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RESEARCH ARTICLE

EVALUATION OF ANTIFUNGAL ACTIVITY OF LICHEN EXTRACTS AGAINST PHYTOPATHOGENIC FUNGI *MACROPHOMINA PHASEOLINA* (TASSI) GOID

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ABSTRACT

Lichens were screened to know their antifungal potentials against phytopathogenic fungi *Macrophomina phaseolina*. Lichen samples like *Heterodermia leucomela*, *Pseudocyphelaria aurata*, *Flavoparmelia caperata*, *Parmotrema tinctorum* and *Parmotrema austrosinensis* were collected from Mysore and Madikeri districts and identified using available standard keys. The extracts of these lichen were obtained with 3 different solvents Methanol, Ethyl acetate and acetone. The antifungal assay was carried out by Agar well diffusion method, Minimum inhibitory concentration and Bioautography technique. The ethyl acetate fraction of all the samples was found to be more effective against the tested fungi. Ethyl acetate extract of *Flavoparmelia caperata* showed maximum inhibition of 18.6±0.5 mm and the least was 10.3±0.5 mm zone of inhibition. The MIC with the lowest concentration of 0.390 mg/ml was in the ethyl acetate extract of *Flavoparmelia caperata*. The TLC bioautographic method showed zone of inhibition in 3 sample extracts. The acetone fraction did not show any results. These lichen extracts tested demonstrated antimicrobial effect which suggests a possibility of their use in the treatment of various diseases caused by these and similar microorganisms.

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INTRODUCTION

Lichens are fungi that live in intimate symbiotic association with green algae or cyanobacteria. Lichens are inherently resistant to microbial infection due to the production of a large number of unique secondary metabolites. Their flexibility in habitat enables them to synthesize unique, naturally occurring secondary metabolites, which not only are different in their chemical structures, but also show differences in their biological activity (Goel *et al.*, 2011). Screening the lichens has revealed the frequent occurrence of metabolites with antibiotics, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic properties (Boustie and Grube, 2005). Lichens are valuable plant resources and are used as medicine, food, fodder, perfume, spice, dyes and for miscellaneous purposes throughout the world (Dayan *et al.*, 2001). Plant fungal pathogens, in particular, pose a major threat to economically valuable crops. Plant pathogenic fungi attack most crops in the field and also post-harvest, thereby decreasing production and shelf life of many agricultural crops (McLaren, 1986). The most important method of protecting plants against fungal attack is the use of fungicides.

The development of resistance of pathogenic fungi towards synthetic fungicides is of great concern. There is, therefore, a motivation to find safe, efficacious and environmental friendly fungicides. Thus, present study was done to evaluate the antifungal activity of lichens *Heterodermia leucomela* (L.) Poelt, *Pseudocyphelaria aurata* (Ach.) Vain., *Flavoparmelia caperata* (L.) Ach., *Parmotrema tinctorum* Nyl. And *Parmotrema austrosinensis* (Zahlbr.) Hale against plant pathogenic fungi *Macrophomina phaseolina* (Tassi) Goid.

MATERIALS AND METHODS

Collection, Identification and processing lichen specimen

The lichen specimen was collected from Mysore district, Karnataka and identified by morphological, anatomical and chemical tests (Awasthi, 1998). Lichens namely *Heterodermia leucomela* (L.) Poelt, *Pseudocyphelaria aurata* (Ach.) Vain., *Flavoparmelia caperata* (L.) Ach., *Parmotrema tinctorum* Nyl. and *Parmotrema austrosinensis* (Zahlbr.) Hale were thoroughly washed 2-3 times with water and shade dried at room temperature. The dried plants were milled to a fine powder with the help of a blender and stored at room temperature in closed containers in the dark until used.

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1g of finely ground lichen material was soaked in 10 ml of Ethyl acetate, Methanol and Acetone solvents in conical flasks and kept on a rotary shaker at 180-200rpm for 24 hrs. Later it was filtered through Whatman No.1 filter paper and were concentrated by air-drying for 4 –5 days preserved at 5°C in airtight bottles until further use.

Antifungal Activity Assay

The phytopathogenic fungi selected for this study were *Macrophomina phaseolina*, which is a causal organism of Charcoal rot disease in Maize. The fungus was isolated from the diseased stem part and maintained on potato dextrose agar (Himedia) at 26-28°C. The standard culture inoculum was prepared on Potato Dextrose Broth by adjusting the spore range of 1×10^6 - 5×10^6 spores /ml (Aberkane *et al.*, 2002). Antifungal activity assays were carried out by Agar well diffusion, Microdilution assay and Direct bioautographic method.

Agar well diffusion

100 µl of fungal suspension was spread on the solidified Potato dextrose agar medium and wells were punched using 5mm cork borer, a concentration of 30 mg/ml extracts of 100 µl was loaded into the wells and the solvents of the same were used as a negative control. The plates were kept for incubation for 4-7 days; the diameter of the zone of inhibition of the tested microorganism by the given extract was measured in millimeters. All experiments were performed in triplicate. To every sample tested, a set of control was run parallel.

Microdilution assay

Determination of Minimum inhibitory concentration [MIC] was carried out by microdilution method. The MIC is to determine the lowest concentration of an antifungal agent that appears to inhibit growth of the fungus (Andrews, 2011). Residues of different extracts were dissolved in respected solvents to a concentration of 50mg/ml. The plant extracts (100µl) were serially diluted 50% with solvents in 96 well flat bottomed microtitre plates. Fungal cultures were transferred into fresh Potato dextrose broth, and 100µl of this was added to each well, 40µl of 2, 3, 5- triphenyltetrazolium chloride [TTC] dissolved in water was added to each of the micro plate wells, as growth indicator. Appropriate solvent blanks as control were included. The micro plates were covered with a cling film and incubated for 2-3 days at 26°C and at 100% relative humidity (Eloff, 1998). The MIC was recorded by visual analysis in microtitre plate wells, where the lowest concentration of the lichen extract that inhibited fungal growth after 48 to 72 hours of incubation will not change its colour to formazan dye.

Direct Bioautography method

Bioautographic method was developed to determine active compounds. Aluminium-backed TLC plates (Aluchrosep Silica Gel 60/UV₂₅₄ for TLC) were loaded with 20µl of 100µg extracts. The TLC plate was developed in solvent system A (180 ml toluene: 60 ml 1-4, dioxin: 8 ml acetic acid) [Culberson, 1972]. The chromatogram was dried for complete removal of solvents. About 25-50ml of inoculum spray solution

was prepared containing approximately 3×10^4 spores/ml of actively growing fungi. The plates were sprayed lightly 3 times with spore suspension and incubated for 24 hr in darkness in a moist chamber at 26°C and then sprayed with 20mg/ml of TTC in boiling water and further incubated overnight. Fungal growth inhibition appeared as clear zones against a dark background (Guleria, 2006). The R_f value of the zone of inhibition is recorded.

RESULTS

In vitro evaluation of five lichen extracts were screened for antifungal activity against phytopathogenic fungi *Macrophomina phaseolina* using Agar well diffusion assay, Microdilution assay and Bioautography technique. A total of five lichens *Heterodermia leucomela*, *Pseudocyphelaria aurata*, *Flavoparmelia caperata*, *Parmotrema tinctorum* and *Parmotrema austrosinensis* with three solvents Methanol, Ethyl acetate and Acetone were extracted in which ethyl acetate extracts showed strong inhibitory activity in most of the lichen species tested. The largest zone of inhibition (18.6±0.5 mm and 12.3±0.5 mm) was measured in the ethyl acetate extract and the methanol extract of *Flavoparmelia caperata* respectively (Table 1).

Table 1. Antifungal activity of lichen extracts by Well diffusion assay

S. No.	Lichen samples	Well diffusion in mm		
		Methanol	Ethyl acetate	Acetone
1	<i>Heterodermia leucomela</i> (L.) Poelt.	10.3±0.5	10.6±0.5	-
2	<i>Pseudocyphelaria aurata</i> (Ach.) Vainio	12.3±0.5	10.6±0.5	-
3	<i>Flavoparmelia caperata</i> (L.) Hale	12.3±0.5	18.6±0.5	-
4	<i>Parmotrema tinctorum</i> (Nyl.) Hale	-	10.3±0.5	-
5	<i>Parmotrema austrosinensis</i> (Zahlbr.) Hale	10.6±0.5	-	-

*results in mean± standard deviation

A poor inhibition of 10.3±0.5 to 10.6±0.5 mm was observed in the ethyl acetate extract of *Heterodermia leucomela*, *Parmotrema tinctorum*, *P.austrosinensis* and in methanol extract of *Heterodermia leucomela* (Fig. 1). Acetone extract did not show any activity. The micro dilution assay was carried out in 96 well flat bottom microtitre plates to record the minimum inhibitory concentration of extract at which the activity of the pathogen will be inhibited. The MIC of all the tested extracts ranged from 0.390-3.125 mg/ml. The lowest concentration of 0.390 mg/ml was observed in the Ethyl acetate extract of *Flavoparmelia caperata* which was able to inhibit the growth of the fungus *Macrophomina phaseolina* (Fig. 2). The next lower concentration was 1.562 mg/ml observed in the methanol extract of *Flavoparmelia caperata*. The technique bioautography was basically used to locate the antifungal compound from the crude extract into the chromatogram. A fungal zone of inhibition was observed in R_f value of 0.5 in methanol and 0.48 in ethyl acetate of *Flavoparmelia caperata*, and at an R_f value of 0.6 in the methanol extract of *Heterodermia leucomela* (Fig. 3).

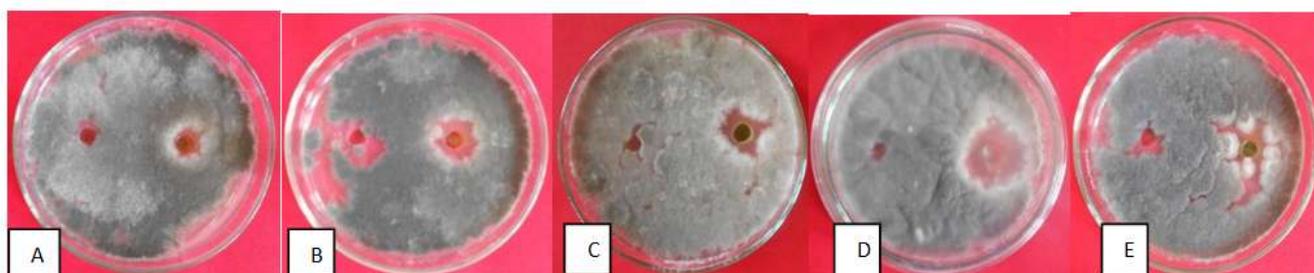


Fig. 1. A: *Heterodermia leucomela* (Ethyl acetate extract), B: *H. leucomela* (Methanol extract), C: *Flavoparmelia caperata* (methanol extract), D: *F. caperata* (Ethyl acetate extract), E: *Parmotrema austrosinensis* (Ethyl acetate extract)

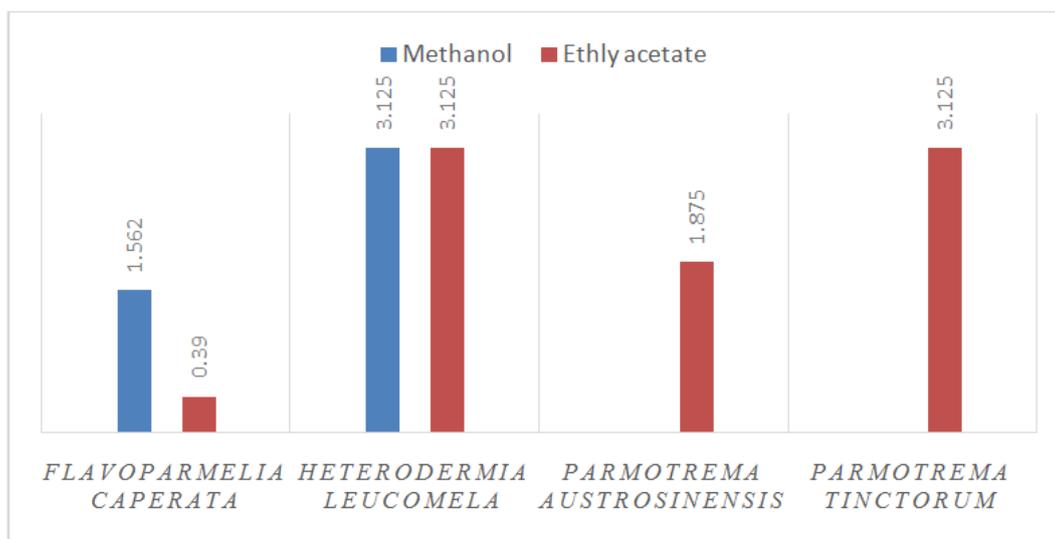


Fig. 2. Results of Minimum Inhibitory Concentration (MIC in mg/ml.) of lichen extracts

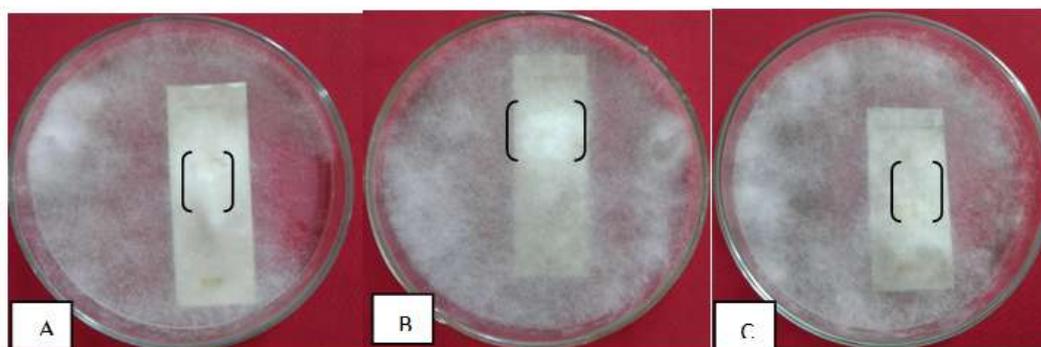


Fig. 3. Results of Bioautography showing inhibition zone on TLC. A-*Heterodermia leucomela* (methanol) R_f -0.6, B- *Flavoparmelia caperata* (Methanol) R_f -0.5, C- *F.caperata* (Ethyl acetate) R_f -0.4

DISCUSSION

Lichens are inherently resistant to microbial infection due to the production of large numbers of unique secondary metabolites. The search for novel bioactive compounds from natural resources to improve pharmaceutical, cosmetic and agriculture applications is an ancient practice and currently it is regaining more rapid importance. The Lichen compounds are not an exception in this field. Currently the interest in the lichen secondary compound is increasing because of ineffectiveness of some known previously reliable drug

(Huneck, 1999). From the present result *Flavoparmelia caperata* showed maximum zone of inhibition with 19mm in the ethyl acetate extract, minimum inhibitory concentration of 0.390 mg/ml and at R_f value 0.48 inhibition zone was observed. *Flavoparmelia caperata* constitutes phytochemicals like Tannins, Flavonoids, proteins, Carbohydrates and Steroids in Methanol and Ethyl acetate extracts (Rashmi and Rajkumar, 2014). Even Ethyl acetate extract of *F. caperata* exhibited maximum inhibitory activity of 18.3 ± 1.5 mm zone against phytopathogenic fungi *Fusarium oxysporum* F. Sp. *Capsici* (Shivanna and Garampalli, 2014). Similar results were obtained

in studying the antifungal activity of lichen extracts (including extracts of the species *Hypogymnia physodes*) against phytopathogenic fungi (Halama and Haluwin, 2004). The aqueous extract of *Heterodermia leucomela* showed broad spectrum antifungal properties at 80 µg ml⁻¹ concentrations against some human and plant pathogens (Shashi *et al.*, 2001). Antifungal activity of *Parmotrema tinctorum*, *P. grayanum* and *P. praesorediosum* showed 40-50% inhibition against *Colletotricum capsici* (Kekuda *et al.*, 2014). Atranorin isolated from *Physcia aipolia* demonstrated an approximate and relatively strong antimicrobial activity of MIC value ranging from 0.031-0.5 µg/ml (Rankovic *et al.*, 2008). The Bioautography showed good results in most of the samples tested, which will be useful in the further isolation and characterization of metabolites (Tay *et al.*, 2004).

Conclusion

The present results showed that lichen extracts tested against the phytopathogenic fungi proved to be a significant biocontrol agent against charcoal rot disease of maize. Thus, the use of lichen extracts with inhibitory activity against fungal plant pathogens could lead to the development of environmentally acceptable fungicides based on the availability of natural products.

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