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IN-VITRO FREE-RADICAL SCAVENGING POTENTIAL OF THREE LIVERWORTS OF DARJEELING HIMALAYA

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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 were also estimated. Qualitative phytochemical screening was carried out to detect the presence of varied phytochemicals. Thin-layer chromatography (TLC) and TLC bioautography assay were 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 a 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, the endogenous antioxidative defense mechanism has evolved to meet this requirement [2].

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The 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 a 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 shown 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.

The 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 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, the emphasis was always given on separating actual sample cautiously to keep a specimen of interest pure. Plant samples were then washed with tap water, air-dried and crushed into a 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*:*

Total Phenol Estimation: The total phenolic content of the extract was estimated by the method of Kadam *et al.,* [7] with few modifications using Folin-Ciocalteu reagent. Gallic acid is used as a 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 h. Absorbance was measured at 725 nm. A standard curve was prepared with different concentrations of gallic acid.

Total Flavonoid Estimation: Total flavonoid content of the 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 min, 0.3 ml of 10% aluminum chloride was added and was left for 6 min. 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 the standard curve was prepared with different concentrations of quercetin. The total flavonol content was expressed as mg quercetin equivalent/g dry weight.

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 the brown bottle. Catechol was used as a 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 the standard curve prepared from working with standard catechol solution at different concentrations.

Antioxidant Activity Determination:

DPPH Scavenging Antioxidant Activity Determination: The effect of the 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 min, and the reduction of the DPPH radical was measured Spectrophoto-metrically at 517 nm against reagent blank. The IC_{50} value was used to express the antioxidant activity of the crude methanolic extract. It is defined as the concentration of extract that results in the 50% reduction of the free-radicals [11]. Lower the IC_{50} value; higher is the antioxidant activity [12]. Scavenging activity of the sample was calculated based on percentage decolorization of the sample according to the following equation:

$$\% \text{ inhibition of DPPH activity} = [(A_0 - A_1) / A_0] \times 100\%$$

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.

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 extracts was incubated at 25 ºC for 150 min. 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 min. The absorbance of the mixture was measured at 540 nm. Following formula was used to calculate the nitric oxide scavenging activity of the extract:

$$\% \text{ inhibition} = [(A_0-A_1) / A_0] \times 100$$

Where, A_0 = Absorbance of control and A_1 = Absorbance of sample

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 min with exposure to fluorescent light, and absorbance was measured at 560 nm. The percentage inhibition was estimated by using the following formula:

$$\text{Superoxide radical scavenging effect (\%)} = [(A_0 - A_1/A_0) \times 100]$$

$$\text{Inhibition \%} = (A_0 - A_1/A_0) \times 100$$

Where A_0 = Absorbance of control and A_1 = Absorbance of the sample.

Metal Chelating Assay: Method described by Dinis *et al.,* [16] was utilized with few modifications for the determination of the metal chelating activity of the extract. The 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 min 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 the following formula:

$$\text{Chelating effect\%} = (A_0 - A_1) / A_0 \times 100$$

Where A_0 = Absorbance of control and A_1 = Absorbance of the sample.

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 ºC for 20 min. After 20 min 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 the methanolic extract to reduce iron by plotting absorbance value against different concentrations.

Lipid peroxidation Assay: The extent of lipid peroxidation in goat liver homogenate was determined by using the standard method of Bouchet *et al.* [18] At first fresh goat, the liver was collected from the slaughterhouse. The liver was cut into small pieces, homogenized in mortar pestle with buffer and filtered through a muslin cloth to get a 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 ºC. 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, the mixture was centrifuged, and the supernatant was separated. Absorbance was measured at 535 nm.

Vitamin E was used as a standard. The percentage of lipid peroxidation inhibition was estimated by comparing with control according to the following formula:

$$ACP\ \% = [(A_F - A_1)/(A_F - A_0)] \times 100$$

Where A_0 = absorbance of the 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. The 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 a 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 was 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 phytochemicals 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 is 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.

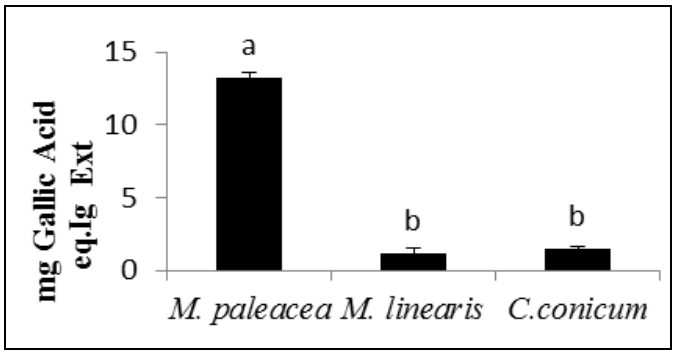

FIG. 1: TOTAL PHENOL CONTENT

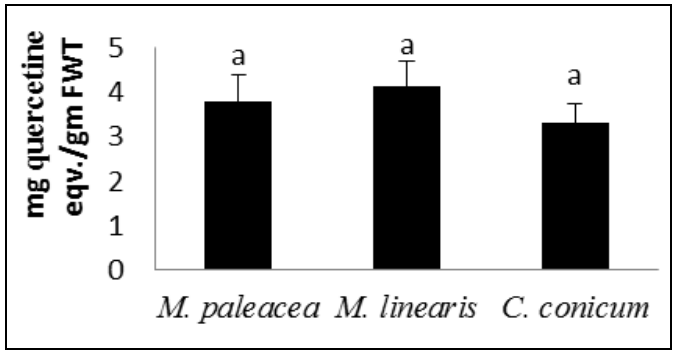

FIG. 2: TOTAL FLAVONOL CONTENT

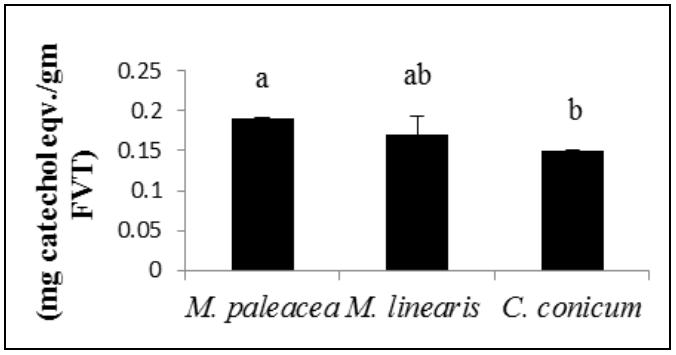

FIG. 3: TOTAL ORTHODIHYDRIC PHENOL CONTENT

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].

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**.

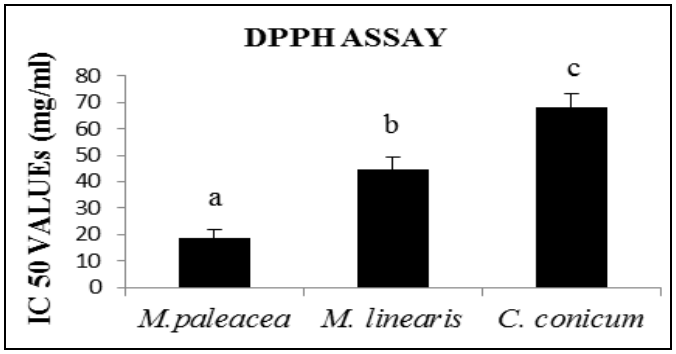

FIG. 4: DPPH RADICAL SCAVENGING ACTIVITY

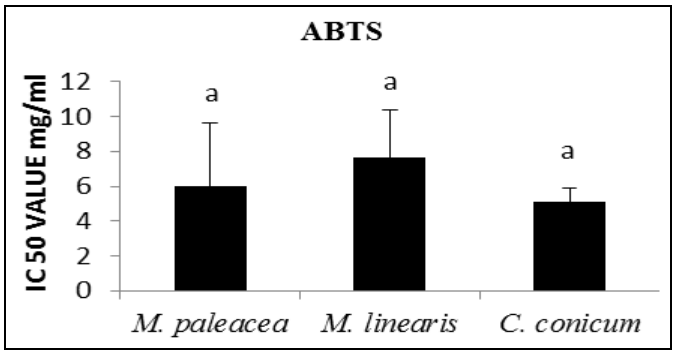

FIG. 5: ABTS RADICAL SCAVENGING ACTIVITY

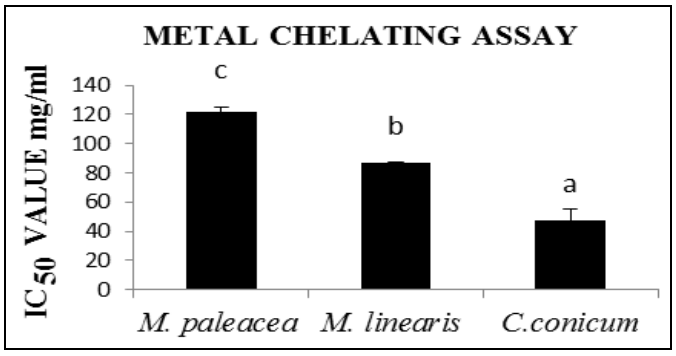

FIG. 6: METAL CHELATING ACTIVITY

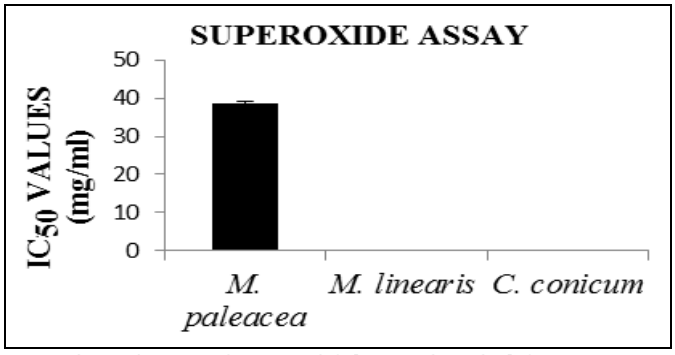

FIG. 7: SUPEROXIDE SCAVENGING ACTIVITY

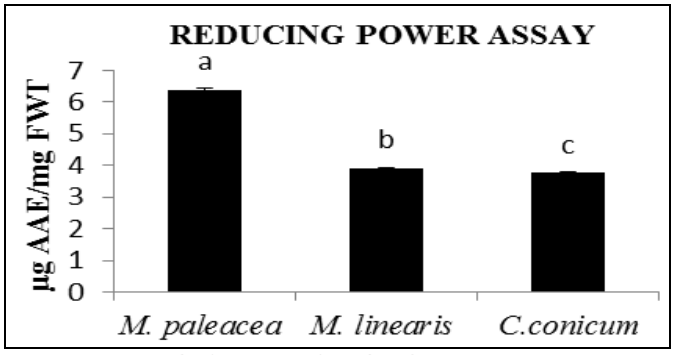

FIG. 8: REDUCING POWER

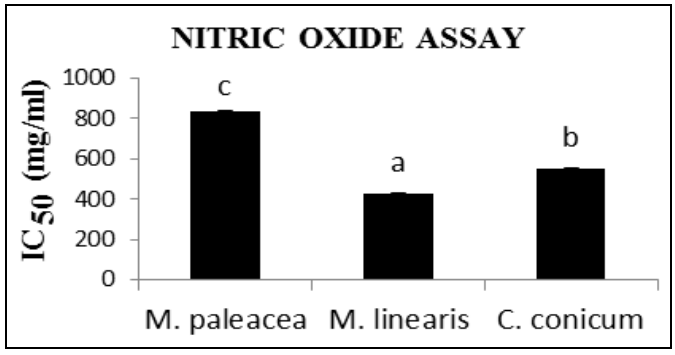

FIG. 9: NITRIC OXIDE SCAVENGING ACTIVITY

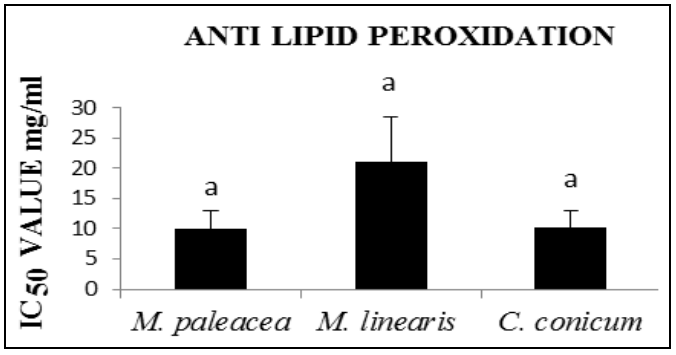

FIG. 10: ANTI-LIPID PEROXIDATION ACTIVITY

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 has revealed that among the three bryophyte samples studied, only *M. paleacea* have superoxide radical scavenging activity (38.41 mg/ml) **Fig. 7**.

The reducing the 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**.

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 a positive correlation with superoxide scavenging activity and iron-reducing ability of the extract. Orthodihydric phenol content shows a positive correlation with metal chelating activity and negative correlation with DPPH scavenging activity. Furthermore, the metal chelating activity of the analyzed bryophyte sample was 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 A 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 a diverse group of biomolecules in these bryophyte species.

TLC Bioautography Assay: Different phyto-chemicals 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 the 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 a 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.

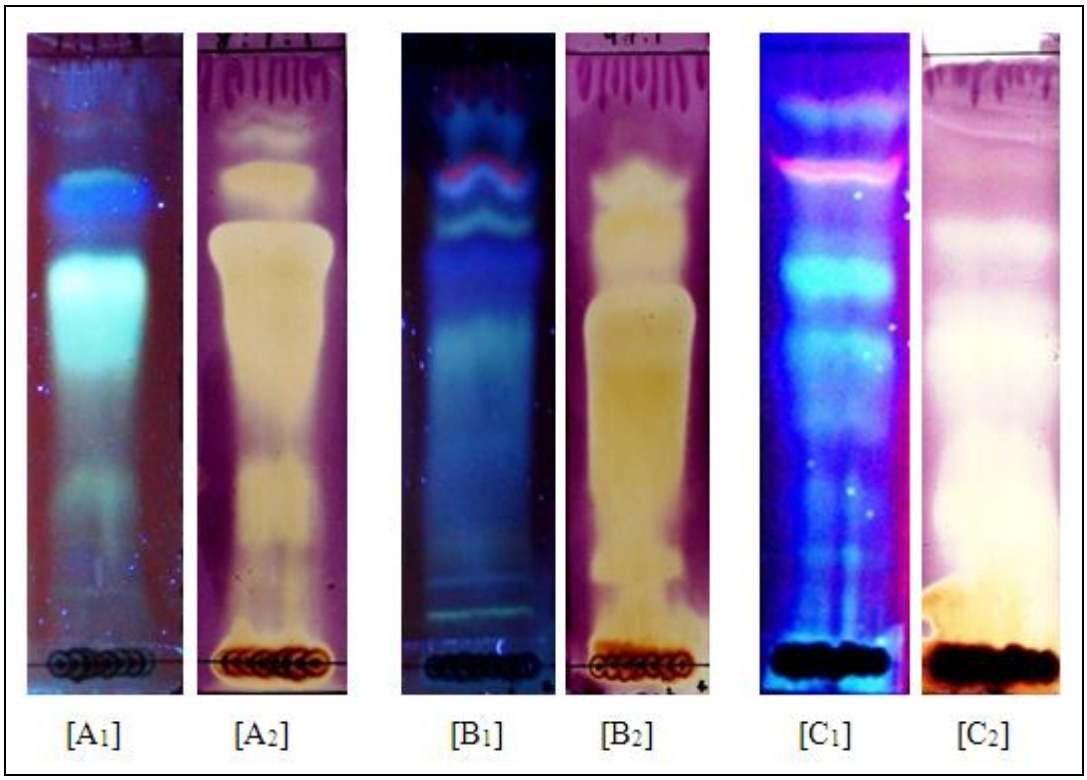

FIG. 11: THIN LAYER CHROMATOGRAPHIC PROFILE OF DIFFERENT SPECIMENS OF MARCHANTIALES: [A] *Marchantia paleacea*; [B] *Marchantia linearis*; [C] *Conocephalum conicum*. [A_1, B_1 and C_1]: Fluorescent bands observed under UV_{365}; [A_2, B_2 and C_2]: DPPH fingerprint of contemporary bands.

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 the 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, the 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 the 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 the 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 is 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, a compound that donates a hydrogen atom by breaking the free radical chain is related to the reducing power [28]. From the present work, it can be concluded that all three bryophytes have significant reducing potential. The highest reducing activity was shown by *C. conicum*. Complexes of ferrozine and Fe^{2+} together with samples have metal chelating activity, and thus the 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 is much higher than that of the methanolic extract of medicinal plant *Leucas plukenetii* [1].

A high correlation between ortho-dihydric phenol and DPPH indicated that ortho-dihydric phenolic components might be responsible for the said activity whereas the 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 the above work, it is evident that all the bryophyte species analyzed here are a 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 the protection of the cellular system against oxidative damage leading to aging 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 the further effort for assessment of bioactive components present in them.

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CONFLICT OF INTEREST: Nil

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