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Antimicrobial Efficacy of Moss *Bryum argenteum* (Hedw.) (Bryales: Bryaceae) against Plant Pathogen *Pseudomonas syringae* (PV.) (Pseudomonadales: Pseudomonadaceae)

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Authors' contributions

This work was carried out in collaboration between both authors. Author GV designed the study, wrote the protocol and the first draft of manuscript and managed the experimental process. Author GSD identified the plant species and managed the literature searches. Both authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Introduction: Bryophytes are considered as a remarkable reservoir of new, natural products or secondary compounds, many of which have shown interesting biological activity. Biological activities of number of liverworts and mosses were studied which suggests that they contain a large number of terpenoids and phenolic compounds.
Aims: To assess antimicrobial activity of moss *Bryum argenteum* (Hedw.) (Bryales: Bryaceae) methanolic crude extract against plant pathogen *Pseudomonas syringae* (pv.) (Pseudomonadales: Pseudomonadaceae).

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Study Design: *In vitro* testing.
Methodology: *In vitro* antimicrobial activity of moss *B. argenteum* was evaluated by using different concentrations of methanolic crude extract for number of bacterial colonies formation and zone of inhibition. Antibiotic drugs such as amoxillin and streptomycin were used as positive control. Phytochemical screening was performed according to the methods described by Raman (2006) to determine the presence of various bioactive antimicrobial compounds. Methanolic crude extract of *B. argenteum* was analyzed for the presence of secondary metabolites using a Perkin Elmer series 200 HPLC system.
Results: Methanolic crude extract of *B. argenteum* had significant antibacterial potential. Number of bacterial colonies decreased with increased concentrations of *B. argenteum* extract. Zone of inhibition was increased from lower to higher concentration of the extract. The results were confirmed with the different concentrations of commercially available antibiotic drugs, amoxillin and streptomycin. Decreased number of colonies and increased size of zone of inhibition of test bacterium *P. syringae* suggests that moss *B. argenteum* possesses high antibacterial efficacy. α-terpineol was confirmed by HPLC analysis.
Conclusion: Present study indicates that natural antimicrobial agents of *B. argenteum* showed inhibitory effect on *in vitro* growth of phytopathogenic bacterium *P. syringae* in form of number of colonies and zone of inhibition.

Keywords: Secondary metabolite; α- terpineol; HPLC analysis; moss; bryophyte.

1. INTRODUCTION

'Bryophyta' represent the simplest extant land plants and are phylogentically very old. They are considered as a "remarkable reservoir" of new, natural products or secondary compounds, many of which have shown interesting biological activity. Traditionally the bryophytes are used throughout the world as drugs and remedies to cure various diseases. Biological activities of a number of liverworts and mosses were described which suggests that they contain a large number of terpenoids and phenolic compounds [1].

The flavonoid compounds are distributed widely in vascular plants and bryophytes, and 5,000 kinds have been reported as naturally occurring substances [2]. The bryophytes are lower group of plants with tremendous potential for antifungal drug developments, are bryophytes. They are closely linked with civilization, culture, beliefs and ethics of humankind and are also used in the ethnomedical field from times immemorial in many parts of the world [3]. The chemistry of bryophytes is poorly known and the results on are very scattered. The reason for this is the difficulty in identification and small amount of the same species available for analyses, usually by sophisticated methods. Therefore, bryophytes are indicated as source of chemically new and unknown Compounds [4].

Bioactivity guided isolation of antifungal compounds from liverwort *Bazzania trilobata* (L.) (Jungermanniales: Lepidoziaceae), was studied. For this purpose thin layer chromatography was used to isolate six antifungal sesquiterpenes and their structure was investigated using extensive NMR spectral evidence [5]. *Pseudomonas syringae* (PV.) (Pseudomonadales: Pseudomonadaceae) is a rod shaped gram negative bacterium generally infects various fruits, vegetables and ornamental plants such as beets, wheat, barley, peas and many other important crops.

In the present study moss *B. argenteum* was investigated for its antibiotic potential against plant pathogenic bacterium *P. syringae.* The plant methanolic extract was also analyzed by high performance liquid chromatography (HPLC) technique to identify the active metabolites responsible for the antimicrobial activity of this primitive land plant.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material and Extract Preparation

The plant material was collected in rainy season from Mount-Abu, District Sirohi, (Raj) from different localities in both vegetative and sporophytic phases. The collected plant material was washed with tap water followed by distilled water till all the debris and soil particles were removed. The collected plant material was identified with the help of moss flora of Rajasthan and the occurrence of bryophytes in Rajasthan

[6,7]. Then the material was dried by pressing in between blotting papers. The plant material was grinded in mortar and pestle with methanol (1:1 v/v) till the formation of fine paste. The smooth paste thus obtained was filtered through muslin cloth. The filtrate was left open in the air for 48 hours to evaporate the organic solvent. The residue was then mixed with equal amount of double distilled water to prepare stock of methanolic extract. It was diluted using double distilled water to prepare various concentrations from 10-100 per cent. All the extract concentrations so prepared were stored in air tight vessels in refrigerator till further use.

2.2 Test Organism

A rod shaped Gram-negative bacterium *P. syringae,* which infect a wide variety of fruits, vegetables and ornamental plants like maple, beets, wheat, barley, peas and many more commercially important crops was chosen to test its sensitivity for bryophyte extract in this study.

2.3 Pure Culture

Pure culture of *P. syringae* (MTCC No. 1604) obtained from the Institute of Microbial Technology (IMTECH), Chandigarh (India).The culture was activated using Nutrient broth and was sub-cultured after every ten days. After incubation for 24 hours at 30±2℃ temperature, the cultures were kept in the refrigerator to cease their further growth.

2.4 Phytochemical Analysis

Phytochemical tests performed according to the methods described by Raman [8] to detect the presence of active secondary metabolites such as alkaloids, falvonoids, sterols, saponins, anthraquinones and cardiac glycosides in the plant extract.

HPLC Analysis: Methanolic extract of *B. argenteum* was analyzed for the presence of secondary metabolites using a PerkinElmer series 200 HPLC system. The mobile phase consisted of solvents; methanol and water in a 65:35 v/v ratio. The column used was Protonsil C-18 ODS AQ (250 mm X 4.6 mm, 5 (j.m). Using flow rate as 1 ml/min and the injection volume as 20 micro liters (Pressure was maintained at 6200PSI). Detection was performed using a diode array detector (PDA) of PerkinElmer series 200 (wavelength range 190-700 nm). Whole operation was done at room temperature. Identification of various compounds was done by comparing retention time (Rt) and UV-Vis spectra of extract chromatogram with reference chromatogram.

2.5 Bioassay of Antibacterial Activity

Amoxicillin and Streptomycin were used to prepare standard stock solutions (10 µg/ml) of antibiotics. These standard stock solutions of drugs were diluted using double distilled water to prepare solutions of different strengths from 10-100 per cent concentrations. All the experiments were set in complete aseptic environment and in triplicates. For each experiment a negative control was also set in which plant extract or antibiotic solution was not added to influence the growth of bacteria. For colony count assay one milliliter of 24 hours old bacterial broth culture was serially diluted up to 10^{-2} dilution. 10 µl of this dilution was then spread evenly over the surface of solidified agar medium containing plant extracts or antibiotic solutions of different concentration in each Petri plate, in a 1:1 ratio. All the Petri dishes were sealed with Para film and incubated at 30±2℃ for 24 hours after which bacterial colonies were counted. For Agar-well diffusion assay medium was inoculated with 100 µl of 24 hours old broth culture of test microorganism.

Once the agar was solidified, it was punched to make a three millimeters diameter well using a sterile cork borer and it was filled with l ml of the plants extracts or antibiotic solutions of different concentrations. The plates were incubated at 30±2℃ for 24 hours. The antimicrobial activity was calculated by counting the number of bacterial colonies appeared after 24 hours and by measuring the diameter of inhibition zone appeared after the incubation period.

3. RESULTS AND DISCUSSION

The results of antimicrobial activity of *B. argenteum* methanolic crude extract and antibiotics streptomycin and amoxicillin are shown in Table 1. It was reported that number of test bacterial colonies decreased with increasing concentrations of crude methanolic extract of *B. argenteum* (Hedw.) (Bryales: Bryaceae) Maximum number of bacterial colonies (117) was reported in 10 per cent concentration whereas; this number of colonies was decreased up to 48 at 100 per cent in comparison to the control (135). Zone of inhibition was 0.5mm at 10 per

cent concentration of the extract whereas, it was maximum (31.5 mm) at 100 per cent concentration. Zone of inhibition increased with increased concentration of extract. No zone of inhibition was observed in the control. The results were confirmed with the different concentrations of commercially available antibiotic drugs amoxillin and streptomycin. In antibiotic drugs maximum 108 and 97 number of test bacterial colonies was reported at 10 per cent concentration of amoxillin and streptomycin respectively. This number of bacterial colony decreased continuously and it was 22 and 8 as a minimum number of colonies at 100 per cent concentration of amoxillin and streptomycin. Zone of inhibition in various concentrations of amoxillin and streptomycin increased with increasing concentrations of both antibiotic drugs.13.5mm and 38.0 mm minimum size of zone of inhibition was noticed at 10 per cent concentration of amoxillin and streptomycin respectively whereas, maximum 76.0 and 90.0 mm zone of inhibition was reported at 100 per cent concentration of drugs.

The results of present study suggested that methanolic crude extract of *B. argenteum* showed high potential of antibacterial activity against pathogenic bacterium *P. syringae*. This was confirmed with the antibacterial activity showed by both the antibiotic drugs used as positive control.

Phytochemical screening of methanolic crude extract of *B. argenteum* showed the presence of flavonoids, terpenoids, phytosterols and cardiac glycosides (Table 2). Due to the presence of these bioactive compounds *B. argenteum* showed antibacterial activity in the present study.

The HPLC chromatograms of the reference compounds and methanol extract of *B. argenteum* and are presented in Figs. 1 and 2 respectively. Based on a comparison of the retention time (Rt) and UV-Vis spectra (190-700 nm) of plant extract and reference solution the presence of α-terpineol was confirmed. The above described results clearly showed that *B. argenteum* posses antibacterial activity owing to the presence of various phytochemicals produced by it. This study indicates that secondary metabolites formed by the plants are natural barriers against pathogens attacking them.

Earlier studies on antimicrobial activities of bryophytes have also proved that these primitive land plants can control growth of microorganisms effectively due to their unique chemical composition in acetone extract of the moss *Pleurochaete squarrosa* (Brid.) Lindb. (Pottiales: Pottiaceae) The extract was tested against eleven bacterial strains, some of which are pathogenous for man. Antibacterial activity of the extract was compared with three reference antibiotics and the extract was found to be active on some Gram-negative strains [9]. Seven pure falvonoids from five moss species were isolated and identified among all these falvonoids, some have shown pronounced antibacterial effects [10].

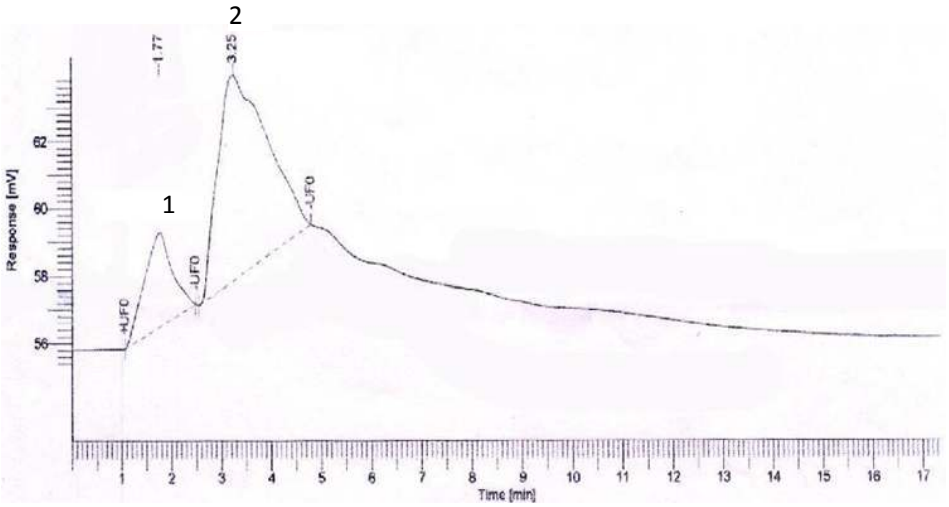

Fig. 1. Reference compounds: Peak (1) 1,8- Cineole (2) α- Terpineol

Table 1. Effect of methanolic crude extract of *Bryum argenteum* (Hedw.) (Bryales: Bryaceae) against plant pathogen *Pseudomonas syringae (*PV.) (Pseudomonadales: Pseudomonadaceae) (After 24 hours of incubation at 30℃)

S.N.	% conc.	*B. argenteum* methanolic extract				Amoxicillin				Streptomycin			
		Number of colonies of *P. syringae*		Zone of Inhibition in mm		Number of colonies of *P. syringae*		Zone of Inhibition in mm		Number of colonies of *P. syringae*		Zone of Inhibition in mm	
		Mean of triplicates	SD	Mean of triplicates	SD	Mean of triplicates	SD	Mean of triplicates	SD	Mean of triplicates	SD	Mean of triplicates	SD
1.	Control	135	3.0	0.0	0.0	131	1.00	0.0	0.0	132	6.00	0.0	0.0
2.	10	117	1.6	9.5	0.5	108	1.91	13.50	0.5	97	1.63	38.0	3.00
3.	20	103	2.0	10.0	0.2	103	3.00	14.00	1.0	58	3.00	39.0	1.00
4.	30	89	2.4	10.5	0.3	100	2.44	15.00	0.3	47	2.44	40.1	0.10
5.	40	79	1.0	11.1	0.1	80	4.00	22.00	0.2	34	4.00	43.0	3.00
6.	50	72	2.0	13.3	0.1	45	4.00	29.20	0.2	25	0.00	46.3	0.30
7.	60	69	1.0	16.0	0.5	32	2.00	37.00	0.2	20	1.00	50.0	1.00
8.	70	62	2.0	19.2	0.2	25	1.00	47.00	0.4	15	2.00	57.1	0.15
9.	80	58	1.9	23.4	0.4	24	1.63	59.10	0.1	12	1.63	66.0	4.00
10.	90	54	3.3	27.0	0.0	23	2.44	71.00	1.0	10	1.63	77.0	2.00
11.	100	48	6.2	31.5	0.4	22	2.00	76.00	1.0	8	0.00	90.0	4.00
12.	GM	80.45	2.40	15.59	0.24	63.00	2.31	34.89	0.44	41.23	2.12	49.88	1.68
13.	CV	2.99%		1.573%		3.66%		1.27%		5.09%		3.213%	

Table 2. Phytochemical tests of *Bryum argenteum* Hedw. (Bryales:Bryaceae)

Class of Phytochemical	Phytochemical test applied	Observations	Results	Intensity of Colour* *B. argenteum* extracts		
				Aqueous	Ethanol	Methanol
Alkaloids	Mayer's and Hager's test	No precipitate	Phytochemical absent	-	-	-
Anthroquinones	Borntrager's test	No layer formation	Phytochemical absent	-	-	-
Cardiac glycoside	Keller Killeni test	Brown ring	Phytochemical present	+	+	++
	Ferric chloride test	Green colour	Phytochemical present	+	++	+++
Flavonoids	Lead acetate and gelatin test	White precipitate	Phytochemical present	+	++	+++
	Alkaline reagent and sodium Hydroxide test	Yellow fluorescent Colour	Phytochemical present	+	++	+++
Saponins	Froth test	No froth	Phytochemical absent	-	-	-
Sterols	Salkowaski test	Reddish brown colour	Phytochemical present	+	++	+++
	Liebermann-Burchardt test	Brown ring	Phytochemical present	+	+	++
Terpenoids	Salkowaski test	Lower layer Turned yellow	Phytochemical present	+	+	++
	Liebermann-Burchardt test	Deep red colour	Phytochemical present	+	+	++
	Phenyl hydrazine test	Yellow-orange colour	Phytochemical present	+	+	++

Note:- Intensity of Colour: (-): No colour; (+): Light colour; (++): More intense colour; (+++): Most intense colour among the three types of extracts tested

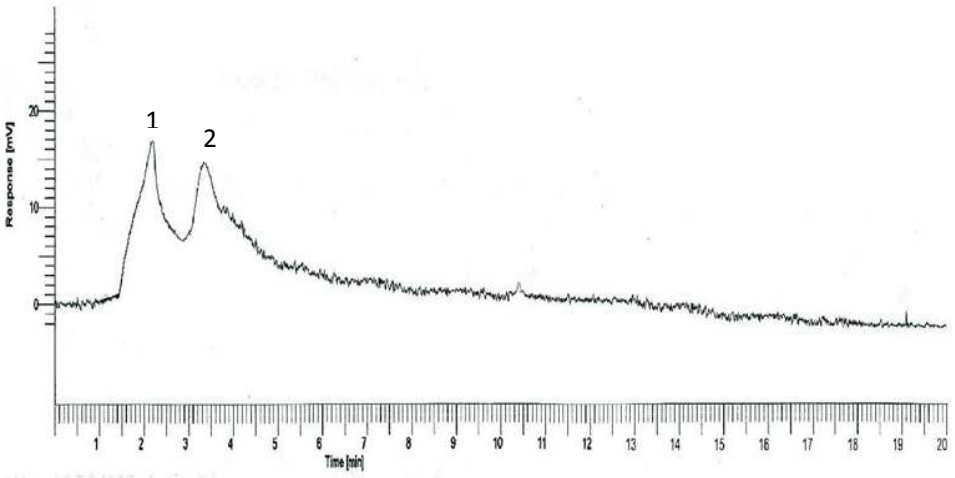

Fig. 2. HPLC Chromatogram of *B. argenteum* (Hedw.) (Bryales: Bryaceae) methanolic extract

The antibacterial activity of methanol extracts of ten moss species were evaluated by disc diffusion method. Out of these ten nine moss species showed antibacterial activity against Gram (+) bacteria [11]. The antibacterial activity of ethanolic extracts of some Indian mosses was investigated against five G (+) and six G (-) bacterial strains. *Entodon prorepens* (Mitt.) Jaeg. (Hypnobryales: Entodontaceae) was found to be most active against all the organisms [12]. Antimicrobial effects of different solvent fractions of selected bryophytes were determined and the results were then compared with the standard antibiotics ampicillin and nystatin (10 ug/ml). Results indicated that bryophyte extracts were found to be very effective against the growth of both bacterial and fungal test organisms [13].

Moss *Philonotis revoluta* (Bosch and Lac) (Eubryales: Bartramiaceae) was studied for its antifungal potential against certain phytopathogenic fungi and reported significant reduction in growth of all the test fungi [14]. The result of this work corresponded to the findings of earlier works reported that the extract of bryophytes such as *Plagiochasma articulatum* (Kash.) (Marchantiales: Atoniaceae), *Anthoceros longii* (Kash.) (Anthocerotales: Anthocerotaceae) and *Fissidens bryoides* (Hedw.) (Fissidentales: Fissidentaceae) showed antibiotic property against *Agrobacterium tumifaciens* [15]. Phytochemical screening of these plants revealed the presence of terpenoids, flavonoids and steroids responsible for their antibiotic action.

Phytotochemical analysis and antimicrobial activity of moss *Bryum celluiare* (Hook.)(Bryales: Bryaceae) against test fungi *Drechslersa maydis* (Drech.) (Pleurosporales: Pleurosporaceae) and *Curvularia lunata* (Wakker) Boedijn) (Pleurosporales: Pleurosporaceae) the causal organisms of leaf blight of *Zea mays* L. (Poales: Poaceae) and leaf spot of wheat respectively and reported that *B. cellulare* is a store house of various bioactive compounds. Some of these compounds showed antifungal property against selected test fungi [16]. Crude aqueous and ethanolic extract of *B. cellulare* strongly inhibited spore germination and mycelial growth of fungus *C. lunata* [17]. All results of the present study confirmed the results obtained in the study of an evaluation of the antimicrobial activity of some Turkish mosses [18].

All these reports along with the present study have clearly proved that bryophytes are natural fighters against plant pathogens and that in future they may serve as potential source of herbal biocides for commercially important crops.

4. CONCLUSION

This study confirms the presence of cardiac glycoside, flavonoids, sterols and terpenoids etc. as main secondary metabolites in *B. argenteum*. Due to the presence of these bioactive compounds this moss showed antibacterial activity against phytopathogenic bacterium *P. syringae*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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