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Research Article

In Vitro Antibacterial Activity, Phytochemical Analysis and Inhibitory Concentration of Bryophytes against Drug Resistant Bacterial Pathogens

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ABSTRACT:
Natural plants especially high altitude plants provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. There is a continuous and urgent need to discover new antimicrobial compounds from the bryophytes from high altitude areas against new and re-emerging infectious diseases. In this study, the epiphytic bryophytes *Homaliodendron montagneanum, Papillaria fuscescens and Campylopus latinervis were* collected separately and was extracted by percolation method using various solvents like acetone, ethyl acetate and distilled water. The antimicrobial activity was evaluated against the different pathogenic bacterial strains using agar well diffusion method. The bryophyte extracts showed remarkable inhibitory activity against drug resistant bacterial pathogens. The extracts were further screened for phytochemical studies and they revealed the presence of alkaloids, flavonoids, phenol, proteins and amino acids, resins, saponins, steroids, tannins, xanthoproteins and sugars. The antimicrobial activities of bryophytes were due to the presence of various secondary metabolites, which may enhance at the high altitudes.. The Minimal Inhibitory Concentration of the acetone extract of *H. montagneanum* against *Staphylococcus aureus* MTCC 3160 is 20mg/ml and Minimal Bactericidal Concentration is 40mg/ml.

Keywords: Antibacterial activity, Bryophytes, High altitudes, *Homaliodendron montagneanum, Papillaria fuscescens, Campylopus latinervis,* phytochemical screening, secondary metabolites *etc*.

[I] INTRODUCTION

Antimicrobial resistance is one of the biggest challenges to face global public health at the beginning of the third millennium. According to WTO, about 440,000 new cases of multi-drug-resistant tuberculosis are reported every year, causing at least 150,000 deaths [1]. The development of novel antimicrobial agents with activity against pathogens that have become resistant to currently available agents is one tactics for combating resistant organisms (Panghal *et al.,* 2011). Therefore the rapid propagation in antibiotic resistance and the increasing interest in natural products have placed medicinal plants back in the front lights as a reliable source for the discovery of active anti-microbial agents and possibly even novel classes of antibiotics (Kenneth, 2009). The bryophytes are recognized as the basal or first diversity lineage of the land

plants[2,3] which includes morphologically and biochemically diverse groups like liverworts, hornworts and mosses. They may expect interesting bioactivities [4,5, 6]. Traditional medicinal use was started around 400 years back in China [7]. According to Umadevi *et al.* [8] (2013), plants growing at high altitudes are subjected to a variety of stressful environments and and hence they may produce a spectrum of secondary metabolites. So in keeping view of all these this study was made on the antibacterial activity and phytochemical analysis of bryophytes against microbial pathogens. The epiphytic mosses, *Homaliodendron montagneanum, Papillaria fuscescens* and *Campylopus latinervis* were collected from the Doddabetta region of Nilgiri Hills. The Nilgiri (blue mountains), are a range of mountains forming a part of the Western Ghats situated in the western part of Tamil Nadu state at the junction of Karnataka and Kerala states in Southern India. There are at least 24 peaks above 2,000 metres (6,600 ft) which make the southwestern edge of the Deccan Plateau. The Doddabetta Peak, 4 km east southeast from Udhagamandalam, 11°24′10″N 76°44′14″E, is the highest point with a height of 2,637 metres (8,652 ft) in the Nilgiris and the southern extent of the range. Prasanth G. Willams *et al.,* [9] reported that there are rane bryophytic diversity on the Western Ghats region. Hence a scientific study on its antimicrobial potential of high altitude bryophytes has not been reported much. Hence, the present investigation is aimed to study the antimicrobial properties of the selected bryophytes and a preliminary investigation was also made on their phytochemical properties. Further the antibacterial activity and find out the Inhibitory (MIC) and bactericidal (MBC) concentrations of the effective extracts were also detected.

[II] MATERIALS AND METHODS

2.1. Collection of bryophytes

The epiphytic mosses, *Homaliodendron montagneanum, Papillaria fuscescens and Campylopus latinervis* were collected from the Doddabetta region of Nilgiri Hills, the Nilgiris District, Tamilnadu. The specimens were brought to the laboratory and identified using Gangulee's mosess of eastern India and adjacent regions"(1969-1980) in the Botany Laboratory and specimen was deposoted on the herbarium, Bishop Moore College, Mavelikara (BMC/BBT-15-01; BMC/BBT-15-02; BMC/BBT-15-03 respectively).

2.2. Extract preparation

All the plant material collected were separately washed with distilled water to remove the adhering soil or extraneous dust particles. The shade dried plants were further ground into a fine power. Organic solvents such as 95 % (v/v) acetone, 95 % (v/v) Ethyl acetate (Nice, Cochin) and distilled water were employed for the extraction of different bioactive principles. In this study, cold extraction (percolation) was done. Powdered plants (50 g) were extracted with 100 ml respective solvents for 96 h at room temperature. The crude extract was prepared by filtering the extracts with whatman filter paper No. 1 followed by evaporating the solvent in open air. Then the extract was collected and stored at 4^{o}C and checked for their antimicrobial property.

2.3. Testing for antibacterial activity

2.3.1. Test cultures

These resistant bacterial strains were procured from Microbial Type Culture Collection (MTCC, Chandigarh, India) were employed in the present study to investigate the antibacterial properties. The antibiotic resistance pattern of bacterial pathogens was deduced from the standard interpretation chart. Almost all the antibiotics are resistant to Penicillin. The Gram negative organisms such as *Escherichia coli* (MTCC 585), *Klebsiella pneumoniae* (MTCC 3040), *Mycobacterium smegmatis* (MTCC 994), *Pseudomonas aeruginosa* (MTCC 7925), *Shigella flexneri* (MTCC 1457), *Xanthomonas campestris* (MTCC 2286) and Gram positive organisms such as *Bacillus subtilis* (MTCC 428), *Staphylococcus aureus* (MTCC 3160) were used as the test pathogens.

2.3.2.Maintenance of Cultures

Bacterial strains were streaked on nutrient agar plate to isolate pure culture. The single pure culture were streaked on nutrient agar slants and stored at 4^0C to keep the strains viable.

2.3.3.Culture Media

The nutrient agar (used for storage) and nutrient broth (used for sub-culturing) were used for the culture maintenance. Sterile Muller Hinton Agar (Hi Media, Mumbai) was used for the antibacterial study of the bryophytes under investigation.

2.3.4.Antimicobial assay

The antimicrobial activity was assessed using the Agar well diffusion assay [10,11]. All bacterial cultures were plated out on Nutrient agar plates and were incubated for 24 h at 37 ± 0.5^0C and colonies from this fresh culture were used for making suspension. 10 ml of sterile nutrient broth were aseptically inoculated with test cultures and incubated at 37±0.5^0C for 18 hours. Fresh inoculum of approximately 10^6 CFU /ml- McFarland Trurbidity Range of tested drug resistant microorganisms was used for the study. 100µl of the bacterial suspension was uniformely spread on sterile Muller Hinton Agar plates. After solidification of the agar, wells were made with a 6 mm sterile cork borer (gel puncher). The extracts were made with 99% (v/v) DMSO (Dimethyl sulfoxide) and 100µl of the bryophyte extracts were pored into separate wells. The plates were incubated for 24 h at 37 ± 0.5^0C and antibacterial activity was observed by measuring the zone of inhibition as diameter in millimeters. Negative controls were made by DMSO alone and Positive controls were made by the antibiotic Streptomycin (25 µg /disc).

2.4.Determination of MIC and MBC

The broth dilution method was adopted [12] to determine the Minimal Inhibitory Concentration of the active extract revealed by previous well assay. The inoculum of each test bacterium was prepared by diluting the overnight culture of the most sensitive test pathogen in Muller Hinton Broth to a level of 1.5 x 10^4 cfu/ml. Two ml of the extract was diluted in two fold dilution with DMSO were added to a sterile glass tube containing 0.5 ml of Muller Hinton broth (the concentration of the bacteria was approximately 1.5x10^7 cfu/ml). The tubes were incubated at 37^0C for 18 to 24 hours. Because of the turbidity and dark colour of the extract, 0.1 ml of the mixture in the tubes were spread onto the surface of Muller Hinton agar. The Minimal Inhibitory Concentration (MIC) was defined as a lowest concentration of extract resulting in the bacterial density lower than 300 colonies /plate. The minimal bactericidal concentration (MBC) is the lowest concentration at which no bacterial growth occurs.

2.5.Preliminary phytochemical analysis

The extracts using different solvents were screened for the qualitative identity of different classes of natural compounds using the methodology of Sofowora [13]. The extract of bryophyte with maximum activity was subjected to GC-MS analysis.

[III] RESULTS

3.1. Identification of bryophyte

The byophytes were identified using character analysis as *Homaliodendron montagneanum, Papillaria fuscescens* and *Campylopus latinervis* and authenticated from the literature (Table-1).

Table 1. Character analysis of the Bryophytes

Characters	Specimen A	Specimen B	Specimen C
Habit	Branched	Branched	Unbranched
Costa	Present	Present	Present
Leaf margin	Serrate	Serrate	Serrate at the tip
Apical cell arrangement	Rhomboid	Rhomboid	Rectangular
Middle cell arrangement	Rhomboid	Rhomboid	Rectangular
Basal cell arrangement	Quadrate	Rectangular	Rectangular

Chloroplast	Parietal	Parietal	Parietal
Distribution	Nilgiri hills	Nilgiri hills	Nilgiri hills
Identification	***Campylopus latinervis***	***Homaliodendron montagneanum***	***Papillaria fuscenscens***

3.2. Antimicrobial activity

Table 2 shows the antibacterial activity of *Homaliodendron montagneanum* using organic solvents acetone, ethylacetate and distilled water. Among the various extracts, maximum activity was found with acetone extract against *Staphylococcus aureus* MTCC 3160 (26mm). *Klebsiella pneumoniae* MTCC 3040 (22mm) and *Xanthomonas campestris* MTCC 2286 (20mm) are moderately sensitive to the acetone extracts. Among the ethylacetate extract, *Mycobacterium smegmatis* MTCC 994 and *Xanthomonas campestris* MTCC 2286 (21mm) are highly sensitive to the bryophytes. *Staphylococcus aureus* MTCC 3160 and *Bacillus subtilis* MTCC 441 (18mm) are moderately sensitive (18mm) to the bryophyte extract. The aqueous extracts of *H. montagneanum* showed maximum antibacterial activity against *Escherichia coli* MTCC 585(19mm).

Table -2. Antibacterial activity of extracts of the bryophyte *Homaliodendron montagneanum*

Microorganisms	**Zone of inhibition (Dia. in mm)**				
	A	**EA**	**DW**	**S +ve control**	**DMSO (-ve control)**
Escherichia coli MTCC 585	15	17	19	27	-
Klebsiella pneumoniae MTCC 3040	22	17	12	22	-
Mycobacterium smegmatis MTCC 994	15	21	11	25	-
Pseudomonas aeruginosa MTCC 424	16	17	12	23	-
Shigella flexneri MTCC 1457	10	12	10	24	-
Xanthomonas campestris MTCC 2286	20	21	15	25	-
Bacillus subtilis MTCC 441	16	18	15	21	-
Staphylococcus aureus MTCC 3160	26	18	11	22	-

S=Streptomycin 25 µg /disc=+ve control; DMSO- Dimethyl Sulfoxide= -ve control, A=Acetone; EA= Ethyl Acetate; DW= Distilled Water; T=Trace (≤ 7mm); -=No activity

Table 3 shows the antibacterial activity of the bryophyte, *Papillaria fuscenscens* extract in various organic solvents like acetone, ethyl acetate and distilled water. Here, all the test pathogens were sensitive to acetone,ethyl acetate and distilled water extracts. The maximum antibacterial activity was found against *Klebsiella pneumoniae* MTCC 3040 (25mm) with acetone extract. Ethyl acetate extract of *P.fuscenscens* against *Pseudomonas aeruginosa* MTCC 424 and *Staphylococcus aureus* MTCC 3160 (25mm) showed equal sensitivity. *Klebsiella pneumoniae* MTCC 3040 and *Xanthomonas campestris* MTCC 2286 are moderately sensitive to ethyl acetate extracts of *P. fuscenscens* (20mm). Aqueous extract of *Papillaria fuscenscens* showed maximum antibacterial activity against *Mycobacterium smegmatis* MTCC 994 (22mm).

Table 3. Antibacterial activity of extract of the bryophye, *Papillaria fuscenscens*

Microorganisms	**Zone of inhibition (Dia. in mm)**				
	A	**EA**	**DW**	**S +ve control**	**DMSO (-ve control)**
Escherichia coli MTCC 585	11	10	10	27	-
Klebsiella pneumoniae MTCC 3040	25	20	15	22	-
Mycobacterium smegmatis MTCC 994	20	16	22	25	-
Pseudomonas aeruginosa MTCC 424	16	25	16	23	-
Shigella flexneri MTCC 1457	17	17	18	24	-
Xanthomonas campestris MTCC 2286	17	20	21	25	-
Bacillus subtilis MTCC 441	20	17	18	21	-
Staphylococcus aureus MTCC 3160	15	25	20	22	-

S=Streptomycin 25 µg /disc=+ve control; DMSO- Dimethyl Sulfoxide= -ve control, A=Acetone; EA= Ethyl Acetate; DW= Distilled Water; T=Trace (≤ 7mm); -=No activity

Table 4 shows the antibacterial activity of the bryophyte, *Campylopus latinervis* extract using various oraganic solvents such as acetone, ethyl acetate and distilled water. *Staphylococcus aureus* MTCC 3160 showed maximum sensitivity to acetone and ethyl acetate extracts (21mm and 22mm) respectevily. Ethyl acetate extracts of *Campylopus latinervis* showed moderate activity against *Mycobacterium smegmatis* MTCC 994 (18mm). *Escherichia coli* MTCC 585 is highly sensitive to the aqueous extract (20mm), whereas *Staphylococcus aureus* MTCC 3160 is moderately sensitive (19mm). In general, all the extracts of the bryophyte *C. latinervis* is active against *Staphylococcus aureus* MTCC 3160.

Table 4.Antibacterial activity of extract of the bryophyte *Campylopus latinervis*

Microorganisms	Zone of inhibition (Dia. in mm)				
	A	EA	DW	S +ve control	DMSO (-ve control)
Escherichia coli MTCC 585	11	12	20	27	-
Klebsiella pneumoniae MTCC 3040	15	14	10	22	-
Mycobacterium smegmatis MTCC 994	-	18	16	25	-
Pseudomonas aeruginosa MTCC 424	15	12	13	23	-
Shigella flexneri MTCC 1457	14	10	15	24	-
Xanthomonas campestris MTCC 2286	14	15	12	25	-
Bacillus subtilis MTCC 441	15	16	13	21	-
Staphylococcus aureus MTCC 3160	21	22	19	22	-

3.3. Deatermination of MIC and MBC

Table 5 shows the Minimal Inhibitory Concentration of *Homaliodendron montagneanum* against *Staphylococcus aureus* MTCC 3160. The Minimal Inhibitory Concentration (MIC) of acetone extract of *H. montagneanum* against *Staphylococcus aureus* MTCC 3160 is 20mg/ml and Minimum Bactericidal Concentration (MBC) is 40 mg/ml.

Table 5.MIC of acetone extract of *H. montagneanum* against *Staphylococcus aureus* MTCC 3160

Concentration of extracts (mg/ml)	Colony counts (CFU/ml)	MIC (mg/ml)	MBC (mg/ml)
10	392	20	40
20	268		
30	28		
40	0		
50	0		

MIC= Minimum concentration at which <300 colonies; MBC= Minimum concentration at which No bacterial growth occurs

3.4. Deatermination of phytochemical analysis

The results of phytochemical screening indicate the presence of some secondary metabolites thay may be responsible for the antibacterial activity of the bryophytes. It reaveals the presence of alkaloides, coumarins, steroides, tannins, saponins, resins, phenols and sugars Table 6.

Table 6. Phytochemical analysis of selected bryophytes

Phytochemical constituents	*H. montagneanum*			*P. fuscensens*			*C. latinervis*		
	A	EA	DW	A	EA	DW	A	EA	DW
Colour	Green	Green	Green	Green	Green	Green	Green	Green	Green
Alkaloids	-	+	-	+	-	-	+	-	-
Carboxylic acids	-	-	-	-	-	-	-	-	-
Coumarins	-	-	+	-	+	-	+	-	+
Flavonoids	-	-	+	+	-	-	-	-	+
Phenol	-	-	+	-	-	+	-	+	-

Proteins & amino acids	-	-	+	-	-	+	-	-	-
Quinones	-	-	-	-	-	-	-	-	-
Resins	+	+	+	+	+	-	-	-	-
Saponins	-	-	+	-	+	+	-	+	+
Steroids	+	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	-	+	+	-
Xanthoproteins	-	+	-	-	-		-	-	-
Sugars	+	+	+	+	+	+	+	+	+

Values are the results of experiments done in triplicates

[IV] DISCUSSION

The byophytes were identified as *Homaliodendron montagneanum, Papillaria fuscescens* and *Campylopus latinervis* and authenticated from the literature. The antibiogram studies revealed that all the tested microorganisms are resistant against Penicillin. The antibacterial activity of the bryophyte extracts are given in Tables 2-4. The acetone extract of the bryophyte *H. montagneanum* reported the maximum antibacterial activity (26 mm) against *Staphylococcus aureus* (MTCC 3160) followed by *Klebsiella pneumoniae* (MTCC 3040) (25 mm) and ethyl acetate extract of *P. fuscensens* (25 mm). The result of this research highlights the fact that the organic solvent extracts exhibited greater antimicrobial activity. The antimicrobial principles were either polar or non-polar and they were extracted only through the organic solvent medium[14] (Locher *et al*., 1995). So the present observation suggests that the organic solvent extraction was suitable to extract antimicrobial properties which are also supported by many other investigators[15,16].

The antibacterial activity of the bryophytes collected from the high altitude areas are remarkable and results are comaprable with standard commercial antibiotics. These may be be due to the stressful envaironment where the plants are inhabited. It is already reported that the Plants interact with stressful environments by physiological adaptation and altering the biochemical profile of plant tissues and producing a spectrum of secondary metabolites [9, 17]**[Fig. 1].**

Figure 2. shows the results of GCMS data of *H. montagneanum*. From the results, it is clear that about 10 compounds are present in the sample and the maximum peak area in the third compound with the peak area of 23.81% (RT 14.35; molecular formula C15H28O and a MW of 224) and the compound is (Z) 6, (Z) 9-Pentadecadien-1-ol.

The results of phytochemical screening indicate the presence of some secondary metabolites thay may be responsible for the antibacterial activity of the bryophytes. It reaveals the presence of alkaloides, coumarins, steroides, tannins, saponins, resins, phenols and sugars. Ahmed *et al*. [18] and Batish *et al.* [19] reported that the phytochemical compounds are responsible for the antimicrobial activity. Kumaraswamy and Sathish [20] reprted that the activity may be due to the presence of various secondary metabolites.

Fig: 1. Antimicrobial activity of bryophyte extracts against drug resistant pathogens

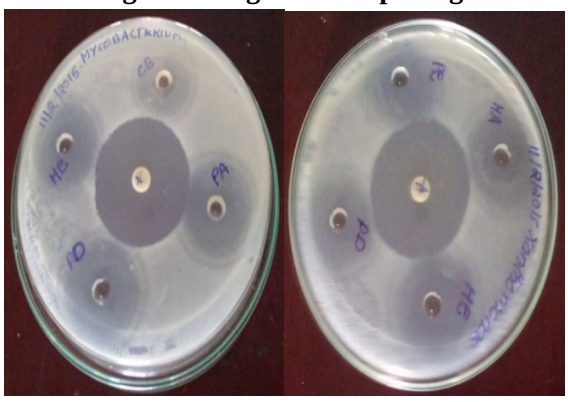
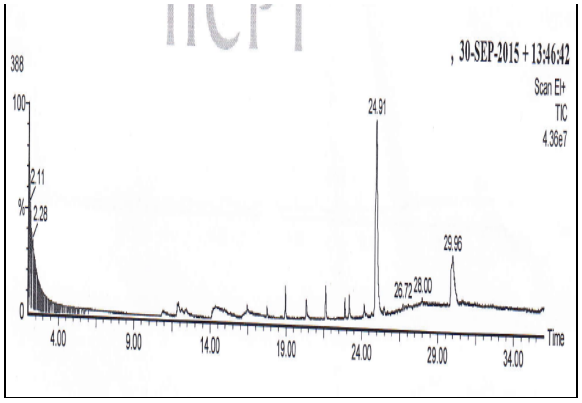


Fig: 2. Figure 2. shows rthe results of GCMS data of *H. montagneanum*



[V] CONCLUSION

The present study identified selected bryophytes from high altitude areas of Nilgiri hills. They are identified as *Homaliodendron montagneanum*, *Papillaria fuscenscens* and *Campylopus latinervis*. The exract were tested against various resistant bacterial pathogens strains using Agar Well Diffusion method. Most of the extract showed good inhibitory activity. The acetone, ethyl acetate and distilled water extracts of bryophyte was used for the study. The acetone extract of *H. montagneanum* showed maximum antibacterial activity against *Staphylococcus aureus* MTCC 3160. The preliminary phytochemical activity revealed the presence of resins, steroids, tannins and sugar in the acetone extracts. The Minimum Inhibitory Concentration of the extract is 20mg/ml and Miminum Bactericidal Concentration is 40mg/ml.

To conclude with, the study revealed that the antibiotic properties of the crude extract of the bryophytes, was found to be comparable with commercial antibiotics. From this study evident that the bryophytes having immense variety of phytochemicals and it is higly warranted to do further research to purity and screen the pharmacological profiles of this plants.

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