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Antioxidant and antimicrobial activities of a common liverwort from Darjeeling Himalaya

Rajib De, Jayati Saha and Prabir K Sarkar*
Microbiology Laboratory, Department of Botany, University of North Bengal, Siliguri 734013, India

Abstract
The study was concerned with an examination of antioxidant and antimicrobial activities of methanolic extract of *Marchantia convoluta* (Merch.) L., collected from Darjeeling Himalaya. The antioxidant activities were evaluated by means of five *in vitro* methods, viz. free radical-scavenging activity, Fe^{3+}-reducing power, metal-chelating ability, trolox equivalent antioxidant capacity (TEAC) and activity in hydroxyl radical-scavenging system. The total phenol content was 1.1 mg gallic acid equivalents g^{-1} dried thallus. After 30 min of reaction, the 100 mg lyophilized extract possessed 6.7% free radical-scavenging activity. The same amount of extract exhibited 13.4% 168.2% metal-chelating and hydroxyl radical-scavenging activities, respectively. The reducing activity was found to be 28.5 mg ascorbic acid equivalents g^{-1} dried thallus. Total antioxidant activity was 0.18 µg TEAC g^{-1} dried thallus. The antimicrobial activity was evaluated by testing the methanolic extract of the samples against five microorganisms including two Gram positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*), two Gram negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*) and one yeast (*Candida* albicans by disc-diffusion assay. *M. convoluta* thallus extract was more or less inhibitory against all of the test bacteria, however did not possess any antifungal property. *S. aureus* was found to be most sensitive target organism.

Keywords: *Markentia Convoluta*, antioxident , antimicrobial, Darjeeling

An imbalance in biological oxidation processes due to uncontrolled rate of metabolic activities leads to the production of greater amount of reactive oxygen species (ROS) in the cells. ROS encompass a variety of diverse chemical species that include free radicals such as superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals (HO) and non-free radicals such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). These radicals cause oxidative damage by oxidizing biomolecules leading to cell death and tissue damage, such as atherosclerosis, cancer, emphysema cirrhosis and arthritis (Kehrer, 1993).

Antioxidants play an important role to protect the living organisms against damages caused by ROS (Halliwell and Gutteridge, 1989). Synthetic antioxidants such as butylhydroxyanisole (BHA), butylhydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) are substituted with natural alternatives due to their probable toxic and carcinogenic effects. Thus, the need for detection of natural antioxidants from various plant resources and evaluation of their properties has drawn the attention of many scientists in recent years (Moktan *et al.*, 2008).

Natural antioxidants are found in almost all plants, microorganisms and even in animal tissues. The majority of natural antioxidants are phenolic compounds and the most important groups of natural antioxidants are flavonoids and phenolics (Yanishlieva *et al.*, 2001). Flavonoids may act as antioxidants by scavenging radicals that include superoxide anions (Robak and Gryglewski, 1988) and hydroxyl radicals (Hussain *et al.*, 1987), singlet oxygen quenching (Criado *et al.*, 1995) and metal chelation (Ramanathan and Das, 1993).

The pathogenic bacteria and fungi cause harmful diseases in plants and animals throughout the world. To control or cope up with these harmful microorganisms different type of chemotherapeutic agents are in use. But nature is undoubtedly the most prolific producer of antimicrobial compounds. *Marchantia* is well known among the bryophytes as it is the type specimen of the liverworts, a group of plants having thalloid plant body (Fig. 1). Among its different species, *Marchantia convoluta* (Merch.) L. can be found quite abundantly in the Darjeeling Himalaya. Though widely used in Chinese herbal medicine (Xiao *et al.*, 2005), *M. convoluta* plants are not much popular for their ethnomedicinal properties among the tribes of this region. This lack of information may be due to the absence of proper attention in this regard. *M. convoluta* flavonoids consist of luteolin 7,4'-di-O-glucuronide, apigenin 7,4'-di-O-glucuronide and apigenin-7-O-b-D-glucuronide (Xiao *et al.*, 2005), and consequently, should possess some antioxidant activities. However, such activities of this plant have not so far been properly investigated.

Watt (1891) first referred to medicinal uses of *Marchantia* sp. A study conducted on the antimicrobial activity of bryophytes shows that *Marchantia* sp. is active against *Salmonella typhi*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Vibrio cholerae*, *Bacillus subtilis*, *Sarcina lutea* and some members of Mycoplasmatales (Banerjee and Sen, 1979). Purified flavonoids of *M. convoluta* have shown inhibition of

*Corresponding author:
E-mail: pksarkar@bsnl.in

growth of several bacteria, like *Sal. typhi*, *Sal. enteritidis*, *Staph. aureus*, *Streptococcus pneumoniae*, *Str. pyogenes* and *Escherichia coli* (Xiuo *et al.*, 2005). *M. convoluta* may possess other type of antimicrobials. However, the antimicrobial principles of the plant have not been thoroughly investigated.

Hence, the objectives of the present study were to evaluate the antioxidative as well as antimicrobial activities of *M. convoluta* to exploit its potentiality against oxidative stress in living system and harmful microorganisms.

Materials and methods

Sampling

Sampling was done from the three hill sub-divisions (altitude, 1200-2300 m ASL) of the district of Darjeeling in West Bengal. Thalli were collected into polyethylene sampling bags and brought immediately to the laboratory where those were washed and taken in porcelain trays to dry in a hot air oven at 45°C for 2 days. The dried samples were ground using an electric grinder (Bajaj, India). The powders were preserved in screw-capped glass bottles (Schott-Duran) at -20°C.

Preparation of freeze-dried extracts

About 10-15 g of the sample was extracted with 15 volume of methanol (SRL, 132977), thrice for 72 h. The mixture was allowed to stand at room temperature, with occasional agitation. The solvent fractions from a single extraction process were pooled and filtered through Whatman No. 1 filter paper and concentrated under reduced pressure at 40 ± 1°C in a rotary evaporator, followed by lyophilization (Eyela freeze-dryer, model FDU-506) to obtain a dry extract which was stored at -20°C. The lyophilized extract was dissolved in methanol and stored at 4°C until use.

Evaluation of Antioxidant activities

Total soluble phenolics in the extracts were assessed using the method described by Yen and Hsieh (1998). The concentration of the total phenolics was expressed as gallic acid equivalents (GAE) g^{-1} lyophilized extract, using a standard curve of gallic acid (HiMedia RM233).

The antioxidant activity of the extracts was measured in terms of hydrogen-donating or radical-scavenging ability using the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH˙) (HiMedia RM2798) method (Sanchez-Moreno *et al.*, 1998). The decrease in absorbance at 517 nm was measured at 1000 µg ml^{-1} concentration after 30 min interval using a spectrophotometer (Systronics, type 118, Ahmedabad). The inhibitory percentage of DPPH˙ was calculated as follows:

$$\% \text{ scavenging} = \frac{A_0 - (A - A_b)}{A_0} \times 100$$

where A_0 is the A_{517} of DPPH˙ without sample (control), A is the A_{517} of the sample and DPPH˙, and A_b is the A_{517} of sample without DPPH˙ (blank).

The trolox equivalent antioxidant assay, based on the reaction of DPPH with trolox, was used to compare the radical-scavenging activity of a compound to those of trolox (Aldrich 23,881-3), a water soluble vitamin E analogue with minor modifications (Van den Berg *et al.*, 1999). The trolox equivalent antioxidant capacity (TEAC) was expressed as µg TEAC g^{-1} dry weight of the sample.

Fig. 1: *Marchantia* thalli

The ability of the extracts to reduce ferric chloride (SRL 64765) was assessed at 700 nm according to the method of Oyaizu (1986). The reducing power was expressed as ascorbic acid equivalents (AAE) g^{-1} lyophilized extract, using a standard curve of ascorbic acid (SRL 0149100).

The metal-chelating ability by the extracts was carried out according to Carter (1971). The ability was monitored by measuring at 562 nm the formation of Fe^{2+}-ferrozine complex. A lower absorbance indicates a stronger metal-chelating ability which was calculated as follows:

$$\textit{Chelating effect } (\%) = \frac{1 - A_s}{A_c} \times 100$$

where A_s is the absorbance of the sample and A_c is absorbance of the control.

The hydroxyl radical-scavenging activity of the extract was measured by the deoxyribose method (Kunchandy and Rao, 1990) and compared with that of mannitol.

Evaluation of Antimicrobial activities

Escherichia coli MTCC 119, *Klebsiella pnumoniae* subsp. *ozaenae* MTCC 2653, *Staphylococcus aureus* MTCC 1430, *Bacillus cereus* HWC 88 and *Candida albicans* MTCC 183, obtained from the Culture Collection of the Microbiology Laboratory of the Department of Botany, University of North Bengal, were used for the this study. The bacterial strains were maintained on nutrient agar (HiMedia, M561) slants and the yeast on yeast malt agar (HiMedia M424) slant. The biological activity was determined by employing the standard disc diffusion technique.

Subculturing of each microorganism was set up 24 h before the assay. A loopful of culture was inoculated into tryptone soya broth (HiMedia M011) for bacteria and 5% malt extract (HiMedia RM004) for the yeast. After 5-6 h of growth under shaking condition (120

rpm), the cultures were used for surface spreading using a sterile swab (Stanbio Reagents, Kolkata) on Mueller-Hinton agar (HiMedia M173) plates for bacterial cultures and Sabouraud dextrose agar (HiMedia M063) plates for the yeast. After 15 min of drying, sterile Whatman No. 1 filter paper discs were impregnated with the desired concentration of the thallus extracts; methanol was completely evaporated in the laminar airflow and the discs were aseptically placed on the agar surfaces. The plates were then incubated at 37°C for 18-24 h (bacterial strains) and at 28-30°C for 5-6 days (yeast strain). A clear zone of inhibited microbial growth surrounding the disc exhibited antimicrobial properties; a zone with a diameter >5.5 mm was considered positive.

Results and discussions

Antioxidant Activities

M. convoluta flavonoids have received considerable attention of late for their potential role in anti-inflammatory activity, diuretic effect and anti-hepatitis B virus activity (Xiao *et al.*, 2005). Flavonoids constitute a large group of naturally occurring plant phenolics. Flavonoids, including flavones, isoflavones, flavonones and chalcones occur in all types of higher plant tissues (Herrmann, 1983). Owing to the polar nature of these compounds, the polar, protic methanol was chosen for extraction in this study.

The sample yielded 1.96 g lyophilized extract 100 g^{-1} dried thallus. The Folin-Ciocalteau phenol reagent is used to obtain a crude estimate of the amount of phenolic compounds present in an extract. Phenolics undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the reagent (Escarpa and González, 2001). As shown in Table 1, the amount of total phenol was 1.1 mg gallic acid equivalents g^{-1} dried thallus. Cuvelier *et al.* (1992) established a relationship between the structure of many phenolic acids and their antioxidant activity. The antioxidant properties of phenolics are mainly due to their redox properties which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. They may also have a metal-chelating potential (Rice-Evans *et al.*, 1995).

Free radical-scavenging is the main mechanism by which antioxidants act. Several methods have been developed in which the antioxidant activity is assessed by the scavenging of synthetic radicals in polar organic solvents, e.g. methanol, at room temperature. DPPH assay is sensitive enough to detect natural antioxidants at low concentrations. In the present study, antioxidant activities of the samples were determined using DPPH method. The total antioxidant activity was 0.18 µg trolox equivalent antioxidant capacity (TEAC) g^{-1} dried thallus (Table 1).

Fe^{3+}-reduction is an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action (Yildirim *et al.*, 2001). The reducing properties are generally associated with the presence of reductants (Duh, 1998). Therefore, the reducing ability of the extract was investigated by reduction of Fe^{3+} in presence of reductants in the tested extract samples. The reducing capacity of the lyophilized samples was evaluated by measuring the formation of Perl's Prussian blue at 700 nm (Oyaizu, 1986). *M. convoluta* demonstrated an effective reducing ability (Table 1).

Transition metal ions have a great importance in the generation of free radicals in cells. They possesses two or more valence states with a suitable oxidation and reduction potential which affect both the speed of auto-oxidation and the direction of hyperoxide breakdown to volatile compounds (Grosch, 1982). The production of free radicals can result into lipid peroxidation of chelating agents. The chelating agents are reported to be effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of metal ion (Gülcin *et al.*, 2007). Fe^{2+} is the most powerful pro-oxidant among various species of metal ions (Halliwell and Gutteridge, 1984). In the presence of chelating agents, the ferrozine-metal complex formation is inhibited and the purple colour of the complex fades. Measuring the colour reduction, it is possible to estimate of the chelating activity of the co-existence chelator. In this assay, the natural compound was inserted with the formation of ferrozine-metal complex, thus suggesting chelating activity (Table 1). Hydroxyl radical-scavenging activity test is important in the sense that hydroxyl radical is the most toxic and reactive free radical formed in biological system and has been implicated as a highly damaging agent to almost every molecule found in living cells (Hochestein and Atallah, 1988). As shown in Table 1, *M. convoluta* exhibited a high level of hydroxyl radical-scavenging activity. All

Table 1. Antioxidant activities[a] of methanolic extract of *Marchantia convoluta*

Parameter	g^{-1} dried thallus	Activity (%)	% decrease over standard[c]
Total phenol (mg GAE)	1.10 ± 0.04		
Free radical-scavenging activity[b]		6.72 ± 0.59	855.2
Total antioxidant activity (µg TEAC)	0.18 ± 0.04		
Reducing activity (mg AAE)	28.47 ± 3.71		
Metal-chelating activity[b]		13.39 ± 5.30	174.3
Hydroxyl radical-scavenging activity[b]		168.20 ± 3.42	50.6

[a]Each value represents mean ± SEM (n = 9).
[b]Present in 100 µg lyophilized extract ml^{-1} methanolic solution.
[c]Trolox, sodium-EDTA and mannitol at 100 µg ml^{-1} for free radical-scavenging, metal chelating and hydroxyl radical-scavenging activities, respectively.

Table 2 Antimicrobial activity[a] of methanolic extract of *M. convoluta*

Test organisms	mg lyophilized extract/disc				
	0.5	1.0	2.0	2.5	3.0
Staphylococcus aureus	7.1 ± 0.12	7.4 ± 0.12	8.0 ± 0.15	ND	8.8 ± 0.27
Bacillus cereus	ND[b]	6.3 ± 0.06	6.5 ± 0.02	ND	7.3 ± 0.03
Escherichia coli	6.2 ± 0.06	ND	6.4 ± 0.09	6.9 ± 0.12	7.2 ± 0.10
Klebsiella pneumoniae	5.5 ± 0	5.7 ± 0.01	5.9 ± 0.02	ND	ND
Candida albicans	ND	ND	ND	ND	5.5 ± 0

[a]Values represent mean ± SEM of diameters of inhibition zones in mm (n = 36). Diameter of antimicrobial disc, 5.5 mm.
[b]ND, not determined

these antioxidant parameters in the plant compared well with the standard antioxidants (Table 1).

Antimicrobial activities

Antimicrobial activities of bryophytes have drawn the attention of botanists and microbiologists only from recent few years. Chopra *et al.* (1965) first observed that bryophytes constitute the least known group of plants from the point of view of poisoning. An interesting feature is that they are relatively free from attack by parasitic microorganisms. Herbarium specimens of these plants need no special treatment like those of higher plants (McCleary and Walkington, 1960). So, it was speculated that the bryophytes might possess antimicrobial activities. In surveys conducted in different parts of India, 50 out of 86 (57%) species of bryophytes tested were found to be antimicrobially active (Banerjee and Sen, 1979). The value compares favourably with the occurrence of antimicrobial-producing organisms among other plant groups.

All the samples of *M. convoluta* were active against all of the tested four species of bacteria; however, none of them possessed antifungal property against the tested yeast (Table 2). The results were in accordance with those of Banerjee and Sen (1979) and Xiao *et al.* (2005). Among the test organisms used, *Staph. aureus* was the most sensitive. The extract was effective against other Gram positive and Gram negative bacteria as well.

Although these results provide a support for the use of *M. convoluta* aerial part in Chinese traditional herbal medicine, the tests were performed under *in vitro* condition. Therefore, purification of the active principles and *in vivo* activity study are warranted.

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