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Evaluation of antimicrobial property of lichen- *Parmelia perlata*

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The species of *Parmelia* mentioned in India Materia Medica are useful in treating a number of ailments and they are used in large quantities as food supplement in India. In this study, the crude extracts obtained from the *Parmelia perlata* by cold and hot extraction method using different solvents were tested for their antimicrobial activity. Results indicated that the antibacterial activity of *P. perlata* crude extracts was more on *Clavibacter michiganensis*, moderate on *Pseudomonas solanacearum* and it was less on *Escherichia coli* than streptomycin. The *Fusarium oxysporum* and *Rhizopus nigricans* were more susceptible, and *Aspergillus niger* was less susceptible to all crude extracts than bavistin. The antibacterial activity was also high in compound-I and compound-II and it was least in compound-III, than streptomycin. The antibacterial activity of compound-II acted highly against *C. michiganensis* and *P. solanacearum*; it was less against to *E. coli.* The compound-I and compound-III were more active against *C. michiganensis*, moderately active against *P. solanacearum* and less active against to *E. coli* when compared to streptomycin. The antifungal activity was more in compound-II and moderate in compound-I and it was less in compound-III .The compound-II was more active against *F. oxysporum* and *R. nigricans* than compound-I and compound-III, and less active against *A. niger* than bavistin. The compound-I and compound-III did not show any activity against *A. niger.* The overall data presented indicates that the crude and isolated compounds of *P. perlata* extracts have antimicrobial property, in that antibacterial activity was found in all extracts than antifungal activity.

Key words: Antimicrobial activity, extraction, agar diffusion.

INTRODUCTION

Lichens are symbiotic organisms composed of a fungal partner (mycobiont) in association with one or more photosynthetic partners (photobiont/ phycobiont). *Parmelia perlata* also called *Parmotrema chinense* belongs to the family Parmeliaceae. *Parmelia* spp. is mentioned in India Materia Medica as useful in treating a number of ailments. The *Parmelia* are collected in large quantities as a food supplement in India. *P. perlata* used to treat wounds, infections, inflammation, skin diseases, diarrhea, dysentery, cough, fever and renal calculi. *P. chinense* in particular, along with *Parmotrema perforatum*, is used medicinally in India as a diuretic, headache remedy, sedative and antibiotics for wounds. India is bestowed with a wealth of medicinal plants used in ages in Ayurveda and Unani systems of medicines. However, there are no scientific studies where the pigments cells of melanophores and melanocytes have been investigated for the effect of various plants extract (Chaudhari et al., 2012). Despite the remarkable progress in synthetic organic chemistry of the twentieth century, over 25% of the prescribed medicines in industrialized countries are derived directly or indirectly from plants (Dabai et al., 2012). Dependence on plants as the source of medicines is prevalent in developing countries where traditional medicine plays a major role in health cure. Medicinal plants over the years have constituted

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indispensable tools for the research and development of new drugs and medications (Gboko et al., 2012).

P. perlata is lichen growing in rosettes or irregularly spreading over the substratum, giving the appearance of a flower (Gollapudi et al., 1994). It is mainly composed of fungal mycelia which form a network enclosing algal cells or gonidia. This lichen is an astringen, bitter, acrid, cooling, anti-inflammatory and aphrodisiac. *P. perlata* contains many chemicals such as atranorin, lecanoric acid, orcin, erythrolein, azolitmin and spaniolitmint. *P. perlata* extract is one of the most common lichen substances that gave positive patch test reactions in eight subjects in a routine series (Ozdemir, 2003). These subjects also reacted to fumarprotocetraric acid and some of them to evernic acid, stictic acid and usnic acid gave negative reactions. In the present study, the antimicrobial property of crude extracts of *P. perlata* and their isolated compounds were tested against three different bacteria and fungi.

MATERIALS AND METHODS

Collection of samples

The lichens were collected from Bhadra Wildlife Sanctuary of the Western Ghats region of Karnataka, India. The collected lichen species was identified as *P. perlata* (Rajnegi and Gadgil, 1996).

Extraction of lichen

The collected lichen materials were brought to the laboratory, air-dried for three days, cleaned free of any other plant materials or mosses and then washed under running tap water. They were oven dried at 40°C for 42 h and ground into powder by using mixer. The powdered samples were stored in sterilized specimen bottles until when needed. Lichen constituents were extracted by two methods of extraction, viz., cold extraction and hot extraction.

Cold extraction

Briefly, 10 g of lichen powder was added to 200 ml of acetone. The mixture was timed thoroughly by using shaker water bath for 5 h, then left at room temperature overnight and filtered using Whatman No. 1 filter paper. The filtrate was collected and solvent was removed using rotary flash evaporator; about 190 mg residues were recovered. The lichen powder that remained on the filter paper was dried and again extracted using 200 ml methanol. From this solvent, about 150 mg residue was recovered (Tay et al., 2004).

Hot extraction

In brief, 100 g of *P. perlata* sample was extracted in Soxhlet apparatus using petroleum ether, chloroform and methanol based on increasing the polarity of the solvent up to 24 h for one solvent. The solvent was removed under reduced pressure at 40±5°C using rotary flash evaporator.

Thin layer chromatography (TLC)

The crude residue obtained from the cold as well as hot extracts were subjected to thin layer chromatography to determine the compounds present, following the protocols used by Culberson (1972). Starting from low polar solvents to higher polarity, the TLC was carried out using solvents in the following order: carbon tetrachloride, petroleum ether, chloroform, ethyl acetate and methanol. Various ratios among these solvents were used as a mobile phase. Petroleum ether ethyl acetate and chloroform; methanol mixture gave a good separation, whereas extraction from other solvents are having more than one compound. Hence, noting all the readings, the crude extracts were subjected to column chromatography (Sao, 2003).

Column chromatography

Silica gel (120-160 mesh) was chosen as the stationary phase. The gel was dried at 100°C for 12 h to activate it. Then the column was filled with the activated silica gel using petroleum ether. The crude residue from methanol (hot) extract was transformed on the bed of silica gel. At first, the column was run by using petroleum ether to remove chlorophyll and some colored pigments. Then petroleum ether: ethyl acetate in the ratio 9:1 was used to remove some unwanted components, followed by ethyl acetate until all the components in that ratio were eluted. Finally, petroleum ether and ethyl acetate in the ratio 6:4 was used to elute 3 fractions that were collected at interval of 5 ml each and were monitored by thin layer chromatography. These obtained fractions were evaporated to dryness and compounds were stored (Yilmaz, 2003).

Antimicrobial activity

Antibacterial activity

The antibacterial activity of the isolated fractions and crude samples for all extracts were screened by the agar well diffusion method against three bacterial species, *Escherichia coli*, *Clavibacter michiganensis* and *Pseudomonas solanacearum*. These isolates were collected from the Department of Microbiology and Department of Applied Botany, Kuvempu University, which were previously identified by following a standard method (Manojrovic and Gritsanapan, 2002).

Agar diffusion method

A sensitive radial diffusion technique was used for the assessment of antibacterial activity of the test samples. Sterilized nutrient agar medium was poured into sterilized Petri dishes. Nutrient broth containing 0.1 ml of 24 h incubated cultures of the respective bacterial strains was spread separately on the agar median. Wells were made using a stainless steel sterilized cork borer under aseptic conditions. Subsequently, 50 µg/100 µL of isolated fractions and crude extracts were loaded into corresponding wells. The standard antibiotic substance - streptomycin was used (50 µg/100 µL of sterile water) in order to compare the result. The plates were incubated for 24 h at 37°C and the diameter of the zone of complete inhibition of the bacteria was measured around the each well and readings were recorded in millimeters.

Antifungal activity

The antifungal activity of lichen *P. perlata* was screened against three fungi viz., *Aspergillus niger, Rhizopus nigricans* and *Fusarium oxysporum.* Spore suspension of different fungi was prepared by using sterile distilled water and 1 ml of inoculum was added into 10 ± 2 ml of potato dextrose agar at 37±3°C and mixed in Petri plates.

Table 1. Yield of crude extracts.

Extraction method	Sample taken for extraction (g)	Solvent	Yield (g)
Cold	10	Acetone	1.9
		Methanol	1.3
Hot	75	Petroleum ether	1.2
		Chloroform	1.5
		Methanol	2.5

Table 2. Detection of number of compounds in crude extracts by thin layer chromatography.

Crude extract	Solvent	Ratio	No. of compounds
Petroleum ether	Chloroform : Methanol	7:3	1
Chloroform	Petroleum ether : Ethyl acetate	6:4	2
Methanol (hot)	Petroleum ether : Ethyl acetate	7:3	3
Acetone	Petroleum ether : Ethyl acetate	7:3	1
Methanol (cold)	Petroleum ether : Ethyl acetate	6:4	1

After solidification at room temperature for a maximum of 20 min, wells were made in the agar with sterile stainless steel cork borer (d = 4 mm). Then, 5 mg of crude extracts and 5 mg of each isolated fractions were dissolved in 5 ml of each respective solvents, then 50 µg /100 µL of the crude extracts and 50 µg /100 µL of the isolated fractions were loaded in the corresponding wells. Petri plates were incubated for 48 h at 28°C. The standard bavistine was used as reference antifungal substances, and inhibition zones were expressed in millimeters as the diameter of clear zones around holes (Modamombe and Afolayan, 2003).

RESULTS

The crude extracts obtained from the *P. perlata* by cold and hot extraction method using different solvents gave varied amounts of yield. These crude extracts were in different colours and have characteristic odours.

Extraction of lichen

Thin layer chromatography

The crude extracts obtained from all the solvents were subjected to thin layer chromatography. The different solvents in different ratios gave a good separation of compounds (Table 1).

Column chromatography

The crude extract from methanol (hot) extraction was subjected to column chromatography using petroleum ether and ethyl acetate. The 2 different ratios of these 2 solvents separated 3 pure fractions (Table 2). The obtained isolated fractions were named as compound-I, compound-II and compound-III.

Antimicrobial activity

The crude extracts as well as isolated constituents were screened for antimicrobial activity against three different bacteria and fungi by agar well diffusion method. The antibacterial activity was high in chloroform and methanol (hot) crude extracts. It was, however, moderate in acetone and methanol (cold) extracts and least in petroleum ether extract, when compare to standard antibiotic (Streptomycin) (Table 3). The antibacterial activity of *P. perlata* crude extracts was more on *C. michiganensis*, moderate on *P. solanacearum* and it was less on *E. coli* than streptomycin. On the other hand, the antifungal activity was more in acetone (cold), methanol (hot) crude extracts and moderate in petroleum ether chloroform crude extracts and it was less in methanol (cold) crude extracts than bavistine (standard antifungal substance) (Table 4). The *F. oxysporum* and *R. nigricans* were more susceptible and *A. niger* was less susceptible to all crude extracts than Bavistin.

The antibacterial activity was also high in compound-I and compound-II and it was least in compound-III, than Streptomycin (Table 5). The antibacterial activity of compound-II was more against to *C. michiganensis* and *P. solanacearum*; it was less against to *E. coli.* The compound-I and compound-III were more active against *C. michiganensis* and moderately active against *P. solanacearum* and less active against to *E. coli* when compared to *Streptomycin.* Moreover, the antifungal

Table 3. Separation of compounds from methanol (hot) extracts by column chromatography.

Solvent	Ratio	No. of compounds separated	Yield of isolated fractions (mg)	Colour of the compound
Petroleum ether:Ethyl acetate	7:3	1	70	Dark brown powder
Petroleum ether:Ethyl acetate	6:4	2	360	Green crystals
			250	Brown paste

Table 4. Antibacterial activity of crude extracts.

Test organism	Inhibition zone diameter in mm						
	Cold extraction		Hot extraction			F	G
	A	B	C	D	E		
Pseudomonas solanacearum	20	22	5	32	33	35	0
Clavibacter michiganensis	21	9	10	30	33	5	0
Escherichia coli	15	19	9	24	28	40	0

A, Acetone; B, methanol; C, pet ether; D, chloroform; E, methanol; F, streptomycin; G, control.

Table 5. Antifungal activity of crude extracts.

Test organisms	Inhibition zone diameter in mm						
	Cold extraction		Hot extraction			F	G
	A	B	C	D	E		
Fusarium oxysporum	30	11	17	20	26	11	0
Aspergillus niger	11	11	13	12	18	30	0
Rhizopus nigricans	19	9	8	11	20	0	0

A, Acetone; B, methanol; C, pet ether; D, chloroform, E, methanol; F, bavistin; G, control.

Table 6. Antibacterial activity of isolated fractions from methanol (hot) extractions.

Test organism	Inhibition zone diameter in mm				
	C-I	C-II	C-III	Streptomycin	Control
Pseudomonas solanacearum	31	44	25	35	0
Clavibacter michiganensis	28	22	12	5	0
Escherichia coli	21	11	8	40	0

C-I, Compound-I; C-II, compound-II; compound-III.

activity was more in compound-II and moderate in compound-I and it was less in compound-III (Table 6). The compound-II was more active against *F. oxysporum* and *R. nigricans* than compound-I and compound-III and less active against *A. niger* than bavistin. The compound-I and compound-III did not show any activity against *A. niger.* The overall data presented indicates that the crude and isolated compounds of *P. perlata* extracts have antimicrobial property, in that antibacterial activity was found in all extracts than antifungal activity (Table 7).

Spectral analysis

Up on ^{1}H NMR, UV, mass spectra analysis and melting point the isolated compounds from methanol (hot extract) were confirmed to be compound-I (Figure 1). The ^{1}H NMR (Figure 2) is as follows: ^{1}H NMR (DMSO-d_6); δ = 2.26 (S, CH_3); δ = 3.34 (S, OCH_3); δ = 3.78 (S, OCH_3); δ= 6.14 (S, NH_3); δ = 9.96 (S, OH); δ = 10.68 (S, CHO). The chromatogram and diagram of the LC-mass spectra are shown in Figures 3 and 4. The molecular weight of the

Table 7. Antifungal activity of isolated fractions from methanol (hot) extractions.

Test organism	Inhibition zone diameter in mm				
	C-I	C-II	C-III	Bavistin	Control
Fusarium oxysporum	14	40	13	11	0
Aspergillus niger	0	18	0	30	0
Rhizopus nigricans	11	27	0	0	0

C-I, Compound-I; C-II, compound-II and C-III, compound-III.

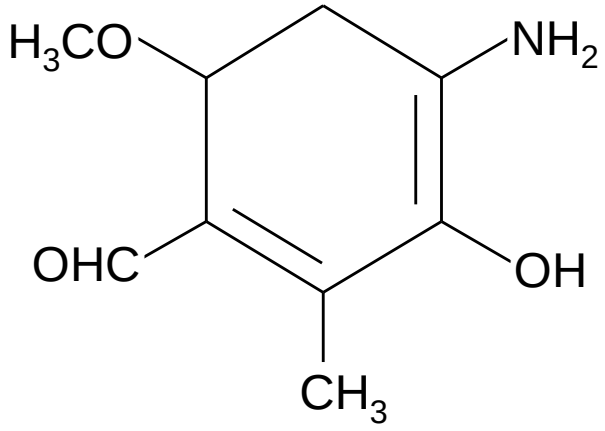


Figure 1. Compound 1, 4-amino-3-hydroxy-6-methoxy-2-methylcyclohexa-1-3-diene-1-carbaldehyde.

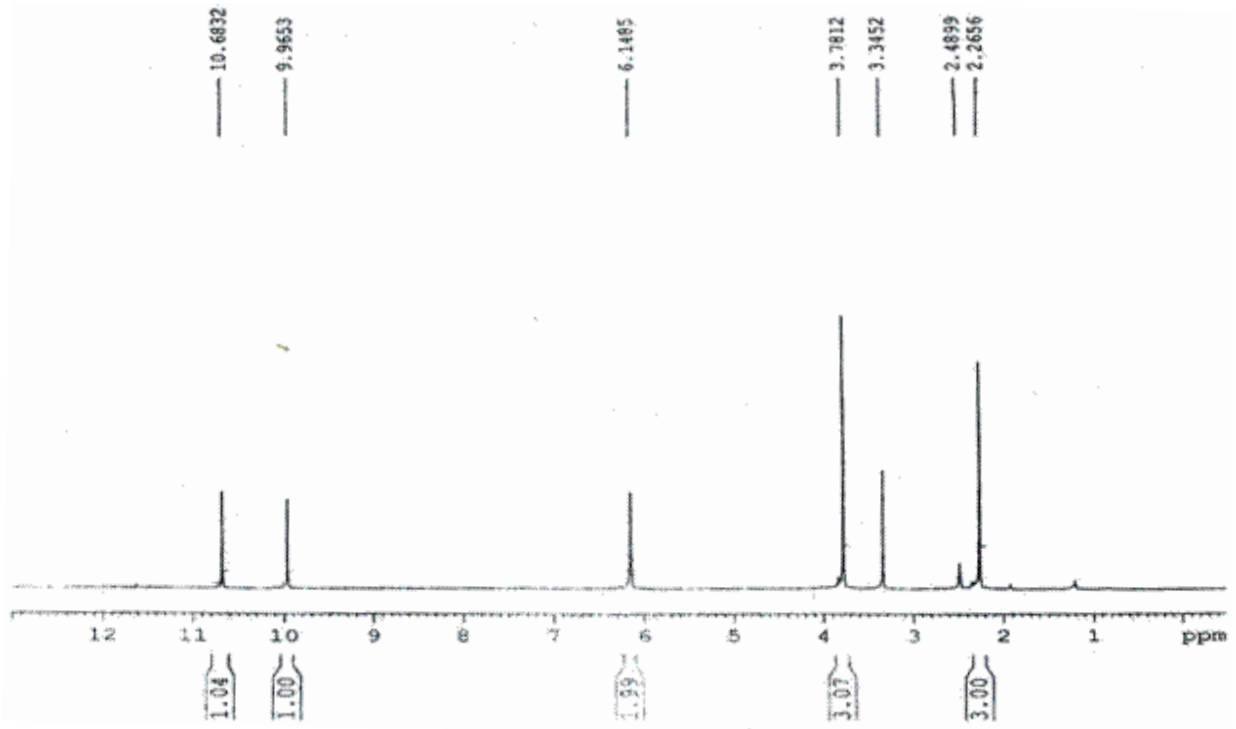


Figure 2. ^{1}H-NMR spectra of compound 1.

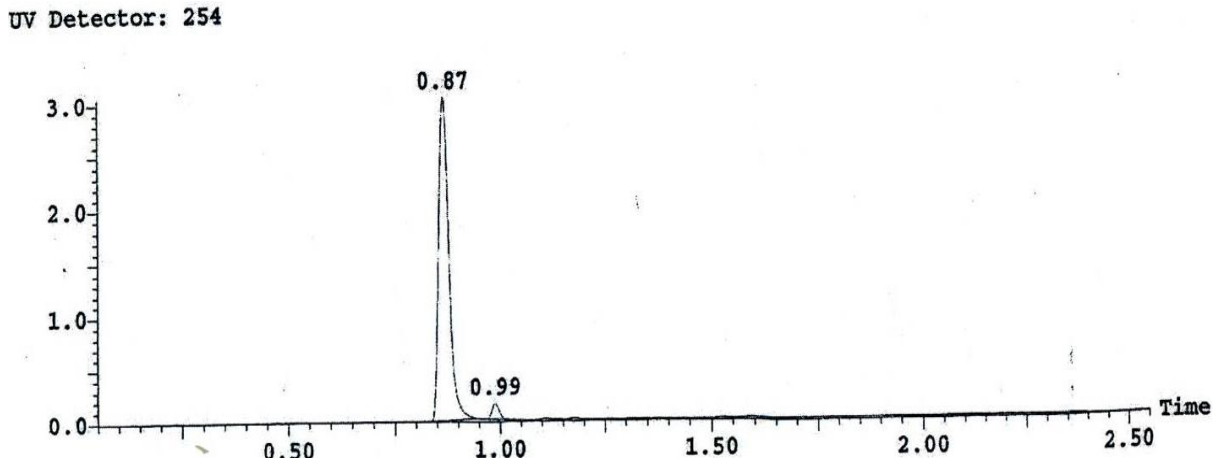


Figure 3. Chromatogram of compound 1.

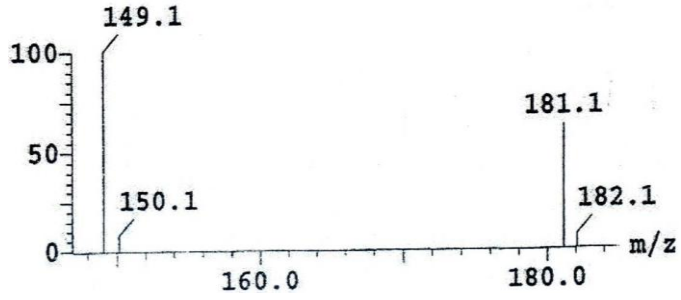


Figure 4. Mass spectra of compound 1.

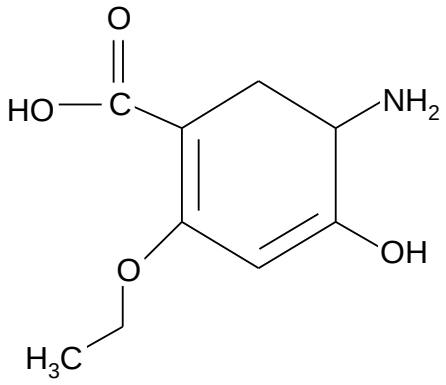


Figure 5. Structure of compound II: 5, amino-2-ethoxy-4-methylcyclohexa-1, 3-diene-1-carboxylic acid.

the compound-I is, LC-ms. = 181.1 m/z $(m+H)^+$, with a melting point of 105°C. The IUPAC name of the compound-I is named as 4-amino-3-hydroxy-6-methoxy-2-methylcyclo-hexa-1, 3-diene-1-carbaldehyde, with molecular formula: $C_9H_{11}NO_3$.

The ^{1}H NMR data for compound II (Figure 6) was obtained as: ^{1}H NMR (DMSO-d_6); δ = 1.62 (S, 3H, CH_3); δ = 2.33 (S, 3H, OCH_3); δ = 2.49 (d, 2HO, CH_2); δ = 6.51-6.33 (d, 2H); δ = 10.00 (S, 1H, OH); δ = 10.33 (COOH). Figures 8 and 9 show the diagram of the chromatogram and LC-mass spectra. The molecular weight of the compound-II is LC-ms = 196.0 m/z $(m+H)^+$ and a melting point of 91°C. The IUPAC name of the compound-II is named as 5, amino-2-ethoxy-4-methylcyclohexa-1, 3-diene-1-carboxylic acid, with molecular formula: $C_9H_{11}NO_4$. The structure of compound-II is shown in Figure 5)

Upon ^{1}H NMR and mass spectra analysis, the isolated compound-III from methanol (hot extract) were confirmed to be 5-methoxy-2-(methoxymethyl)-3 methylpyrazine (Figure-9). The ^{1}H NMR is as follows: δ = 1.222 (S, CH3), δ = 3.51 – 3.65 (m, OCH3), δ= 3.096 (d, CH2, 2H), δ = 4.13 – 4.42 (m, CH) (Figure-10). The diagram of the LC-mass spectra are shown in the (Figure-11 and 12). The molecular weight of the compound-III is LC-ms-m/z =168 (M+H). The IUPAC name of the compound-III is 5-methoxy-2-(methoxymethyl)-3 methylpyrazine, with the Molecular formula: $C_8H_{12}N_2O_2$.

DISCUSSION

Lichen compounds are known to show some biological activities against microorganisms (Hank, 2001). In the present study, the lichen *P. perlata* were taken. It is mainly used for medicinal purpose and as a food supplement in India (Brodo et al., 2001). The extracts of this lichen showed antimicrobial activity against six test organisms. The secondary metabolites of the *P. perlata* are extracellular, low molecular weight crystals and they are insoluble in water and they can be extracted using different organic solvents (Gulluce et al., 2007). The compound extraction from *P. perlata* was done by using 2 different methods – cold extraction and hot extraction

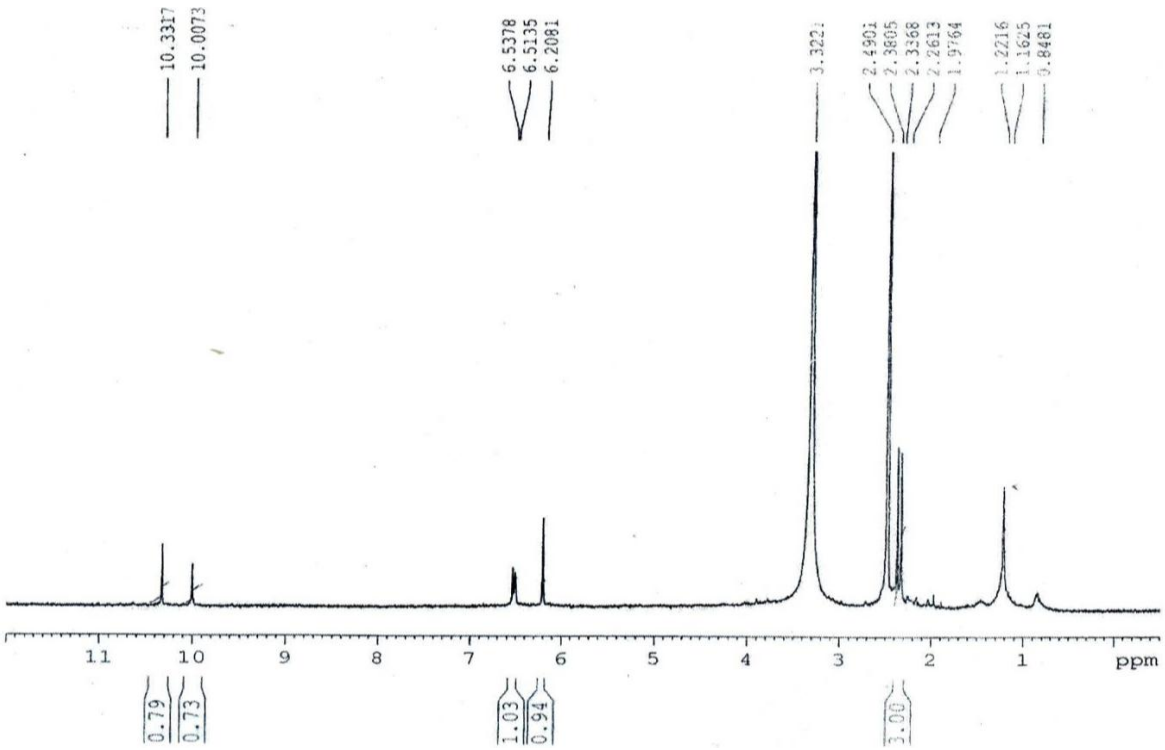


Figure 6. [1]H-NMR spectra of compound II.

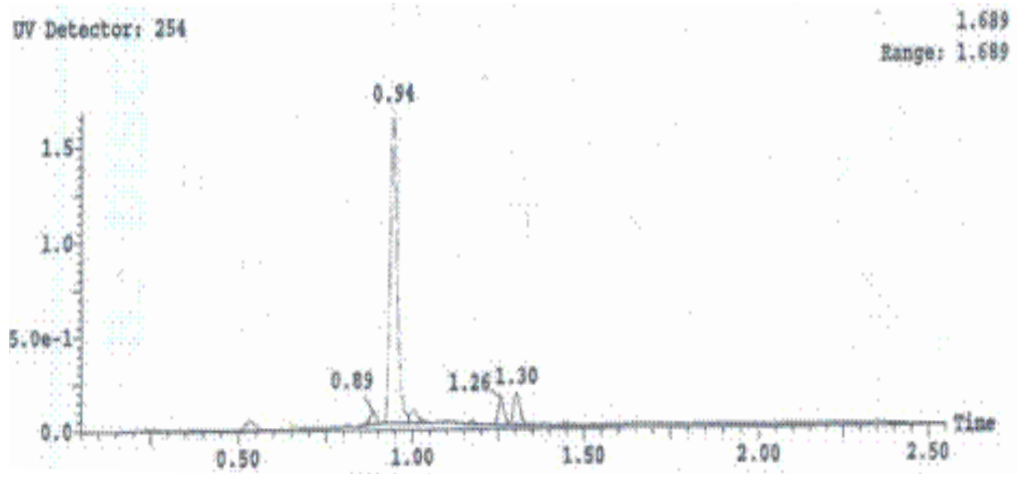


Figure 7. Chromatogram of compound II.

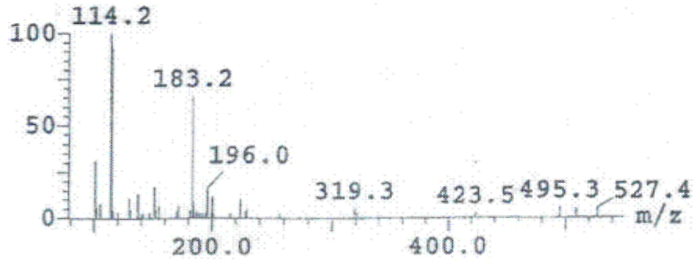
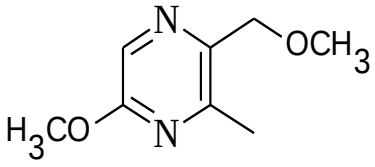


Figure 8. Mass spectra of compound II.



5-methoxy-2-(methoxymethyl)-3-methylpyrazine

Figure 9. Structure of compound-III: 5-methoxy-2-(methoxymethyl)-3 methylpyrazine.

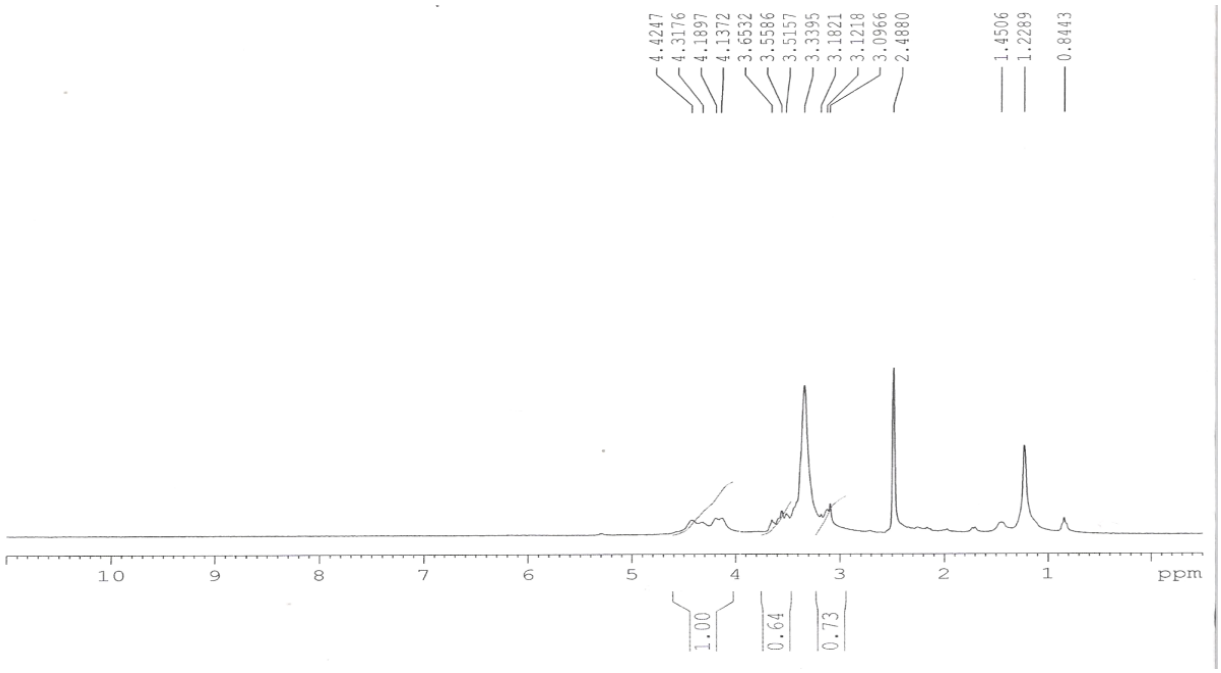


Figure 10. ^{1}H NMR spectra of compound-III of methanol extract.

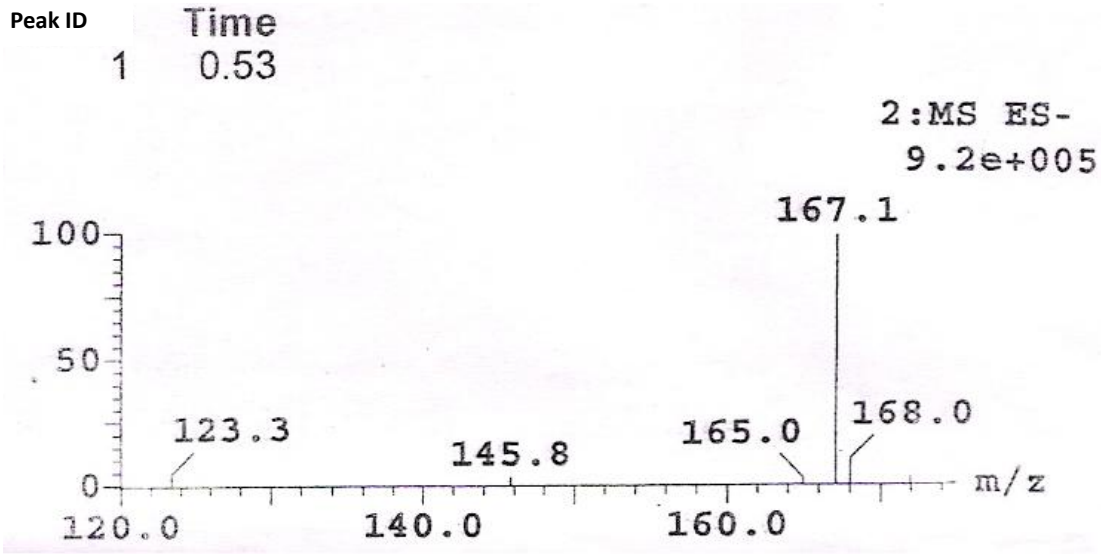


Figure 11. LC-Mass spectra of compound-III of methanol extract.

method. The cold extraction method requires less amount of solvent and less time to perform (Turk et al., 2006), but from this method, a minimum number of compounds can be extracted. Whereas in hot extraction method, more number of compounds can be extracted but it requires more solvents and also more time to perform. The hot extraction was done using Soxhlet apparatus and different solvents based on increased polarity.

In the present study, there was a comparison between these two methods- the antimicrobial activity of methanol extracts (both crude and isolated fractions) from hot extraction was more compared to methanol (cold) extracts because three compounds were extracted by methanol (hot) extraction method, whereas only one compound was extracted by methanol (cold) extraction method. The secondary metabolites of different lichens, the species of

Parmelia, Clodonia etc., showed antibacterial activity against only few Grams positive cocci and anaerobic bacteria like *Clostridium* spp., but it did not show any activity against gram negative rods (Lauterwein and Octhinger, 1995). These results were compared with present study, the extracts of *P. perlata* showed growth inhibition against Gram negative rods. The activity of the extracts against *P. solanacearum* was higher when compared to that against *E. coli*.

The antimicrobial activity of extracts of *Pseudevernia furfuraceae* and their chemical constituents like atranorin, chloroatranorin was not found against *E. coli*, *Pseudomonas* spp. and filamentous fungi (Turk et al., 2006). These results were compared with present study; the antimicrobial activity of *P. perlata* was good against *P. solanacearum* and filamentous fungi like *F. oxysporum* and *R. nigricans*, but less against *E. coli* and *A. niger.* The antimicrobial property of *P. perlata* is due to the presence of compounds like 4-amino-3-hydroxy-6- methoxy-2-methylcyclohexa-1-3-diene-1-carbaldehyde, 5-amino-2-ethoxy-4-methylcyclohexa-1-3-diene-1-carboxylic acid and some other compounds that may also be involved.

Conclusion

The *P. perlata* is one of the important foliose lichen that has antimicrobial activity against different bacteria and fungi. It would be advantageous to standardize the methods of extraction and *in vivo* testing so that the search could be more systematic and it may facilitate to control the pathogenic microorganisms, which have already become resistant to existing antibiotics. Hence, further investigations on the antimicrobial activity as well as the economical and fast isolation of the metabolite from the Lichen are needed.

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