Phenolic profiles, antimicrobial and antioxidant potentiality of methanolic extract of a liverwort, *Plagiochila beddomei* Steph.

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The present study was carried out to evaluate antioxidant and antimicrobial activities of methanol extract of *Plagiochila beddomei* Steph. - a liverwort (Bryophyte). Total phenolics was fractionated by high performance liquid chromatography, showed the presence of a pool of phenolic acids such as coumaric, ferulic, gallic, caffeic, protocatechol, cinnamic, sinapate, chlorogenate and hydroxyl benzoate. Methanolic extract showed broad spectrum antimicrobial activity against various bacteria such as *Salmonella typhimurium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Bacillus cereus*, *B. subtilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and fungi like *Candida albicans*, *Cryptococcus neoformans*, *Trichophyton rubrum*, *Aspergillus niger*, *A. flavus*, *A. terreus* and *Mucor indicus*. Survivorship curve of bacteria and fungal spore germination were also analyzed. *P. beddomei* displayed significant antioxidant activity evaluated by 1,1-diphenyl-2-picrylhydrazyl free radical-scavenging ability, ferric-reducing antioxidant power, ferric thiocyanate assay and hydroxyl radical-scavenging activity. Phenols and flavonoids were found to be present in high levels. Total phenol content was shown to provide the highest correlation with FRAP assay (R² 0.966). MTT assay revealed the non-toxic nature of the extract.

**Keywords**: Antimicrobial, Antioxidant, Bryophyte, Flavonoids, Liverwort, *Plagiochila beddomei*, Phenols.

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**Introduction**

Bryophytes, the oldest land plants possess medicinally important bioactive compounds and are traditionally used in Chinese, Europeans, North American and Indian medicine, to treat illness of cardiovascular system, tonsillitis, bronchitis, tympanitis (inflammation of inner ear), skin diseases and burns. Compounds like polygodial from *Porella*, Norpiguison from *Conocephalum conicum* and Lunularin from *Lunularia cruciata*, 4-hydro-3-methoxybibenzyl and α and β pininealloromadendrine from *Plagiochila stevensoniana* are useful as anticancer and antimicrobial compounds. *Plagiochila fascicula* shown inhibitory effect on P388 cells (Leukemia), *Herpes Simplex type 1*, *Poliovirus type 1*, *Bacillus subtilis*, *Escherichia coli*, *Candida albicans*, *Trichophytion mentagrophytes* and *Cladosporium resinat*. The antifungal activity of *Herberta aduncus* against *Botrytis cinerea*, *Rhizoctonia solani*, *Pythium debaryanum* is well illustrated. Members of *Fissidens* and *Polytrichum* were used as diuretic and hair stimulating drugs. Generally, bryophytes are not damaged by insects, snails, slugs and other small animals. The biological activities of bryophytes are due to these lipophilic mono, sesqui and diterpenoids, aromatic compounds (bibenzyls, bis-bibenzyls, benzoates, cinnamates, long-chain alkyl phenols, naphthlenes, phthalides and isocoumarins) and acetogenins which constitute the oil bodies. In recent years, many possible sources of natural antibiotics have been in use for several infectious diseases, mostly bacterial and fungal. In this respect, the most investigated taxa are from angiosperms whereas very little data is currently available about other groups of plants, especially bryophytes. The purpose of the present study was to evaluate the *in vitro* antioxidant and antimicrobial activities of methanolic extract of *Plagiochila beddomei* Steph.

**Material and Methods**

**Plant material**

Fresh thallus of *P. beddomei* was collected from Nilgiri hills of Tamil Nadu, India. Taxonomic identity was confirmed by comparing with authenticated

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herbarium specimen (MCN 120257) at Department of Botany Herbaria, University of Calicut, Kerala. A voucher specimen of the plant is kept in the herbarium of the institute.

**Preparation of extracts**
Fresh thallus (100 g) was chopped and successively extracted with 300 ml of hexane, ethyl acetate, methanol and water for 6 h by hot continuation extraction using Soxhlet apparatus. The supernatants were concentrated using rotavapour at 50°C. The yields of the extract were, hexane (0.55 g), ethyl acetate (0.40 g), methanol (5.7 g) and water (4.5 g), respectively. The methanolic residue was lyophilized and stored at −20°C.

**Phytochemical screening**
Methanolic extract was subjected to various tests in order to detect the presence of different phytochemicals such as phenols, flavonoids, carbohydrates, glycoproteins, alkaloids, sterols and triterpenes outlined by Harborne.

**Total phenols assay**
Total phenols was determined using Folin Ciocalteu reagent at 765 nm and expressed in terms of gallic acid equivalent (mg/g of dry mass).

**Reverse Phase High Performance Liquid Chromatography (RP-HPLC) of phenols**
Phenolic components of the extract were further fractionated following the method of Beta et al. The separation of phenolic acids was performed in a Waters 2690 HPLC system equipped with a Waters AF on-line degasser and connected to a Waters model 996 photodiode array detector. Instrument control and data analysis were carried out using Millennium 3.20 software. Separation of phenolic acids was performed on a reverse-phase Waters Symmetry C-18 (250 mm × 4.6 mm, 5mm) (Millipore, Milford, MA) column at 30°C. Standard phenolic acids such as gallic, vanillic, caffeate, p-hydroxybenzoic, ferulic, chlorogenic, sinapic, coumaric, protocatechol and cinnamic acids were injected into the column separately. Phenolic acids in the sample were identified by comparing with the retention time of the standards. Area of the peaks was taken for quantification.

**Quantification of total flavonoids**
Flavonoid was quantified following the method of Chang et al. The calibration curve was prepared by quercetin at concentrations 12.5 to 100 µg/ml in methanol.

**Test microorganisms**
Salmonella typhimurium (CCM 583), Staphylococcus aureus (MTCC 740), Klebsiella pneumoniae (MTCC 109), Escherichia coli (MTCC 42), Bacillus cereus (MTCC 430), B. subtilis (MTCC 47), Proteus vulgaris (MTCC 426), Pseudomonas aeruginosa (MTCC 429) and fungi like Candida albicans (MTCC 183) Cryptococcus neoformans (MTCC6333) Trichophyton rubrum (MTCC 296), Aspergillus Niger (MTCC 16404), A. flavus ( MTCC 1973), A. terreus (MTCC 782) and Mucor indicus were identified and procured from Institute of Microbial Technology (IMTECH-CSIR), Chandigarh, India.

**Antimicrobial assay**
Microdilution method was used to determine the MIC of the crude methanolic extract. Each well of microtiterplates was filled with 50 µl of test organism and 200 µl extract. For bacteria and fungi the microtiterplates were incubated at 37±2°C and 30±2°C for 24 h and 48 h, respectively. One hour before the end of incubation 40 µl of a 0.2% solution of Iodo-Nitro Tetrazolium (INT) (Merck, Germany) was added to the wells and the plate was incubated for another hour. Since the colourless tetrazolium salt is reduced to a red coloured product by biologically active organisms, the inhibition of growth can be detected when the solution in the well remains clear after incubation with INT. The assay was run three times. After the incubation period the plates were read at 620 nm using ELISA reader. Minimum inhibitory concentration (MIC) was determined as the lowest concentration of the extract inhibiting the growth of the organism, based on the readings.

Different concentrations of the extract prepared were; 0.0625, 0.25, 0.5, 0.75, 1, 2 and 4 mg/ml. To determine minimum killing concentration, 50 µl broth was taken from each well and inoculated in 200 µl nutrient broth for 24 h at 37°C for bacteria and 48 h at 30°C for the fungal cells. Minimum killing concentration is defined as the lowest concentration of the extract inhibiting the growth of the organism, based on the readings. Each test was performed in triplicate and repeated twice. Streptomycin was used as positive controls for bacteria and Fluconazole for fungi. Each plate had a set of controls: a column with antibiotic as positive control and another control with
all solutions with the exception of the test compound, and a column with all solutions with the exception of the bacterial solution.

**Bacterial survival graph study**

Mid-logarithmic phase cultures of all bacterial strains were transferred into 50 ml portions of pre-warmed nutrient broth (Hi-Media) containing MIC, 2 \( \times \) MIC, 4 \( \times \) MIC concentrations of extract, to yield final concentrations of \( 10^5 \) cfu/ml. The broths were maintained, with agitation, on a water bath at 30°C.

Aliquots (1 ml) were drawn, after 0, 1, 2, 3, 4, 5, 6, 12 and 24 h and appropriately diluted in nutrient broth to neutralize the effect of the extract and viable counts determined by pour plate techniques. Each of the diluted culture suspensions (1 ml) was added to sterile petri dishes and approximately 20 ml, were melted and cooled (45°C), nutrient broth was added and mixed. Control broths containing 1 \% \( \text{v/v} \) Dimethyl sulfoxide (DMSO) were also set up for each test organism. All experiments were performed in triplicate. Colonies were counted and the number of survivors was calculated after incubation at 30°C for 48 hours. Survivor-time curves were drawn for the test organisms, exposed to the various concentrations, as well as the control.

**Inhibition of fungal spore germination**

Spores of all fungal species were incubated in Sabauroud Dextrose Agar (SDA) in microtitre plates in the presence of increasing concentrations of extract at 30±2°C. Growth inhibition was determined after 24 h incubation by microscopic observation and after 48 h by measuring the density of the germinating spores at 580 nm.

**MTT assay**

Cytotoxic activity was studied by the MTT assay using a microplate ELISA reader described by Mosmann. Plant extracts were tested in independent assays at concentrations of 500 to 2000 \( \mu \text{g/ml} \). Parthenolide (100 \( \mu \text{M} \)) was used as positive control and DMSO 1\% (\( \text{v/v} \)) as negative control.

**Antioxidant activity**

**Ferric reducing/antioxidant power FRAP assay**

Antioxidant activity was estimated by the assay method of Benzie and Strain.

**Free radical scavenging ability by the use of a stable DPPH radical**

Free radical scavenging activity of methanolic extract was determined using the stable radical DPPH (1,1-diphenyl-2-picrylhydrazyl).

**Total antioxidant capacity (TAC) determination**

Total antioxidant activity was measured by ferric thiocyanate (FTC) method described by Saha et al.

**Hydroxyl radical-scavenging activity**

2-Deoxyribose is oxidized by the Fenton reaction and degraded to malondialdehyde.

**Statistical analysis**

All the experiments were designed with six replications. Statistical analyses were conducted using SPSS version 12.0 for Windows. ANOVA and student - \( t \) test were performed to compare the data. The confidence limits used in this study were based on 95\% (\( P < 0.05 \)).

**Results and Discussion**

**Phytochemical analysis**

Preliminary phytochemical analysis showed the presence of carbohydrates, glycoproteins, flavonoids, alkaloids, sterols, phenols and triterpenes. The methanolic extract showed significant amount of total phenols (19.3 mg/g) and flavonoids (16.9 mg/g) compared to the other solvent extracts.

**Fractionation of total phenols**

RP-HPLC fractionation of phenols in \( P. \) beddomei (Fig. 1) revealed the presence of phenolic acids such as gallate (199.4 \( \mu \text{g/g} \)), vanilate (75.3 \( \mu \text{g/g} \)), chlorogenate (200 \( \mu \text{g/g} \)), cinnamate (212.2 \( \mu \text{g/g} \)), protocatechol (16121.7 \( \mu \text{g/g} \)), coumarate (289.7 \( \mu \text{g/g} \)), ferulate (232.7 \( \mu \text{g/g} \)), sinapic (222 \( \mu \text{g/g} \)), caffeate (322.4 \( \mu \text{g/g} \)) and hydroxyl benzoate (1.5 \( \mu \text{g/g} \)). The retention time of standards and sample was listed in Table 1. Values of retention time were in agreement with those published by Beta et al.

A positive correlation was observed between the phenolic acids and total phenols in the plant suggesting their role as precursor of many of the secondary metabolites. Cinnamate, coumarate, gallate, ferulate and hydroxy benzoate has proven antioxidant potentiality, which inturn supports the antioxidant significance of the plant. Chlorogenate can regenerate oxidized vitamin E via caffeate and it also acts as a pro oxidant in the propagation phase of LDL oxidation. Coumarate is a precursor of flavonoids and also binds with nitric acid and its derivatives before they combine with protein amines to form nitrosamine radical. Similarly cinnamate has antibacterial, antifungal and antiparasitic properties.
and gallate and its derivatives exhibit higher free radical scavenging properties.

**Antimicrobial activity**

Antimicrobial assay exhibited different degree of growth inhibition against tested bacterial and fungal species such as *Salmonella typhimurium, Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Bacillus cereus, B. subtilis, Proteus vulgaris, Pseudomonas aeruginosa* and fungi like *Candida albicans, Cryptococcus neoformans, Trichophyton rubrum, Mucor indicus, Aspergillus niger, A. flavus and A. terreus* (Table 2). The microbicidal effect of the extract was further visualized as inhibition zone by treating the pathogens with methanolic extract and then spreading the cells on agar plates. Among the pathogens tested species of *Aspergillus, Trichophyton rubrum* and *K. pneumoniae* were the most sensitive and *Pseudomonas aeruginosa, Bacillus cereus* and *S. typhimurium* are the resistant species. Protective effect of the extract was comparable to that of *Piper nigrum* Linn. Antimicrobial activity of phenolic compounds is likely exerted primarily by its ability to act as a nonionic surface-active agent therefore disrupting the lipid-protein interface or by the denaturation of proteins and inactivation of enzymes in the pathogens. Secondly, phenols alter the permeability of the membrane that could result in the uncoupling of oxidative phosphorylation, inhibition of active transport and loss of metabolites due to membrane damage. Gallic acid has proven anti-fungal and antiviral properties.

**Survival-time studies**

The means of survivors obtained at each concentration plated for each organism followed more or less a similar pattern (Data not shown). All the tested bacteria exhibited a decline in survivors in the initial 12-18 h of exposure to the extract in

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**Table 1 — Comparison of retention time of phenolic acids of Standards and methanolic extract of Plagiochila beddomei**

<table>
<thead>
<tr>
<th>Phenolic acids</th>
<th>Retention time (Rt) in minutes.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>standard</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>2.6</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>4.5</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>3.8</td>
</tr>
<tr>
<td>p-HBA</td>
<td>6.4</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>5.7</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>6.2</td>
</tr>
<tr>
<td>ProtoCatechol</td>
<td>3.9</td>
</tr>
<tr>
<td>Coumeric acid</td>
<td>7</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>9.89</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Note: A gradient consisting of solvent A (2.5:97.5 v/v methanol-double distilled water at pH 3 with acetic acid) and solvent B (50:50 v/v methanol-double distilled water at pH 3 with acetic acid) was applied at a flow rate of 1 ml/min. Injection volume of both the standards and the samples was 20 µl.
concentration dependent manner whilst the control showed a steady rise in population within the same periods, followed by a marginal level of recovery of the organisms even at higher concentrations. This depicts a biostatic mode of action of the extract. The recoveries may be due to the inactivation of the bioactive constituents of the extract with time in the nutrient medium.

**Effect on fungal spore germination**

Antifungal effect of the extract was examined by incubating *Candida albicans*, *Cryptococcus neoformans*, *Trichophyton rubrum*, *Mucor indicus*, *Aspergillus flavus*, *A. niger*, *A. terreus* spores (2×10³) in microtitre plates for 24 h with increasing concentrations of extract. After 20 h incubation, the spore viability was determined by measuring absorbance at 570 nm. *Aspergillus niger*, *A. flavus* and *A. terreus* showed 50% inhibition at 0.16 mg/ml. But *Candida albicans*, *Cryptococcus neoformans*, *Trichophyton rubrum* and *Mucor indicus* the inhibitory concentration varied between 0.3 to 0.4 mg/ml. Higher concentrations resulted in 100% inhibition of spore germination (Figs 2 and 3).

**Fibroblast bioassays**

MTT assay was adapted to evaluate the biosafety of *P. beddomei* extract. L 929 mouse fibroblast cells were incubated with 500 to 2000 µg/ml extract at 37 ± 2°C for 24 and 48 h under aseptic condition. After incubation, the cell culture was examined under microscope in order to reveal the cellular response acquired by the cells after treatment with the extract. As control the fibroblast cells were cultured without any sample. It could be seen from the figure that the cultured cells did not exhibit any morphological change in comparison with the control at 500 and 1500 µg/ml concentrations (Fig. 4). The preliminary results indicate the non interference of the extract on the growth of L 929 cells which in turn confirm the non toxic nature of the extract. Since the cells are found metabolically active in the test sample the physiological status of the cell was measured by succinate dehydrogenase (SDH) assay. The assay detects mitochondrial succinate dehydrogenase (SDH) activity as a determinant of mitochondrial function and cell viability. SDH activity is measured by its capacity to convert 3- [4, 5-dimethylthiazol-2-yl]-2,5-diphenyltratrazolium bromide (MTT) to a blue crystallized compound. Sample/control absorbance values higher than 100% were accepted to have growth stimulant activity. It is clear from the data that the cell culture treated with 500 µg/ml extract showed 102% metabolically active cells whereas, at 1500 µg/ml the value was 118% (Fig. 5). Thus, the study provides a clear indication of the non toxic property of *P. beddomei* in maintaining the metabolic activeness of cell line culture.

### Table 2 — Antimicrobial activity- MIC and MBC of *Plagiochila beddomei* methanolic extract* against tested bacteria and fungi.

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>MIC (mg/ml)</th>
<th>MBC/MFC (mg/ml)</th>
<th>Streptomycin (mg/ml)</th>
<th>MBC/MFC (mg/ml)</th>
<th>Fluconazole (mg/ml)</th>
<th>MBC/MFC (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em> (+)</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> (+)</td>
<td>0.75</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> (-)</td>
<td>0.125</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (-)</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em> (-)</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em> (-)</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> (+)</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> (-)</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td>0.5</td>
<td>0.75</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>0.0625</td>
<td>0.25</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>Aspergillus terreus</em></td>
<td>0.125</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>0.0625</td>
<td>0.25</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>0.75</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>Trichophyton rubrum</em></td>
<td>0.25</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>Mucor indicus</em></td>
<td>0.5</td>
<td>0.75</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

*Gram positive (+) and Gram negative (-) bacteria. MIC, minimal inhibitory concentration which corresponds to the minimum *Plagiochila beddomei* methanolic extracts concentration capable to inhibit the visible growth of the micro-organism. MBC/MFC, minimal bactericide/fungicide concentration which corresponds to the minimum concentration of the extract capable to reduce the number of CFU for 0.1% of the initial inoculums.
Antioxidant potentiality

Antioxidant potentiality and stabilization of lipid peroxidation by phenols is mainly due to their redox properties of hydroxyl groups, which allow them to act as reducing agents/hydrogen donors/singlet oxygen quenchers\(^1\). Gallate, Coumarte, ferulate, Caffeate and hydroxy benzoate are potential established antioxidants\(^1\).

**DPPH\(^{•}\) radical scavenging activity**

DPPH\(^•\) is one of the stable organic nitrogen free lipophilic radical, which has been used to test the free radical-scavenging ability\(^1\). Antioxidants, on interaction with DPPH\(^•\) either transfer an electron or hydrogen atom to DPPH, thus neutralizing its free radical character. The degree of discolouration indicates the radical scavenging potential of the antioxidant\(^2\). \(P. \) beddomei extract exhibited significant linear increase in activity with concentration \(r^2 = 0.8699\) \((P < 0.05)\) (Table 3). The relative activities of DPPH inhibition of the extract and the standards are in the following order: ascorbate > BHT > \(P. \) beddomei. Similar result was also found in IC\(_{50}\) value (concentration of sample required to scavenge 50% free radical or to prevent lipid peroxidation by 50%) was found to be 1.62 ± 0.09.

Fig. 2 — Differences in fungal spore germination of *Candida albicans* (A & B), *Cryptococcus neoformans* (C & D), and *Trichophyton rubrum* (E & F) (control treated with sterile buffer; and test with methanolic extract of *Plagiochila beddomei*, A, C, E – controls; B, D, F – experimentals, 40 x.)
Hydroxyl radical-scavenging activity

Hydroxyl radical is the most reactive molecule among reactive oxygen species (ROS) and it bears the shortest half-life compared with other ROS\textsuperscript{19}. The scavenging abilities of \textit{P. beddomei} extract on hydroxyl radical inhibition by the 2-deoxyribose oxidation method are shown in Table 3. The results are indicated as the inhibition rate. Methanolic extract showed good hydroxyl radical-scavenging activity (70\%) at 1 mg/ml in the reaction mixture. Hydroxyl radical-scavenging activity was increased with increasing concentration of the extract but lower than
that of 1 mg/ml ascorbate. The value of IC$_{50}$ of the extract was 2.96 ± 0.04 mg/ml (Table 3).

**Ferric reducing activity based on FRAP assay**

FRAP assay measures the reducing potential of an antioxidant with ferric tripyridyltriazine (Fe$^{3+}$–TPTZ) complex to its ferrous coloured form tripyridyltriazine (Fe$^{2+}$–TPTZ)$^{12}$. Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom. The FRAP values of the extract varied from 156.8 ± 0.33 to 496.8 ± 0.26/ g DW. FRAP showed the highest activity which was in the increasing order BHT > ASC > P. beddomei. The values are comparable with antioxidant potentialities of many angiosperms$^{30}$.

**Antioxidant capacity by FTC method**

FTC method measures the amount of peroxide generated at the initial stage of linoleic acid emulsion during incubation. Here, peroxide reacts with ferrous chloride to form ferric chloride, which in turn reacts with ammonium thiocyanate to produce ferric thiocyanate, a reddish pigment. Low absorbance values measured via the FTC method indicate high antioxidant activity. Table 3 shows the

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**Fig. 4** — Effect of methanolic extract of *Plagiochila beddomei* on L929 cell morphology at various concentrations. A is the control L929 cells; B & C, cells after 24 and 48 h treatment with 500 µg/ml; D control cells; E & F, cells after 24 & 48 h with 1500 µg/ml exposure, respectively.
hydroperoxides inhibitory activity of *P. beddomei* through FTC test. As shown in the Table 3, almost all fractions significantly retarded the formation of hydroperoxides in the linoleic acid emulsion throughout the incubation period as compared to the control i.e. showed the highest percent of inhibition of lipid peroxidation 85.6 ± 0.22. This result suggests that the methanolic fraction might contain primary antioxidant compounds, which are able to react aggressively with free radicals, particularly hydroxyl radicals, thereby terminating the radical-chained reaction and retard the formation of hydroperoxides suggesting its lipid peroxidation inhibitory potential. The overall inhibitory activity against hydroperoxides formation can be established in the following descending order *P. beddomei* > BHT > ascorbate.

### Table 3 — Antioxidant activities of *Plagiochila beddomei* with different methods

<table>
<thead>
<tr>
<th>Conc. (mg/ml)</th>
<th>DPPH assay</th>
<th>Hydroxyl radical scavenging</th>
<th>FRAP assay</th>
<th>FTC Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Inhibition ± SD</td>
<td>% Inhibition ± SD</td>
<td>(µmol FeSO$_4$/g dry wtt)</td>
<td>Inhibition of lipid peroxidation (%) ± SD</td>
</tr>
<tr>
<td>0.1</td>
<td>14.8 ± 0.54</td>
<td>19.2 ± 0.11</td>
<td>156.8 ± 0.33</td>
<td>23.6 ± 0.21</td>
</tr>
<tr>
<td>0.2</td>
<td>43.9 ± 0.32</td>
<td>32.5 ± 0.02</td>
<td>238 ± 0.01</td>
<td>41.5 ± 0.34</td>
</tr>
<tr>
<td>0.4</td>
<td>61.6 ± 0.08</td>
<td>46 ± 0.42</td>
<td>296 ± 0.23</td>
<td>58.6 ± 0.09</td>
</tr>
<tr>
<td>0.6</td>
<td>74 ± 0.01</td>
<td>59 ± 0.65</td>
<td>378 ± 0.06</td>
<td>64.9 ± 0.03</td>
</tr>
<tr>
<td>0.8</td>
<td>88 ± 0.12</td>
<td>63 ± 0.72</td>
<td>426 ± 0.02</td>
<td>76.5 ± 0.42</td>
</tr>
<tr>
<td>1</td>
<td>93 ± 0.76</td>
<td>70 ± 0.07</td>
<td>496.8 ± 0.26</td>
<td>85.6 ± 0.22</td>
</tr>
<tr>
<td>IC$_{50}$ (mg/ml)</td>
<td>1.62 ± 0.09</td>
<td>2.96 ± 0.04</td>
<td>3.99 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>Ascorbate(1mg)</td>
<td>96%</td>
<td>76%</td>
<td>490 ± 0.01</td>
<td>69 ± 0.08</td>
</tr>
<tr>
<td>BHT(1mg)</td>
<td>94%</td>
<td>69.6%</td>
<td>336 ± 0.02</td>
<td>80.5 ± 0.1</td>
</tr>
</tbody>
</table>

Experiments were designed with six replications. Confidence limits used in this study were based on 95% (*P* < 0.05). Capability to scavenge the DPPH radical was (%) = \(\left(\frac{A_c - A_t}{A_c}\right) \times 100\), where *A*$_c$ is the absorbance of the control reaction and *A*$_t$ is the absorbance in presence of the sample of the extracts. Antioxidant activity was expressed as IC$_{50}$ as the concentration in mg of dry material / ml that inhibits the formation of DPPH radicals by 50%. FTC as % inhibition = \(\left(\frac{A_0 - A_t}{A_0}\right) \times 100\), where *A*$_0$ is the absorbance of the control reaction and *A*$_t$ is the absorbance of the sample. OH-scavenging activity was calculated as the inhibition rate of 2-deoxyribose. OH-scavenging activity % = 1 - \(\left(\frac{A_b - A_{bs}}{A_{bc} / A_{bo}}\right) \times 100\). FRAP assay was calculated from standard curve plotted using the FeSO$_4$·7H$_2$O linear regression equation. Butylated hydroxyl toluene (BHT) and ascorbate were used as standards.
The inhibition of lipid peroxidation by BHT (standard) was 80.5 ± 0.1 (Table 3). The high amount of total phenolics including the major phenolic acid such as caffeic acid and chlorogenic acid and flavonoids it is likely responsible from the high radical scavenging activity of the methanolic extract. Moreover, caffeic acid (322.4 µg/g) was proven DPPH scavenging activity very close to that of ascorbic acid. The high antioxidant capacity by FTC method may be attributed by the high total phenolics content or cooperative effect of phenolics and/or synergistic effects by phenols and flavonoids.

Correlation of total phenol and antioxidant potentials

Total phenol content in the present study shows highest correlation with FRAP assay ($R^2 = 0.966$) and was superior to bittergourd. The result was also in agreement with Sun and Ho, who found a strong positive correlation between total phenols and FRAP assay. Similar results were also found for hydroxyl radical-scavenging activity ($R^2 = 0.897$) and DPPH ($R^2 = 0.845$) as shown in Table 4. Yu et al. reported a significant correlation between total phenolics and scavenging ability of buckwheat extracts on DPPH radicals. By contrast, a study by Othman et al. found no correlation between scavenging activity and the total phenolic content.

Conclusion

It is concluded that methanolic fraction of *P. beddomei* showed significant value of inhibition of DPPH radical (93% ± 0.76), relative to butylated hydroxytoluene (BHT). Similarly, the extract displayed highest FRAP value (496.8 ± 0.26 µmol FeSO$_4$/g DW) and also inhibition of peroxidation (85.6 ± 0.22). So, it was promising to say that *P. beddomei* is a potentially valuable source of bioactive materials, which will be expected to protect against peroxidative damage in living systems in relation to ageing and carcinogenesis by the additive roles of natural antioxidants like phenolics and flavonoids. Parallely, the methanolic extract showed potent antimicrobial activity as demonstrated by the *in vitro* growth inhibition of important pathogenic bacteria and fungi.

<table>
<thead>
<tr>
<th>Correlation between total phenol content and antioxidant activity determined by different assays.</th>
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</thead>
<tbody>
<tr>
<td>TPC</td>
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<tr>
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</tr>
<tr>
<td>TPC</td>
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<tr>
<td>DPPH</td>
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<td>Hydroxyl</td>
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<tr>
<td>FRAP</td>
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<tr>
<td>FTC</td>
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</tbody>
</table>

Total polyphenols is expressed as gallic acid equivalent (mg/g of dry mass); (FTC: Ferric thiocyanate, TPC: total phenol content significantly correlated at $P < 0.01$, n = 12).

Table 4 — Correlation between total phenol content and antioxidant activity determined by different assays.

References


