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Original Research

Antimicrobial Activity of Selected Corticolous Macrolichens

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Abstract

The present study was conducted to screen antimicrobial activity of three foliose macrolichens *viz.*, *Parmotrema reticulatum* (Taylor) M. Choisy (Parmeliaceae), *Heterodermia obscurata* (Nyl.) Trevis (Physciaceae) and *Dirinaria consimilis* (Stirt.) D.D. Awasthi (Physciaceae) and two fruticose macrolichens *viz.*, *Ramalina pacifica* Asahina (Ramalinaceae), and *Ramalina hossei* H. Magn & G. Awasthi (Ramalinaceae) growing on barks of areca trees. The lichens were identified on the basis of morphological, anatomical and color tests and the secondary metabolites (lichen substances) were identified by thin layer chromatography. Antibacterial activity of lichen extracts was evaluated against three Gram positive and five Gram negative bacteria by Agar well diffusion assay. The lichen extracts were effective in inhibiting all test bacteria. Overall, extract of *D. consimilis* and *P. reticulatum* displayed marked and least antibacterial efficacy. Marked inhibitory activity was observed against *Klebsiella pneumoniae*. Antifungal effect of lichen extracts was determined against four molds by Poisoned food technique. Among fungi, the growth of *Colletrotrichum capsici* was suppressed to higher extent by lichen extracts. The observed inhibitory activity of lichens could be ascribed to the presence of lichen substances.

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INTRODUCTION

Lichens are nonvascular cryptogams and represents stable, successful and self-supporting symbiotic relationship between mycobiont (fungus) and photobiont partners (algae/cyanobacteria) which together form an independent physiological unit. The photobiont provides the mycobiont with nutrients by its photosynthetic activity whereas the mycobiont helps in absorption of water and nutrients from surroundings and also provide protection to the photobiont. Out of nearly 80000 fungi reported in nature, approximately 17% have been lichenized. The fungi that are present in lichens are called lichenized fungi. Vast majority of mycobiont belongs to Ascomycetes. Lichens are omnipresent and are known to occupy every possible ecological niche and can easily cope up with extreme conditions. The lichens are known to be early land colonizers and have a slow growth rate. Lichens occur in various growth forms such as crustose, foliose and fruticose. All over world, lichens are used as medicine, spice and in the manufacture of dyes. Lichens are used in several systems of traditional medicine such as Ayurveda and Unani. Lichens are said to cure dyspepsia, bleeding piles, bronchitis, stomach disorders, scabies and many disorders of blood and heart. Lichens have been considered as indicators of air pollution and are used as biomonitors of atmospheric pollution in different zones. Lichens are also used as food especially during scarcity and famine. The natural thalli of these lichens contain a variety of secondary metabolites (lichen substances) such as depsides, depsidones, dibenzofurans and pulvinic acid which seldom occur in other organisms. These metabolites are predominantly produced by mycobiont. Lichen extracts containing usnic acid have been utilized for medicinal, perfumery, cosmetic and other applications. Usnic acid is formulated in creams, toothpaste, mouthwash, deodorants and sunscreen products. Lichen extracts and the secondary metabolites are known to exhibit a number of bioactivities such as antioxidant, antimicrobial, antimycobacterial, antiviral, antiinflammatory, antioxidative, analgesic, antipyretic, antiproliferative, insecticidal, anthelmintic, and cytotoxic effects (Nayaka *et al*., 2007; Upreti and Nayaka, 2008; Vinayaka *et al*., 2009; Hoskeri *et al*., 2010; Verma *et al*., 2012; Zambare and Christopher, 2012; Kekuda *et al*., 2012; Ghate *et al*., 2013; Upreti *et al*., 2013; Balaji and Hariharan, 2013; Shukla *et al*., 2014; Kambar *et al*., 2014a).

Lichens grow on various substrates such as barks (corticolous), rocks (saxicolous), leaves (follicolous), moss (muscicolous) and soils (terricolous) and manmade substrates like iron, cloth, glass, leather which provides relative performance and surface stability. Most lichen taxa are substrate specific while few can grow on a variety of substrates. Lichens growing on the barks of tress are known as corticolous lichens. In India, >70% lichens are corticolous (Nayaka *et al*., 2007; Kambar *et al*., 2014a). Barks of a number of trees support the growth of epiphytic lichens (Rambo, 2010; Rout *et al*., 2012; Kambar *et al*.,

2014a; Dudani *et al*., 2015). *Areca catechu* (betel nut) is grown widely in southern India. The nature and texture of bark of areca seems to be suitable for the luxuriant growth of lichens (Nayaka *et al*., 2006). In the present study, we carried out antimicrobial activity of three foliose macrolichens *viz*., *Parmotrema reticulatum* (Taylor) M. Choisy (Parmeliaceae), *Heterodermia obscurata* (Nyl.) Trevis (Physciaceae), *Dirinaria consimilis* (Stirt.) D.D. Awasthi (Physciaceae) and two fruticose lichens *viz*., *Ramalina pacifica* Asahina (Ramalinaceae), and *Ramalina hossei* H. Magn & G. Awasthi (Ramalinaceae) growing on barks of areca trees.

MATERIALS AND METHODS

Collection and Identification of Lichens

The lichens *viz*., *D. consimilis*, *R. hossei*, *R. pacifica*, *P. reticulatum* and *H. obscurata* grown on the barks of areca trees were collected in and around Tarikere, Chikkamagaluru district, Karnataka during January 2015. The collected lichens were identified on the basis of morphological, anatomical and color tests. Morphological characters were studied under dissection microscope. Color tests were done by spot tests using chemical reagents viz., aqueous potassium hydroxide (K), Steiner's stable paraphenylinediamine (P) and aqueous calcium hypochlorite (C). Secondary metabolites were detected by thin layer chromatography using solvent system A (Culberson and Kristinsson, 1970; Culberson, 1972; Walker and James, 1980; Awasthi, 2000).

Extraction

The lichens were powdered in a blender. A known quantity (10g) of each lichen material was subjected to maceration process. The powder was immersed in 100ml of methanol and left for 48 hours with occasional stirrings. The content of flask was filtered through muslin cloth followed by Whatman no. 1 filter paper. The filtrates were evaporated to dryness and used for bioactivity determinations (Kambar *et al*., 2014a).

Antibacterial Activity of Lichen Extracts

The inhibitory efficacy of extracts of selected lichens against test bacteria (Gram positive bacteria *viz*., *Staphylococcus aureus*, *Bacillus subtilis* and *B. coagulans* and Gram negative bacteria *viz*., *Klebsiella pneumoniae*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Ralstonia solanacearum*) was tested by Agar well diffusion method. 24 hours old Nutrient broth cultures of test bacteria were swabbed uniformly on sterile Nutrient agar plates. Wells of 8mm diameter were punched in the inoculated plates using sterile cork borer. Using sterile pipettes, lichen extracts (20mg/ml of 25% Dimethyl sulfoxide [DMSO]), standard antibiotic (Chloramphenicol, 1mg/ml of sterile distilled water) and DMSO (25%, in sterile distilled water) were dispensed into labeled wells. The plates were incubated in upright position for 24 hours at 37^{o}C. The zones of inhibition were measured using a ruler (Kambar *et al*., 2014a).

Antifungal Activity of Lichen Extracts

The antifungal potential of extract of selected lichens was evaluated by Poisoned food technique against fungi namely *Colletotrichum capsici* (isolate from anthracnose of chilli), *Fusarium oxysporum* f.sp. *zingiberi* (isolate from rhizome rot of ginger) and *Alternaria alternata* and *Aspergillus flavus* (isolates from moldy grains of sorghum). The control and poisoned (0.5mg extract/ml of medium) Potato dextrose agar plates were aseptically inoculated at the centre of plates by point inoculation technique. The plates were incubated in upright position for 72 hours at room temperature. The diameter of fungal colonies in mutual perpendicular directions was measured using a ruler. Antifungal activity (in terms of inhibition of growth) was calculated using the formula:

Inhibition of mycelial growth (%) = (C – T / C) x 100, where C and T refers to diameter of fungal colonies on control and poisoned plates respectively (Kambar *et al*., 2014a).

RESULTS AND DISCUSSION

Characteristics of Lichens Selected

The result of color tests and the secondary metabolites (as detected by TLC) of selected lichens is shown in Table 1.

Table 1: Result of color tests and secondary metabolites of lichens

Name	Color test	Secondary metabolites
P. reticulatum	Cortex K+ yellow, C-, KC-, P+ yellow; Medulla K+ yellow turning deep red, C-, KC-, P+ orange	Atranorin, Chloroatranorin, Salazinic acid, Consalazinic acid
H. obscurata	Cortex K+ yellow C-, KC-, P+ yellow; Medulla K-, C-, KC-, P-	Atranorin, Chloroatranorin, Zeorin
R. pacifica	Medulla K-, C-	Usnic acid, Salazinic acid
D. consimilis	Cortex K+ yellow, C–, KC–, P+ yellow; Medulla K–, C–, KC–, P–	Atranorin, Chloroatranorin, Sekikaic acid
R. hossei	Medulla K-, C-	Usnic acid, sekikaic acid

Antibacterial Activity of Lichen Extracts

The discovery of antibiotics is considered as one the major milestones in chemotherapy and represents a fundamental triumph of science of medicine. However, the indiscriminate use of these antibiotics resulted in the emergence of resistant pathogens. The prevalence of these resistant bacterial strains increased in various countries. Antibiotics such as penicillins, cephalosporins and aminoglycosides are no longer effective against certain pathogenic bacteria. The resistance development increases the potential of bacteria to cause diseases and poses a serious challenge to health. This also increases morbidity and mortality and the costs for the treatment. The natural products derived from animals, plants and microorganisms have been considered as suitable alternatives to antibiotics (Chait *et al*., 2012; Chaves *et al*., 2015). Lichens appear to be promising resources of bioactive compounds having activity against many pathogens. It is shown that lichen extracts and their compounds exhibit antimicrobial activity. The secondary metabolites of lichens are shown to possess antimicrobial activity. Atranorin (Yilmaz *et al*., 2004; Verma *et al*., 2011), Chloroatranorin (Turk *et al*., 2006), Lecanoric acid (Verma *et al*., 2011), Usnic acid (Yilmaz *et al*., 2004) and

Protolichesterinic acid (Ingolfsdottir *et al*., 1997; Turk *et al*., 2003) were shown to exhibit antimicrobial activity.

In the present study, we evaluated the inhibitory effect of selected lichens against Gram positive and Gram negative bacteria by Agar well diffusion assay. The result of antibacterial effect is shown in Table 2 and Figure 1. All lichens were effective in inhibiting test bacteria as evidenced by the presence of zones of inhibition around wells. However, the antibacterial effect observed varied among lichens. Overall, extract of *D. consimilis* and *R. pacifica* exhibited marked antibacterial activity. Least inhibitory effect was observed in case of *P. reticulatum*. Among bacteria, *K. pneumoniae* was inhibited to high extent. Reference antibiotic caused higher inhibition of test bacteria when compared to lichen extracts. DMSO (vehicle) did not cause inhibition of test bacteria. The efficacy of lichens to inhibit bacteria is demonstrated by several researchers. The solvent extracts of *R. pacifica* were shown to exhibit inhibitory activity against multi-resistant bacterial strains (Hoskeri *et al*., 2010). Extract of *Everniastrum cirrhatum* was shown to inhibit the growth of Gram positive and Gram negative bacteria (Swathi *et al*., 2010). *Parmotrema nilgherrense* was shown to possess inhibitory activity against drug resistant pathogens (Javeria *et al*., 2013). Methanol extract of macrolichens namely *R. hossei*, *Heterodermia diademata*, *Roccella montagnei* and *Leptogium burnetiae* inhibited clinical isolates of *S. aureus* and *Streptococcus mutans* (Kambar *et al*., 2014b). The study of Vivek *et al*. (2014a) showed the inhibitory effect of three *Parmotrema* species against Gram positive and Gram negative bacteria. Similarly, studies by Karthikaidevi *et al*. (2009), Karagöz *et al*. (2009), Ranković *et al*. (2010), Paudel *et al*. (2012), Pavithra *et al*. (2013), Kekuda (2014), Kambar *et al*. (2014a) and Anjali *et al*. (2015) showed the antimicrobial efficacy of lichens.

Table 2: Antibacterial activity of extracts of selected lichens

Test bacteria	Zone of inhibition in cm						
	P.reticulatum	***H.obscurata***	***R.pacifica***	***D.consimilis***	***R.hossei***	**Antibiotic**	**DMSO**
S. aureus	1.7	2.1	2.0	2.3	2.3	3.2	0.0
B. subtilis	1.8	1.8	2.2	2.2	2.2	3.0	0.0
B. coagulans	1.6	2.2	2.3	2.3	2.2	2.8	0.0
K. pneumoniae	2.1	2.3	3.2	2.6	2.6	2.8	0.0
S. typhi	1.8	2.0	2.4	2.4	2.2	2.6	0.0
P. aeruginosa	1.8	2.0	2.5	2.5	2.3	2.8	0.0
E. coli	1.7	1.9	2.1	2.2	2.0	2.7	0.0
R. solanacearum	1.8	1.8	2.0	2.3	2.0	2.4	0.0

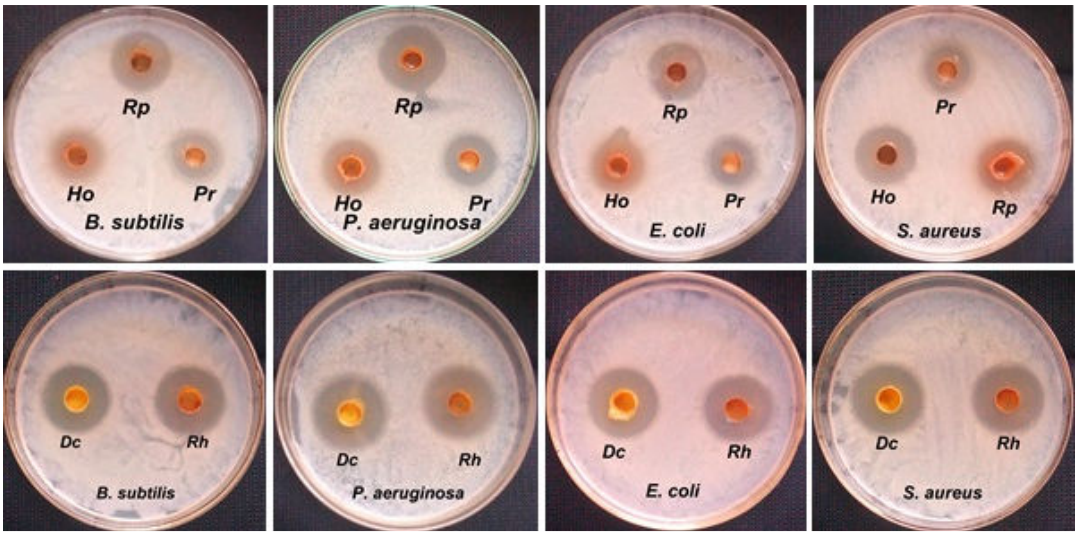

Figure 1: Inhibition of bacteria by lichens (Rp- *R.pacifica*; Ho- *H.obscurata*; Dc- *D.consimilis*; Pr- *P.reticulatum*; Rh- *R.hossei*)

Antifungal Activity of Lichen Extracts

Plants are susceptible to infections caused by many pathogens such as bacteria, viruses, nematodes and fungi. Among the various aetiological agents causing plant diseases, fungi appear to be the major pathogens causing a great number of diseases leading to enormous quantity of loss in agricultural and horticultural crops. The fungal diseases results in considerable loss of crop yield (in many cases 50% or higher). These pre- and post-harvest losses are greater in developing countries. Moreover, certain fungi are known to produce toxins (for example Aflatoxins) which cause adverse health effects on consumption. In order to counteract the fungal diseases, chemical fungicides are widely used. The use of these chemicals is associated with certain drawbacks such as resistance development in fungi, high cost and adverse effects on non-target organisms (including humans). Natural products including extracts and purified metabolites from lichens are considered to be alternative strategies for prevention and control of fungal diseases (Wei *et al*., 2008; Park *et al*., 2008; Shukla *et al*., 2011; Goel *et al*., 2011; Kambar *et al*., 2014a).

In the present study, we evaluated inhibitory efficacy of selected lichens by Poisoned food technique which is one of the widely used methods to evaluate the potential of various kinds of samples to inhibit the growth of fungi. Table 3 and Figure 2-4 shows the result of potential of lichen extracts to inhibit test fungi. Overall, *C. capsici* was inhibited to higher extent among test fungi by lichen extracts (with >50% inhibition). *C. capsici* and *F. oxysporum* were inhibited to higher extent by *R. pacifica*. *D. consimilis* inhibited *A. alternata* to higher extent. Extract of *R. hossei* and *D. consimilis* inhibited *A. flavus* to high extent when compared to other lichens. Among lichens, *P. reticulatum* was found to inhibit test fungi to least extent. It has been shown that lichen extract exhibit inhibitory efficacy against a variety of phytopathogenic fungi. Vivek *et al*. (2014b) found antifungal effect of extract of three *Parmotrema* species against *Sclerotium rolfsii* (isolate from foot rot of finger millet) and *Helminthosporium* sp., *Alternaria* sp., and *Aspergillus flavus* isolated from moldy grains of sorghum. In a study by Kambar *et al*. (2014a) and Kekuda *et al*. (2014), marked inhibition of *Colletotrichum capsici* was exhibited by extracts of macrolichens. Vinayaka *et al*. (2014) showed the potential of extract of *Usnea pictoides* to inhibit the mycelial growth of *F. oxysporum* f.sp. *zingiberi* and *Pythium aphanidermatum* isolated from rhizome rot of ginger. Shivanna and Garampalli (2015) evaluated antifungal efficacy of methanol and ethyl acetate extracts of certain lichens and found their inhibitory effect against *Fusarium solani* causing rhizome rot of ginger. The studies by Madamombe and Afolayan (2003), Rankovic *et al*. (2009), Tiwari *et al*. (2011), Karabulut and Ozturk (2015), Devi *et al*. (2015) and Babiah *et al*. (2015) also highlighted the potential of lichens to inhibit various fungi.

Table 3: Colony diameter of fungi on control and poisoned plates

Treatment	Colony diameter in cm (inhibition of growth in %)			
	C. capsici	***A. alternata***	***F. oxysporum***	***A. flavus***
Control	4.0	3.8	4.6	3.3
P. reticulatum	1.8 (55.00)	2.8 (26.31)	2.8 (39.13)	2.4 (27.27)
H. obscurata	0.8 (80.00)	1.1 (71.05)	2.1 (54.34)	2.1 (36.36)
R. pacifica	0.7 (82.50)	2.2 (42.10)	1.9 (58.69)	1.6 (51.51)
D. consimilis	1.2 (70.00)	1.0 (73.68)	2.0 (56.52)	1.2 (63.63)
R. hossei	1.2 (70.00)	1.6 (57.89)	2.3 (50.00)	1.2 (63.63)

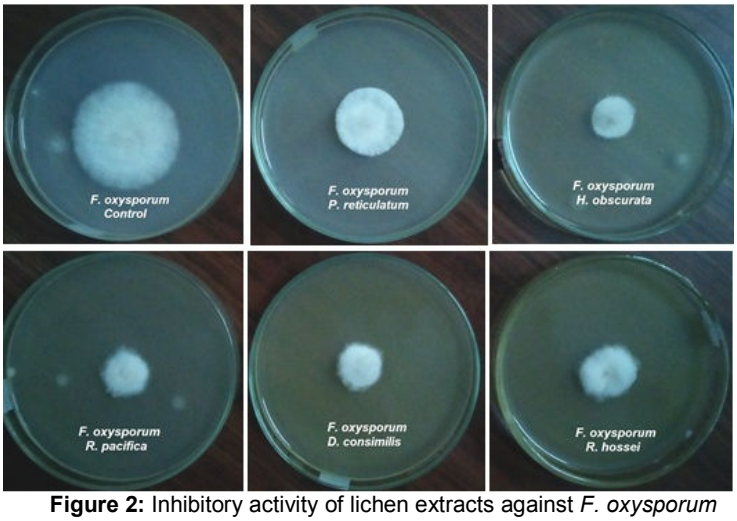

Figure 2: Inhibitory activity of lichen extracts against *F. oxysporum*

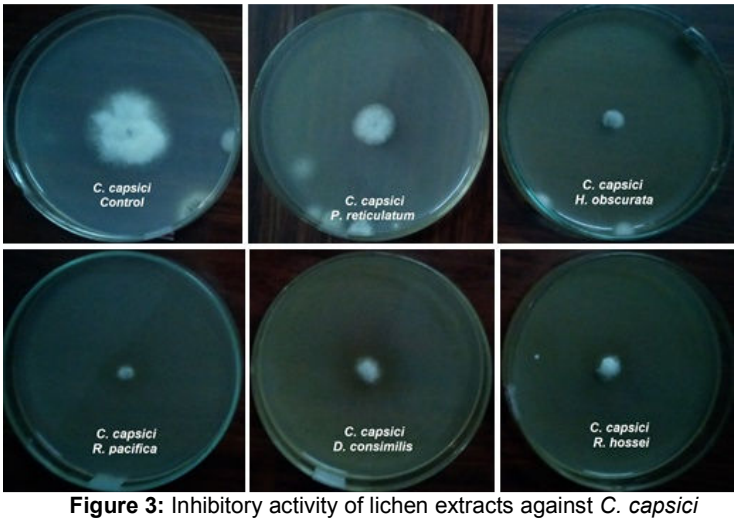

Figure 3: Inhibitory activity of lichen extracts against *C. capsici*

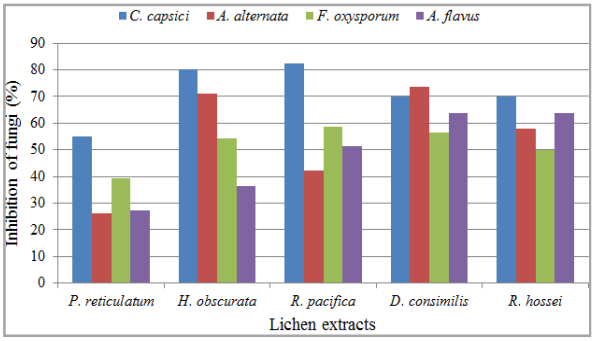

Figure 4: Inhibition (%) of test fungi by extract of selected lichens

CONCLUSION

The lichens of the present study appear to be promising sources of bioactive compounds with potent antimicrobial activity. The observed bioactivities could be ascribed to the presence of bioactive lichen substances which are to be isolated and tested for inhibitory activity. The bioactive agents from these lichens could be used as natural antimicrobial agents and these lichens may be used to develop new therapeutic agents effective against infectious microorganisms.

Conflict of Interest

Conflict of interest none declared.

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