Comparative evaluation of antimicrobial activity of methanolic extract and phenolic compounds of a liverwort, *Reboulia hemispherica*

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Abstract

Comparative evaluation of antimicrobial activity of methanolic extract and phenolic compounds of a liverwort, *Reboulia hemispherica* was carried out by Agar well diffusion technique. The Gram positive bacteria were more sensitive than the Gram negative ones, while the fungal species were least sensitive. *R. hemispherica* extract exhibited best results against *Staphylococcus aureus*, although it was active against all tested microbes. The antimicrobial activity increased with the increase in the concentration of the extract except in *Klebsiella* sp. *S. aureus*, *E. faecalis* and *Bacillus cereus* were inhibited more by the crude methanol extract of *R. hemispherica* than the phenolic compounds isolated from the extract. *A. niger* was inhibited equally by the crude methanol extract as well as the phenolic compounds. *B. subtilis*, *E. coli*, *P. aeruginosa*, *K.* sp. and *P. notatum* were inhibited more by phenolic compounds than the crude methanol extract of *R. hemispherica.* Thus phenolic compounds of *R. hemispherica* should be studied further for use as antimicrobial agent.

Keywords: Antimicrobial, methanol extract, liverwort, *Reboulia hemispherica.*

1. Introduction:

Rapid multiplication rates, large population sizes and the ability of these populations to respond to environmental changes enable bacteria to rapidly acquire resistance to antibiotics. This has led to the widespread emergence of drug resistant microbe such as methicillin-resistant *S. aureus* (MRSA), and multidrug resistant strains of *K. pneumonia* and *P. aeruginosa.* It does potentially pose a serious concern and challenge the scientific community to discover alternative and more efficient antimicrobials for the treatment of such nosocomial infections. Such alternative antimicrobial molecules include substances from natural sources like some medicinal plants because of multiple reasons-(a) have antimicrobial activity against wide range of microbial strains (Botelho *et al*.(2007a,b, 2008), Didrey *et al*., (2000), Fine *et al*., (2000), Takrada *et al*.,(2004) (b) reduce the risk of developing microbial resistance (c) biodegradable (d) renewable in nature (e) safe for human health(Edris, 2007). For centuries, higher plants have been used for this purpose. But the present attempt was made with respect to lower group of plants so called bryophytes.

An interesting feature of bryophytes is that they are relatively free from attack by parasitic microorganisms. Unlike higher plants, herbarium specimens of these plants need no special treatment (Mc Cleary and Walkintgton, 1966). Bryophytes are ecologically important as

good indicators of environmental conditions. Many botanists and microbiologists have documented the presence of antibiotic substances and biologically active compounds such as terpenoids, phenols, glycosides, and fatty acids in bryophytes(Banerjee and Sen, 1979, Van wagen and Cardellina, 1986, Glime and Saxena 1991, Sabovljevic *et al.*, 2009 and Zhu *et al.,* 2006). They also possess anticancer activity due to their unique chemical constituents (Asakawa, 1990). Reports on traditional use of bryophytes as medicine are available (Harris, 2008) but very few attempts have been taken to exploit the pharmacological, clinical and medicinal potential of bryophytes. Ethnic use of bryophytes when scientifically verified, might serve an exciting aspect to explore the immense bioactive potential of this plant group. The present study aims to explore antibiotic potential of liverwort taxa *R. hemispherica* against some human pathogens.

2. Material and methods:

2.1 Plant material and Methanolic extraction Preparation:

Thalli of *R. hemispherica* were collected from March to October 2012 from different sites largely in and around Jammu city. Dried material was crushed with pestle and mortar for extraction. Methanol was used for extraction. 20 g of dried crushed tissue was mixed with 100 ml methanol and kept for 24 h at room temperature followed by shaking the extracts at 200 rpm for 14 h in Incubator Orbital Shaker. They were then filtered using Whatman filter paper (No.1). The solvents were evaporated at 55-60 ^{o}C on water bath. The stock was prepared by dissolving 100 mg of the dry residue in 1 ml of dimethyl sulfoxide (DMSO). Different concentrations (1-100 %) of the plant extract were prepared by diluting the stock solution in DMSO. The extracts were then sterilized by filtration using 0.45µm Millipore filters.

2.2 Extraction of phenols:

10 ml of sodium hydroxide (1 M) was added to 1 g of methanol extract. It was then shaken well and kept overnight. The solution was filtered and 1M hydrochloric acid was added slowly to the filtrate till the solution became acidic. The above solution was then taken in a separating flask and 60 ml ethyl acetate was added to it. Two layers were formed in the solution; upper ethyl acetate and the lower aqueous. The latter one was discarded. To the ethyl acetate layer an estimated amount of anhydrous sodium sulphate was added and kept overnight. Next day, it was filtered and the solvent was evaporated on boiling water bath. 100 mg of dry residue was dissolved in 1 ml of DMSO to get a concentration of 100 mg/ml and sterilized by filtration using 0.45µm Millipore filters.

2.3 Microbial strains tested

Test microorganisms comprised seven bacterial species including four Gram positive (*S. aureus*, clinical isolate; *E. faecalis*, clinical isolate; *B. subtilis*, MTCC 121; *B. cereus*, MTCC 430) and three Gram negative (*E. coli*, MTCC 1673; *P. aeruginosa*, MTCC 1934; *K.* sp., clinical isolate). Fungal species included *A. niger*, MTCC 1344 and *P. notatum*, MTCC 1898. The bacterial and fungal cultures were maintained on Luria Bertani Agar and Sabouraud Dextrose Agar, respectively.

2.4 Preparation of inoculums

Single isolated colony of bacteria was inoculated in Luria Bertani Broth and incubated at 35-37 ^{o}C in incubator for 2-8 hours until light to moderate turbidity developed. The inoculum turbidity was compared with standard 0.5 Mc Farland (1×10^{6}-5×10^{6} cells/ml). For fungal cultures, inoculum was prepared by transferring single colony from 24 hour old culture grown on

Sabouraud Dextrose Agar with a needle to 5 ml of Sabouraud Dextrose Broth. The inoculum turbidity was compared with standard 0.5 Mc Farland (1×10^6-5×10^6 cells/ml).

2.5 Antimicrobial Assay

The antibacterial and antifungal activity of the plant extract and phenol fraction was determined by Agar Well Diffusion method using Mueller Hinton Agar and Czapek's Dox medium respectively (Bauer *et al.*, 1966, Perez *et al.*, 1990). 50 µl of each concentration of plant extract, 50 µl of phenol fraction were used in the well. DMSO and Methanol were taken as negative control, whereas, Ampicillin (10 mg/ml), Ofloxacin (10 mg/ml) in case of bacteria and Griseofulvin (10 mg/ml) in case of fungi were used as positive controls. Plates were incubated overnight at 37 °C. Diameter of zone of inhibition was recorded in mm. The assays were replicated and the mean value of 3 experiments were recorded (n=3) with SEM.

3. Results and discussion

The results obtained showed that all test microorganisms were sensitive to the methanol extract of *R. hemispherica.* The Gram positive bacteria were however, more sensitive followed by Gram negative bacteria and fungi. Similar observations were earlier made on methanol extract of mosses (Veljic *et al.*, 2008). Previous studies on the contrary, found Gram negative bacteria to be more sensitive than Gram positive ones (Basile *et al*., 1999) Observations of Subhisha and Subramoniam (2005) however, were in contrast to those made presently because they found that fungal species were inhibited more than bacteria by a steroid from *Pallavicinia lyellii*.

In the present study, the Gram positive bacteria were sensitive to the extracts of *R. hemispherica.* The best results were obtained for *S. aureus* followed by *E. faecalis*, *B. subtilis* and *B. cereus.* These results are in contrast to those obtained by Singh *et al*. (2006) as they found maximum inhibition of *B. subtilis* followed by *B. cereus* and *S. aureus.*

Among Gram negative bacteria, the extract of *R. hemispherica* exhibited the largest zones of inhibition against *E. coli* followed by *Klebsiella* sp. and *Pseudomonas aeruginosa* (Table 1). These observations are in conformity with those of Singh *et al*. (2006) who also found *Escherichia coli* to be most sensitive followed by *K. pneumoniae* and *P. aeruginosa.* Singh *et al*. (2011)

However, found *P. aeruginosa* to be more sensitive among the Gram negative bacteria tested in chloroform fractions of liverworts (*P. appendiculatum* and *Conocephalum conicum*).

Both fungal species tested, *A. niger* and *P. notatum* were least sensitive to the extract. Similarly, Vashistha *et al*. (2007) observed that the inhibition of *A. niger* was not very much marked as it showed only 46.6 % inhibition at 100 % concentration of *P. appendiculatum* extract. In contrast, Singh *et al*. (2006) reported *Aspergillus niger* to be inhibited more than *K. pneumoniae, P. aeruginosa, B. subtilis, B. cereus* and *S. aureus.*

3.1 Antimicrobial activity exhibited by phenolic compounds

The phenolic compounds isolated from the methanolic extract of *R. hemispherica* also exhibited antimicrobial potential. Out of the various microorganisms tested presently, *E. coli* was most sensitive to phenols whereas *Bacillus cereus* and the two fungal species (*A. niger* and *P. notatum*) were least sensitive. *S. aureus*, *E. faecalis* and *B. cereus* showed less sensitivity to phenolic compounds as compared to crude methanol extract which might be suggesting that instead of phenols, other chemical entities in the extract contributing more towards antimicrobial activity. Similarly *E. coli, P. aeruginosa, Klebsiella* sp., *B. subtilis* and *P. notatum* were more sensitive to phenolic compounds than the extract suggesting that phenolic compounds contribute more towards antimicrobial activity against gram negative bacteria. Previously, in extract some component(s) might be inhibiting/blocking the action of the phenols. *A. niger* exhibited equal sensitivity against phenolic compounds and methanol extract of *R. hemispherica*

Phenolic compounds are known to be synthesized by plants in response to microbial infection (Doughari *et al.*, 2008). It is therefore logical that they have been found in vitro to be effective antimicrobial substances against a wide array of micro-organisms. The antimicrobial activity in bryophytes has been attributed to the presence of flavonoids, steroids, terpenoids, triterpenoids, mono-, di- sesquiterpenoids, unsaturated lipids, fatty acid esters, phenolics and other polyphenolic compounds (Tutschek and Rudolph, 1971). Chemical compounds having antimicrobial potential have earlier been analysed from *P. appendiculatum, R. hemispherica, M. palmata* and *M. polymorpha* (Dixit and Banerjee, 2007).

Comparison of antimicrobial activity of phenolic compounds with the standard antimicrobial drugs (Ampicillin, Ofloxacin and Griseofulvin) revealed that the antifungal drug, Griseofulvin was more effective than the phenols and the extracts. In the present study, the antibacterial as well as the antifungal drugs were found to be more effective than the extract and phenolic compounds. *E. coli* was however, inhibited equally by Ofloxacin and phenolic compounds but both were used at different concentrations. (Table 1). Thus the result of present study suggested that phenolic compounds from bryophytes could be the choice of molecule to inhibit the *E. coli* and other pathogens. Further work can be undertaken in the direction.

In present study, the Gram positive bacteria were more sensitive than the Gram positive ones, while the fungal species were least sensitive. *R. hemispherica* extract exhibited best results against *Staphylococcus aureus*, although it was active against all test microbes. The antimicrobial activity increased with the increase in the concentration of the extract except in *Klebsiella* sp. *S. aureus, E. faecalis* and *Bacillus cereus* were inhibited more by the crude methanol extract of *R. hemispherica* than the phenolic compounds isolated from the extract. *A. niger* was inhibited equally by the crude methanol extract as well as the phenolic compounds isolated from the extract. *B. subtilis*, *E. coli, P. aeruginosa*, *K.* sp. and *P. notatum* were inhibited more by phenolic compounds than the crude methanol extract of *R. hemispherica.*

Acknowledgements
Thanks are due to the Head, Department of Botany, University of Jammu, and tissue culture division of IIIM, Jammu for their help and cooperation.

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Table 1. Antimicrobial activity of *Reboulia hemispherica* expressed as inhibition zones (diameter in mm).

Concentration of methanol extract (%)	**BACTERIA**							**FUNGI**	
	EC	**PA**	**KS**	**SA**	**EF**	**BS**	**BC**	**AN**	**PN**
1	2	1	6	4	4	4	-	3	2
10	2	1	6	19	8	6	6	3	2
20	2	2	6	19	10	8	6	4	2
30	4	2	4.5	19	10	10	8	4	2
40	4	2	4.5	19.5	12	12	10	4	4
50	4	4	4.5	19.5	12	12	11	4	4
60	6	6	4.5	19.5	14	12	11	4	4
70	6	6	4.5	19	14	12	11	4	4
80	8	6	2.5	19	14	12	11	4	4
90	8	6	2.5	19	14	12	11	4	4
100	8	6	2.5	19	14	12	11	4	4
Positive and negative controls	**BACTERIA**							**FUNGI**	
	EC	**PA**	**KS**	**SA**	**EF**	**BS**	**BC**	**AN**	**PN**
Methanol	-	-	-	-	-	-	-	-	-
Dimethyl sulfoxide	-	-	-	-	-	-	-	-	-
Ampicillin(10mg/ ml)	40	24	30	24	24	36	40	-	-
Ofloxacin (10mg/ ml)	18	40	33	33	24	39	42	-	-
Phenols (100 mg/ml)	18	12	10.5	12	10.5	13.5	8	4	6
Griseofulvins(10 mg /ml)	-	-	-	-	-	-	-	8	10

EC-*Escherichia coli*; **PA**-*Pseudomonas aeruginosa*; **KS**-*Klebsiella* sp; **SA**-*Staphylococcus aureus*; **EF**-*Enterococcus faecalis*; **BS** *Bacillus subtilis*; **BC** *Bacillus cereus*; **AN**-*Aspergillus niger*; **PN**-*Penicillium notatum*