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Original Research Article

An *in vitro* analysis of antifungal potential of lichen species *Parmotrema reticulatum* against phytopathogenic fungi

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A B S T R A C T

Parmotrema reticulatum (Taylor) M. Choisy, a common foliose lichen was screened *in vitro* for its antifungal activity against *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum* and *Fusarium solani*. The antifungal properties of lichen were tested in acetone, methanol and chloroform solvents by Disc diffusion method. The percentage inhibition (PI %) was also calculated along with the Minimum Inhibitory concentration (MIC) which was determined by the Broth Tube Dilution method. The acetone extracts exhibited maximum activity followed by methanol against all the pathogens. Least activity was shown by the chloroform extracts. Maximum inhibition was shown by acetone extract against *Fusarium solani* (17.0±0.0) which was higher than the standard control Ketoconazole (15.3±0.2) and the lowest activity was exhibited by chloroform extract against the same pathogen (7.6±0.5). The MIC value ranged from 6.25 to 50 mg/ml while the range for percent inhibition was 35–89%. Thus, the present investigation indicated that the tested lichen extracts have potential antifungal effect and may be exploited further in development of potent antifungal biomolecule.

Introduction

Medicinal plants continue to be major resources for therapeutic compounds and are receiving greater attention, as synthetic drugs and pesticides have emerged to pose harmful for environment and human health. Plant products when compared to their synthetic counterparts minimise the adverse side effects. Moreover, resistance by pathogens to fungicides has rendered certain fungicides ineffective. There is a need to develop new management systems to reduce the dependence on the synthetic agrochemicals. Recent trends favour the use of alternative substances derived from natural plant extracts to control diseases. Similar to higher plants, lichens have been used as natural drugs since antiquity (Barner, 2000).

Lichens are symbiotic associations of a fungus (mycobiont) and a photosynthetic partner (photobiont) which can either be green algae or cyanobacteria (Ahmadjian, 1993). Lichens are well known for their prolific sources of biologically active natural products, as they produce a diverse range of secondary metabolites which are not found in any other plant groups. Slow growth and often harsh living make production of protective metabolites a necessity to lichens and are believed to serve as antigrowth, antiherbivore, antimicrobial (Hale, 1983; Rankovic *et al.,* 2008). The lichen secondary compounds are depsides, depsidones, dibenzofurans and pulvinic acid derivatives having low molecular weight (Turk *et al.,* 2006). The chemically diverse secondary compounds are unique to lichen symbiosis and help the organism to survive in extreme environmental conditions (Oksanen *et al.,* 2006). Lichens synthesize a wide variety of secondary metabolites "lichen substances" mostly from fungal metabolism and are crystallized on the fungal hyphae being extracellular products. They are usually insoluble in water and can be extracted into organic solvents (Otzurk *et al.,* 1999).The antibiotic, antioxidant, antiallergen, antimycobacterial, antiviral, anti inflammatory, cytotoxic, antipyretic and antibacterial activities of the lichen secondary compounds are well known (Molner and Farkas, 2010). Therefore, these compounds attracted great attention of investigators due to their marked medicinal properties as a new significant source of antimicrobial agents (Ingolfsdottir, 1998). India being a megadiversity country exhibit rich diversity of most of the plant groups including lichens. The Himalayan region in northern India and Western Ghats in south India represents luxuriant and diverse growth of a number of lichen species. So far, few lichen species are screened for their antifungal activities from India (Shahi *et al.,* 2001; Behera *et al.,* 2010; Tiwari *et al.,* 2011). Owing to the rich diversity of lichens in the country there is a lot of scope for such studies. Hence, the aim of the present study is to evaluate the fungicidal potential of lichen *Parmotrema reticulatum* especially against phytopathogenic fungi, which are responsible for the huge loss of agricultural yield throughout the world. It is very important to search for new leads to combat plant diseases effectively and lichens can be excellent alternatives for new pesticide development.

Materials and Methods

Collection and Identification of Lichen samples

The Lichen sample of *Parmotrema reticulatum* (Taylor) M. Choisy, growing luxuriantly in temperate and alpine regions of India was collected from bark, rocks and soil in Uttarkashi district of Uttarakhand state in North Western Himalaya, India. The collected samples were identified chemically and morpho-anatomically using relevant literature (Awasthi, 2007; Orange *et al.,* 2001). The voucher specimens of the selected lichen were deposited at the Lichen herbarium (LWG), CSIR- National Botanical Research Institute, Lucknow, India.

Description: *Parmotrema reticulatum* is typically characterized by foliose thalli.

Habitat: corticolous, saxicolous or terricolous, 20 cm across; Lobes 5-10 mm wide, ciliate.

Upper surface: grey to darker, densely white maculate, maculate eventually reticulately fissured; soralia either capitates on short lacinules of palmate lobes or marginal to sub-marginal on rounded or involute lobes.

Lower surface: centrally black, marginal zone white mottled or brown and nude or lower side black, rhizinate upto the margin, medulla white.

Apothecia: rare to 5 mm in diameter, perforate or not, ascospores 15-18×6-10µm.

Spot test: medulla K+ yellow turning red, C-, P+ orange-red.

Secondary metabolites: salazinic acid and consalazinic acids as secondary compounds. The species distributed in tropical regions to sub-temperate regions of the world. (D.D. Awasthi, 2007).

Extraction from Lichen sample

The harvested lichen sample was sorted, cleaned of substratum, washed with distilled water and dried for extraction. The air dried lichen sample was pulverised mechanically to powder, and then extracted using Soxhlet Extractor equipped with a Reflux condenser (Soxhlet, 1879, Harwood and Moody, 1989), in 3 different solvents viz., Acetone, Methanol and Chloroform, differing in polarity. The solvent extraction was carried out at the specific boiling temperature of the solvents, i.e. (acetone- 56ºC, methanol- 65ºC and chloroform- 61.2ºC) for 48 hours for complete extraction of secondary compounds. The crude lichen extracts obtained were filtered and then concentrated to dryness in vacuo at 50ºC using Rotary vacuum Evaporator (Buchi Rotavapour R-200™). Extracts were stored at -80ºC for further assays.

Micro-organisms and media

Four strains of plant pathogenic fungi were used as test organisms in the study, viz., *Aspergillus niger, Aspergillus flavus, Fusarium oxysporum, Fusarium solani,* which were obtained from mycological collection maintained by Babasaheb Bhimrao Ambedkar University, Lucknow. The fungal cultures (slants) were maintained on Potato Dextrose Agar (PDA) and were transferred to Sabouraud Dextrose Broth (SDB) for experimental purposes. All cultures were stored at -4ºC and subcultured every 15 days.

Determination of antifungal activity

A standard Disk-Diffusion method, i.e. the Kirby-Bauer disk diffusion assay (Kirby *et al.,* 1957; Bauer *et al.,* 1959 ; National Committee for Clinical Standards (NCCLS, 1993) was employed to determine the Antifungal activity of acetone, methanol, chloroform extracts of lichen *Parmotrema reticulatum* against selected four test phytopathogenic fungi.

The fungal strains were inoculated onto sterilized potato dextrose agar (PDA media) plate (10 spores/ml). Test solutions of lichen substances were prepared by dissolving recovered lichen extracts in 10ml of their respective solvents. Experimental diffusion disks were prepared by soaking the disks (6 mm in diameter) individually in 50 µl of lichen extract. For each solvent 15 such disks were prepared, allowing the solvent to evaporate between applications and leaving the lichen extracts on disks without the solvent. All the three lichen extracts (i.e, acetone, methanol, chloroform) were loaded following the same procedure. These disks were laid on the test plant pathogenic fungi inoculated plates. Commercially available synthetic standard antifungal drug Ketoconazole was used as positive control. The plates were incubated for 3 days at 25ºC. The antifungal activity was evaluated by measuring the Inhibition zone diameter (mm) observed (NCCLS). All the experiments were performed in triplicates.

Percentage Inhibition of mycelia growth against pathogens by bio-control lichen extracts as compared to control was calculated according to the formula:

Percent Inhibition (PI) = Fungal diameter in control – Fungal diameter in treatment / Fungal diameter in control × 100

Determination of Minimum Inhibitory Concentration (MIC value)

The Minimal Inhibitory Concentration (MIC) is the lowest concentration of a material which inhibits the growth of an organism. It was determined by the standard Broth Tube Dilution method (NCCLS, 1998). A series of dilutions with concentrations ranging from 50 - 0.1 mg/ml was used in the experiment with each extract for every microorganism tested. Two-fold dilution was performed. The boundary dilutions without any visible growth was defined as the minimal inhibitory concentration (MIC) for the tested micro-organism at the given lichen extract concentration. Negative control of solvent influence was realized by parallel. The last test tube carrying no visible growth of micro-organism was rechecked by Agar Plate method in triplicates. The plates were incubated for 48 hours at 27ºC. No growth of micro-organism confirmed the MIC value of the lichen extract. The results were expressed in milligrams per millilitres (mg/ml).

Statistical analysis

The results of experimental antifungal activities are expressed as Mean ± SE of three replicates determinations in each sample. Statistically significant differences among the four fungal pathogens and also among the three extracts used for activity were measured using Two-way analysis of variance (ANOVA).

Results and Discussion

The antifungal potential of differential extracts of *Parmotrema reticulatum* were tested against four major fungal plant pathogens, viz, *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum*, *Fusarium solani*. The results of the screening on test isolates were positive and were effective in inhibiting the mycelial growth of the pathogens to a significant level. The amount of growth inhibition was recorded by calculating the Percentage inhibition (PI) and the data are represented in Table 2.

Among the three solvents used for extraction acetone extracts showed highest inhibition activity followed by methanol while chloroform extract showed the least activity. The assessment of antifungal activity was based on the measurement of Inhibition zone diameter observed (mm). The highest activity exhibited by acetone extract against *Fusarium solani* (17.0±0.0) was higher than the standard control Ketoconazole (15.3±0.2) and the lowest activity was exhibited by chloroform extract against the same pathogen (7.6±0.5).

The acetone extract also showed good activity against *Aspergillus niger* (15.6±0.5), followed by *Aspergillus flavus* (14.0±1.0) and *Fusarium oxysporum* (11.6±1.5). The methanolic extracts showed highest activity against *Aspergillus flavus* (13.6±0.5) followed by *Aspergillus niger* (12.6±1.1), *Fusarium solani* (11.0±1.0) and *Fusarium oxysporum* (10.0±0.0). Among the methanolic extracts, the screening results among the pathogens were in close proximity to eachother with minor variations in contrast to the results given by acetone extracts. Both acetone and methanol extracts showed more or less higher antifungal activity than chloroform extracts. Among

the chloroform extracts the highest activity was exhibited against *Aspergillus flavus* (9.3±0.5) followed by *Aspergillus niger* (8.3±0.5), *Fusarium oxysporum* (8.0±1.0) and *Fusarium solani* (7.6±0.5). The screening results with chloroform extracts against pathogens were more or less similar. As compared to control the acetone extracts showed promising results than methanol and chloroform extracts. The sequence of activity for all the four pathogens is, *Aspergillus niger* □ *Aspergillus flavus* □ *Fusarium solani* □ *Fusarium oxysporum.*

Analysis of Variance (ANOVA) revealed that there were significant differences among the activities of three solvent extracts tested, which proved the effectiveness of the extracts significantly.

The results of Percentage Inhibition (PI %) confirmed that the *Parmotrema reticulatum* extracts tested were significantly effective in inhibiting the mycelia growth of pathogenic micro-organisms. The inhibition percentage as compared to the control ranged from 89 – 35 %. The minimum inhibitory concentration values ranged from 6.25 – 50 mg/ml, showing highest MIC for *Aspergillus niger* (6.25 mg/ml.) and lowest for *Fusarium oxysporum* (50 mg/ml.). *Aspergillus flavus* and *Fusarium solani* showed equivalent MIC of 25 mg/ml (Fig. 2).

The present study revealed and confirmed the presence of fungicidal substances in the tested extracts of lichen *Parmotrema reticulatum.* The antifungal activity with varying zones of inhibition reveals the antifungal potency of the species. The ethnobotanical usage of *Parmotrema reticulatum* is also known, as for preparing tea to relieve discomfort from kidney disorder or veneral diseases. Gupta et al (2007) reported the antibacterial activity of this species against virulent strain of *Mycobacterium tuberculosis*. In this study, all selected fungal pathogens were susceptible to the extract, but showed variations depending on the type of extracting solvent and on the type of lichen species and pathogenic microorganisms selected, since bioactive components of any medicinal plant have different solubility in different extracting solvent (Oloke and Kolawole, 1998). The acetone and methanol extracts were more effective in the inhibition of pathogenic growth as compared to the chloroform extracts. It is suggested that the polar solvents acetone and methanol are most successful in extracting secondary metabolites responsible for antimicrobial property than non-polar solvents (Banso *et al.*, 2007). Due to the rising demand of new environmentally safe drugs and medicines, not only for human as well as plant pathogens there is a need for search of new alternatives to meet the demand which can combat pathogens naturally and effectively. Thus, the *In vitro* evaluation of lichens for their antimicrobial properties is a good initiative towards achieving the goal for developing eco-friendly management of infectious diseases.

It may be concluded from the present study that bioactive compounds from *Parmotrema reticulatum* can be employed in the formulation of antimicrobial agents for the treatment of various pathogenic infections. Furthur studies related to, isolation, identification, purification and characterization of the potential compounds is required to determine the pharmacologically active phytoconstituents responsible for fungicidal activity. Consequently, determination of the therapeutic compounds and the investigation of their full spectrum of efficacy is necessary to be explored, which will be a great help in the field of disease management.

Table.1 Results of zone of inhibition (mm) of extracts of *Parmotrema reticulatum* against tested pathogens

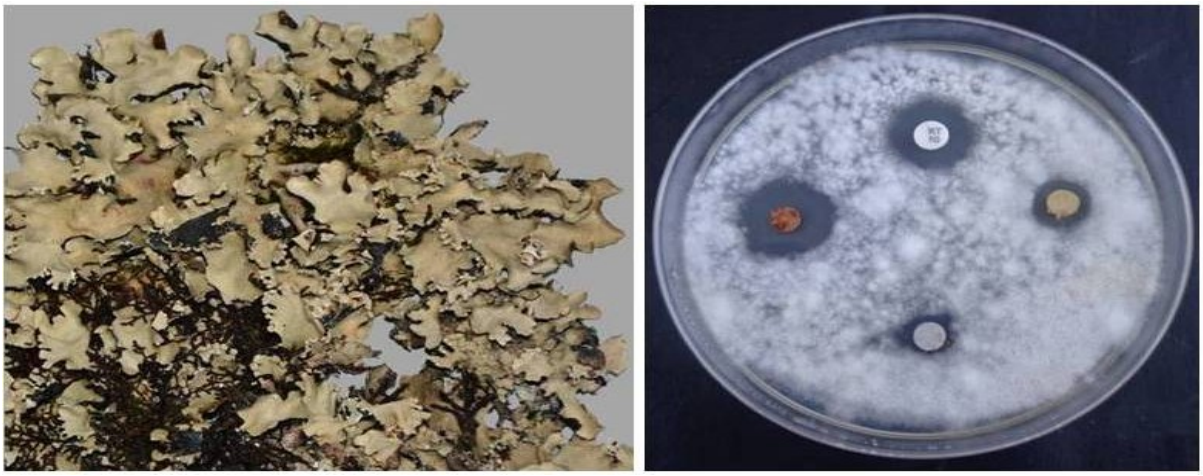
S.No.	Phytopathogenic fungi	*Parmotrema reticulatum* Extracts			Standard
		cetone	Methanol	Chloroform	Ketoconazole
1.	*Aspergillus niger*	15.6±0.5	12.6±1.1	8.3±0.5	24.3±0.3
2.	*Aspergillus flavus*	14.0±1.0	13.6±0.5	9.3±0.5	22.0±0.0
3.	*Fusarium oxysporum*	11.6±1.5	10.0±0.0	8.0±1.0	17.3±0.3
4.	*Fusarium solani*	17.0±0.0	11.0±1.0	7.6±0.5	15.3±0.2

(Values are in arithmetic mean ± standard error) The data analysed statistically by Two-way ANOVA shows significant differences among the activities of the three solvent extracts (F value = 18.77 □ F crit = 3.86).

Table.2 Effect of different solvent extracts of lichen *Parmotrema reticulatum* on growth inhibition of Pathogenic fungi (Percent inhibition of mycelial growth) as compared to control

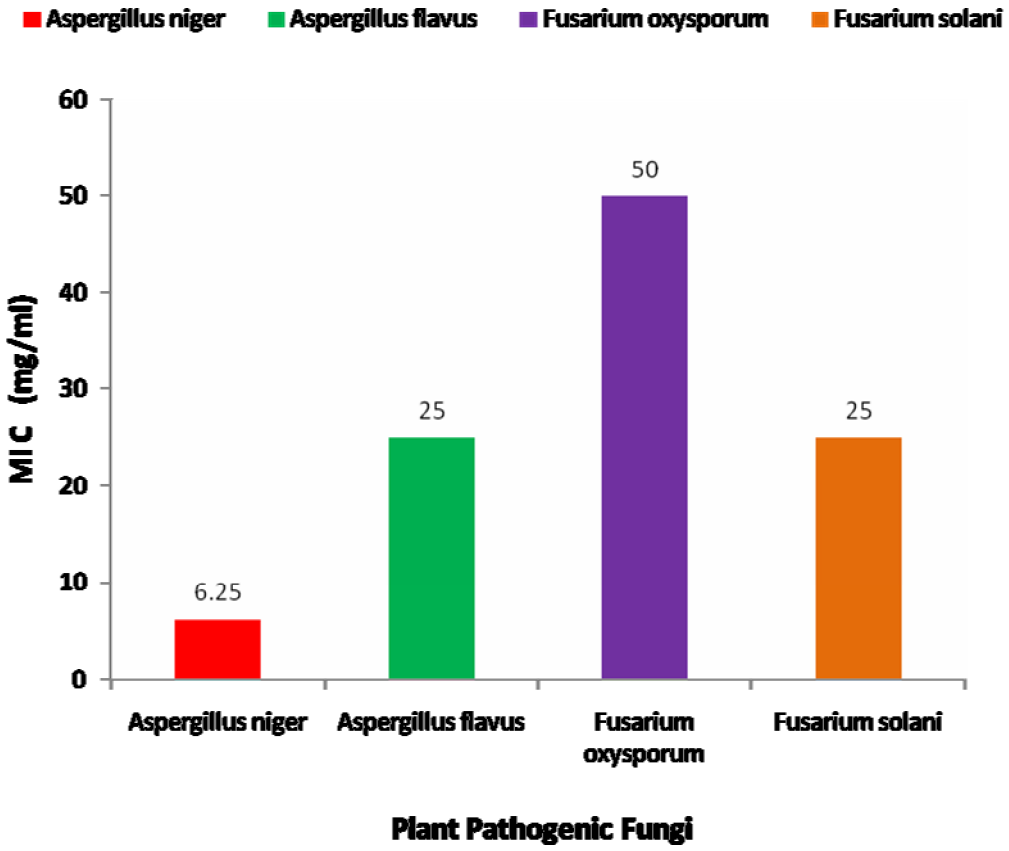
S.No.	**Pathogenic Fungi**	**Inhibition Rate (%) PI**		
		Acetone (values in %)	**Methanol** (values in %)	**Chloroform** (values in %)
1.	*Aspergillus niger*	65	52	35
2.	*Aspergillus flavus*	64	62	43
3.	*Fusarium oxysporum*	68	58	47
4.	*Fusarium solani*	89	72	50

Fig.1 A Thallus of *Parmotrema reticulatum* (Taylor) M. Choisy. B) The inhibition zones of tested lichen extract against pathogen *Fusarium solani*, for which highest activity was recorded in comparison to standard



A) **B)**

Fig.2 Results of Minimum Inhibitory Concentration (MIC in mg/ml.) of *Parmotrema reticulatum* against test four fungal phytopathogens



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