Antifungal Activity of a Common Himalayan Foliose Lichen *Parmotrema tinctorum* (Despr. ex Nyl.) Hale.

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Abstract: *In-vitro* antifungal activity of acetone, methanol and chloroform extracts of *Parmotrema tinctorum* (Despr.ex.Nyl.) Hale. was investigated against ten plant pathogenic fungi viz. *Aspergillus niger*, *Aspergillus flavus, Aspergillus fumigatus, Alternaria alternata, Fusarium oxysporum, Fusarium solani*, *Fusarium roseum, Ustilago spp., Albugo candida and Penicillium citrinum* , with reference to commercially available synthetic antifungal drug Ketoconazole (positive control) using disk diffusion assay. Methanol extract was most effective against all investigated fungi followed by acetone and chloroform extract. Principal component analysis (PCA) concluded that though Ketoconazole was effective against five of the investigated fungi, the extracts of *Parmotrema tinctorum* were more effective against rest of the five broad spectrum plant pathogenic fungi (*Aspergillus fumigatus,Fusarium solani, Fusarium roseum, Penicillium citrinum* and *Ustilago spp.*).
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Key Words: *Parmotrema tinctorum*, Principal component analysis, Himalayan lichen, lichen extract, antifungal activity, secondary metabolites.

1. Introduction

Lichens have been used widely in traditional medicines in various parts of the world (Richardson 1991; Perry *et al.* 1999). Lichens produce exclusive characteristic secondary metabolites that are unique with respect to those of higher plants (Hale 1983; Lawrey 1986). Lichen substances are extracellular products of relatively low molecular weight crystallized on the hyphal cell walls, they are usually insoluble in water and can be extracted into organic solvents (Ötzürk *et al*., 1999). Lichen metabolites have shown to have manifold biological and pharmaceutical activity such as antimicrobial, antiviral, cytotoxic, antitumor, allergic, plant growth inhibitory, antiherbivore, ecological roles and enzyme inhibitory (Dülger *et al.* 1997, 1998; Huneck 1999; Ozturk 1999; Aslan *et al.* 2001; Perry *et al.* 1999; Manojlovic *et al.* 2002)

In India Parmeloid lichens are extensively used in traditional medicine to treat several diseases and disorders e.g. headache, skin diseases, urinary trouble, boils, vomiting, diarrhea, dysentery, heart trouble, cough, fever, leprosy and as blood purifier (Chandra and Singh, 1971; Kumar and Upreti 2001). Antibiotic and antifungal activity screenings of Indian lichens have been initiated recently (Shahi *et al.* 2001; Balaji & Hariharan 2007; Sati & Joshi 2011). Lichen screening against plant pathogenic fungi is still an unexplored field.

Parmelioid lichens, which constitute major biomass of lichens in the Himalayan forest, can be used for screening as antifungal agents. Thus, the present investigation was undertaken to evaluate *in-vitro* antifungal activity of common Himalayan Parmelioid lichen *Parmotrema tinctorum*, against ten plant pathogenic fungi.

2. Materials and Methods

2.1Collection and identification of lichen sample

The lichen sample of *Parmotrema tinctorum* were collected from bark in Pithoragarh district of Uttarakhand state in north western Himalaya, India. The identification was done morpho-anatomically using a Labomed ™ stereomicroscope and Leica ™ DM 500 optical microscope and chemically with the help of thin-layer chromatography (Elix *et al.* 1993; Orange *et al.* 2001). Identification was done using relevant key and monographs (Divakar & Upreti 2005; Awasthi 2007) .The voucher specimens were deposited at the lichen herbarium (LWG), National Botanical Research Institute (NBRI), Lucknow, India.

2.2 Extraction from lichen sample

Lichen samples were sorted, cleaned of substratum and dried for extraction. Three different solvent systems i.e. acetone, methanol and chloroform were used for extraction.

Table1. Antifungal activity of Acetone, Methanol and Chloroform extracts of *Parmotrema tinctorum.*

Plant Pathogenic Fungi	**Diameter of inhibition zone (mm)***			
	Acetone	**Methanol**	**Chloroform**	**Ketoconazole**
Aspergillus flavus	12.6±0.3	11.3±1.7	8.0±0.5	20.0±0.3
Aspergillus niger	14.7±0.3	19.0±0.6	0.0±0.0	22.6±0.3
Alternaria alternata	8.3±1.2	08.7±0.3	0.0±0.0	21.0±1.3
Aspergillus fumigatus	12.0±0.5	18.7±0.8	19.6±0.3	10.0±1.2
Fusarium solani	10.3±0.3	17.6±0.3	0.0±0.0	0.0±0.0
Fusarium roseum	14.0±1.5	18.0±0.5	0.0±0.0	0.0±0.0
Fusarium oxysporum	15.6±0.3	17.6±0.3	6.3±0.5	20.0±0.3
Penicillium citrinum	14.3±0.6	24.3±1.2	0.0±0.0	11.0±0.7
Ustilago sp.	28.3±0.8	33.0±1.5	14.7±0.3	11.0±0.3
Albugo candida	13.0±0.6	18.3±1.2	10.0±0.6	20.0±0.6

*values are in Arithmetic mean± Standard error

Lichen substances were extracted using Soxhlet extractor equipped with a reflux condenser (Soxhlet 1879; Harwood & Moody 1989) in selected solvents (acetone, methanol and chloroform) and further recovered through gentle removal of solvents from lichen samples by evaporation using rotary evaporator (Büchi Rotavapor R-200 ^{TM}). The solvent extraction was carried out at the specific boiling temperature of the solvents (acetone-56^{o}C, methanol-65^{o}C and chloroform-61.2^{o}C) for 48h for complete extraction of secondary compounds.

2.3 Microorganisms and media:

Ten plant pathogenic fungal strains were procured from the mycological collection maintained by the Mycological Laboratory, department of microbiology at Kanpur University. The fungi used as test organisms were: *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Alternaria alternata*, *Fusarium oxysporum*, *Fusarium solani*, *Fusarium roseum*, *Ustilago spp.*, *Albugo candida* and *Penicillium citrinum.* Fungal cultures were maintained on Potato Dextrose agar (PDA).

2.4 Determination of antimicrobial activity:

The antimicrobial activity of lichen extracts against test fungi was determined employing disk diffusion method (Kirbey *et al* 1957; Bauer *et al* 1959, 1966; Larkin 1982; National Committee for Clinical Laboratory Standards, 1993). Fungal strains were inoculated onto potato dextrose agar plate (10^8 spores/ml) in triplicate.

Test solutions of lichen substances were prepared by dissolving recovered lichen substances in 10 ml of their respective solvents. Experimental diffusion discs were prepared by loading five milliliters of lichen extract, 1 ml in each load on filter paper disks (6 mm in diameter), allowing the solvent to evaporate between each loading and leaving the lichen extracts on disk without the solvent. All the three lichen extracts (i.e. acetone, methanol and chloroform) were loaded in this manner. Loaded discs were planted on test plant pathogenic culture plate in triplicate. Commercially available synthetic antifungal drug Ketoconazole was used as positive control. The plates were incubated for 5 days at 20^{o} to25^{o}C. Growth was evaluated visually by comparing a particular plate with the control plates. The antimicrobial activity was evaluated by measuring the inhibition zone diameter (in millimeter) observed (National Committee for Clincal Laboratory Standards Nccls Document, 1997).

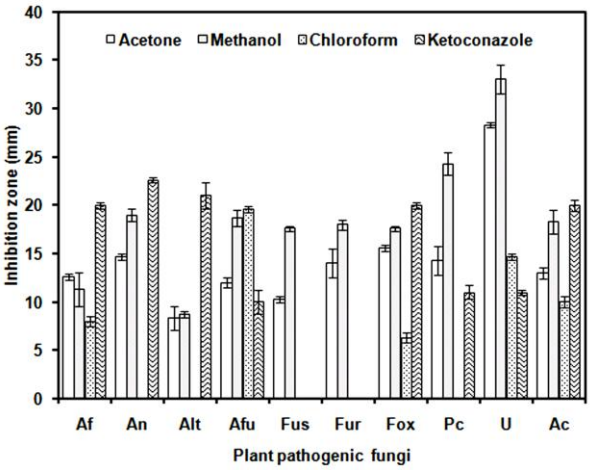

Figure1. Comparative antifungal activity of Acetone, Methanol, and Chloroform extracts of *Parmotrema tinctorum* against selected plant pathogenic fungi (Af=*Aspergillus flavus* , An= *Aspergillus niger*, Alt=*Alternaria alternata* , Afu= *Aspergillus fumigatus*, Fus= *Fusarium solani*, Fur =*Fusarium roseum* , Fox =*Fusarium oxysporum* , Pc= *Penicillium citrinum* , U=*Ustilago* sp., Ac=*Albugo candida*)

2.4 Data analysis:

Indirect gradient ordination method, principal component analysis (PCA) was used to summarise the effect of three solvent extracts of *Parmotrema*

tinctorum on test plant pathogenic fungi with reference to positive control Ketoconazole (Gauch 1982; ter Braak and Prentice 1988). PCA was done on the basis of inhibition zone (mm) produced on test fungi colonies, utilizing correlation matrix in the data set. PC was done using multivar option in PAST 2.07 (Hammer 2001; Hall 2005).

3. Results:

3.1 Differential activity of the extracts:

Disc diffusion assay of different solvent extracts of *Parmotrema tinctorum* showed that acetone and methanol extracts were active against all the test plant pathogenic fungi, while chloroform extract and Ketoconzole exhibited activity against five out of ten pathogenic fungi (Table1, Figure 1).

Methanol extract exhibited highest antifungal activity against all the ten tested fungi followed by acetone extract and Ketoconazole (Table1, Figure 1). The chloroform extract was least active among the three extracts (Table1, Figure 1). All the three test extracts were found effective against five of the broad spectrum plant pathogenic fungi- *Aspergillus flavus, A.fumigatus, Fusarium oxysporum, Ustilago spp* and *Albugo candida.*

Ketoconazole and Chloroform extract of *Parmotrema tinctorum* were found ineffective against *Fusarium solani* and *Fusarium roseum* while acetone and methanol extracts were effective against the two (Table 1, Figure1). Chloroform extract was also found ineffective against *Aspergillus niger*, *Alternaria alternata* and *Penicillium citrinum* (Table 1, Figure1).

The overall antifungal activities of acetone and methanol extracts were better than Ketoconazole whereas chloroform extract was comparatively less effective than the other two. All the three solvents extracts were active against *Ustilago spp*. and exhibited zones of inhibition greater than that of Ketoconazole (Table1, Figure.1).

3.2 Principal component analysis (PCA):

PCA analysis required four components (axis) to account for 100% variance in the data set. The first two components (axis) of PCA explained 81.38 % of variance, and each of the two axis explained 53.67% and 27.72 % variance respectively. The PCA biplot

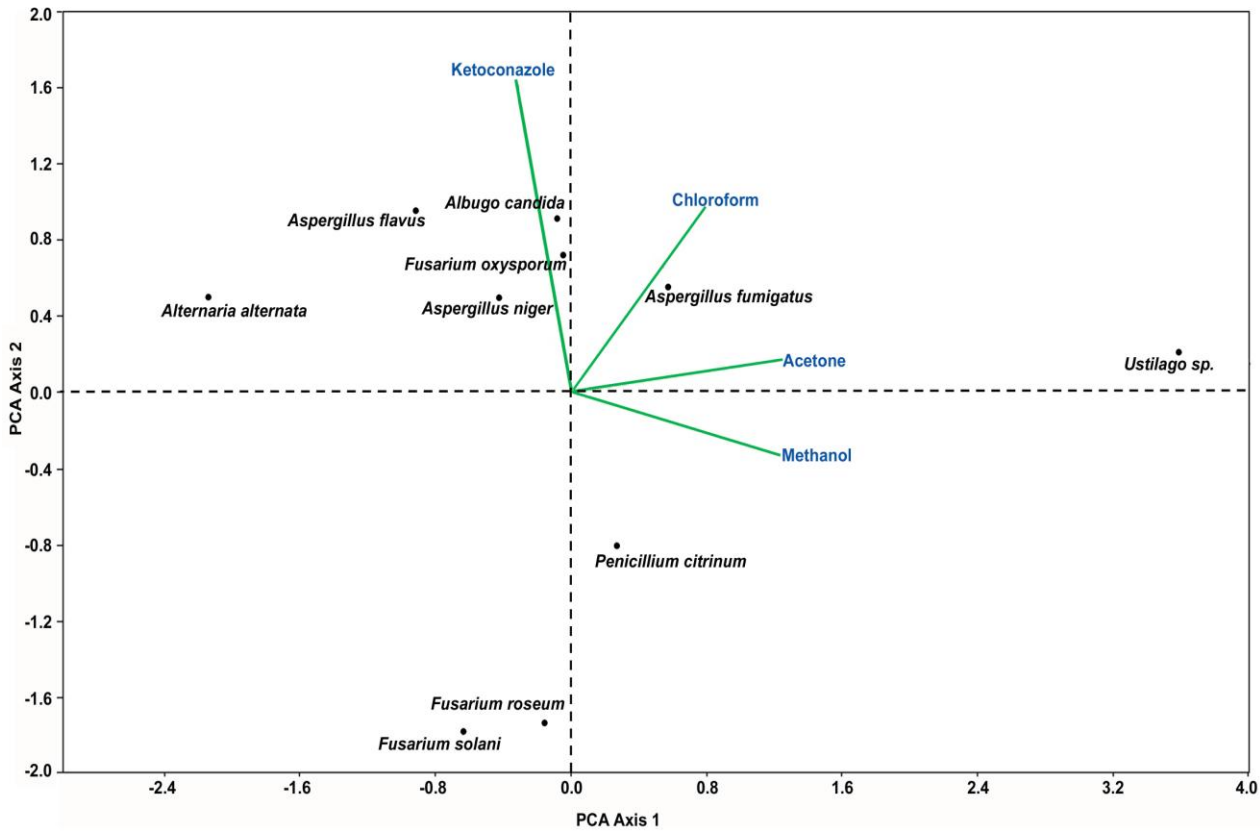

Figure 3: PCA biplot depicting effect of extracts of *Parmotrema tinctorum* against all investigated plant pathogenic fungi.

(Figure 3) shows that though positive control Ketoconazole was more effective against five of the ten test fungi i.e. *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus niger*, *Fusarium oxysporum* and *Albugo candida* lichen extracts were more effective against rest of the five test fungi i.e. chloroform extract against *Aspergillus fumigatus*, acetone extract against *Ustilago spp.* and methanol extract against *Penicillium citrinum*, *Fusarium roseum* and *Fusarium oxysporum*.

4. Discussion:

Lichen substances as bioactive compounds are gaining edge over traditionally known chemicals due to their improved effectiveness over synthetic compounds (Huneck1999). Extracts of lichen thalli proved to have strong antifungal activity against various plant pathogenic fungi (Gulluce *et al.*2006 ; Halama and Van Haluwin 2004).

In present study the comparative better effectiveness of methanol and acetone extract of *Parmotrema tinctorum* against some well known plant pathogenic fungi, can be attributed to lichen substances like lecanoric and orsellinic acid, known for their antifungal properties (Gomes *et al.* 2002; Ranković 2008, 2010)

5. Conclusion:

The selective antifungal effect of acetone and methanol extracts over chloroform extracts can be attributed to differential solubility of constituent secondary metabolites in these extracts (Goel *et.al.*2011 , Halama and Haluwin 2004).

The better performance of lichenic extracts against broad spectrum plant pathogenic fungi (i.e. *Fusarium roseum*, *Fusarium solani*, *Ustilago* and *Penicillium citrinum*) suggests their superior potentials as antifungal substances.

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