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In vitro Antibacterial Activity of Himalayan Lichenized Fungi

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Abstract

The increase in the new strains of multi drug-resistant pathogens, the standard drugs have become less effective, thereby increasing the demand for the search of novel natural bioactive compounds from lichenized fungi. Despite rich diversity of lichenized fungi in Kumaon Himalaya, only a few of them have been screened for their biological activities. Present communication deals with antimicrobial activity of ethyl acetate extracts of *Everniastrum cirrhatum*, *Usnea longissima*, *Flavoparmelia caperata* and *Ramalina conduplicans* against five pathogenic bacteria. Among these, *U. longissima* and *F. caperata* extracts have revealed significant activity against all the bacteria while *U. longissima* was more active against *Escherichia coli* (13.0 mm; MIC=7.5 mg/mL) and *Bacillus subtilis* (11.6 mm; MIC=7.5 mg/mL). The *F. caperata* extract was active against *E. coli* (15.3 mm; MIC=15 mg/mL). Hence, there is an interest in the potential uses of antibiotics derived from lichenized fungi for pharmaceutical purposes.

Keywords: Lichenized fungi; *Usnea longissima*; *Flavoparmelia caperata*; Antibacterial activity; Kumaun Himalaya

Introduction

India is a rich centre of biodiversity contributing nearly 15% of the 13,500 species of lichenized fungi [1]. Of these, several lichenized fungi of the Himalayan region are said to effectively cure dyspepsia, bleeding piles, bronchitis, scabies, stomach disorders and many disorders of blood and heart [2-4]. *Flavoparmelia caperata* is part of Ayurvedic and Unani medicines under the name 'Chharila' as carminative and aphrodisiac and considered useful in treatment of intestinal worms and burns [5], *Everniastrum cirrhatum* relieves headache [6,7]. *Ramalina conduplicans* was put on wounds to stop bleeding, cure jaundice [8,9]. *U. longissima* has been used in the treatment of bone fractures and strains, and ulcers [10,11]. It is also used as a simple drug to stimulate menstruation or induce abortion. Reports on floristic, monographic, revisionary, pollution monitoring studies of lichenized fungi exist but little attention has been paid to the detailed chemical analysis and biological activity of lichenized fungi native to high altitude region of Himalaya.

Lichenized fungi are known to produce a great variety of bioactive secondary metabolites. Recent developments in analytical techniques have resulted in the identification of about 1050 lichen substances [12] *viz*. usnic acid, phenolic compounds, anthraquinones, dibenzofurans, depsides, depsidones, depsones, triterpenes, γ-lactones and pulvinic acid derivatives [13]. These exhibit a multiple biological activity, such as: antiviral, antibiotic, antitumor, allergenic, plant growth inhibitory and enzyme inhibitory [14-16].

The aim of present study is to investigate the antibacterial activity of extracts from Himalayan lichenized fungi *viz. E. cirrhatum*, *U. longissima*, *F. caperata* and *R. conduplicans*.

Experimental Section

Plant materials

Lichenized fungi were collected from Kumaun (Uttarakhand) Himalaya during March 2015. The lichen specimens were identified with the help of published flora [17]. Voucher specimens have been deposited in Department of Botany, Kumaun University, and Almora.

Chemicals and reagents

All chemicals and reagents used were of analytical grade. Nutrient Agar (NA) and Mueller Hinton Broth (PDB) were obtained from Hi-Media, India.

Preparation of lichen extracts

Each lichen sample was washed to remove debris, dried, ground to powder and stored in a sterile glass bottle in the refrigerator. The powder (2-3 g) was added to 10 ml of ethyl acetate and left for 10 days at room temperature. The crude extract was filtered with Whatman No. 42 and solvent was evaporated to obtain dried extract. The extract was stored in refrigerator at ~4°C.

Bacterial strains

The *in vitro* antibacterial activity was evaluated against pathogenic and clinically isolated 5 bacterial strains *Pseudomonas aeruginosa* (MTCC No. 424), *Escherichia coli* (MTCC No. 443), *Klebsiella pneumoniae* (MTCC No. 3384), *Salmonella typhimurium* (MTCC No. 3224) and *Bacillus subtilis* (MTCC No. 441). The test strains were provided by the Department of Biotechnology, Bhimtal, Kumaun University which were procured from the Institute of Microbial Technology, Chandigarh. Microbial Technology Culture Collection (MTCC) numbers represent the standard strain numbers assigned to these microorganisms. The cultures of bacteria were maintained on their appropriate nutrient agar at 4°C throughout and used as stock cultures.

Antibacterial activity by disc diffusion

Evaluation of antibacterial activity of lichenized fungi extracts was carried out by disc-diffusion method [18]. The samples were dissolved

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in Dimethyl Sulfoxide (DMSO) to prepare desired concentrations. Inoculums of the microbial strains (1×10^6 CFU/mL) were plated using sterile swabs into petri dishes (90 mm) with 20 mL of Nutrient Agar, and then discs of Whatman paper-42 were soaked in sample solution (15 mg/mL) and placed onto inoculated petri dishes. Standard antibiotic streptomycin (15 mg/mL) was used as positive control and DMSO as negative control. The petri dishes were pre-incubated for 3 h at room temperature, allowing the complete diffusion of the samples and then, incubated at 37°C ± 1°C for 24 h [19]. The zones of inhibition were measured.

Antibacterial activity evaluation by agar dilution method

The evaluation of MICs was done using the agar dilution method with slight modifications described by the National Committee for Clinical Laboratory Standards [20]. Equal volume of each microbial strain culture, containing approximately 1×10^6 CFU/mL, was applied onto MHB supplemented with the extract at concentration ranging from (0.46-30 mg/mL) in tubes. These cultures were then incubated at 37°C for 24 h then cultures were finally inoculated on nutrient agar media to determine the growth of bacteria. Controls of bacteria without the extract were also applied. The concentration at which no visible growth was observed is considered as MICs.

Statistical analysis

Data were subjected to one-way Analysis of Variance (ANOVA) and the means were compared by Duncan Multiple Range tests at a level of significance of $p<0.05$ using SPSS 16.0 statistical software. The Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were performed using PAST statistical computer software package for evaluating correlation between antibacterial activity and extract.

Results and Discussion

The antibacterial activity data of lichenized fungal extract against five bacteria are presented in Tables 1 and 2. The maximum zone of inhibition was recorded against *E. coli* (19.0 mm), *K. pneumoniae* (16.3 mm) and *B. subtilis* (15.3 mm). Activity data revealed *U. longissima* and *F. caperata* to be more active against almost all the bacteria. *U. longissima* showed higher activity against Gram-negative bacteria *E. coli* (13.0 mm; MIC=7.5 mg/mL) and Gram-positive bacteria *B. subtilis* (11.6 mm; MIC=7.5 mg/mL), while *F. caperata* was more active against *E. coli* (15.3 mm; MIC=15 mg/mL) followed by *K. pneumoniae* (13.6 mm; MIC=15 mg/mL). The extract of *E. cirrhatum* was effective against *E. coli* (19.0 mm; MIC=15 mg/mL). The extract of *R. conduplicans* was found more active against *B. subtilis* (15.3 mm; MIC=15 mg/mL). It is interesting to note that the growth of *S. typhimurium* remained unaffected by any of the lichenized fungal extracts. The activity of extracts was noticed in the following descending order *E. coli*>*K. pneumonia*>*B. subtilis*>*P. aeruginosa*>*S. typhimurium*.

The inhibition data were subjected to PCA and HCA analysis (Figures 1 and 2). Group I, composed of the Gram-negative bacteria (*E. coli* and *K. pneumonia*), is characterized by high sensitivity to the extracts (13-19 mm). Group II is represented by Gram-positive bacteria *B. subtilis* distinguishable in the PCA as a distinct group (8.3-15.3 mm). Group III, which constituents Gram-negative bacteria *P. aeruginosa* and *S. typhimurium* was characterized by relatively resistant to all the extracts, especially *S. typhimurium* strain which showed high resistance to the extracts (<7 mm).

Secondary metabolites derived from natural products possess various benefits including antimicrobial properties against pathogenic and spoilage microbes. Major groups of compounds that are responsible for antimicrobial activity from plants include phenolics, phenolic

Lichenized fungi	Diameter of inhibition zone (mean ± SD) mm[a]				
	Bacterial strains				
	B. subtilis	***P. aeruginosa***	***E. coli***	***S. typhimurium***	***K. pneumoniae***
E. cirrhatum	8.3 ± 1.1[a]	10.0 ± 1.7[b]	19.0 ± 3.0[c]	7.3 ± 1.5[a]	13.3 ± 2.0[a]
F. caperata	12.0 ± 1.0[b]	12.3 ± 1.5[b]	15.3 ± 2.5[b]	7.3 ± 1.5[a]	13.6 ± 1.1[a]
R. conduplicans	15.3 ± 2.5[c]	11.0 ± 1.0[b]	10.6 ± 0.5[a]	6.6 ± 1.1[a]	10.0 ± 1.0[a]
U. longissima	11.6 ± 0.5[b]	7.6 ± 0.5[a]	13.0 ± 0.5[a,b]	7.0 ± 1.0[a]	16.3 ± 1.1[a]
Streptomycin (Reference antibiotic)	31.3 ± 1.1[d]	21.6 ± 1.1[c]	20.0 ± 0.5[c]	24.0 ± 1.0[b]	21.3 ± 0.5[b]

[a]Mean (± SD) value (at 15 mg/mL) followed by different letters in the same column differ significantly at p ≥ 0.05 according to Duncan test.

Table 1: Antibacterial activity of lichenized fungal extracts against test organisms.

Lichenized fungi	Minimum inhibitory concentration (mg/mL)				
	Bacterial strains				
	B. subtilis	***P. aeruginosa***	***E. coli***	***S. typhimurium***	***K. pneumoniae***
E. cirrhatum	15.0	30.0	15.0	30.0	15.0
F. caperata	15.0	15.0	15.0	30.0	15.0
R. conduplicans	15.0	15.0	15.0	30.0	15.0
U. longissima	7.5	30.0	7.5	30.0	15.0
Streptomycin (Reference antibiotic)	3.7	3.7	0.9	3.7	3.7

Table 2: Minimum Inhibitory Concentration (MIC) of lichenized fungal extracts.

Lichenized fungi	Chemical constituents	References
E. cirrhatum	Salazinic acid, protolichesterinic acid	[17]
F. caperata	Usnic acid, atraric acid, arabinitol, atranol, orcinol, lichesterol, ergosterol, protocetraric acid, caperatic acid	[21,22]
R. conduplicans	Usnic acid, salazinic acid, sekikaic acid	[17,23]
U. longissima	Usnic acid, 8-hydroxydiffractaic acid, isostrepsilic acid	[17,23]

Table 3: The major constituents present in the lichenized fungi.

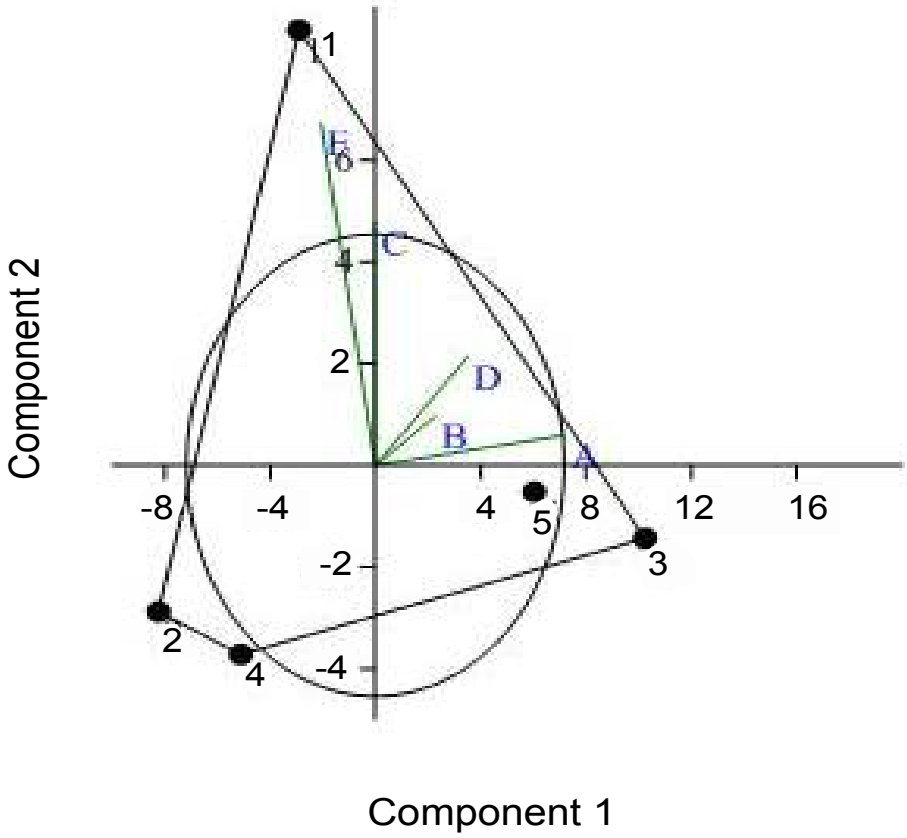

Figure 1: PCA of the antimicrobial activity of lichenized fungal extract against five bacteria (1: *B. subtilis*; 2: *P. aeruginosa*; 3: *E. coli*; 4: *S. typhimurium*; 5: *K. pneumoniae*; A: *E. cirrhatum*; B: *F. caperata*; C: *R. conduplicans*; D: *U. longissima*; *E: Streptomycin*).

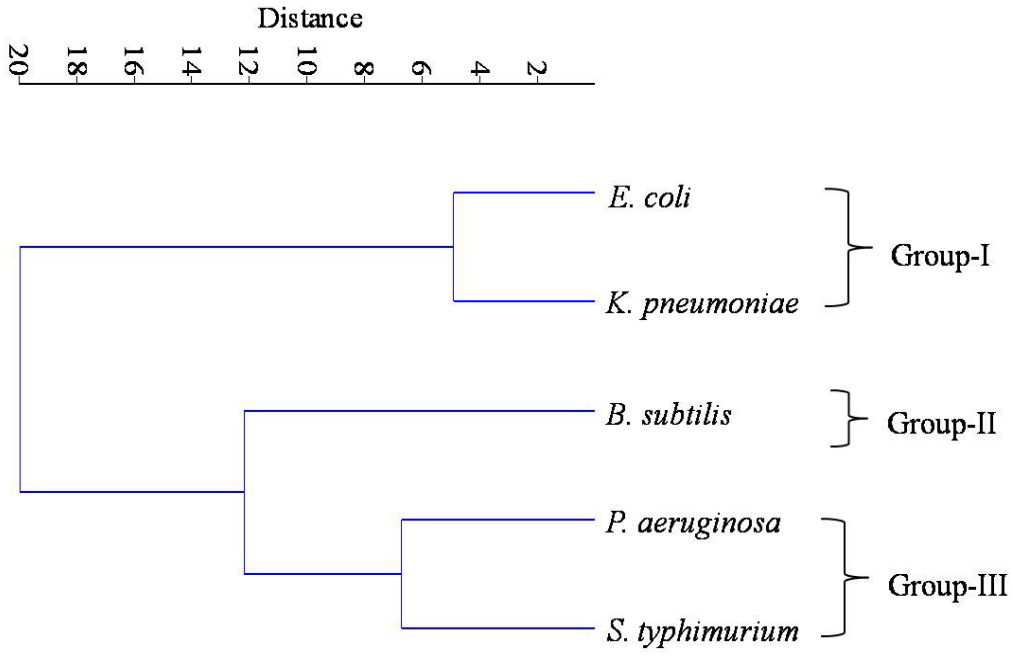

Figure 2: HCA based on the Euclidean distance between groups of the antibacterial activity of lichenized fungal extract.

acids, quinones, saponins, flavonoids, tannins, coumarins terpenoids, and alkaloids. Variations in the structure and chemical composition of these compounds result in differences in their antimicrobial action. Antimicrobial activity depends not only on chemical composition but also on lipophilic properties, the potency of functional groups or aqueous solubility and the mixture of compounds. This action involves membrane disruption by lipophilic compounds resulting in inhibition of electron transport, protein translocation, phosphorylation, and other enzymatic activity which ultimately destroy the cell membrane integrity resulting in the death of microorganisms [21-24]. The major constituents of *E. cirrhatum, F. caperata, R. conduplicans* and *U. longissima* are given in the Table 3. Higher activity of *U. longissima* and *F. caperata* could be assigned to the presence of usnic acid, caparatic acid, protocetraric acid, barbatic acid, evernic acid and fumarprotocetraric acid present in their extracts.

Conclusion

There has been recurrence on the natural product chemistry researches in the recent years for new bioactive molecules that can replace synthetic additives and their potential use in the food and pharmaceutical industries. The results of the present study showed that some of the lichenized fungi extracts possess significant antibacterial activity. Further investigations of antimicrobial potential of these particular lichenized fungi in relation to human pathogens can be of pharmacological importance. Hence, there is an interest in the potential uses of antibiotics derived from lichenized fungi for the pharmaceutical industry.

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