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Original Research Article

Fungicidal Efficacy of a Foliose Lichen *Flavoparmelia caperata* (L.) Hale against Phytopathogenic Fungi

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Abstract

Fungicidal efficacy of lichen *Flavoparmelia caperata* was evaluated against pathogenic strains of fungi- *Fusarium oxysporum*, *Fusarium solani*, *Aspergillus niger* and *Aspergillus flavus.* The methanol, acetone and chloroform extracts were used to determine the antifungal properties of lichen by the Disc-diffusion technique given by Kirby and Bauer and Broth Tube dilution assay proposed by NCCLS. The maximum mycelial inhibition was manifested by acetone extract against *Aspergillus niger* (12.6±0.5 mm) and methanol extract (12.0±0.0 mm) against the same pathogen. The MIC values ranged from 3.12–12.5 mg/ml. The obtained results indicated that acetone and methanol extract of investigated lichen manifested significant and selective antifungal activity which was further statistically analysed by analysis of variance. The results were promising and supported the traditional use of lichens as antimicrobial agents against various pathogenic microorganisms and infections. The efforts of this study reinforce bioprospecting of lichens as a potential drug candidate for the future pharmacological industries.

Keywords

Antifungal
Aspergillus niger
Biocontrol
Flavoparmelia caperata
Lichens

Introduction

Lichens are symbiotic organisms comprised of a fungal partner (mycobiont) and an algal partner (photobiont), which may be either a green algae or cyanobacteria (Nash, 1996) lichens are usually grow on rocks, non-fertile ground, as well as epiphytes on the trees and leaves. These organisms are used for human nutrition, animal nutrition, for getting colours, perfumes and alcohol. Lichens have also, for hundreds of years, been used in many countries as a cure for diseases of humans. For example, *Lobaria pulmonaria* and *Parmelia sulcata* have been used in the treatment of pulmonary and cranial diseases, respectively. Similarly, *Xanthoria parietina* was used to cure jaundice and *Letharia vulpina* in stomach diseases. The usage of some lichens for many years in the traditional medicine

was later justified by numerous researches that confirmed their various biological activities. But as far as plant diseases are concerned the lichens are very less explored and especially, against fungal plant pathogens there is very little information reported till yet. So far, the antifungal properties of lichens have been studied by Land and Lundstrom (1998), Shahi et al. (2001), Halama and Haluwin (2004), Rankovic et al. (2007), Marijana et al. (2010), Verma et al. (2011) and Tiwari et al. (2011).

Lichens hold potential medicinal value because they produce the unique secondary metabolites which are not found in any other plant resources. These secondary metabolites fall into various chemical classes including: diterpene, triterpene, dibenzofuran, depsides, depsidones, anthraquinones, xanthones, usnic acid and pulvinic acid derivatives (Dayan and Romagni, 2001). These metabolites sometimes make even more than 30% of the dry mass of thallus (Galun et al., 1988).

Lichens and their metabolites have manifold biological activities: antioxidant, analgesic, antibacterial, cytotoxic, antimicrobial, antiviral, antibiotic, antitumor, allergenic, plant growth inhibitory, antiherbivore, ecological roles and enzyme inhibitory (Hidalgo et al., 1994; Okuyama et al., 1995; Neamati et al., 1997; Ingolfsdottir et al., 1998; Dayan and Romagni, 2001). It is well known fact that lichens are the resources of promising drugs for many diseases. Several lichen species have been used in folk medicine for treatment of stomach diseases, diabetes, whooping cough, skin diseases (Baytop, 1999; Huneck, 1999; Richardson, 1991). Continuous and uncontrolled use of synthetic drugs has led to the need to find new preparations of natural origin in the control and prevention of various human, animal and plant diseases. It is known that long-term use of synthetic drugs often causes numerous side effects and sometimes resistance. Unlike synthetic drugs, bioactive natural products have beneficial effect on the whole organism and without causing unwanted effects. In search of new bioactive preparations of natural origin, lichens are the subject of many research teams.

India exhibits rich diversity of lichens, found in all phytogeographical regions of the country. Out of more than 2300 species of lichens, the biological screening of only few lichens is available, therefore to search new biomolecules for development of natural and safe products lichens can play a vital role and the present study on *in vitro* antifungal activity of extracts of *Flavoparmelia caperata* against plant pathogenic fungi is initiated with this aim.

Materials and methods

Collection and identification of lichen samples

Samples of lichen *Flavoparmelia caperata* (L.) Hale, were collected from bark and rocks in Uttarkashi district of Uttarakhand state in North Western Himalaya, India (Table 1). The Identification was done 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 (NBRI), Lucknow, India.

Extraction from Lichen sample

The lichen material was collected freshly from nature, cleaned, shade dried, powdered mechanically to a uniform powder, and extracted in 3 different solvents viz, acetone, methanol and chloroform differing in polarity using Soxhlet extraction unit (Soxhlet, 1879; Harwood and Moody, 1989) for 48 h at room temperature at the specific boiling temperature of the solvents, i.e. (acetone: 56ºC, methanol: 65ºC and chloroform: 61.2ºC) for complete extraction of secondary compounds. The lichen extracts obtained were filtered and concentrated to dryness in vacuum under reduced pressure using Rotary Vacuum Evaporator (Buchi Rotavapour R-200™). Extracts were stored at -80ºC for further assays.

Micro-organisms and media

The plant pathogenic fungi used as test organisms in the study, viz., *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum* and *Fusarium solani*, 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 sub-cultured every 15 days.

Determination of antifungal activity

The standard Kirby-Bauer disc diffusion technique (Kirby et al., 1957; Bauer et al., 1959; NCCLS, 1993) was used to test the sensitivity of test pathogenic fungi to the acetone, methanol, chloroform extracts of lichen species *Flavoparmelia caperata*.

The fungal suspensions were inoculated and spread onto sterilized potato dextrose agar plate (10 spores/ml). Test solutions of lichen substances were prepared by dissolving recovered lichen extracts in 10ml of their respective solvents. Experimental diffusion discs were prepared by soaking the discs (6 mm in diameter) individually in 50 µl of lichen extract. For each solvent 15 such discs were prepared, allowing the solvent to evaporate between applications and leaving the lichen extracts on discs without the solvent.

All the three extracts (i.e., acetone, methanol, chloroform) of *Flavoparmelia caperata* were loaded following the same procedure. These discs 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 diameter of the zone of inhibition (mm). All the experiments were performed in triplicates.

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, 2002). 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 parallelly. 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 h at 25ºC. 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 at $p<0.05$ level among the four fungal pathogens and also among the three extracts used for activity were measured using Two-way analysis of variance (ANOVA).

Table 1. Description of lichen *Flavoparmelia caperata* (L.) Hale screened for its fungicidal efficacy.

Morpho-anatomical characters	Description
Habitat	Corticolous, occasionally saxicolous or terricolous.
Thallus	Closely adnate (separation from substrate may lead to tearing through medulla), to 20 cm across; Lobes to 10 mm wide; Upper surface: plicate, pustules on ridges developing into discrete or confluent soralia, soredia granular; Lower surface: black, narrow, marginal zone brownish and shiny; rhizines short, simple; medulla white. Apothecia: rare to 3mm in diameter; ascospores: 16-20×7-10 µm.
Spot test	upper cortex : K −; medulla: K −, C −, KC −, P + orange-red.
TLC	Usnic acid in cortex, atranorin, caperatic and protocetraric acids in medulla.

Results

The fungicidal potential of methanol, acetone and chloroform extracts of lichen *Flavoparmelia caperata* against the tested micro-organisms was estimated on the basis of presence or absence of inhibitory zones, their diameters and values of minimum inhibitory concentration. Extracts of the lichen acted selectively on the fungi tested as the acetone extract showed maximal activity followed by methanol and least activity was given by chloroform extract in relation to the pathogens. All the solvent extracts showed highest activity against *Aspergillus niger*.

The acetone extract acted effectively on *Aspergillus niger* by showing the highest activity with zones of inhibition 12.6±0.5 mm followed by *Fusarium oxysporum* (11.6±0.5 mm) and *Fusarium solani* (10.3±0.5 mm) and lowest for *Aspergillus flavus* (9.0±1.5 mm). For methanolic extract highest activity was manifested by *Aspergillus niger* (12.0±0.0 mm), lowest for *Aspergillus flavus* (8.6±1.0 mm) and moderate activities were observed against *Fusarium oxysporum* (9.3±0.5 mm) and *Fusarium solani* (7.6±0.5 mm). The chloroform extract showed inhibition zone of 6.6±0.5 and 8.6±1.1 mm against *Aspergillus niger* and *Aspergillus flavus* respectively (Table 2).

Table 2. Results of zone of inhibition (mm) of extracts of *Flavoparmelia caperata* (L.) Hale against tested fungal pathogens.

Phytopathogenic fungi	*Flavoparmelia caperata* Extracts			Standard
	Acetone	Methanol	Chloroform	Ketoconazole
Aspergillus niger	12.6±0.5	12.0±0.0	6.6±0.5	24.3±0.3
Aspergillus flavus	9.3±1.5	8.6±0.5	8.6±1.1	22.0±0.0
Fusarium oxysporum	11.6±0.5	9.3±0.5	7.6±1.1	17.3±0.3
Fusarium solani	10.3±0.5	7.6±0.5	7.0±0.0	15.3±0.2

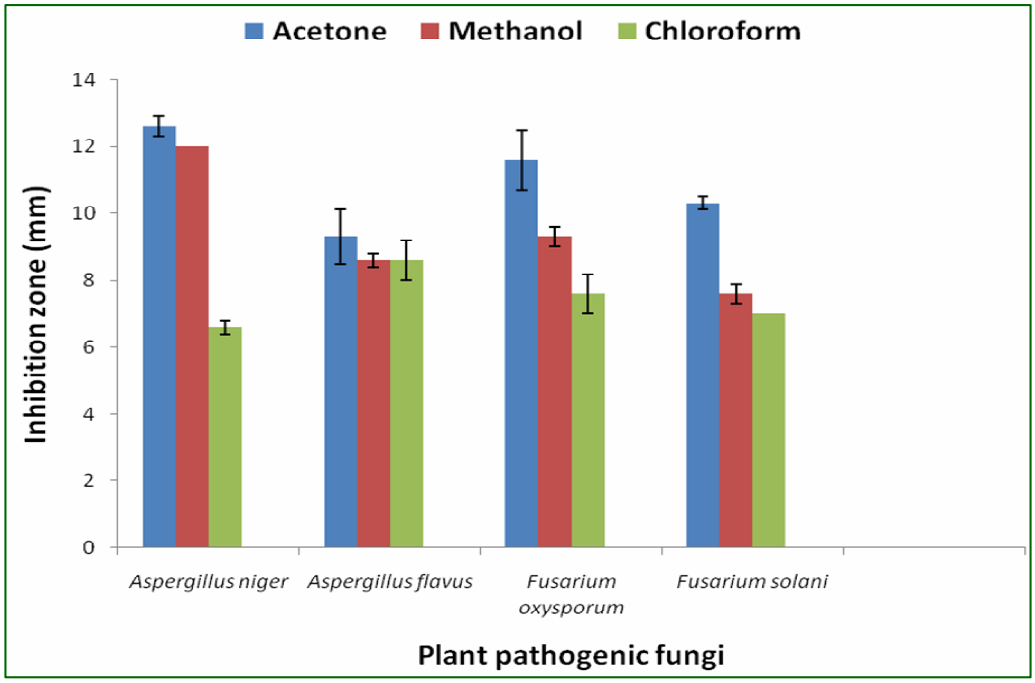
Values are in arithmetic mean ± standard error;
The data analysed statistically by Two-way ANOVA shows significant differences among the activities of the three solvent extracts (F-value = 26.17 > F crit = 3.86).

The comparative antifungal activity of all the solvent extracts of *Flavoparmelia caperata* reveals that there is significant differences among the inhibitory activity of differential solvents and the intensity of pathogenic inhibition shown by the standard was more or less similar to the selected lichen (Fig. 1). The largest area of inhibition zone for ketoconazole was observed against *Aspergillus niger* (24.3±0.3 mm) and *Aspergillus flavus* (22.0±0.0 mm) followed by *Fusarium oxysporum* (17.3±0.3 mm) and *Fusarium solani* (15.3±0.2 mm) having lowest area of inhibition zone. *Aspergillus flavus, Fusarium oxysporum* and *Fusarium solani* showed minimum inhibition (MIC) at a concentration ranged from 6.25–12.5 mg/ml, while *Aspergillus niger* showed significant result at a concentration of 3.12 mg/ml (Fig. 2). Statistical analysis of variance (ANOVA) showed that there is significant difference among the activity of the three solvent extracts tested (F value = 26.17 > F crit = 3.86).

Discussion

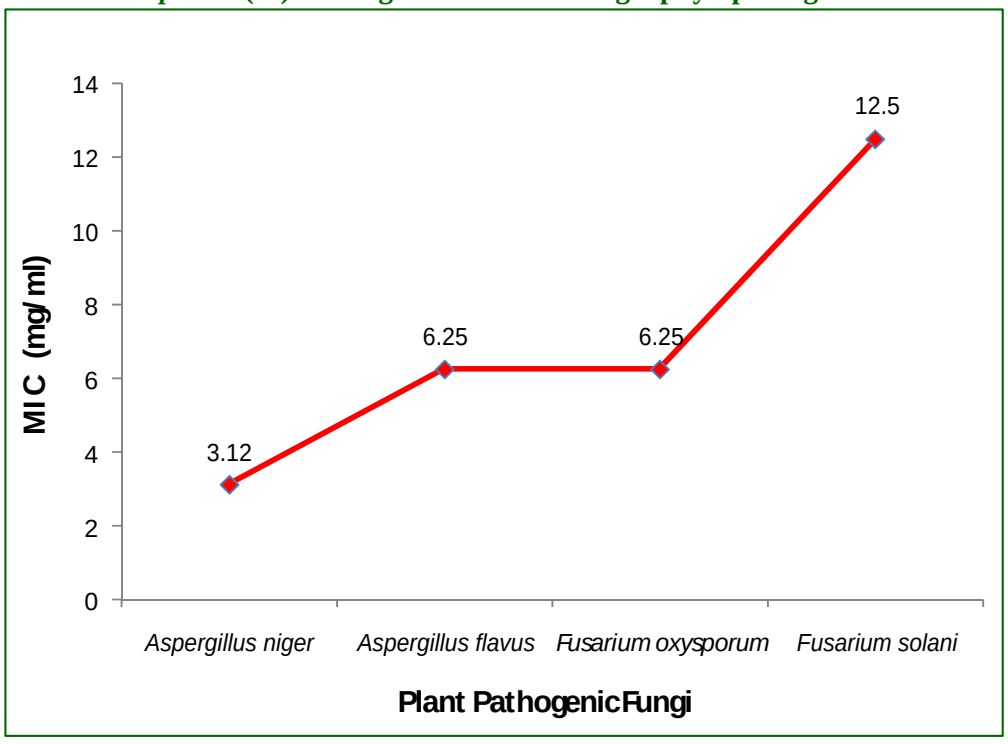
The results obtained in the present study indicate that the lichen *Flavoparmelia caperata* possess potential antifungal properties. The antifungal activity with varying zones of inhibition reveals the fungicidal potency of this lichen which is presumed to be due to the bioactive compounds present in this lichen. Similar to the studies of Madamombe and Afolayan (2003), Yilmaz et al. (2005), Rankovic et al. (2007) the results of this study indicate that differences in antifungal activity between the extracts is mainly dependent on the type of extracting solvent and also on the type of lichen species and pathogenic microorganisms selected. There was variation in the zones of different solvents which indicated variation in the concentration of their chemical constituents extracted. The results are in agreement with the suggestion of Oloke and Kolawole (1998) that bioactive components of any medicinal plant have different solubility in different extracting solvent.

Fig. 1: The data represents the comparative analysis of antifungal activity of *Flavoparmelia caperata* (L.) Hale among the extracts of three test solvents.



(The results are mean values ± SE (error bars) of three independent replicates. Two-way ANOVA shows significant differences among the values of the extracts at $p<0.05$).

Fig. 2: Results of Minimum Inhibitory Concentration (MIC in mg/ml.) of *Flavoparmelia caperata* (L.) Hale against test four fungal phytopathogens.



The antifungal potential of *Flavoparmelia caperata* also studied by Shivanna and Garampalli (2014) showed similar antifungal potential of lichen against *Fusarium oxysporum.* The effective antifungal activity of lichen extracts against phytopathogenic fungi was also carried out by Halama and Haluwin (2004). Shahi et al. (2003) studied strong antifungal activity of *Parmelia cirrhatum* in relation to filamentous fungi. Kumar et al. (2010) also demonstrated antifungal activities of lichens. The lichen compounds (protocetraric acid, caperatic acid, atranorin) present in *Flavoparmelia caperata* showed significant and effective results which suggest the biochemicals of the studied lichen as strong alternative to synthetic drugs. The preliminary antifungal activities studied on the lichen will provide important leads for further investigation for identification, isolation and characterization of active compounds to formulate new and more potent antimicrobial drugs of natural origin which are safe for environment as well as for well-being.

Conclusion

The results of the present study showed that the test lichen extracts demonstrated significant antifungal activity relative to the test pathogenic fungi which could be of significant use in combating plant diseases. Further investigations of antimicrobial potential of this particular lichen in relation to plant as well as human pathogens is required along with the economical and fast isolation of bioactive metabolites from lichens which can be of pharmacological interest.

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