*. 113: 859–71.

Chandra S, Chandra D, Barh Anupam, Pankaj, Pandey RK, Sharma IP. Bryophytes: Hoard of remedies, an ethno-medicinal review. *Journal of Traditional and Complementry Medicine.* 7: 94-98.

Cheng HW, He JG, He WY. 2018. Anticancer properties of Semen Euphorbiae towards ACHN human renal adenocarcinoma cells by inducing apoptosis. *Saudi Journal of Biological Sciences*. 25: 1146-53.

Cheng X, Xiao Y, Wang X, Wang P, Li H, Yan H *et al*. 2012. Anti-tumor and pro-apoptic activity of ethanolic extract and its various fractions from *Polytrichum commune* L. Ex Hedw in L1210 cells. *Journal of Ethnopharmacology*. 143: 49–56.

Chiueh CC. 1999. Neuroprotective properties of nitric oxide. *Annals of the New York Academy of Sciences*. 890: 301–11.

Chobot V, Kubicova L, Nabbout S, Jahodar L, Hadacek F. 2008. Evaluation of antioxidant activity of some common moss species. *Zeitschrift fur Naturforschung C.* 63: 476–82.

Cosa P, Vlietinck A J, Berghe D V, Maes L. 2006. Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'. *Journal of Ethnopharmacology*. 106: 290–302.

Cowan MM. 1999. Plant products as antimicrobial agents. *Clinical Microbiology Review.* 12: 564–82.

Cushnie TP, Lamb AJ. 2005. Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents*. 26: 343-56.

de Souza RF, De Giovani WF. 2004. Antioxidant properties of complexes of flavonoids with metal ions. *Redox Report*. 9: 97–104.

Dean RT, Gieseg S, Davies MJ. 1993. Reactive species and their accumulation on radical damaged proteins. *Trends in Biochemical Sciences.* 18: 437–41.

Denizot F, Lang R. 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *Journal of Immunological methods.* 89: 271-7.

Deora GS, Suhalka D. 2017. Evaluation of bryophyte for green fungicides as alternative treatment to control plant pathogen. *International Journal of Pharmacognosy and Phytochemical Research*. 9: 1373-9.

Dey A, De A, Ghosh P, Mukherjee S. 2013. Altitude and tissue type influence antioxidant potential of *Pellia endiviifolia* from Darjeeling Himalaya. *Pakistan Journal of Biological Sciences*. 13: 707-11.

Dey A, De JN. 2012. Antioxidative potential of bryophytes stress tolerance and commercial perspectives: a review. *Pharmacologia*. 3: 151–59.

Di Mascio P, Kaiser S, Sies H. 1989. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Archives of Biochemistry and Biophysics*. 274: 532–8.

Ding H, 1982. Zhong guo Yao Yun Bao zi Zhi Wu. Kexue Jishu Chuban She: Shanghai; 1–409.

Dinis TCP, Madeira VM, Almeida LM. 1994. Action of phenolic derivates (acetoaminophen, salicylate and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Archives of Biochemistry Biophysics*. 315:161-9.

Dowling S, Regan F, Hughes H. 2010. The characterisation of structural and antioxidant properties of isoflavone metal chelates. *Journal of Inorganic Biochemistry*. 104: 1091-8.

Dulger B, Ugurlu E, Aki C, Suerdem TB, Camdeviren A, Tazeler G. 2005. Evaluation of Antimicrobial Activity of Some Endemic Verbascum., Sideritis., and Stachys. *Species from Turkey*. 43: 270-4.

Ejele AE, Iwu IC, Enenebeaku CK, Ukiwe IN, Okolue BN. 2012. Bioassay guided isolation, purification and partial characterization of antimicrobial compound from basic metabolite of *Garcinia kola*. *Journal of emerging Trends in Engineering and Applied Sciences*. 4: 668-72.

Erhardt JG, Meisner C, Bode JC, Bode C. 2003. Lycopene, β-carotene, and colorectal adenomast. *The American Journal of Clinical Nutrition*. 78: 1219–24.

Escudier B, Eisen T, Porta C, Patard JJ, Khoo V, Algaba F *et al*. 2012. Renal cell carcinoma: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Annals of Oncology*. 23: 65-71.

Ferhat R, Lekbir A, Ouadah H, Kahoul MA, Khalfa L, Laroui S et al. 2017. Effect of extraction solvent on total phenolic content, total flavonoid content, and antioxidant activities of Algerian pomace olive oil. *International Food Research Journal*. 24: 2295-303.

Fernando TM, Cristina E, Isabel M, Adriaan E. 2008. Are soil lichen communities structured by biotic interactions? A null model analysis. *Journal of Vegetation Science*. 19: 261–6.

Fidyt K, Fiedorowicz A, Strzadala L, Szumny A. 2016. β-caryophyllene and β-caryophyllene oxide- natural compounds of anticancer and analgesic properties. *Cancer Medicine*. 5: 3007-17.

Freeman LC. 1977. A set of measures of centrality based on betweenness. *Sociometry*. 40: 35-41.

Freeman LC. 1979. Centrality in Networks: I. Conceptual Clarification. *Social Networks*. 1: 215-39.

Friedel A, Oheimb GV, Dengler J, Hardtle W. 2006. Species diversity and species composition of epiphytic bryophytes and lichens – a comparision of managed and unmanaged beech forests in NE Germany. *Feddes Repertorium*. 117: 172-85.

Fu P, Lin S, Shan L, Lu M, Shen YH, Tang J *et al*. 2012. Constituents of the moss *Polytrichum commune*. *Journal of Natural Products*. 72: 1335–7.

Fu W, Chen J, Cai Y, Lei Y, Chen L, Pei L *et al*. 2010. Antioxidant, free radical scavenging, anti- inflammatory and hepatoprotective potential of the extract from *Parathelypteris nipponica* (Franch. et Sav.) Ching. *Journal of Ethnopharmacology*. 130: 521-8.

Gaff DF. 1997. Mechanisms of desiccation tolerance in resurrection vascular plants. In: Basra AS, Basra RK, eds. *Mechanisms of environmental stress resistance in plants*. Harwood Academic Publishers: Netherlands; 43-58.

Gaikwad SA, Kamble GS. Devare S, Deshpande NR, Salvekar JP. 2011. *In vitro* evaluation of free radical scavenging potential of *Cassia auriculata* L. *Journal of Chemical and Pharmaceutical Research.* 3: 766-772.

Ghafourifar P, Cadenas, E. 2005. Mitochondrial nitric oxide synthase. *Trends Pharmacological Sciences*. 26: 190–5.

Ghasemzadeh A, Jaafar HZE, Rahmat A. 2011. Effects of solvent type on phenolics and flavonoids content and antioxidant activit5ies in two varieties of young ginger (Zingiber officinale Roscoe) extracts. *Journal of Medicinal Plants Research*. 5: 1147-54.

Ghosal S, Singh SK, Srivastava RS. 1988. Shilajit part 2. Biphenyl metabolites from *Trifolium repens*. *Journal of Chemical Research*. 196: 165-66

Glime JM. 2007. Economic and Ethnic Uses of Bryophytes. In: *Flora of North America*. New York & Oxford; 27: 14- 41.

Gokbulut A, Satilmis B, Batcioglu K, Cetin B, Sarer E. 2012. Antioxidant activity and luteolin content of *Marchantia polymorpha* L. *Turkish Journal of Biology*. 36: 381–85.

Gomathi D, Kalaiselvi M, Uma C. 2012. In vitro α-amylase and α-glucosidase inhibitory effects of ethanolic extract of *Evolvulus alsinoides* (L.). *International Research Journal of Pharmacy*. 3: 226–29.

Gough DR, Cotter TG. 2011. Hydrogen peroxide: a Jekyll and Hyde signalling molecule. 2: e213.

Gulcin I, Oktay M, Kirecci E, Kufrevioglu OI. 2003. Screening of antioxidant and antimicrobial activities of anise (*Pimpinella anisum* L.) seed extracts. *Food Chemistry.* 83: 371–82.

Gulcin I. 2009. Antioxidant activity of L-adrinaline: an activity structure insight. *Chemico-Biological Interaction*. 179: 71-80.

Gunatilakaa AAL, Kingston DGI. 1997. DNA-damaging natural products with potential anticancer activity. *Studies in Natural Products Chemistry*. 20: 457-505.

Gupta SS. 1966. Effect of Shilajit, *Ficus bengalensis* & ant. Pituitary extract on glucose tolerance in rats. *Indian Journal of Medical Research*. 54: 354-66.

Hadjeri M , Peiller EL , Beney C , Deka N , Lawson MA , Dumontet C *et al*. 2004. Antimitotic activity of 5-hydroxy-7-methoxy-2-phenyl-4-quinolones. *Journal of Medicinal Chemistry*. 47: 4964-70.

Halliwell B, de Rycker J. 1978. Superoxide and peroxidase-catalysed reactions. Oxidation of dihydroxyfumarate, nadh and dithiothreitol by horseradish peroxidase*. *Photochemistry and Photobiology*. 28: 757-62.

Halliwell B, Gutteridge JM. 1990. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods in Enzymology.* 186: 1–85.

Halliwell B, Gutteridge JMC. 1999. Free radicals in biology and medicine (3rd edn). Oxford University Press: Oxford; 1-25

Harborne JB. 1998. Textbook of phytochemical methods (5th edn). A Guide to Modern Techniques of Plant Analysis. Chapman and Hall Ltd: London, UK; 21-72

Harinantenaina L, Asakawa Y. 2007. Malagasy liverworts, source of new and biologically active compounds. *Natural Product Communications*. 2: 701–9.

Harman-Ware AE, Sykes R, Peter GF, Davis M. 2016. Determination of terpenoid content in pine by oraganic solvent extraction and Fast-GC analysis. *Frontiers in Energy*. 4: 1-9.

Hasan SZ, Misra V, Singh S, Arora G, Sharma S, Sharma S. 2009. Current status of herbal drugs and their future perspectives. *Biological Forum*. 1: 12-7.

Hettenbergerova E, Hajek M, Zeleny D, Jirouskova J, Mikulaskova E. 2013. Changes in species richness and species composition of vascular plants and bryophytes along a moisture gradient. *Preslia*. 85: 369-88.

Ho YL, Huang WSS, Deng JS, Lin YH, Chang YS, Huang GJ. 2012. In-vitro antioxidant properties and total phenolics contents of wetland medicinal plants in Taiwan. *Botanical Studies*. 53: 55-66.

Hsiao G, Teng CM, Wu CL, Ko FN. 1996. Marchantin H as a natural antioxidant and free radical scavenger. *Archives of Biochemistry and Biophysics*. 334: 18–26.

Huang WJ, Wu CL, Lin CW, Chi LL, Chen PY, Chiu CJ *et al*. 2010. Marchantin A, a cyclic bis(bibenzyl ether), isolated from the liverwort *Marchantia emarginata* sub sp. *tosana* induces apoptosis in human MCF-7 breast cancer cells. *Cancer Letters*. 291: 108–19.

Ibrahim HA, Ibrahim H. 2009. Phytochemical screening and toxicity evaluation on the leaves of *Argemone mexicanalinn* (Papararaceal). *International Journal of Pure and Applied Science and Technology*. 3: 39-43.

Ilhan S, Savaroglu F, Colak F, Filik-Iseen C, Erderngil FZ. 2006. Antimicrobial activities of *Palustriella commutate* (Hedw.) ochyra extracts (Bryophyta). *Turkish Journal of Biology*. 30: 149–52.

Ivanova V, Kolarova K, Domberger KJ, Heartl A, Moellmann U, Dahse HM, Chipev N. 2007. Sanionins: anti-inflammatory and antibacterial agents with weak cytotoxicity from the Antartic moss *Sanionia georgico-uncinata*. *Preparative Biochemistry and Biotechnology*. 37: 343–52.

Jackson ML. 1973. Soil chemical analysis. Prentice Hallof India Pvt. Ltd.:New Delh; 1-498.

Jacob RA. 1995. The Integrated Antioxidant System. *Nutrition Research*. 15: 755-66.

Jakovlev V, Thiemer K, Kunde R. 1979. Pharmacological investigations with compounds of chamomile. II. New investigations on the antiphlogistic effects of (-)bisabolol and bisabolol oxides. *Plant Medica*. 35: 125-40.

Jassbi AR, Mirzaei Y, Firuzi O, Chandran JN, Schnneider B. Bioassay guided purification of cytotoxic natural products from red alga *Dichotomaria obtusata*. *Brazilian Journal of Pharmacognosy*. 26: 705-9.

Jayaprakasha GK, Selvi T, Sakariah KK. 2003. Antibacterial and antioxidant activities of grape (*Vitis vinifera*) seed extracts. *Food Research International*. 36: 117–22.

Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. 2009. Cancer statistics. *CA: A Cancer Journal for Clinicians*. 59: 225-249.

Jian-Bo X, Feng-Lian R, Xu M. 2005. Anti-Hepatitis B Virus Activity of Flavonoids from *Marchantia convoluta*. *Indian Journal of Pharmacology and Therapeutics*. 4: 128-31.

Joshi GG, Tewari KC, Pande NK, Pande G. 1994. Bryophyte, the source of the origin of Shilajit- a new hypothesis. *Bulletin of Medico-Ethno Botanical Research*. 15: 106-111.

Kadam PS. 2017. Phytochemical screening and antibacterial activity of bryophytes. *International Journal of Life Science*. 5: 405-8.

Kadam VB, Momin RK, Fatima S, Kadam UB. 2013. Estimation of total phenol in different plant parts of genus Sesbania in Maharastra. *International Journal of Pharmaceutical Research and Bio-Science*. 2: 202-6.

Kaidanovich O, Eldar-Finkleman H. 2002. The role of glycogen synthase kinase-3 in insulin resistance and type 2 diabetes. *Expert Opinion Therapeutic Targets*. 6: 555-61.

Khanam R, Chaudhary BL, Khanam S, Kumar P. 2011. Antibacterial activity of *Marchantia palmate*. *Asian Journal of Biochemical and Pharmaceutical Research*. 22: 2231-560.

Khiev P, Oh SR, Chae HS, Kwon OK, Ahn KS, Chin YW, Lee HK. 2011. Anti-inflammatory diterpene from *Thyrsanthera suborbicularis*. *Chemical Pharmaceutical Bulletin (Tokyo)*. 59: 382-4.

Kim DH. 2001. Factors controlling natural VOC emissions in a southeastern US pine forest. *Atmospheric Environment*. 35: 3279-92.

Kim YM, Jeong YK, Wang MH, Lee W, Rhee H. 2005. Inhibitory effect of pine extract on α-glucosidase activity and postprandial hyperglycemia. *Nutrition*. 21: 756-61.

Klavina L, Springe G, Nikolajeva V, Martsinkevich I, Nakurte I, Dzabijeva D, Steinberga I. 2015. Chemical composition analysis, antimicrobial activity and cytotoxicity screening of moss extract (Moss Phytochemistry). *Molecules*. 20: 7221- 43.

Klavina, L. 2014. Polysaccharides from Lower Plants: Bryophytes. In: Ramawat KG, Merillon JM, Eds. *Polysaccharides, Bioactivity and Biotechnology*. Cham: Springer International Publishing: Switzerland; 1-14.

Kletzien RF, Clarke SD, Ulrich RG. 1992. Enhancement of adipocyte differentiation by an insulin-sensitizing agent. *Molecular Pharmacology*. 41:393–8.

Kokpol U, Chittawong V, Miles DH. 1984. Chemical constituents of the roots of *Acanthus ilicifolius* and biological activity .*Journal of Natural Product*. 49:355-7.

Koleva II, Van Beek TA, Linssen JPH, de Groot A, Evstatieva LN. 2002. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. *Phytochemical Analysis*.13: 8-17.

Komala I, Ito T, Nagashima F, Yagi Y, Asakawa Y. 2010. Cytotoxic, radical scavenging and antimicrobial Activities of sesquiterpenoids from the Tahitian liverwort *Mastigophora diclados* (Brid.) Nees (Mastigophoraceae). *Journal of Natural Medicines*. 64: 417–22.

Kranner I, Lutzoni F. 1999. Evolutionary consequences of transition to a lichen symbiotic state and physiological adaptation to oxidative damage associated with poikilohydry. In: Lerner HR, ed. *Plant responses to environmental stresses. From phytohormones to genome reorganization*. Marcel Dekker: New York, NY, USA; 591 -628.

Kris-Etherton PM, Hecker KD, Bonanome A, Coval SM, Binkoski AE, Hilpert KF, Griel AE, Etherton TD. 2002. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *American Journal of Medicine.* 113: 71– 88.

Krishnan R, Murugan K. 2013. In-vitro anticancer properties of flavonoids extracted from cell suspension culture of *Marchantia linearis* Lehm & Lindenb. (Bryophyta) against SW 480 colon cancer cell lines. *Indo American Journal of Pharmaceutical Research*. 3: 1437-72.

Krishnan R, Murugan K. 2014a. Axenic culture of bryophytes: a case study of liverwort *Marchantia linearis* Lehm. & Lindenb. *Indian Journal of Biotechnology*. 13: 131-5.

Krishnan R, Murugan K. 2014b. Comparison of GC-MS analysis of phytochemicals in the ethanolic extracts of *Marchantia linearis* Lehm & Lindenb. and *Marchantia polymorpha* L. (Bryophyta). *International Journal of Pharmaceutical Sciences and Research.* 5: 1981-7.

Ku KM, Kim HS, Kim SK, Kang YH. 2014. Correlation analysis between antioxidant activity and phytochemicals in Korean colored corns using Principal Component Analysis. *Journal of Agricultural Science.* 6: 1-9.

Kumar A, Ilavarasan R, Jayachandan T, Decaraman M, Aravindhan P *et al*. 2009. Phytochemical investigation on a tropical plant, *Syzygiumcuminifrom kattuppalayam*, Erode District, Tamil Nadu, South India. *Pakistan Journal of Nutrition*. 8: 83-5.

Kumar K, Singh KK, Asthana AK, Nath V. 2000. Ethnotherapeutics of bryophyte *Plagiochasma appendiculatum* among the Gaddi Tribes of Kangra valley, Himachal Pradesh, India. *Pharmaceutical Biology*. 38: 353-56.

Kumari M, Jain S. 2015. Screening of Potential Sources of Tannin and Its Therapeutic Application. *World Acadamy of Science, Engineering and Technology*. 9: 820-3.

Kumari M. 2013. Phytochemical screening and antioxidant activity of in-vitri grown plants *Clitoria ternatea* L., using DPPH assay. *Asian Journal of Pahrmaceutical and Clinical Research*. 6: 38-42.

Kuppusamy P, Zweier JL. 1989. Characterization of free radical generation by xanthine oxidase. Evidence for hydroxyl radical generation. *Journal of Biological Chemistry*. 264: 9880–4.

Kuppuswamy KM, Jonnalagadda B, Arockiasamy S. 2013. GC-MS analysis of chloroform extract of *Croton bonplandianum*. *International Journal of Pharma and Bio Science*. 4: 613-7.

Lani R, Hassandarvish P, Shu MH, Phoon WH, Chu JJ. Higgs S *et al*. 2016. Antiviral activity of selected flavonoids against Chikungunya virus. *Antiviral Research*. 133: 50-61.

Lappas M. 2014. GSK3β is increased in adipose tissue and skeletal muscle from women with gestational diabetes where it regulates the inflammatory response. *Plos 1*. DOI:10.1371/journal.pone.0115854.

Laranjinha J, Vieira O, Madeira V, Almeida L. 1995. Two related phenolic antioxidants with opposite effects on vitamin E content in low density lipoproteins oxidized by ferrylmyoglobin: consumption versus regeneration. *Archives of Biochemistry and Biophysics*. 323: 373-81.

Larson RA. 1988. The antioxidants of higher plants. *Phytochemistry*. 27: 969-78.

Li XC, Wu XT, Huang L. 2009. Correlation between antioxidant activities and phenolic contents of Radix *Angelicae sinensis* (Danggui). *Molecules*. 14: 5349-61.

Liu S, Lin J, Wang C, Chen H, Yang D. 2009. Antioxidant properties of various solvent extracts from lychee (*Litchi chinenesis* Sonn.) flowers. *Food Chemistry*. 114: 577-81.

Lobel S, Snall T, Rydin H. 2006. Species richness patterns and metapopulation processes – evidence from epiphyte communities in boreonemoral forests. *Ecography*. 29:163–82.

Lorimeres SD, Perry NB, Tangney RS. 1993. An antifungal bibenzyl from the New Zealand liverwort, *Plagiochila stephensoniana*. Bioactivity- directed isolation synthesis and analysis. *Journal of Natural Products*. 56: 1444-50.

Lu ZQ, Fan PH, Ji M, Lou HX. 2006. Terpenoids and bisbibenzyls from Chinese liverworts *Conocephalum conicum* and *Dumortiera hirsuta*. *Journal of Asian Natural Products Research*. 8: 187-92.

Lubaina AS, Meenu Krishnan VG, Murugan K. 2013. Induction of oxidative stress and antioxidative response mechanisms in *Octoblepharum albidum* HEDW. a bryophyte under desiccation – rehydration stress. *Indian Journal of Plant Sciences*. 2: 12-22.

Lukaszkiewicz J, Kosmala M. 2008. Determining the age of streetside trees with diameter at breast height based mulifactorial model. *Arboculture and Urban Foresty*. 34: 137-43.

Mackawy AMH, Ahmed EA, Badawy MEH. 2015. Association of protein tyrosine phosphatase 1B (PTPN1) gene polymorphisms (1023C>A and 467T>C) with Type 2 Diabetes: A case control study. 2016. *Journal of Clinical and Medical Genomics*. 315: DOI: 10.4172/2472-128X.1000135.

Madhu M, Sailaja V, Satyadev TNVSS, Satyanarayana MV. 2016. Quantitative phytochemical analysis of selected medicinal plant species by using various organic solvents. *Journal of Pharmacognosy and Phytochemistry*. 5: 25-9.

Madsen GC, Pates AL. 1952. Occurrence of antimicrobial substances in chlorophyllose plants growing in Florida. *Botanical Gazette*. 113: 293-300.

Mahadevan A, Sridhar R. 1982. Methods in physiological plant pathology. Sivakami Publication: Chennai; 1-316.

Manivannan A, Soundararajan P, Park YG, Jeon, BR. 2015. In vitro propagation, phytochemical analysis, and evaluation of free radical scavenging property of *Scrophularia kakudensis* Franch tissue extracts. *Hindawi Publishing Coporation*. 2015: 1-11.

Manoj GS, Krishnan R, Murugan K. 2012. *In vitro* antioxidant potentiality and total phenolic content in selected bryophytes. *Asian Pacific Journal of Tropical Biomedicine*. 2: 1–8.

Manoj GS, Murugan K. 2012a. Phenolic profiles, antimicrobial and antioxidant potentiality of methanolic extract of a liverwort, *Plagiochila beddomei* Steph. *Indian Journal of Natural Products and Resources*. 3: 173-83.

Manoj GS, Murugan K. 2012b. Wound healing potential of aqueous and methanolic extracts of *Plagiochila beddomei* steph. – a bryophyte. *International Journal of Pharmacy and Pharmaceutical Sciences.* 4: 222-7.

Marcocci L, Packer L, Droy-Lefaix MT, Sekaki A, Grades-Albert M. 1994. Antioxidant action of *Ginkgo biloba* extracts EGb 761. *Methods in Enzymology*. 234: 462-75
.
Maritim AC, Sanders RA, Watkins JB. 2003. Diabetes, oxidative stress, and antioxidants: a review. *Journal of Biochemical* and *Molecular Toxicology*. 17: 24–38.

Mayaba N, Minibayeva F, Beckett RP. 2002. An oxidative burst of hydrogen peroxide during rehydration following desiccation in the moss *Atrichum androgynum*. *New Phytologist*. 155: 275-83.

McGee GG and Kimmerer RW. 2002. Forest age and management effects on epiphytic bryophyte communities in Adirondack, northern hardwood forests, New York, U.S.A. *Canadian Journal of Forest Research*. 32: 1562–76.

McIntyre M, Bohr DF, Dominiczak AF. 1999. Endothelial function in hypertension. *Hypertension*. 34: 539–45.

Meir S, Kanner J, Akiri B, Philosoph-Hadas S. 1995. Determination and involvement of aqueous reducing compounds in oxidative defense systems of various senescing leaves. *Journal of Agricultural and Food Chemistry*. 43: 1831-9.

Mezaka A and Znotiņa V. 2006. Epiphytic bryophytes in old growth forests of slopes, screes and ravines in north-west Latvia. *Acta Universitatis Latviensis*. 710: 103–16.

Mezaka A, Guntis B, Piterans A. 2008. The distribution of epiphytic bryophyte and lichen species in relation to phorophyte characters in Latvian natural old-growth broad leaved forests. *Folia Cryptogamica Estonica, Fascicles*. 44: 89-99.

Michelson AM, McCord JM, Fridovich I. 1977. Superoxide and Superoxide Dismutases. Academic Press: London; 1-320.

Miller DM, Buettner GR, Aus SD. 1990. Transition metals as catalysts of "autoxidation" reactions. *Free Radical Biology and Medicine*. 8: 95–108.

Mirza MA, Alam MN, Faiyazuddin M, Mahmood D, Bairwa R, Mustafal G. 2010. Shilajit: An ancient panacea. *International Journal of Current Pharmaceutical Review and Research*. 1: 3-11.

Mittermeier RA, Gil PR, Mittermeier CG. 1997. Megadiversity: Earth's Biologically Wealthiest Nations. Conservation International, Cemex: Mexico City.

Mohan N, Jassal PS, Kumar V, Singh RP. 2011. Comparative in-vitro and in-vivo study of antioxidants and phytochemical content in *Bacopa monnieri*. *Recent Research in Science and Technology*. 3: 78-83.

Mohsen SM, Ammar ASM. 2009. Total phenolic contents and antioxidant activity of corn tassel extracts. *Food Chemistry*. 112:595-8.

Mousinho NMHDC, Tonder JJV, Vanessa S. 2013. In vitro anti-diabetic activity of *Sclerocarya birrea* and *Ziziphus mucronata*. *Natural Product Communications*. 8: 1279-84.

Mukhopadhyay ST, Mitra S, Biswas A, Das N, Poddar-Sarkar M. 2013. Screening of antimicrobial and antioxidative potential of selected Eastern Himalayan Mosses. *European Journal of Medicinal Plant*. 3: 422–28.

Nagashima F, Kondoh M, Kawase M, Simizu S, Osada H, Fujii M *et al.* 2003. Apoptosis-inducing properties of ent-kaurene-type diterpenoids from the liverwort *Jungermannia truncata*. *Planta Medica*. 69: 377-79.

Nagashima F, Nondo M, Uematsu T, Nishiyama A, Sato S, Sato M, Asakawa Y. 2002. Cytotoxic and apoptosis-inducing ent-kaurane-type diterpenoids from the Japanese liverwort *Jungermannia truncata* Nees. *Chemical and Pharmaceutical Bulletin*. 50: 808-13.

Nandy S, Paul HS, Barman NR, Chakraborty B. 2012. *In vitro* evaluation of antioxidant activity of *Leucas plukenetii* (Roth) Spreng. *Asian Journal of Plant Science and Research.* 2:254–62.

Neves M, Ferreira M, Terreaux C, Hostettmann K, Morais R. 2001. Phytochemical profiles of *Targionia lorbeeriana*: grown in vitro and in the open. *Zeitschrift für Naturforschung C*. 56: 726-30.

Ngbede J, Yakubu RA, Nyam DA. 2008. Phytochemical screening for active compound in *Canaruim schweinfurthii* (Atile) leaves from Ios North, Plateau state, Nigeria. *Research Journal of Biological Science*. 3: 1076-78.

Ngo TV, Scarlett CJ, Bowyer MC, Ngo PD, Vuong QV. 2017. Impact of different extraction solvents on bioactive compounds and antioxidant capacity from the root of *Salacia chinensis* L. *Journal of Food Quality*. 2017: 1-8.

Niedowicz D, Daleke D. 2005. The role of oxidative stress in diabetic complications. *Cell Biochemistry and Biophysics*. 43: 289-330.

Nikolova M, Petrova M, Zayova E, Vitkova A, Evstatieva L. 2013. Comparative study of in vitro, ex vitro and in vivo grown plants of *Arnica montana* – polyphenols and free radical scavenging activity. *Acta Botanica Croatica*. 72: 13-22.

Niu C, Qu JB, Lou HX. 2006. Antifungal bisbibenzylsfrom the Chinese liverwort *Marchantia polymorpha*. *Chemistry & Biodiversity.* 3: 34–40.

Nordberg J, Arner ES. 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radicical Biology and Medicine.* 31: 1287–312.

Nuryanti S, Puspitasari DJ. 2017. Screening of metabolites secondary compounds in extract of moringa fruit and determination of inhibitory effect on growth of the fungus *Candida albicans*. *AIP Conference Proceedings*. 1868: doi.org/10.1063/1.4995092.

Ode OJ, Asuzu IU, Ajayi LE. 2011. Bioassay guided fractionation of the crude methanol extract of *Cassia singueana* leaves. *Journal of Advanced Scientific Research*. 2: 81-6.

Odor P, Van Hees ADFM. 2004. Preferences of dead wood inhabiting bryophytes for decay stage, log size and habitat types in Hungarian beech forests. *Journal of Bryology*. 26: 79-95.

Oh C, Li M, Kim EH, Park JS, Lee JC, Ham SW. 2010. Antioxidant and radical scavenging activities of ascorbic acid derivatives conjugated with Organogermanium. *Bulletin of Korean Chemical Society*. 12: 3513-14.

Ohta Y, Katoh K, Takeda R. 1990. Growth and secondary metabolites in cultured cells of liverworts. In: Chopra RN, Bhatla SC (eds) Bryophyte development: physiology and biochemistry. CRC Press, Boston. 209–223.

Ojo OO, Ajayi AO, Anibijuwon II. 2007. Antibacterial potency of methanol extracts of lower plants. *Journal Zhejiang University Sciences B.* 8: 189–91.

Oki T, Masuda M, Furuta S, Nishibia Y, Terahara N, Suda I. 2002. *Food and Chemical Toxicology*. 67: 1752-6.

Oliver MJ, Tuba Z, Mishler BD. 2000. The evolution of vegetative desiccation tolerance in land plants. *Plant Ecology*. 151: 85-100.

Orjan F, Mats N, Churski M. 2009. Tree age is a key factor for the conservation of epiphytic lichens and bryophytes in beech forests. *Applied Vegetation Science*. 12: 93-106.

Packer L, Witt EH. 1995. Antioxidant Properties and Clinical Implications of Alpha-Lipoic Acid. In Packer L, Cadenas E. eds. *Biothionls in Health and Disease*. New York: Marcel Dekker, Inc; 479-516.

Pande SK. 1958. Some aspects of Indian Hepaticology (Presidential address). *Journal of the Indian Botanical Society.* 15: 221–233.

Panichayupakarananta P, Issuriya A, Sirikatitham A, Wang W. 2010. Antioxidant assay guided purification and LC determination of ellagic acid in pomegranate peel. *Journal of Chromatographic Science*. 1125: 204-10.

Pansera MR, Iob GA, Atti-Santos AC, Rossato M, Atti-Serafini L, Cassel E. 2004. Extraction of tannin by *Acacia mearnsii* with supercritical fluids. *Brazilian Archives of Biology and Technology*. 47: 995-8.

Pant G, Kumar G, Karthik L, Gyana Prasuna R, Bhaskara Rao KV. 2011. Antioxidant activity of methanolic extract of blue green algae *Anabaena* sp. (Nostocaceae). *European Journal of Experimental Biology*. 1: 156-62.

Pant GB , Tewari SD, Joshi S. 1992. An assessment of vanishing rare bryophytes in Kumaon Himalaya- thalliod liverworts. *The Bryological Times*. 68: 8-10.

Pant GB, Tewari SD, Joshi S.1994. Vanishing greenery in Kumaon Himlaya: observation on bryoflora. *Geophytology*. 23: 253-57.

Pant GB, Tewari SD.1989. Various human uses of bryophytes in the Kumaun region of Northwest Himalaya. *The Bryologist*. 92: 120-22.

Parul R, Kundu SK, Saha P. 2013. In Vitro nitric oxide scavenging activity of methanol extracts of three Bangladeshi medicinal plants. *The Pharma Innovation – Journal*. 12: 83-8.

Pejin B, Bogdanovic-Pristov J. 2012. ABTS Cation scavenging activity and total phenolic content of three moss species. *Hemijska Industrija.* 66: 723–26.

Perry NB, Burgess EJ, Baek SH, Weavers RT, Geis W, Mauger AB. 1999. 11-oxygenated cytotoxic 8,9-secokauranes from a New Zealand liverwort, *Lepidolaena taylorii*. *Phytochemistry*. 50: 423-33.

Perry NB, Burgess EJ, Tangney RS. 1996. Cytotoxic 8,9-secokaurane diterpenes from a New Zealand liverworts, *Lepidolaena taylorii*. *Tetrahedron Letters*. 37: 9387-90.

Perry NB, Foster LM, Lorimer SD, May BC, Weavers RT. 1996. Isoprnyl phenyl ethers from liverworts of the genus Trichocolea: cytotoxic activity, structural corrections, and synthesis. *Journal of Natural Product*. 59: 729-33.

Pham-Huy LA, He H, Pham-Huy C. 2008. Free radicals, antioxidants in disease and health. *International Journal of Biomedical Science*. 4: 89-96.

Phillips M, Cataneo RN, Cheema T, Greenberg J. 2004. Increased breath biomarkers of oxidative stress in diabetes mellitus. *Clinica Chimica Acta*. 344: 189-94.

Pietta PG. 2000. Flavonoids as antioxidants. *Journal of Natural Products*. 63: 1035-42.

Qusti SY, Abo-Khatwa AN, Bin Lahwa MA. 2010. Screening of antioxidant activity and phenolic content of selected food items cited in the Holly Quran. *European Journal of Biological Sciences*. 2: 40-51.

Rado MC, Rios JL, Villar A. 1989. A review of some antimicrobial compounds isolated from medicinal plants. *Phytotherapy Research.* 3: 117–25.

Rai MV, Pai VR, Kedilaya HP, Hedge S. 2013. Preliminary phytochemical screening of members of Lamiaceae family: *Leucas linifolia, Coleus aromaticus* and *Pogestemon patchouli*. *International Journal Pharmaceutical Sciences Review and Research*. 21: 131-7.

Rebaya A, Belghith SI, Baghdikian B, Leddet VM, Mabrouki F, Oliver E et al. 2015. Total phenolic, total flavonoids, tannin content and antioxidant capacity of *Halimium halimifolium* (Cistaceae). *Journal of Applied Pharmaceutical Science.* 5: 52-57.

Remesh R, Manju CN. 2009. Ethnobryological notes from Western Ghats, India. *The Bryologist*. 112: 532- 37.

Revathi D, Rajeswari M. 2015. In vitro evaluation of nitric oxide scavenging activity of *Guettarda speciosa* Linn. *International Journal of Science and Research*. 4: 962-5.

Ribeiro AB, Bonafe EG, Silva BC, Montanher PF, Junior OOS, Boeing JS *et al.* 2013. Antioxidant capacity, total phenolic content, fatty acids and correlation by Principal component analysis of exotic and native fruits from Brazil. *Journal of the Brazilian Chemical Society*. 24: 797-804.

Rinaldi MVN, Diaz IEC, Suffredini IB, Moreno PRH. 2017. Alkaloids and biological activity of beriba (*Annona nypoglauca*). *Brazillian Journal of Pharmacognosy*. 27: 77-83.

Robards K, Prenzler PD, Tucker, G, Swatsitang P, Glover W. 1999. Phenolic compounds and their role in oxidative processes in fruits. *Food Chemistry*. 66: 401-36.

Rodrigues-Correa KCS, Lima JC, Fett-Neto AG. 2013. Pine oleoresin: tapping green chemicals, biofuels, food protection, and carbon sequestration from multipurpose trees. *Food and Energy Security*. 1: 81-93.

Rodriguez-Amaya DB. 2010. Quantitative analysis, in vitro assessment of bioavailability and antioxidant activity of food carotenoids—a review. *Journal of Food Composition and Analysis*. 23: 726-40.

Ross SA, Gulve EA, Wang M. 2004. Chemistry and biochemistry of type 2 diabetes. *Chemical Reviews*. 104: 1255–82.

Russo A, Acquaviva R, Campisi A, Sorrenti V, Giacomo CD, Virgata G *et al.* 2000. Bioflavonoids as antiradicals, antioxidants and DNA cleavage protectors. *Cell Biology and Toxicology*. 16: 91–8.

Sabovljevic A, Sabovljevic M. 2008. Bryophytes, a source of bioactive and new compounds. In: Govil JN, Singh VK (eds.). Phytopharmacology and Therapeutic Values IV, the Series "Recent Progress in Medicinal Plants". *Studium Press LLC, USA*, 9–25.

Sabovljevic A, Sokovic M, Glamoclija J, Ciric A, Vujicic M, Pejin B, Sabovljevic M. 2011. Bio-activities of extracts from some axenically framed and naturally grown bryophytes. *Journal of Medicinal Plants Research*. 5: 565-71.

Sadamori M. 2009. Studies on the new biologically active substances of Tahitian and Tokushima's Plagiochila genus, Master thesis. Tokushima Bunri University: Tokushima, Japan; 1–70.

Saeed N, Khan MR, Shabbir M. 2012. Antioxidant activity, total phenolic and total flavonoids contents of whole plant extracts *Torilis leptophylla* L. *Complementary and Alternative Medicine*. doi: 10.1186/1472-6882-12-221

Saha J, Sarkar PK, Chattopadhyay S. 2011. A survey of ethnomedicinal plants of Darjeeling hills for their antimicrobial and antioxidant activities. *Indian Journal of Natural Products and Resources*. 24(2): 478–92.

Sahoo S, Ghosh G, Das D, Nayak S. 2013. Phytochemical investigation and in vitro antioxidant activity of an endogenous medicinal plant *Alpinia nigra* B.L, Burtt. *Asian Pacific Journal Tropical Biomedicine*. 3: 871-6.

Saravanam S, Parimelazhagan T. 2014. In-vitro antioxidant, antimicrobial and anti-diabetic properties of polyphenols of *Passiflora ligularis* Juss. fruit pulp. *Food Science Human Wellness*. 3: 56-64.

Sauerwein M, Becker H. 1990. Growth terpenoid production and antibacterial activity of an in vitro culture of a liverwort *Fossombronia pusilla*. *Planta Medica*. 56: 364- 7.

Savaroglu F, Ilhan S, Filik-Iscen C. 2011. An evaluation of the antimicrobial activity of some Turkish mosses. *Journal of Medicinal Plants Research*. 5: 3286–92.

Savluchinske-Feio S, Curto M, Gigante B, Roseiro JC. 2006. Antimicrobial activity of resin acid derivatives. *Applied Microbiology and Biotechnology*. 72: 430-6.

Scher JM, Burgess EJ, Lorimer SD, Perry NB. 2002. A cytotoxic sesquiterpene and unprecedented sesquiterpene-bisbibenzyl compounds from the liverwort *Schistochila glaucescens*. *Tetrahedron*. 58: 7875-82.

Schumacher A. 2000. Mosses in Central European beech forests: their ecology and the effects of forestry on them. *Dissertationes botanicae*. 331: 1–176.

Schwartner C, Bors W, Michel C, Franck U, Muller-Jakic B, Nenninger A *et al.* 1995. Effect of marchantins and related compounds on 5-Lipoxygenase and Cyclooxygenase and their antioxidant properties: a structural activity relationship study. *Phytomedicine*. 2: 113-7.

Schwartner C, Michel C, Stettmaier K, Wagner H, Bors W. 1996. Marchantins and related polyphenols from liverwort: Physicochemical studies of their radical-scavenging properties. *Free Radical Biology and Medicine*. 20: 237–44.

Seeger T, Geiger H, Zinsmeister HD. 1993. Bioflavonoids from the moss *Homalthecium lutescens*. *Phytochemistry*. 34: 295-6.

Senarath RMUS, Karunaratha BMAC, Senarath WTPSK, Jimmy GC. 2017. In vitro propagation of *Kaempferia galanga* (zingiberaceae) and comparison of larvicidal activity and phytochemical identities of rhizomes of tissue cultured and naturally grown plants. *Journal of Applied Biotechnology and Bioengineering*. 2: 1-6.

Serafini M, Peluso I, Raquzzini A. 2010. Flavonoids as anti-inflammtory agents. *Proceedings of the Nutrition Society*. 69: 273-8.

Sesti G. 2006. Pathophysiology of insulin resistance. *Best Practice & Research Clinical Endocrinology & Metabolism*. 20: 665-79.

Setzer WN, Setzer MC. 2003. Plant-derived triterpenoids as potential antineoplastic agents. *Mini Reviews in Medicinal Chemistry*. 3: 540-56.

Shen J, Li G, Liu Q, He Q, Gu J, Shi Y, Lou H. 2010. Marchantin C: a potential anti-invasion agent in glioma cells. *Cancer Biology and Therapy*. 9: 33–39.

Sherwin ER. 1990. Antioxidants in Food Additives, (ed. Branen R.). Marcel Dekker: New York; 139–93
.

Shi Y, Zhu CJ, Yuan H, Li B, Gao J, Qu X, Bin S, Cheng Y, Li S, Li X, L HX. 2009. Marchantin C, a novel microtubule inhibitor from liverwort with anti-tumor activity both in vivo and in vitro. *Cancer Letters*. 276: 160-70.

Shi YQ, Liao YX, Qu XJ, Yuan HQ, Li S, Qu JB *et al.* 2008a. Marchantin C, a macrocyclic bisbibenzyl, induces apoptosis of human glioma A172 cells. *Cancer Letters*. 262: 173-82.

Shi YQ, Qu YX, Liao YX, Xie CF, Cheng YN, Li S *et al*. 2008b. Reversal effect of a macrocyclic bisbibenzyl plagiochin E on multidrug resistance in adriamycin resistant K567/A02 cells. *European Journal of Pharmacology*. 584: 66-71.

Shirwaikar A, Prabhu KS, Punitha IS. 2006. In vitro antioxidant studies of *Sphaeranthus indicus* (Linn). *Indian Journal of Experiment Biology*. 44: 993-6.

Shyur LF, Tsung JH, Chen JH, Chiu CY, Lo CP. 2005. Antioxidant properties of extracts from medicinal plants popularly used in Taiwan. *International Journal of Applied Science and Engineering*. 3: 195-202.

Sidduraju P, Mohan P, Becker K. 2002. Studies on the antioxidant activity of Indian *Laburnum Cassia* fistula L): a preliminary assesment of crude extracts from stem bark, leaves, flowers and fruit pulp. *Food Chemistry*. 79: 61-7.

Sies H, Stahl W, Sundguist AR. 1992. Antioxidant Function of Vitamins. *Annals of the New York Academy Science*. 669: 7-20.

Singh A. 2011. Herbalism, phytochemistry and ethnopharmacology. CRC Press Book: USA; 1-403.

Singh DK 2001. Diversity in Indian Liverworts: their status, vulnerability and conservation, in Nath V, Asthana AK (eds). *Perspectives in Indian Bryology*. BSI: Dehradun; 325 – 54.

Singh DK, Singh SK, Singh D. 2016. Liverworts and hornworts of India: an annotated checklist (1st edn). Botanical Survey of India: Kolkata; 1-404.

Singh DK. 1992. Liverworts (Hepaticae) diversity in India and its conservation. In: *Status Report of Biodiversity Conservation in India*. Ministry of Environment and Forests: New Delhi; 92.

Singh DK. 1997. Liverworts. In: Mudgal V and Hajra PK, P.K. (eds.) I, *Floristic studies and conservation strategies in India*. BSI: Dehra Dun; 235-300.

Singh DK. 1999. Indian Hepaticae : Status and Strategies. *Annals of Forestry.* 72: 199–211.

Singh DK. 2008. Red-listing of Hepaticae and Anthocerotae in India. In: Mohamed H, Baki BB, Nasrullaq-Boyce A, Lee PKY (eds.) *Bryology in the New Millenium*. University of Malaya: Kuala Lumpur; 451– 458.

Singh H, Dixit S, Verma PC, Singh PK. 2014. Evaluation of total phenolic compounds and insecticidal and antioxidant activities of tomato hairy root extract. *Journal of Agriculture and Food Chemistry*. 62: 2588-94.

Somova LI, Shode FO, Moodley K, Govender Y. 2001. Cardiovascular and diuretic activity of kaurene derivatives of *Xylopia aethiopica* and *Alepidea amatymbica*. *Journal of Ethnopharmacology*. 77: 165-74.

Sonwa MM, Konig WA. 2003. Chemical constituents of the essential oil of the hornwort *Anthoceros caucasicus*. *Flavour and Fragrance Journal*. 18: 286-9.

Sorbo S, Basile A, Cobianchi RC. 2004. Antibacterial, antioxidant and allelopathic activities in bryophytes. *Recent Research Developments in Phytochemistry*. 8: 69-82.

Soumia K, Tahar D, Lynda L, Saida B, Chabane C, Hafidha M. 2014. Antioxidant and antimicrobial activities of selected medicinal plants from Algeria. *Journal of Coastal Life Medicine*. 2: 478-83.

Souri E, Amin G, Farsam H, Tehrani M. 2008. Screening of antioxidant activity and phenolic content of 24 medicinal plant extracts. *DARU Journal of Pharmaceutical Sciences*. 16: 83-7.

Stahl W, Sies H. 2004. Antioxidant activity of caretenoids. *Molecular Aspects of Medicine*. 24: 345-5.

Stone JR, Yang S. 2006. Hydrogen peroxide: a signalling messenger. *Antioxidants and Redox Signaling*. 8: 243-70.

Suba V, Murugesan T, Rao RB, Ghosh L, Pal M, Mandal SC, Saha BP. 2004. Antidiabetic potential of *Barleria lupulina* extract in rats. *Fitoterapia.* 75: 1-4.

Suganya Devi P, Saravanakumar M, Das SM. 2012. DNA damage protecting activity and free radical scavenging activity of anthocyanins from red Sorghum (*Sorgum bicolour*) Bran. *Biotechnology Research International*. doi:10.1155/2012/258787.

Suh SJ, Kwak CH, Chung TW, Park SJ, Cheeeei M, Park SS *et al.* 2012. Pimaric acid from Aralia cordata has an inhibitory effect on TNF-α-induced MMP-9 production and HASMC migration via down-regulated NF-KB and AP-1. *Chemico-Biological Interactions*. 199:112-9.

Sultan A, Raza AR. 2015. Steroids: A diverse class of secondary metabolites. *Medicinal Chemistry*. 5: 310-17.

Suzuki I, Kondoh M, Nagashima F, Fujii M, Asakawa Y, Watanabe Y. 2004a. A comparison of apoptosis and necrosis induced by ent-kaurene-type diterpenoids in HL-60 cells. *Planta Medica.* 70: 401-46.

Suzuki I, Kondoh M, Harada M, Koizumi N, Fujii M, Nagashima F *et al.* 2004b. An ent-kaurene diterpene enhances apoptosis induced by tumor necrosis factor in human leukemia cells. *Planta Medica*. 70: 723-27.

Tag H, Das A K, Hari L. 2007. Anti-inflammatory plants used by the Khamti tribe of Lohit district in Eastern Arunachal Pradesh India. *Natrual Product Radiance*. 6: 334-40.

Taira Z, Takei M, Endo K, Hashimoto T, Sakiya Y, Asakawa Y. 1994. Marchantin A trimethyl ether: its molecular structure and tubocurarine-like skeletal muscle relaxation activity. *Chemical and Pharmaceutical Bulletin*. 42: 52-6.

Tazaki H, Ito M, Miyoshi M, Kawabata J, Fukushi E, Fijita T *et al.* 2002. Subulatin, an antioxidic caffeic acid derivative isolated from the in vitro cultured liverworts, *Jungermannia subulata, Lophocolea heterophylla*, and *Scapania parvitexta*. *Bioscience Biotechnology and Biochemistry*. 66: 255–61.

Theng KB, Korpenwar AN. 2013. Quantitative estimation of some phytochemical and determination of metallic elements from *Pueraria tuberose* (Roxb. Ex Willd.) DC. Tuber. *International Journal of Science and Research*. 4: 2396-9.

Thiapong K, Boonprakob U, Crosby K, Cisneros-Zevallos L, Byrne DH. 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *Journal of Food Composition and Analysis*. 19: 669-75.

Thimmaiah SK. 1999. Standard method of biochemical analysis. Kalyani Publishers: New Delhi; 1-534.

Tiwari VP, Tiwari KC, Joshi PJ. 1973. An interpretation of Ayurvedika findings on Shilajit. *Journal of Research in Indigenous Medicine*. 8: 57.

Toyota M, Asakawa Y, Frahm JP. 1990. *Ent*sesquiterpenoids and cyclic bis (bibenzyls) from the german liverwort *Marchantia polymorpha. Phytochemistry.* 29: 1577-84.

Trease G, Evans WC. 1983. Textbook of Pharmacognosy, (12th Edn). Balliere Publishers: London.

Trease GE, Evans WC. 1989. Pharmacology (11th Edn). Bailliere Tindall Ltd.: London; 60-75.

Tusevski O, Kostovska A, Iloska A, Trajkovska L, Simic SG. 2014. Phenolic production and antioxidant properties of some Macedonian medicinal plants. *Central European Journal of Biology*. 9: 889-80.

Udar R, Srivastava SC. 1983. Rare and endangered liverworts of India. In: S.K. Jain and R.R. Rao (eds.). *An assessment of threatened plants of India*. Botanical Survey of India: Botanic Garden, Howrah; 303-12.

Unnikrishnan PS, Suthindhiran K, Jayasri MA. 2015. Alpha-amylase Inhibition and Antioxidant Activity of Marine Green Algae and its Possible Role in Diabetes Management. *Pharmacognosy Magazine*. 11: 511-15.

Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. 2006. Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry and Cell Biology*. 39: 44-84.

Valko M, Morris H, Cronin MTD. 2005. Metals, toxicity and oxidative stress. *Current Medical Chemistry*. 12: 1161–208.

Van Breusegem F, Vranovà E, Dat JF, Inze D. 2001. The role of active oxygen species in plant signal transduction. *Plant Science*. 161: 405–14.

Varsha KK, Shilpa G, Devendra L, Nampoothiri KM. 2015. 2,4-Di-tert-butyl phenol as the antifungal, antioxidant bioactive purified from a newly isolated *Lactococcus* sp. *International Journal of Food Microbiology*. 211: 45-50.

Vasco C, Ruales J, Eldin AK. 2008. Total phenolic compounds and antioxidant capacities of major fruits from Ecuador. *Food Chemistry*. 111: 816-23.

Vats S, Alam A. 2013. Antioxidant activity of *Barbula javanica* Doz. et Molk. A relatively unexplored bryophyte. *Elixir Applied Botany*. 65: 20103–4.

Velioglu YS, Mazza G, Gao L, Oomah BD. 1998. Antioxidant activity and total phenolics in Selected fruits, vegetables, and grain products. *Journal of Agriculture and Food Chemistry*. 46: 4113-7.

Veljic M, Ciric A, Sokovic M, Janackovic P, Marin PD. 2010. Antibacterial and antifungal activity of the liverwort (*Ptilidium pulcherrimum*) methanol extract. *Archives of Biological Sciences*. 62: 381–95.

Virranen R, Johnston AE, Crawley MJ, Edwards GR. 2000. Bryophyte biomass and species richness on the Park Grass Experiment, Rothamsted, UK. *Plant Ecology*. 151: 129-41.

Vitale DC, Piazza C, Melilli B, Drago F, Salomone S. 2013. Isoflavones: estrogenic activity, biological effect and bioavailability. *European Journal of Drug Metabolism and Pharmacokinetics*. 38: 15-25.

Vujcic V, Brkanac SR, Redovnikovic IR, Ivankovic S, Stojkovic R, Zilic I *et al.* 2017. Phytochemical and bioactive potential of in vivo and in vitro grown plants of *Centaurea ragusina* L. – detection of dna/rna active compounds in plant extracts via thermal denaturation and circular dichroism. *Phytochemical Analysis*. 28: 584-92.

Wagner H, Bladt S. 1996. Plant Drug Analysis: A Thin Layer Chromatography Alas. (2nd Edn) Spinger-Verlag: Berlin, Heidelberg.

Wang X, Yu W, Lou H. 2004. Antifungal constituents from the Chinese moss *Homalia trichomanoides*. *Chemistry and Biodiversity*. 2 : 139-45.

Waterbeemd VD, Gifford E. 2003. ADMET in silico modelling: towards prediction paradise? *Nature Reviews Drug Discovery*. 2: 192-204.

Weisany W, Sohrabi Y, Heidari G, Siosemardeh A, Golezani K G. 2012. Changes in antioxidant enzymes activity and plant performance by salinity stress and zinc application in soybean (*Glycine max* L.). *Plant Omics Journal*. 5: 60–7.

Werner H, Goddert VO, Christina W. 2003. The effects of light and soil conditions on the species richness of the ground vegetation of deciduous forests in northern Germany (Schleswig-Holstein). *Forest Ecology and Management*. 182: 327-38

Whitelaw M, Burton MAS. 2015. Diversity and distribution of epiphytic bryophytes on Bramley's seedling trees in East of England apple orchards. *Global Ecology and Conservation*. 4: 380-7.

Williams JC, Read A, Norton S, Dovers M, Burgman W, Proctor H *et al*. 2001. Biodiversity, Australia state of the environment report 2001 (Theme Report). CSIRO: Canberra.

Wink DA, Mitchell JB. 1998. Chemical biology of nitric oxide: insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radical Biology and Medicine*. 25: 434–56.

Wirasathien L, Boonarkart C, Pengsuparp T, Suttisri R. 2006. Biological activities of alkaloids from *Pseuduvaria setose*. *Pharmaceutical Biology*. 44: 274-8.

Wong CC, Li HB, Cheng KW, Chen F. 2006. A systematic survey of antioxidant activity of 30 Chinese medicinal plants using the ferric reducing antioxidant power assay. *Food Chemistry*. 97: 705-11.

Woodgett JR, Cohen P. 1984. Multisite phosphorylation of glycogen synthase. Molecular basis for the substrate specifi city of glycogen synthase kinase-3 and casein kinase-II (glycogen synthase kinase-5). *Biochimica et Biophysica Acta*. 788: 339 – 47.

Wurger G, McGaw LJ, Eloff JN. 2013. Tannin content of leaf extracts of 53 trees used traditionally to treat diarrhoea is an important criterion in selecting species for further work. *South African Journal of Botany*. 90: 114-117.

Xiao J, Chen X, Zhang YW, Xu M. 2006. Cytotoxicity of *Marchantia convulata* leaf extracts to human liver and lung cancer cells. *Brazilian Journal of Medical and Biological Research*. 39: 731-738.

Xiao J, Jiang X, Chen X. 2005. Antibacterial, anti-inflammatory and diuretic effect of flavonoids from *Marchantia convoluta*. *African Journal of Traditional Comlpementary and Alternative Medicine*. 2: 244-52.

Xie CF, Lou HX. 2009. Secondary metabolites in Bryophytes: an ecological aspec. *Chemistry and Biodiversity*. 6: 303-12.

Yang J, Ou X, Zhang X, Zhou Z, Ma L. 2017. Effect of different solvents on the measurement of phenolics and the antioxidant activity of mulberry (*Morus atropurpurea* Roxb.) with accelerated solvent extraction. *Journal of Food Science*. 82: 605-12.

Yang T, Gao X, Sandberg M, Zollbrecht C, Zhang XM , Hezel M *et al*. 2015. Abrogation of adenosine A1 receptor signalling improves metabolic regulation in mice by modulating oxidative stress and inflammatory responses. *Diabetologia.* 58: 1610-20.

Yoshida T, Hashimoto T, Takaoka S, Kan Y, Tori M, Asakawa Y. 1996. Phenolic constituents of the liverwort: Four novel cyclic bisbibenzyl dimers from *Blasia pusilla* L. *Tetrahedron*. 52: 14487–500.

Yuliana P, Laconi EB, Wina E, Jayanegara A. 2014. Extraction of tannins and saponins from plant sources and their effects on in vitro methanogenesis and rumen fermentation. *Journal of the Indonesian Tropical Animal Agriculture*. 39: 91-7.

Yumrutas O, Saygideger SD. 2012. Determination of antioxidant and antimutagenic activities of *Phlomis armeniaca* and *Mentha pulegium*. *Journal of Applied Pharmaceutical Science*. 2: 36-40.

Zarena AS, Sankar KU. 2009. A study of antioxidant properties from *Garcinia mangostana* L. pericarp extract. *Acta Scientiarum Polonorum Technologia Alimentaria*. 8: 23-34.

Zhang Y, Seeram NP, Lee R, Feng L, Hebe D. 2008. Isolation and identification of strawberry phenolics with antioxidant and human cancer cell antiproliferative properties. *Journal of Agricultural and Food Chemistry*. 56: 670-5.

Zima TS, Fialova L, Mestek O, Janebova M, Crkovska J, Malbohan I *et al*. 2001. Oxidative stress, metabolism of ethanol and alcohol-related diseases. *Journal of Biomedical Science*. 8: 59–70.

INDEX

A

$ABTS^+$	17, 38, 47, 49, 52, 57, 62, 83, 85, 96, 102, 111, 173, 205, 215, 239
Antioxidant	2, 17, 18, 38, 47, 83, 91, 93, 204, 207, 214, 228, 238, 240
Anti-diabetic	6, 13, 20, 40, 48, 49, 53, 65, 83, 86, 88, 91, 209, 212, 240
Abundance	23, 25, 26, 27, 53, 192, 194

B

BHT	3, 16

C

Cytotoxicity	4, 210, 219, 239
Correlation	26, 55, 56, 74, 192, 194, 215, 232

D

DPPH	17, 38, 49, 57, 96, 173
Druggability	162

E

Extractive weight	54, 70, 97, 211
Epiphytic liverwort	55, 112, 232

F

Free radicals	2, 13, 86, 88, 96, 205, 213, 238, 240
Flavonoids	9, 10, 24, 41, 54, 97, 100, 176, 193, 198, 212

G

Gallic acid	41, 54, 70 ,97, 193, 211

M

Metal chelating	47, 52, 58, 74, 83, 97, 207, 215

N

Nitric oxide	39, 57, 96, 204, 215
Network pharmacology	50, 122, 137, 220, 236

O

Oxidative stress	3, 15, 18, 210, 237, 238

P

Purification	6, 47, 100, 217, 240
Phenol	9, 10, 20, 40, 53, 74, 97, 193, 209, 231, 227
Phytochemical	9, 12, 41, 50, 79, 91, 211, 226, 230

Q
Quercetine 13, 16, 97

R
Reactive oxygen species 2, 206, 216

S
Seasonal variation 6

T
Tannin 10, 42, 83, 242

Appendix A

LIST OF CHEMICALS USED

CHEMICAL USED

A

Alpha-amylase enzyme
Alpha-glucosidase
2,2α azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) ABTS
2,2´-di-p-nitrophenyl-5,5´-diphenyl-(3,3´-dimethoxy-4,4´-diphenylene)di-tetrazolium chloride,
2-deoxyribose solution
Acetic acid
Acetic anhydride
Acetone
Aluminum chloride
Antimony-II-chloride

Butanol
6-Benzylaminopurine

C

Chloroform
Citrate buffer (pH 4.5)
Conc.H_2SO_4
Cupper acetate

D

Dichloromethane
Dichromatic acetic acid
DNS (3,5-dinitrosalicylic acid) reagent
DPPH (2,2-diphenyl-1- picrylhydrazyl)
Dragendroff's reagent
MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Diphenylboric acid-β-ethylamino ester
Diethyl ether

E

Ethylenediaminetetraacetic acid
Ethanol
Ethyl Acetate
Ethylenediamine tetraacetic acid

Etoposide

F

$FeCl_2$
$FeCl_3$

Fehling's solution I (A)

Fehling's solution II (B)
Ferric chloride
Ferrozine
$FeSO_4$, 7H2O
Folin-Ciocalteu reagent
Formic Acid
Ferrous chloride
Formazan
Fast blue reagent

G

Gallic acid
Glacial Acetic Acid
Glibenclamide
Glucose
Gamborg G5 medium

H

Hexane
Heptane
Hydrated ferrous sulphate
Hydrochloric acid
Hydrogen peroxide

Hydroxylamine hydrochloride
Hydrogen sulphate

I

Isopropanol

K

Knop's medium

L

Lead acetate
Liebermann Burchard's Reagent

M

Methanol
Murashige and Skoog medium

N

Na_2CO_3
Na_2SO_4
NaOH
Napthylethylenediamine dihydrochloride
n-butanol
Nicotinamide-adanine dinucleotide phosphate (NADPH)
Ninhydrin reagent
Nitro-blue tetrazolium (NBT)
1-Naphthaleneacetic

P

Petroleum ether
Phenazine methosulphate (PMS)
p-nitrophenol-a-D-glucopyranoside
Potassium dichromate
Potassium ferricyanide
Potassium hydroxide
Potassium persulfate
Pyridine
Polyethylene glycol-4000
Potassium hexacyanoferrate

S

Sodium carbonate
Sodium hydroxide
Sodium nitroprusside
Sulfanilamide
Sodium phosphate
Sodium nitrite
Solasodine
Silica gel 200-400
Sodium hypochlorite

T

Thiobarbituric acid (TBA)
Trichloroacetic acid (TCA)

V

Vanillin

Appendix B

ABREVIATION AND SYMBOLS USED

ABBREVIATION

A

A	Acetone
AA	Amino acid
$ABTS^{+}$	2,2’ azinobis-(3-ethylbenzoline-6-sulfonic acid
ABUN	Abundance
ACHN	Kidney cancer cell line
ALK	Alkaloid
ALP	Anti-lipid peroxidation
ANT	Anthraglycoside
ANT	Anthraglycoside
AKR1B1	Aldo-Keto Reductase Family 1 Member B
ADORA1	Adenosine A1 Receptor
ADMET	Absorption, distribution, metabolism and excretion-toxicity
AR	Androgen receptor
ALOX15	Arachidonate 15-lipoxygenase
ALOX5	Arachidonate 5-lipoxygenase

B

B	Butanol
BAP	6-Benzylaminopurine
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BindingDB	Binding database
BCHE	Butyrylcholinesterase
BACE1	Beta secretase 1
BBB	Blood brain barrier

C

CAT	Catalase
CE	Catechol equivalent
CG	Cardiac glycoside
COX	Cyclooxygenase
CYP1	Cytochrome P450
CNS	Central Nervous System

D

DBH	Diameter at breast height
DE	Diethyl ether
DM	Diabetes mellitus
DMRT	Duncan’s multiple test
$DPPH^{\bullet}$	2,2-diphenyl-1-pyicrihydrazil
D	Dark

E

ESR1	Estrogen receptor alpha
EA	Ethyl acetate
EW	Extractive weight

F

Fe^{2+}	Ferrous ion
FLA	Flavonoid
FLAV	Flavonoids
FRAP	Ferric reducing ability of plasma
fwt	Fresh weight

G

GAE	Gallic acid equivalent
GIR	Girth
GLY	Glycoside
GC-MS	Gas Chromatography Mass Spectrometry
GSK3B	Glycogen synthase kinase 3

H

H	Heptane
H_2O_2	Hydrogen peroxide
HMGCR	3-hydroxy-3-methylglutaryl-coenzyme A
HSDII	11 beta hydroxysteroid dehydrogenase
hERG	ether-a-go-go gene

I

IBA	Indole -3- butyric acid
IR	Infrared

K

KEGG	Kyoto Encyclopedia of Genes and genomes

L

LCMS	Liquid chromatography mass spectrometry
LIG	Light
LOX	5-lipoxygenase
L	Light

M

MC	Metal chelating
MS	Murashige and Skog medium
MOIST	Moisture
MS/2	Half strength Murashige and Skoog medium
MIF	Macrophage migration inhibitory factor
MRTD	Maximum recommended tolerated dose

N

NAA	Napthalenecetic acid
NADPH	Nicotinamide-adanine dinucleotide phosphate
NMR	Nuclear magnetic resonance
NO	Nitric oxide

O

$O_2^{\bullet}$	Superoxide
ODP	Orthodihydric phenol
$OH^{\bullet}$	Hydroxyl radical
OPC	Orthodihydric phenol content
OCT2	Organic Cation Transporter 2

P

PA	Protected area
PC1	Principal component one
PC2	Principal component two
PCA	Principal component analysis
PHEN	Phenol
PTPN1	Protein Tyrosine Phosphatase, Non-Receptor Type 1
PPARG	Peroxisome proliferator activated receptor gamma

Q

QE	Quercetin equivalent

R

RCC	Renal cell carcinoma
RES	Resin
Rf	Retention value
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RP	Reducing power
RS	Reducing sugar

S

SMILE	Smart molecule input line entry system
SO	Superoxide
ST	Steroid
ST	Steroid
STRING	Search tool for the retrieval of interacting genes/proteins
SUG	Total sugar

T

TAN	Tannin
TANN	Tannin
TBA	Thiobarbituric acid
TBHQ	Gallates and tertiary butyl hydroquinone
TCA	Trichloroacetic acid

TERPEN	Terpenoid
TF	Total flavonoids
TLC	Thin layer chromatography
TN	Tannin
TP	Total phenol
TT	Triterpenoids
TTD	Therapeutic target database

V

VEGFR	Vascular endothelial growth factor

α

α-A	Alpha amylase
α-G	Alpha glycosidase

Appendix C

LIST OF PUBLICATIONS FROM THE THESIS

LIST OF PUBLICATIONS

S Mukhia, P Mandal, DK Singh, D Singh. 2014. *In-vitro* free radical scavenging potential of three liverworts of Darjeeling Himalaya. *International Journal of Pharmaceutical Sciences and Research.* 5: 4552-61.

S Mukhia, P Mandal, DK Singh, D Singh. 2015. Evaluation of antidiabetic, antioxidant activity and phytochemical constituents of liverworts of Eastern Himalaya. *Journal of Chemical and Pharmaceutical Research.* 7: 890-900.

S Mukhia, P Mandal, DK Singh, D Singh. 2017. Study of bioactive phytoconstituents and *in-vitro* pharmacological properties of thallose liverworts of Darjeeling Himalaya. *Pharmacy Research.* 11: 490-501.

SEMINARS ATTENDED

NATIONAL

National symposium on Recent Trends in Plant and Microbial Research, March 22-23, 2013

National Conference of Plant Physiology, December 13-16, 2013

National symposium on Advances in Plant and Microbial Research, December 12-13, 2014

INTERNATIONAL

2nd International Conference on Bridging Innovations in Pharmaceutical, Medical and Bio-Science, February 2017 11-12, 2017

Advances in Development, delivery systems and Clinical Monitoring of Drugs, September 23-24, 2017

Appendix D

PUBLISHED ARTICLES

IJPSR (2014), Vol. 5, Issue 10 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 24 March, 2014; received in revised form, 25 April, 2014; accepted, 11 July, 2014; published 01 October, 2014

IN-VITRO FREE-RADICAL SCAVENGING POTENTIAL OF THREE LIVERWORTS OF DARJEELING HIMALAYA

Sumira Mukhia [1], Palash Mandal*[1], D.K. Singh [2], Devendra Singh [2] and Dibakar Choudhury [3]

Plant Physiology and Pharmacognosy Research Laboratory [1], Department of Botany, North Bengal University, P.O: NBU, Raja Rammohunpur, District: Darjeeling, West Bengal, India
Botanical Survey of India [2], CGO Complex, 3rd MSO Building, Salt Lake Sector I, Kolkata, West Bengal, India
Plant Taxonomy and Environmental Biology Laboratory [3], Department of Botany, North Bengal University, P.O: NBU, Raja Rammohunpur, Dictrict: Darjeeling, West Bengal, India

Keywords:

Bryophytes, Antioxidants, DPPH; ABTS, Lipid peroxidation, Phenolics

Correspondence to Author:

Palash Mandal

Plant Physiology and Pharmacognosy Research Laboratory, Department of Botany, North Bengal University, P.O: NBU, Raja Rammohunpur, District: Darjeeling, West Bengal, India

E-mail: nbubotanypalash@rediffmail.com

ABSTRACT: The present study was aimed to screen the antioxidant activity and phytochemical content of bryophyte species *Marchantia paleacea* Bertol., *Marchantia linearis* Lehm. & Lindenb. and *Conocephalum conicum* (L.) Underw. collected from Darjeeling hills of Eastern Himalaya, India. The free radical scavenging activities were measured *in-vitro* by DPPH radical, metal chelating, superoxide, $ABTS^+$, reducing power, anti-lipid peroxidation and nitric oxide scavenging activity. Total phenol, flavonoids and ortho-dihydric phenol present in the samples was also estimated. Qualitative phytochemical screening was carried out to detect the presence of varied phytochemicals. Thin layer chromatography (TLC) and TLC bioautography assay was also performed to confirm the presence of different bioactive compounds and its free radical scavenging potential. All the tested bryophyte species showed potential antioxidant activity and the existence of different phytochemicals. The results obtained from this work indicated that all the three bryophyte species analyzed are potent source for antioxidants and can be pharmaceutically explored in future.

INTRODUCTION: Oxidative stress caused by reactive oxygen species (ROS) generated from molecular oxygen as by products during different metabolic pathways, are the cause of many degenerative diseases [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.5(10).4552-61

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.5(10).4552-61

Thus, for the survival of all life forms detoxification of reactive oxygen species is highly essential. As such, endogenous antioxidative defense mechanism has evolved to meet this requirement [2].

Antioxidant can be either natural or synthetic, however synthetic antioxidants are considered harmful for health [3]. Therefore, there is a need to look for new natural sources with potential pharmaceutical and antioxidant capabilities [4]. Bryophytes are now increasingly being considered as new source of pharmaceuticals [5].

They possess different therapeutic activities and have been reported to be medicinally used by different ethnic communities [6]. Screening of different bryophyte species has showed their ability to be used as a possible source of antioxidants for medicinal and cosmetic purposes. However in comparison to angiosperms, bryophytes are used much less as medicinal plants. Present study is the report on free-radical scavenging potential of *Marchantia paleacea* Bertol., *Marchantia linearis* Lehm. & Lindenb., *Conocephalum conicum* (L.) Underw. collected from Darjeeling hills of Eastern Himalaya.

MATERIALS AND METHODS

Collection of Plant materials: Fresh thalli of all the three tested liverwort samples: *Marchantia paleacea* Bertol., *Marchantia linearis* Lehm. & Lindenb., *Conocephalum conicum* (L.) Underw. were collected from Singamari, Darjeeling hills, India in the month of January 2013. The taxonomic identification of collected sample was done by Dr. D.K. Singh and Dr. Devendra Singh, Botanical Survey of India, Kolkata, West Bengal and the voucher specimen was deposited in the Central National Herbarium, Kolkata, West Bengal, India.

Methods of extraction: Collected samples were carefully inspected to remove contaminants like soil and other plant materials. As different bryophyte species grow in close association with each other forming mixed culture, emphasis was always given on separating actual sample cautiously to keep specimen of interest pure. Plant samples were then washed with tap water, air dried and crushed into fine paste. Extraction was done with methanol by reflux technique for three hours. The extracts were filtered, concentrated and then used for further investigation.

Preliminary phytochemical analysis*:*

1. **Total Phenol estimation:** The total phenolic content of extract was estimated by the method of Kadam *et al* [7] with few modifications using Folin-Ciocalteu reagent. Gallic acid is used as standard to estimate total phenols present. 1ml 95% ethanol, 5 ml distilled water and 0.5 ml 50% Folin Ciocalteu reagent was added with 1 ml extract. After 5 minutes, 1 ml of 5 % sodium carbonate was added and the mixture was incubated for 1 hours. Absorbance was measured at 725 nm. Standard curve was prepared with different concentrations of gallic acid.

2. **Total Flavonoid estimation:** Total flavonoid content of extract was determined by the method of Atanassova *et al* [8] with little modification. 4 ml distilled water and 0.3 ml 5% sodium nitrite was added to 0.5 ml extract. After 5 minutes 0.3 ml of 10% aluminium chloride was added and was left for 6 minutes. Then 2 ml of 1.0 M sodium hydroxide, 2.4 ml of distilled water was added sequentially and vortexed well. Absorbance was measured at 510 nm and standard curve was prepared with different concentrations of quercetin. The total flavonol content was expressed as mg quercetin equivalent/g dry weight.

3. **Orthodihydric Phenol estimation:** Total ortho-dihydric phenol present in the bryophyte samples was estimated by the method of Mahadevan and Sridhar [9] with few modifications. Arnow's reagent was prepared by dissolving 10 g of sodium nitrite and 10 g of sodium molybdate in 100 ml of water and stored in brown bottle. Catechol was used as standard for estimation of orthodihydric phenol content of the sample. 0.5 ml of Arnow's reagent was added to 0.5 ml extract. Then 5 ml water and 1 ml of 1(N) NaOH was added. Absorbance was measured at 515 nm and the total amount of orthodihydric phenol present in the sample was estimated by using standard curve prepared from working with standard catechol solution at different concentrations.

Antioxidant activity determination:

1. **DPPH Scavenging Antioxidant Activity determination:** The effect of crude methanolic extract on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical was determined according to the procedure described by Sharma and Goyal [10] with some modifications. 4 μg DPPH was dissolved in 100 ml of methanol to prepare

DPPH solution. 200 µl extract was taken, to which 2 ml of DPPH solution was added. The mixture was then incubated for 20 minutes and the reduction of the DPPH radical was measured Spectrophotometrically at 517 nm against reagent blank. IC_{50} value was used to express the antioxidant activity of crude methanolic extract. It is defined as concentration of extract that results in the 50% reduction of the free-radicals [11]. Lower the IC_{50} value, higher is the antioxidant activity [12]. Scavenging activity of the sample was calculated based on percentage decolorization of the sample according to following equation:

% inhibition of DPPH activity= $[(A_0-A_1)/A_0] \times 100$ %

Where A_0 is the absorbance value of the control reaction or blank sample and A_1 is the absorbance value of the tested sample.

2. **Nitric Oxide Scavenging Activity:** Nitric oxide scavenging activity of the tested bryophyte sample was estimated by the method of Marcocci *et al* [13] with few modifications. 2 ml 20 mM sodium nitroprusside, 0.5 ml phosphate buffer and 0.5 ml extract was incubated at 25°C for 150 minutes. After two and half hours of incubation, 3 ml Griess reagent was added to the mixture and allowed to stand at room temperature for 30 minutes. The absorbance of the mixture was measured at 540 nm. Following formula was used to calculate the nitric oxide scavenging activity of the extract:

% inhibition = $[(A_0-A_1)/ A_0]$ x100

Where, A_0 = Absorbance of control and A_1 = Absorbance of sample

3. **Superoxide radical scavenging assay:** Determination of superoxide scavenging activity of extracts was done by the method described by Fu *et al* [14] with few modifications.

To 1 ml extract, 1 ml nitroblue tetrazolium chloride (312 µM in phosphate buffer, 7.4) was added followed by the addition of 1 ml nicotinamide adenine dinucleotide (936 µM prepared in phosphate buffer, pH-7.4) after 5 minutes. The mixture was centrifuged to remove the precipitation developed after the addition of nitroblue tetrazolium chloride. The reaction mixture was again left for 5 minutes. Later 10 µl phenazine methosulphate was added to the mixture to start the reaction. The reaction mixture was then incubated for 30 minutes with exposure to fluorescent light and absorbance was measured at 560 nm. The percentage inhibition was estimated by using following formula:

Superoxide radical scavenging effect (%) = $[(A_0 - A_1/A_0)$ x 100]

Where, A_0 = Absorbance of control and A_1 = Absorbance of sample

4. **2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)/($ABTS^+$) scavenging antioxidant assay:** 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical scavenging activity of extract was determined by following the method of Li *et al* [15] with minor modifications. At first, 7 mM $ABTS^+$ stock was reacted with 2.45 mM potassium persulfate to prepare $ABTS^+$ radical cation and was stored in room temperature for 6 hours in a dark place. The $ABTS^+$ solution was then diluted with sodium phosphate buffer (0.1 M, pH 7.4) to give an absorbance of 0.750 ± 0.025 at 734 nm. After that 2 ml of $ABTS^+$ solution was added to sample and the mixture was incubated for 10 minutes. Scavenging activity of the sample was calculated based on percentage inhibition of absorbance at 734 nm against the reagent blank by the following formula:

Inhibition % = $(A_0 - A_1/A_0) \times 100$

Where, A_0 = Absorbance of control and A_1 = Absorbance of sample.

5. **Metal chelating assay:** Method described by Dinis *et al* [16] was utilized with few modifications for the determination of metal chelating activity of extract. Crude extract (400 µl) was mixed with 1600 µl methanol, 40 µl of 2 mM $FeCl_2$, 80 µl of 5 mM Ferrozine and was allowed to equilibrate for 10 minutes before measuring the absorbance. The metal chelating activity was measured by the decrease of the absorbance at 562 nm of the iron (II) – ferrozine complex. The ability to chelae metal ion by the sample was calculated relative to control using following formula:

Chelating effect% = $(A_0 - A_1) / A_0 \times 100$

Where, A_0 = Absorbance of control and A_1 = Absorbance of sample.

6. **Reducing power assay:** Iron reducing ability of methanolic extract was determined by the method of Gulcin [17] with few modifications. 1 ml extract was mixed with 2.5 ml of phosphate buffer (0.2 M) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50° C for 20 minutes. After 20 minutes of incubation, 2.5 ml of 10% trichloroacetic acid was added. The mixture is then allowed to cool and centrifuged at 3000 rpm for 10 minutes. 2.5 ml upper layer was collected, to this 2.5 ml of distilled water and 250 µl of 0.1% $FeCl_3$ was added. The absorbance was measured at 700 nm. Linear regression analysis was carried out to determine the ability of methanolic extract to reduce iron by plotting absorbance value against different concentrations.

7. **Lipid peroxidation assay:** The extent of lipid peroxidation in goat liver homogenate was determined by using standard method of Bouchet *et al* [18]. At first fresh goat liver was collected from slaughter house. Liver was cut into small pieces, homogenized in mortar pestle with buffer and filtered through muslin cloth to get clear solution. The solution was then centrifuged and refrigerated. Lipid peroxidation was initiated by adding 0.1 ml of $FeSO_4$, 2.8 ml 10% liver homogenate and 100 µl extract. The mixture was incubated for 30 min at 37°C. After that 1 ml reaction mixture was taken in a test tube and 2 ml of thiobarbituric acid-trichloroacetic acid (10% TCA and 0.67% TBA) was added. The mixture was heated in a boiling water bath for 1 hour. After heating, mixture was centrifuged and supernatant was separated. Absorbance was measured at 535 nm. Vitamin E was used as standard. The percentage of lipid peroxidation inhibition was estimated by comparing with control according to the following formula:

ACP %= $[(A_F - A_1)/(A_F - A_0)] \times 100$

Where A_0 = absorbance of control, A_1= absorbance of sample and A_F = absorbance of Fe^{+2} induced oxidation.

Qualitative phytochemical analysis: Extract obtained by reflux method were screened for the presence of different phytochemicals according to the method of Kumar *et al* [19], Ngbede *et al* [20], Ibrahim [21], Trease and Evans [22] with few modifications.

Thin layer chromatography: 40g thallus each of *M. paleacea*, *M. linearis* and *C. conicum* were extracted with 2 molar 100 ml hydrochloric acid for 30 minutes. Extract was filtered, mixed with diethyl ether and separated in a separating funnel. The extract was then concentrated and after complete solvent evaporation, solvent extracts were dissolved in 2 ml of methanol and subjected to TLC analysis. Silica gel 60 F_{254} pre-coated plates (Merck, Darmstadt, Germany) was employed as stationary phase and mixture of chloroform, methanol and acetic acid (8:1:1 v/v for *M. paleacea*; 9:1:1 v/v for *M. linearis*; 15:1:1 v/v for *C. conicum*) was used as mobile phase. Using a micropipette, 20 µl sample extract were spotted in the form of bands gradually over the plate and air dried. The plate was allowed to develop to a distance of 80 mm and was evaluated under UV light at 365 nm.

TLC bioautography assay: TLC plate developed was used for TLC bioautography assay. Plates were immersed for 1 second in 0.05% DPPH methanolic solution. Plates were removed quickly and excess DPPH was removed. The plates were scanned in a scanner and the images were stored for further processing.

RESULTS:

Preliminary quantitative phytochemical analysis: Quantitative phytochemical analysis determines the total amount of phytochemical present in the plant. Preliminary quantitative analysis in *M. paleacea*, *M. linearis* and *C. conicum* have revealed that total phenol content of the three bryophytes are 13.27, 1.18 and 1.47 GA eq/ g fresh weight tissue (**Fig. 1**) respectively. Similarly as seen in **Fig. 2, Fig. 3** total flavonol and orthodihydric phenol content ranged between 3.31-4.13 mg quercetin eqv/g FWT and 0.19-0.15 mg catechol eqv/g FWT respectively. Phenolic compounds present in plants are considered to be responsible for the significant free radical scavenging activity [23]. This free radical scavenging activity is considered to be due to their redox properties [24].

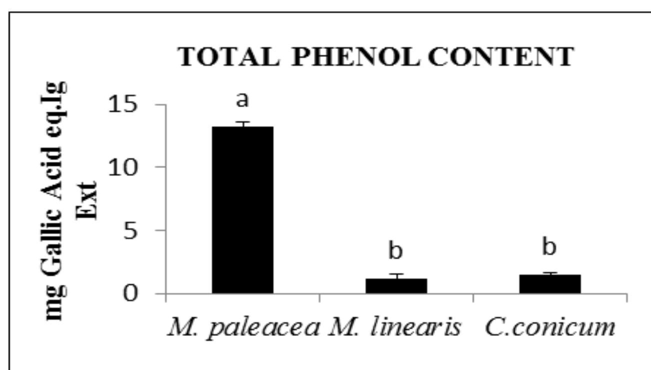


FIG. 1: TOTAL PHENOL CONTENT

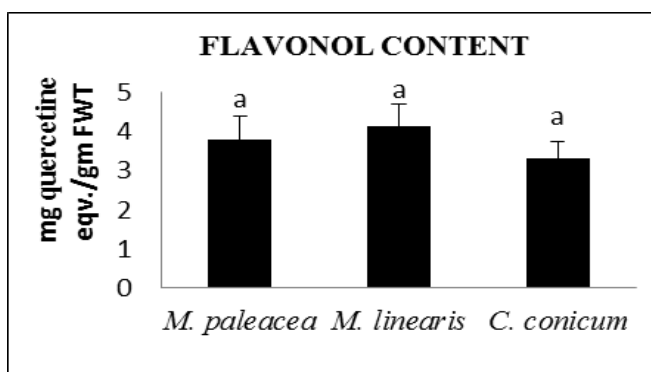


FIG. 2: TOTAL FLAVONOL CONTENT

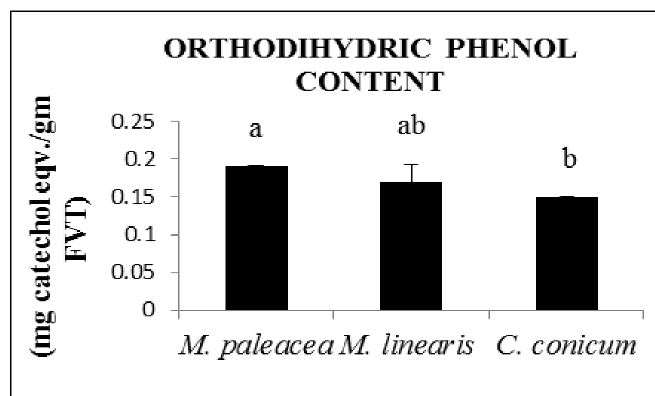


FIG. 3: TOTAL ORTHODIHYDRIC PHENOL CONTENT

Antioxidant activity: Three bryophyte samples analyzed in the present work showed strong free radical scavenging potentiality. DPPH radical scavenging activities of *M. paleacea*, *M. linearis* and *C. conicum* was found to be 18.82 mg/ml, 44.88 mg/ml , 68.44 mg/ml respectively (**Fig. 4**) and $ABTS^+$ radical scavenging activity was 5.97 mg/ml, 7.68 mg/ml, 5.14 mg/ml respectively (**Fig. 5**).

IC_{50} value of metal chelating activity was found to be 122.13 mg/ml for *M. paleacea*, 86.52 mg/ml for *M. linearis* 47.32 mg/ml for *C. conicum* (**Fig. 6**). Superoxide assay have revealed that among the three bryophyte samples studied, only *M. paleacea* have superoxide radical scavenging activity (38.41mg/ml) (**Fig. 7**).

Reducing capacity of an extract serves as a significant indicator of its antioxidant activity. Reducing potential of antioxidant present in *M. paleacea*, *M. linearis* and *C. conicum* was observed 6.38, 3.92, 3.76 µg Ascorbic acid equivalent /mg FWT respectively (**Fig. 8**) while, nitric oxide scavenging activity was found to be 838.96 mg/ml, 425.86 mg/ml, 552.66 mg/ml respectively (**Fig. 9**).

Present work revealed that *M. paleacea, M. linearis* and *C. conicum* have the high potential to inhibit lipid peroxidation. Ability to inhibit peroxidation of lipid by *M. paleacea* was 10.05 mg/ml, *M. linearis* was 10.18 mg/ml and *C. conicum* was 21.01 mg/ml (**Fig. 10**).

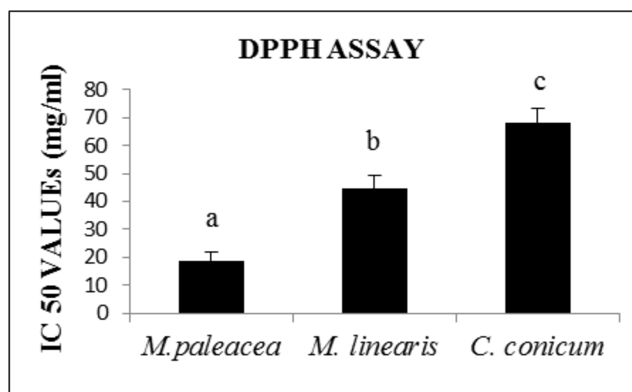


FIG. 4: DPPH RADICAL SCAVENGING ACTIVITY

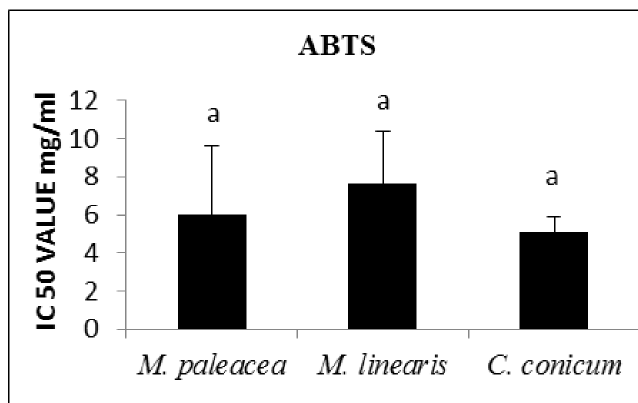


FIG. 5: ABTS RADICAL SCAVENGING ACTIVITY

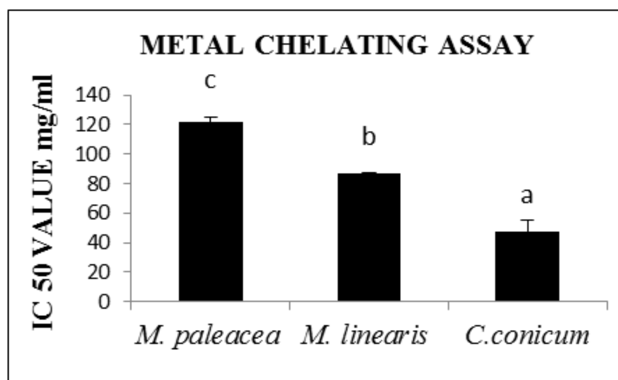


FIG. 6: METAL CHELATING ACTIVITY

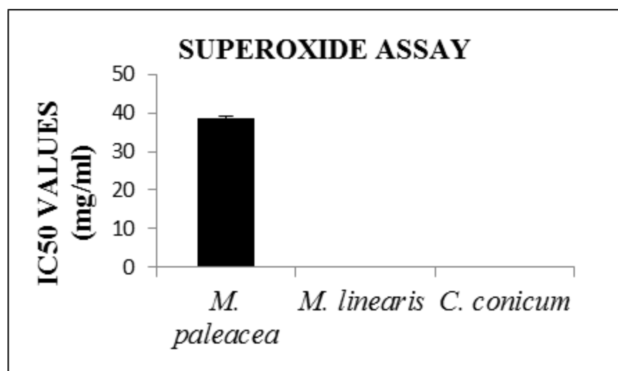


FIG. 7: SUPEROXIDE SCAVENGING ACTIVITY

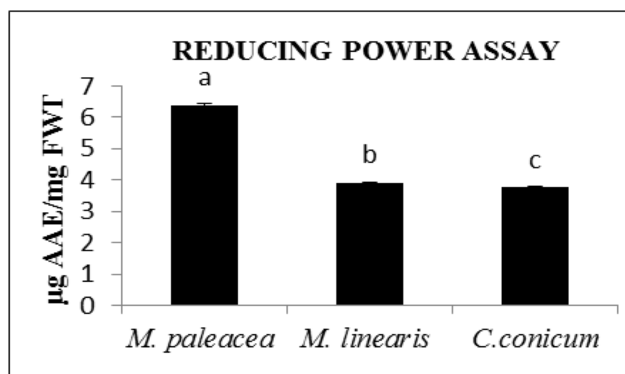


FIG. 8: REDUCING POWER

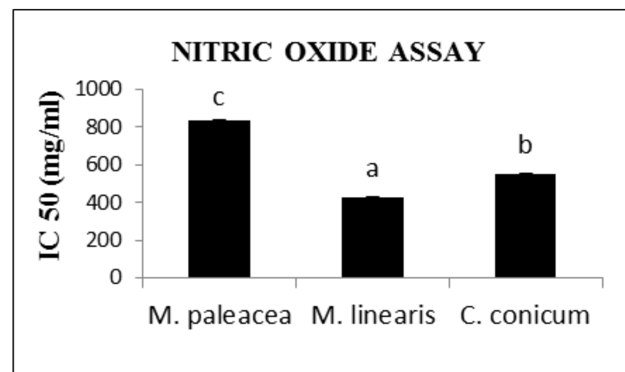


FIG. 9: NITRIC OXIDE SCAVENGING ACTIVITY

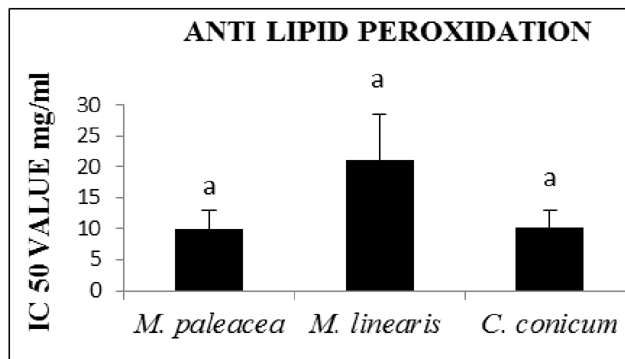


FIG. 10: ANTI-LIPID PEROXIDATION ACTIVITY

Qualitative phytochemical analysis: The preliminary phytochemical analysis gives information regarding the presence of primary or secondary metabolites in plant extract having clinical importance. Preliminary phytochemical analysis in *M. paleacea, M. linearis* and *C. conicum* have revealed the presence of resins, amino acid, phytosterol, tannin, flavonoid, cardiac glycoside and reducing sugar; while glycosides, anthraquinones and alkaloids were completely absent in the tested species. Triterpenoid was found to be present in *M. palaecea* and *M. linearis* only (**Table 1**).

TABLE 1: PRELIMINARY PHYTOCHEMICAL ANALYSIS IN *M. PALEACEA*, *M. LINEARIS* AND *C. CONICUM*, *MARCHANTIA PALEACEA, MARCHANTIA LINEARI, CONOCEPHALUM CONICUM*

Triterpenoid	+	+++	-
Resins	++	++	++
Glycosides	-	-	-
Amino acid	+	+++	+
Anthraquinones	-	-	-
Phytosterol	+++	++	++
Tannin	++	++	++
Flavonoid	+++	++	++
Cardiac Glycoside	++	+	+
Alkaloids	-	-	-
Reducing sugar	+	+	+

Correlation of total phenol, flavonol, orthodihydric phenol content and antioxidant potential: Total phenol content of the bryophyte samples studied in this work shows positive correlation with superoxide scavenging activity and iron reducing ability of the extract. Orthodihydric phenol content shows positive correlation with metal chelating activity and negative correlation with DPPH scavenging activity. Furthermore, metal chelating activity of the analyzed bryophyte sample has found to be negatively correlated with DPPH scavenging property (**Table 2**).

TABLE 2: Correlation between total phenol, flavonol, orthodihydric phenol content and antioxidative activity determined by different assay in *M. paleacea*, *M. linearis* and *C. conicum*

	DPPH	ABTS	MC	SO	RP	NO	ALP	TP	TF
ABTS	-0.293								
MC	**-0.998***	0.347							
SO	-0.880	-0.196	0.852						
RP	-0.904	-0.143	0.879	**.999***					
NO	-0.698	-0481	0.656	0.954	0.937				
ALP	0.040	0.944	0.017	-0.509	-0.462	-0.744			
TP	-0.870	-0.217	0.840	**1.000***	**.997***	0.960	-0.528		
TF	-0.540	0.963	0.587	0.076	0.130	-0.226	0.819	0.055	
OP	**-1.000***	0.320	**1.000***	0.866	0.892	0.676	-0.011	0.855	0.564

* indicates that the correlation is significant at the 0.05 level (2-tailed).
Abbreviations used: Total phenol (TPC), flavonoid (TFC) and orthodihydric phenol content (TOC), Free-radicals: DPPH, $ABTS^{+}$, superoxide (SO), nitric oxide (NO); metal chelating (MC), reducing power (RP) and Antilipid peroxidation (ALP).

Thin layer chromatography: TLC profiling of *M. paleacea*, *M. linearis* and *C. conicum* extract in Chloroform: methanol: acetic acid solvent system showed the presence of florescent bands of different colors at 365 nm. These bands confirm the presence of diverse group of bio molecules in these bryophyte species.

TLC bioautography assay: Different phytochemicals present in the studied bryophyte species are separated by thin layer chromatography and TLC bioautography assay was performed to determine the free radical scavenging activity of these bands. The bands with antioxidative activity were determined *in situ* with DPPH reagent. Yellowish bands produced on the purple background of the plates are considered to be produced due to free radical scavenging activity of phytochemicals present in corresponding bands [25]. In the present study, all the three plates showed yellowish bands due to bleaching of DPPH radical (**Fig. 11**), proving that the phytochemicals present in the three bryophyte species have free radical scavenging activity.

DISCUSSION: Preliminary phytochemical analysis can help to detect chemical constituents of plant that may have pharmacological importance. Preliminary phytochemical analysis of methanolic extract of *M. paleacea*, *M. linearis* and *C. conicum* revealed the presence of terpenoid, resin, amino acid, phytosterol, tannin, cardiac glycoside, flavonoid and reducing sugar.

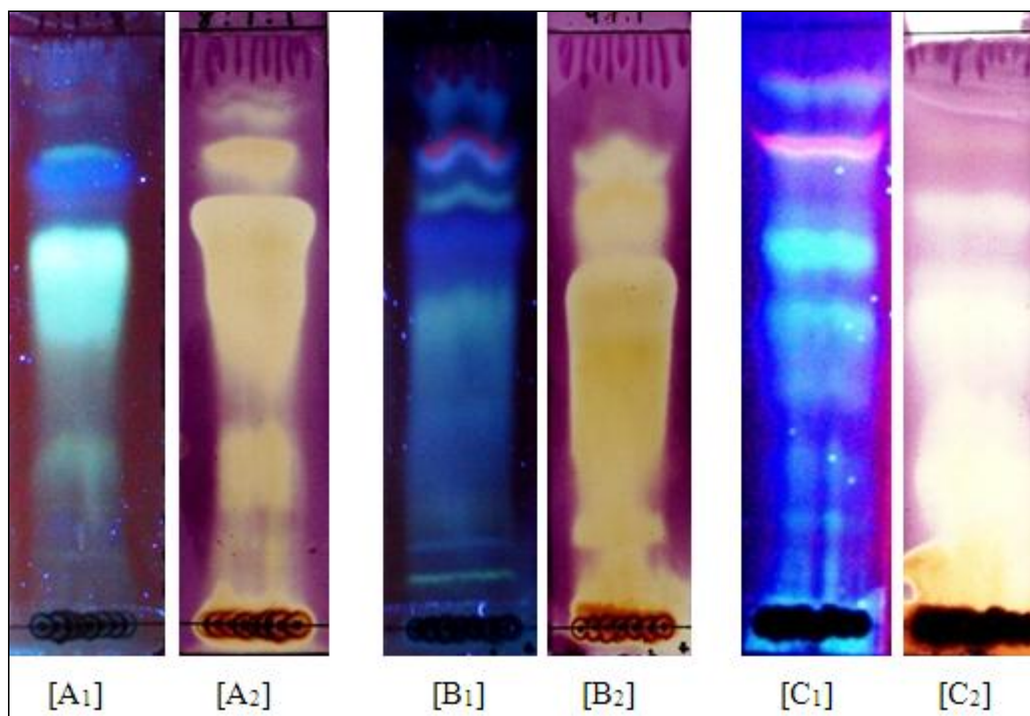


FIG. 11: THIN LAYER CHROMATOGRAPHIC PROFILE OF DIFFERENT SPECIMENS OF MARCHANTIALES: [A] *Marchantia paleacea*; [B] *Marchantia linearis*; [C] *Conocephalum conicum*. [A_1, B_1 and C_1]: Fluorescent bands observed under UV_{365}; [A_2, B_2 and C_2]: DPPH fingerprint of contemporary bands.

TLC profiling of all the three species further confirmed the presence of diverse bioactive natural products. Many workers [26, 27] stated that different phytochemicals present in the plant are responsible for its antioxidant activities. This has proved to be correct in this work where TLC bioautography assay had shown yellowish bands on plate, which showed the potentiality of different phytochemicals separated on TLC plate to scavenge DPPH free radicals. Through quantitative phytochemical analysis significant and varied levels of phenols and flavonoids were detected in the bryophyte samples analyzed.

Moreover quantity of extractable ortho-dihydric phenols also varied greatly among the bryophyte species investigated. All the species under analysis revealed the presence of antioxidant activity. The stable organic nitrogen free lipophilic radical DPPH is commonly used to investigate the scavenging activities of various sample extracts. Electron or hydrogen atom transferred from antioxidants normally neutralizes the DPPH radical. Here highest DPPH scavenging activity was shown by *M. paleacea*. DPPH radical scavenging activity of *M. polymorpha* extract was found to be higher [5] than the bryophyte sample studied in the present work. Manoj *et al* [28] and Dey *et al* [23] stated that the presence of phenolic compounds might be the cause of significant DPPH scavenging activities. This is at par with the findings of present work where *M. paleacea* with highest phenol content showed optimum DPPH scavenging activity.

In plants nitric oxide (NO) is highly essential signaling molecule [29] but the incessant nitric oxide radical production has enormous ill effects on health of all living forms. Different plant products may be effectual in neutralizing NO generation. All bryophyte species studied were found to possess the nitric oxide scavenging activity, although the nitric oxide scavenging activity was much lesser compared to other radical scavenging activities of the same bryophyte sample.

Superoxide radicals are one of the most powerful reactive oxygen species that are accountable for production of other radicals like hydroxyl radical. Present work shows that only *M. paleacea* have the potential to scavenge the superoxide radical. Superoxide radical scavenging activity of these bryophytes was found to be higher than that of medicinal plant *Vitis thunbergii* studied by Shyur *et*

al [30]. ABTS assay are primarily based on inhibition of absorbance of radical cation $ABTS^+$ by antioxidants. $ABTS^+$ radical showed higher profile in *C. conicum* than the other two. However $ABTS^+$ scavenging activity of studied bryophyte species was much less significant than that of *M. polymorpha* [5].

Fe^{+3}- Fe^{+2} reducing capacity of an extract is an important indication of its antioxidant activity. Generally, compound that donates hydrogen atom by breaking the free radical chain is related with the reducing power [28]. From the present work it can be concluded that all three bryophytes have significant reducing potential. Highest reducing activity was shown by *C. conicum*. Complexes of ferrozine and Fe^{2+} together with samples have metal chelating activity, and thus probability for the production of complexes to yield hydroxyl radical will be decreased. The methanolic extract of *C. conicum* showed the highest degree of metal chelating activity.

Metal chelating activity of tropical fruits [31] was found to be more or less similar to the present work. Lipid peroxidation is the oxidative degradation of lipids in which $OH^{\cdot}$ radicals cause cell membrane damage and initiate peroxidation of lipids. High chance of damage of cell membrane exists if this process is not terminated fast enough [32]. All the three bryophyte species displayed high ability to prevent peroxidation of lipid. In our work *M. paleacea* showed highest lipid peroxidation activity and the ability to prevent peroxidation lipid by these bryophyte samples are much higher than that of the methanolic extract of medicinal plant *Leucas plukenetii* [1].

High correlation between ortho-dihydric phenol and DPPH indicated that ortho-dihydric phenolic components might be responsible for the said activity whereas significant correlation between total phenol and reducing power establishes the fact that free phenols present in the sample might regulate redox potential of the system *in-vitro*. Similar findings were also obtained by Lai and Lim [33] and Kumar *et al* [34], who stated that phenolic compounds are powerful free radical scavengers and reducing agents.

CONCLUSION: From the results of above work, it is evident that all the bryophyte species analyzed here are potential source for antioxidants and can be pharmaceutically explored in future. The extract displayed strong inhibition of peroxidation of lipids and other free-radicals but their ability to scavenge superoxide was comparatively weaker. So, it is not erroneous to say that *M. paleacea*, *M. linearis* and *C. conicum* are the potentially valuable sources of bioactive materials which will be effective in protection of cellular system against oxidative damage leading to ageing and carcinogenesis. This test has opened the path for screening more genera of bryophytes taking into account their therapeutic and medicinal utility and to make further effort for assessment of bioactive components present in them.

ACKNOWLEDGEMENTS: We are highly indebted to "University Grant Commission" for funding this work under "Maulana Aazad National Fellowship" scheme and the director, Botanical Survey of India, Kolkata for facilitating the identification of specimens.

REFERENCES:

1. Nandy S, Paul HS, Barman NR, Chakraborty B: *In vitro* evaluation of antioxidant activity of *Leucas plukenetii* (Roth) Spreng. Asian Journal of Plant Science and Research 2012; 2(3): 254- 262.
2. Dey A, De JN: Antioxidative potenrial of Bryophytes: stress tolerance and commercial perspective: A review. Pharmacologia 2012; 3(6): 151-159.
3. Lobo V, Patil A, Phatak A, Chandra N: Free radicals, antioxidants and functional foods: impact on human health. Pharmacognosy Review 2010; 4(8): 118-126.
4. Sharathchandra K, Rajashekhar M: antioxidant activity in the four species of cyanobacteria isolated from a sulfur spring in the Western Ghats of Karnataka. International Journal of Pharma and Bio Sciences 2013; 4(1): 275-285.
5. Gokbulut A, Satilmis B, Batcioglu K, Cetin B, Sarer E: Antioxidant activity and luteolin content of *Marchantia polymorpha* L. Turkish Journal of Biology 2012; 36: 381-385.
6. Dey A, De JN: Nitric oxide inhibitory activity of bryophytes. Insight Ethnopharmacology 2012; 2(1): 3-4.
7. Kadam VB, Momin RK, Fatima S, Kadam UB: Estimation of total phenol in different plant parts of genus *Sesbania* in Maharashtra. International Journal of Pharmaceutical Research and Bio-Science 2013; 2(4): 202-206.
8. Atanassova M, Georgieva S, Ivancheva K: Total phenolic and total flavonoid contents, antioxidant capacity and biological contaminants in medicinal herbs. Journal of the University of Chemical Technology and Metallurgy 2011; 46(1): 81-88.
9. Mahadevan A, Sridhar R: Methods in physiological plant pathology. Sivakami Publication, Chennai 1986; 183-184.

10. Sharma SK, Goyal N: *In vitro* antioxidant activity of root extracts of *Heliotropium eichwaldi* Stued. Research Journal of Pharmaceutical, Biological and Chemical Sciences 2012; 3(1): 444-449.
11. Gaikwad SA, Kamble GS, Devare S, Deshpand R, Salvekar JP: *In-vitro* evaluation of free radical scavenging potential of *Cassia auriculata* L: Journal of Chemical and Pharmaceutical Research 2011; 3(4): 766-722.
12. Ho YL, Huang SS, Deng JS, Lin YH, Chang YS, Huang GJ: *In vitro* antioxidant properties and total phenolic contents of wetland medicinal plants in Taiwan. Botanical Studies 2012; 53: 55-66.
13. Marcocci L, Packer L, Droy-Lefaix MT, Sekaki A, Grades-Albert M: Antioxidant action of *Ginkgo biloba* extracts EGb 761. Methods in Enzymology 1994; 234: 462-475.
14. Fu W, Chen J, Cai Y, Lei Y, Chen L, Pei L, Zhou D, Liang X, Ruan J: Antioxidant, free radical scavenging, anti-inflammatory and hepatoprotective potential of the extract from *Parathelypteris nipponica* (Franch. Et Sav.) Ching. Journal of Ethnopharmacology 2010; 130(3): 521-528.
15. Li XC, Wu XT, Huang L: Correlation between antioxidant activities and phenolic contents of Radix Angelicae Sinensis (Danggui). Molecules 2009; 14: 5349-5361.
16. Dinis TCP, Madeira VM, Almeida LM: Action of phenolic derivates (acetoaminophen, salicylate and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Archives of Biochemistry Biophysics 1994; 315: 161-169.
17. Gulcin I: antioxidant activity of L-adrinaline: an activity structure insight. Chemico-Biological Interaction 2009; 179: 71-80.
18. Bouchet N, Barrier L, Fameoneau B: Radical scavenging activity of tannins from *Guier senegalen* (Combretaceae). Phyotherapy Research 1998; 12: 159-162.
19. Kumar A, Ilavarasan R, Jayachandan T, Decaraman M, Aravindhan P, Padmanabhan N: Phytochemical investigation on a tropical plant, *Syzygium cumini* from kattuppalayam, Erode District, Tamil Nadu, South India. Pakistan Journal of Nutrition 2009; 8(1): 83-85.
20. Ngbede J, Yakubu RA, Nyam DA: Phytochemical screening for active compound in *Canaruim schweinfurthii* (Atile) leaves from Ios North, Plateau state, Nigeria. Research Journal of Biological Science 2008; 3(9): 1076-1078.
21. Ibrahim HA, Ibrahim H: Phytochemical screening and toxicity evaluation on the leaves of *Argemone mexicana* linn (Papararaceal). International Journal of Pure and Applied Science and Technology 2009; 3(2): 39-43.
22. Trease G, Evans WC: Ed Textbook of Pharmacognosy. Balliere Publishers, 12th Edition 1983.
23. Dey A, De A, Ghosh P, Mukherjee S: Altitude and tissue type influence antioxidant potential of *Pellia endiviifolia* from Darjeeling Himalaya. Pakistan Journal of Biological Science 2013; 1-5.
24. Shahriar M, Hossain MI, Sharmin FA, Akhter S, Haque MA, Bhuiyan MA: *In-vitro* antioxidant and free radical scavenging activity of *Withania Somnifera* root. Iosr Journal of Pharmacy 2013; 3(2): 38-47.
25. Rumzhum NN, Rahman MM, Kazal MK: Antioxidant and cytotoxic potential of methanol extract of *Tabernaemontana. divaricata* leaves. International Current Pharmaceutical Journal 2012; 1(2): 27–31.
26. Kumar JK, Prasad AGD, Richard SA: Antioxidant activity and preliminary phytochemical analysis of medicinal legumes. Journal of Pharmacy Research 2012; 5(6): 3059-3062.
27. Sahoo S, Ghosh G, Das D, Nayak S: Phytochemical investigation and *in vitro* antioxidant activity of an endogenous medicinal plant *Alpinia nigra* B.L. Burtt. Asian Pacific Journal of Tropical Biomedicine 2013; 3(11): 871-876.
28. Manoj GS, Krishnan R, Murugan K: *In vitro* antioxidant potentiality and total phenolic content in selected bryophytes. Asian Pacific Journal Tropical Biomedicine 2012; 1-8.
29. Popova L, Tuan T: Nitric oxide in plants: properties, biosynthesis and physiological functions. Iranian Journal of Science and Technology Transaction A 2010; 34:173-183
30. Shyur LF, Tsung JH, Chan JH, Chiu CY, Lo CP: Antioxidant properties of extracts from medicinal plants popularly used in Taiwan. International Journal of Applied Science and Engineering 2005; 3(3): 195-202.
31. Lim YY, Lim TT, Tee JJ: Antioxidant properties of several tropical fruits: A comparative study. Food Chemistry 2007; 103: 1003-1008.
32. Mohan SC, Balamurugan V, Elayaraja R, Prabakaran AS: Antioxidant and phytochemical potential of medicinal plant *Kalanchoe pinnata*. International Journal of Pharmaceutical Science Research 2012; 3(3): 881-885.
33. Lai HY, Lim YY: Evaluation of antioxidant activities of the methanolic extracts of selected ferns in Malaysia. International Journal of Environment and Sustainable Development 2011; 2(6): 442-447.
34. Kumar KS, Ganesam K, Rao PVS: Antioxidant potential of solvent extracts of *Kappaphycus alvarezi* (Doty): an edible seaweed. Food Chemistry 2011: 107; 289-295.

How to cite this article:
Mukhia S, Mandal P, Singh DK, Singh D and Choudhury D: *In vitro* free-radical scavenging potential of three liverworts of Darjeeling, Himalaya. Int J Pharm Sci Res 2014; 5(10): 4552-61.doi: 10.13040/ IJPSR.0975-8232.5(10).4552-61

All © 2014 are reserved by International Journal of Pharmaceutical Sciences and Research. This Journal licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License.

This article can be downloaded to **ANDROID OS** based mobile. Scan QR Code using Code/Bar Scanner from your mobile. (Scanners are available on Google Playstore)

1.

Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(10):890-900

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Evaluation of anti-diabetic, antioxidant activity and phytochemical constituents of liverworts of Eastern Himalaya

Sumira Mukhia[1], Palash Mandal[1*], D. K. Singh[2] and Devendra Singh[2]

[1]*Plant Physiology and Pharmacognosy Research Laboratory, Department of Botany, North Bengal University, Siliguri, West Bengal, India*
[2]*Botanical Survey of India, CGO Complex, 3rd MSO Building, Salt Lake Sector I, Kolkata, West Bengal, India*

ABSTRACT

This work is an attempt to study the in-vitro anti-diabetic and antioxidant activity along with the analysis of phytochemical constituents of liverworts Ptychanthus striatus (Lehm. & Lindenb.) Nees (Lejeuneaceae), Pellia epiphylla (L.) Corda (Pelliaceae) and Bazzania oshimensis (Steph.) Horik. (Lepidoziaceae). Antioxidant activities were analyzed in-vitro by seven different assays: DPPH radical, metal chelating, superoxide, $ABTS^{+}$, reducing power, anti-lipid peroxidation and nitric oxide assay. Anti-diabetic potential was analysed by estimating α-amylase and α-glucosidase inhibitory activity of the plant. Detection and estimation of the constituent phytochemicals was done through qualitative test, quantitative test and TLC analysis. Results indicated significant anti-diabetic activity, antioxidant activity and occurrence of varied phytochemicals in studied plants. This finding paved the way for further analysis on these liverworts for obtaining phytochemicals of significant clinical and cosmetic importance.

Keywords: Anti-diabetic, antioxidant, phytochemicals, liverworts, TLC

INTRODUCTION

Diabetes mellitus (DM) is one among the major worldwide health problems of 21st century. Either incapability of pancreas to produce enough insulin (T1DM) or failure of body cell to respond to insulin (T2DM) results in diabetes [1]. Oxidative stress plays major role in both insulin secreting β-cell dysfunction and insulin resistance [2]. Multiple sources of oxidative stress are identified, viz. non-enzymatic, enzymatic and mitochondrial pathways. Non-enzymatic sources are: i) auto-oxidation of glucose generating $^{\bullet}OH$ radicals ii) glucose protein reaction during non-enzymatic glycation producing advanced glycosylation end products (AGEs) and iii) enhanced metabolism of glucose through polyol pathway resulting in $O_2^{\bullet-}$ production [3]. Generation of $O_2^{\bullet-}$ during mitochondrial respiratory chain is another non-enzymatic source of reactive species (RS) [4]. Persistent elevated hyperglycemia enhances glucose flux through glycolysis and tricarboxylic acid cycle and leads to an overdrive of mitochondrial electron transport chain and elevation of proton gradient, resulting generation of more $^{\bullet}O_2^{-}$ than mitochondrial antioxidant enzyme superoxide dismutase (SOD) can dismute. Enzymatic sources are enhanced activity of nitric oxide synthase (NOS), NAD(P)H oxidase and xanthin oxidase generating greater RS [3]. RS cause insulin resistance and disinfection of insulin secreting β-cells as they are low in free radical quenching enzymes like superoxide dismutase, catalase and glutathione peroxidase [5]. Antioxidants can, therefore, be considered effective in reducing increased blood sugar level [1]. Two enzymes α–amylase and α–glucosidase play key role in diabetes. By inhibiting these enzymes, the rate of glucose absorption and post-prandial blood sugar levels can be reduced.

Herbal remedies for diabetes and other oxidative stress related diseases are favoured due to least side effects [6]. Liverworts with its record of being used in several traditional medicine [7], have so far lagged behind in terms of exploration of its pharmacological activity and phytochemical constituents. Life strategies of bryophytes are considered to be the system of co-evolved adaptive qualities. They can survive extreme environmental conditions.

Most plants die when their relative water content falls below 20-50%. Only few plants, including bryophytes, can dry up to 4-13% and can still be resurrected and hence are referred as the desiccation tolerant plants [8]. During extreme dryness, they enter a stage of little intracellular water and almost no metabolic activity resulting in irreversible damage to lipids, protein and nucleic acids through production of RS. One of the important strategies shown by desiccation tolerant plants to limit damage to a repairable level is the production of antioxidants [9]. Considering that bryophytes can survive extreme climate and resurrect under favourable condition, it is assumed that this group of plant might possess strong antioxidative mechanism. Thus, in this work an attempt has been made to study the antioxidative and anti-diabetic activity along with phytochemical content of three important liverworts namely *Ptychanthus striatus, Pellia epiphylla* and *Bazzania oshimensis*. To our knowledge, present study is the first report detailing antidiabetic activity and phytochemical constituents of *P. striatus, P. epiphylla* and *B. oshimensis* found in the Darjeeling hills of Eastern Himalaya, India.

EXPERIMENTAL SECTION

Collection and identification

Liverwort samples were collected from Sinchel, Darjeeling in the month of July, 2013. The taxonomic identification was done by Dr. D.K. Singh, Scientist G and Dr. Devendra Singh Scientist C, Botanical Survey of India, Kolkata and voucher specimens have been deposited in the Central National Herbarium of the Botanical Survey of India, Howrah, India (CAL).

Animal material

Goat liver used for anti-lipid per oxidation assay was collected immediately after slay from slaughter house and experiment was conducted within 1 hour.

Chemicals used

Methanol (M), 2.2-diphenyl-1-picryl hydrazyl (DPPH), reduced nicotinamide adenine dinucleotide sodium salt monohydrate (NADH), nitro blue tetrazolium (NBT), sulfanilamide, phenazine methosulphate (PMS), ferrous chloride, trichloroacetic acid (TCA), ferrozine, thiobarbituric acid (TBA), glacial acetic acid, napthylethylene diamine dihydrochloride, $FeSO_4.7H_2O$, potassium dihydrogen phosphate (KH_2PO_4), potassium hydroxide (KOH), potassium ferricyanide, ethylene-diamine tetraacetic acid (EDTA), 2-deoxyribose, ferric chloride ($FeCl_3$), hydrogen peroxide (H_2O_2), sodium nitroprusside, gallic acid, Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), ninhydrin, lead acetate, aluminium chloride ($AlCl_3$), petroleum ether, copper acetate, chloroform, sodium hydroxide (NaOH), sulphuric acid, Dragendorff's reagent, hydrochloric acid, pyridine, α-glucosidase, α-amylase, pNPG (p-Nitrophenyl-α-D-glucopyranoside) were either purchased from Sigma Chemicals (USA) or of Merck analytical grade.

Methods of extraction

Collected liverworts were cautiously inspected to remove soil and other plant materials attached to it. As different species grow in close association with each other, sample of interest is cautiously separated from other associated taxa. It was then washed, air dried, crushed into powder and extracted with methanol.

Anti-diabetic assay

α-Glucosidase inhibitory activity

Alpha-glucosidase inhibitory activity was assessed according to the method described previously [10], with few modifications. Reaction was initiated by incubation of 2.5 ml phosphate buffer, reduced glutathione and 0.1 ml enzyme for 15 min followed by addition of 0.5 ml sample and 0.25 ml P-NPG. The mixture was then left for 15 minutes and finally reaction was stopped by adding 0.1 M Na_2CO_3. The absorbance was taken at 405 nm and the α-glucosidase inhibitory activity was calculated using formula:

$\% \ I = [1 - (A_s - A_b) / A_c] \times 100$, where

As = absorbance of sample, Ab = absorbance of blank, Ac = absorbance of control.

α- Amylase inhibitory activity

Method of Kim *et al.* [10] with few modifications was followed to study α- amylase inhibitory activity. Aqueous extract at various concentrations, 0.02 M sodium phosphate buffer containing α- amylase and starch were mixed and incubated for 10 min. The reaction was terminated by 1 ml dinitrosalicylic acid. Absorbance was measured at 540 nm by the following formula:

$\% \ I = [A_{540} C - A_{540} E] / [A_{540} C] \times 100$, where

A_{540} C = absorbance of control, A_{540} E = absorbance of extract

Determination antioxidant activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

DPPH scavenging activity was estimated by the method of Sidduraju *et al.* [11]. To 200 µl extract 2 ml DPPH solution was added and the reduction in solution colour was measured spectrophotometrically at 517nm. Scavenging activity of the sample was calculated by using following formula:

$\% I = [(A_0 - A_1) / A_0] \times 100$, where

I = inhibition, A_0 = absorbance of blank, A_1 = absorbance of test sample.

Nitric oxide (NO) scavenging activity

Nitric oxide scavenging activity was estimated following earlier method [12] with few modifications. To 2 ml sodium nitroprusside, 0.5 ml phosphate buffer and 0.5 ml extract were mixed and incubated at 25°C for 150 minutes, then 3 ml Griess reagent was added and allowed to stand at room temperature for 30 minutes. The absorbance was measured at 540 nm. Nitric oxide scavenging activity was calculated by the following formula:

$\% I = [(A_0 - A_1) / A_0] \times 100$, where

A_0 = absorbance of control and A_1 = absorbance of sample.

Superoxide radical (SO) scavenging assay

Superoxide scavenging activity was analysed following the method of Fu *et al.* [13] with few modifications. 1 ml sample and 1 ml nitroblue tetrazolium chloride, 1 ml nicotinamide adenine dinucleotide and 10 µl phenazine methosulphate were mixed and incubated for 30 min under fluorescent light. Absorbance was measured at 560 nm. Superoxide scavenging activity was estimated by using following formula:

$\% I = [(A_0 – A_1) / A_0 \times 100]$, where

A_0 = absorbance of control and A_1 = absorbance of sample.

$ABTS^+$ (2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) scavenging assay

$ABTS^+$ scavenging activity was studied by slightly modified method of Li *et al.* [14]. 1 ml sample and 2 ml $ABTS^+$ solution was incubated for 10 minutes. Scavenging activity was calculated based on percentage inhibition of absorbance at 734 nm by using formula

$\% I = [(A_0 – A_1) / A_0 \times 100]$, where

A_0 = absorbance of control and A_1 = absorbance of sample.

Metal chelating assay

Method of Dinis *et al.* [15] with slight modification was implemented for metal chelating activity estimation. 400 µl sample, 1.6 ml methanol, 40 µl of $FeCl_2$ and 80 µl Ferrozine were mixed and kept for 10 minutes before measuring the absorbance at 562 nm. Metal chelating ability was measured using formula

$\% I = (A_0 – A_1) / A_0 \times 100$, where

A_0 = absorbance of control and A_1 = absorbance of sample.

Reducing power assay

Iron reducing ability was studied by using Gulcin [16] method with few modifications. 1 ml sample, 2.5 ml phosphate buffer, 2.5 ml potassium ferricyanide were mixed and incubated for 20 min at 50° C. Then, 2.5 ml 10% trichloroacetic acid was added and centrifuged. 2.5 ml upper layer was collected, to this 2.5 ml of distilled water and 250 µl of 0.1% $FeCl_3$ was added and absorbance was measured at 700 nm.

Lipid peroxidation assay

Lipid peroxidation inhibition activity was studied following standard method of Bouchet *et al*. [17]. Goat liver was homogenized with phosphate buffered saline. The assay mixture contained in volume 0.1 ml of $FeSO_4$, 2.8 ml 10% liver homogenate and 100 µl sample. After 30 min incubation at 37°C, 1 ml reaction mixture was taken and to this, 2

ml of thiobarbituric acid-trichloroacetic acid was added and heated for 1h in water bath. Supernatant was used to measure absorbance at 535 nm. The percentage of lipid peroxidation inhibition was estimated using formula:

$\% I = [(A_F - A_1) / (A_F - A_0)] \times 100$, where

A_0 = absorbance of control, A_1= absorbance of sample and A_F = absorbance of Fe^{+2} induced oxidation.

Preliminary phytochemical analysis

Total phenol estimation

Method of Kadam *et al.* [18] with few modifications was implicated for phenol estimation. To 1 ml sample, 1 ml 95% ethanol, 5 ml distilled water and 0.5 ml 50% Folin ciocalteau reagent were added followed by addition of 1 ml 5% Na_2CO_3 after 5 min. The absorbance was measured at 725 nm. Standard curve was calibrated using different concentrations of gallic acid.

Total flavonoid estimation

Flavonoid content was estimated following method described by previous authors [19] with few modifications. 0.5 ml extract, 4 ml distilled H_2O and 0.3 ml 5% $NaNO_2$ were mixed followed by the addition of 0.3 ml of 10% $AlCl_3$ and 2 ml of 1.0 M NaOH after 5 and 6 min respectively. Finally, 2.4 ml of distilled water was added and absorbance was measured at 510 nm. Standard curve was prepared using different concentrations of quercetin.

2.7.3 Ortho-dihydric phenol estimation

Method of Mahadevan and Sridhar [20] with minor changes was followed to estimate ortho-dihydric phenol content. 0.5 ml extract, 0.5 ml of Arnow's reagent (10g each of $NaNO_2$ and sodium molybdate in 100ml H_2O), 5 ml H_2O and 1 ml of 1(N) NaOH were mixed. Absorbance was measured at 515 nm. Standard curve was prepared with different concentrations of catechol.

Qualitative phytochemical tests

Tests for steroid, tannin, triterpenoid, amino acid, resin, cardiac glycoside, alkaloid, flavonoid, reducing sugar, anthraquinones and glycosides were carried out according to the methods mentioned earlier in different literatures with few modifications [21, 22, 23, 24].

Thin layer chromatography

To screen the presence of secondary metabolites, TLC analysis (Silica gel 60 F_{254} pre-coated plates, Merck, Darmstadt, Germany) was performed using different solvent system ($CH_3COOC_2H_5$: CH_3OH : H_2O :: 100:13.5:10); ($CH_3COOC_2H_5$: CH_2O_2 : CH_3COOH : H_2O :: 100:11:11:26); ($CHCl_3$: CH_3COOH : CH_3OH : H_2O :: 64:32:12:8); (C_7H_8 : $CH_3COOC_2H_5$ :: 93:7). The developed TLC plates were then air dried and observed at 254 nm and 366 nm UV light. It was then sprayed with different spraying reagent. Spray reagents used were: KOH reagent, Berlin blue, Dragendorff's reagent, NP/PEG reagent, 10% ethanolic KOH, vanillin-sulphuric acid reagent, Fast blue salt reagent for detection of anthraglycoside, arbutin, alkaloids, flavonoid, coumarin, saponins and phenols respectively [25]. The movement of the phytochemical was determined by its retention factor (R*f*)

$$Rf = \frac{\text{Distance travel by solute}}{\text{Distance travel by solvent}}$$

Statistical analysis

All statistical analysis was performed using standard software SPSS (ver. 15.0). Data was expressed as mean ± standard deviation and its difference was compared using one-way analysis of variance (ANOVA). Duncan's Multiple Range Test (*P≤0.05*) was also used to find out the significant difference in values. Correlation and Principal Component Analysis was done by using XLSTAT 2009 (Addinsoft) and Smith's Statistical Package.

RESULTS AND DISCUSSION

Tight control of post ingestion glucose level is important therapeutic strategy for the management of diabetes. The inhibition of carbohydrate hydrolyzing enzyme, α-amylase and α-glucosidase is an important strategy to tackle diabetes. Several synthetic α-glucosidase and α-amylase inhibitors are in clinical use but their prices are high and have many side effects [26]. All the analysed plant in this work samples showed α-amylase and α-glucosidase inhibitory activity with the highest activity shown by *B. oshimensis* in both cases. Table 1 and Table 2 represent dose dependent α-glycosidase and α-amylase inhibition potential of *P. striatus, P. epiphylla* and *B. oshimensis*. It is hypothesized that higher polyphenol content of the extract of *B. oshimensis* might be responsible for this inhibitory

activity. Similar kind of finding was also reported by Saravanam and Parimelazhagan [27]. In DM patients during persistent hyperglycemia, low density lipoprotein oxidation by the overproduction of RS contributes to oxidative protein damage and, therefore, to the pathogenesis of diabetic's complication like arteriosclerosis. All three studied liverwort extracts prevented oxidation of lipid (Figure 1). This property might be attributed to their ability to scavenge $OH^{\cdot}$ that causes peroxidation of lipid. *B. oshimensis* displayed lower IC_{50} value compared to *P. epiphylla* and *P. striatus*, which implies that the former has better lipid peroxidation inhibitory potential.

Table 1: The percent inhibition of yeast alpha-glucosidase by methanolic extracts of *P. striatus, P. epiphylla* and *B. oshimensis* at varying concentration

Concentration (mg/ml)	% Inhibition by *P. striatus*	IC_{50} mg/ml	% Inhibition by *P. epiphylla*	IC_{50} mg/ml	% Inhibition by *B. oshimensis*	IC_{50} mg/ml
1	45.89		42.89		43.20	
2	50.17		46.89		51.03	
4	54.32	2.18	67.91	1.88	65.23	1.74
7	65.88		83.46		78.95	
10	71.21		90.83		88.96	

Table 2: The percent inhibition of yeast alpha-amylase by methanolic extracts of *P. striatus*, *P. epiphylla* and *B. oshimensis* at varying concentration

Concentration (mg/ml)	% Inhibition by *P. striatus*	IC_{50} mg/ml	% Inhibition by *P. epiphylla*	IC_{50} mg/ml	% Inhibition by *B. oshimensis*	IC_{50} mg/ml
1	49.49		43.89		38.26	
2	51.65		48.19		58.39	
4	56.16	1.72	71.94	1.58	66.32	1.53
7	65.88		86.93		86.93	
10	71.21		95.21		92.13	

As studies suggest that oxidative stress imposed by hyperglycemia induced RS play major role in the pathogenesis of T2DM, antioxidative therapies reducing oxidative stress can be effective in controlling diabetic complications [28]. Figure 1and 2 represents the potential of *P. striatus, P. epiphylla* and *B. oshimensis* to scavenge different free radicals that might be generated in the living system. On examination of antioxidant activity, studied plant extracts potentially scavenged DPPH radical. *B. oshimensis* extract showed high scavenging activity of DPPH radical, which might be attributed to their ability to donate electron or hydrogen radical to $DPPH^{\bullet}$ and stabilize them. Similarly, ABTS radical scavenging activity is also greater in case of *B. oshimensis* than other two. The result suggests that plant extracts produce sufficient donor hydrogen molecules that reduce $DPPH^{\bullet}$ and $ABTS^{+}$ radicals. Similar quantum of activity was also shown by moss *Polytrichastrum alpinum* [29].

Free ferrous ions are the most powerful pro-oxidants and thus its reduction is important for protection against oxidative damage and lipid peroxidation by Fenton reaction [27]. The metal chelating assay shows that *P. epiphylla* has higher chelating activity. Fe^{2+} and ferrozine forms complexes to generate hydroxyl radical, in this assay plant extract may have interfered in the complex formation resulting in above mentioned chelating effect. Superoxide is considered one among strongest radicals, as it acts as a precursor for other RS like H_2O_2, O_2^{-} and $OH^{\bullet}$ that are extremely reactive and capable of damaging bio-molecules of living system. Three liverwort sample scavenged superoxide in the following order *P. striatus* > *B. oshimensis* > *P. epiphylla*.

Reducing power is considered as an effective assay for assessment of antioxidant reducing potential. It is the ability to reduce Fe^{3+}-ferricyanide complex to Fe^{2+}. It is assumed that reductive ability of plant extract might be due to the presence of compounds that are electron donors having the capability to reduce oxidized Fe^{3+} to Fe^{2+} [30]. *B. oshimensis* have the highest capability to reduce oxidized Fe^{3+} to Fe^{2+} than other two plant samples. Nitric oxide (NO) is an important bio-molecule, but its sustained level is toxic to tissue. NO generates spontaneously from sodium nitroprusside in aqueous solution and reacts with oxygen to form nitrite (NO_2^{-}) anion. Scavengers of NO compete with oxygen to inhibit the formation of nitrite [31]. Present study demonstrated that the methanolic extract of *B. oshimensis* has better NO scavenging activity than *P. striatus* and *P. epiphylla*.

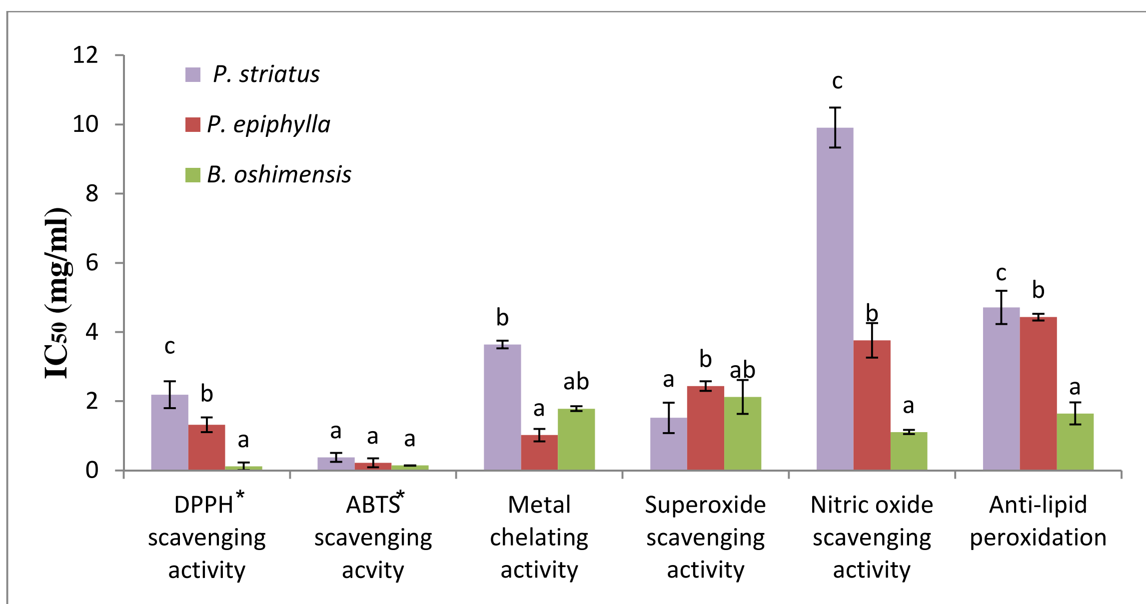


Figure 1: Radical scavenging activity of crude extracts of *P. striatu, P. epiphylla* and *B. oshimensis*

Values with different letters (a, b, c) are significantly ($p < 0.05$) different from each other by Duncan's multiple range test (DMRT)

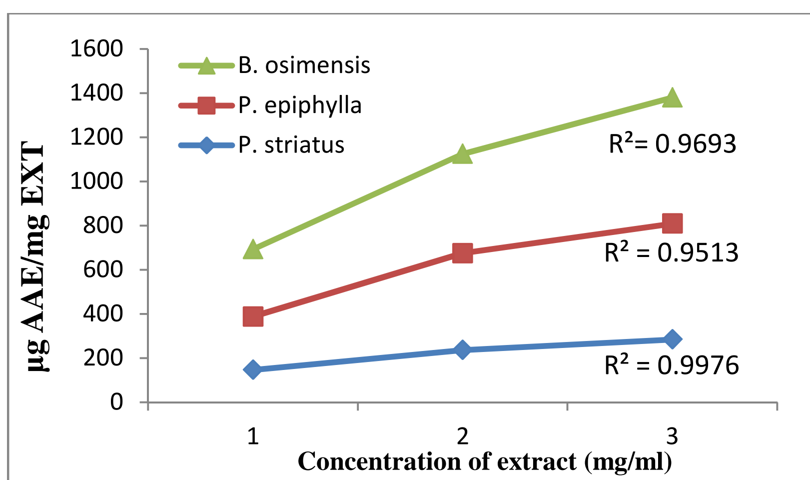


Figure 2: Reducing potential of crude extract of *P. striatus, P. epiphylla* and *B. oshimensis*

Table 3: Preliminary phytochemical analysis of *P. striatus, P. epiphylla* and *B. oshimensis*

	Ptychanthus striatus	*Pellia epiphylla*	*Bazzania oshimensis*
Steroid	+++	++	+
Tannin	++	+++	+
Triterpenoids	+++	+++	+
Amino acid	++	+++	+
Resin	++	++	-
Cardiac glycoside	+++	++	++
Flavonoids	++	++	++
Alkaloid	-	-	-
Reducing sugar	++	+	+
Anthraquinones	+	+	+
Glycosides	-	-	-

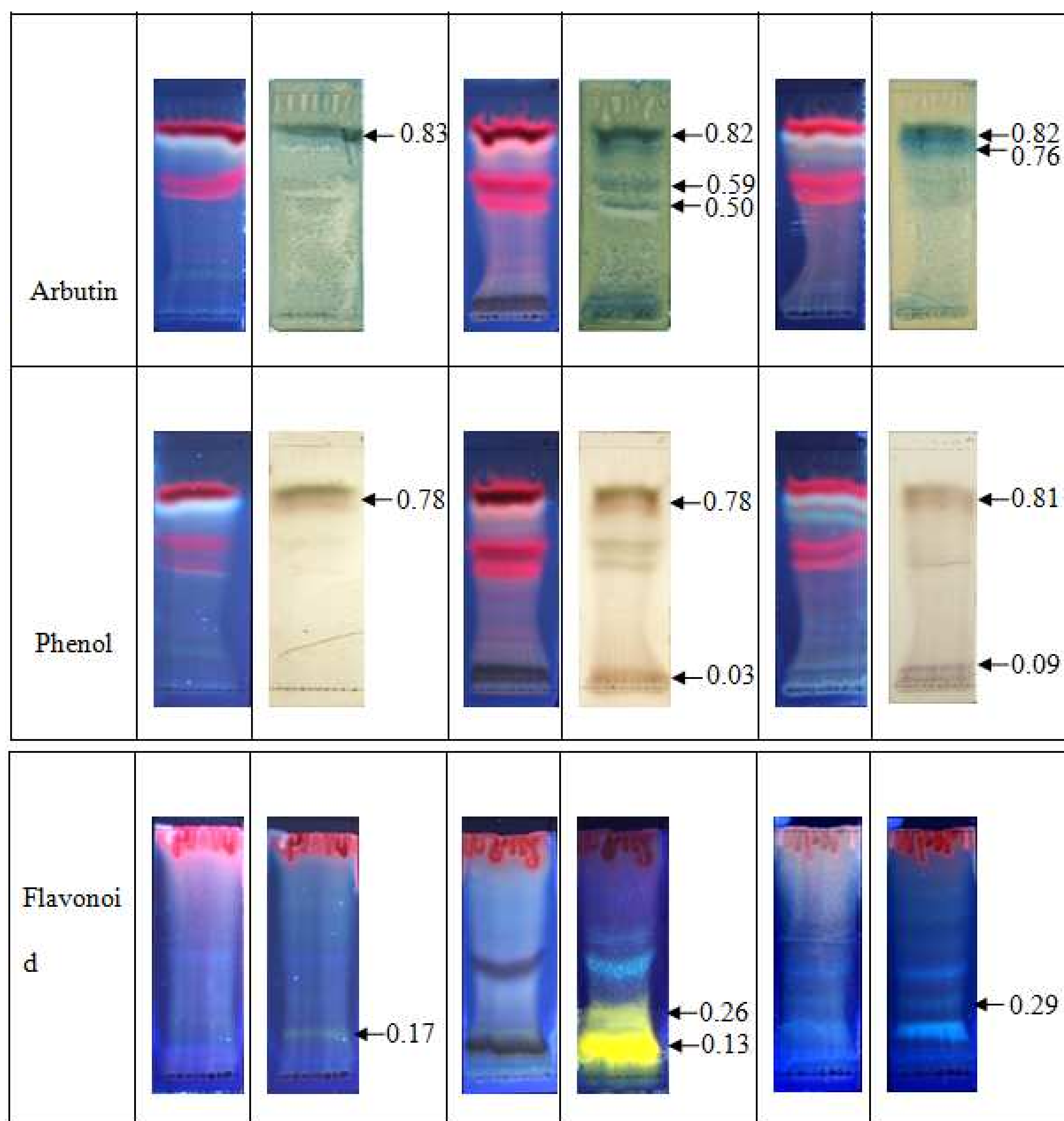
(+++) appreciable amount; (++) moderate; (+) trace amount; (-) completely absent.

Study claims that phytochemicals especially phenols to be accountable for oxidative stress reducing ability [32]. An insight into chemical nature can present rich data in understanding correlation between phenolic compound and stress reducing ability. By qualitative analysis, the phytochemicals like steroid, tannin, triterpenoids, amino acids, resin, cardiac glycoside, flavonoids, reducing sugar and anthraquinones are proven to occur in all studied hepatics (Table 3). These secondary metabolites present in the plant are accountable for the displayed antioxidative [33] and α-glucosidase inhibitory [28] activity by such plants. TLC profiling provides impressive information regarding the

existence of different phytochemicals. TLC analysis of *P. striatus*, *P. epiphylla* and *B. oshimensis* further confirmed the presence of phenolic compounds like coumarin, alkaloid, anthraglycoside, arbutin, phenol and flavonoids (Table 4). Different phyto-compounds encompass unique R_f values in different solvent system providing an important clue in understanding their polarity.

Table 4: Determination of chemical constituents of *P. striatus, P. epiphylla* and *B. oshimensis* by TLC

	Ptychanthus striatus		*Pellia epiphylla*		*Bezzania oshimensis*	
	Pre Detection	**Post Detection**	**Pre Detection**	**Post Detection**	**Pre Detection**	**Post Detection**
Coumarin		←0.78		←0.13		←0.70 ←0.14
Alkaloid		←0.78		←0.04		←0.76
Anthraglycosides		←0.86 ←0.58 ←0.52		←0.85 ←0.57 ←0.52		←0.86 ←0.58 ←0.52



The phytochemical screening indicated that the phenolic compounds to be major components of the liverwort extract. The quantitative estimation of crude chemical compounds present in the studied hepatics is summarized in Table 5. Quantification of total phenol, flavonoid and ortho-dihydric phenol demonstrated that *B. oshimensis* extract has higher phenol, flavonoid and ortho-dihydric phenol content compared to *P. striatus* and *P. epiphylla.* High phenolic compound content of *B. oshimensis* extract justifies the higher antioxidant activity showed by the plant. Flavonoids are considered as good inhibitors of α-glucosidase and also the regulators of oxidative stress induced diabetic complications [34]. Result from our work also supported this fact, where *B. oshimensis* with higher flavonoid content showed higher α-glucosidase inhibitory activity.

Table 5: Total phenol, flavonoid and orthodihydric phenol content of crude methanolic extract of *P. striatus, P. epiphylla* and *B. oshimensis*

	TPC (mg gallic acid eqv / g EWT)	TFC (mg quercetin eqv / g EWT)	OPC (mg catechol eqv / g EWT)
P. striatus	32.27±0.21	9.78±0.06	2.37±0.06
P. epiphylla	41.29±0.18	13.56±0.05	6.066±0.06
B. oshimensis	63.33±0.19	17.88±0.05	6.18±0.07

Abbreviation used: Extractive weight (EWT), Total phenol (TPC), flavonoid (TFC) and ortho-dihydric phenol content (OPC)

Data in the literature are sometimes contradictory regarding correlation between antioxidant activity of the plants and their polyphenol content. Strong correlation was observed between the two by some authors [28][35] while others observed no such correlation [36][37] or very weaker one only. From the Pearson correlation test (Table 6), significant positive correlation was absorbed between alpha glucosidase inhibitory activity and ABTS radical scavenging activity. While, flavonoid content and DPPH• scavenging activity; superoxide scavenging and metal chelating activity; reducing power and ABTS radical scavenging activity; reducing power and nitric oxide scavenging activity were found to be negatively correlated with each other. It is difficult to explain relationship between antioxidant activity and phenolic content using statistical tools because antioxidant potential of single compound within a group can differ extremely and thus an equal amount of phenolics doesn't always show similar radical scavenging activity. Moreover different antioxidant assay used as well as synergism of antioxidant compounds with each other may also affect antioxidant activity significantly [38].

Table 6: Correlation between antioxidant content and radical scavenging assay of three liverwort samples

	DPPH•	$ABTS^+$	NO	MC	SO	RP	LP	α-G	TPC	TFC
$ABTS^+$	0.961									
NO	0.931	0.996								
MC	0.617	0.812	0.862							
SO	-0.569	-0.776	-0.830	**-0.998***						
RP	-0.944	**-0.998***	**-0.999***	-0.843	0.810					
LP	0.949	0.824	0.768	0.338	-0.281	-0.791				
α-G	0.969	**0.999***	0.992	0.792	-0.754	-0.996	0.843			
TPC	-0.989	-0.910	-0.868	-0.497	0.444	0.885	-0.985	-0.924		
TFC	**-0.999***	-0.974	-0.949	-0.658	0.612	0.960	-0.931	-0.981	0.980	
OPC	-0.802	-0.936	-0.965	-0.965	0.948	0.954	-0.572	-0.924	0.706	0.832

**. Correlation is significant at the 0.05 level (2-tailed).*

Abbreviation used: superoxide (SO), nitric oxide (NO), metal chelating (MC), Antilipid peroxidation (ALP), α-glucosidase (αG), α-amylase (αA)

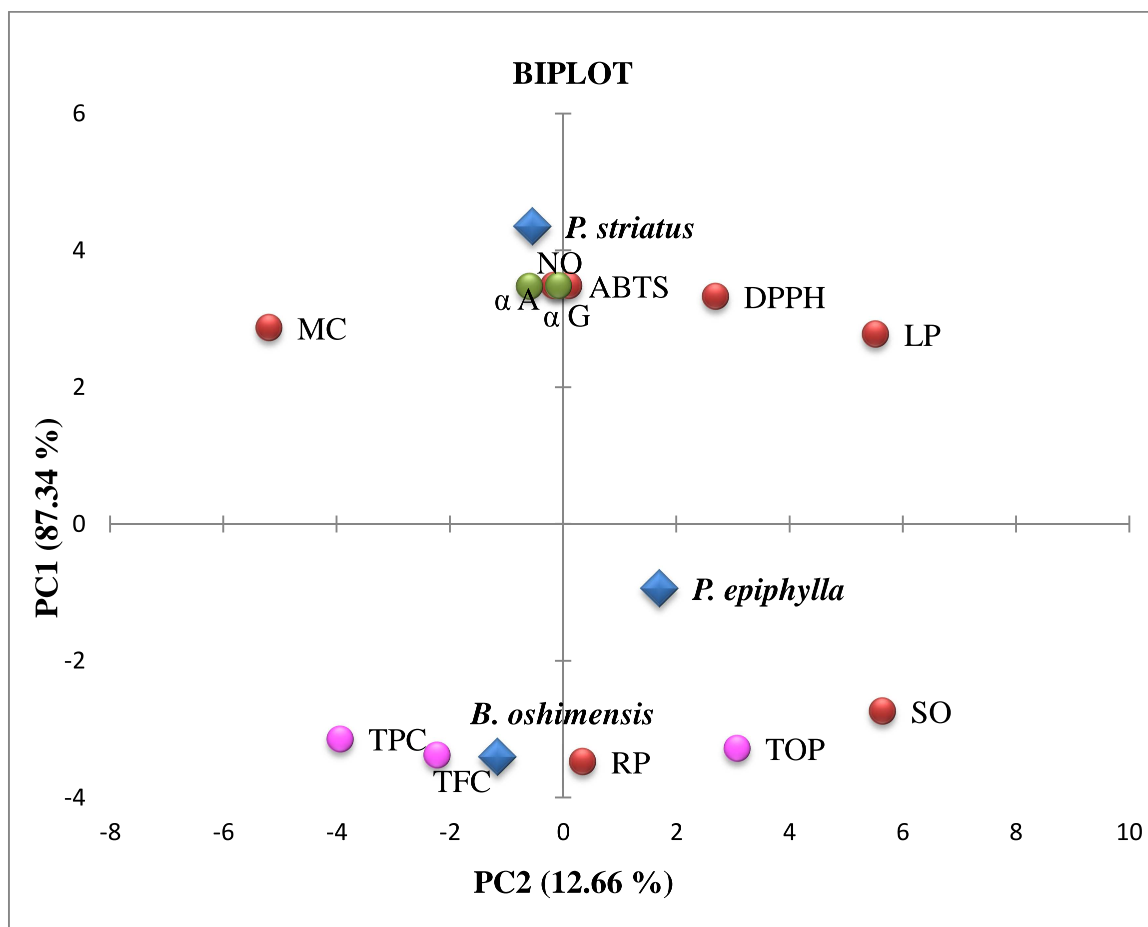


Figure 3: Principal component analysis of radical scavenging activity, anti-diabetic activity and phenolic compounds

For better understanding of relationship among the analyzed variables, Principal Component Analysis (PCA) was applied. First two principal components (PC1 and PC2) obtained after statistical analysis accounted for 87.34 % and 12.66 % of the variance respectively. The loading plots of first and second principle components are shown in figure 3. The loading of PC1 had a strong correlation with *in vitro* anti-diabetic and radical scavenging but not with the phenolic compounds indicating these antioxidant and antidiabetic activity to be controlled by compounds other than phenolics. Whereas the Principal Component 2 (PC2) had a high correlation with reducing power and super oxide scavenging activity as well as total phenolic compounds.

CONCLUSION

Till date, higher group of plants like angiosperm have been mostly investigated for the drug development, but studies suggest that plants belonging to same group have similar phytochemical constituents. Thus for the discovery of new therapeutic substances, there’s a constant need to search for novel resources within unexplored group of plants like liverworts (bryophytes). At present, only about 5% percent of liverworts are chemically studied worldwide [39]. Similarly, in India only recently, studies have been initiated to screen the bioactivities as well as phytochemicals of liverworts. This work confirms the *in vitro* anti-diabetic and free-radical scavenging potential as well as the existence of versatile groups of bioactive phytochemicals in three liverworts namely *P. striatus, P. epiphylla* and *B. oshimensis* collected from Eastern Himalaya.

Acknowledgement
We are highly indebted to “University Grant Commission" for funding this work under “Maulana Aazad National Fellowship” scheme and the Director, Botanical Survey of India, Kolkata for facilitating the identification of specimens.

REFERENCES

[1] NMH Da Costa Mousinho; JJ Van Tonder and V Steenkamp. *Nat Prod Res*., **2013**, 8, 1279-1284.
[2] G Drews; P Krippeit-Drews and M Dufer. *Pflugers Arch*., **2010**, 460, 703-718.
[3] JS Johansen; AK Harris; JR David and A Ergul. *Cardiovasc Diabetol*., **2005**, 4, 5.
[4] AY Andreyev; YE Kushnareva and AA Starkov. *Biochem (Mosc)*., **2005**, 70, 200-214.
[5] M Tiedge; S Lortz; J Drinkgern and S Lenzen. *Diabetes*., **1997**, 46, 1733-1742.
[6] P Mandal; Mitali G; MT Kumar and DP Prasad. *Int J. Drug Res*., **2010**, 2, 830-843.
[7] S Mukhia; P Mandal; DK Singh; D Singh and D Choudhury. *Int J. Pharm Sci Res*., **2014**, 5, 4552-4561.
[8] P Scott. *Ann Bot*., **2000**, 85, 159-166.
[9] I Kranner; RP Beckett; S Wornik; M Zorn and HW Pfeifhofer. *Plant J*., **2002**, 31, 13-24.
[10] YM Kim; YK Jeong; MH Wang; W Lee and H Rhee. *Nutrition*., **2005**, 21, 756-761.
[11] P Sidduraju; P Mohan and K Becker. *Food Chem*., **2002**, 79, 61-67.
[12] L Marcocci; L Packer; MT Droy-Lefaix; A Sekaki and M Grades-Albert. *Methods Enzymol*., **1994**, 234, 462-475.
[13] W Fu; J Chen; Y Cai; Y Lei; Chen L; Pei L, *et al*. *J. Ethnopharmacol*., **2010**, 130, 521-528.
[14] XC Li; XT Wu and L Huang. *Molecules*., **2009**, 14, 5349-5361.
[15] TCP Dinis; VM Madeira and LM Almeida. *Arch Biochem Biophys*., **1994**, 315, 161-169.
[16] I Gulcin. *Chem Biol Interact*., **2009**, 179, 71-80.
[17] N Bouchet; L Barrier and B Fameoneau. *Phytother Res*., **1998**, 12, 159-162.
[18] VB Kadam; RK Momin; Fatima S and UB Kadam. *Int J. Pharm Res Biosci*., **2013**, 2, 202-206.
[19] M Atanassova; S Georgieva and K Ivancheva. *J. Chem Technol Metall*., **2011**, 46, 81-88.
[20] A Mahadevan and R.Sridhar. Methods in physiological plant pathology. Sivakami Publication, Chennai, **1986**.
[21] A Kumar; R Ilavarasan; T Jayachandan; M Decaraman; P Aravindhan; N. Padmanabhan and MRV Krishnan. *Pak J. Nutr*., **2009**, 8, 83-85.
[22] J Ngbede; RA Yakubu and DA Nyam. *Res J. Biol Sci*., **2008**, 3, 1076-1078.
[23] HA Ibrahim and H Ibrahim. *Int J. Pure Appl Sci Technol*., **2009**, 3, 39-43.
[24] G Trease and WC Evans. Ed Textbook of Pharmacognosy, 12th Edition, Balliere Publishers, **1983**.
[25] H Wagner and S Bladt. Plant Drug Analysis: A Thin Layer Chromatography Alas. 2nd Edition, Spinger-Verlag. Berlin, Heidelberg, **1996**, 349-362.
[26] D Gomathi; M Kalaiselvi and C Uma. *Int Res J. Pharm*., **2012**, 3, 226-229.
[27] S Saravanam and T Parimelazhagan. *Food Sci Hum Wellness*., **2014**, 3, 56-64.
[28] M Ghosal and P Mandal. *Int J. Pharma Biol Sci*., **2013**, 4, 671-684.
[29] HD Bhattarai; B Paudel; HL Lee; H Oh and HY Joung. *Z Naturforsch C*., **2009**; 64: 197-200.
[30] P Jayanthi and P Lalitha. *Int J. Pharm Pharm Sci*., **2011**, 3, 126-128.
[31] P Mandal and M Ghosal. *Int J. Pharm Sci Rev Res*., **2012**, 13, 39-47.

[32] A Subba and P Mandal. *Free Rad Antiox*., **2015**, 5, 6-12.
[33] S Sahoo; G Ghosh; D Das and S Nayak. *Asian Pac J. Trop Biomed*., **2013**, 3, 871-876.
[34] CV Montefusco-Pereira; MJ de Carvalho; AP de Araujo Boleti; LS Teixeira; HR Matos and ES Lima. *Appl Biochem Biotechnol*., **2013**, 170, 1367-1378.
[35] C Coman; OD Rugina and C Socaciu. *Not Bot Horti Agrobo*., **2012**, 40, 1842-4309.
[36] E Souri; G Amin; H Farsam and M Tehrani. *DARU J. Pharm Sci*., **2008**, 16, 83-87.
[37] SY Qusti, AN Abo-khatwa and MAB Lahwa.. *Eur J. Biol Sci* **2010**, 2, 40-51.
[38] MP Kahkonen; AI Hopia and H Marina. *J. Agric Food Chem*., **2001**, 49, 4076-4082.
[39] Y Asakawa. Maechantiophyta: a good source of biologically active compounds, In: H. Mohamed, B. B. Baki, A.N. Boyce & P.K.Y. Lee (Eds), Bryology in the New Millennium, University of Malaya, Kuala Lampur, **2008**, 367.

Research Article
ISSN: 0974-6943

Available online through
http://jprsolutions.info

Study of Bioactive Phytoconstituents and *In-Vitro* Pharmacological Properties of Thallose Liverworts of Darjeeling Himalaya

Sumira Mukhia[1], Palash Mandal[1]*, D. K. Singh[2], Devendra Singh[2]

[1]Plant Physiology and Pharmacognosy laboratory, Department of Botany, University of North Bengal, Siliguri, West Bengal, India.
[2]Botanical Survey of India, CGO Complex, 3rd MSO Building, Salt Lake Sector I, Kolkata, West Bengal, India.

Received on:12-04-2017; 18-05-2017;Accepted on: 26-05-2017

ABSTRACT

Background: Study on bryophytes has long been neglected although many biological activities and many phytochemicals unique to plant kingdom are reported from them. **Objective:** This work aims to study the *in vitro* antioxidant along with anti-diabetic activities and phytochemical constituents of three liverworts of Marchantiales group namely *Plagiochasma cordatum*, *Marchantia subintegra* and *Marchantia emarginata*. Furthermore an effort was made to find out the solvent system for extraction of phytochemicals showing better pharmacological activities and also the correlation between pharmacological activities and bioactive components. **Materials and methods:** For the said purpose DPPH, ABTS, nitric oxide, superoxide, metal chelating, reducing power, α-amylase inhibitory and α-glucosidase inhibitory assay were performed. Quantitative test for phenol, flavonoid, ortho-dihydric phenol, steroid and tannin was done. Qualitative test and thin layer chromatographic analysis was performed for detection of constituent phytochemicals. Pearson correlation analysis, Principle component analysis and Heatmap were done for studying the association between the bioactive components of the studied plants and their pharmacological activities. **Results:** All the studied plants showed free radical scavenging and anti-diabetic activity but the activity of *Plagiochasma* sp. was superior to *Marchantia* spp. Among different extracts, diethyl ether extract showed significant potential to scavenge different free radicals analyzed, thus suitable for extraction of bioactive phytoconstituents for oxidative stress management. **Conclusion:** Potent pharmacological activities of this less explored group of plants pave the pathway for isolation of bioactive compounds of therapeutic and medicinal value.

KEY WORDS: Liverworts, antioxidants, anti-diabetic, phytochemicals

1. INTRODUCTION

Bryophytes are reported of having different biological activities like antimicrobial, antifungal, cytotoxic, DNA polymerase-β, α-glucosidase, NO production inhibitory, antioxidant and muscle relaxing activities[1] triggered by bioactive components they possess[2]. Liverworts contain lipophilic mono-sesqui, diterpenoids, aromatic compounds like bibezyles, bisbibenzyles, benzoates, cinnamates, long chain phenols, napthalenes, phthalides, coumarins that contribute to these biological activities.

Various compounds isolated from liverworts are potential antioxidants. Herbertene -1,2-diol, mastigophorene C, mastigophorene D isolated from *Mastigophora diclados* showed strong antioxidative activity[3].

***Corresponding author**
Dr.Palash Mandal
Plant Physiology and Pharmacognosy laboratory, Department of Botany, University of North Bengal, Siliguri, West Bengal, India.

Phenolics like marchantin, riccardins and pleatins isolated from different species of liverworts also demonstrated antioxidative capacity[4,5]. Antioxidants are compounds that neutralizes free radicals which causes direct damage to biological molecules such as DNA, proteins, lipids, carbohydrates leading to many chronic diseases, such as atherosclerosis, cancer, diabetes, aging, and other degenerative diseases in humans. Diabetes mellitus type 2 is characterized by postprandial hyperglycemia resulting from impaired insulin secretion. Hyperglycemia increases free radical generation leading to oxidative damage and diabetic complications. Thus antioxidant therapy targeting diabetes induced oxidative stress is worth considering for prevention of downstream diabetes complications[6]. Digestive enzymes α-amylase and α-glucosidase hydrolyses starch into simple monosaccharides. Increased activity of these enzymes results in post prandial hyperglycemia, therefore, by inhibiting the activity of these enzymes postprandial glucose level can be reduced.

Bryophytes, liverworts in particular, has been used in Native American, Indian and Chinese traditional medicine since ancient time[7], however, fewer efforts has been made to explore biological activity of this plant group worldwide. Darjeeling hills, the part of Eastern Himalayan region of India, favour the luxuriant growth of various species of liverworts. However, till date only eight liverwort species are studied for their biological activities. This study was performed to determine the phytochemical constituents and antioxidative, anti-diabetic activity of liverworts *Plagiochasma cordatum*, *Marchantia subintegra*, *Marchantia emarginata* from Darjeeling Himalaya.

2. MATERIALS AND METHOD

2.1. Collection and identification

Liverwort samples were collected from Darjeeling in the month of July, 2015. The taxonomic identification was done by Dr. D.K. Singh, Scientist G and Dr. Devendra Singh Scientist C, Botanical Survey of India, Kolkata and voucher specimens have been deposited in the Central National Herbarium of the Botanical Survey of India, Howrah, India (CAL).

2.2. Methods of extraction

Collected liverworts were air dried and crushed into fine powder. 10 gm powder of each plant was then successively extracted with 200 ml of heptane, diethyl ether, ethyl acetate, acetone, butanol and methanol based on their polarity. The extracts were then evaporated, reconstituted in methanol and used for the analysis.

2.3. Anti-diabetic assay

2.3.1. α-Glucosidase inhibitory activity

Method of Kim *et al.*[8] with few modifications was followed. 2.5 ml 0.2 mM phosphate buffer, 0.1 ml 3 mM reduced glutathione and 0.1 ml enzyme (10 µg/ml) incubated for 15 min at 37º C. To this 0.5 ml sample and 0.25 ml 3 mM ρ-NPG was added and left for 15 minutes. Reaction was stopped by adding 0.1 M Na_2CO_3. Absorbance measured at 405 nm. Formula used:

$$\% I = [1- (A_s-A_b) / A_c] \times 100$$

where A_s = absorbance of sample
A_b = absorbance of blank
A_c = absorbance of control.

2.3.2. α- Amylase inhibitory activity

Earlier described method of Kim *et al.*[8] with few modifications was followed. 0.1 ml extract, 0.3ml 0.02 M sodium phosphate buffer and 0.1ml α- amylase were mixed. After 10 min 500 µl of starch solution was added. Finally 1 ml dinitrosalicylic acid was added to terminate the reaction. The mixture was heated at 100ºC for 15 min. Absorbance measured at 540 nm. Formula used:

$$\% I = [A_{540} C - A_{540} E] / [A_{540} C] \times 100$$

where $A_{540} C$ = absorbance of control
$A_{540} E$ = absorbance of extract

2.4. Antioxidant assay

2.4.1. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

Method of Sidduraju *et al.*[9] was followed. To 200 µl extract, 2 ml DPPH was added. Absorbance was measured spectrophotometrically at 517nm. Formula used:

$$\% I: (A_0-A_1) / A_0] \times 100$$

where I = inhibition
A_0 = absorbance of control
A_1 = absorbance of test sample

Antioxidant activity was expressed using IC_{50} value which is defined as concentration of the extract that results in 50% reduction of free radicals.

2.4.2. Nitric oxide (NO) scavenging activity

Method of Marcocci *et al.*[10] was followed with few modifications. 2 ml 20 mM sodium nitroprusside, 0.5 ml phosphate buffer, 0.5 ml extract and 3 ml Griess reagent were mixed and incubated for 30 minutes. Absorbance was measured at 540 nm. Inhibition percentage was calculated as described previously.

2.4.3. Superoxide radical (SO) scavenging assay

SO scavenging activity was measured by earlier described method of Fu *et al.*[11]. To 1 ml plant extract, 1 ml nitroblue tetrazolium chloride, 1 ml nicotinamide adenine dinucleotide and 10 µl phenazine methosulphate were added. Absorbance was measured at 560 nm after 30 min. Inhibition percentage was calculated as described previously.

2.4.4. $ABTS^+$ (2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) scavenging assay

Method of Li *et al.*[12] was followed. 1 ml extract and 2 ml $ABTS^+$ solution were mixed. Absorbance was measured at 734 nm. Formula used:

$$\% I= [(A_0 - A_1) / A_0] \times 100.$$

where I = inhibition
A_0 = absorbance of control
A_1 = absorbance of test sample

2.5. Metal chelating assay
Previously described method of Dinis *et al.*[13] with few modifications was followed. To 400 µl extract, 1.6 ml methanol, 40 µl of 2 mM $FeCl_2$ and 80 µl of 5 mM Ferrozine were added. Absorbance was measured at 562 nm for 10 min. Inhibition percentage was calculated as described previously.

2.6. Reducing power assay
Previously described method of Gulcin [14] was followed. To 1 ml sample, 2.5 ml phosphate buffer and 2.5 ml 1% potassium ferricyanide were added. After 20 min incubation 2.5 ml 10% trichloroacetic acid, 2.5 ml of distilled water and 250 µl of 0.1% $FeCl_3$ was added. Absorbance was measured at 700 nm.

2.7. Preliminary phytochemical analysis

2.8. Total phenol estimation
For total phenol estimation, method of Kadam *et al.*[15] with few modifications was followed. To 1 ml extract, 1 ml ethanol, 5 ml distilled water, 0.5 ml 50% Folin ciocalteau reagent and 1 ml 5 gallic acid % Na_2CO_3 were added. Absorbance was measured at 725 nm. Gallic acid was used as standard.

2.9. Total flavonoid estimation
Flavonoids estimation was done following the method of Atanassova *et al.*[16] To 0.5 ml extract, 4 ml distilled H_2O, 0.3 ml 5% $NaNO_2$, 0.3 ml 10% $AlCl_3$ and 2 ml 1.0 M NaOH were added. Absorbance was measured at 510 nm. Quercetin was used as standard.

2.10. Orthodihydric phenol estimation
For Orthodihydric phenol content estimation, method of Mahadevan and Sridhar[17] was followed. To 0.5 ml extract, 0.5 ml of Arnow's reagent, 5 ml H_2O and 1 ml of 1(N) NaOH were added and absorbance was measured at 515 nm. Catechol was used as standard.

2.11. Tannin estimation
Tannin estimation was done by method described by Thimmaiah[18]. To 0.1 sample 5 ml water and 1 ml sodium carbonate were added. Absorbance was measured at 700 nm after 30 min incubation. Tannic acid was used as standard.

2.12. Steroid estimation
Method described by Rai *et al*.[19] was followed for steroid estimation. To 1 ml extract 4 ml chloroform was added. The mixture was shaken vigorously. 1 ml chloroform layer was taken and evaporated. To this 2 ml Liebermann Burchard's Reagent (0.5 ml H_2SO_4 in 10 ml acetic anhydride) was added. Absorbance was measured at 640 nm. Solasodine was used as standard.

2.13. Qualitative phytochemical tests
For steroid, tannin, triterpenoid, amino acid, resin, cardiac glycosides, alkaloids, flavonoids, reducing sugar, anthraquinones and glycosides test method described by Kumar *et al.*[20] and Ngbede[21] was followed.

2.14. Thin layer chromatography
To screen the presence of secondary metabolites, TLC analysis (Silica gel 60 F_{254} pre-coated plates, Merck, Darmstadt, Germany) was performed using different solvent system (Ethyl acetate : Methanol : Water :: 100:13.5:10); ($CH_3COOC_2H_5 : CH_2O_2 : CH_3COOH : H_2O$ :: 100:11:11:26); ($CHCl_3 : CH_3COOH : CH_3OH : H_2O$ :: 64:32:12:8); ($C_7H_8 : CH_3COOC_2H_5$ :: 93:7). The developed TLC plates were then air dried and observed at 254 nm and 366 nm UV light. It was then sprayed with different spraying reagent for detection of different phytochemicals[22]. The movement of the phytochemical was determined by its retention factor (R_f)

$$R_f = \frac{\text{Distance travel by solute}}{\text{Distance travel by solvent}}$$

3. RESULTS
Result obtained in the present study showed that diethyl ether extract of all studied liverworts has the higher phenol content (TPC) than other solvent extracts **(Fig 1)**. In addition to phenolics, higher flavonoid (TFC) level was also recorded in diethyl ether extracts **(Fig 2)**. While ortho-dihydric phenol (TOPC) was recorded to be present in higher concentration in acetone extract of studied plants **(Fig 3)**. Steroid content was found to be higher in extracts extracted with heptane, diethyl ether, acetone and butanol **(Fig 4)**. Heptane, diethyl ether, ethyl acetate and acetone extract of all the three studied liverworts showed better tannin content when analyzed **(Fig 5)**. *M. emarginata* showed the presence of higher amount of tannin among three samples. Qualitative test and thin layer chromatographic analysis has shown the presence of phytochemicals like steroid, tannin, triterpenoids, cardiac glycoside, flavonoids, resin, reducing sugar, amino acid, glycoside, anthraglycoside, arbutin, phenol, bitter principle, coumarin. Study suggests that phenolic compounds present in plant are accountable for their antioxidative and other biological activities. Phenol scavenges the free radicals by donating an electron or hydrogen atom. DPPH‡ are mostly used in the model system for investing the antioxidative properties of natural compounds. In this study, highest DPPH radical scavenging activity was displayed by acetone extract of *P. cordatum* **(Fig 6)**. In the ABTS‡scavenging assay, diethyl ether extract in *P. cordatum* and *M. subintegra*, ethyl acetate extracts in *M. emarginata* displayed highest activity **(Fig 7)**. Sustained level of nitric oxide (NO) is toxic to tissue as it generates harmful NO_2^- anion. In our experiment, NO scavenging activity of phytochemicals extracted in acetone from *P. cordatum* was highest **(Fig 8)**. Heptane, diethyl ether and ethyl acetate extracts of all three plants showed effective activity against ferrous ion **(Fig 9)**. Reduction of ferrous ion is important as it leads to oxidative

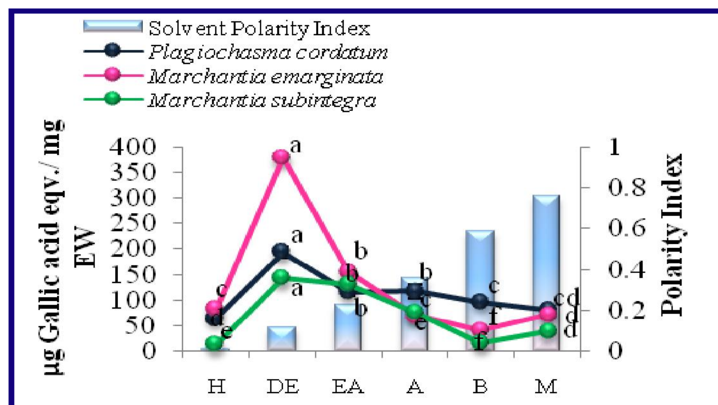


Fig 1: Total Phenol Content of studied liverworts

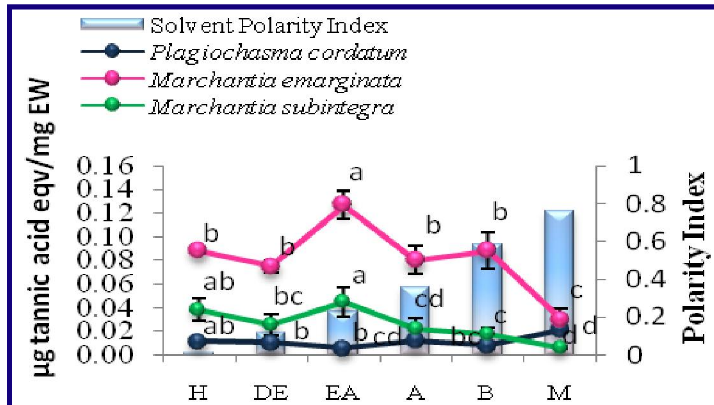


Fig 5: Tannin content of studied liverworts

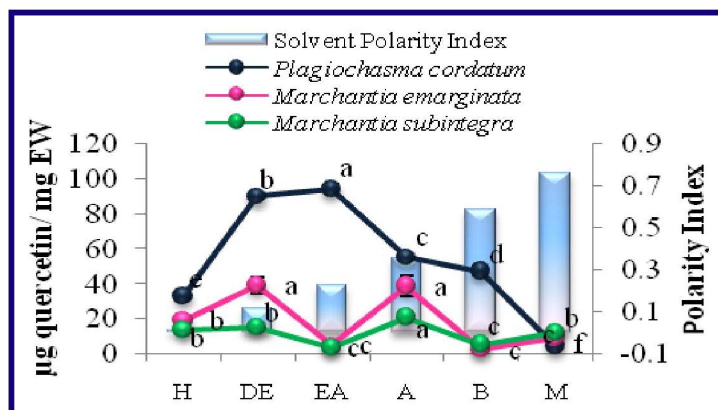


Fig 2: Total Flavonol Content of studied liverworts

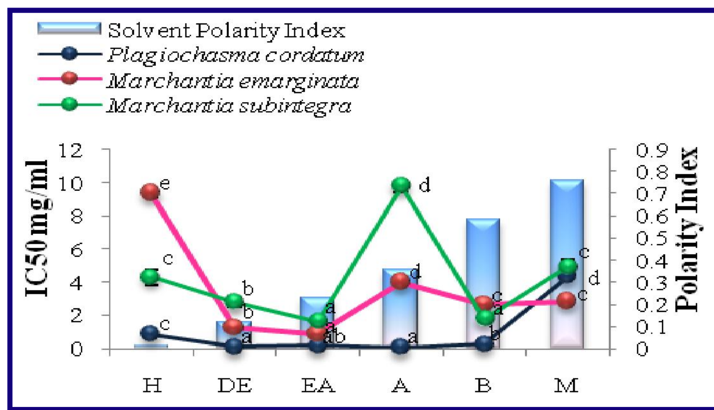


Fig 6: DPPH scavenging activity of studied liverworts

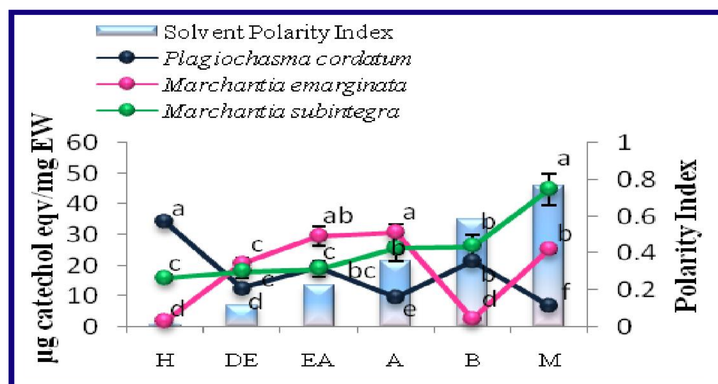


Fig 3: Total Orthodihydric Phenol Content of liverworts

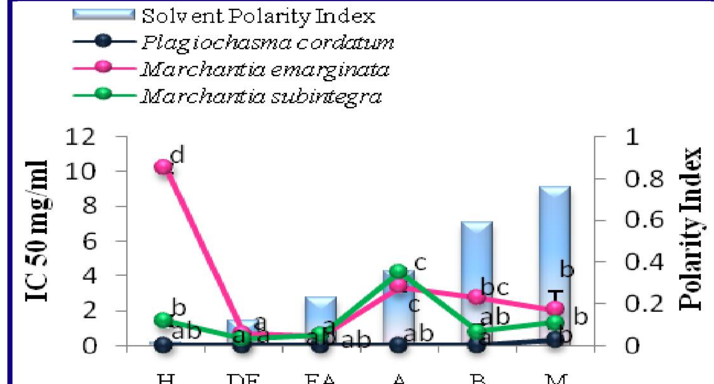


Fig 7: ABTS scavenging activity of studied liverworts

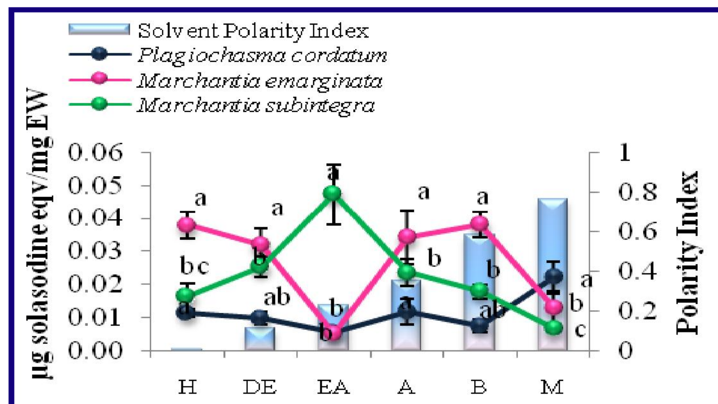


Fig 4: Steroid content of studied liverworts

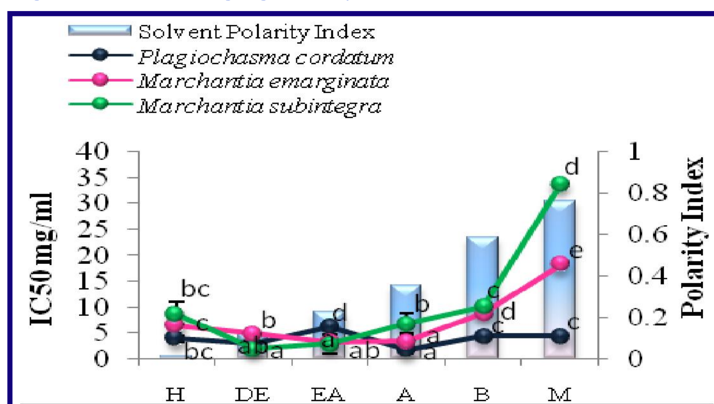


Fig 8: NO scavenging activity of studied liverwort

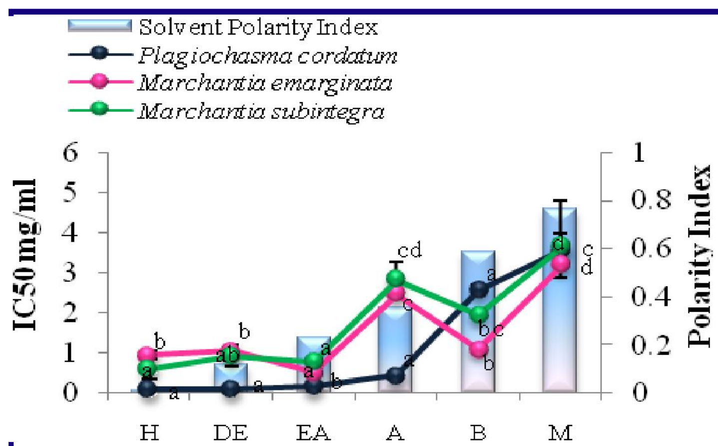


Fig 9: Metal Chelating activity of studied liverworts

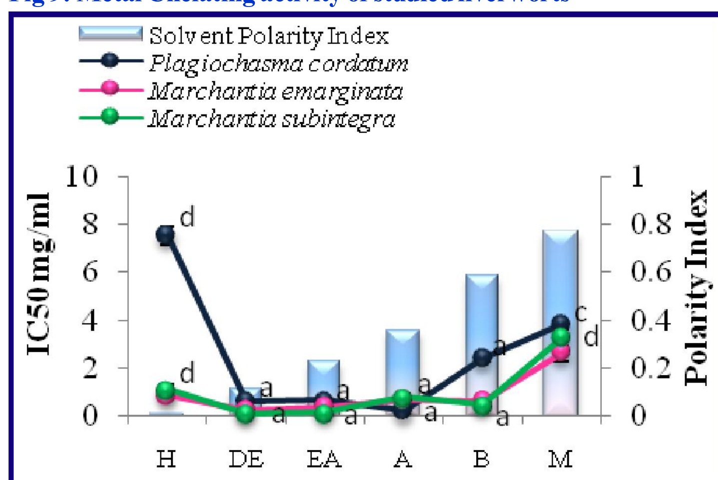


Fig 10: SO scavenging activity of studied liverworts

damage and lipid peroxidation by Fenton reaction. Superoxide (SO) is the most damaging radical in biological system. In our study, diethyl ether and ethyl acetate extracts displayed highest SO scavenging

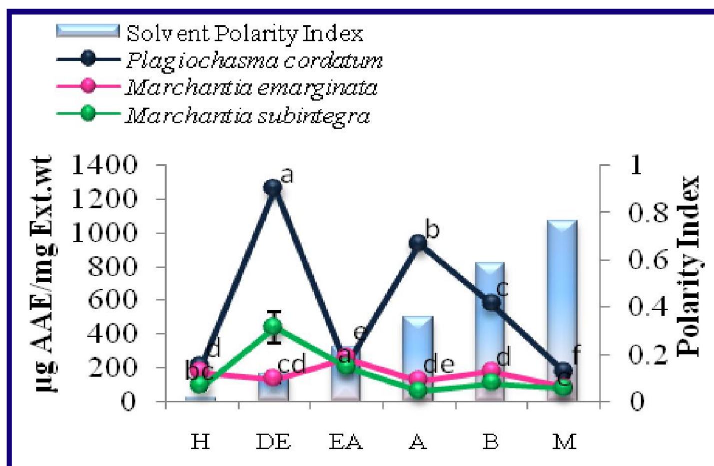


Fig 11: Reducing potential of studied liverworts

Values with different letters (a, b, c, d. e. f) are significantly ($p < 0.05$) different from each other by Duncan's multiple range test (DMRT)

activity in *M. subintegra* and *M. emarginata* while in *P. cordatum* acetone extract was most active **(Fig 10)**. Reducing power of bioactive compounds provides direct assessment to their antioxidative property. Diethyl ether extract of *P. cordatum* and *M. subintegra* showed highest Fe^{3+} reducing activity and in *M. emarginata*, ethyl acetate has higher activity **(Fig 11)**. This result is at par with the notion that reducing potential is correlated with its role as radical scavengers.

Diabetes mellitus Type 2 one of the chronic diseases worldwide is characterized by the reduced insulin sensitivity by body cells and postprandial hyperglycemia. Highest α-glucosidase and α-amylase inhibitory activity was shown by diethyl ether extract of *M. subintegra* **(Table 1 & 2)**.

Table 1: Alpha-glycosidase inhibitory activity of different solvent extracts of *P. cordatum, M. subintegra and M. emarginata*

	H	DE	EA	A	B	M
P. cordatum	0.78±0.03^{bc}	0.66±0.02^{b}	0.62±0.09^{b}	1.07±0.05^{d}	0.34±0.14^{a}	0.83±0.04^{c}
M. subintegra	0.19±0.039^{ab}	0.06±0.01^{a}	0.58±0.013^{b}	4.67±0.26^{d}	0.51±0.04^{ab}	3.79±0.42^{c}
M. emarginata	0.25±0.004^{a}	0.21±0.08^{a}	0.18±0.088^{a}	0.17±0.089^{a}	0.41±0.07^{a}	3.66±0.103^{b}

H= Heptane, DE= Diethyl ether, EA= Ethyl acetate, A= Acetone, B= Butanol, M= Methanol

Table 2: Alpha-amylase inhibitory activity of different solvent extracts of *P. cordatum, M. subintegra and M. emarginata*

	H	DE	EA	A	B	M
P. cordatum	1.27±0.26^{a}	1.27±0.24^{a}	1.12±0.34^{a}	1.22±0.13^{a}	1.08±0.34^{a}	1.35±0.11^{a}
M. subintegra	0.17±0.06^{a}	0.06±0.01^{a}	0.51±0.09^{b}	3.6±0.103^{d}	0.44±0.002^{b}	2.7±0.047^{c}
M. emarginata	0.20±0.01^{a}	0.19±0.01^{a}	0.12±0.02^{a}	0.17±0.07^{a}	0.49±0.04^{b}	3.17±0.17^{c}

H= Heptane, DE= Diethyl ether, EA= Ethyl acetate, A= Acetone, B= Butanol, M= Methanol

4. DISCUSSION

Solvents' polarity is an important factor for different nature of phytochemicals extracted from plants. Akinmoladun *et al*.[23] while working on *Ocimum gratissimum* found the presence of anthraquinones only in aqueous extract while alkaloids are detected in methanolic extract only. Bryophytes are composed of different types of polysaccharides[24]. Lower polarity substances like lipids and polar substances like carbohydrates, polyphenols are identified as the secondary metabolites that are present in bryophytes. The amount of polar substances extracted is lower in case of mosses than the non polar compounds[25]. In our study, the studied liverwort showed the presence of both non polar substances (Heptane, diethyl ether) and polar substances (methanol extracts), when extracted by sequential extraction approach **(Table 3)**. While, in mosses presence of higher amount of nonpolar compounds are reported by Klavina *et al*.[25] In higher plant groups higher extraction yield is obtained in polar solvents like methanol, ethanol and water[26]. The present studies have shown that different kinds of free radicals generated in the body are scavenged by phytochemicals extracted in different solvent. For example heptane extract of all three plants showed significant metal chelating activity. Butanol and acetone extracts showed better ABTS, DPPH and NO scavenging activity, while diethyl ether and ethyl acetate extracts of all the three liverworts showed significant potential to scavenge all free radicals. It has been reported that terpenoids and aromatic compounds present in the liverwort are responsible for different biological activities[27]. Terpenoids are naturally occurring non polar compounds derived from isoprene unit. They are extracted by using non polar compounds[28]. In our study, better antioxidant activity of dithyl ether and ethyl acetate extract can be assumed due to the presence of non-polar compounds like terpenoids in the extract which is similar with the findings of other workers[27].

Table 3: Extractive weight of different solvent extracts of *P. cordatum*, *M. subintegra* and *M. emarginata*

Solvents	*P. cordatum*	*M. subintegra*	*M. emarginata*
Heptane	3.3 mg/ml	2.3 mg/ml	1.0 mg/ml
Diethyl ether	3.9 mg/ml	1.5 mg/ml	1.2 mg/ml
Ethyl acetate	4 .0mg/ml	0.8 mg/ml	0.7 mg/ml
Acetone	3.2 mg/ml	2.1 mg/ml	1.1 mg/ml
Butanol	5.1 mg/ml	1.6 mg/ml	0.1 mg/ml
Methanol	1.7 mg/ml	5.6 mg/ml	2.9 mg/ml

Decrease of postprandial hyperglycemia is important for treatment of diabetes and this can be achieved by inhibiting the activities of carbohydrate hydrolyzing enzymes like α-amylase and α-glucosidase[29]. In this work, *M. emarginata* showed good α-glucosidase and α-amylase inhibitory activity in almost all the solvent extract used. Antidiabetic activity shown is reported to be due to α-amylase and α-glucosidase inhibitory activity of flavonoids and tannin present in plant[30]. Finding of this work is in agreement with the present statement, where *M. emarginata* showing better α-glucosidase inhibitory activity also has higher tannin content than *P. chordatum* and *M. subintegra*.

Several bioactive phytochemicals are present in plant. These phytoconstituents are responsible for different biological activity displayed by plants. Different solvent extracts potential to scavenge different free radicals might be due to extraction of different polarity phytocompounds like steroid, tannin, triterpenoids, cardiac glycoside, flavonoids, resin, reducing sugar, amino acid, glycoside, anthraglycoside, arbutin, phenol, bitter principle, coumarin from studied liverworts as detected by qualitative phytochemical test and thin layer chromatographic analysis **(Table 4** and **8)**; while alkaloids were absent in all the extracts of studied liverworts. Jockovic *et al*.[31] also reported the absence of alkaloid in bryophytes. Studies suggest that the phenolic compounds are determinants of radical scavenging activity. Poyphenol content of the studied liverwort showed significant positive correlation with the free radical scavenging activity. The result obtained supports the previous findings[32,33] stating role of phenolic compound as free radical scavengers. We have also found strong positive correlation between phenolic compound and reducing potential of analyzed liverwortsn **(Table 5,6,7)**. For better understanding of the relationship between variables and clustering group, Principle Component Analysis test was done **(Fig 12)**. First two principal components accounted for 29.37% and 23.89 % of the data variance. Variables were clustered in four groups A, B, C and D. Cluster A shows that IC_{50} value of DPPH and ABTS inhibiting activity is strongly correlated with steroid and tannin content of the plant. Nitric oxide scavenging and metal chelating activities are found to be related with glycoside and orthodihydric content suggesting that the glycoside and orthodihydric phenol present in the plant are more responsible for scavenging nitric oxide and ferrous ion in the assay (Cluster B). Variables in cluster C is directly correlated with each other which interprets that terpenoids present in liverworts are mainly responsible for superoxide scavenging activity. Moreover, it is established that reducing potential of the plant is the measure of antioxidant activity[34,35]. Cluster D shows strong correlation between polyphenol content and reducing potential of studied liverworts. Phenolic compounds are proven to have radical scavenging activity[36,37], so here correlation between polyphenol content and reducing potential of the plant is justifiable.

In order to visualize and decipher the relationship between the extracting solvent, phytochemical and pharmacological attributes, a heatmap is produced using R-software and data visualization

Table 4: Phytochemical analysis of *P. cordatum*, *M. subintegra* and *M. emarginata*

		ANT	STE	TAN	TRITER	C G	ALK	FLA	RES	R S	A A	GLYC
P. cordatum	H	-	++	+	++	+++	-	-	-	++	-	+
	DE	-	++	+	++	+++	-	-	++	++	-	+
	EA	-	++	+	++	+++	-	+	-	++	-	+
	A	-	+	+	-	+++	-	-	++	++	-	+
	B	-	++	+	++	+++	-	+	++	++	-	+
	M	-	++	++	+	-	-	-	-	-	-	++
M. subintegra	H	-	+	+	+	+	-	-	-	++	-	+
	DE	-	+	+	+	+	-	+	+	+	-	+
	EA	-	+	+	+	-	-	-	-	+	+	+
	A	-	+	+	-	-	-	+	-	+	+	+
	B	-	+	++	+	-	-	-	+	+	-	+
	M	-	+	++	+	+	-	+	+-	++	+	++
M. emarginata	H	-	+	+	+	+	-	-	-	-	-	+
	DE	-	+	+	+	+	-	+	-	-	-	+
	EA	-	++	+	+	+	-	-	-	-	-	+
	A	-	+	+	-	-	-	-	-	-	-	+
	B	-	+	+	-	-	-	-	-	-	-	+
	M	-	+	+++	-	-	+	-	+	-	-	++

STE= steroid, TAN= tannin, GLYC= glycosides, AA= amino acid, RS= reducing sugar, RES= resine, CG= cardiac glycoside, FLA=flavonoid, TRITER= triterpenoids, ANT= anthraglycosides, ALK= alkaloids

Table 5: Correlation between Phytochemical content and antioxidant activity in *P. cordatum*

	DPPH ‡	ABTS	N O	M C	S O	RP	TPC	TFC	TOPC	STE
ABTS *	0.98**									
NO	0.17	0.12								
MC	0.75*	0.75*	0.16							
SO	0.22	0.09	0.17	-0.16						
FRAP	-0.29	-0.32	-0.14	-0.15	-0.340					
TPC	-0.45	-0.38	-0.31	-0.42	-0.650	0.780*				
TFC	-0.78*	-0.73*	0.11	-0.71	-0.461	0.469	0.749*			
TOPC	-0.58	-0.59	0.52	-0.08	-0.348	0.239	0.135	0.550		
STE	0.92**	0.93**	-0.22	0.62	0.215	-0.277	-0.351	-0.819*	-0.817*	
TAN	0.91**	0.93**	-0.25	0.64	0.151	-0.261	-0.310	-0.798*	-0.811*	0.99**

***. Correlation is significant at the 0.01 level (1-tailed); *. Correlation is significant at the 0.05 level (1-tailed). NO= Nitric oxide scavenging activity, MC= Metal chelating activity, SO= super oxide scavenging activity, RP= Reducing power, TPC= Total phenol content, TFC= Total flavonoid content, TOPC= Total orthodihydric phenol content, STE = steroid, TAN= tannin*

Table 6: Correlation between Phytochemical content and antioxidant activity in *M. subintegra*

	DPPH‡	ABTS‡	N O	M C	S O	RP	TPC	TFC	TOPC	STE
ABTS*	0.945**									
NO	0.159	0.034								
MC	0.531	0.500	0.773*							
SO	0.307	0.146	0.973**	0.735*						
FRAP	-0.424	-0.552	-0.493	-0.522	-0.517					
TPC	-0.106	-0.187	-0.510	-0.331	-0.498	0.774*				
TFC	0.861*	0.715	0.017	0.312	0.173	-0.017	0.026			
TOPC	0.238	0.158	0.933**	0.920**	0.869*	-0.442	-0.345	0.051		
STE	-0.266	-0.152	-0.707	-0.562	-0.714	0.370	0.726	-0.379	-0.589	
TAN	-0.247	-0.187	-0.707	-0.855*	-0.618	0.208	0.350	-0.268	-0.830*	0.746*

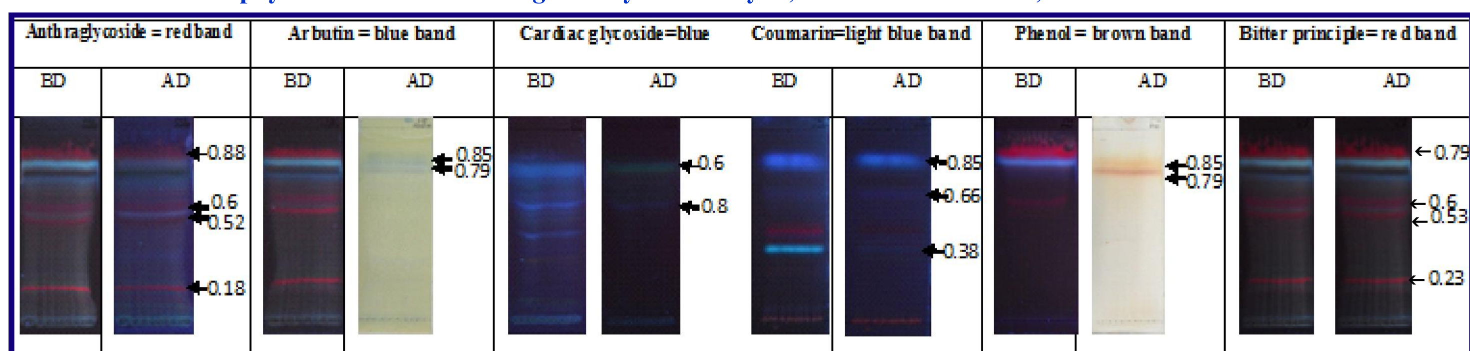
***. Correlation is significant at the 0.01 level (1-tailed); *. Correlation is significant at the 0.05 level (1-tailed).*

Table 7: Correlation between phytochemical content and antioxidant activity in *M. emarginata*

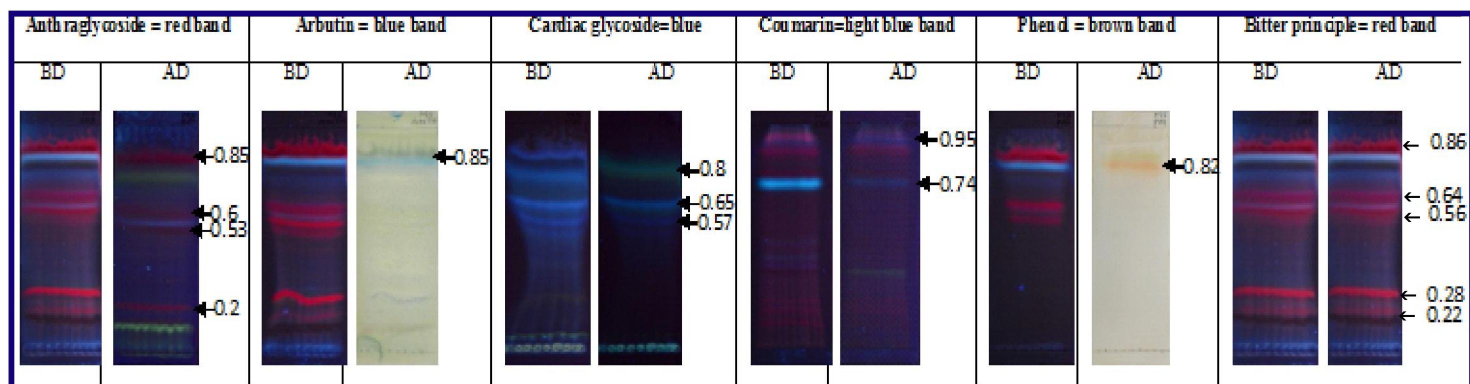
	DPPH*	ABTS*	NO	MC	SO	RP	TPC	TFC	TOPC	STE
ABTS*	0.994**									
NO	0.004	-0.034								
MC	0.007	-0.092	0.660							
SO	0.123	0.061	0.935**	0.802						
FRAP	-0.110	-0.021	-0.583	-0.838*	-0.614					
TPC	-0.416	-0.407	-0.339	-0.351	-0.441	0.002				
TFC	0.087	0.029	-0.428	0.174	-0.310	-0.454	0.531			
TOPC	-0.575	-0.638	-0.089	0.401	0.113	-0.108	0.224	0.301		
STE	0.550	0.582	-0.331	-0.318	-0.445	-0.051	-0.070	0.275	-0.766	
TAN	-0.052	0.026	-0.784	-0.823*	-0.772	0.953**	0.017	-0.219	-0.062	0.105

***. Correlation is significant at the 0.01 level (1-tailed); *. Correlation is significant at the 0.05 level (1-tailed)*

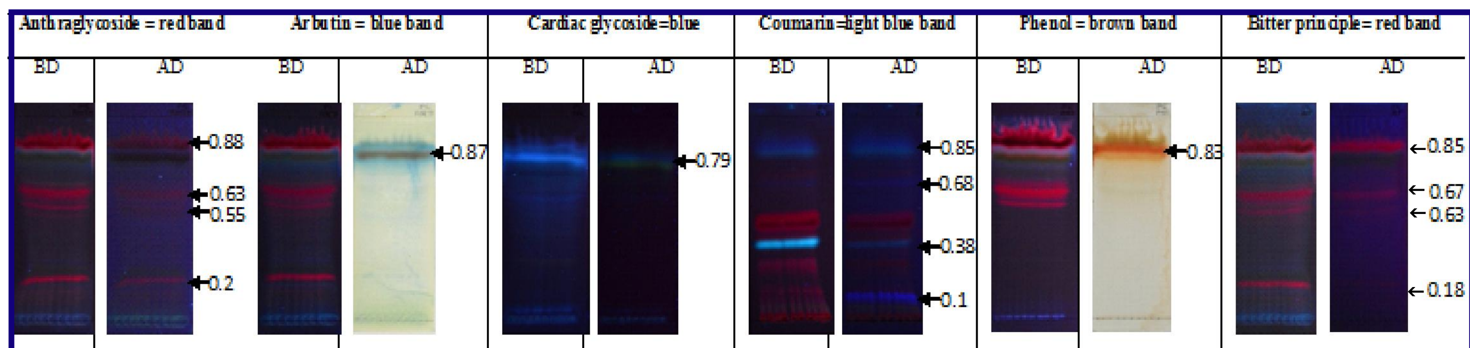
Table 8: Detection of phytochemicals in *M. emarginata* by TLC analysis; BD= before detection, AD= after detection

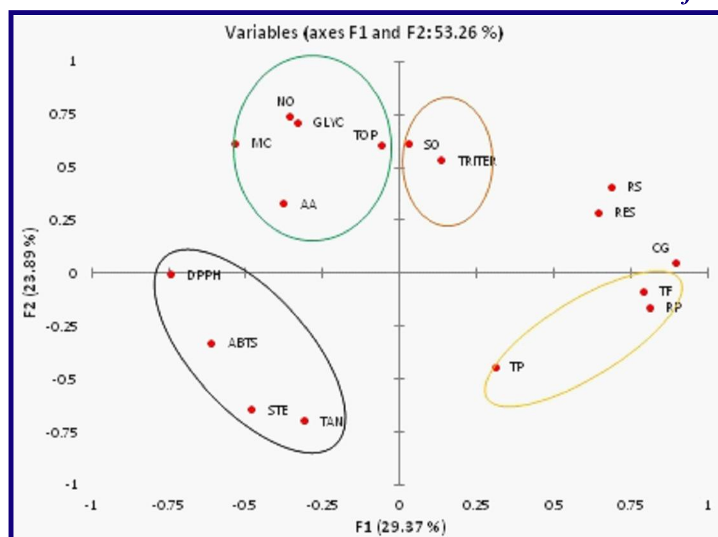


Detection of phytochemicals in *M. subintegra* by TLC analysis



Detection of phytochemicals in *P. cordatum* by TLC analysis



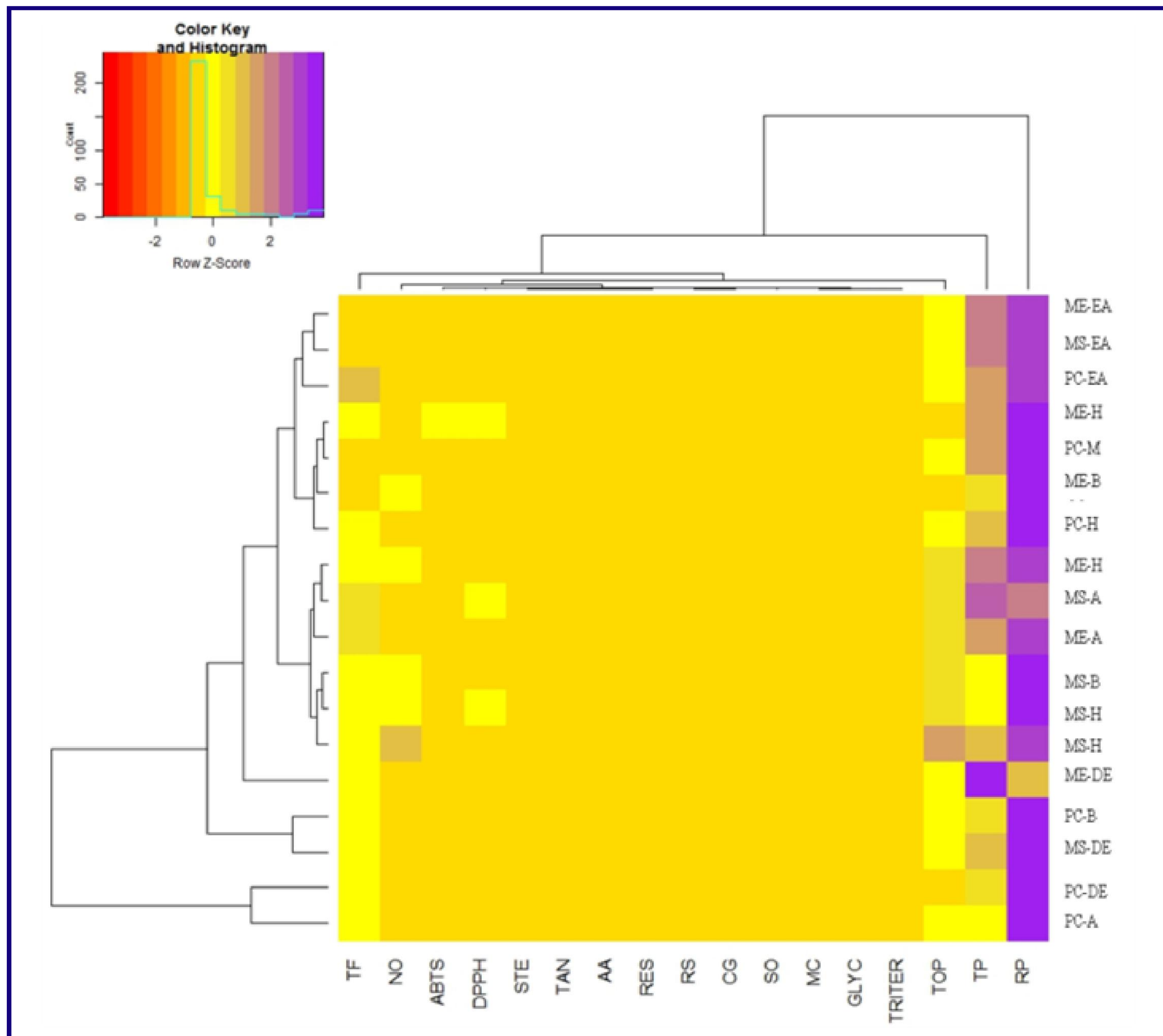


STE= steroid, TAN= tannin, GLYC= glycosides, AA= amino acid, RS= reducing sugar, RES= resine, CG= cardiac glycoside, TF= total phenol, TF= total flavonoid, TOP= total ortho-dihydric phenol, TRITER= triterpenoid

Fig 12: Principal component analysis of phytochemicals, radical scavenging activity, anti-diabetic activity of studied liverworts.

(Fig 13). Analysis depicted that flavonoids are extracted better in solvents like diethyl ether, ethyl acetate and butanol in the studied case. In *M. emarginata*, it can be assumed that flavonoids contribute to its DPPH and ABTS scavenging activity. Higher steroid content are found in the diethyl ether, ethyl acetate, heptane, acetone and butanol extracts. Extracts showing higher steroid content has showed better DPPH, ABTS, NO and MC activity. Similar solvents also extracted higher amount of tannin from studied liverworts. Through Z-score test in our study, it has been observed that steroid and tannin has potential to scavenge similar kind of free radicals. Ortho-dihydric phenols are high in extracts extracted with diethyl ether, ethyl acetate, acetone, butanol and methanol. These extracts, with higher ortho-dihydric phenol content has found to have greater potential to scavenge different kinds of radicals analyzed in our study. Moreover, in this case diethyl ether and ethyl acetate are proven as better solvents for extracting phenolic compounds from studied liverworts. In addition, acetone and butanol extracts also showed significant phenol content. Analysis result also showed that reducing potential is higher of extracts with higher phenol content suggesting possible role of phenolic compound in reducing free radicals.

Fig 13: Z-score (coloumn) heatmap (left) and Z-score (row) heatmap of the extracting solvent, phytochemical and pharmacological attributes



PC-H= P. cordatum heptane extract, PC-DE= P. cordatum diethyl ether extract, PC-EA= P. cordatum ethyl acetate extract, PC-A= P. cordatum acetone extract, PC-B= P. cordatum butanol extract, PC-M= P. cordatum methanol extract.
ME-H= M. emarginata heptane extract, ME-DE= M. emarginata diethyl ether extract, ME-EA= M. emarginata ethyl acetate extract, ME-A= M. emarginata acetone extract, ME-B= M. emarginata butanol extract , ME-M= M. emarginata methanol extract.
MS-H= M. subintegra heptane extract, MS-DE= M. subintegra diethyl ether extract, MS-EA= M. subintegra ethyl acetate extract, MS-A= M. subintegra acetone extract, MS-B= M. subintegra butanol extract, MS-M= M. subintegra methanol extract.

5. CONCLUSION

Liverworts are reported of having many biological activities and also many phytochemicals unique to plant kingdom are reported from them. Studied liverworts showed antioxidant and anti-diabetic property. Many vital phytocompounds like steroid, tannin, triterpenoids, cardiac glycoside, flavonoids, resin, reducing sugar, amino acid, glycoside, anthraglycoside, arbutin, phenol, bitter principle and coumarin were found in these liverworts. Higher extractive weight of diethyl ether suggested presence of phytochemicals of moderate polarity in greater amount in studied liverworts. Phytochemicals present were found to be correlated with pharmacological activities. Further research focusing on the isolation

of particular bioactive compounds with potent pharmacological activities from these less explored group of plants are suggested.

ACKNOWLEDGEMENT

We are grateful to "University Grant Commission" for funding this work under "Maulana Aazad National Fellowship" scheme and the Director, Botanical Survey of India, Kolkata for facilitating the identification of specimens.

REFRENCES

1. Asakawa Y. Biologically active compounds from bryophytes. *Pure. Appl. Chem*., 79(4), 2007, 557-80.
2. Jensen S, Omarsdottir S, Bwalya AG, Morten AN, Tasdemir D, Olafsdottir ES. Marchantin A, a macocycylic bisbibenzyl ether, isolated from the liverwort *Marchantia polymorpha*, inhibits protozoal growth *in vitro*. *Phytomedicine*, 19(13), 2012, 1191-5.
3. Komala I, Ito T, Nagashima F, Asakawa Y. Cytotoxic, radical scavenging and antimicrobial activities of sesquiterpenoids from the Tahitian liverwort *Mastigophora diclados* (Brid.) Nees (Mastigophoraceae). *J. Nat. Med*., 64(4), 2010, 417-22.
4. Schwartner C, Bros W, Michel C, Franck U, Mukker-Jakic B, Nenninger A, et al. Effect of Marchantins and relate compounds on 5-Lipoxygenase and cyclooxygenase and their antioxidant properties: a structure activity relationship study. *Phytomedicine*, 2(2), 1995, 113-7.
5. Schwartner C, Michel Christa, Stettmaier K, Wagner H, Bros W. Marchantins and related polyphenols from liverwort: Physico-chemical studies of their radical scavenging properties. *Free. Radic. Boil. Med*., 20(2), 1996, 237-44.
6. Da Costa Mushino NMHD, Van Tonder JJ, Steenkamp V. *In vitro* anti-diabetic activity of *Sclerocarya birrea* and *Ziziphus mucronata*. *Nat. Prod. Commun.* 8(9), 2013, 1279-84.
7. Bodade RG, Borkar PS, Md Saiful A, Khobragade CN. *In vitro* screening of bryophytes for antimicrobial activity. *J. Med. Plants,* 7, 2008, 23-8.
8. Kim Ym, Jeong YK, Wang MH, Lee W, Rhee H. Inhibitory effect of pine extract on α-glucosidase activity and postprandial hyperglycemia. *Nutrition*, 21(6), 2005, 756-61.
9. Sidduraju P, Mohan P, Becker K. Studies on the antioxidant activity of Indian Laburnum *Cassia fistula* L): a preliminary assesment of crude extracts from stem bark, leaves, flowers and fruit pulp. *Food. Chem.*, 79(1), 2002, 61-7.
10. Marcocci L, Packer L, Droy-Lefaix MT, Sekaki A, Grades-Albert M. Antioxidant action of *Ginkgo biloba* extracts EGb 761. *Methods. Enzymol*., 234, 1994, 462-75.
11. Fu W, Chen J, Cai Y, Lei Y, Chen L, Pei L, et al. Antioxidant, free radical scavenging, anti- inflammatory and hepatoprotective potential of the extract from *Parathelypterisnipponica*(Franch. et Sav.) Ching. *J. Ethnopharmacol*., 130(3), 2010, 521-8.
12. Li XC, Wu XT, Huang L. Correlation between antioxidant activities and phenolic contents of Radix AngelicaeSinensis (Danggui). *Molecules*, 14(12), 2009, 5349-61.
13. Dinis TCP, Madeira VM, Almeida LM. Action of phenolic derivates (acetoaminophen, salicylate and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch. Biochem. Biophys*., 315(1): 1994, 161-9.
14. Gulcin I. Antioxidant activity of L-adrinaline: an activity structure insight. *Chem. Biol. Interact.*, 179(2-3), 2009, 71-8.
15. Kadam VB, Momin RK, Fatima S, Kadam UB. Estimation of total phenol in different plant parts of genus *Sesbania*in Maharastra. *IJPRBS*, 2, 2013, 202-6.
16. Atanassova M, Georgieva S, Ivancheva K. Total phenolic and total flavonoid contents, antioxidant capacity and biological contaminants in medicinal herbs. *J. chem. Technol. Metall*., 46(1), 2011, 81-8.
17. Mahadevan A, Sridhar R. Methods in physiological plant pathology. Sivakami Publication, Chennai, 1986.
18. Thimmaiah SK. Standard method of biochemical analysis. Kalyani Publishers. New Delhi, 1999.
19. Rai MV, Pai VR, Kedilaya HP, Hedge S. Preliminary Phytochemical Screening of Members of Lamiaceae Family: *Leucas linifolia, Coleus aromaticus* and *Pogestemon patchouli*. International Journal Pharm. *Sci. Rev. Res.*, 21(1), 2013, 131-7.
20. Kumar A, Ilavarasan R, Jayachandan T, Decaraman M, Aravindhan P, Padmanabhan N, et al. Phytochemical investigation on a tropical plant, *Syzygiumcumini*from kattuppalayam, Erode District, Tamil Nadu, South India. *Pakistan J. Nutr.,* 8(1), 2009, 83-5.
21. Ngbede J, Yakubu RA, Nyam DA. Phytochemical screening for active compound in *Canaruim schweinfurthii*(Atile) leaves from Ios North, Plateau state, Nigeria. *Res. J. Biol. Sci.*, 3 (9), 2008, 1076-8.
22. Wagner H, Bladt S. Plant Drug Analysis: A Thin Layer Chromatography Alas. 2nd Edition, Spinger-Verlag, Berlin, Heidelberg, 1996.
23. Akinmoladun AC, Ibukun EO, Afor E, Obuotor EM. Phytochemical constituent and antioxidant ectract from the leaves of *Ocimum gratissimum*. *Sci. Res. Essays*, 7(5), 2007, 163-6.

24. Klavina, L. Polysaccharides from Lower Plants: Bryophytes. In *Polysaccharides*; Ramawat, K.G., Mérillon, J.M., Eds.; Springer International Publishing: Cham, Switzerland, 2015.
25. Klavina L, Springe G, Nikolajeva V, Martsinkevich I, Nakurte I, Dzabijeva D, et al. Chemical composition analysis, antimicrobial activity and cytotoxicity screening of moss extract (Moss Phytochemistry). *Molecules*, 20(9), 2015, 7221-43.
26. Saeed N, Khan MR, Shabbir M. Antioxidant activity, total phenolic and total flavonoids contents of whole plant extracts *Torilis leptophylla* L. *Complement. Altern. Med.*, 12(221), 2012, 161-12.
27. Asakawa Y. Biologically active compounds from bryophytes. *Pure. Appl. Chem.*, 79 (4), 2007, 557-80.
28. Harman-Ware AE, Sykes R, Peter GF, Davis M. Determination of terpenoid content in pine by oraganic solvent extraction and Fast-GC analysis. *Front. Energy. Res.*, 4 (2): 2016, 1-9.
29. Ali H, Houghton PJ, Soumyanath A. α-Amylase inhibitory activity of some Malaysian plants used to treat diabetes; With particular reference to *Phyllanthus amarus*. *J. Ethnopharmacol.*, 107(3), 2006, 449–55.
30. Suba V, Murugesan T, Rao RB, Ghosh L, Pal M, Mandal SC, et al. Antidiabetic potential of Barleria lupulina extract in rats. *Fitoterapia*, 75(1), 2004, 1-4.
31. Jockovic N, Pavlovic M, Sabovljevic M, Kovacevic N. Contribution to the chemical constituents of Balkan bryophytes: phenolic acids, flavonoids, triterpenes and alkaloids. *Natura. Montenegrina.*, 6, 2007, 123-29.
32. Wong CC, Li HB, Cheng KW, Chen F. A systematic survey of antioxidant activity of 30 Chinese medicinal plants using the ferric reducing antioxidant power assay. *Food Chem.*, 97(4), 2006, 705-11.
33. Tusevski O, Stanoeva JP, Stefova M, Simic SG. Phenolic profile of dark grown and photoperiod-exposed *Hypericum perforatum* L. Hairy root culture. *Scientific World J.*, 2013, 1-9.
34. Meir S, Kanner J, Akiri B, Hadas SP. Determination and involvement of aqueous reducing compounds in oxidative defense systems of various senescing leaves. *J. Agric. Food. Chem.*, 43 (7), 1995, 1813-7.
35. Bimla N, Punita B. Aluminium-induced imbalance in oxidant and antioxidant determinants in brain regions of female rats: protection by centrophenoxine. *Toxicol. Mech. Methods.* 16 (1), 2006, 21-5.
36. Bhattarai HD, Paudel B, Lee HS, Lee YK, Yim JH. Antioxidant activity of *Sanioniauncinata*, a polar moss species from King George Island, Antartica. *Phytotherapy. Res.*, 22(12), 2008, 1635-9.
37. Adedapo AA, Jimoh FO, Afolayan AJ, Masika PJ. Antioxidant properties of the methanol extracts of the leaves and stems of *Celtisafricana*. *Rec. Nat. Prod.* 3(1), 2009, 23-31.

Source of support: UGC,New Delhi,India **, Conflict of interest: None Declared**