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PHYTOCHEMICAL ANALYSIS AND BACTERICIDAL POTENTIALITY OF *POGONATUM MICROSTOMUM* SCHW.

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ABSTRACT

Bryophytes include mosses, liverworts and hornworts and are the second largest group of plants, with approximately 28.000 species worldwide. Bryophyte chemistry is poorly documented, and are scattered. They are considered as remarkable reservoir of new, secondary compounds, many of which have shown interesting biological activities. Therefore, the present investigation was undertaken to analyse the various phytochemicals qualitatively and quantitatively using various solvents such as water, ethyl acetate and petroleum ether of the moss *Pogonatum microstomum* and its bactericidal activities. Initially the different extracts revealed a pool of phytochemicals. Quantification data revealed significant levels of phenols and flavonoids. In the second phase, the total phenols were fractionated by RP- HPLC analysis, which revealed the presence of phenolic acids such as sinapic acid, hydroxycinnamic acid, vanillate, gallate, chlorogenate, ferullate, phloroglucinol and catechol. Varying levels of bactericidal potentialities was displayed by the three different extracts against *Streptococcus haemolyticus, Staphylococcus aureus, Klebsiella pneumoniae,* and *Escherichia coli* using disc diffusion method and the results were comparable with synthetic antibiotic. Further studies are warranted to elucidate, purify the lead molecule and to explore their bactericidal mechanism.

KEYWORDS: Phytochemicals, bactericidal activity, RP- HPLC analysis, *Klebsiella pneumoniae*, *Streptococcus haemolyticus*.

INTRODUCTION

Bryophytes synthesize an array of phytochemicals to combat against the unhospitable environmental conditions including predation, UV radiation, high temperature, pest and

pathogens. They are potential source of natural bioactive compounds such as secondary matabolites and are commercially used in many pharmaceutical preparations. Flavonoids and phenolic acids are the most important groups of secondary metabolites in bryophytes.[1] Flavonoids are proven antioxidants constitute a wide range of molecules that play important role in protecting biological systems against the harmful effects of oxidative processes on macromolecules such as carbohydrates, proteins, lipids and DNA.[2] In recent years the search for plants with antimicrobial activity has gained increasing importance, due to worldwide concern about the alarming increase in the rate of infection by antibiotic resistant microorganisms. Studies have been conducted with the extracts of bryophytes for screening antimicrobial activity as well as for the discovery of new antimicrobial compounds. Since the most infectious diseases are of microbial origin, there has been an increasing demand for natural antimicrobial therapeutics. In spite of accumulating knowledge regarding the phytochemicals from plant origin, only fewer reports were documented from bryophytes. Regarding ethno medicinal utility it has become essential to study the chemical entities as well as pharmacological evaluation of bryophytes. The purpose of the present study was to evaluate phytochemicals and antibacterial activities of water, ethyl acetate and petroleum ether extracts of the moss *Pogonatum microstomum.*

MATERIALS AND METHODS

Plant material

Fresh thallus of *Pogonatum microstomum* were collected from Munnar hills of Kerala, India. The specimen was taxonomically identified and confirmed by comparing with authenticated herbarium specimen at Department of Botany Herbaria, University of Calicut, Kerala. A voucher specimen of the plant is kept in the herbarium of our institute (CCB 014).

Preparation of extracts: Fresh thallus (50g) was chopped, air dried at room temperature, finely powdered and successively extracted with 100 ml of ethyl acetate, petroleum ether and water for 6 h using soxhlet hot continuous extraction method. The extracts were filtered and concentrated using rotary evaporator at 50°C.

Phytochemical screening: All the three extracts were subjected to various tests in order to detect the presence of different phytochemicals such as glycosides, tannins, coumarins, alkaloids, saponins, flavonoids, phenols, steroids, reducing sugar and terpenoids.[3]

Quantification of phenols and flavonoids

Total phenols of ethyl acetate, petroleum ether and aqueous extracts were isolated and quantified by the method of [4] and the total flavonoid content determined by $AlCl_3$ method.[5]

Reverse phase high performance liquid chromatography (RP-HPLC) of phenols

Phenolic components of the extract were further fractionated following the method.[6] 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 x 4.6 mm, 5mm) (Millipore, Milford, MA) column at 30^0C. 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.

Test microorganisms

Gram-positive bacteria *(Streptococcus haemolyticus,* and *Staphylococcus aureus)* and Gram-negative bacteria (*Klebsiella pneumonia* and *Escherichia coli*) were collected and identified from research laboratory, Department of Microbiology, Government Medical College, Thiruvananthapuram. They were maintained on Mueller–Hinton Agar medium.

Antibacterial activity

The various extracts of *Pogonatum microstomum* at different concentration were subjected to explore its effects on bacteria. Disc diffusion method was performed to study the antibacterial activity. The study mainly focused on *Klebsiella pneumonia, Streptococcus haemolyticus, Escherichia coli* and *Staphylococcus aureus*. The strains were sub-cultured on to nutrient agar and fresh cultures were grown in peptone water for 2 h and the concentration was adjusted to 10^8cfu/ml. Each labelled nutrient agar plate was uniformly inoculated with the test organism by using a sterile cotton swab rolled in the suspension to streak the plate surface. The extracts were properly distributed in three concentrations (250, 500, 1000 µg/ml). The extract discs were prepared by soaking paper disc into the extract for ample time period. A sterile disc (diameter 5 mm, Whatman paper No.3) was impregnated with test concentrationsof the compounds and inoculated on plates containing test microorganism. The inoculated plates were kept in refrigerator for 1 h to allow the extracts to diffuse into the agar

and were incubated at 37°C for 24 h. Antimicrobial activity was determined by measuring the diameter of zones of inhibition (mm) produced after incubation.Each microorganism was tested in triplicate and ampicillin was used as a positive control.

Statistical analysis

The data was statistically analysed by one way analysis of variance (ANOVA) and t-test ($p < 0.05$). The results are average of five replications and were represented as mean ± SD.

RESULTS AND DISCUSSION

Table 1: Preliminary phytochemical analysis using ethyl acetate, petroleum ether and water extracts from *P. microstomum*.

Phytochemicals	Petroleum ether	Ethyl acetate	Water
Phenols	++	++	+++
Glycosides	++	+	++
Tannins	++	+	+++
Comarins	++	+	+++
Alkaloids	+	+	+++
Saponins	-	-	-
Flavanoids	++	++	+++
Steroids	++	++	++
Terpenoids	++	-	++

Strong positive: + + +; moderately positive: + +; Low positive: +; negative test: -

Table 2: Total phenols and flavonoids of ethyl acetate, petroleum ether and water extracts from *P. microstomum*. Values are mean ± SD of three independent replications.

Solvent	TPC (mg/g tissue)	Flavonoids (mg/g tissue)
Ethyl acetate	54.4 ± 0.57	28.6 ± 0.37
Petroleum ether	84.3± 0.67	36.3 ± 0.78
water	91.2 ± 0.81	54.6 ± 0.14

Table 3: RP-HPLC fractionation of phenols in *P. microstomum*

Name of phenolic acid	Retention time	Amount (µg/g tissue)
Sinapic acid	4.234	3171.04
Hydroxycinnamic acid	3.319	3194.96
Vanillate	3.319	2319.96
Gallate	3.391	2394.43
Chlorogenate	3.391	2793.50
Ferullate	4.509	2529.62
Phlonogucinol	4.234	2379.70
Catechol	3.319	2982.81

Table 4: Antibacterial activity of *P. microstomum* ethyl acetate, petroleum ether and water extracts against selected bacteria

Microorganism	Extract	Concentration of the extract (µg/ml)		
		250	500	1000
Klebsiella pneumoniae	Ethyl acetate	0	1	2
	Petroleum ether	0	3	4
	Water	0	4	5.2
	Ampicilin	3	5	6.1
Streptococcus haemolyticus	Ethyl acetate	0	3	3
	Petroleum ether	0	4	4
	Water	0	4	5
	Ampicilin	4	5	6
Escherichia coli	Ethyl acetate	0	0	1
	Petroleum ether	0	1	3
	Water	0	2	4
	Ampicilin	4	5	5.2
Staphylococcus aureus	Ethyl acetate	0	0	1
	Petroleum ether	0	2	2
	Water	0	2	3
	Ampicilin	4	4.8	5

Values of zone of inhibition in mm are mean of three replicates

0 = No zone of inhibition

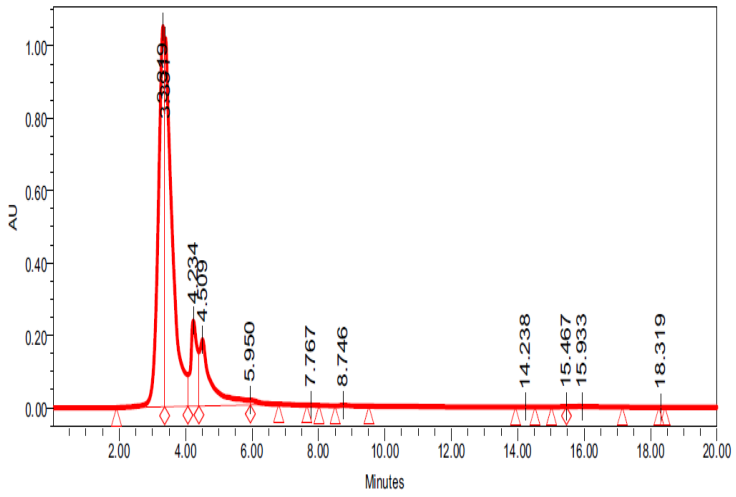
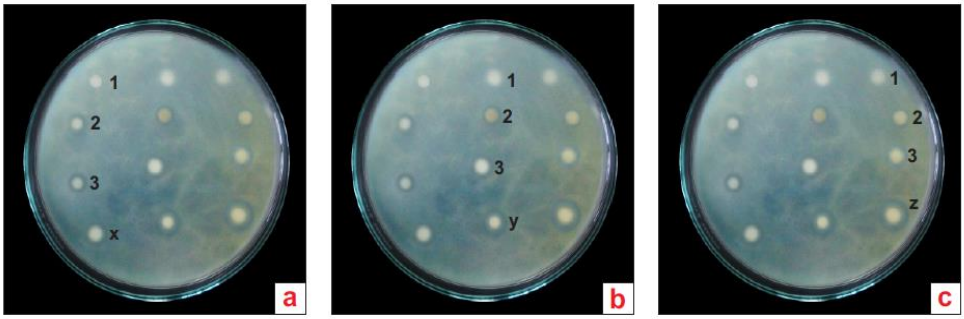
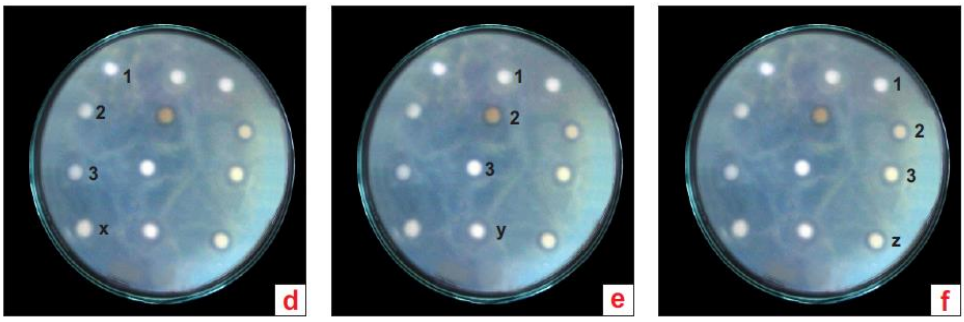


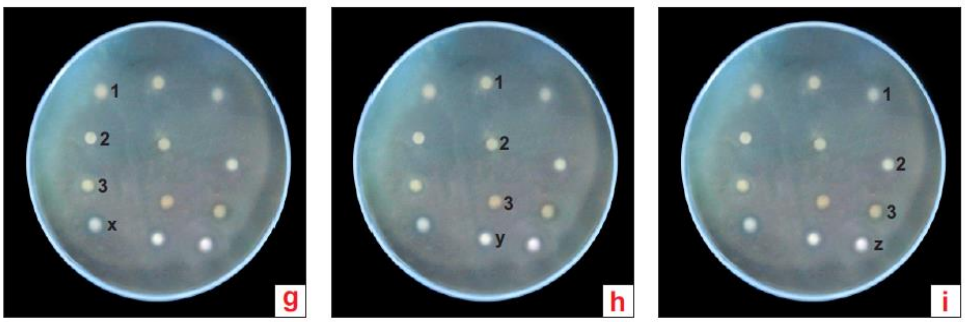
Fig: 1 Phenolic acid profile of *Pogonatum microstomum* by RP- HPLC



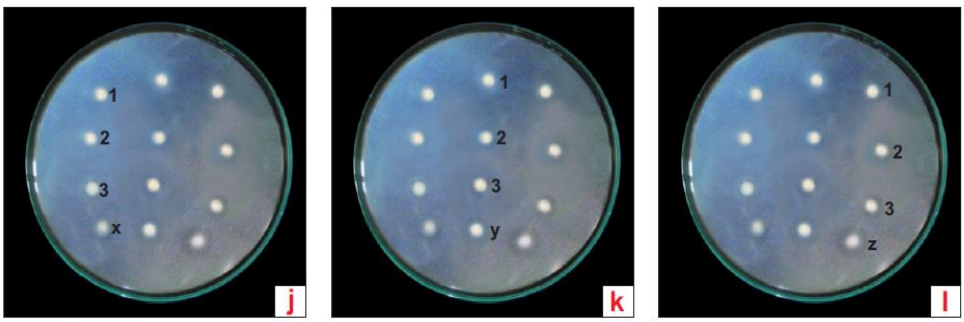
I. *Klebsiella pneumonia* (a)Ethyl acetate extract (b)Petroleum ether extract (c)Aqueous extract
1. 250 µg/ml, 2. 500 µg/ml, 3. 1000 µg/ml, x. Ampicillin 250 µg/ml y. Ampicillin 500 µg/ml z. Ampicillin 1000 µg/ml



II. *Streptococcus haemolyticus* (d)Ethyl acetate extract (e)Petroleum ether extract (f)Aqueous extract
1. 250 µg/ml, 2. 500 µg/ml, 3. 1000 µg/ml, x. Ampicillin 250 µg/ml y. Ampicillin 500 µg/ml z. Ampicillin 1000 µg/ml



III. *Escherichia coli* (g)Ethyl acetate extract (h)Petroleum ether extract (i)Aqueous extract
1. 250 µg/ml, 2. 500 µg/ml, 3. 1000 µg/ml, x. Ampicillin 250 µg/ml y. Ampicillin 500 µg/ml z. Ampicillin 1000 µg/ml



IV. *Staphylococcus aureus* (j)Ethyl acetate extract (k)Petroleum ether extract (l)Aqueous extract
1. 250 µg/ml, 2. 500 µg/ml, 3. 1000 µg/ml, x. Ampicillin 250 µg/ml y. Ampicillin 500 µg/ml z. Ampicillin 1000 µg/ml

Fig 2 a-l: Antibacterial activity by disc diffusion assay of *Pogonatum microstomum* against *Klebsiella pneumonia*, *Streptococcus haemolyticus*, *Escherichia coli* and *Staphylococcus aureus.*

Phytochemical analysis

Bryophytes, the oldest land plants are endowed with various bioactive compounds such as terpenoids, phenolics, lignins, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other metabolites which are proven antioxidants . Studies have shown that many of these phytochemicals possess anti-inflammatory, anti-atherosclerotic, antitumor, anti-mutagenic, anti-carcinogenic, antibacterial, and antiviral activities and will reveal novel new molecules, some of which are not synthesizable by higher plants. The biosynthesis and degradation of these chemicals play important roles in the ecology and physiology of them. The phytochemistry of bryophytes has been neglected for a long time because they are small and difficult to collect in large amounts as pure samples.

The percentage yield in the sequential extraction of the moss *Pogonatum microstomum* was in the range of 0.39- 0.72%. The yield of water extract of was higher (0.72%) than that of petroleum ether (0. 51%) and ethyl acetate (0.39%) extract. The highest yield of water extract from *Pogonatum microstomum* established in the present analysis reveal the greater efficiency of water as extracting solvent due to its high polar nature. The qualitative analysis of phytochemical constituents in petroleum ether, ethyl acetate and water extracts of the *P. microstomum* revealed a marked variability in the presence of glycosides, tannins, coumarins, alkaloids, saponins, flavonoids, phenols, steroids and terpenoids (Table 1). The therapeutic value of plants lies in the principle component that has potential physiological role in the human body. Structural peculiarities of phytochemicals show wide variation and in turn the range of activities, which may safe guard against chronic diseases.[7]

Spectrophotometric analysis of total phenol and flavonoid content showed remarkable level in aqueous extracts compared to ethyl acetate and petroleum ether extracts. The total phenolic content in water extract of *Pogonatum microstomum* was 91.2 ± 0.81mg/g tissue, whereas in petroleum ether 84.3 ± 0.67 mg/g tissue and that of ethyl acetate was 54.4 ± 0.57 mg/g tissue. These values were extremely higher than *Centella asiatica* L.[8] Similar to phenols, flavonoids also showed significant amount in water extract (54.6 ± 0.14 mg/g tissue) followed by petroleum ether (36.3 ± 0.78 mg/g tissue) and ethyl acetate (28.6 ± 0.37 mg/g tissue) respectively (Table 2). The antioxidant activity of plants might be due to the presence of phenolic and flavonoid compounds. Phenolic compounds undergo complex redox reaction with phosphotungstic and phosphomolybdic acids present in the Folin-Ciocalteu reagent. It should be noted that some chemical group of amino acids, proteins, organic acids, sugars and

aromatic amines could react with the reagent. Flavonoids are one of the most diverse and widespread group of natural compounds and are the most important natural phenolics. These compounds possess broad spectrum of chemical and biological activities including free radical scavenging properties, inhibition of hydrolytic and oxidative enzymes.[9]

The phenolics and flavonoids are important plant phytochemicals that has several hydroxyl groups. These hydroxyl groups have been shown to be responsible for these chemicals radical scavenging ability. They have been found to show inhibitory effects on inflammation, allergies, mutagenesis and carcinogenesis in humans, prevent the decomposition of hydroperoxides into free radicals and slow down the rate of conjugated diene formation, retard oxidative degradation of lipids thereby improve the nutritional value and quality of food.[10]

The continued search among plant secondary metabolites as natural antioxidants has gained importance in recent years because of the increasing awareness of herbal remedies as potential sources of phenolic oxidants. It is well known that phenolic compounds contribute to quality and nutritional value in terms of modifying colour, taste, aroma and flavor besides providing health beneficial effects.[11]

Phenolic acids in *P. microstomum*

The role of phenolic acids as antioxidant is well established by several authors (Kim *et al*., 2006; Robbins, 2003). The chromatogram represents the phenolic acid profile of *P. microstomum* (Fig 1). The major phenolic acid noticed in the plant was represented in the Table 3. Interestingly, the moss shows significant profile of phenolic acids such as sinapic acid (3171.04 µg/g tissue), hydroxycinnamic acid (3194.96 µg/g tissue), catechol(2982.81 µg/g tissue) and chlorogenate (2793.50 µg/g tissue).

Phenolic compounds such as quercetin, rutin, narigin, catechin, caffeic acid, gallic acid and chlorogenic acid are important plant constituents. Bryophytes known to produce diverse substances possessing antioxidant properties having ability to protect the human body against cellular oxidation. Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radicals inducing oxidative stress. Polyphenols also enhance the level of cellular antioxidative system and induce the cytochrome P-450 resulting in detoxifying the activity of carcinogens intracellularly. The abundance in polyphenol compounds would confirm the therapeutic properties that they are assigned in ethnotherapy.[12]

The antioxidant activity of phenolic acids is related to the acid moiety and the number and relative positions of hydroxyl groups on the aromatic ring structure. Hydroxycinnamic acids are more effective antioxidants than hydroxybenzoic acids due to increased possibilities for delocalization of the phenoxyl radical.[13] Di- and trihydroxylation increase the activity over a single hydroxyl group with the position of the hydroxyl groups being the most important factor. Hydroxylation in the 2- and 4-positions or in the 3-, 4- and 5-positions confers the greatest antioxidant activity. Adjacent hydroxyl groups, as found in proto catechuic acid, are less favourable for antioxidant activity than those meta-orientated with respect to each other, as in the case of cc-resorcylic acid.[14]

Similarly, phenolic acids, deriving both by direct absorption from food consumption and as a result of the cleavage of flavonoids by gut microflora. These compounds acted as chain-breaking antioxidants, with different effectiveness, in membrane models and were able to scavenge intracellular ROS raise due to exogenous oxidative stress in both leukemia and normal cells. Moreover, it is observed that phenolic acids were able to scavenge reactive oxygen species in HELA cells, characterized by very high intracellular ROS levels.[15]

Antimicrobial activity

Bryophytes exhibit antimicrobial effects against bacteria and fungi. Generally they are not damaged by insects, fungi, bacteria, slugs and snails because of the presence of biological compounds like oligosaccharides, polysaccharides, sugar, alcohols, amino acids, fatty acids, aliphatic, aromatic and phenolic substances in them. The biological activities of bryophytes are due to these compounds. Bryophytes are important source of potentially useful new chemotherapeutic agents. Their usage in traditional medicine as curatives for several diseases is most popular for 80% of world population in Asia, Africa and South America. There has been an increasing interest in the exploration of antibacterial plant products having mechanisms of action different from those of the conventional chemical drugs.This aspect is effectively evaluated by ethyl acetate, petroleum ether and aqueous extract of a bryophyte, *P. microstomum*, on the selected bacterial strains.

Bactericidal activity of the ethyl acetate, petroleum ether and aqueous extract of *P. microstomum* exhibited varied susceptibility against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli* and *Streptococcus haemolyticus* at different concentrations tested (Table 4). The microbicidal potential of the extract was visualized as inhibition zone by treating the pathogens with the extracts and then spreading the cells on agar plates by disc

diffusion assay (Fig 2a-l). Among the pathogens tested *K. pneumonia* and *Streptococcus haemolyticus* were the most resistant species with *P. microstomum.* On the other hand, water extract of *P. microstomum* showed highest antibacterial potential with all tested bacterial strains. The mechanism of antibiosis indicated by synthetic antibiotic ampicillin was comparable against the entire tested bacterial isolates. The effectiveness of an antibacterial agent is measured by its ability to inhibit and kill bacteria. At higher concentration of the extract more bacteria were killed. The antibacterial activity revealed by the extracts might be due to presence of flavonoids, terpenoids and other polyphenolic compounds. Due to the variation in composition of active compounds in various extract of *Pogonatum* species resulted in significant difference on the level of bactericidal activity (inhibitory zone) against the tested bacterial strains.

A steroid isolated from alcohol extract of *Pallavicinia lyellii* is able to inhibit the growth of *Aspergillus fumigatus*.[16] The drug inhibits the germination of the spore as well as the multiplication of mycelia. Ethanol extracts of *Sphagnum magellanicum* showed antibacterial potentiality against *Enterobacter aerogenes, Escherichia coli, Salmonella typhii* and *Staphylococcus aureus*.[17] Extracts of *Targionia, Marchantia, Plagiochasma, Rhodobryum,* and *Plagiomnium* inhibit the growth of *Biden biternata*.[18] Flavonoids isolated from *Marchantia convoluta* extracts could inhibit the growth of *Staphylococcus aureus, Bacillus enteridis, Streptococcus hemoliticus* type B and *Diplococcus pneumonia*.[19] Extract of bryophytes contains isoflavonoid, flavonoid, biflavonoid and terpenoids. Those compounds have been reported to have antimicrobial activity. In recent years, many possible sources of natural antibiotics have been used against 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 in bryophytes. Presence of several bioactive compounds from bryophytes like polygodial from *Porella,* norpiguisone from *Conocephalum conicum* and lunularin from *Lunularia cruciata,* 4-hydro-3-methoxybibenzyl and α and β pinineallоromadendrine from *Plagiochila stevensoniana* are useful anticancer and antimicrobial compounds.[20,21] *Plagiochila fascicula* shows inhibitory effect on *Bacillus subtilis, Escherichia coli, Candida albicans, Trichophyton mentagrophytes* and *Cladosporium resinae*.[22] The antifungal activity of *Herberta aduncus* against *Botrytis cinerea, Rhizoctonia solani, Pythium debaryanum* is well illustrated.[21] Species of *Fissidens* and *Polytrichum* were used as diuretic and hair stimulating drugs. There are reports regarding the broad spectrum of antifungal activity of bryophytes like *Porella, Makinoa , Lunularin*

cruciate, Septoria nodorum, Dumortiera hirsute, Sphagnum portoricense and *Orthotrichum rupestre*.[20] The reason for varied activities between gram negative and gram positive bacteria towards plant extracts could be due to their morphological difference. Gram positive bacteria should be more susceptible, since they have only an outer peptidoglycan layer, which is not an effective permeability barrier.[23]

The mechanism of antimicrobial activity of phenolic compounds is primarily by its ability to act as non-ionic 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 antifungal and antibacterial properties.[24] Polyphenols inhibit bacterial DNA gyrase by binding to the ATP binding site.[25] Similarly, tannins exert antimicrobial activities by iron deprivation, hydrogen binding or specific interactions with vital proteins, such as enzymes in microbial cells, bind to adhesions, complexation with cell wall, other membranes and metal ion complexes. Flavonoids are also possessing antimicrobial potential by link to adhesions or complexes with the cell wall, inactivation of enzymes and inhibition of HIV reverse transcriptase.[26]

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