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RESEARCH ARTICLE

BACTERICIDAL POTENTIALITY OF SELECTED BRYOPHYTES PLAGIOCHILA BEDDOMEI,LEUCOBRYUM BOWRINGII AND OCTOBLEPHARUM ALBIDUM.

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Abstract

A general lack of commercial value, small size, and inconspicuous place in the ecosystem have made the bryophytes appear to be of no use to common man. It is common knowledge that bryophytes are not infected by pathogens, even though most live in close proximity with the forest floor or organic decomposing substrates. Bryophytes contain numerous potentially useful compounds, including sugars, sugar alcohols, amino acids, fatty acids, aliphatic compounds, phenylquinones, flavonoids and phenolic substances, but much work remains to link medical efficacy with specific bryophyte species or compounds. Relatively little attention has been paid to correlate secondary metabolites of bryophytes with therapeutic uses. Hence the present investigation on selected bryophytes has been an attempt to screen the secondary metabolites and their potential roles as antibacterial. Initially, a phytochemical screening was attempted revealed a pool of secondary metabolites. Phenols and flavonoids were present in significant levels. Subsequently, phenols were fractionated revealed the presence of phenolic acids such as gallic, vanillic, p-hydroxybenzoic, ferulic, chlorogenic, sinapic, para coumarate and cinnamic acids. As the last phase, the antibacterial potentiality was analyzed. Methanolic extracts of the bryophytes strongly inhibited pathogenic microbial strains, including Salmonella typhimurium, Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Bacillus cereus, Bacillus subtilis, Proteus vulgaris, Pseudomonas aeruginosa as revealed by ELISA technique and disc diffusion method. Further, the bacterial survival-time analysis substantiated the MIC and MBC data. Further studies are warranted to analyze the mechanism of microbicidal action of the bryophytes.

Introduction:

Bryophytes, an unexplored group of lower non vascular plants comprise about 45,000 species concentrated mostly in humid environment around the world. For the last two decades, the chemistry of bryophytes has been studied by various researchers. Their studies indicated that bryophyte species contain mainly phenols, flavonoids and their derivatives including biflavonoids and glycosides. Terpenoids are considered to be characteristic metabolites of liverworts which include bi benzyls and their bi and tri tetramers. Among the secondary metabolites isolated from the mosses, biflavonoids are the characteristic compounds. This lead scientist to believe that bryophytes are not primitive embryo plants, but are more related to higher plants.

The biological activity of bryophyte compounds has been documented in several species. Numerous bryophyte species, especially liverworts are now cultured under axenic conditions, and substantial number of compounds have

been synthesized (Martin, 1996). Inspite of accumulating information regarding the natural products from plant origin, there are not many reports of these from the bryophytes. So the main goal of the present study is to elucidate the major secondary metabolites and its therapeutic evaluation of antimicrobial potentiality. Plant materials selected for the present study are Plagiochila beddomei, Leucobryum bowringii and Octoblepharum albidum and evaluated their bactericidal potentialities.

Materials and methods:-

Fresh samples of Plagiochila beddomei, Leucobryum bowringii and Octoblepharum albidum are collected from Ponmudi during June to August. Soxhlet hot continuous extraction.100 g of fresh thallus of Plagiochila beddomei, Leucobryum bowringii and Octoblepharum albidum was chopped, air dried and successively extracted with 300 ml of hexane, chloroform, petroleum ether, acetone, methanol (Merck, India) and water for 6 h using soxhlet hot continuous extraction method. The supernatants were concentrated using rotavapour at 50°C. The yields of the extract were displayed in the table 1. The methanolic and water residues of P .beddomei, L. bowringii and O. albidum were further lyophilized and stored at -20°C.

Preliminary Phytochemical Screening:-

The various solvent extracts from soxhlet hot continuation method were screened for the presence of phytochemicals according to the method of Khandelwal (2007).

Quantification of total phenol and total flavonoids:-

Total phenol content in the samples was estimated by the method of Mayer et. al., (1995). Flavonoids react with vanillin (1 % in 70 % sulphuric acid) to produce a coloured product, which can be measured spectrophotometrically (Mervat, 2009).

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

Quantitative fractionation of various phenolic acids in the samples was studied by RP-HPLC analysis (Beta et. al., 1999).

Antimicrobial assay by MIC:-

Antimicrobial assay was performed in 96 well, sterile, flat bottom microtiter plates, based on broth microdilution assay, which is an automated colorimetric method, using the optical density of cultures in a microtiter plate (Santos et. al., 2009). After the experimental period, the plates were read at 620 nm using ELISA reader. Minimum inhibitory concentration (MIC) was determined as the lowest concentration of the extract inhibiting the growth of the organism, based on the readings.

To determine minimum killing concentration, 50 µl broths was taken from each well and inoculated in 200 µl nutrient broth, containing methanolic or aqueous extract of L.bowringii, P.beddomei, O.albidum separately for 24 h at 37°C for bacteria. The minimum killing concentration is defined as the lowest concentration of the extract at which inoculated microorganism was completely killed. Each test was performed in triplicate and repeated twice. Streptomycin was used as positive controls for bacteria and Fluconazole for fungi. As negative control, 200 µl of different concentrations of the extract in 50 µl nutrient broth was used.

Antimicrobial assay by disc diffusion method:-

Petriplates were prepared with 20 ml of sterile MHA (Hi-media). The test cultures were swabbed on the top of the solidified media and allowed to dry for 10 min. The tests were conducted at three different concentrations of the crude extract (2, 1 and 0.5 mg per disc) in triplicates. The loaded discs were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. Negative control was prepared using respective solvent. Streptomycin (10 µg/disc) was used as positive control. The plates were incubated for 24 h at 37°C. Zone of inhibition was recorded in millimeters and the experiment was repeated twice (Murray et. al., 1995).

Survival-time study:-

Mid-logarithmic phase cultures of all bacterial strains were transferred into 50 ml portions of pre-warmed MHA nutrient broth (Hi-Media), containing MIC, 2 x MIC, 4 x MIC concentrations of extract, to yield final concentrations of 10^5 cfu/ml. The broths were maintained, with agitation, on a water bath at 30°C. Aliquots (1 ml) were drawn, after 0, 6, 12, 24, 30 and 36 h and appropriately diluted in MHA broth to neutralize the effect of

the methanolic extracts of P.beddomei and O.albidum and viable counts determined by pour plate techniques. Colonies were counted and the number of survivors was calculated after incubation at 30°C for 48 h. Survivor-time curves were drawn for the test organisms, exposed to the various concentrations, as well as the control.

Results and discussion:-

Preliminary phytochemical analysis:-

The results of preliminary phytochemical study are tabulated in Tables 2a, b and c. The phytochemical study in Plagiochila beddomei, Leucobryum bowringii and Octoblepharum albidum revealed the presence of phenols, saponins, alkaloids, flavonoids, glycosides and tannins. Thus, the preliminary phytochemical tests are helpful in finding chemical constituents in the bryophytes that may lead to their quantitative estimation and also in locating the source of pharmacologically active chemical compounds.

Table 1:- The yield of the extracts (g/100g tissue) in different solvent system in P. beddomei, L. bowringiiand O. albidum

	Hexane	Chloroform	Petroleum ether	Acetone	Methanol	Aqueous
P. beddomei	0.55	0.24	0.40	0.3	9.6	6.8
L. bowringii	0.64	0.19	0.56	0.24	8.9	5.9
O. albidum	0.47	0.2	0.55	0.29	7.5	5.2

Table 2a:-Data of preliminary phytochemical analysisin Plagiochilabeddomei.

	Hex	Chloro	P. Ether	Acetone	Methanol	Aqueous
Phenolics						
$FeCl_3$ test	-	-	+	+	+++	+
Lead acetate test	+	-	-	-	++	+
Flavonoids						
NaOH test	-	+	-	-	++	+
Shinoda test	-	-	+	-	+	+
H_2SO_4 test	-	-	-	-	+	+
Saponins						
Foam test	-	-	+	+	-	-
Glycosides						
KellarKillani test	-	-	-	-	+	-
Alkaloids						
Mayer's test	-	-	+	-	+	-
Wagner's test	-	-	-	-	-	-
Dragendorff's test	+	+	+	-	+	-
Tannins						
Ferric chloride test	-	-	+	+	+	+
Gelatin test	+	-	-	-	-	+

Table 2:- b Data of preliminary phytochemical analysisin Leucobryumbowringii.

	Hex	Chloro	P. Ether	Acetone	Methanol	Aqueous
Phenolics						
$FeCl_3$ test	-	+	+	-	++	+
Lead acetate test	+	-	-	+	+	-
Flavonoids						
NaOH test	-	-	-	+	+	+
Shinoda test	-	-	+	-	+++	++
H_2SO_4 test	-	+	-	-	++	+
Saponins						
Foam test	-	+	+	-	-	+
Glycosides						
KellarKillani test	+	+	+	+	+	-
Alkaloids						
Mayer's test	-	-	-	-	+	-
Wagner's test	-	-	-	-	-	-
Dragendorff's test	-	-	-	-	+	-
Tannins						
Ferric chloride test	+	-	-	+	+	+
Gelatin test	-	-	+	-	-	-

Table 2:- c Data of preliminary phytochemical analysisin O.albidum.

	Hex	Chloro	P. Ether	Acetone	Methanol	Aqueous
Phenolics						
$FeCl_3$ test	-	-	+	+	++	+
Lead acetate test	-	-	-	-	-	-
Flavonoids						
NaOH test	-	-	-	-	++	+
Shinoda test	-	+	+	-	+++	+
H_2SO_4 test	-	+	-	+	++	+
Saponins						
Foam test	-	+	-	+	+	+
Glycosides						
KellarKillani test	+	-	+	+	++	+
Alkaloids						
Mayer's test	-	-	-	-	+	-
Wagner's test	-	-	-	-	-	-
Dragendorff's test	-	-	-	-	+	-
Tannins						
Ferric chloride test	-	++	+	+	-	+
Gelatin test	-	-	-	+	-	+

The results of quantitative estimation of proteins, phenols and flavonoids are tabulated in Table 3. It was evident that the protein content was more in the thallus of O. albidum followed by P. beddomei and the least in L. bowringii. The protein values in the present study are comparable with that of Racomitrium crispipilum (Baron et. al., 2009) and Leptodictiyum riparium (Barbara et. al., 2007). The maximum amount of phenols was detected in the thallus of P.beddomei followed by L. bowringii. HPLC-DAD of phenolics in Lunularia cruciata, Brachytheciastrum velutinum and Kindbergia praelaonga are comparatively lower than the phenolics of the present bryophytes analysed (Jockvoic et. al., 2008). The phenolic compounds play an important role in giving protection to the plants against deleterious effects of UV rays and also against phytopathogenic microbes. The flavonoids showed a high profile than others. Basile et al., (2011) estimated total flavonoids in Leptodictyum riparium and were at par with the present results. The quantitative estimation of proteins, phenols and flavonoids in P. beddomei, L. bowringii and O. albidum gives an insight into their chemical nature quantitatively, which can provide rich data in understanding certain basic pattern of growth and metabolism. At the same time, proteins, phenols and flavonoids

can be used as chemical markers in taxonomic studies (Agrawal et. al., 2012).

Table 3:- Data of quantitative estimation of proteins, phenols and flavonoids in P. beddomei, L. bowringiiand O. albidum

	P.beddomei	L.bowringii	O.albidum
Proteins (mg/g)	2.87 ± 0.98	1.96 ± 1.1	4.72 ± 0.39
Phenols (mg/g)	19.3 ± 0.36	13.52 ± 0.43	12.48 ± 0.57
Flavonoids (mg/g)	16.9 ± 0.13	24.7 ± 0.26	29.4 ± 0.11

Fractionation of total phenols:-

RP-HPLC fractionation of phenols in P. beddomei (Fig. 1) revealed the presence of phenolic acids such as gallate (199.4 µg/g), vanilate (75.3 µg/g), chlorogenate (200 µg/g), cinnamate (212.2 µg/g), protocatechol (161121.7 µg/g), coumarate (289.7 µg/g), ferulate (232.7 µg/g), sinapic (222 µg/g), caffeate (322.4 µg/g) and hydroxyl benzoate (1.5 µg/g). The retention time of standards and sample was listed in Table 4 a.

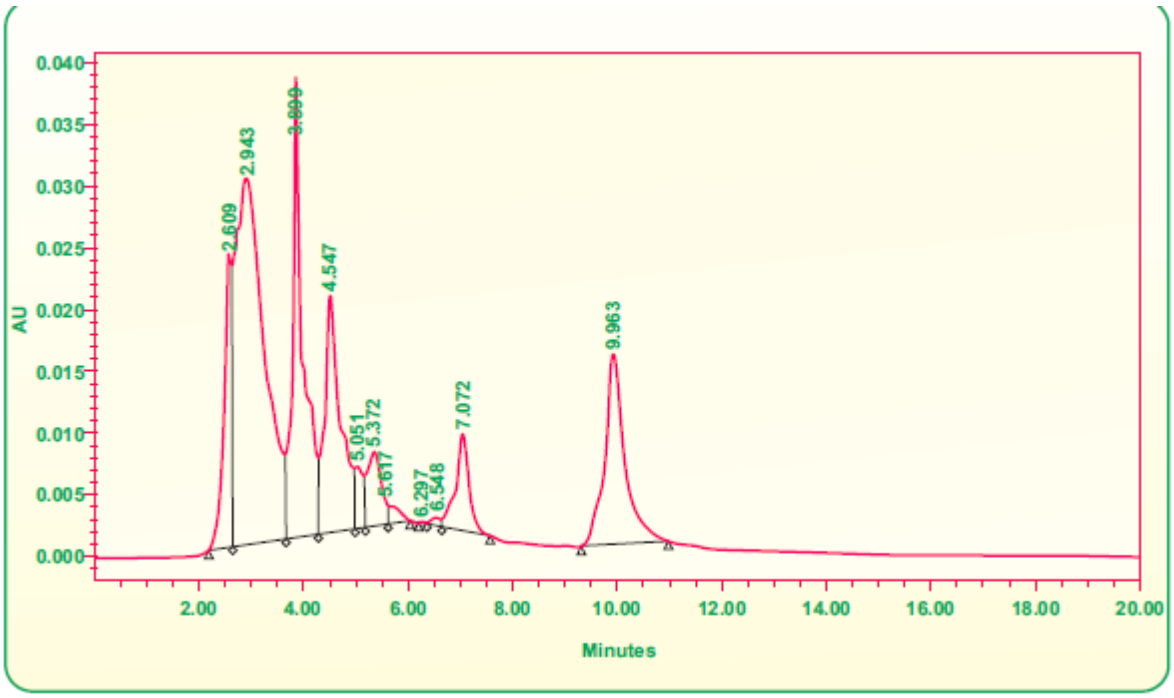


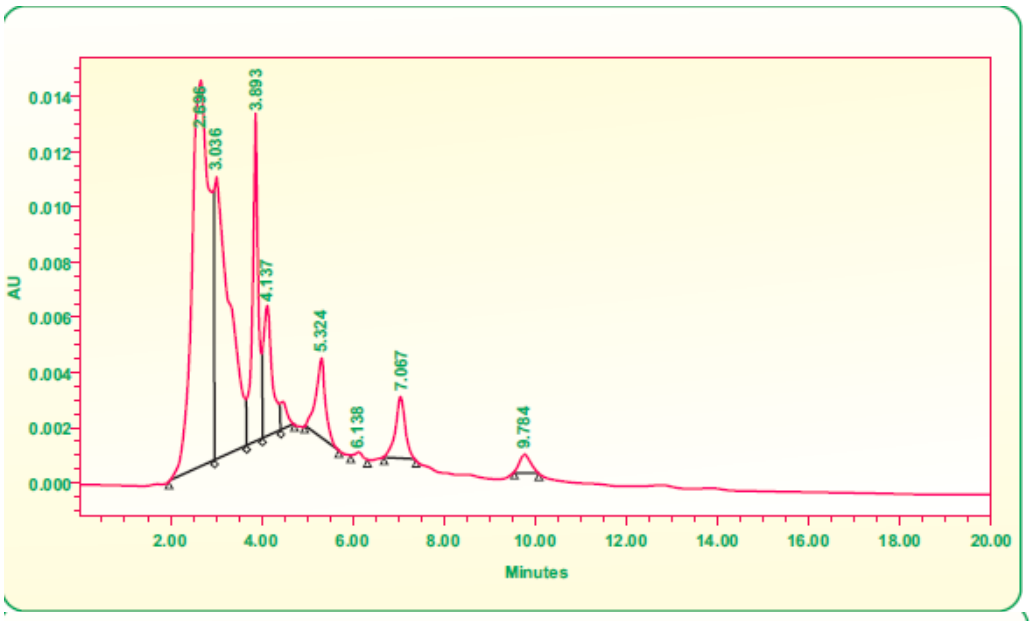
Table 4:-a.

Phenolic acids	Retention time (Rt) in min.	
	standard	sample
Gallic acid	2.6	2.609
Vanillic acid	4.5	4.547
Caffeic acid	3.8	3.899
p-HBA	6.4	6.548
Ferulic acid	5.7	5.617
Sinapic acid	6.2	6.297
ProtoCatechol	3.9	3.899
Coumeric acid	7	7.072
Cinnamic acid	9.89	9.963
Chlorogenic acid	2.9	2.943

Fractionation of phenols in L. bowringii (Fig. 2) revealed the presence of phenolic acids such as gallate (10.4 µg/g), vanilate (11.3 µg/g), chlorogenate (11.2 µg/g), cinnamate (11.1 µg/g), protocatechol (13.4 µg/g), coumarate (114 µg/g), ferulate (34.8 µg/g), sinapic (87.4 µg/g) and hydroxyl benzoate (0.65 µg/g). The retention time of standards

and sample was listed in Table 4b. Similarly, the total phenols in O.albidum (Fig. 3) revealed the presence of phenolic acids such as gallate (11.5 µg/g), vanilate (12.4 µg/g), chlorogenate (12.3 µg/g), caffeate (48.6 µg/g), protocatechol (14.7 µg/g), and ferulate (19.8 µg/g). The retention time of standards and sample was listed in Table 4c. The other phenolic acids such as cinnamate, hydroxy benzoate, sinapate and coumarate showed a low retention time compared to the standards. The values of retention time were in agreement with those published by Beta et. al. (1999). The chromatogram of phenolics studied by Jockvoic et. al. (2008) in selected bryophytes showed more or less similar trend. A positive correlation was observed between the phenolic acids and total phenols in the plants suggesting their role as precursor of many of the secondary metabolites. Cinnamate, coumarate, gallate, ferulate and hydroxy benzoate has proven antioxidant potentiality, which inturn supports the antioxidant significance of the plant. Chlorogenate can regenerate oxidized vitamin E via caffeate and it also acts as a pro oxidant in the propagation phase of LDL oxidation. Coumarate is a precursor of flavonoids and also binds with nitric acid and its derivatives before they combine with protein amines to form nitrosamine radical. Similarly, cinnamate has antibacterial, antifungal and antiparasitic properties (Boudet, 2007). Gallate and its derivatives also exhibit higher free radical scavenging properties.

Fig. 1 a, b and c RP-HPLC profile of phenolic acids of P. beddomei, L. bowringii and O. albidum



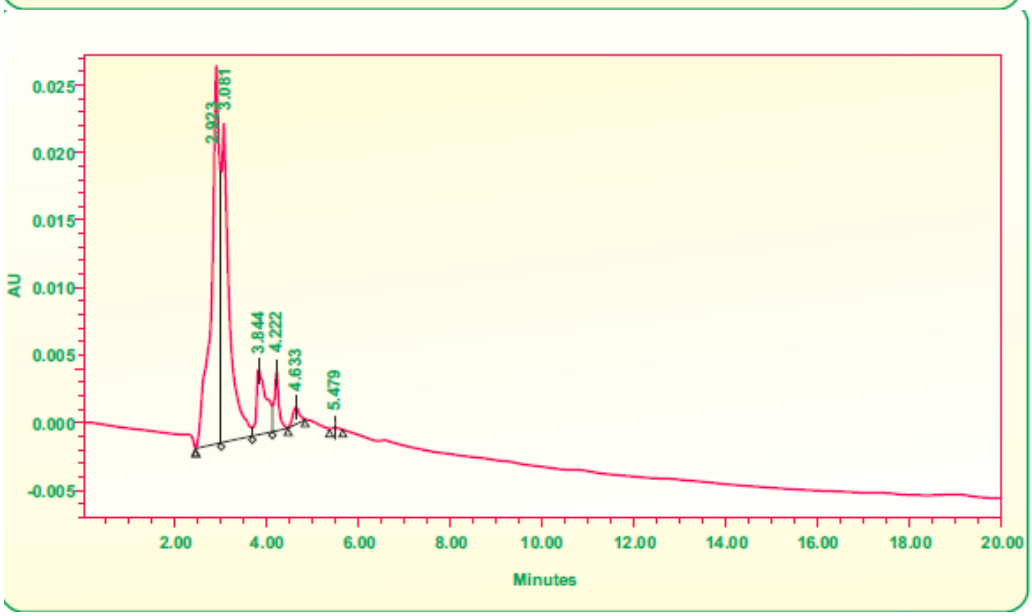


Table 4:-b

Table 4:-

c.

Phenolic acids	Retention time (Rt) in min.	
	standard	sample
Gallic acid	2.6	2.696
Vanillic acid	4.5	4.472
p-HBA	6.4	6.13
Ferulic acid	5.7	5.324
Sinapic acid	6.2	6.138
ProtoCatechol	3.9	3.893
Coumeric acid	7	7.607
Cinnamic acid	9.89	9.784
Chlorogenic acid	2.9	3.036
Phenolic acids	Retention time (Rt) in min.	
	standard	sample
Gallic acid	2.6	3.08
Vanillic acid	4.5	4.22
Caffeic acid	3.8	3.844
Ferulic acid	5.7	5.479
ProtoCatechol	3.9	3.8
Chlorogenic acid	2.9	2.923

Comparison of retention time of Phenolic acids of standards and methanolic extract of (a) P.beddomei, (b)L.bowringii and (c) O. albidum

A gradient consisting of solvent A (2.5:97.5 v/v methanol-double distilled water at pH 3 with acetic acid) and solvent B (50:50 v/v methanol-double distilled water at pH 3 with acetic acid) was applied at a flow rate of 1ml/min. Injection volume of both the standards and the samples was 20 µl.

Bryophytes, the oldest land plants possess medicinally important bioactive compounds and are traditionally used in Chinese, European, North American and Indian medicine, to treat illness of cardiovascular system, tonsillitis, bronchitis, tympanitis, skin diseases and burns (Khanam et. al., 2011). Compounds 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 (Bodade et. al., 2008; Asakawa, 2011). Plagiochila fascicula shows inhibitory effect on P388 cells (Leukemia), Herpes, Simplex type1, Polio type1, Bacillus subtilis, Escherichia coli, Candida albicans, Trichophyton mentagrophytes and Cladosporium resinae (Lorimeres and Perry, 1994). The antifungal activity of Herberta aduncus against Botrytis cinerea, Rhizoctonia solani, Pythium debaryanum is well illustrated (Asakawa, 2011). Species of Fissidens and Polytrichum were used as diuretic and hair stimulating drugs. Generally, bryophytes are not damaged by insects, snails, slugs, and other small animals. The biological activities of bryophytes are due to these lipophilic mono, sesqui, and diterpenoids, aromatic compounds (bibenzyls, bis-bibenzyls, benzoates, cinnamates, long-chain alkyl phenols, naphthalenes, phthalides, isocoumarins, and acetogenins), which constitute the oil bodies (Bodade et. al., 2008). In recent years, many possible sources of natural antibiotics have been in use for several infectious diseases, mostly bacterial, fungal and viral. In this respect, the most investigated taxa are from angiosperms, whereas little data is currently available about other groups of plants, especially bryophytes (Subhisha and Subramoniam, 2005). The purpose of this part of the study was to evaluate the in vitro antibacterial activities of methanolic and aqueous extracts of P.beddomei, O. albidum and L. bowringii.

P. beddomei, O. albidum and L. bowringii extracts exhibited different degree of growth inhibition against tested bacterial species such as Salmonella typhimurium, Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Bacillus cereus, Bacillus subtilis, Proteus vulgaris, Pseudomonas aeruginosa (Tables 5, 6 and 7). The values of MIC ranged between 0.0625 to 3.00 mg/ ml and that of MBC were 0.25 to 3.00 mg/ml for P. beddomei, O. albidum and L. bowringii extracts. Streptomycin was used as positive controls for bacteria. Range of MICs for streptomycin was from 0.5 to 1.0 mg/ml while MBC was from 1 to 2 mg/ml. Although all the extracts showed varying levels of activity against all the tested bacteria, the methanolic extract was found to be more significant than aqueous extracts. This may be due to the variations in chemical composition of particular species of

bryophytes, which can also vary according to the geographical origin. The antibacterial activity revealed by the extracts might be due to presence of flavonoids, terpenoids and other polyphenolic compounds. The microbicidal effects of the extracts were further visualized as inhibition zone by treating the pathogens with extracts and then spreading the cells on agar plates. Among the pathogens tested against P. beddomei methanolic extract K. pneumoniae was the most sensitive and Pseudomonas aeruginosa, Bacillus cereus and S. typhimurium are the resistant species. In the case of O. albidum methanolic extract, the pathogens such as Pseudomonas aeruginosa and Bacillus cereus were the sensitive and K. pneumoniae, the resistant species. Meanwhile, L. bowringii methanolic extract displayed a moderate antimicrobial activity against almost all the tested pathogens. The present results of the protective effect of the bryophyte extracts against microbial pathogens were comparable to that of antibacterial activity of black pepper (Karsha and Lakshmi, 2010). Similarly, the antimicrobial potentiality of P. beddomei, O. albidum and L. bowringii extracts are superior to that of dimethyl sulfoxide extract from Rhodobryum ontariense (Pejin et. al., 2011). According to Argaez et. al. (2007) MIC value of 100 to 200 µg/ml is a fair one for plant extract in the search of new anti-infectious agents. Most research studies of Indian bryophytes revealed no antifungal activity, while the results from other countries provides a broad spectrum of antifungal activity related to bryophytes like Porella, Makinoa , Lunularin cruciate, Septoria nodorum, Dumortiera hirsute, Sphagnum portoricense, Orthotrichum rupestre and Uromyces fabae (Bodade et. al., 2008). The reasons for varied activities between gram negative and Gram positive bacteria could be due to their morphological difference. Gram negative bacteria have an outer phospholipidic membrane carrying the structural lipopolysaccharide components. This makes the cell wall more permeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic Gram positive bacteria should be more susceptible, since they have only an outer peptidoglycan layer, which is not an effective permeability barrier as reported by Gradisar et. al. (2007). But in this study, contradicting results are observed. Some extracts are active on both Gram positive and Gram negative, hence bryophytes can be considered as having broad spectrum of activity and present a potential agent for control of drug resistant strains.

Table 5:- a.Antimicrobial activity in terms of MIC and MBC of P. beddomei methanolic extract* against tested bacteria

Pathogens	MIC (mg/ml)	MBC (mg/ml)	Streptomycin	
			MIC (mg/ml)	MBC (mg/ml)
Staphylococcus aureus (+)	0.5	1	0.5	1
Bacillus subtilis(+)	0.75	1	1	2
Klebsiellapneumoniae(-)	0.125	0.5	1	2
Escherichia coli (-)	0.5	1	0.5	1
Proteus vulgaris (-)	0.5	1	1	2
Salmonella typhimurium (-)	1	2	1	2
Bacillus cereus (+)	1	2	1	2
Pseudomonas aeruginosa(-)	1	2	1	2
Cryptococcus neoformans	0.5	0.75	-	-

*Gram-positive (+) and Gram-negative (-) bacteria. MIC, minimal inhibitory concentration which corresponds to the minimum P. beddomeimethanolicextract concentration capable to inhibit the visible growth of the micro-organism. MBC, minimal bactericide concentration which corresponds to the minimum concentration of the extract capable to reduce the number of CFU for 0.1% of the initial inoculums.

Table 5:- b. Antimicrobial activity, MIC and MBC of P. beddomei aqueous extract* against tested bacteria.

Pathogens	MIC (mg/ml)	MBC (mg/ml)	Streptomycin	
			MIC (mg/ml)	MBC (mg/ml)
Staphylococcus aureus (+)	1.0	1.5	0.5	1
Bacillus subtilis(+)	1.5	2	1	2
Klebsiellapneumoniae(-)	0.5	1.0	1	2
Escherichia coli (-)	1.0	1.75	0.5	1
Proteus vulgaris (-)	1.0	1.5	1	2
Salmonella typhimurium (-)	1.75	2.5	1	2
Bacillus cereus (+)	2.0	2.75	1	2
Pseudomonas aeruginosa(-)	1.75	2.5	1	2
Cryptococcus neoformans	1.0	1.5	-	-

*Gram-positive (+) and Gram-negative (-) bacteria. MIC, minimal inhibitory concentration which corresponds to the minimum P. beddomeiaqueous extract concentration capable to inhibit the visible growth of the micro-organism. MBC, minimal bactericide concentration which corresponds to the minimum concentration of the extract capable to reduce the number of CFU for 0.1% of the initial inoculums.

Table 6:- a. Antimicrobial activity in terms of MIC and MBC of O.albidum methanolic extract* against tested bacteria

Pathogens	MIC (mg/ml)	MBC (mg/ml)	Streptomycin	
			MIC (mg/ml)	MBC (mg/ml)
Staphylococcus aureus (+)	0.75	1.25	0.5	1
Bacillus subtilis(+)	1.0	1.25	1	2
Klebsiellapneumoniae(-)	2.0	2.5	1	2
Escherichia coli (-)	0.75	2.0	0.5	1
Proteus vulgaris (-)	0.75	1.5	1	2
Salmonella typhimurium (-)	2.0	3.0	1	2
Bacillus cereus (+)	0.0625	0.25	1	2
Pseudomonas aeruginosa(-)	0.0625	0.25	1	2
Cryptococcus neoformans	1.5	1.75	-	-

*Gram-positive (+) and Gram-negative (-) bacteria. MIC, minimal inhibitory concentration which corresponds to the minimum O.albidummethanolic extract concentration capable to inhibit the visible growth of the micro-organism. MBC, minimal bactericide concentration which corresponds to the minimum concentration of the extract capable to reduce the number of CFU for 0.1% of the initial inoculums.

Table 6:-b. Antimicrobial activity in terms of MIC and MBC of O.albidumaqueous extract* against tested bacteria

Pathogens	MIC (mg/ml)	MBC (mg/ml)	Streptomycin	
			MIC (mg/ml)	MBC (mg/ml)
Staphylococcus aureus (+)	1.75	2.5	0.5	1
Bacillus subtilis(+)	1.75	2.5	1	2
Klebsiellapneumoniae(-)	2.5	3.0	1	2
Escherichia coli (-)	1.75	2.5	0.5	1
Proteus vulgaris (-)	1.5	2.5	1	2
Salmonella typhimurium (-)	2.5	3.0	1	2
Bacillus cereus (+)	0. 25	0.5	1	2
Pseudomonas aeruginosa(-)	0. 25	0.5	1	2
Cryptococcus neoformans	2.5	2.75	-	-

*Gram-positive (+) and Gram-negative (-) bacteria. MIC, minimal inhibitory concentration which corresponds to the minimum O.albidumaqueous extract concentration capable to inhibit the visible growth of the micro-organism.

MBC, minimal bactericide concentration which corresponds to the minimum concentration of the extract capable to reduce the number of CFU for 0.1% of the initial inoculums

Table 7:- a. Antimicrobial activity in terms of MIC and MBCof L.bowringiimethanolic extract* against tested bacteria

Pathogens	MIC (mg/ml)	MBC (mg/ml)	Streptomycin	
			MIC (mg/ml)	MBC (mg/ml)
Staphylococcus aureus (+)	1.75	2.5	0.5	1
Bacillus subtilis(+)	1.75	2.5	1	2
Klebsiellapneumoniae(-)	2.5	3.0	1	2
Escherichia coli (-)	1.75	2.5	0.5	1
Proteus vulgaris (-)	1.5	2.5	1	2
Salmonella typhimurium (-)	2.5	3.0	1	2
Bacillus cereus (+)	0. 25	0.5	1	2
Pseudomonas aeruginosa(-)	0. 25	0.5	1	2
Cryptococcus neoformans	2.5	2.75	-	-

*Gram-positive (+) and Gram-negative (-) bacteria. MIC, minimal inhibitory concentration which corresponds to the minimum L.bowringii methanolic extract concentration capable to inhibit the visible growth of the micro-organism. MBC, minimal bactericide concentration which corresponds to the minimum concentration of the extract capable to reduce the number of CFU for 0.1% of the initial inoculums.

Table 7:- b. Antimicrobial activity in terms of MIC and MBC of L.bowringii aqueous extract* against tested bacteria

Pathogens	MIC (mg/ml)	MBC (mg/ml)	Streptomycin	
			MIC (mg/ml)	MBC (mg/ml)
Staphylococcus aureus (+)	2.0	2.5	0.5	1
Bacillus subtilis(+)	3.0	3.0	1	2
Klebsiellapneumoniae(-)	3.0	3.0	1	2
Escherichia coli (-)	2.5	3.0	0.5	1
Proteus vulgaris (-)	2.75	3.0	1	2
Salmonella typhimurium (-)	3.0	3.0	1	2
Bacillus cereus (+)	1.75	2.25	1	2
Pseudomonas aeruginosa(-)	1.75	2.25	1	2
Cryptococcus neoformans	3.0	3.0	-	-

*Gram-positive (+) and Gram-negative (-) bacteria. MIC, minimal inhibitory concentration which corresponds to the minimum L.bowringii aqueous extract concentration capable to inhibit the visible growth of the micro-organism. MBC, minimal bactericide concentration which corresponds to the minimum concentration of the extract capable to reduce the number of CFU for 0.1% of the initial inoculums.

The mechanism of antibacterial activity of phenolic compounds is likely exerted primarily by its ability to act as a 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 microbicidal and antiviral properties (Boudet, 2007). Korir et. al, (2012) proved that the polyphenols inhibit bacterial DNA gyrase by binding to the ATP binding site of the gyrase B subunit. Specific binding to the N-terminal fragment of gyrase B was determined by them using fluorescence spectroscopy and confirmed using heteronuclear two-dimensional NMR spectroscopy of the EGCG–^{15}N-labelled gyrase B fragment complex. 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 (Akinpelu et. al., 2008). Plants that have tannins are astringent in nature and are used for the treatment of intestinal disorders, such as diarrhoea and dysentery and further support the use of Garcinia kola among the medicinal plants for the treatment of microbial infections (Dharmananda, 2003).

Similarly, tannins have been observed to have remarkable activity in cancer prevention (Lee and Xiao, 2005). 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 (Akinpelu et. al., 2008).

Survival-time studies:

The bacteria which are more sensitive to methanolic extracts of P.beddomei and O.albidum were further evaluated for bacterial survival-time studies. The means of survivors obtained at each concentration plated for each organism followed more or less a similar pattern. All the tested bacteria exhibited a decline in survivors in concentration dependent manner, whilst the control showed a steady rise in population within the same periods. (Tables 8a, b, c). This depicts the active phase of the bioactive constituents of the extract with time in the nutrient medium.

Table 8:- a. Survivorship of K. pneumoniae cells against methanolic extract of P.beddomei.

Time (h)	0	0.5 mg/ml	1 mg/ml	1.5 mg/ml	2 mg/ml
0	4.0	3.6	3.4	3.1	3.1
6	5.6	3.2	3.1	2.9	2.8
12	7.8	3	2.8	2.5	2.4
18	8.1	2.6	2.2	2.0	1.8
24	8.4	1.8	1.7	1.5	1.4
30	9.0	1.3	1.2	1.0	1.0
36	10	0.24	0.17	0.11	0.1

Table 8:- b. Survivorship of P.aeruginosa cells against methanolic extract of O.albidum.

Time (h)	0	0.5 mg/ml	1 mg/ml	1.5 mg/ml	2 mg/ml
0	4.4	4.1	4	3.7	3.5
6	5.9	3.6	3.2	3.2	3.0
12	8.1	2.9	1.67	1.55	1.43
18	8.5	2.1	1.3	1.3	1.2
24	9	1.45	0.9	0.88	0.9
30	9.5	1.2	0.8	0.6	0.6
36	10.4	0.19	0.14	0.1	0.09

Table 8:- c. Survivorship of B.cereus cells against methanolic extract of O.albidum.

Time (h)	0	0.5 mg/ml	1 mg/ml	1.5 mg/ml	2 mg/ml
0	4.7	4.5	4.2	3.8	3.1
6	5.8	4.0	3.9	3.0	2.0
12	6.6	3.47	3.2	2.7	1.5
18	7.9	2.6	2.5	2.0	1.2
24	8.2	2.2	2.0	1.4	1.0
30	9.4	1.6	1.0	1.0	0.9
36	10.3	0.2	0.15	0.1	0.07

Most significantly, the number of Klebsiella pneumoniae cells reduced in the presence of the P. beddomei methanolic extract. After 12 h incubation, there was a decrease of over 16.6 fold in the numbers of cells. During 24 h incubation, the number of cells fell further by about 50 % compared with the controls. At 36 h, the cells declined to 70 %. In the case of O.albidum methanolic extract, the number of Pseudomonas aeruginosa cells reduced sharply at 24 h (66.6 %) and 36 h (83.3 %) incubation. Similarly, the Bacillus cereus cells also showed significant levels of reduction in the number of survivors. The present study confirmed that bryophyte methanolic extracts maintained their inhibitory activity towards selected pathogens over a period of time. It is recognized that microbial community is influenced by a range of plant secondary compounds, including tannins, saponins (Wallace et. al., 1994) and phenolic acids (Chesson et. al., 1982). The screening of plants or their compounds for antibacterial effects on pathogens has been performed mainly using higher plants or their constituents such as tea catechins (Toda et. al., 1989) and essential oils (Hammer et. al., 1999). However, little attention has been paid related to the bactericidal properties in lower plants. The results of the time kill assay showed that 1-2 mg/ ml of bryophyte

extract killed the bacteria, confirming the potent antibacterial activity of the bryophyte extracts. Similarly, the present results are comparable with the work of Jnior et. al. (2011) and Kiruthika et. al. (2011).

Conclusion:-

Thus, the present results suggest the antibacterial impact of the bryophytes with varied levels. Future studies are warranted to purify the lead molecule and analyze the mode of action of the phytochemical as bactericidal.

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