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

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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

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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]. 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].

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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 de-colorization 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 = $[(A_0-A_1)/ A_0] \times 100$

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 (%) = $[(A_0 – A_1/A_0) \times 100]$

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% = $(A_0 – A_1) / A_0$ x 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 %= $[(A_F - A_1)/(A_F – A_0)]$x 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].

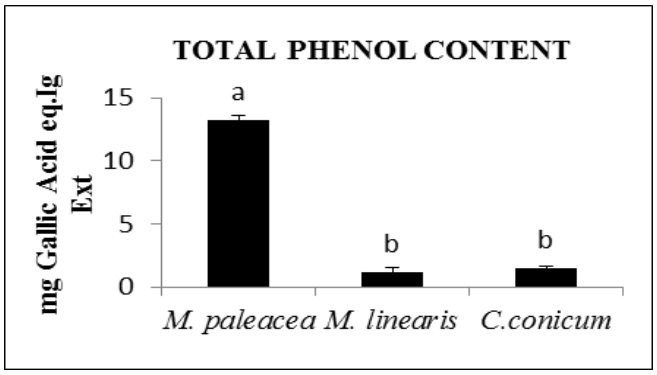

FIG. 1: TOTAL PHENOL CONTENT

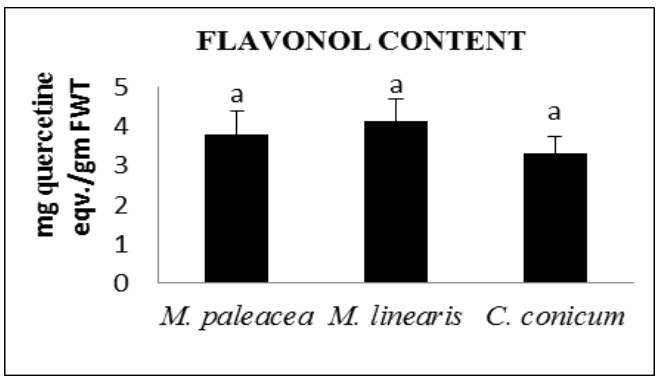

FIG. 2: TOTAL FLAVONOL CONTENT

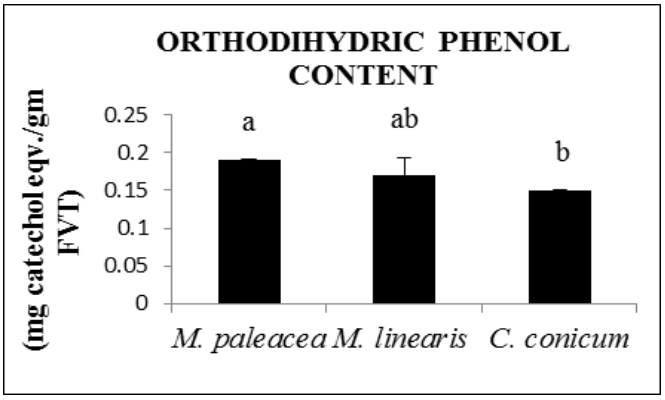

FIG. 3: TOTAL ORTHODIHYDRIC PHENOL CONTENT

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**).

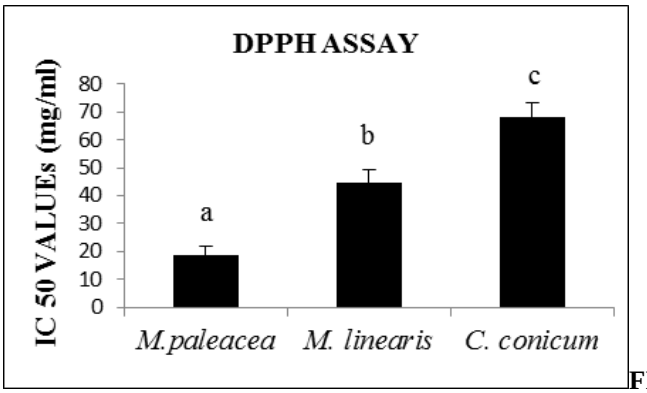

FIG. 4: DPPH RADICAL SCAVENGING ACTIVITY

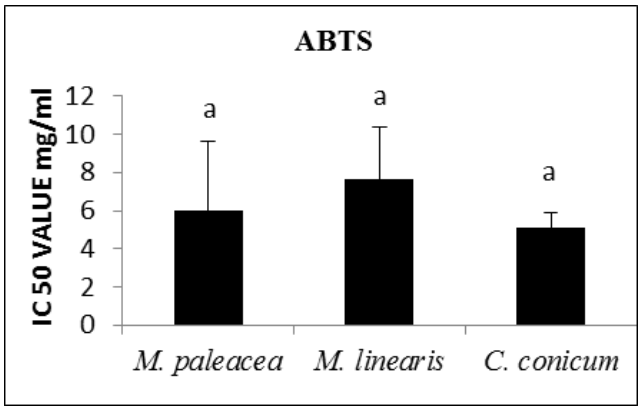

FIG. 5: ABTS RADICAL SCAVENGING ACTIVITY

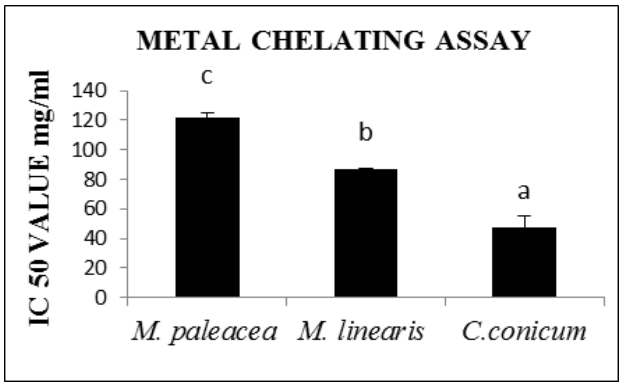

FIG. 6: METAL CHELATING ACTIVITY

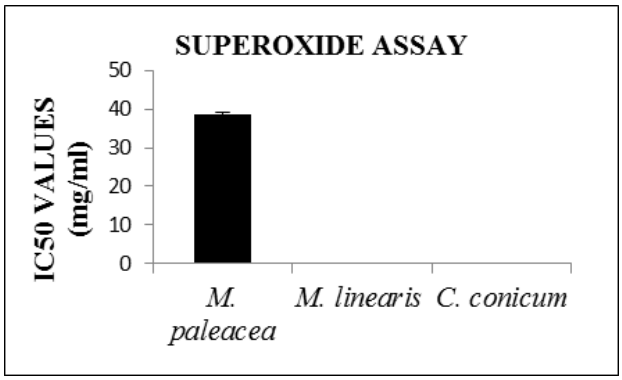

FIG. 7: SUPEROXIDE SCAVENGING ACTIVITY

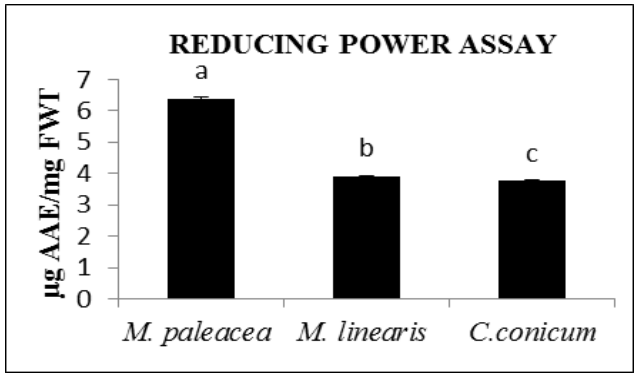

FIG. 8: REDUCING POWER

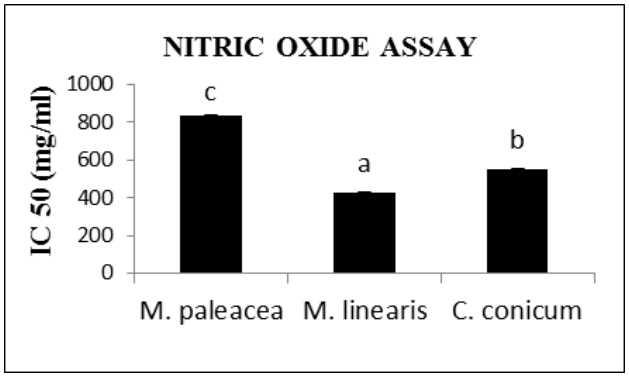

FIG. 9: NITRIC OXIDE SCAVENGING ACTIVITY

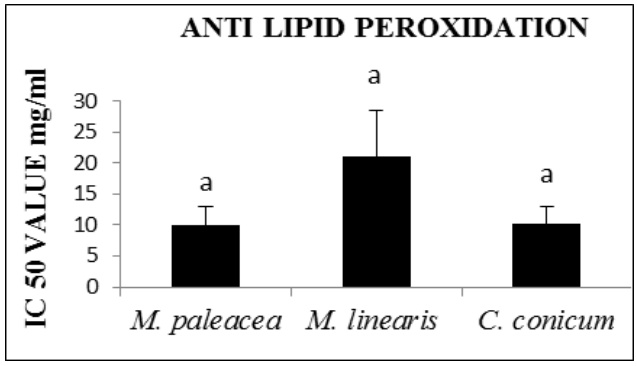

FIG. 10: ANTI-LIPID PEROXIDATION ACTIVITY

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.

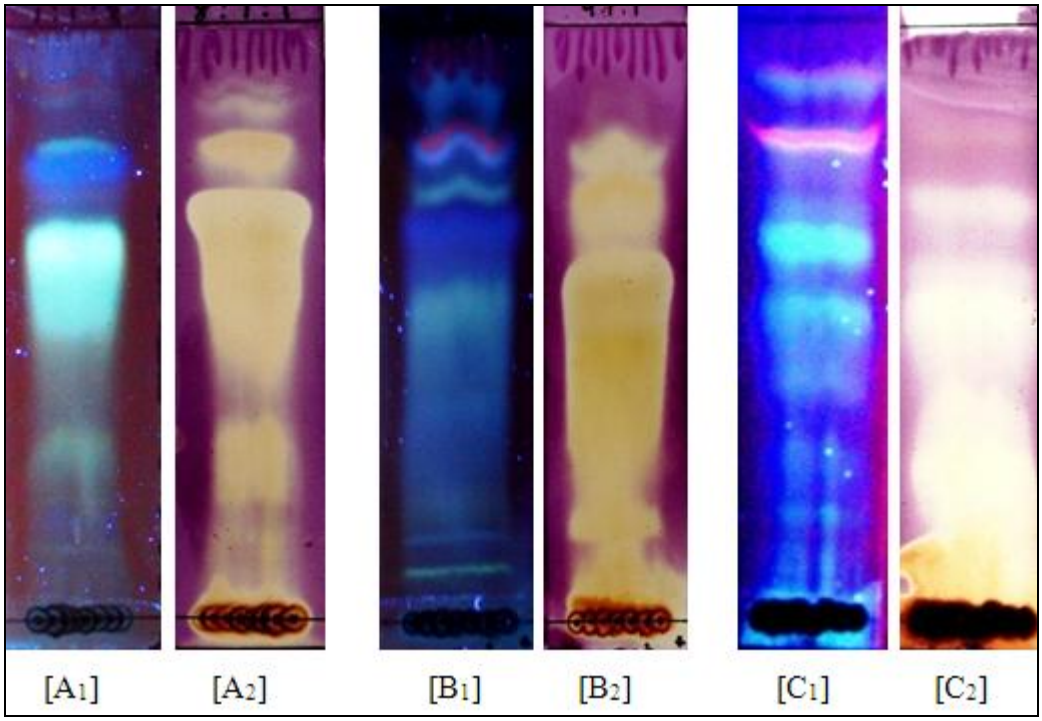

FIG. 11: THIN LAYER CHROMATOGRAPHIC PROFILE OF DIFFERENT SPECIMENS OF MARCHANTIALES: [A] *Marchantia paleacea*; [B] *Marchantia linearis*; [C] *Conocephalum conicum*. [A_1, B_1 and C_1]: Fluorescent bands observed under UV_{365}; [A_2, B_2 and C_2]: DPPH fingerprint of contemporary bands.

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.

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Evaluation of anti-diabetic, antioxidant activity and phytochemical constituents of liverworts of Eastern Himalaya

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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^{o}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^{o}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 ($P \leq 0.05$) 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*.

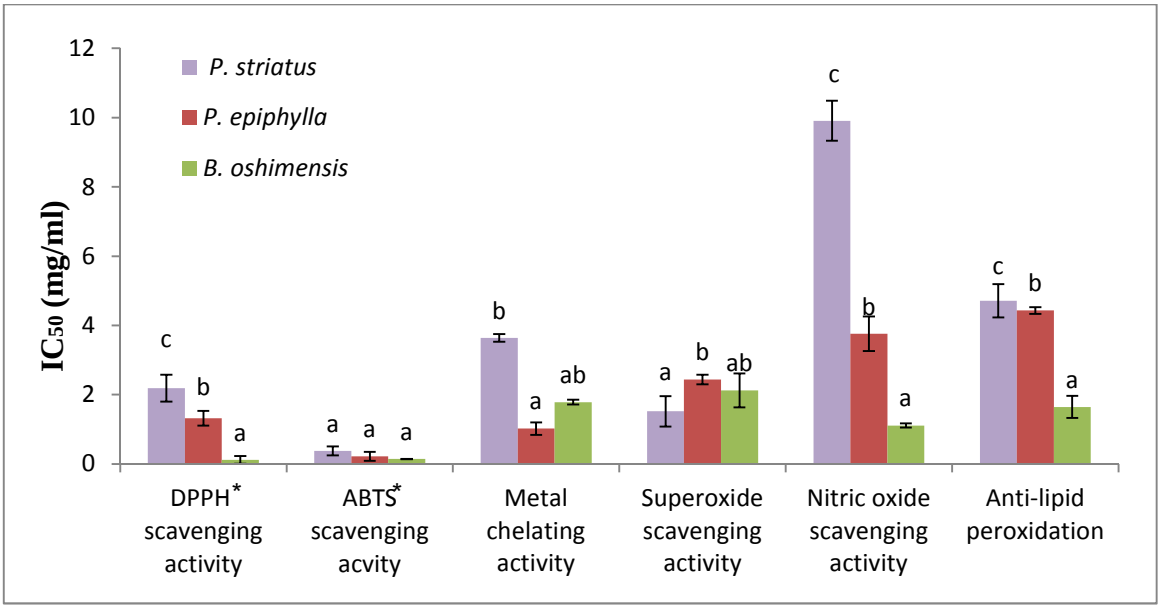


Figure 1: Radical scavenging activity of crude extracts of *P. striatu, P. epiphylla* and *B. oshimensis*
Values with different letters (a, b, c) are significantly ($p < 0.05$) different from each other by Duncan's multiple range test (DMRT)

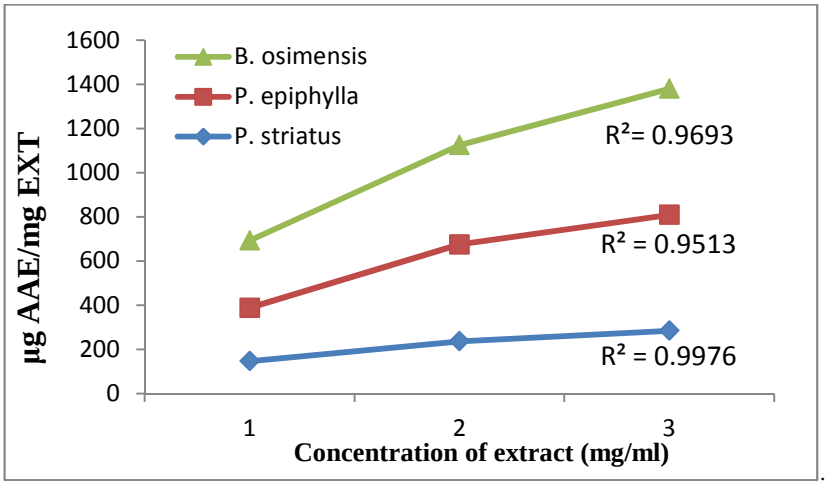


Figure 2: Reducing potential of crude extract of *P. striatus, P. epiphylla* and *B. oshimensis*

Table 3: Preliminary phytochemical analysis of *P. striatus, P. epiphylla* and *B. oshimensis*

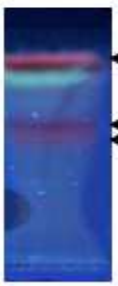
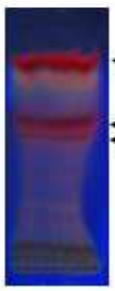
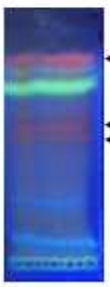
	Ptychanthus striatus	*Pellia epiphylla*	*Bazzania oshimensis*
Steroid	+++	++	+
Tannin	++	+++	+
Triterpenoids	+++	+++	+
Amino acid	++	+++	+
Resin	++	++	-
Cardiac glycoside	+++	++	++
Flavonoids	++	++	++
Alkaloid	-	-	-
Reducing sugar	++	+	+
Anthraquinones	+	+	+
Glycosides	-	-	-

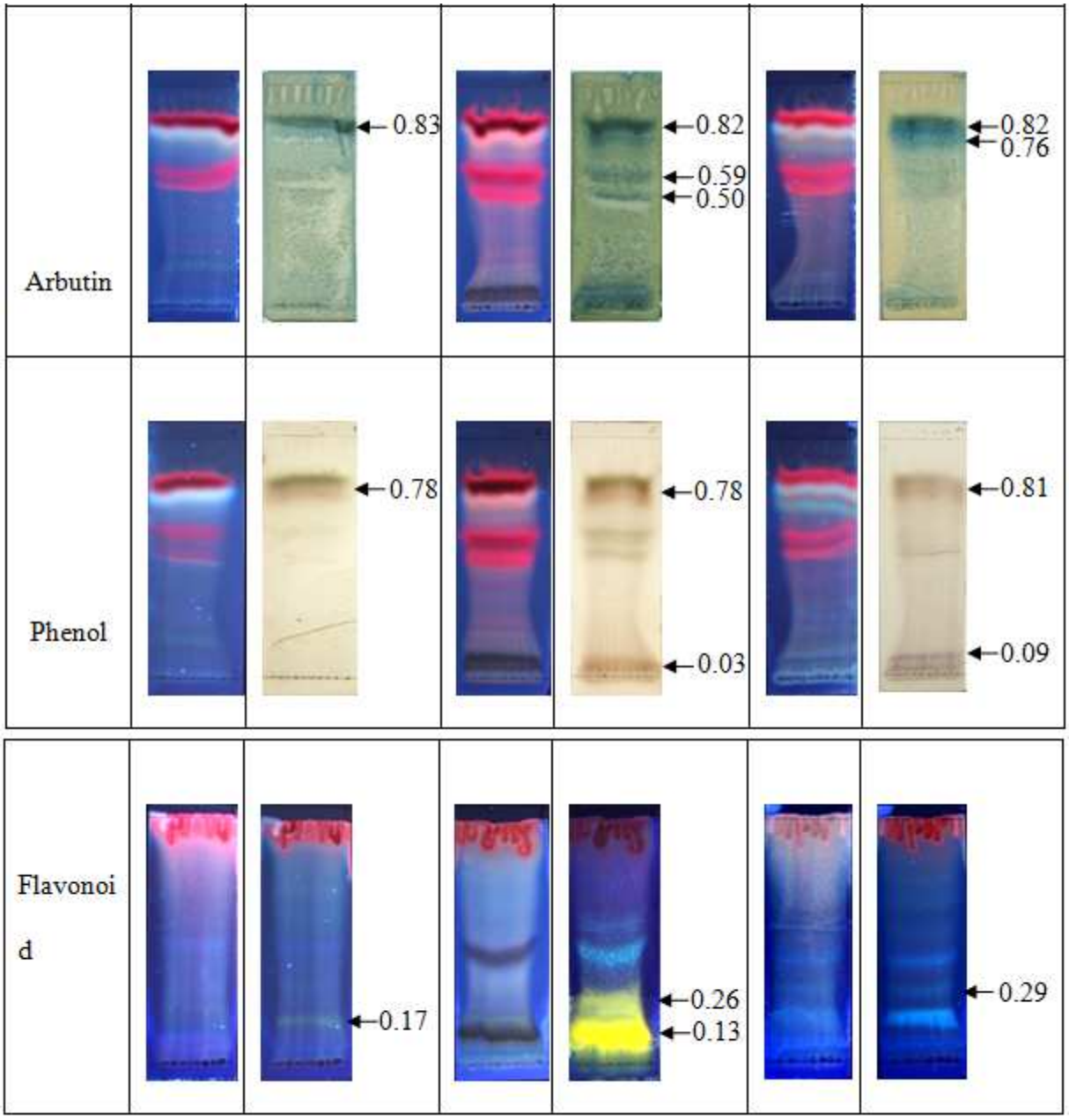
(+++) appreciable amount; (++) moderate; (+) trace amount; (-) completely absent.

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						
Alkaloid						
Anthraglycosides						



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$^{\bullet}$ 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$^{\bullet}$	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)

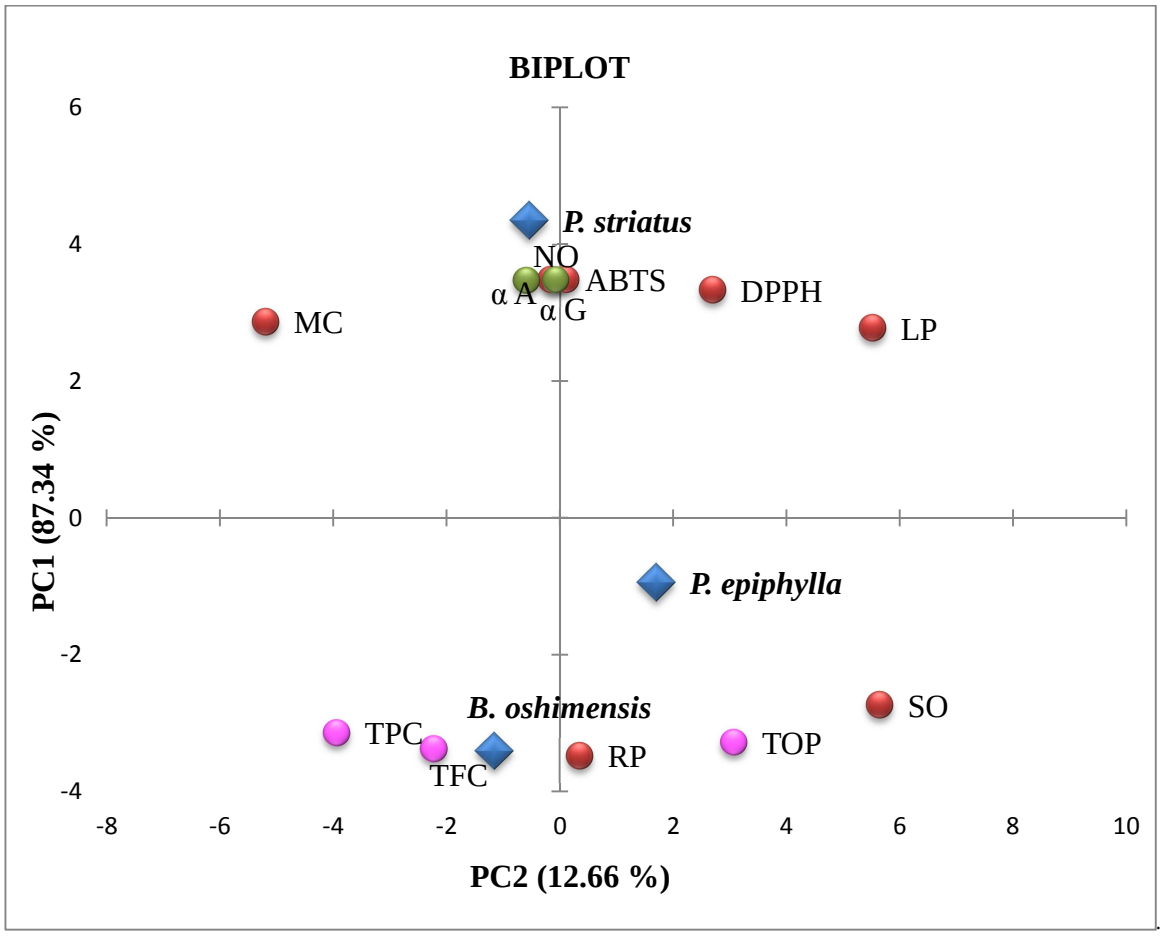


Figure 3: Principal component analysis of radical scavenging activity, anti-diabetic activity and phenolic compounds

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.

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Study of Bioactive Phytoconstituents and *In-Vitro* Pharmacological Properties of Thallose Liverworts of Darjeeling Himalaya

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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]. 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.

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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 37° 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 100°C 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

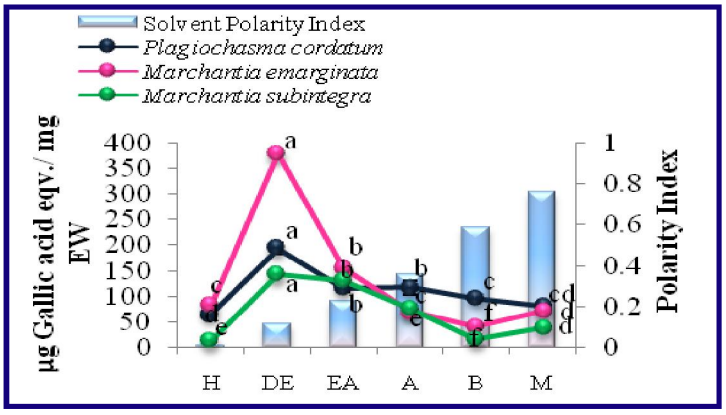


Fig 1: Total Phenol Content of studied liverworts

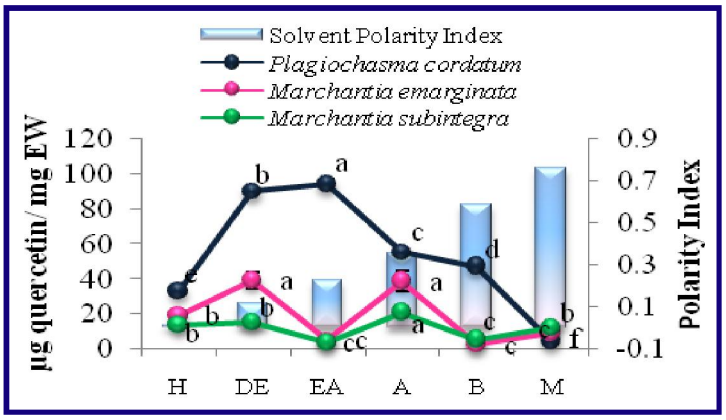


Fig 2: Total Flavonol Content of studied liverworts

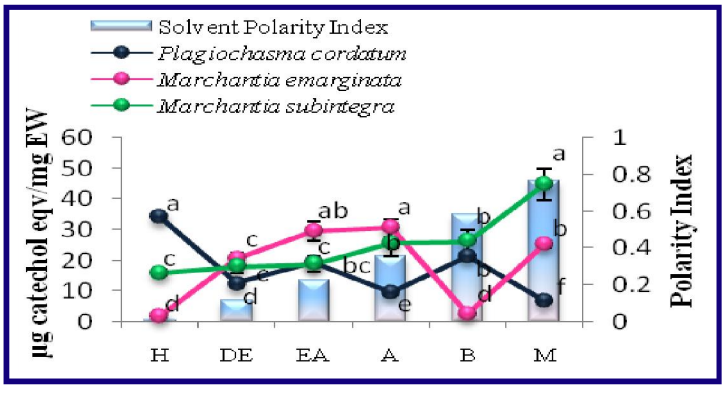


Fig 3: Total Orthodihydric Phenol Content of liverworts

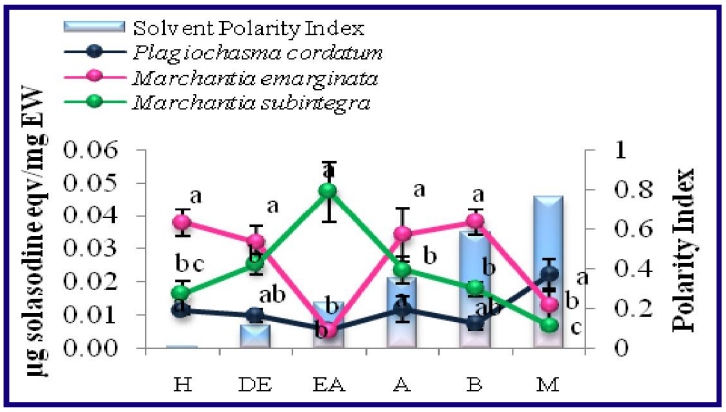


Fig 4: Steroid content of studied liverworts

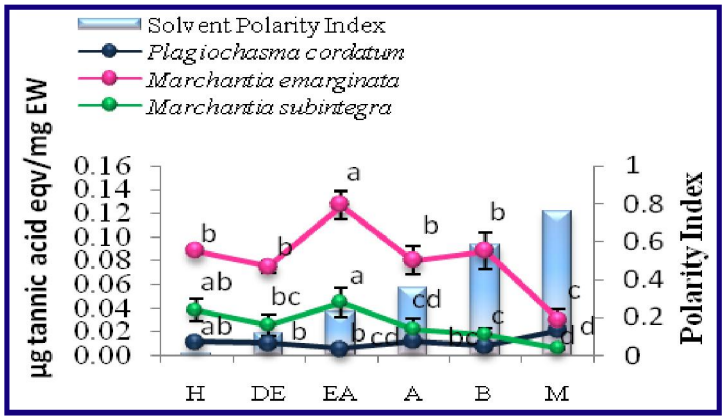


Fig 5: Tannin content of studied liverworts

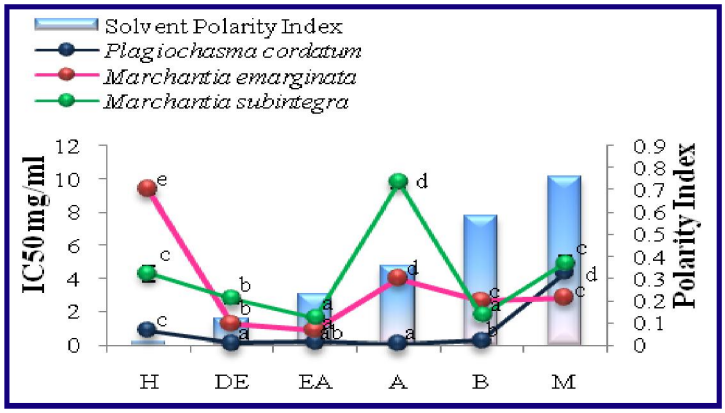


Fig 6: DPPH scavenging activity of studied liverworts

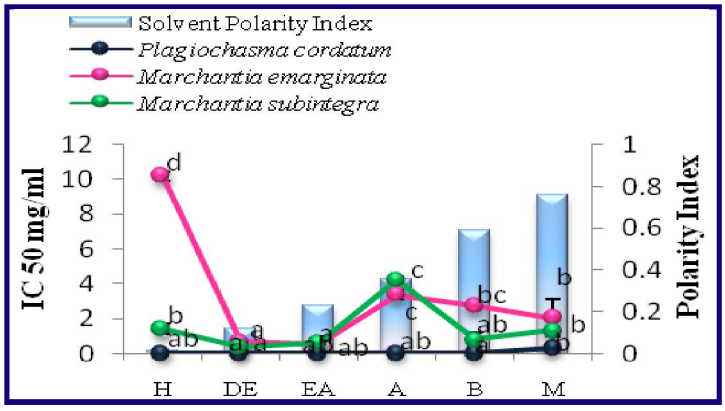


Fig 7: ABTS scavenging activity of studied liverworts

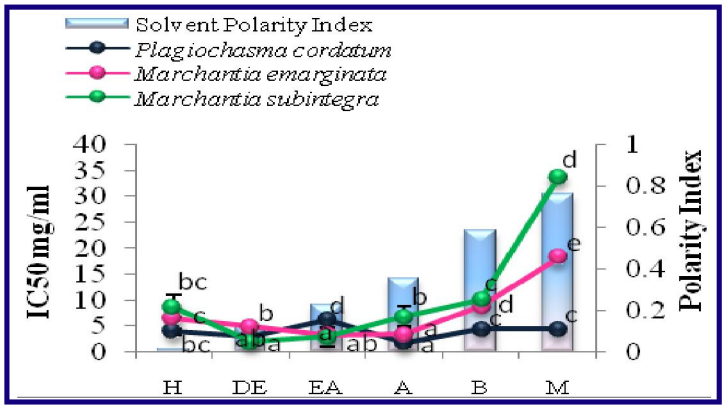


Fig 8: NO scavenging activity of studied liverwort

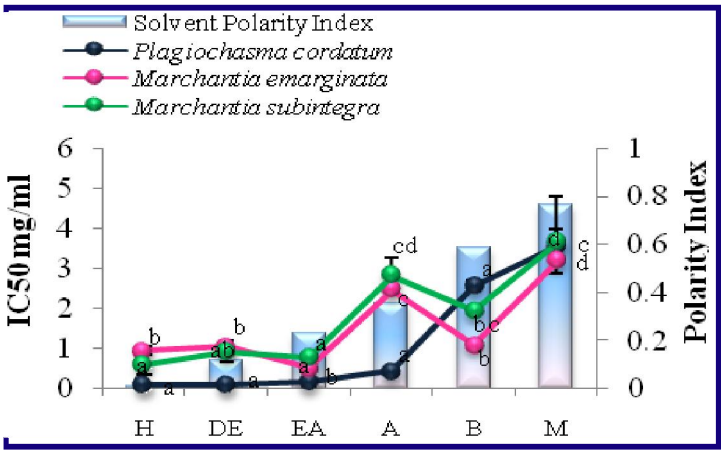


Fig 9: Metal Chelating activity of studied liverworts

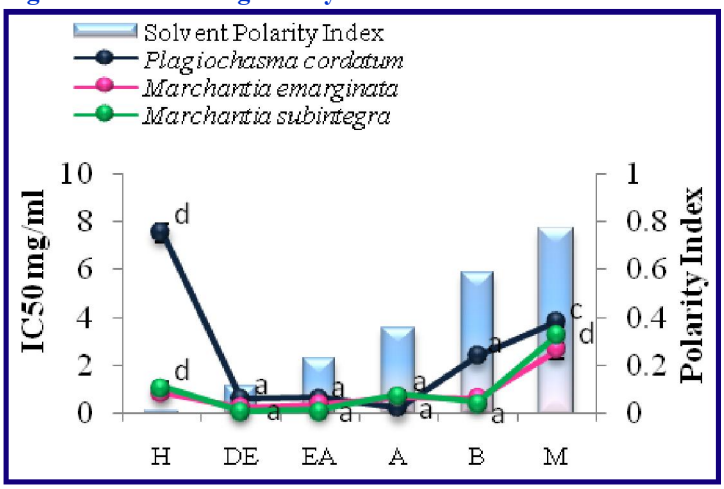


Fig 10: SO scavenging activity of studied liverworts

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

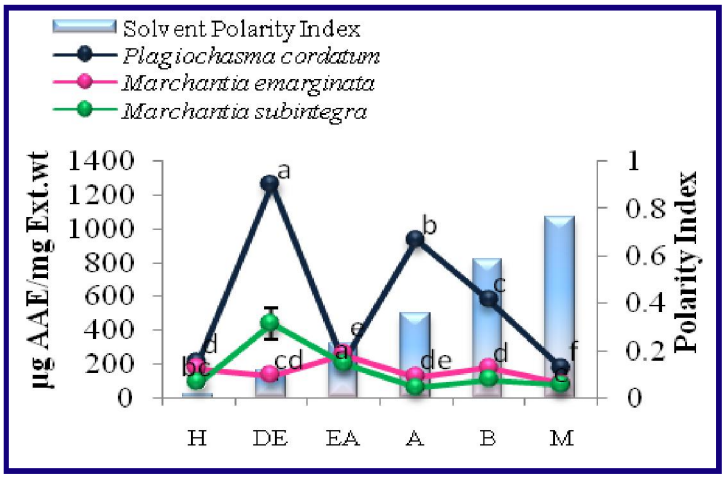


Fig 11: Reducing potential of studied liverworts

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 phyto-constituents 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 phyto-compounds 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	RS	AA	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	NO	MC	SO	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‡	NO	MC	SO	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*

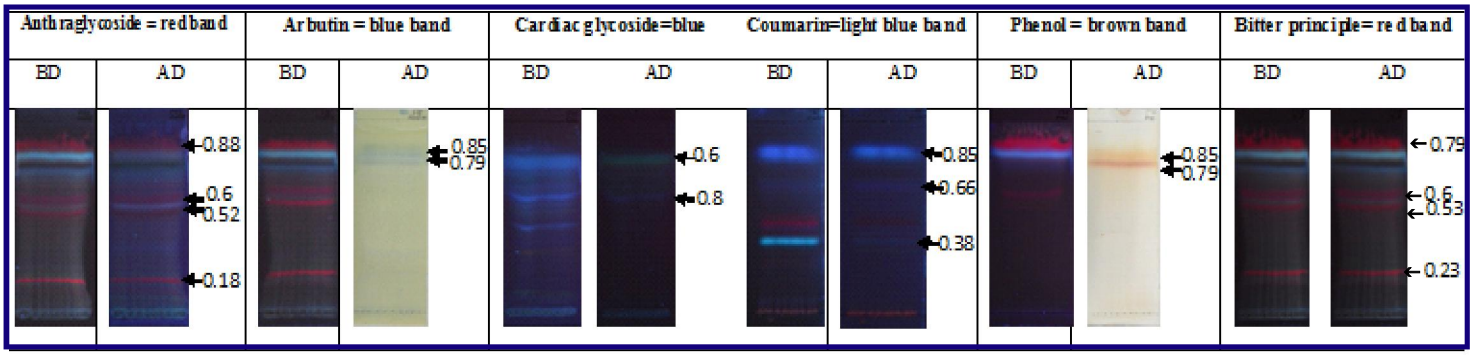
*****. Correlation is significant at the 0.01 level (1-tailed); *. Correlation is significant at the 0.05 level (1-tailed).***

Table 7: Correlation between phytochemical content and antioxidant activity in *M. emarginata*

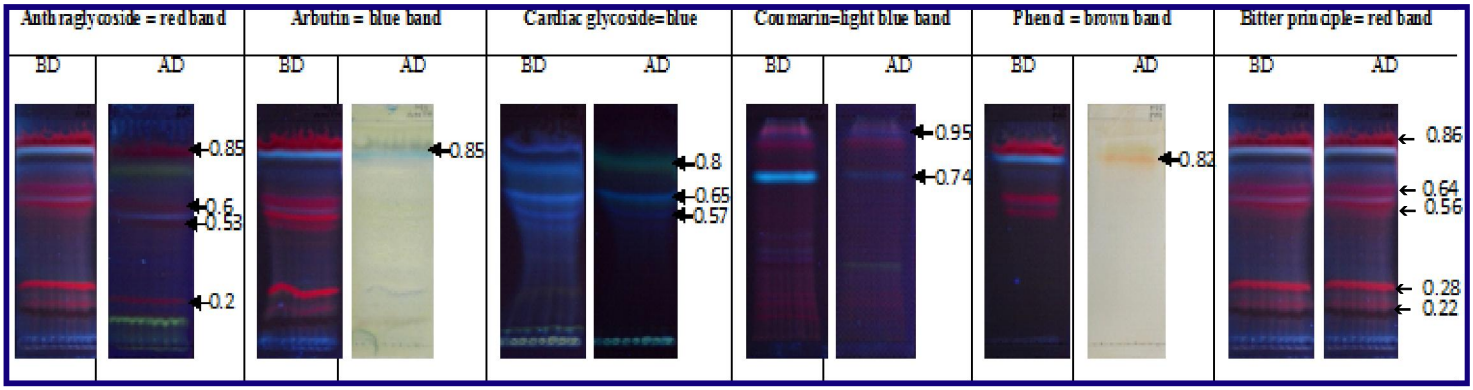
	DPPH*	ABTS*	NO	MC	SO	RP	TPC	TFC	TOPC	STE
ABTS*	0.994**									
NO	0.004	-0.034								
MC	0.007	-0.092	0.660							
SO	0.123	0.061	0.935**	0.802						
FRAP	-0.110	-0.021	-0.583	-0.838*	-0.614					
TPC	-0.416	-0.407	-0.339	-0.351	-0.441	0.002				
TFC	0.087	0.029	-0.428	0.174	-0.310	-0.454	0.531			
TOPC	-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)

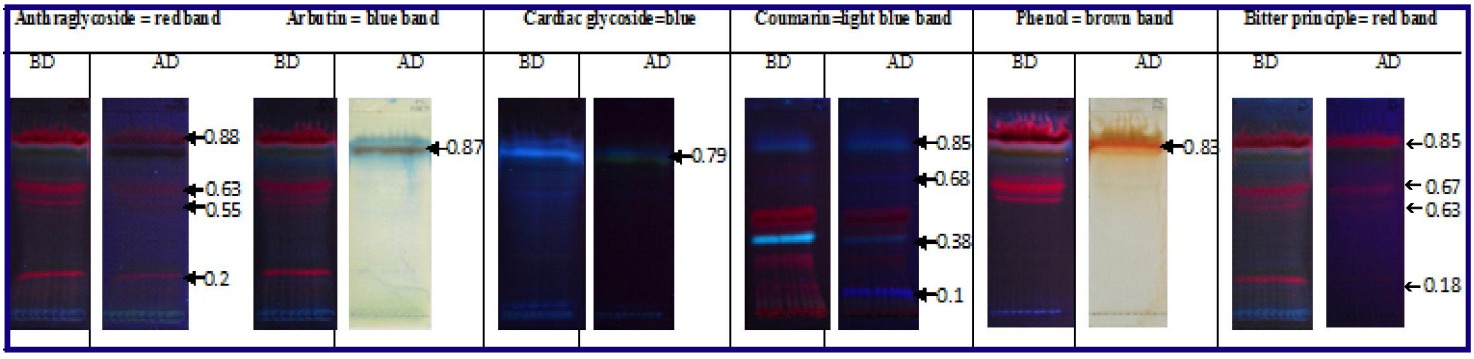
Table 8: Detection of phytochemicals in *M. emarginata* by TLC analysis; BD= before detection, AD= after detection

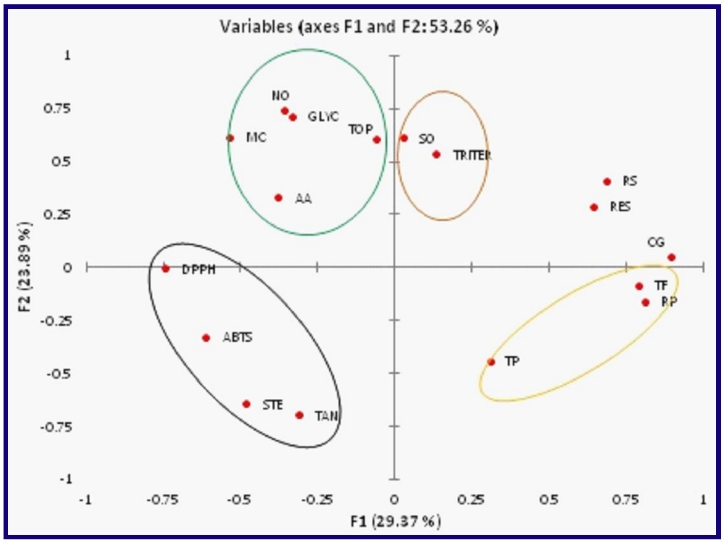


Detection of phytochemicals in *M. subintegra* by TLC analysis



Detection of phytochemicals in *P. cordatum* by TLC analysis



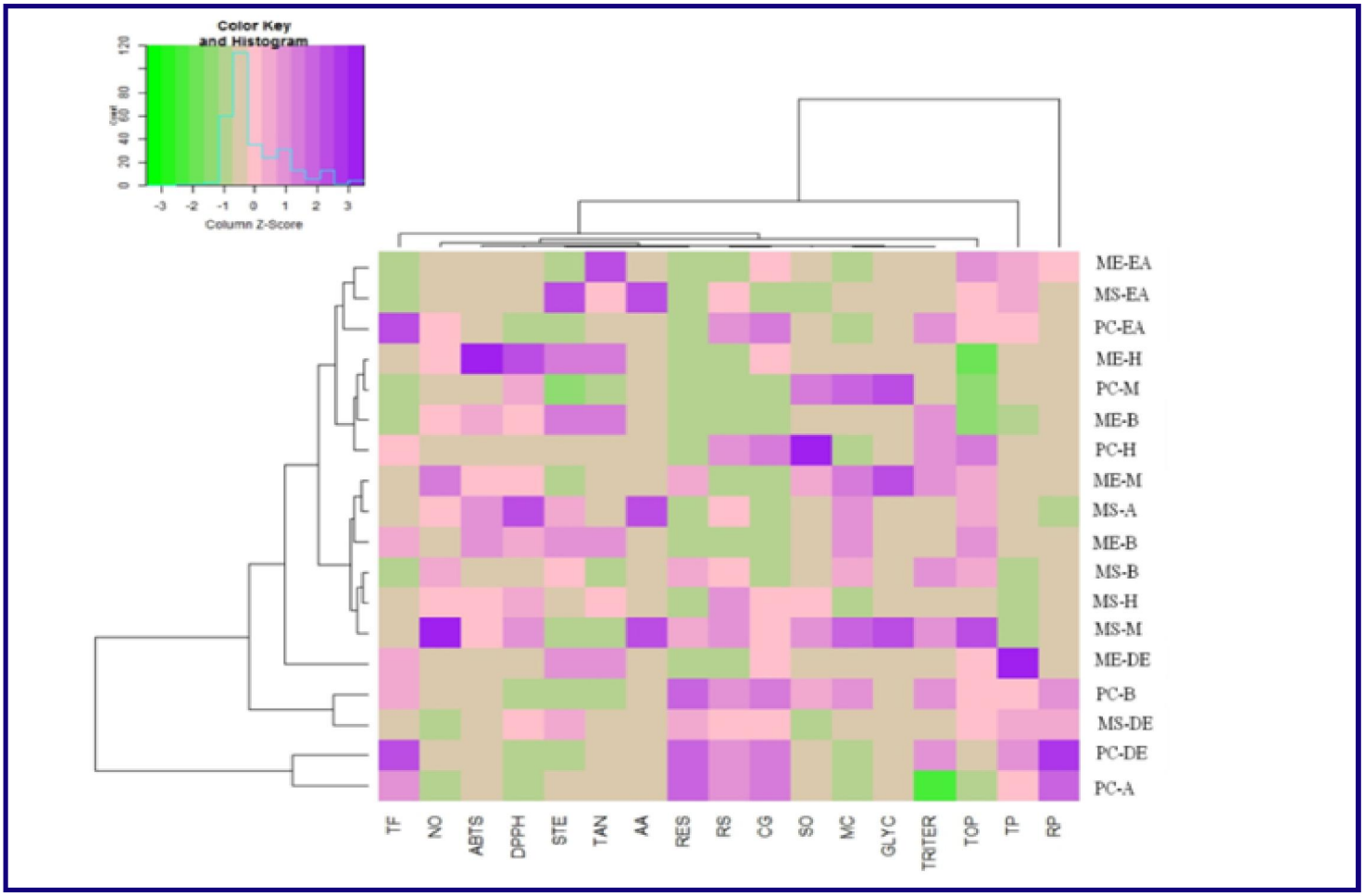


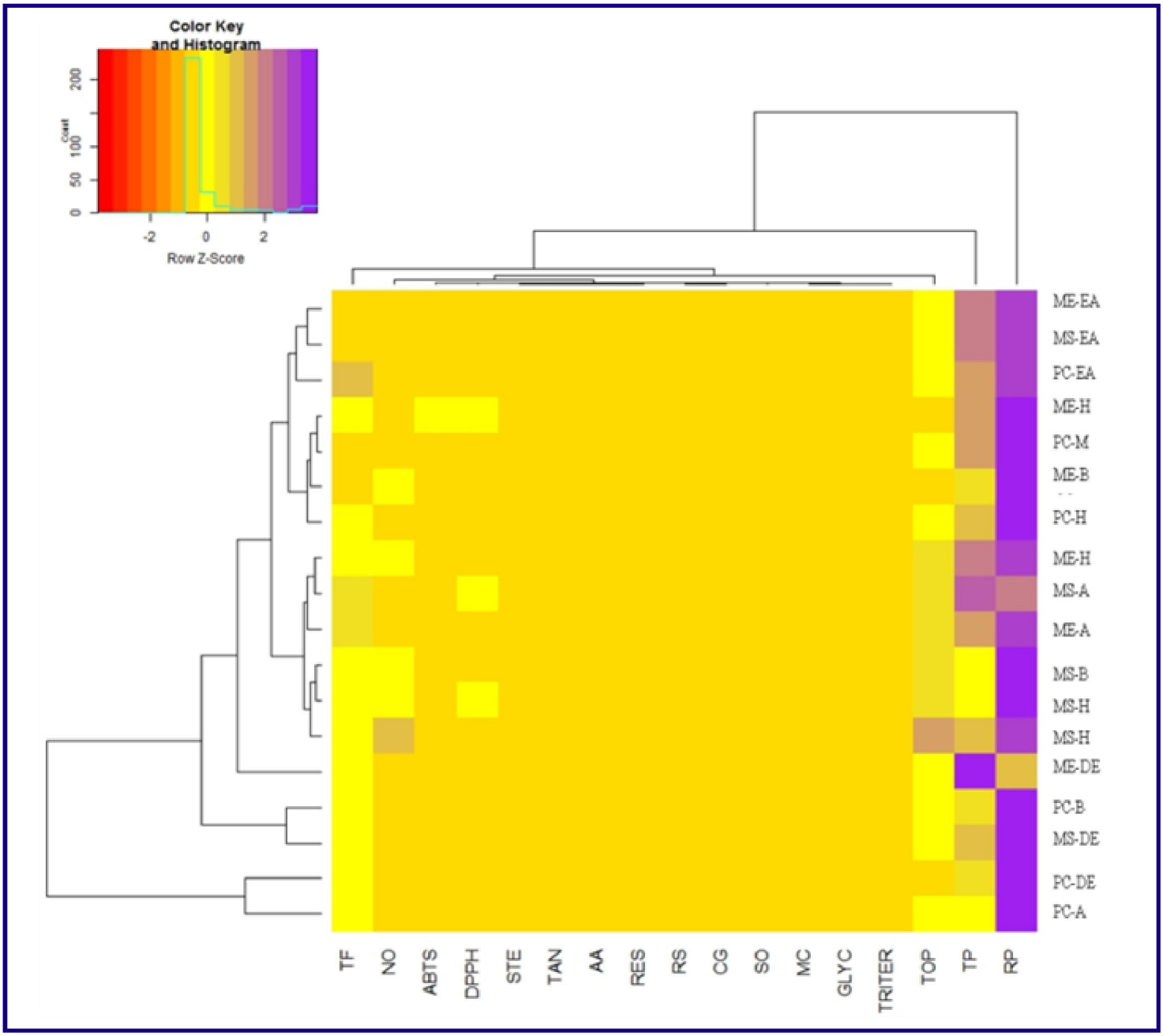
STE= steroid, TAN= tannin, GLYC= glycosides, AA= amino acid, RS= reducing sugar, RES= resine, CG= cardiac glycoside, TF= total phenol, TF= total flavonoid, TOP= total ortho-dihydric phenol, TRITER= triterpenoid

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.

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