ORIGINAL ARTICLE

Antimicrobial activity of *Heterodermia incana* (Stirt.) D.D. Awasthi

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Abstract

Objectives: Lichens represent one of the most successful symbiotic interactions and are formed from a photobiont and a mycobiont. The foliose lichen genus *Heterodermia* is one of the cosmopolitan lichen genera. The present study was conducted to investigate antibacterial and antifungal activity of *Heterodermia incana* (Stirt.) D.D. Awasthi, a foliose macrolichen belonging to the family *Physciaceae*. **Materials and Methods:** Extraction of dried and powdered lichen was carried out by maceration process. Antibacterial activity of the extract was evaluated against 2 Gram-positive and 2 Gram-negative bacteria by agar well diffusion assay. Antifungal activity of extract was determined against 3 seedborne fungi by poisoned food technique. **Results:** Extract was effective in inhibiting the growth of all test bacteria in a concentration dependent manner with marked activity against Gram-positive bacteria. *Bacillus cereus* (zone of inhibition 2.26 ± 0.05 cm) and *Pseudomonas aeruginosa* (zone of inhibition 1.76 ± 0.05 cm) were inhibited to higher extent among Gram-positive and Gram-negative bacteria, respectively, at 10 mg/ml extract concentration. The extract was effective in inhibiting the mycelial growth of test fungi in a concentration dependent manner. Among three fungi, the susceptibility to extract was in the order: *Fusarium* sp. > *Curvularia* sp. > *Alternaria* sp. At extract concentration 1 mg/ml, >60% inhibition of all test fungi was observed. **Conclusion:** The lichen *H. incana* is a promising resource of antimicrobial agents. The observed bioactivities could be attributed to the presence of secondary metabolites such as atranorin and zeorin present in the extract. In suitable form, the lichen can be used as anti-infective agent and in the management of seedborne fungal diseases.

Key words: Agar well diffusion, antimicrobial, *Heterodermia incana*, lichens, poisoned food technique

INTRODUCTION

Lichens are non-vascular cryptogams and comprise a self-supporting, symbiotic association between a photobiont (an alga or a cyanobacterium) and a mycobiont (an ascomycetes or basidiomycetes fungus). They represent one of the most stable and successful symbiotic interactions among organisms. Lichens are cosmopolitan and found distributed in almost every type of habitats on earth such as Arctic region, desserts, high mountains elevations, tropical and temperate forests, and others. Together with mosses, lichens form a dominant group of organisms covering over 8-10% of terrestrial habitats, especially at higher elevations. Lichens occur in any one of the three morphological forms such as crustose (spreading over surface of substratum), foliose (leafy and often loosely attached to substratum), and fruticose (bush like hanging and attached to substratum at a single point). Lichens are capable of growing on various substrates such as rock (saxicolous), bark (corticolous), soil (terricolous), plastic (plasticolous), and leaves (follicolous). Lichens are considered as one of the best indicators of air quality.[1-9] Since time immemorial, lichens have been used as sources of food, spice, medicine, and dye. Lichens have been considered to be a part of traditional medicine and are used to treat several human and veterinary ailments by various tribes of several countries.[10,11] Lichens produce a number of low molecular weight compounds (secondary metabolites, often termed as lichen substances) which do not occur in other organisms. Lichen extracts and the secondary metabolites of lichens are known to exhibit a range of bioactivities such as antimicrobial, antioxidant, insecticidal, anthelmintic,

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antiviral, anthelmintic, antiproliferative, anti-inflammatory, analgesic, and enzyme inhibitory activities.[3,6,7,9,12-16]

The lichen genus *Heterodermia* belongs to *Physciaceae* and is one among the most common lichens in tropical regions. The genus *Heterodermia* is distinguished from other foliose lichen genera of *Physciaceae* as the genus is characterized by the presence of the prosoplectenchymatous upper cortex in combination with atranorin as a cortical substance. Moreover, many species are characterized by lacking a lower cortex and producing abundant marginal cilia that resembles rhizenes. Zeorin is one of the major lichen substances in *Heterodermia*. Some species of *Heterodermia* are known to have ethno-medicinal and traditional uses and are shown to exhibit bioactivities such as antimicrobial, antiviral, antioxidant, enzyme inhibitory, and anthelmintic activity.[11,17-25] *Heterodermia incana* is a corticolous (and rarely saxicolous) macrolichen. The lichen is reported from subtropical to lower temperate regions of India, Nepal and Sri Lanka and also found in China, Taiwan, and Thailand. Thallus is white to whitish gray on the upper side and the lower side is white, veined with marginal rhizenes. Thallus is about 6 cm across and branched with corticated on the upper side only. Lobes are spathulate and are apically 5 mm wide. Soredia and isidia are lacking. Apothecia are pedicellate with distinct margin (lecinulate) and about 8 mm in diameter.[19] The study of Behera *et al*.[9] has shown the anti-lipoxygenase, antimicrobial and antioxidant potential of *H. incana*. In this study, we evaluated antibacterial and antifungal potential of an extract of *H. incana*.

MATERIALS AND METHODS

Collection and Identification of Lichen

The corticolous foliose lichen *H. incana* [Figure 1] was collected at outskirts of Sagara, Shivamogga district, Karnataka, India, during February 2017. The collected lichen was identified on the basis of the result of morphological, anatomical, and color (K [potassium hydroxide], C [calcium hypochlorite], and Pd [paraphenylene diamine]) tests. Secondary metabolites in lichens were detected by thin layer chromatography (TLC) using solvent system A that comprised toluene, 1,4-dioxane and acetic acid.[26-28] A voucher specimen (KFGCS0756) was kept in the herbaria maintained in the Department of Botany, KFGC, Shikaripura, Karnataka, India.

Extraction of Powdered Lichen Material

The lichen was dried and powdered. Maceration process was used for extraction. In stoppered container, 10 g of powdered lichen material was left for 48 h in 100 ml of methanol, and the container was stirred occasionally. The content was filtered through Whatman No. 1 filter paper, and the filtrate was subjected for evaporation to get crude extract. The extract, thus, obtained was stored in the refrigerator.[29,30]

Figure 1: *Heterodermia incana* (Stirt.) D.D. Awasthi

Test Bacteria

A total of 4 bacteria which included two Gram-positive bacteria (*Bacillus subtilis* NCIM 2063 and *Bacillus cereus* NCIM 2016) and two Gram-negative bacteria (*Escherichia coli* NCIM 2065 and *Pseudomonas aeruginosa* NCIM 2200) were used. The pure cultures of these bacteria were obtained from National Chemical Laboratory, Pune, India. The cultures were maintained on nutrient agar slants under refrigeration conditions.

Antibacterial Activity of Lichen Extract

The test bacteria were seeded into sterile nutrient broth tubes and incubated overnight at 37°C to obtain broth cultures. Antibacterial activity of lichen extracts was evaluated by agar well diffusion assay. Using sterile swabs, the broth cultures of test bacteria were inoculated all over the surface of sterile nutrient agar plates. Using a sterile cork borer, wells (8 mm diameter) were punched in the plates. Respective wells were filled with 100 µl of lichen extract (5 and 10 mg/ml of dimethyl sulfoxide [DMSO]), reference antibiotic (chloramphenicol, 1 mg/ml of sterile distilled water), and DMSO. The plates were incubated for 24 h at 37°C. Zones of inhibition formed were measured.[30,31]

Test Fungi

Three fungi, viz., *Alternaria* sp., *Fusarium* sp., and *Curvularia* sp., isolated previously from moldy grains of sorghum were used. The fungal cultures were maintained on Potato dextrose agar slants under refrigeration conditions.

Antifungal Activity of Lichen Extract

Poisoned food technique was carried out to investigate antifungal potential of lichen extract. The test fungi were allowed to grow on control (without extract) and poisoned

Potato dextrose agar (0.5 and 1.0 mg extract/ml of medium) plates for 4 days at room temperature. After incubation, the diameter of fungal colonies was measured in mutual perpendicular directions. Antifungal potential of lichen extract, assessed in terms of inhibition of mycelial growth of test fungi, was determined using the formula:

Inhibition of mycelial growth (%) = (Dc−Dt/Dc) × 100, where "Dc" and "Dt" denotes the colony diameter of test fungi on control and poisoned plates, respectively.[8,30]

Statistical Analysis

All experiments were done in triplicates. The results are represented as mean ± standard deviation of three trials.

RESULTS AND DISCUSSION

The details on morphological characteristics and result of color test and secondary metabolites are shown in Table 1. TLC showed the presence of a despide (atranorin) and a terpenoid (zeorin).

Antibacterial Activity of *H. incana*

Antibiotics have revolutionized the field of medicine as they have saved countless lives from infectious diseases caused by pathogenic bacteria. However, indiscriminate use of antibiotics and the ability of bacteria to transmit resistance trait to susceptible strains resulted in the emergence of antibiotic resistant bacteria. These resistant bacteria are of serious threat in both nosocomial and community settings. Treatment of diseases caused by resistant bacterial strains is often difficult. Hence, there is a great need for developing antibacterials from other sources. Natural products are shown to be one of the important alternatives for developing antimicrobial agents. Lichens and their metabolites are shown to possess antibacterial activity against various pathogenic bacteria including antibiotic resistant bacteria.[9,32-38] In this study, we evaluated the potential of *H. incana* to inhibit Gram-positive and Gram-negative bacteria by agar well diffusion method. The presence of an inhibition zone around the well is an indication of antibacterial potential of extract. The result of antibacterial activity of lichen extract is shown in Table 2 and Figure 2. The extract exhibited inhibitory activity against test bacteria in a concentration dependent manner. Overall, extract showed marked inhibition of Gram-positive bacteria when compared to Gram-negative bacteria. *B. cereus* (zone of inhibition 2.36 ± 0.05 cm) and *P. aeruginosa* (zone of inhibition 1.76 ± 0.05 cm) were inhibited to a higher extent among Gram-positive and Gram-negative bacteria, respectively. Least inhibitory activity of extract was observed against *E. coli* (zone of inhibition

Table 1: Morphological features, color test, and TLC of lichen

Characteristics	*H. incana*
Morphological features	Thallus (5-6 cm across) branched, upper side whitish gray, lower side is white, veined with marginal rhizenes, corticated on upper side only, lobes spathulate, soredia and isidia absent, apothecia pedicellate
Color test	Medulla K+ yellow; C -; P + yellow
Secondary metabolites	Atranorin, zeorin

H. incana: Heterodermia incana, TLC: Thin layer chromatography

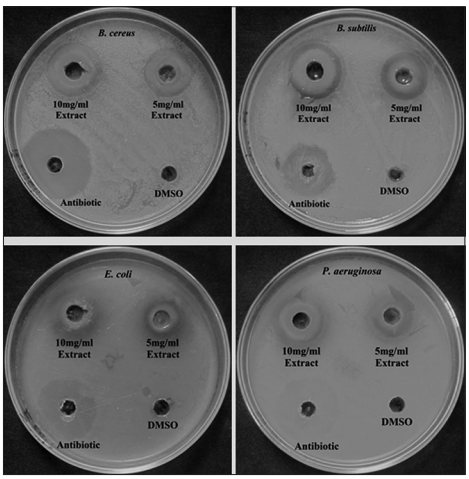

Figure 2: Inhibition of test bacteria by extract of *Heterodermia incana*

Table 2: Antibacterial activity of *H. incana*

Test bacteria	Zone of inhibition in cm (mean±SD)			
	DMSO	Chloramphenicol	Lichen extract	
			5 mg/ml	10 mg/ml
E. coli	0.00±0.00	2.90±0.10	1.20±0.10	1.50±0.10
P. aeruginosa	0.00±0.00	2.90±0.10	1.46±0.05	1.76±0.05
B. subtilis	0.00±0.00	2.90±0.00	2.00±0.10	2.20±0.10
B. cereus	0.00±0.00	3.70±0.10	2.10±0.10	2.36±0.05

E. coli: *Escherichia coli*, *P. aeruginosa*: *Pseudomonas aeruginosa*, *B. subtilis*: *Bacillus subtilis*, *B. cereus*: *Bacillus cereus*, *H. incana*: *Heterodermia incana*, DMSO: Dimethyl sulfoxide, SD: Standard deviation

Table 3: Colony diameter of test fungi in control and poisoned plates

Concentration of extract	Colony diameter of test fungi (mean±SD)		
	***Alternaria* sp.**	***Curvularia* sp.**	***Fusarium* sp.**
0.0 mg/ml (control)	4.26±0.05	4.83±0.11	4.60±0.00
0.5 mg/ml	2.30±0.10	1.80±0.10	1.66±0.05
1.0 mg/ml	1.33±0.05	1.13±0.05	1.00±0.00

SD: Standard deviation

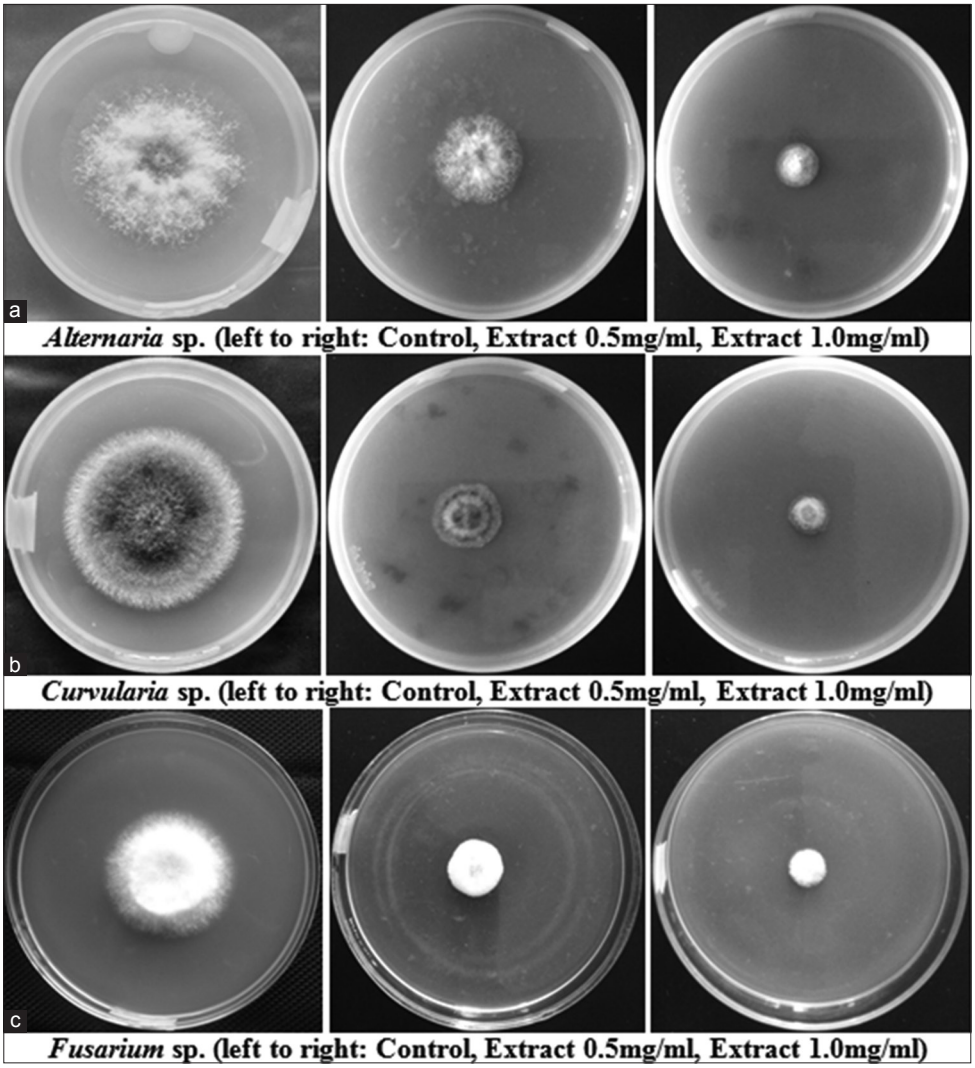

Figure 3: Growth of test fungi on control and poisoned plates (a) *Alternaria* sp. (left to right: Control, extract 05 mg/ml, and extract 1.0 mg/ml), (b) *Curvularia* sp. (left to right: Control, extract 0.5 mg/ml, and extract 1.0 mg/ml), (c) *Fusarium* sp. (left to right: Control, extract 0.5 mg/ml, and extract 1.0 mg/ml)

1.40 ± 0.10 cm). Reference antibiotic exhibited marked inhibitory activity against test bacteria when compared to lichen extract. No inhibitory activity against test bacteria was observed in case of DMSO. In an earlier study, Behera *et al*.[9] showed the antibacterial effect of ethyl acetate extract of *H. incana* against *Streptococcus faecalis* with an minimum inhibitory concentration value of 1.624 mg/ml. Studies have shown the antibacterial potential of some species of *Heterodermia* such as *Heterodermia diademata,*[9,36] *Heterodermia flabellata,*[9] *Heterodermia pseudospeciosa,*[9] *Heterodermia podocarpa,*[39] *Heterodermia Obscurata,*[8,40] and *Heterodermia boryi*.[15,41]

Antifungal Activity of *H. incana*

Fungi are known to represent the dominant group of phytopathogenic organisms causing several diseases in crops. Fungi cause damage to crop in field conditions as well as during storage. Fungal diseases of plants result in decreased

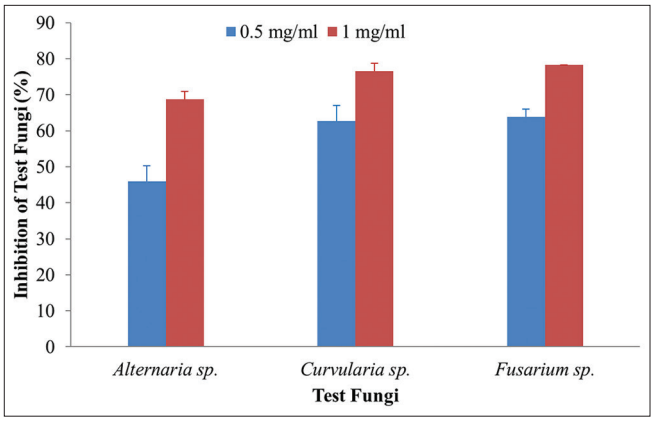

Figure 4: Extent of inhibition of test fungi by lichen extract

productivity and huge economic losses in severe cases. Fungal genera such as *Aspergillus, Fusarium, Helminthosporium, Curvularia, Alternaria, Rhizopus, Cercospora, Pyricularia,* and *Rhizoctonia* are often present on seeds of several crops. Seeds are passive carriers of several pathogenic fungi which are capable of causing infections in seedlings and later stages of growth. Management of phytopathogenic fungi is usually accomplished with the use of synthetic fungicides. However, indiscriminate use of these chemical agents is deleterious to environment and results in toxic effects on nontarget organisms. Besides, high cost and emergence of fungicide resistant strains of pathogens triggered immense interest in scientific community to search for alternatives for management of fungal diseases. Natural products from plants, lichens and microorganisms are shown to inhibit a range of phytopathogenic fungi. Lichens and their metabolites have shown to possess antifungal activity against various fungi including phytopathogenic fungi.[42-48] In this study, we evaluated antifungal potential of two concentrations of an extract of *H. incana,* viz., 0.5 mg/ml and 1 mg/ml by poisoned food technique. Poisoning of medium with extract resulted in drastic reduction in mycelial growth of test fungi [Table 3 and Figure 3]. The extract exhibited concentration dependent antifungal activity. The susceptibility of fungi to extract is in the order: *Fusarium* sp. > *Curvularia* sp. > *Alternaria* sp. At extract concentration of 1mg/ml, the extent of inhibition of *Fusarium* sp., *Curvularia* sp., and *Alternaria* sp. was 78.26%, 76.60%, and 68.77%, respectively [Figure 4]. Earlier studies have shown the potential of some *Heterodermia* species such as *H. boryi,*[15] *H. diademata,*[36,49] *H. comosa,*[50] *H. leucomelos,*[42,51] *H. microphylla,*[43] and *H. obscurata*[8] to inhibit phytopathogenic fungi.

It is known that lichens produce diverse secondary metabolites that seldom occur in other organisms. These compounds are predominantly produced by mycobiont, and more than 800 lichen substances have been recognized. Metabolic pathways such as polyketide pathway, acetyl-polymalonyl pathway, mevalonic acid pathway, and shikimic acid pathway are involved in the biosynthesis of lichen substances. Most of these compounds are phenolic compounds. These metabolites play several ecological roles such as light screen, allelopathic, chemical weathering, and defense against herbivores. These compounds are useful in lichen taxonomy. Many of these substances are shown to exhibit a range of biological activities.[3,52-57] TLC that uses several solvent systems is commonly used to detect the characteristic secondary metabolites in lichens.[26,58,59] In this study, the TLC showed the presence of two major compounds, viz., atranorin and zeorin. It is shown that atranorin[60-62] and zeorin[34] exhibit antimicrobial activity. The observed antimicrobial potential of methanolic extract of *H. incana* could be endorsed to the presence of these secondary metabolites.

CONCLUSIONS

The result of this study clearly indicated the possible use of the foliose lichen *H. incana* as a resource of bioactive agents. The observed antibacterial and antifungal activity could be attributed to the presence of atranorin and zeorin which were identified by TLC. The lichen can be used to treat infectious agents and manage seedborne fungal diseases of plants. Further, studies concerned with the purification of lichen substances and their antimicrobial activity determination are to be carried out.

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