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Antimicrobial property of bioactive factor isolated from *Parmelia perlata*

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Abstract

Parmelia is previously mentioned in India Materia Medica as a treatment for a number of ailments and hence they are being used in large quantities as a food supplement in India. The crude hot extracts of *Parmelia perlata* and the isolated compounds were evaluated for their antimicrobial activity. The antibacterial activity of *Parmelia perlata* crude hot extracts revealed that the extracts showed significant effect against *Clavibacter michiganensis* (33 ± 0.06), moderate against *Pseudomonas solanacearum* (33 ± 0.32) and less potent against *Escherichia coli* (28 ± 0.10) when compared to the standard drug Streptomycin. The *Fusarium oxysporum* (26 ± 0.38) and *Rhizopus nigricans* (20 ± 0.06) were more susceptibility to wards the treatment of hot extracts, whereas *Aspergillus niger* (18 ± 0.15) demonstrated less susceptibility to crude hot extracts, but stardard antifungal drug bavistin was potent against all the fungal pathogens used in the study. Compound-I and compound-II isolated from the crude extract of *Parmelia perlata* showed efficient antibacterial activity. The antibacterial activity of compound-II was significant against to *Clavibacter michiganensis* (22 ± 0.17) and *Pseudomonas solanacearum* (44 ± 0.21), but less against to *Escherichia coli* (11 ± 0.17). Compound-I were more active against *Pseudomonas solanacearum* (31 ± 0.06) and moderately active against *Clavibacter michiganensis* (28 ± 0.05) and less active against to *Escherichia coli* (21 ± 0.23) when compared to *Streptomycin.* The antifungal activity of compound-II was better than compound-I. Compound-II was significant against *Fusarium oxysporum* (40 ± 0.05), *Rhizopus nigricans* (27 ± 0.02) and less active against to *Aspergillus niger* (18 ± 0.02) than compared to compound-I. The compound-I did not impotent against *Aspergillus niger.* The present investigation indicated that the crude hot extracts and the isolated compounds of *Parmelia perlata* have potential antimicrobial property.

Keywords: *Parmelia perlata,* Lichen, Antimicrobial activity, Extraction, Agar diffusion Method

INTRODUCTION

Lichens are symbiotic organisms composed of a fungal partner (mycobiont) in association with one or more photosynthetic partners (Bransislav and Sukdolak, 2007). *Parmelia perlata* is also called *Parmotrema chinense* or Stone flower belongs to family Parmeliaceae. *Parmelia* sp. is mentioned in India Materia Medica as useful in treating a number of ailments (Halama and Haluwin, 2004). The Species of *Parmelia* are collected in large quantities as a food supplement in India. *Parmelia perlata* used to treat wounds, infections, inflammation, skin diseases, diarrhea, dysentery, cough, fever and renal calculi (Tay *et al*., 2004). *Parmotrema chinense* in particular, along with *Parmelia perforatum*, is used medicinally in India as a diuretic, headache remedy, sedative and antibiotics for wounds (Dayan and Joanne, 2001; Zeytinoglu *et al*., 2007). *Parmelia 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. *Parmelia perlata* contains many chemicals, atranorin, lecanoric acid, orcin, erythrolein, azolitmin, spaniolitmint. *Parmelia perlata* extract is one of the most common lichen substances gave positive patch test reactions in eight subjects in a routine series (Ozdemir, 2003; Khatwa *et al*., 1996). 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 *Parmelia perlata* and their isolated compounds were tested against three different bacteria and fungi.

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MATERIALS AND METHODS

Collection of the sample

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

Extraction of lichen

The collected lichen materials were brought to the laboratory and air dried for three days and cleaned free of any other plant materials or mosses and washed under running tap water. They were oven dried at 40^{0}C for 42hrs and ground into powder by using mixer. The powdered samples were stored in sterilized specimen bottles until when needed. Extractions of Lichen constituents were carried out by two methods, viz., cold extraction and hot extraction.

Hot extraction

A 100gm sample was extracted in Soxhlet apparatus using

petroleum ether, chloroform and methanol based on increasing the polarity of the solvent, upto 24 hr for one solvent. The solvent was removed under reduced pressure at 40±5^{0}C using rotary flash evaporator.

Thin layer chromatography

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 i.e. by 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. Extraction from other solvents having more than one compound. Hence, nothing all the readings, the crude extract was subjected to column chromatography (Sao Paulo, 2003).

Column chromatography

Silica gel (120-160 mesh) was chosen as the stationary phase. The gel was dried at 100^{0}C for 12 hours 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 ration 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 on interval of 5ml 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 Dept. 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 hr 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. 50 µg/100µl of isolated fractions and crude extracts were loaded into corresponding wells. The standard antibiotic substance i.e. streptomycin was used (50µg/100µl of sterile water) in order to compare the result. The plates were incubated for 24 h at 37^{0}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 *Parmelia 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 potatodextrose agar at 37±3^{0}C and mixed in Petriplates. After solidification at room temperature for a maximum of 20 minutes, wells were made in the agar with sterile stainless steel cork borer (d=4mm). 5 mg of crude extracts and 5mg of each isolated fractions were dissolved in 5ml of each respective solvents. 50µg /100µl of the crude extracts 50µg /100µl of the isolated fractions were loaded in the corresponding wells. Petriplates were incubated for 48 hrs at 28ºC. The standard Bavistine was used as reference antifungal substances inhibition zones were expressed in millimeters as the diameter of clear zones around holes (Modamombe and Afolayan, 2003; Alexopoulos, 1999).

RESULTS
Extraction of lichen

The crude extracts obtained from the *Parmelia perlata* by hot extraction method using different solvents. These crude extracts were in different colours and have characteristic odours.

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.

Column chromatography

The crude extract from methanol hot extraction was subjected to column chromatography using petroleum ether and ethyl acetate. The two different ratios of these three solvents separate two pure fractions. The obtained isolated fractions were named as 4- amino-3-hydroxy-6-methoxy-2-methylcyclo-hexa-1, 3-diene-1-carbaldehyde (compound-I), and, 5- amino- 2- ethoxy-4-methylcyclohexa-1, 3-diene-1-carboxylic acid (compound-II).

Antimicrobial activity

The crude hot 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 and least in petroleum ether extract, when compared to standard Streptomycin antibiotic. The antibacterial activity of *Parmelia perlata* crude extracts was more on *Clavibacter michiganensis*, moderate on *Pseudomonas solanacearum* and it was less on *Escherichia coli* when compared to Streptomycin (Table-1).

The antifungal activity was more in methanol hot crude extracts and moderate in petroleum ether chloroform crude extracts than Bavistin (standard antifungal substance). The *Fusarium oxysporum* and *Rhizopus nigricans* were more susceptible and

Aspergillus niger was less susceptible to all crude extracts than Bavistin (Table-2).

The antibacterial activity was also high in compound-I and compound-II than Streptomycin. The antibacterial activity of compound-II was more against to *Clavibacter michiganensis* and *Pseudomonas solanacearum*; it was less against to *Escherichia coli.* The compound-I were more active against *Clavibacter michiganensis* and moderately active against *Pseudomonas solanacearum* and less active against to *Escherichia coli* when compared to Streptomycin (Table-3).

The antifungal activity was more in compound-II and moderate in compound-I. The compound-II was more active against *Fusarium oxysporum* and *Rhizopus nigricans* than copared to compound-I and less active against *Aspergillus niger* than Bavistin. The compound-I did not show any activity against *Aspergillus niger.* The overall data presented indicates that the crude and isolated compounds of *Parmelia perlata* extracts have antimicrobial property. In that antibacterial activity was found in all extracts than antifungal activity (Table-4).

Table 1. Antibacterial activity of crude hot extracts

Test organisms	Inhibition zone diameter in mm				
	Hot extraction				
	Petroleum ether	Chloroform	Methanol	Streptomycin	Control
Pseudomonas solanacearum	5 ± 0.10	32 ± 0.53	33 ± 0.32	35 ± 0.06	-
Clavibacter michiganensis	10 ± 0.29	30 ± 0.07	33 ± 0.06	5 ± 0.29	-
Escherichia coli	9 ± 0.32	24 ± 0.07	28 ± 0.10	40 ± 0.07	-

Table 2. Antifungal activity of crude hot extracts

Test organisms	Inhibition zone diameter in mm				
	Hot extraction				
	Petroleum ether	Chloroform	Methanol	Bavistin	Control
Fusarium oxysporum	17 ± 0.25	20 ± 0.33	26 ± 0.38	11 ± 0.07	-
Aspergillus niger	13 ± 0.66	12 ± 0.32	18 ± 0.15	30 ± 0.75	-
Rhizopus nigricans	8 ± 0.17	11 ± 0.25	20 ± 0.06	-	-

Table 3. Antibacterial activity of isolated fractions from methanol hot extraction.

Test organisms	Inhibition zone diameter in mm			
	C-I	C-II	Streptomycin	Control
Pseudomonas solanacearum	31 ± 0.06	44 ± 0.21	35±0.17	-
Clavibacter michiganensis	28 ± 0.05	22 ± 0.17	5±0.11	-
Escherichia coli	21 ± 0.23	11 ± 0.17	40±0.21	-

Note: 4-amino-3-hydroxy-6-methoxy-2-methylcyclo-hexa-1, 3-diene-1-carbaldehyde (Compound-I) and 5-amino-2-ethoxy-4-methylcyclohexa-1, 3-diene-1-carboxylic acid (Compound- II).

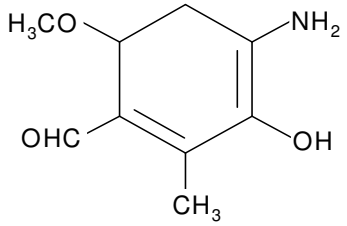
Table 4. Antifungal activity of isolated fractions from methanol hot extraction.

Test organisms	Inhibition zone diameter in mm			
	C-I	C-II	Bavistin	Control
Fusarium oxysporum	14 ± 0.01	40 ± 0.05	11 ± 0.20	-
Aspergillus niger	-	18 ± 0.02	30 ± 0.05	-
Rhizopus nigricans	11 ± 0.21	27 ± 0.02	-	-

Note: Compound-I and Compound-II

Spectral analysis

Upon ^{1}H NMR, UV, mass spectra analysis and melting point the isolated compounds from methanol (hot extract) were confirmed to be, 4-amino-3-hydroxy-6-methoxy-2-methylcyclo-hexa-1, 3-diene-1-carbaldehyde (Compound-I) and 5, amino-2-ethoxy-4-methylcyclohexa-1, 3-diene-1-carboxylic acid (Compound-II) (Figure-1 & 2).



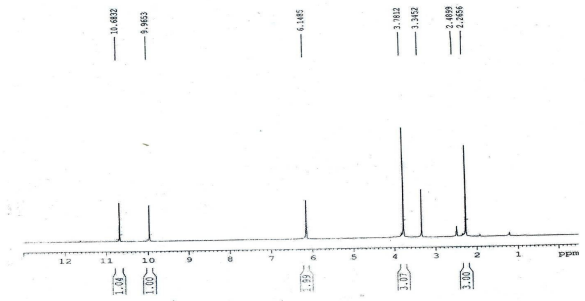


Fig. 3. ^{1}H-NMR spectra of compound-I

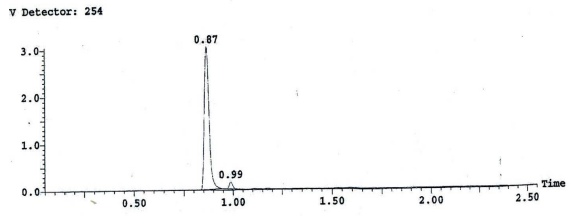


Fig. 4. Chromatogram of compound-I

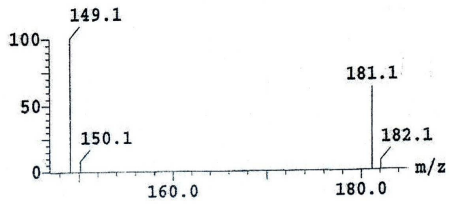
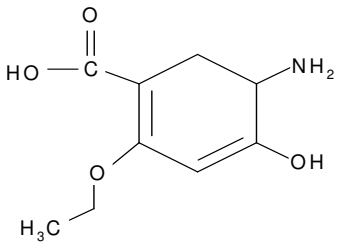


Fig. 5. Mass spectra of compound-I

Fig 1. 4-amino-3-hydroxy-6-methoxy-2-methylcyclo-hexa-1, 3-diene-1-carbaldehyde (Compound-I)



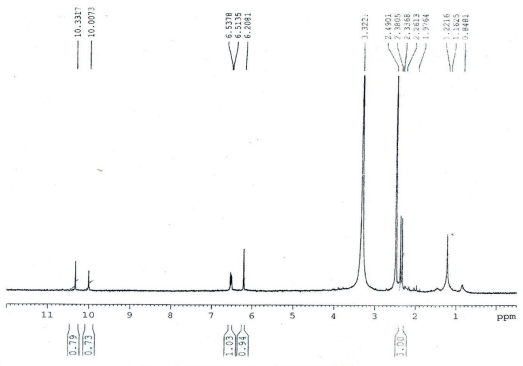


Fig. 6. ^{1}H-NMR spectra of compound-II

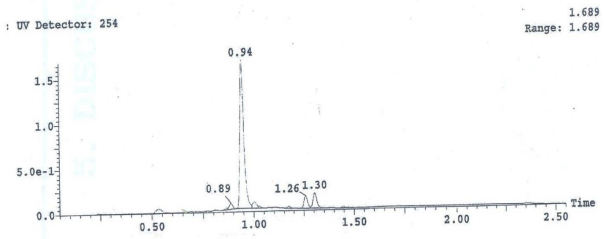


Fig. 7. Chromatogram of compound-II

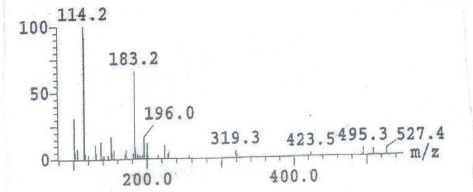


Fig. 8. Mass spectra of compound-II

Figure 2. 5-amino-2-ethoxy-4-methylcyclohexa-1,3-diene-1-carboxylic acid (Compound- II)

DISCUSSION

Lichen compounds are known to show some biological activities against microorganisms (Hank Becker, 2001; Cocchietto *et al*., 2002). In the present study the lichen *Parmelia perlata* was taken and to check the Antimicrobial activity (Ingolfsdottir *et al*., 1985). It is mainly used for medicinal purpose and as a food supplement in India (Bransislav and Sukdolak, 2007). The extracts of this lichen showed antimicrobial activity against six test organisms. The secondary metabolites of the *Parmelia perlata* are extracellular, low molecular weight crystals and they are insoluble in water and they can be extracted using different organic solvents (Gulluce, 2007; Singh *et al*., 2007). The compound extraction from *Parmelia perlata* was done by using hot extraction method (Turk *et al*., 2003). The hot extraction was done using Soxhlet apparatus and different solvents based on increased polarity.

In the present study the antimicrobial activity of methanol extracts (both crude and isolated fractions) from hot extraction method. Two compounds were extracted by methanol hot 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* sp., but it did not show any activity against gram negative rods (Lauterwin *et al*., 1995). The extracts of *Parmelia perlata* showed growth inhibition against Gram negative rods. The activity of the extracts against *Pseudomonas solanacearum* was higher when compared to that against *Escherichia coli*.

The antimicrobial activity of extracts of *Pseudevernia furfuraceae* and their chemical constituents like atranorin, chloroatranorin was not found against *Escherichia coli*, *Pseudomonas* sp. and filamentous fungi (Turk *et al*., 2006; Zovko *et al*., 2001). These results were compared with present study; the antimicrobial activity of *Parmelia perlata* was good against *Pseudomonas solanacearum* and filamentous fungi like *Fusarium oxysporum* and *Rhizopus nigricans*, but less against *Escherichia coli* and *Aspergillus niger.* The antimicrobial property of *Parmelia perlata* is due to the presence of two compounds like 4-amino-3-

hydroxy-6-methoxy-2-methylcyclohexa-1-3-diene-1-carbaldehyde and 5-amino-2-ethoxy-4-methylcyclohexa-1-3-diene-1-carboxylic acid and some other compounds may also be involved.

CONCLUSION

The *Parmelia perlata* is one of the important foliase Lichen, which 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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