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Original Article

EVALUATION OF ANTIBIOTIC AND BIOCHEMICAL POTENTIAL OF BRYOPHYTES FROM KUMAUN HILLS AND TARAI BELT OF HIMALAYAS

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ABSTRACT

Objective: Today, one of the major problems in the treatment of disease is the development of resistance against conventional antibiotics. One way to curb the problem of increasing antibiotic resistance is to use botanicals. Bryophytes, one of the earliest land inhabitants, are generally not known to get affected by any disease in nature owing to their unique chemical constituents. Therefore, the study was aimed to test the efficacy of bryophytes as an alternative to the synthetic drugs by exploring their antimicrobial and biochemical potential.

Methods: Antibacterial, biochemical and antioxidant characterization of 2 liverworts, *Reboulia hemisphaerica* L. (Raddi), *Marchantia palmata* Reinw., Nees & Blume and one moss species, *Hydrogonium gracilantum* (Mitt). P. C. Chen was done under laboratory conditions.

Results: Both acetone and ethanol extracts of the collected bryophytes inhibited the growth of *Escherichia coli, Bacillus cereus, Erwinia chrysanthemi* and *Pseudomonas aeruginosa* on an agar plate. The ethanol extract of *H. gracilantum* was the most potent inhibitor of *E. chrysanthemi* followed by ethanol extract of *R. hemisphaerica* against *E. coli*.

Conclusion: *E. chrysanthemi* was the most sensitive pathogen to ethanol extract of *H. gracilentum* while *E. coli* and *B. cereus* were inhibited most by ethanol extract of *R. hemispherica*. However, *P. aeruginosa* was most sensitive to acetone extracts of *M. palmata* and *H. gracilantum* and ethanol extract of *R. hemisphaerca*. All the plant extracts had moderate content of phenols and flavonoids. The antioxidant activity of corresponding extracts could be related with the total phenol and flavonoid contents.

Keywords: Bryophytes, *Reboulia hemisphaerica*, *Marchantia palmata, Hydrogonium gracilantum*, Phenols, antioxidants, Flavonoids, Antibacterial

INTRODUCTION

The oldest land plants on earth, *i.e.*, "bryophytes" comprise of three phyla: the liverworts (Marchantiophyta), the mosses (Bryophyta) and the hornworts (Anthocerophyta). Despite the rich diversity and unique characteristics of bioactive molecules of bryophytes, their medicinal importance is negligibly explored. Interestingly, the plants are being used in the ethnomedical field from times immemoriall [1]. However, the group has drawn the attention of plant and chemical scientists for application potential only in the last few decades [2, 3]. Pharmacological investigations of bryophytes have proved that the active principles of the plants are very unique and have the remarkable potential of therapeutic applications. Presently, over 400 new compounds have been isolated and characterized for their biochemical and antimicrobial properties. The enzymatic machinery of these plants also has antioxidative property which helps them to withstand the extreme climate and stress conditions particularly desiccation [4]. An important observation about the bryophytes is that these are rarely infected by microorganisms through their habitat, being moisture rich, is usually very prone to microbial attacks.

Bryophytes, particularly liverworts, are known to show antibacterial and antifungal activity [5]. Both *Reboulia hemisphaerica* (Aytoniaceae) and *Marchantia palmata* used in the present study are thalloid liverworts. *Marchantia* spp are known to be used in folk remedies for cancer [6]. The moss, *Hydrogonium gracilantum* grows as a pure or mixed patch on the hills. All these plants used in the present study have been unexplored for their biochemical and antioxidant potential. There is not much information available in the literature on the antioxidative and biochemical potential of these bryophytes. The present study is focused on the preliminary evaluation of phytochemicals, assessment of antioxidative and antibacterial potential of aqua-ethanolic and aqua-acetonic extracts of the three bryophytes *viz*., *Reboulia hemisphaerica*, *Marchantia palmata and Hydrogonium gracilantum* under *in vitro* conditions for analyzing their potential as a therapeutic source on the basis of plant type and the solvent system used for extraction.

MATERIALS AND METHODS

Collection of plant material

Gametophytes of *Reboulia hemisphaerica* and *Hydrogonium gracilantum* were collected from Dwarahat (29.78 °N latitude,79.43°E longitude at an altitude of 1499m asl), Almora distt. and *Marchantia palmata* was collected from Pantnagar (29.02 °N latitude, 79.30 °E longitude at an alt. of 243m asl) in U. S. Nagar distt. of Uttarakhand, India. Voucher specimens of the bryophytes have been deposited in the herbarium maintained at Department of Biological Sciences.

Preparation of plant extract

The plants with rhizoids were extensively washed with running tap water, spread on the paper sheet, shade dried, pulverized and extracted by cold percolation (10 g/100 ml) in 80 % of ethanol and acetone solvents. The extracts were filtered and concentrated using rotary evaporator (Biogen). Different concentrations of the crude extract (100, 400, 700 and 1000 µg/ml) were prepared and used for the further study.

Microorganisms

To test the antibacterial activity of the plant extracts, 4 common pathogenic bacteria were used. The test bacteria *viz*., *Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 424), and *B. cereus* (MTCC 430) were procured from Institute of Microbial Technology, Chandigarh. *Erwinia chrysanthemi* was kindly provided by Department of Microbiology, G. B. Pant University of Agriculture & Technology, Pantnagar.

Antibacterial activity

Antibacterial activity of all the plant extracts was assessed by agar well diffusion method according to Kumar & Chaudhary [7]. Different concentrations of crude ethanol and acetone extracts (40 µl) were pipetted out into the wells of bacteria seeded nutrient agar

plates. Overnight actively growing bacterial culture(s) with an optical density of 0.8 were used to make a smooth lawn. The plates were incubated at 37 °C and zones of inhibition (mm) were measured after 24 h. Standard antibiotic solutions of streptomycin, chloramphenicol and tetracycline were used as a positive control whereas ethanol and acetone were kept as negative control. Total of 5 replicates were used for all the experiments, and each experiment was performed twice.

Determination of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration)

Broth dilution test was used to determine the MIC of the ethanol and acetone extracts against the test bacteria [8]. Freshly prepared nutrient broth (NB) was used as diluent. Test bacteria, grown overnight in nutrient broth (OD =0.8) were used after 100 folds dilution. Different concentrations of the plant extracts (1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.94, 0.98 µg/ml in two-fold serial dilutions) were added to the test tubes containing the bacterial cultures to know the inhibitory concentration of the extracts. All the tubes were incubated at 37 °C for 24 h. The tubes were examined for visible turbidity, and optical density of cultures was determined at 620 nm using nutrient broth as a control. The lowest concentration that inhibited visible growth of the test organism was recorded as MIC. Subculturing of the aliquots of the inoculums on to the antibiotic free media was done to determine minimum bactericidal concentrations (MBC).

Antioxidant activity

Antioxidant activity of the plant extracts was evaluated in terms of 2, 2'-diphenyl-l-picrylhydrazyl (DPPH) radical scavenging ability of the extracts using BHT as a standard following the method of Blois [9]. Different concentrations of the extracts (20, 40, 60, 80, 100 µg/ml) were mixed with 1 ml of DPPH soln. (0.1 mM of DPPH in methanol) individually. The mixture was incubated at room temperature for 30 min. and the absorbance was measured at 517 nm in a uv-vis spectrophotometer (Thermo Scientific). Lower absorbance indicated higher radical scavenging power. DPPH radical scavenging activity was calculated by the following equation:

% DPPH radical scavenging activity = (1-As/Ac) × 100 (Where, As is the absorbance of the sample and Ac is the absorbance of the control at 517 nm). The experiment was conducted in triplicate.

Total phenolic content (TPC)

Total phenolic content of the plant extracts was determined by the method given by Singleton & Rossi [10] using gallic acid as standard. Absorbance was measured at 765 nm. A standard curve was prepared using various concentrations of gallic acid, and results were expressed as gallic acid equivalents (GAE µg/g).

Total flavonoids

Estimation of total flavonoids in the plant extracts was carried out using the method of Ordon Ez *et al.* [11] taking quercetin as the standard. Total flavonoid content was calculated as quercetin equivalent (mg/g) using quercetin calibration curve, y = 0.020x, where x denotes absorbance and y was the quercetin equivalent (mg/g).

RESULTS

All the crude extracts showed varying degree of antibacterial activity against all the test bacteria irrespective of Gram reaction [fig.1]. Antibiotic activity of the crude extracts was compared with that of the standard antibiotics. Tetracycline was the most inhibitory of all the antibiotics for all the test bacteria. Stronger and broader spectrum of antimicrobial activity was observed with ethanol extracts of the bryophytes.

All the extracts except ethanolic extract of *H. gracilantum* showed larger inhibition zones against *P. aeruginosa* compared to Chloramphenicol, which showed inhibition zone of 15.0±0.58 mm. Maximum antibacterial activity was observed for the ethanol extract of *H. gracilantum* against *E. chrysanthemi* with maximum zone size of 20.0±0.58 mm. However, acetone extracts of *M. palmata* and *H. gracilantum* showed better activity against *P. aeruginosa* with a maximum zone of inhibition of 17.67±0.33. Ethanol extract of *R. hemisphaerica* showed a maximum zone of inhibition against *E. coli* (17.67±0.33) and *B. cereus* (16.0±0.58) respectively.

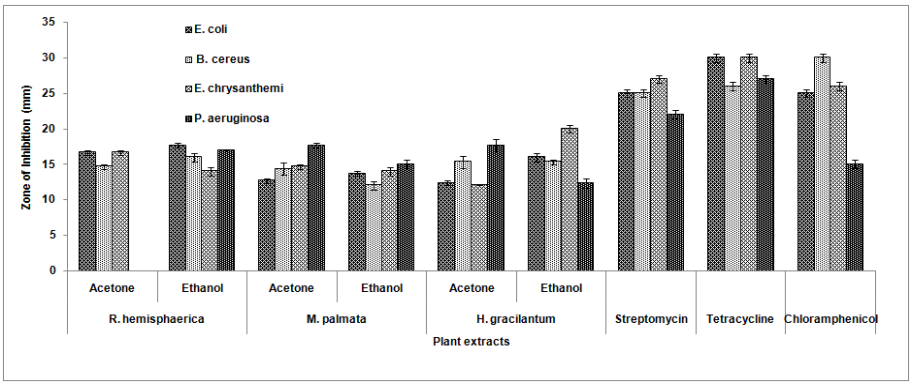

Fig. 1: Antibacterial activity of bryophytes extracts

Table 1: Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of different extracts of bryophytes against different pathogens (µg/ml)

Plant extracts	Test organisms							
	E. coli		*B. cereus*		*E. chrysanthemi*		*P. aeruginosa*	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
RA	31.25	125	31.25	1000	125	250	-	-
RE	7.81	62.5	125	500	125	500	3.91	15.63
MA	125	1000	62.5	250	7.81	250	62.5	125
ME	7.81	62.5	1000	-	125	500	125	500
HA	15.63	500	62.5	125	15.63	500	62.5	62.5
HE	3.91	250	125	500	1.94	62.5	125	500

Here, RA, RE = Aqua acetonic, Aqua-ethanolic extracts of *Reboulia hemisphaerica*; MA, ME= Aqua acetonic, Aqua-ethanolic extracts of *M. palmata*; HA, HE = Aqua acetonic, Aqua-ethanolic extracts of *H. gracilantum*.

Minimum inhibitory concentrations (MIC) of different extracts against the test microorganisms ranged from 1.94 µg/ml to 1000 µg/ml [table 1]. Ethanolic extract of *H. gracilantum* showed very low MIC for *E. chrysanthemi* (1.94 µg/ml) and *E. coli*(3.91 µg/ml) inconsistent with the results of ZI [fig. 1]. Ethanolic extracts of *R. hemisphaerica* also exhibited a lower MIC for *P. aeruginosa* (3.91 µg/ml) and *E. coli* (7.81 µg/ml) respectively. Similarly, ethanolic and acetonic extracts of *M. palmata* showed low MIC (7.81 µg/ml) against *E. coli* and *E. chrysanthemi*. Minimum bactericidal concentration (MBC) ranged from 15.63 to 500 µg/ml for ethanol extracts and 62.5 to 1000 µg/ml for acetone extracts respectively. The ethanolic extract of *R. hemisphaerica* showed lowest MBC against *P. aeruginosa* (15.63 µg/ml) and *E. coli* (62.5 µg/ml). Similarly, ethanolic extracts of *M. palmata* and *H. gracilantum* showed low MBC (62.5 µg/ml) against *E. coli* and *E. chrysanthemi* respectively.

Results of antioxidant activity suggest that all the extracts were able to reduce DPPH radical in a concentration-dependent manner [table 2]. Ethanol (80%) and acetone extracts (80%) exhibited highest % DPPH radical scavenging activity in *R. hemisphaerica* (78.62±0.17) and *M. palmata* (65.62±0.32) respectively at 100 µg/ml. *H. gracilantum* showed comparatively low % DPPH radical scavenging activity in both the extracts. Total phenolic content (TPC) of the crude extracts of bryophytes also increased with increasing concentration of the tested extracts. The range of TPC values (µg GAE) in acetone extracts (17.33±0.60-62.50±1.00) was comparatively higher than that of ethanol extracts (8.89±0.29-27.56±0.29) [table 3]. TPC was highest in *R. hemisphaerica* in both acetone (62.50±1.00) and ethanol extracts (27.56±0.29) at the concentration of 100 µg/ml.

This clearly suggested that *R. hemisphaerica*, among the three bryophytes, possessed higher phenol content in acetone extracts. Total phenol content and % DPPH radical scavenging activity of both ethanol and acetone extracts of all the bryophytes showed positive correlation [fig.3]. The total flavonoid content (mg/gquercetin) was highest in *R. hemisphaerica* for both acetone and ethanol extracts followed by an aqua-acetone extract of *H. gracilantum* [fig. 2].

Table 2: Percent DPPH radical scavenging activity of different species of bryophytes in acetone and ethanol extracts

Conc. of plant extracts (µg/ml)	% DPPH radical scavenging activity±SE							
	Aqua-acetone (80 %) extract				Aqua-ethanol (80 %) extract			
	R	M	H	BHT	R	M	H	BHT
20	29.02±0.48	20.39±0.16	17.71±0.14	74.45±0.61	53.02±0.35	24.60±0.16	31.79±0.14	75.77±4.33
40	41.05±0.16	43.92±0.16	32.06±0.14	78.42±0.38	65.96±0.34	32.67±0.16	39.80±0.85	77.61±0.13
60	62.35±0.32	61.05±0.32	37.27±0.28	88.82±0.00	72.15±0.34	55.82±0.32	52.88±0.17	82.29±0.25
80	62.35±0.32	64.97±0.32	50.35±0.28	90.43±0.12	76.51±0.17	59.92±0.16	54.57±0.17	89.11±0.12
100	64.31±0.16	65.62±0.32	54.75±0.42	94.10±0.12	78.62±0.17	66.27±0.49	55.84±0.17	92.16±0.12
	S Em		CD (at 5%)		SEm		CD (at 5%)	
Plant extract(P)	0.14		0.40		0.36		1.04	
Concentration(C)	0.16		0.44		0.41		1.16	
P × C	0.31		0.89		0.81		2.32	

Data given in as mean±SEM of 5 replicates, where R= *R. hemisphaerica;* M= *M. palmata;* H= *H. gracilantum*, BHT: Standard, P=Plant extract, C=Concentration

Table 3: Total phenolic content (µg GAE) of different species of bryophytes in different solvents

Plant extract	Aqua-acetone (80 %) extract			Aqua-ethanol (80 %) extract		
Conc. (µg/ml)	*R. hemisphaerica*	*M. palmata*	*H. gracilantum*	*R. hemisphaerica*	*M. palmata*	*H. gracilantum*
20	30.50±0.50	28.33±0.73	17.33±0.60	13.67±0.51	10.33±0.51	8.89±0.29
40	31.50±0.29	28.67±0.44	19.17±0.33	13.89±0.40	10.78±0.40	10.56±0.48
60	44.17±0.83	32.33±0.17	23.33±0.33	19.78±0.48	12.00±0.33	12.78±0.29
80	53.67±0.17	40.00±0.50	28.83±0.44	22.78±0.29	15.11±0.29	16.11±0.44
100	62.50±1.00	43.83±1.17	32.33±0. 44	27.56±0.29	16.67±0.19	16.78±0.68
	SEm	CD (at 5%)		SEm	CD (at 5%)	
Plant extract (P)	0.27	0.77		0.18	0.53	
Concentration(C)	0.35	1.00		0.24	0.69	
P × C	0.60	1.73		0.41	1.19	

Data given in as mean±SEM of 5 replicates, Conc. = Concentration, GAE= Gallic Acid equivalents

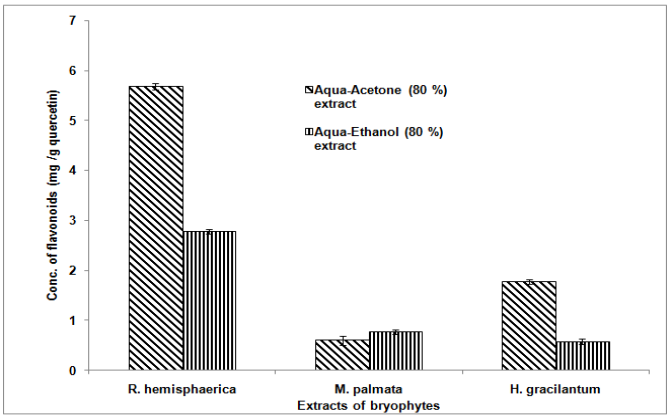

Fig. 2: Total flavonoid content (mg/g quercetin) of different species of bryophytes

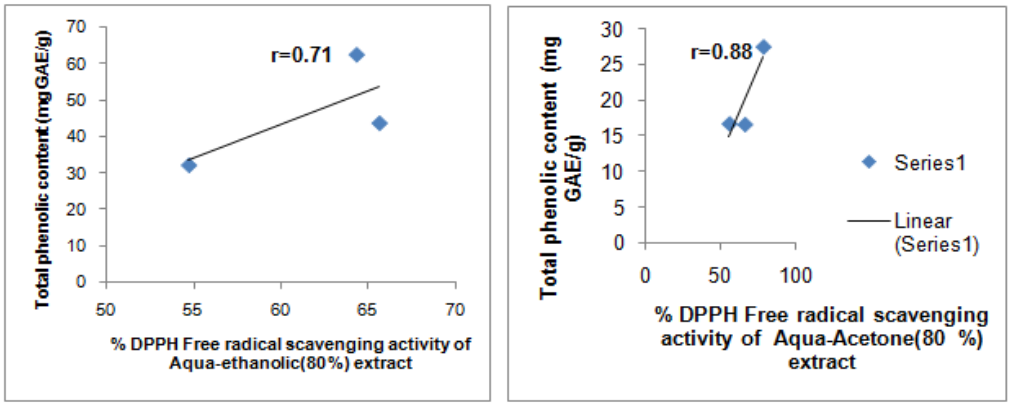

Fig. 3: Correlation between total phenolic content (mg GAE/g) and % DPPH free radical scavenging activity

DISCUSSION

Therapeutic potential of plants can be assessed by many means. One such means is to explore their antimicrobial and antioxidant potential along with their pool of chemical compounds. In this study, crude ethanol and acetone extracts of three bryophytes were used to inhibit *in vitro* growth of four pathogens *viz., E. coli, B. cereus*, *E. chrysanthemi* and *P. aeruginosa*. Results of antimicrobial effect clearly suggested their immense potential as antibacterial agents.

In the present study, ethanol extract showed a broader range of ZI and thus exhibited good antibacterial action against most of the bacteria. The result is in conformity with the other studies that also reported stronger and broader spectrum of antimicrobial activity in an ethanolic extract of *Plagiochasma appendiculatum* and *Rhynchostegium vagans* respectively [12, 13]. This may be due to extraction of specific antibacterial compounds in the ethanolic extracts [14]. Krishnan *et al.* [15] also reported good antimicrobial activity of methanolic and water fractions of *Targionia hypophylla* and *Bryum* sp. Similarly, Manoj and Murugan [16] also reported broad spectrum antimicrobial activity of methanolic extracts of *Plagiochila beddomei* Steph. However, Savaroglu *et al.* [17] reported significant inhibition of *P. aeruginosa* by all the extracts of *Polytrichum juniperinum* and *Tortella tortuosa*.

The results of MIC proved the greater efficacy of these extracts against *E. chrysanthemi* and *P. aeruginosa*. The high MIC value (1000 µg/ml) for ethanol extract of *M. palmata* against *B. cereus* indicates that either the plant extract is less effective for a respective microorganism or that the organism has the potential of developing antibiotic resistance [18]. Lowest MBC (15.63 µg/ml) for ethanolic extract of *R. hemisphaerica* suggested that it has got the necessary compound in the adequate quantity required for inhibiting/killing *P. aeruginosa*. It also gets support from Zhu *et al.* [19] who suggested the presence of diverse oil bodies in liverworts which are responsible for their biological and medicinal properties. Low MIC and MBC clearly indicate that the plant extracts are effective at very low dosage [20, 21]. Similar values of MIC and MBC (62.5 µg/ml) for an acetonic extract of *H. gracilentum* against *P. aeruginosa* suggested its bactericidal nature inconsistent with the study of *M. polymorpha* by Gahtori *et al.* [22]. Rest of the other extracts showed different values of MIC and MBC implying that most of the extracts are bacteriostatic in nature. The difference in the antimicrobial activity may be due to potential differences in the strains of bacteria and differences between extraction and experimental procedures. The different antimicrobial activity of different bryophyte species may also be attributed to the presence of a number of antimicrobial substances with different spectra of action and intensity [23] in different plant extracts.

Ethanol extracts, compared to the acetone extracts, exhibited higher activity to scavenge DPPH radicals. This may be due to different abilities of different solvents to extract different active compounds depending on their solubility or polarity in the solvent [24]. Ethanol extract in this study might have had a higher solubility for more number or more concentration of active compounds and therefore exhibited higher activity. However, acetone extracts exhibited the higher content of phenolics and flavonoids, clearly suggesting a contribution of other metabolites in addition to phenolics and flavonoids towards the antioxidant activity. The higher values of TPC and total flavonoids in *R. hemisphaerica* suggests it to be possessing good antioxidant potential. Interestingly, all the bryophytes in the present study showed good antimicrobial potential against the test organisms which may again involve the synergistic effect of several other compounds in addition to phenolics and flavonoids.

CONCLUSION

Lower plants, particularly bryophytes are one of the most neglected group of plants owing to their little food value. However, their chemical nature makes them a unique group in the plant kingdom that is rarely affected by any disease. The study led to the conclusion that this relatively unexplored plant group is truly a rich storehouse of many bioactive compounds *viz*., phenolics and flavonoids which may be responsible for both antimicrobial and antioxidant properties. It also concludes that both antibacterial and antioxidant activity is dependent on individual plants and the solvent system used for extraction. Hence, this group including both liverworts and mosses can be further explored chemically for their biocontrol potential in plants as well as veterinary diseases.

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CONFLICT OF INTERESTS

Declared none

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