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Assessment of Antifungal Activity of Some Himalayan Foliose Lichens against Plant Pathogenic Fungi

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ABSTRACT

In vitro antifungal activity of the acetone, methanol and chloroform extracts of four lichen species viz, Bulbothirx setschwanensis, Everniastrum nepalense, Heterodermia diademata, Parmelaria thomsonii were investigated against seven plant pathogenic fungi (Aspergillus flavus, A. fumigatus, Alternaria alternata, Fusarium oxysporum, F. solani, F. roseum and Penicillium citrinum) with reference to commercially available synthetic antifungal drug Ketoconazole (positive control). Lichen secondary metabolites were extracted using Soxhlet extractor and were further recovered through gentle evaporation of solvents in rotary evaporator. Antifungal activity was analysed employing Bauer-Kirby disc diffusion assay. Acetone and methanol extracts of lichenized fungi were found more effective against tested plant pathogenic fungi. Principal component analysis concluded that though, Ketoconazole was effective against four of the tested plant pathogenic fungi, acetone and methanol extracts of lichens were comparatively more effective against some broad spectrum plant pathogenic fungi (Fusarium oxysporum, F. solani, F. roseum).

***Keywords*:** *Acetone Extract, Antifungal Activity, Bauer-Kirby Disc Diffusion Assay, Foliose, Himalayan Lichen, Methanol Extract, Rotary Evaporator, Secondary Metabolites, Soxhlet Extractor*

1. Introduction

The utilization of lichen in medicine has been cited in different pharmacopoeias of the world. During the middle-ages lichens figured prominently among the herbs used by medicinal practitioners [1]. *Lobaria pulmonaria*, *Cetraria islandica*, and *Cladonia* species are reported to be effective in the treatment of pulmonary tuberculosis [2].

Lichens synthesize a wide range of primary (polysaccharides) and secondary organic compounds that show manifold bioactivities from nematocidal, antimicrobial, cytotoxic, antimutagenic and antiproliferative to immunostimulatory effects [3-5]. Out of 700 secondary metabolites so far known from lichens 550 are unique to them [6]. The lichen extracts and their components have a distinguished antimicrobial activity [2,5,7-9]. On the other hand, it is well known that microorganisms have well developed resistance to many antibiotics. This creates enormous problems in the treatment of infectious disease, and investigators therefore seek new antimicrobial substances from different sources so new sources of bioactive substances have been searched for, such as medicinal herbs, fungi and lichens [10,11].

India being a mega diversity country exhibit rich diversity of different plant groups.The medicinal properties of higher group of plants is well known from the country. However, despite of the manifold medicinal uses of lower plants by traditional and ethnic groups the medicinal potential of these plants are not studied upto a greater extent. Thus, present study was done to evaluate the *in vitro* anti- fungal activity of four foliose lichens *Bulbothrix setschwanensis* (Zahlbr.) Hale, *Parmelaria thomsonii* (Stirton) D.D. Awasthi, *Everniastrum nepalense* (Taylor) Hale, *Heterodermia diademata* (Taylor) D.D. Awasthi, against some common plant pathogenic fungi.

2. Materials and Methods

2.1. Collection and Identification of Lichen Samples

The lichen specimens of *Bulbothrix setschwanensis* (Zahlbr.)

Hale, *Parmelaria thomsonii* (Stirton) D.D. Awasthi, *Everniastrum nepalense* (Taylor) Hale, *Heterodermia diademata* (Taylor) D.D. Awasthi, growing luxuriantly in temperate regions of India were collected from different locations of Pithoragarh district, Uttarakhand. 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 [12,13]. Identification was done using relevant key and monographs [14,15]. 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. Lichen substances were extracted using Soxhlet extractor equipped with a reflux condenser [16,17] 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™). The solvent extraction was carried out at the specific boiling temperature of the solvents (acetone-56˚C, methanol-65˚C and chloroform -61.2˚C) for 48 h for complete extraction of secondary compounds.

2.3. Microorganisms and Media

Seven fungal strains were procured from the mycological collection maintained by the Mycological Laboratory within the Department of microbiology Kanpur University. The fungi used as test organisms were: *Aspergillus flavus*, *Aspergillus fumigatus*, *Alternaria alternata*, *Fusarium oxysporum*, *Fusarium solani*, *Fusarium roseum and Penicillium citrinum.* Fungal cultures were maintained on Potato Dextrose agar (PDA) and were transferred to Sabourad dextrose agar for experimental purposes.

2.4. Determination of Antimicrobial Activity

The antimicrobial activity of lichen extracts against test fungi was determined employing disk diffusion method [18-21]. Fungi strains were inoculated on 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 fungi culture plate in triplicate. Commercially available synthetic antifungal drug Ketoconazole was used as positive control. The plates were incubated for 5 days at 20˚C to 25˚C. Growth was evaluated visually by comparing a particular plate with the negative control plates (having only plant pathogenic fungi). The antimicrobial activity was evaluated by measuring the inhibition zone diameter (in millimeter) observed (National Committee for Clincal Laboratory Standards Nccls Document [22]).

2.5. Data Analysis

Indirect gradient ordination method, principal component analysis (PCA) was used to summarise the effect of three solvent extracts of test lichens on test plant pathogenic fungi with reference to positive control Ketoconazole [23, 24]. PCA was done on the basis of inhibition zone (mm) produced on test fungi colonies, utilizing correlation matrix, using multivar option in PAST 2.09 [25,26].

3. Results

3.1. General Patterns of Antifungal Activity

Disc diffusion assay of the crude extracts of all the four lichens *Bulbothrix setschwanensis*, *Parmelaria thomsonii*, *Heterodermia diademata* and *Everniastrum nepalense* showed antifungal activity against most of the tested fungi (**Figures 1** and **2**). A differential activity of the three extracts (*i.e.* Acetone, Methanol and Chloroform) was observed. Among the three acetone and methanol extracts were more effective than the chloroform extract.

Lichen extract in acetone was found more effective in *Bulbothrix setschwanensis*, *Parmelaria thomsonii* and *Heterodermia diademata*, whereas in *Everniastrum nepalense* methanol extract was found more effective than other solvents. Positive control Ketoconazole was ineffective in case of *Fusarium roseum*, *Fusarium solani* and *Fusarium oxysporum* whereas lichen extracts were found active against these pathogens. The chloroform extracts of all the four lichens though were sporadically more effective than some extracts but overall Chloroform extracts were not as effective as acetone and methanol extracts.

The acetone and methanol extracts of *Bulbothrix setschwanensis* showed activity against *Fusarium roseum* and *Fusarium solani* while the chloroform extract and the positive control Ketoconazole exhibited no activity. The methanol extract of *Bulbothrix setschwanensis* showed maximum zone of inhibition of 15 mm while the Ketoconazole exhibited 20 mm zone of inhibition against *Penicillium citrinum*. The chloroform extract exhibited no activity against *Fusarium solani*, *Fusarium roseum* and *Alternaria alternata*.

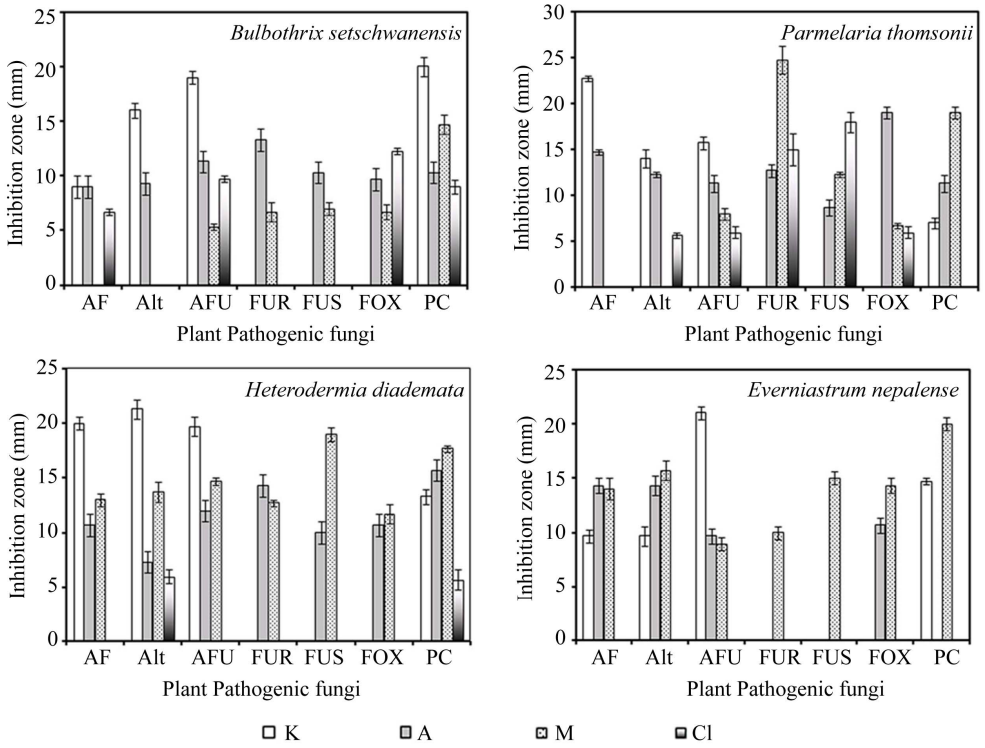

Figure 1. Results of comparative antifungal screening of the different solvent extracts (A = Acetone, M = Methanol, and Cl = Chloroform) of Himalayan foliose lichens and commercially available fungicide Ketoconazole(K), against selected plant pathogenic fungi (AF = *Aspergillus flavus*, Alt = *Alternaria alternata*, AFU = *Aspergillus fumigatus*, FUR = *Fusarium roseum*, FUS = *Fusarium soloni*, FOX = *Fusarium oxysporum*, and PC = *Penicilium citrinum*). Reported values are in Arithmetic mean ± Standard error.

The acetone extracts of *Parmelaria thomsonii* were active against all the seven tested fungi. Though all the three lichen extracts in acetone methanol and chloroform exhibited inhibition zones of 19 mm, 7 mm and 6 mm respectively, the positive control showed no antifungal activity against *Fusarium oxysporum*. The maximum zone of inhibition 24 mm exhibited by methanol extract of *Parmelaria thomsonii* against *Fusarium roseum* was better than Ketoconazole. No activity was recorded by Methanol and chloroform extracts against *Aspergillus flavus*, *Alternaria alternata* and *Penicillium citrinum*.

Acetone and methanol extracts of *Heterodermia diademata* were active against all the seven tested fungi while the chloroform extract showed activity against only two pathogenic fungi *Alternaria alternata* and *Penicillium citrinum*. The maximum zone of inhibition 19 mm was exhibited by methanol extract.

The methanol extracts of *Everniastrum nepalense* showed antifungal activity against all the tested fungi, while acetone extract exhibited activity against only four pathogenic fungi. Maximum zone of inhibition 20 mm was showed by methanol extract which is better than the inhibitory zone of Ketoconazole 15 mm against *Penicillium citrinum*.

3.2. Principal Component Analysis (PCA)

PCA analysis required four components (axis) to account for 100% variance in dataset for all the four lichenized fungi. The first two components (axis) of PCA explained maximum variation (*Bulbothrix setschwanensis*-72%; *Parmelaria thomsonii*-83%; *Heterodermia diademata*-70%; *Everniastrum nepalense*-90%) and were taken into account in the study (**Figure 3**). PCA biplots concluded that though positive control Ketoconazole showed higher degree of antifungal activity against some of the plant pathogenic fungi (*Alternaria alternata*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Penicillium citrinum*), the lichen extracts were more effective against broad spectrum plant pathogenic fungi (*Fusarium oxysporum*, *F. roseum*, *F. soloni* (**Figure 3**).

4. Discussion

Bioactive compounds in recent past are gaining edge over traditionally known drugs because of their improved effectiveness against pathogens, the lichen compounds are not an exception in this field [27]. Extracts of lichen thalli proved to have strong antifungal activity against various plant pathogenic fungi [9,28].

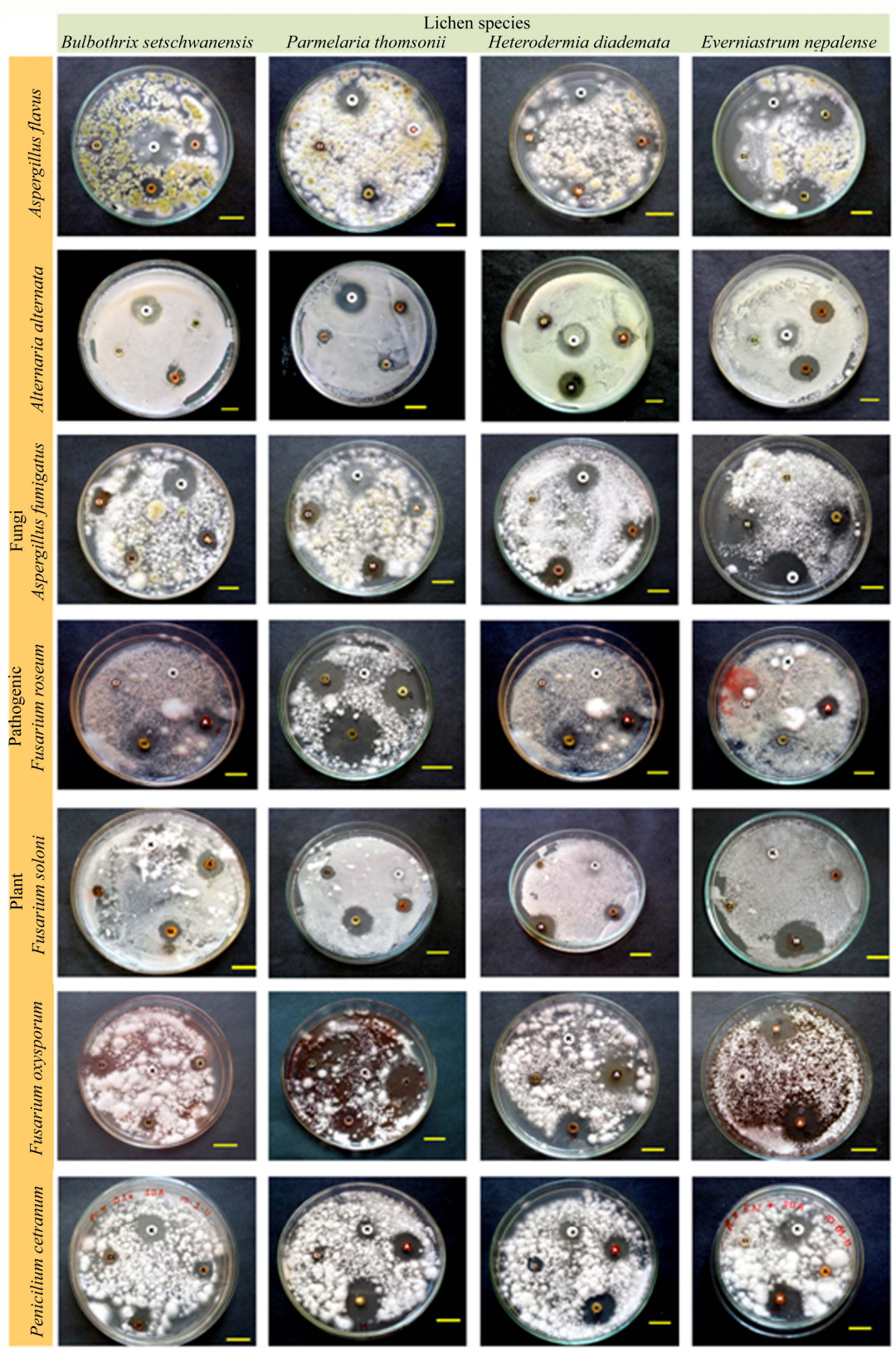

Figure 2. The inhibition zones of tested lichen extracts (A = Acetone, M = Methanol and Cl = Chloroform) against selected plant pathogenic fungi and commercially available fungicide Ketoconazole (K). Bar = 10 mm.

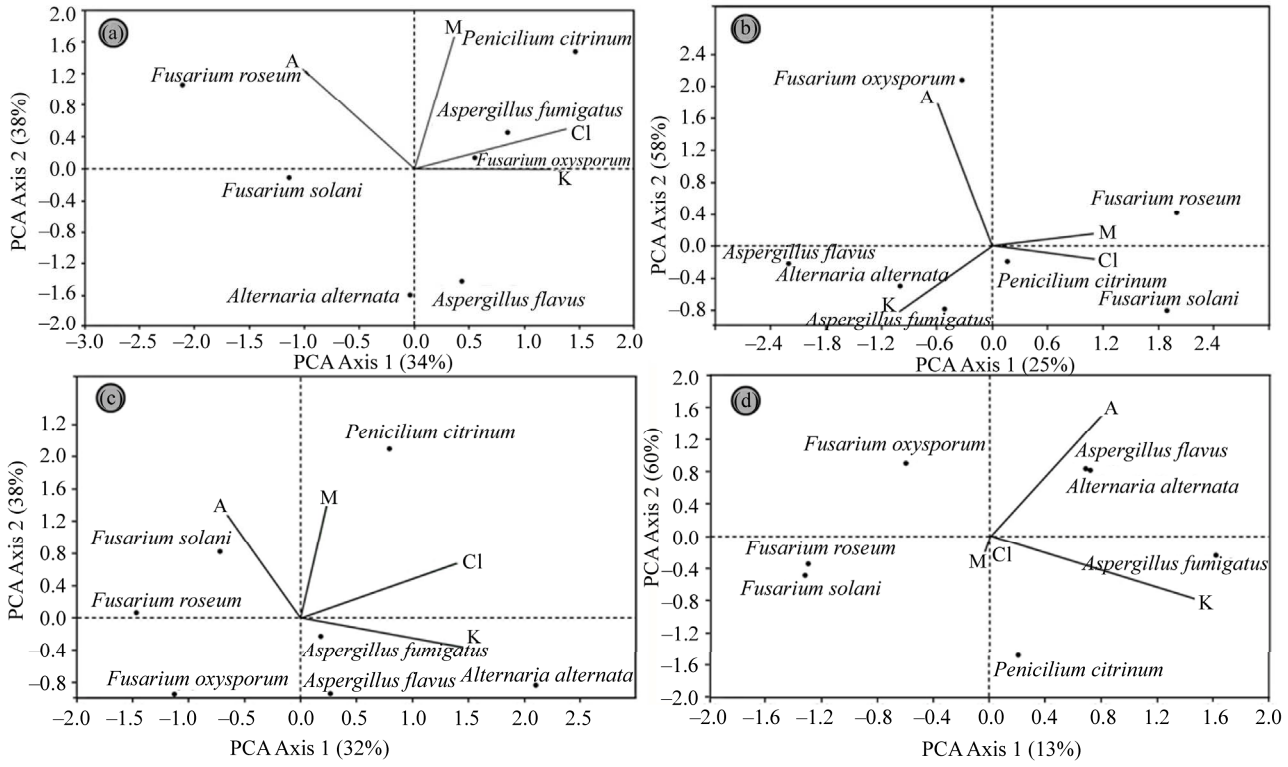

Figure 3. PCA Biplots of selected lichen ((a) = *Bulbothrix setschwanensis*, (b) = *Parmelaria thomsonii*, (c) = *Heterodermia diademata* (d) = *Everniastrum nepalense*) extracts (A = Acetone, M = Methanol, Cl = Chloroform) and commercially available fungicide Ketoconazole(K) on different plant pathogenic fungi.

The present study with different solvent extracts of *Bulbothrix setschwanensis*, *Parmelaria thomsonii*, *Heterodermia diademata* and *Everniastrum nepalense* showed promising results against some well known plant pathogenic fungi. The selective antifungal effect of acetone and methanol extracts of test lichens over chloroform extracts can be attributed to the presence of different constituent secondary metabolites in lichen thalli [28,29].

The better performance of lichenic extracts against commercially available antifungal Ketoconazole against some (*Fusarium roseum*, *Fusarium solani* and *Fusarium oxysporum*) plant pathogenic fungi suggests their superior potentials as fungicides.

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