Antimicrobial role of *Cladonia rangiferina* against pathogenic microorganisms

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Abstract

An attempt was made to study the antimicrobial activity of foliose lichen *Cladonia rangiferina* under invitro conditions. The antimicrobial activities of Methanol, Ethanol, Ethyl Acetate and Acetone extracts of *Cladonia rangiferina* were assayed against nine pathogenic microorganisms using standard well diffusion method. The Ethyl acetate extract was found most effective antibacterial as well as antifungal against most of organisms. The maximum inhibition zone was recorded in *E.coli* , *Staphylococcus aureus and Agrobacterium tumefaciens* with inhibition zone 22 mm. Fungal pathogens showed their inhibition zones in varying levels as 20 mm in *Candida albicans* and *Fusarium oxysporium*. The present study reveals that extracts obtained from *C. rangiferina* have potential compounds that can lead to control human pathogenic microorganisms in future.

Keywords :Lichens; well diffusion ;inhibition zone ; antimicrobial ; *Cladonia rangiferina*

Introduction

The search for novel natural bioactive compounds leading to new drug discovery is increasing as reliable standard drugs becomes less effective against new strains of multi drug resistant pathogens (Muller,2001). Lichens are considered as potential resource since these compounds function as chemical defence against biotic and abiotic stresses and they are antibacterial (Lawrey,1986)., anticancer (Williams et al.,1998), anti HIV (Huneck and Yoshimura,1996), analgesic and antipyretic (Muller, 2001). It has been documented that more than 1050 secondary metabolites were found so far (Huneck and Yoshimura, 1996) and among them 550 are unique in

lichens. Lichens produce large number of primary and secondary metabolites. Most lichen substances are phenolic compounds, dibenzofuranes and usnic acids, depsidones, depsones, lactones, quinines and pulvinic acid derivatives (Boustie and Grube, 2005). Lichen extracts have been used for various remedies in folk medicines and screening of compounds has shown potentiality as antimicrobial, anticancer, antioxidant, antitumour and analgesic. India is among the richest biodiversity centres contributing about 15% of 13,500 species of the world (Negi, 2000). Total of 2450 species of lichens were present in India and were abundant in temperate and alpine regions of Peninsular India (Nayaka et al, 2010).

Cladonia rangiferina, also known as **reindeer lichen** (c.p. Sw. *renlav*), lat., is a light-colored, fruticose lichen belonging to the Cladoniaceae family. It grows in both hot and cold climates in well-drained, open environments. Found primarily in areas of alpine tundra, it is extremely cold-hardy. Reindeer lichen, like many lichens, is slow growing . Thalli are fruticose, and extensively branched, with each branch usually dividing into three or four (sometimes two). The photobiont associated with the reindeer lichen is *Trebouxia irregularis*. It grows on humus, or on soil over rock. it also grows mostly in taiga and the tundra.The lichen is used as a traditional remedy for removal of kidney stones. It can also be used for food by crushing the dry lichen and then boiling it or soaking it in hot water until it becomes soft and also boil reindeer lichen and drink the juice as a medicine for diarrhea. Due to acids present in lichens, their consumption may cause an upset stomach, especially if not well cooked.It can act against Klebsiella pneumonia and very much effective as antifungal. It has been used in the treatment of pulmonary and cranial diseases. During the Middle Ages lichens figured prominently as the herbs used by practitioners. Northern native people used it to treat colds, arthritis, fevers and other problems. It was also used to relieve the aches of arthritic joints. In addition it could also help with constipation, convulsions, tuberculosis and a useful remedy of whooping cough.

Materials and Methods

Fig 1. *Cladonia rangiferina* sample was crushed to powdered form.

Lichen material : *C. rangiferina* thalli was collected from Sela Pass and was identified based on standard literature (Awasthi, 1988; Swinscow and Krog,1988). The collected material was washed thoroughly with distilled water followed by tween 80 and made air dried. The dried material was weighed and made into powdered form.

Extraction of Lichen Material : The powdered lichen (10gms) was wrapped in 8 x 6 cm cylindrical pouch made of Whatmann filter paper grade 1 and kept inside the extracter arm of Soxhlet apparatus(Balaji, 2005). A series of solvents as Methanol, Ethanol, Ethyl acetate and Acetone were used for extraction based on their polarity and each extraction was carried out at the specific boiling temperature for a priod of 48 hrs for the complete extraction of secondary compounds. The final filtrate of each of the extraction obtained was concentrated using Rotatory Evaporator or Rotavapour.

Culture Media : Nutrient Agar (NA) and Potato Dextrose Agar (PDA) medium were used to culture pathogens and for bacterial and fungal susceptibility test (Balaji,2005).

Microorganisms source : Total of Six bacterial cultures (*Pseudomonas aeruginosa, Agrobacterium tumefaciens, Escherichia coli, Streptococcus mutans, Staphylococcus aureus and Klebsiella pneumoniae*) and three fungal cultures of *Aspergillus niger , Candida albicans and Fusarium oxysporium* was used in this testing and screening process. All the cultures were

obtained from the Pharmacological Laboratory, National Botanical Research Institute (NBRI), Lucknow. The cultures were maintained at 4 degrees Celsius and subcultured in solid and semisolid nutrient agar slants.

Determination of Antimicrobial Activity : Antimicrobial activity was tested using well-diffusion method (Bauer et al.,1966). The Nutrient Agar medium was transferred into one fourth volume of petriplates for antibacterial activity. Potato Dextrose Agar medium was transferred into one fourth volume of petriplates for antifungal activity. Inoculation of cultures (100 mg/ml) to this medium was carried out uniformly using glass spreader. Five wells were made in each petriplate. Different concentration of crude extracts of Methanol, Ethanol, Ethyl acetate and Acetone (i.e. 2.5%, 5%, 10%,15% and 20%) were prepared as individual stock solutions by mixing Dimethyl Sulfoxide (DMSO) and Distilled Water. These stock solutions of different concentrations were filled in their respective wells alongwith DMSO as negative control and Streptomycin (in antibacterial testing) and Ketoconazole (in antifungal testing) as positive control. The plates were labeled and incubated for 24 hrs at 37 degree Celsius in BOD.

Results and Discussion

The inhibitory zones were recorded and measured with the help of Hi-Antibiotic Zone Scale. The results of antimicrobial activity of extracts are given in Table.1. Among the four different extracts, Ethyl acetate extract exhibited growth inhibition on all the nine organisms whereas Methanolic and Acetonic extracts exhibited growth inhibition on seven organisms and no inhibition against *Streptococcus mutans and Aspergillus niger.* There was least inhibitory activity for Ethanolic extract against *Agrobacterium tumefaciens.*

The Ethyl acetate extract inhibited the growth of all the organisms tested and specially exhibited 20-24 mm zones of inhibition against *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Candida albicans and Fusarium oxysporium.* The various concentrations (5-20%) of Ethyl acetate extracts exhibited more effective zone of inhibition compared to the antibiotic standard Streptomycin (20-24 mm) against *Staphylococcus aureus* and *Escherichia coli (*28-32 mm) also compared to the antifungal standard Ketoconazole (12-14 mm) against *Candida albicans and Fusarium oxysporium* (18-20 mm).

The antimicrobial potential of Ethyl acetate extracts is much more than the methanolic and acetonic extracts of *C.rangiferina*.

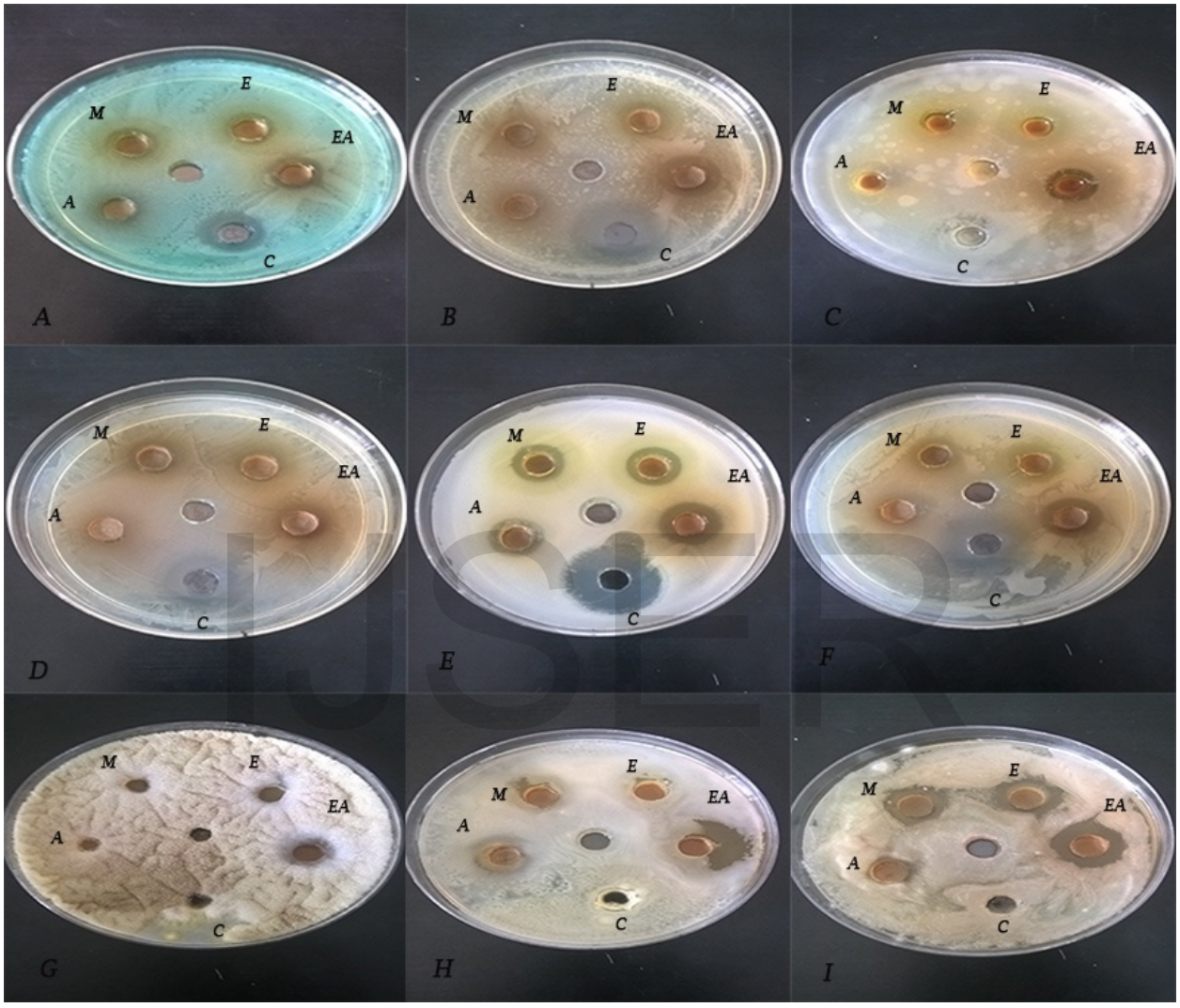


Fig 2. Inhibition zones after Antimicrobial test activity where, A. *Pseudomonas aeruginosa* B. *Staphylococcus aureus* C. *Streptococcus mutans* D. *Agrobacterium tumefaciens* E. *Escherichia coli* F. *Klebsiella pneumonia* G. *Aspergillus niger* H. *Candida albicans* and I. *Fusarium oxysporium.*

In the figure 2. M denotes Methanolic Extract, E denotes Ethanolic extract and A denotes Acetonic extract. C is the positive control used as Streptomycin for antibacterial and

Ketoconazole for antifungal testing. In the middle, DMSO or DimethylSulphoxide has been used as negative control in antibacterial as well as antifungal testing.

The results of antimicrobial activities of extracts are given in table 1.

	BACTERIAL and FUNGAL PATHOGENS	DIAMETER OF INHIBITION ZONES (mm)				
			Solvent systems			
		Control	Methanol	Ethanol	Acetone	Ethyl Acetate
1.	*Pseudomonas aeruginosa*	16 ± 1.4	18 ± 0.7	16 ± 0.7	14 ± 0.7	16 ± 1.4
2.	*Staphylococcus aureus*	20 ± 0.7	18 ± 0.7	20 ± 0.7	18 ± 1.4	22 ± 2.1
3.	*Streptococcus mutans*	14 ± 0.0	18 ± 1.4	16 ± 0.7	0.0 ± 0.7	20 ± 1.4
4.	*Agrobacterium tumefaciens*	20 ± 1.4	22 ± 2.1	20 ± 0.7	16 ± 1.4	22 ± 0.7
5.	*Escherichia coli*	24 ± 1.4	20 ± 0.7	20 ± 1.4	18 ± 1.4	22 ± 0.7
6.	*Klebsiella pneumonia*	18 ± 0.7	16 ± 0.7	15 ± 0.0	14 ± 0.7	20 ± 1.4
7.	*Candida albicans*	14 ± 0.7	15 ± 1.4	14 ± 0.7	16 ± 1.4	20 ± 0.7
8.	*Aspergillus niger*	14 ± 0.0	16 ± 0.7	16 ± 1.4	0.0 ± 0.0	18 ± 0.7
9.	*Fusarium oxysporium*	14 ± 1.4	18 ± 0.7	18 ± 1.4	15 ± 0.7	20 ± 1.4

Table 1. Inhibitory zones of extracts of lichen

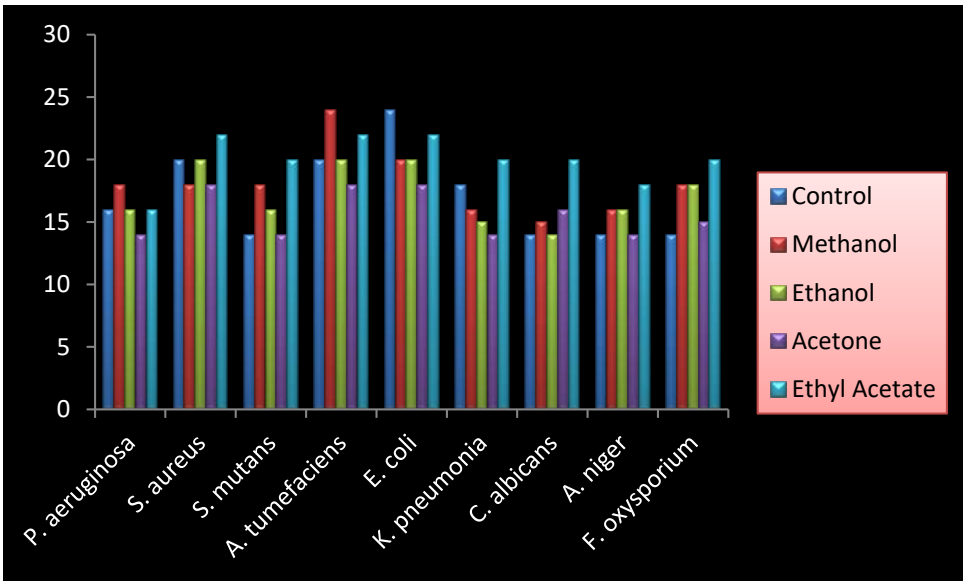
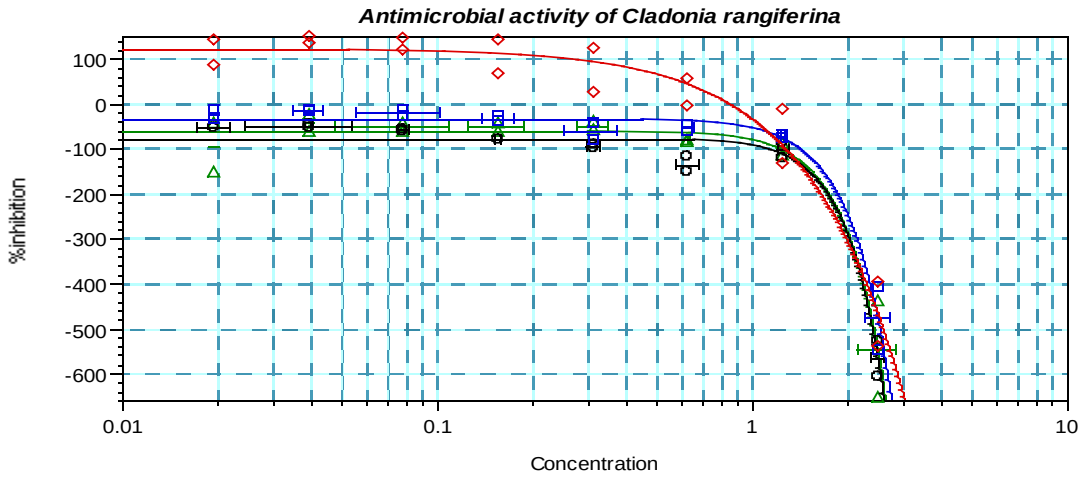


Fig.3 . Yield of concentration of different lichen extracts against 9 pathogens

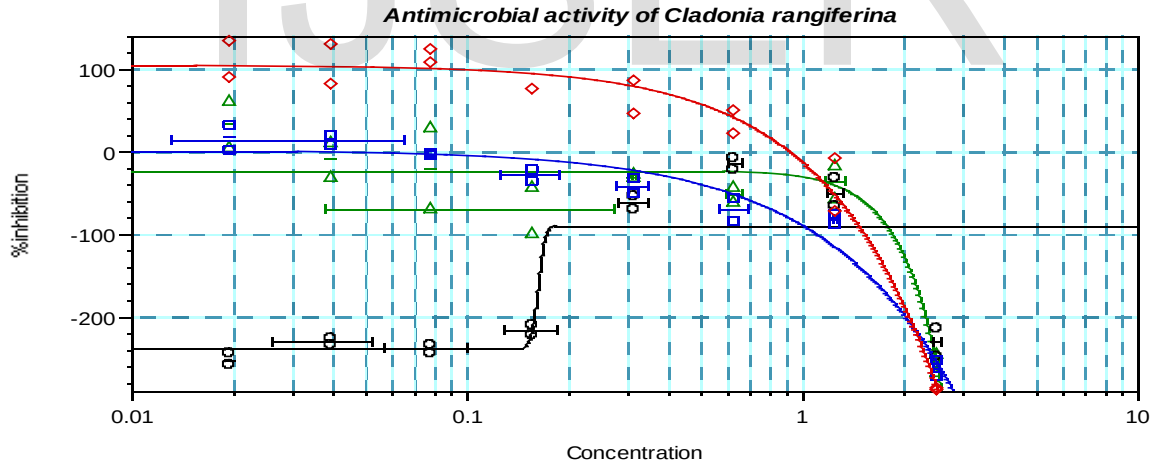
Assayed microtitre plates incubated at 35 + 2 degree Celsius for 24 hours. After incubation, Optical density or OD taken at 492 nm from Spectramax Plus 384 spectro for growth inhibition and quantitative data, in form of IC and MIC (mg/ml). All the results in form of Standard deviation error calculated by Softmax Pro-5 software. Lichens studied for antibacterial against observation on basis of antibacterial susceptibility assay of lichen belongs to family Roccellaceae with acids Roccellic acid, Lecanoric acid, Lepraric acid and Pulvinic acid as Lichen acids. Tested against Bacteria as well as fungi. Lichen was found to have activity only against *Pseudomonas aeruginosa, Streptococcus mutans, Staphylococcus aureus, Agrobacterium tumefacienss, Escherichia coli, Klebsiella pneumonia, Candida albicans, Fusarium oxysporium* with MIC values percentage growth inhibition at various concentrations and graph for growth inhibitory activity as in figures 4, 5 and 6. Absorbance data depicts colour of the drug can be a factor hindering spectrophotometer means of quantitative analysis.



4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
Against Pseudomonas aeruginosa (Group#1: Conce...	-60.7	3.52	61.5	-3.87e+07	0.92
Against Agrobacterium tumefaciens (Group#2: Conc...	-76.7	3.81	58.4	-8.02e+07	0.966
Streptococcus mutans (Group#3: Concentration vs ...	-33.2	3.36	88.5	-7.13e+07	0.959
Plot#4 (group#4: Concentration vs %inhibition)	122	1.45	2.18e+04	-3.03e+08	0.948

Curve Fit Option - Fixed Weight Value

Fig.4. MIC against bacterial pathogens



4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
Against Staphylococcus aureus (Group#1: Concentr...	-22.9	3.7	60.8	-3.13e+07	0.8
Against Escherichia coli (Group#2: Concentration v...	-240	44.1	0.162	-88.9	0.594
Against Klebsiella pneumoniae (Group#3: Concentr...	1.88	1.12	6.05e+05	-2.83e+08	0.941
Plot#4 (group#4: Concentration vs %inhibition)	105	1.31	7.37e+04	-2.72e+08	0.971

Curve Fit Option - Fixed Weight Value

Fig.5. MIC against bacterial pathogens

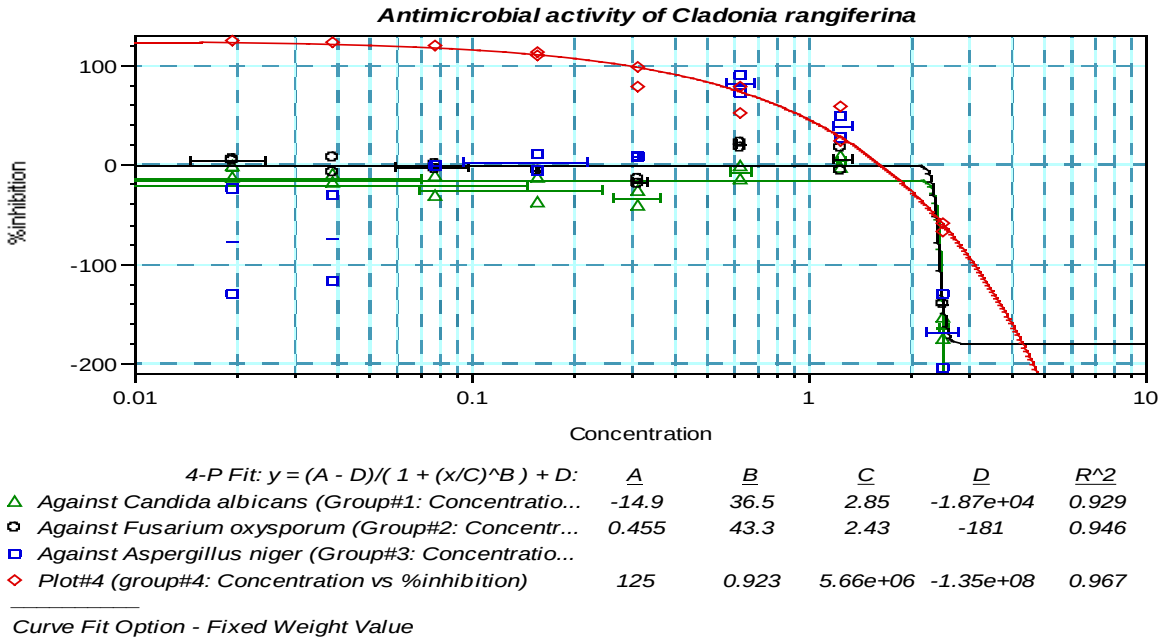


Fig.6. MIC against fungal pathogens

Conclusions

The ethanolic extract (Standard Lichenological Procedure) showed minimum inhibitory effect or activity against the pathogens tested. However, The Methanolic and acetonic extracts showed significant antimicrobial activity while the Ethyl acetate extract showed maximum inhibitory zones against the pathogens tested. Therefore, this study proves the antimicrobial potential of ethyl acetate extracts of *C. rangiferina* and in the discovery of the novel potential biomolecules from lichens , application of different solvents in combination with extraction procedures. Further processing and investigation into fractionation and purification of ethyl acetate extract may result in the isolation of viable alternate source to the presently available antibiotics. Lichens hold great potential that needs to be fully explored and utilized for the benefit of human health and our society. This will definitely provide a new base and ray of light for the future perspectives and highlight the need for further studies of this promising source to harvest more beneficial in the field of bioprospection. This work is intended to contribute in the current research and development trends in the bioprospection of lichens and their bioactive compounds in the applications of commercial interest as well.

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