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Research Article

IN VITRO MICROBICIDAL POTENTIALITY OF *TARGIONIA HYPOPHYLLA* L. AND *BRYUM* SPECIES- BRYOPHYTES

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ABSTRACT

Gosnite Indians use bryophytes as one of the candidates known to be effective for the treatment of inflammation, hyperlipemia, arteriosclerosis, rheumatism, skin diseases, osteoporosis and bone resorption. The present study investigated the antimicrobial activity of benzene, chloroform, petroleum ether, methanol and water extract fractions of *Targionia hypophylla* L. and species of *Bryum* such as *B. argenteum* Hedw.; *B. cellulare* Hook.*; B.coronatum* Schwaegr. *; B. plumosum* Dozy & Molk.; *B. pseudotriquertum* (Hedw) Schwaegr. and *B.capillare* Hedw. against bacterial and fungal species. The methanolic and water fractions were demonstrated as strong microbicidal activity than the other extracts. The results of the fractions were compared with ampicillin and gentamicin. We suggest that *Targionia hypophylla* could be employed as a natural antimicrobial agent in skin care products.

Keywords: Bryophytes, Antimicrobial activity, Minimum inhibitory concentrations/Minimum Bactericidal concentrations

INTRODUCTION

Historically, a large number of medicinal plants were identified and used by the aboriginal people to treat various ailments. The application ranged from topical, to prevent infection, to internal consumption, facilitating relief from nausea and other disorders. However, there have been a few studies to screen and confirm the medicinal potential of bryophytes in Kerala for antibiotic activity. The efficacies of these medicinal plants are due to the presence of secondary metabolites such as tannin, flavonoids, alkaloids and phenols function as microbicidal against fungi, bacteria, insects and viruses[1].

Bryophytes are one of the most significant and promising sources of antibiotics and biologically active compounds in nature. Flowers[2] reported the use of mosses in the treatment of burns and open wounds. Banerjee[3], reported the use of 40 bryophytes in China for the treatment of ulcer, bronchitis, tympanitis, cystitis as well as skin diseases and burns, Kang et al.,[4] reported that antibiotic principle against at least one bacterium have been found in extract of 55% of the mosses investigated. The compounds responsible for the observed antibiotic activity in bryophytes have not been isolated and purified[3].

Hundreds of medicinal bryophytes have been identified and classified in ethnobotanical literature as potential antimicrobial agents[5]. Many of these species are prevalent in temperate zones. However, few studies have been concerned with an in-depth analysis of the antimicrobial activity and efficacy of phytochemicals in different species of bryophytes. To explore the bryophytic potential in pharmaceutical applications, a quantitative and qualitative screening of bryophytes against Gram-positive and Gram-negative bacteria will be used as evidence for antimicrobial action and efficacy. This study aims to determine whether bryophytes show significant difference in microbicidal potentialities.

MATERIAL AND METHODS

The aim of the present work was to study the antimicrobial activity of *Targionia hypophylla* L. and species of *Bryum* such as *B. argenteum* Hedw.; *B. cellulare* Hook.*; B.coronatum* Schwaegr. *; B. plumosum* Dozy & Molk.; *B. pseudotriquertum* (Hedw) Schwaegr. and *B.capillare* Hedw. extracts in different solvents like benzene, chloroform, petroleum ether, methanol and water against both human and plant pathogenic bacteria and fungi.

Preparation of extracts

Fresh thalli were washed thoroughly under running tap water, shade dried 72-120 h and were homogenized to a fine powder and stored in airtight containers. 25 g of powder were extracted with 100 ml of non polar to polar solvents (benzene, chloroform, petroleum ether, methanol, and water) for 24 h by using Soxhlet apparatus. The extract was dried in a rotary evaporator for 30 min and the dry powder was considered 100%. Different concentrations; 0.25, 0.5, 1, 2, 4, 6, 8, 10, 20, 30, 40, 50, 60, 80 and 100 mg/ml were prepared by redissolving the extracted powder in the same solvent which was used in the extraction.

Test microorganisms

Selected pathogenic bacteria; *Bacillus subtilis, Escherichia coli, Pseudomonas solanacearum, Xanthomonas axonopodis, Xanthomonas vesicatoria* and fungi like *Aspergillus ochreaceous, Aspergillus flavipes, Fusarium verticilloides* and *Penicillium notatum* were obtained from culture collection of the Department of Applied Botany and Biotechnology, University of Mysore, Mysore, India.

All the test bacterial species were maintained on nutrient agar media. 36 h old bacterial culture were inoculated into nutrient broth and incubated at 35±2°C on a rotary shaker (Janked and Kunkel, Germany) at 100 rpm. After 36 h incubation, the bacterial suspension was centrifuged at 10,000 rpm for 15 min. The pellet was resuspended in sterile distilled water and the concentration was adjusted to 1 x 10^8 cfu/ml by reading the OD of the solution at 610 nm using UV-visible spectrophotometer and was used for further studies. Fungal colonies were harvested from 9 - 10 day old cultures, which were maintained on Potato dextrose agar. The spores were suspended in sterile distilled water and the spore suspensions were adjusted to 1 x 10^8 spores/ml.

Antimicrobial assay

Antimicrobial assay was performed in 96 well, sterile, flat bottom microtiter plates, based on broth microdilution assay, which is an automated colorimetric method, uses the absorbance of cultures in a microtiter plate[6]. Each well of microtiter plates was filled with 200 µl of nutrient broth/potato dextrose broth, 1 µl of test organism and 15 µl different concentrations extracts. For bacteria and fungi the microtiterplates were incubated at 35±2°C for 24 h. After the incubation period the plates were read at 465 nm using ELISA reader (ELX 800 MS, USA). Minimum inhibitory concentration (MIC), which was determined as the lowest concentration of plant extracts inhibiting the growth of the organism, was determined based on the readings.

RESULTS AND DISCUSSION

The ethnobotanical screening tests of *Targionia hypophylla* L. and species of *Bryum* such as *B. argenteum* Hedw.; *B. cellulare* Hook.*;*

B.coronatum Schwaegr. ; *B. plumosum* Dozy & Molk.; *B. pseudotriquertum* (Hedw) Schwaegr. and *B.capillare* Hedw. in different solvents against both human and plant pathogenic bacteria and fungi using microdilution technique are displayed in Table 1. The extracts of different species show differential response against bacteria and fungi. Both benzene and chloroform extracts were found to be ineffective or showed poor inhibition of bacterial and fungal growth.

The thallus extract of *T. hypophylla* showed significant microbicidal potentiality against most of the pathogens i.e., methanol showed MIC of 2 mg/ml against all tested bacteria except *X. vesicatoria* (4 mg/ml). Petroleum ether extract showed MIC of 4 mg/ml against *B. subtilis*, *E. coli* and *X. vesicatoria*, whereas MIC of 1 and 2 mg/ml were found against *P. solanacearum* and *X. axonopodis*, respectively (Table 1).

	B. argenteum					***B. cellular***					***B. coronatum***					***B. plumosum***					***B. pseudo triquertum***					***T. hypophylla***				
	B	**C**	**PE**	**M**	**W**	**B**	**C**	**PE**	**M**	**W**	**B**	**C**	**PE**	**M**	**W**	**B**	**C**	**PE**	**M**	**W**	**B**	**C**	**PE**	**M**	**W**	**B**	**C**	**PE**	**M**	**W**
Bacillus subtilis	6	2	4	2	2	6	4	4	2	2	6	4	4	1	2	4	4	6	2	2	6	4	4	2	1	5	2	4	2	1
Escherichia coli	6	4	4	2	4	6	4	4	2	2	6	4	4	2	2	4	6	6	2	4	6	6	4	4	4	5	2	4	2	2
Pseudomonas Solanacearum	6	4	4	2	4	6	4	4	4	4	6	4	2	4	4	6	4	4	4	2	4	6	6	2	2	5	2	1	2	1
Xanthomonas axonopodis	6	4	4	2	2	6	4	4	2	2	4	4	4	2	4	6	6	4	4	4	4	4	6	4	4	5	2	2	2	1
Xanthomonas vasicatoria	6	4	4	2	4	6	4	4	1	2	6	4	2	4	2	6	4	6	6	4	6	4	4	2	6	5	4	4	4	4
Aspergillus ochreaceous	6	4	6	4	4	6	4	4	4	2	4	4	2	1	4	6	2	6	2	6	4	6	6	4	2	4	1	4	2	1
Aspergillus flavipes	6	4	6	1	4	6	4	4	2	2	6	2	4	4	2	6	4	4	2	2	6	6	6	4	2	5	4	4	2	2
Fusarium verticilloides	6	2	6	4	4	6	4	4	2	4	6	4	4	4	4	6	2	2	2	2	6	4	4	4	4	4	6	4	2	4
Penicillium notatum	6	2	6	4	4	6	4	4	4	4	6	4	4	2	4	6	2	2	2	6	6	4	4	4	4	4	2	4	2	2

The MIC of 2 mg/ml was found against *E. coli*, *P. solanacearum* and *X. axonopodis* when chloroform extract was used also found to be effective against *B. subtilis*, *P. solanacearum* and *X. axonopodis* when water extract was used (Table 1).

A stronger and broader spectrum of antimicrobial activity was observed in methanolic and water extract. The MIC of 2 and 4 mg/ml was found against all the tested bacteria when methanol, water and chloroform extracts of *B. argenteum* and *B.coronatum* were used. Whereas chloroform, petroleum ether extract showed MIC of 4 mg/ml against all the test bacteria except for *E. coli* (2 mg/ml) (Table 1).

T. hypophylla extract showed MIC of 2 and 4 mg/ml against all the tested fungi when extracted in methanol and petroleum ether, where the MIC ranged between 1 to 6 mg/ml when the thallus extracted with chloroform and water were used (Table 1). *B. plumosum* and *B. pseudotriquertum* extracted with methanol also showed MIC of 2 and 4 mg/ml respectively against all the tested fungi. The thallus extract of *B. argenteum* with methanol showed MIC of 4 mg/ml against *A. ochreaceous*, *F. verticilloides* and *Penicillium notatum* (Table 1). Petroleum ether and benzene extracts were weakly active against all the tested bacteria and fungi (Table 1).

Preliminary results of screening the extracts of selected species of bryophyte tested against bacteria and fungi showed that there is a distinct difference between the antibiotic activities of the tested species in relation to the solvents used. Most species showed antibiotic activity when methanolic/water extraction is used, in comparison to other solvent extractions. In addition, none of the species showed prominent antibiotic activity against benzene extraction. *B. argenteum, B. cellulare*, *B.coronatum, B. plumosum* and *B. pseudotriquertum,* showed antibiotic activity using methanolic or petroleum ether extraction. This suggests that specific antibacterial compound(s), effective against the selected bacterial species, tend to be isolated more effectively from liverworts and mosses using methanol. Table 2 displays the phytochemicals which have been extracted from the bryophytes with methanolic system.

Table 2: Phytochemical screening in *T. hypophylla* and different *Bryum* species using methanol solvent system

	B.argenteum	***B. cellular***	***B. coronatum***	***B. plumosum***	***B. pseudotriquertum***	***T. hypophylla***
Alkaloids	-	-	-	+	-	-
Flavonoids	+	+	+	+	+	+
Saponins	+	-	-	-	-	-
Tannins	+	+	+	+	+	+
Glycosides	-	-	+	-	-	+
Phenols	+	+	+	+	+	+

Furthermore, this also suggests that one antibiotic compound may be responsible for the antimicrobial qualities of a genus or species. This clearly demonstrates the importance of using a multitude of extraction compounds and methods to isolate antibiotic compounds. A failure to do so could result in an inability to retrieve valuable information, and incorrectly dismissing potentially beneficial species. McCutcheon et al.[7] suggests that when a relatively limited number of bacteria are used to screen for antibiotic qualities, potentially beneficial species can be overlooked.

The occurrence of antibiotic activity definitely appears to favour liverworts over mosses. This suggests that detectable antibacterial compounds are present in most taxa of liverworts[8]. *T. hypophylla* demonstrated the greatest MIC i.e., it showed significant inhibition (MIC) than others, depending on the extraction method. These values are approximately one third of the inhibition of reference antibiotics. Zhu et al.[1] suggests that the reason for this is the diverse cellular oil bodies that are found in liverworts. These contain vast array of lipophilic terpenoids, aromatic compounds, and acetogenins which may be responsible for the extraordinary array of bioactivities and medicinal properties[9]. Presently, over 400 novel compounds have been isolated with antibiotic potentialities[10]. Furthermore, Asakawa[11] suggests that oil bodies are easily extracted with alcoholic extraction methods, while the other classes of bryophytes lack oil bodies, making antibiotic activity more difficult extract.

Gram-positive bacteria, *B. subtilis*, showed the greatest sensitivity to bryophyte extracts, while Gram-negative showed lowest sensitivity. Physiologically, Gram-negative bacteria have an outer membrane while the Gram-positive bacteria only have peptidoglycan layer[12]. The outer membrane is responsible for protecting the bacteria from an array of different antibiotics, detergents, and enzymes that would normally damage the inner member or peptidoglycan cell wall[12].

The effectiveness of liverworts and mosses, solely against Gram-positive bacteria supports the notion that they are more vulnerable[13]. On the other hand, McCutcheon et al.,[7] states that there are few known natural antibiotic compounds with a high antibacterial activity against Gram-negative bacteria, which parallel our results. Mc-Cutcheon et al.,[7] also states that Gram-negative bacteria *K. pneumoniae* is particularly resistant to antibiotic therapy. Further research will be needed to confirm indications that *T. hypophylla* has antibiotic activity against *E. coli*.

Studies indicate evidence of antibiotic properties of selected bryophyte species, incongruent with our results. For example, Castaldo-Cobianchi[9] showed high antibacterial activity of *Conocephalum conicum* against pathogenic bacteria. *Atricum selwynii* and *Sphagnum palustre* have also shown activity against a variety of Gram-positive and Gram-negative bacteria species[14-16]. Lastly, *Hylocomium splendens* has shown antibiotic activity against nine Gram-positive bacteria[17]. Similarly, *Bryum* species displayed variable microbicidal activity. According to the literature, many bryophyte species should have shown antibiotic activity against *B. subtilis*[9]. Therefore, potential differences in species, seasonal collection, locality, or extraction and experimental procedure may have been factors the results. Therefore, further studies will be needed to explore the factors affecting bryophyte activity.

Plants that possess alkaloids are pharmacologically active as they have physiological effects on man and other animals and serve as therapeutic and antimalarial drugs. Saponins are glycoside components often referred to as natural detergent because of their foamy nature[18, 19]. Pawar and Arumugam [20] reported that saponins have anticarcinogenic properties (antioxidant effect direct and selective, cytotoxicity of cancer cells, immune modulation, acid and neutral sterol metabolism and regulation of cell proliferation) and health benefits (inhibition of the growth of cancer cells, cholesterol lowering agents an immune booster and a natural antibiotic). He further reported that cardiac glycosides inhibit the Na\K and ATPase pumps in mammals. *Digitalis* spp and *Strophantus* spp contain cardiac glycoside, which is responsible for its use in treating heart problems[20]. Since these screened moss species contain alkaloid, phenoiics, tannins, saponins and cardiac glycoside, it would therefore be correct to assume, their relevance in the production of drugs- against heart problems and other ailments. A further research is needed to isolate these secondary metabolites and identify their specific types. Singh et al., [13] also reported the antibacterial activity of alcoholic and aqueous extracts from Marchantiales.

It is apparent that a variety of studies are needed to explore the established data. Therefore, further studies could include performing more antibiotic screenings with variety of potential bacterial species. This would provide evidence to support current results. Furthermore, re-testing bryophytes that performed differently from documented results could allow for greater understanding of the factors affecting activity. This would also allow one to test for broad spectrum antibiotic and narrow spectrum antibiotics. Once significant antibiotic activities have been isolated, MIC and MBC would be performed to determine antibiotic efficacy. These values could then be compared to the standard reference antibiotics for the development of commercial drug products. In addition, isolating the antibiotic compounds by NMR and MS, GC-MS allows for examination of compound structure. This could lead to further studies in synthetic production or chemical development. Also, these compounds would be subjected to animal and human studies to determine their effectiveness on whole-organism systems, including toxicity and normal microbiota studies. Animal and human studies would also include infection prevent and treatment.

In conclusion petroleum ether, methanolic and water extracts of bryophytes inhibited bacterial and fungal growth suggesting that ethanopharmcological approach in selecting the plants for study may be useful. Further work is needed to isolate the active principle from the plant extracts and to carry out pharmaceutical studies.

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