Plant Archives Volume 20 No. 2, 2020 pp. 6959-6965 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

A COMPARATIVE PHYTOCHEMICAL AND ANTIBACTERIAL ANALYSIS OF TWO SELECTED LIVERWORTS (*LUNULARIA CRUCIATA (L.) DUM EX.LINDB* AND *MARCHANTIA EMARGINATA*) OF KERALA

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Abstract

Bryophytes are a miniature group of plants preferred to grow in wet and damp places. Many studies brings to light the presence of a large array of biologically active compounds present in them and these active compounds are responsible for their antibacterial, antifungal, cytotoxic, antitumor and insecticidal properties. In the present study, we have selected two liverworts, *Marchantia emarginata* and *Lunularia cruciata* L. Dum ex.Lindb and a comparative assay of their phytochemical and antibacterial properties were worked out. Alcoholic and acetonic extracts of both *Marchantia emarginata* and *Lunularia cruciata* revealed the presence of carbohydrates, proteins, diterpenes, phytosterols and anthocyanin while flavonoids were present in the alcoholic extract and phenol in the acetonic extract of *Marchantia emarginata* only. Against *E coli* bacteria, acetonic and alcoholic extract of *Lunularia cruciata* exhibited highest inhibition (18±0mm and 14±0mm) followed by alcoholic extract of *Marchantia emarginata* (8±0mm). Against *Klebsiella pneumoniae*, both acetonic and alcoholic extracts of *Lunularia cruciata* showed high inhibition (12.33±0.57mm and 11±0mm) followed by acetonic and alcoholic extracts of *Marchantia emarginata* (9±0mm and 10±0mm). Against *Staphylococcus aureus* bacteria, acetonic extract of *Lunularia cruciata* was the only one which showed inhibition and the zone of inhibition obtained was 10±0 mm while acetonic extract of *Marchantia emarginata* and alcoholic extracts of both *Lunularia cruciata* and *Marchantia emarginata* was not exhibiting inhibition at all. Lunularic acid, a compound very commonly seen in several species of *Lunularia* may be responsible for the greater antimicrobial efficiency *Lunularia cruciata* compared to that of *Marchantia emarginata.*

Key Words: Liverworts; Phytochemicals; Secondary Metabolites; Antibacterial; Inhibition zone.

Introduction

Bryophytes are a distinct group of plants that prefer to grow in wet and damp places. Since they require water for their fertilization, they are often referred to as the 'amphibians of the plant kingdom'. Bryophytes are differentiated into Liverworts, Hornworts and Mosses based on their characteristic thallus structure and represented by around 15,000 species around the world. In India, bryophytes represented by about 17.27% of the total bryophytes recorded in the world with about 25.64% of endemism (Chandra *et al*., 2017).

Even though these bryophytes are very simple morphological and anatomical structures and are found everywhere in the world from desert to ice cold polar regions except seas, they are less known to most people due to their small size and unawareness regarding their potentialities. Some bryophytes are ecological indicators of pollution and other heavy metals, all of them has the ability to control soil erosion as they often form a mat over the soil surface and bind soil with their rhizoids, some are used as fuel (Peat). Many bryophytes like sphagnum yield sphagnol which is used in medicine and some are used as food and fodder sources. Peat moss obtained from Sphagnum is used for horticultural purposes like packaging seedlings, bulbs etc during shipment due to its ability to store water several times of its body weight.

Many studies brings out the presence of secondary metabolites in them and these active compounds of bryophytes are widely considered as having antibacterial, antifungal, cytotoxic, antitumor and insecticidal properties (Pant and Tewari, 1990; Asakawa, 2007; Ulka and Karadge, 2010; Nikolajeva *et al*., 2012).

Bryophytes are the second largest group of plants but there is only very little knowledge available about

medicinal properties of these plants. Several bryophytes are considered to be ethnobotanically significant. Bryophytes are a popular remedy among the tribal people of different parts of the world. Liverworts like *Marchantia polymorpha* has long been used in hepatic disorders (Miller and Miller, 1979). Similarly oil expressed from *Polytrichum commune* was used by woman of ancient times on their hair (Glime, 2007). People of Gaddi tribes of Himachal Pradesh, India used *Plagiochasma appendiculatum* for treating skin diseases (Kumar *et al*., 2000). Due to long stemmed and hair like thallus of *Frullania ericoides* this liverwort is applied for hair related afflictions by tribal people of South India (Remesh and Manju, 2009). In the present study, we have selected two liverworts *Marchantia emarginata* and *Lunularia cruciata* and an enquiry into the phytochemical analysis and antimicrobial properties of them were carried out to have an understanding about their potentials against dreadful human pathogens.

Materials and Methods

Phytochemical Screening

(a) Collection of Plant specimens.

Fresh plant specimens of *Marchantia emarginata* and *Lunularia cruciata* were collected from Wayanad and Munnar areas of Kerala. *Marchantia emarginata* were plentifully found in the road sides while *Lunularia cruciata* were comparatively scarce and collected from a Botanical garden as a weed inside the flower pots. Specimens were identified and authenticated by Dr Manju. C. Nair. The green thalloid portions of the bryophytes were carefully lifted and washed many a times to remove mud and after thorough washing, the specimens were spread in a clean sheet of paper and kept in shade for drying. Dried specimens were subjected to grinding in a mortar and pestle. Then the dried powder is stored in air tight containers for future studies.

b) Preparation of Extract

Five grams of powdered *M. emarginata* and *L.cruciata* were weighed accurately and separately dissolved in 50 ml ethyl alcohol and acetone for 48-72 hours and it is centrifuged. The supernatant of each is collected, labelled and kept in the refrigerator and used for further analysis. The extract of selected bryophytes were of 10% concentration and it is used for both phytochemical analysis and antibacterial studies.

c) Phytochemical Analysis

Alcohol and acetone extracts of *Marchantia emarginata* and *Lunularia cruciata* were used for phytochemical studies as per standard procedures (Harborne, 1978).

Antimicrobial Assay

Methodology

a) Preparation of culture medium:

28 g of Nutrient agar was weighed and transferred into beaker containing 1 litre distilled water. Gently heated the contents to dissolve the medium and covered the mouth of the beaker with aluminium foil. Petriplates and nutrient agar containing beaker were placed in autoclave and sterilized. Further operations were done in laminar air flow chamber. The sterilized agar medium was poured into petridishes and allowed to solidify at room temperature and kept in an incubator in inverted position for 24 hours.

b) Selection of Bacterial strains

Three strains of bacteria were used for the study. The bacterial strains selected for study were *E.coli, Klebsiella pneumoniae* and *Staphylococcus aureus*. *S. aureus* is gram positive and the rest were gram negative. The bacterial inoculums available at Maharaja's College Botany laboratory were selected for study and they were originally collected from the Cochin University of Science and Technology, Kalamassery, Ernakulam.

c) Inoculation and Incubation of Microbes selected for study

The experiment was done in a laminar air flow chamber. The bacterial culture in nutrient broth was swabbed using buds over the solidified agar medium. A cork borer was used to prepare well in the medium. The medium was kept in the incubator for 2 to 3 hours. Extracts of the bryophytes (5g in 50ml acetone and alcohol) of 10% concentration were filled in the wells prepared. Ampicillin antibiotic solution was kept as the positive control and the solvent in which the plant extracts were prepared (alcohol, acetone and chloroform) were taken as the negative control respectively. 1.005 g powder of ampicillin dissolved in 100 ml of distilled water(1% antibiotic concentration) was kept as positive control and the solvent in which the liverwort extracts were prepared was taken as negative control ie acetone and alcohol. Extracts of liverworts, Ampicillin (antibiotic) solution and negative control (the solvent in which the extract is prepared –acetone and alcohol respectively) were filled in the corresponding wells prepared and kept in the incubator for 24 hours The petriplates were then inverted and kept in the incubator at 37^0C for 24 hours for the optimum growth of microorganisms. After the stipulated period of time, petriplates were taken out, zone of inhibition was recorded using scale.

Calculation of Inhibition Zone

Well method was used to investigate the antibacterial activity of plant extracts. A scale was used to measure the diameter of the zone of inhibition of the experimental specimens as well as the positive (Ampicillin Solution) and negative control (acetone/alcohol in which the extract was prepared) in millimetres including the size of the well. Each extract was tested thrice and the mean value with standard deviation was calculated.

Results and Discussion

Phytochemical Analysis

The phytochemical analysis of extracts of two liverworts studied showed almost similar results. The alcoholic and acetonic extract of both *Marchantia emarginata* and *Lunularia cruciata* showed the presence of carbohydrates, proteins, diterpenes, phytosterols and anthocyanin in common while alcoholic extract of *Marchantia emarginata* obtained flavonoids and acetonic extract of the same yielded phenol in addition to the above mentioned ones. Qualitative tests and TLC analysis of *Marchantia emarginata* has shown the presence of phytochemicals like steroids, tannins, triterpenoids, cardiac glycosides, flavonoids, resin , reducing sugar, aminoacid glycoside, phenol and coumarin (Mukhia *et al*., 2014). Our study is in confirmation with the presence of steroids, diterpenes, flavonoids, phenols and proteins with the above study mentioned.

On the other hand, *Lunularia cruciata* extracts did not yield flavonoid or phenol in any of its extracts. Other secondary metabolites like alkaloids, saponins, tannins were altogether absent in both alcoholic and acetonic extracts of both *Marchantia emarginata* and *Lunularia cruciata*. The phytochemical analysis of *Marchantia emarginata* and *Lunularia cruciata* were presented in table 1 and table 2.

Antibacterial Studies

Antibacterial studies of plant extracts were done. Against *Escherichia coli* bacteria, acetonic extract of *Lunularia cruciata* obtained higher inhibition of 18±0mm while *Marchantia emarginata* acetonic extract was not able to produce inhibition. Here, the antibiotic solution produced 13mm inhibition zone which in fact is lower than that of the effect produced by the acetonic extract of *Lunularia cruciata*. The alcoholic extract of *Lunularia cruciata* was also capable of inhibiting *E.coli* (14±0 mm) while the alcoholic extract of *M.emarginata* produced an inhibition zone of 8±0 mm. Here the positive control obtained 12 mm inhibition zone. In short, against *E coli* bacteria, acetonic and alcoholic extract of *Lunularia cruciata* exhibited highest inhibition (18±0mm and 14±0mm) followed by alcoholic extract of *Marchantia emarginata*. (8±0mm) (Plate 1, 2 and table 3).

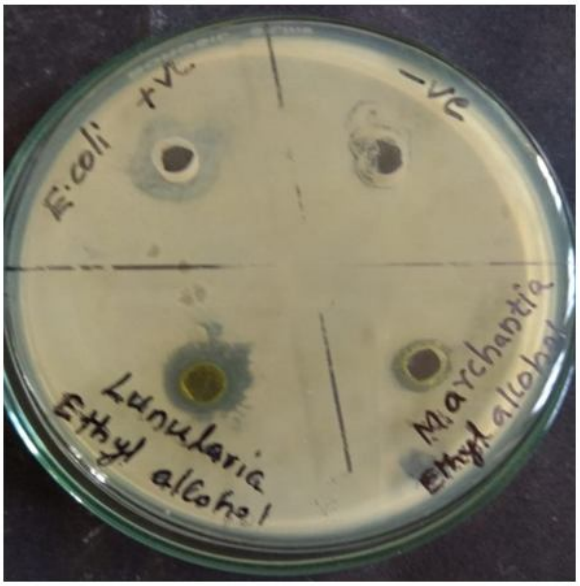
Table 1: Phytochemical analysis of alcoholic and acetonic extracts of *Marchantia emarginata.*

Phytocons-tituents	Test/ Reagent	In Alcohol	In Acetone
Alkaloids	Mayer's test	-	-
	Hager's test	-	-
	Wagner's test	-	-
Terpenoids	Copper acetate test	+	+
Proteins	Millon's test	+	+
	Xanthoprotein test	+	+
Phytosterols	Salkowski's test	+	+
	Libermann Burchard test	-	-
Saponins	Froth test	-	-
Flavonoids	Ferric chloride test	+	-
	Lead acetate test	-	-
Phenol	Lead acetate test	-	+
Carbohydrate	Benedict's test	-	-
	Molisch's test	+	+
Tannins	Ferric chloride test	-	-
	Lead acetate test	-	-
Anthocyanins	Con.H_2SO_4 test	+	+

Table 2: Phytochemical analysis of alcoholic and acetonic extracts of *Lunularia cruciata.*

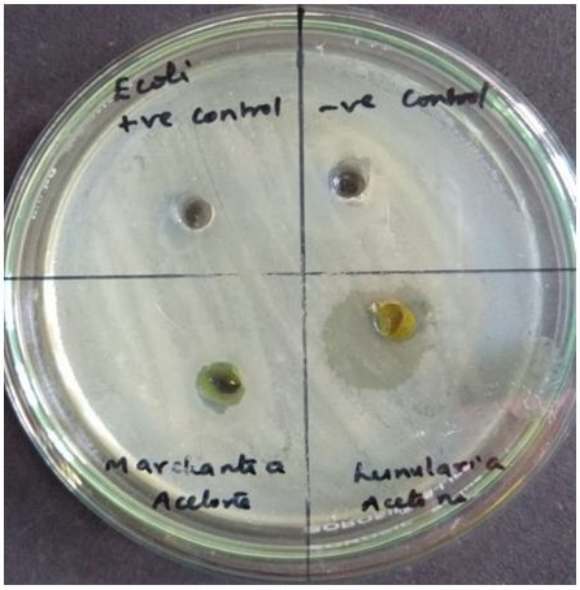
Phytocons-tituents	Test/ Reagent	In Alcohol	In Acetone
Alkaloids	Mayer's test	-	-
	Hager's test	-	-
	$FeCl_2$	-	-
Diterpenes	Copper acetate test	+	+
Proteins	Millon's test	-	-
	Xanthoprotein test	+	+
Phytosterols	Salkowski's test	+	+
	Libermann Burchard test	-	-
Saponins	Froth test	-	-
Flavonoids	Ferric chloride test	-	-
	Lead acetate test	-	-
Phenol	Lead acetate test	-	-
Carbohydrate	Benedict's test	-	-
	Molisch's test	+	+
Tannins	Ferric chloride test	-	-
	Lead acetate test	-	-
Anthocyanin	Con.H_2SO_4	+	+

According to a study conducted by Dhondiyal *et al*., 2013 ethanol extracts of *L.cruciata* obtained 18±0.33 mm against *E.coli* bacteria while in our study the ethanol extracts obtained 14±0 mm. In our study acetonic extract of *L.cruciata* obtained 18±0 mm while they obtained 9±0



A. Against *E.coli* bacteria-*Marchantia emarginata* (Alcohol) extract—8±0mm; *Lunularia cruciata* (Alcohol) extract—14±0mm

Plate 1: Alcoholic extracts of *M.emarginata* and *L.cruciata* against *E.coli* bacteria.



B. Against *E coli* bacteria—*Marchantia emarginata*(Acetone)extract—0mm; *Lunularia cruciata* (Acetone) extract—18±0mm.

Plate 2: Acetonic extracts of *M.emarginata* and *L.cruciata* against *E.coli* bacteria.

Table 3: Antibacterial properties of both *M.emarginata* and *L.cruciata* against *E.coli* bacteria.

Against *E.coli* Bacteria			
Ethyl alcohol	**Zone of Inhibition**	**Acetone**	**Zone of Inhibition**
Positive control	12±0mm	Positive control	13±0mm
Negative control	0mm	Negative control	0mm
Lunularia cruciata extract	14±0mm	*Lunularia cruciata* extract	18±0mm
Marchantia emarginata extract	8mm	*Marchantia emarginata* extract	0mm

mm only. Their study also stated that the order of inhibitory activity of antibiotic potential of different extracts of *L.cruciata* was ethanol > methanol > acetone > chloroform but in our studies we found that acetonic extracts were more active in inhibiting the bacteria than the alcoholic extracts. The differences in the effectiveness of different solvent extracts of *Lunularia cruciata* could be attributed to the relative solubility of secondary metabolites in the different solvents.

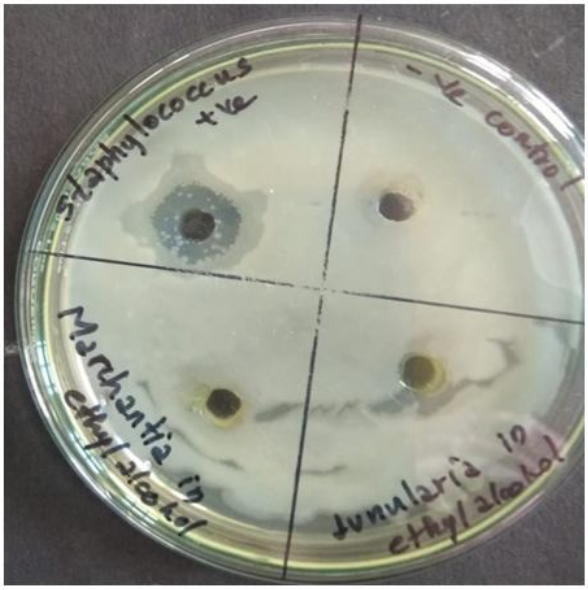
Basile in 1998, evaluated the action of acetone extract of *L.cruciata* against 13 bacteria and found substantial antibacterial activity against *Pseudomonas areuginosa, E.coli, Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella pneumoniae, Salmonella typhi, Proteus vulgaris, Proteus mirabilis, Enterobacter cloacae, Enterobacter aerogenes, Proteus mirabilis, Bacillus subtilis, Citrobacter diversus and Streptococcus faecalis* . Our studies are in confirmation with the above said studies regarding the antibacterial abilities of *Lunularia cruciata* against *Staphylococcus aureus, E.coli* and *Klebsiella pneumoniae.*

Neelam Mewari and Padmakumar in 2008 reported that the crude methanol and flavonoid extracts of *Marchantia polymorpha* L was found effective against *E.coli, Proteus mirabilis* and *Staphylococcus aureus* and the best activity was against *S.aureus* with a zone of inhibition of 20.6 and 19.6mm. A species of *Marchantia -M. paleacea* showed the broadest spectrum of antibiotic activity and among the test organisms, *Salmonella typhi* was found to be the most sensitive. In our studies, *Marchantia emarginata* ethyl alcoholic extract exhibited highest inhibition against *Klebsiella pneumoniae* (10±0mm) followed by *E.coli* (8±0mm). From our studies it can be concluded that the *M.emarginata* extracts were not having much antimicrobial properties when compared to *M. polymorpha* or *M.paleacea*. Further it can be concluded that both the alcoholic and acetonic extracts of *M.emarginata* showed no inhibition or sensitivity

against *S.aureus* bacteria, but showed moderate inhibition against both *E.coli* and *Klebsiella pneumoniae* bacteria (Plate 3 , 4 and table 4).

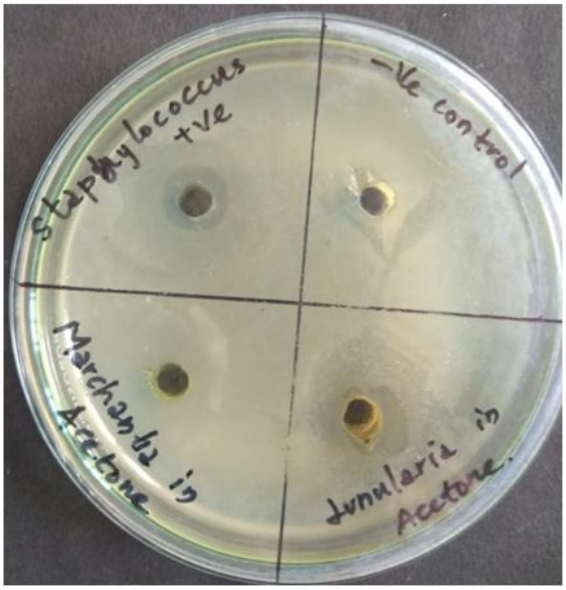
As per the studies of Kavita Negi and Preeti Chaturvedi, ethanol extracts of *M. papillata* showed highest zone of inhibition of 31mm and the zone of inhibition was found superior over the zone of inhibition of used antibiotics ie. Streptomycin and Chloramphenicol. GC-MS data obtained from its ethanolic extract showed a high percentage of sesquiterpenes/diterpenes, steroids, fatty acids and alcohol derivatives attributing to its antibacterial potential. But in our studies, using *M.emarginata* even though phytochemical analysis revealed the presence of steroids and diterpenes, antibacterial effect was not exhibited by both ethyl alcoholic and acetonic extracts of *Marchantia emarginata* against *Staphylococcus aureus*. It may be due to the differences in the species of the bryophytes selected for the study.

Against *Staphylococcus aureus* bacteria, acetonic extracts of *Lunularia cruciata* was the only one extract which produced inhibition of 10±0mm. Here, the antibiotic produced an inhibition of 11±0mm.Either the alcoholic extracts of both the liverworts or the acetonic extract of *M.emarginata* could not produce inhibition against *S.aureus* bacteria (Plate 3, 4 and table 4). As per the studies of Joshi (1993), the water and DMSO extracts of *Dumortiera hirsuta* and *Lunularia cruciata* possessed antibacterial activity against *S.aureus, E.coli, Klebsiella pneumoniae* and *B.subtilis*. The geographical conditions under which these plants were growing may also play a crucial role in determining their production of secondary metabolites thereby leading to their antimicrobial efficiency. Further , it was stated that the synthesis of chemical constituents in the tissues of plants may be an adaptation to their ecological set up (Anderson *et al*., 1974; Karunen, 1978). Besides the variation in ageing and the selection of solvents may be responsible for the



C. Against *Staphylococcus aureus* bacteria—*Marchantia emarginata*(Alcohol) extract—0mm; *Lunularia cruciata* (Alcohol)extract—0mm.

Plate 3: Alcoholic extracts of *M.emarginata* and *L.crucuiata* against *S.aureus* bacteria.



D. Against *Staphylococcus aureus* bacteria—*Marchantia emarginata* (Acetone)extract—0mm; *Lunularia cruciata* (Acetone) extract—10 ±0 mm.

Plate 4: Acetonic extracts of *M.emarginata* and *L.cruciata* against *S.aureus* bacteria.

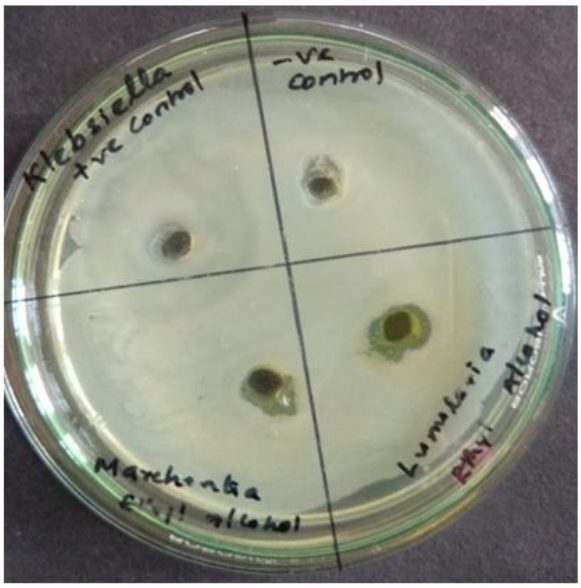
Table 4: Antibacterial properties of both *M.emarginata* and *L.cruciata* against *S.aureus* bacteria.

Against *Staphylococcus aureus* Bacteria			
Ethyl alcohol	**Zone of Inhibition**	**Acetone**	**Zone of Inhibition**
Positive control	13±0mm	Positive control	11±0mm
Negative control	0mm	Negative control	0mm
Marchantia emarginata extract	0mm	*Marchantia emarginata* extract	0mm
Lunularia cruciata extract	0mm	*Lunularia cruciata* extract	10±0mm

variation of the results (Banerjee and Sen, 2001).

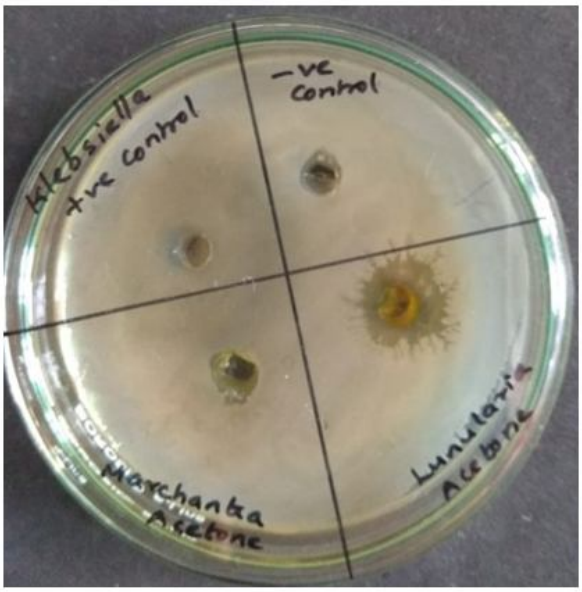
Against *Klebsiella pneumoniae* bacteria, acetonic extracts of *Lunularia cruciata* obtained 12.33±0.57mm inhibition zone while *M.emarginata* obtained 9±0 mm inhibition zone. Here the positive control got 18±0 mm and the negative control obtained no inhibition at all. Alcoholic extracts of *L.cruciata* and *M. emarginata* against *Klebsiella pneumoniae* bacteria showed maximum inhibition of 11±0 mm and10±0mm respectively. Here the positive control (antibiotic solution) obtained 19±mm respectively and negative control exhibited no inhibition zone at all. Here the positive control (antibiotic solution) obtained 18mm and 19mm inhibition zones which is higher, but the antibiotic potential exhibited by liverwort extracts showed a comparable result with that of the antibiotic solution (Plate 5, 6 and table 5). Previous reports of Karpinski and Adamczak in 2017 regarding the antibacterial activity of ethanolic extracts of mosses against *Klebsiella pneumoniae* showed that the mean Zone of Inhibition was 8.2mm and the highest zone of inhibition obtained against *Klebsiella pneumoniae* was 12.3mm by the moss *Dryptodon pulvinatus*) In this context, both *Marchantia emarginata* and *Lunularia cruciata* liverwort extracts exhibited comparatively good inhibition against the bacteria and more or less the same value as that of the mosses. The occurence of antibiotic substances seems to be more frequent in hepatics than in mosses and anthocerotopsids. Solubility data and antibiotic spectra of the active plants indicate the occurrence of antibiotic substances mong bryophytes (Banerjee and Sen,1979).

Both acetonic and alcoholic extracts produced inhibition against different bacteria, but more appreciable results were found in acetonic extracts. The effectiveness of different extracts of *L.cruciata* differed largely due to relative solubility of various secondary metabolites in



E. Against *Klebsiella pneumoniae* bacteria—*Marchantia emarginata* (Alcohol) extract—10±0mm; *Lunularia cruciata* (Alcohol) extract—11±0mm.

Plate 5: Alcoholic extracts of *M.emarginata* and*L.cruciata* against *Klebsiella pneumoniae* bacteria.



F. Against *Klebsiella pneumoniae* bacteria—*Marchantia emarginata* (Acetone) extract—9±0mm; *Lunularia cruciata* (Acetone)extract—12.33±0.57mm.

Plate 6: Acetonic extracts of *M.emarginata* and *L.cruciata* against *Klebsiella pneumoniae*.

Table 5: Antibacterial properties of both *M. emarginata* and *L.cruciata* against *Klebsiella pneumoniae* bacteria.

Against *Klebsiella pneumoniae* Bacteria			
Ethyl alcohol	**Zone of Inhibition**	**Acetone**	**Zone of Inhibition**
Positive control	19±0mm	Positive control	18mm
Negative control	0mm	Negative control	0mm
Marchantia emarginata extract	10±0mm	*Marchantia emarginata* extract	9±0mm
Lunularia cruciata extract	11±0mm	*Lunularia cruciata* in extract	12.33±0.57mm

different solvents and it appears that these metabolites were more soluble in acetone than alcohol, resulting in the comparative less antibacterial activity in alcoholic extracts.

Analysis of both the phytochemical properties and antibacterial efficiencies revealed that the phytochemicals like terpenoids, phytosterols and anthocyanins which were present in *Lunularia* cruciata and *Marchantia emarginata* may be responsible for their antibacterial effects. Further many studies revealed that many liverworts especially *Lunularia* species possess lunularic acid which may be responsible for their comparatively higher antibacterial potency than *Marchantia emarginata* .The liverwort *Marchantia emarginata* was reported to have anticancer activity due to the presence of a compound Marchantin A. Besides good antioxidant activities has also been reported by many workers. Antibacterial properties of *Marchantia emarginata* has not yet been conducted so far and this work may be considered as a first attempt to unweil the antibacterial potency of the plant.

Conclusion

In the present study, it was found that bryophytes contain many significant secondary metabolites in them which have profound antibiotic potentials comparable to that of an antibiotic. In short, *Lunularia cruciata* exhibited high antibacterial potency against dreadful *Staphylococcus aureus*, *E.coli* & *Klebsiella pneumoniae* and *Marchantia emarginata* produced moderate level of inhibition against *Klebsiella pneumoniae* and *E.coli.*

Acknowledgment

The authors greatly acknowledge KSCSTE (Kerala State Council for Science, Technology and Environment) for the financial aid given for carrying out the work.

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