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Original Research

Antimicrobial Activity of *Ramalina conduplicans* Vain. (Ramalinaceae)

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Abstract

The members of the genus *Ramalina* (Ramalinaceae) are fruticose lichens growing on various types of substrata. The present study was conducted with an aim of determining antimicrobial activity of *Ramalina conduplicans* Vain. The powdered lichen material was extracted sequentially using petroleum ether, ethyl acetate and ethanol. The solvent extracts were screened for antibacterial activity by Agar well diffusion assay against 15 bacteria which included reference strains and isolates from burn, dental caries and urinary tract infections. Poisoned food technique was performed to determine antifungal effect of solvent extracts against fungal isolates from anthracnose of chilli, foot rot of finger millet and mouldy grains of sorghum. Usnic acid, Salazinic acid and Sekikaic acid were detected in the lichen. The solvent extracts were shown to exhibit dose dependent inhibitory activity against test bacteria. Ethyl acetate extract inhibited reference bacterial strains to higher extent. Among solvent extracts, only ethanol extract inhibited all urinary tract bacteria. *S. aureus* isolates from burn were inhibited to higher extent when compared to *S. mutans* isolates from dental caries. The solvent extracts exhibited varied inhibitory activity against test fungi. Ethyl acetate extract inhibited *Alternaria* sp., *Aspergillus flavus* and *Sclerotium rolfsii* to higher extent while *Colletotrichum capsici* and *Helminthosporium* sp. were inhibited to higher extent by petroleum ether and ethanol extract respectively. The observed inhibitory potential of solvent extracts of *R. conduplicans* could be ascribed to the presence of secondary metabolites. The lichen can be used in the treatment of bacterial infections and to manage plant pathogenic fungi.

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INTRODUCTION

Lichens represent self-supporting symbiotic association comprising of a Photobiont (algae or blue-green algae) and a Mycobiont (fungi). The lichens grow on rocks, roofs, tree trunks, leaves etc. and occur in different growth forms namely crustose, foliose and fruticose. They lack specialized organs such as roots, leaves etc., which allows them to survive in harsh environmental conditions. Lichens are considered as the primary colonizers of terrestrial ecosystem. Lichens are the indicators of air pollution and are valuable resources of medicine, food, fodder, perfume, spices and dyes. They are consumed in certain parts of the world as food especially during famine. Several lichen species are often used as spice and flavoring agents in certain foods. Lichens are traditionally used worldwide to treat various ailments. Lichens produce a number of secondary metabolites called lichen substances which seldom occur in other organisms. Most of these metabolites are of fungal origin. These metabolites are useful in the lichen taxonomy and have diverse bioactivities such as antimicrobial, antioxidant, enzyme inhibitory, cytotoxic, antiherbivore, phytotoxic, analgesic, wound healing, antitermite, antiinflammatory etc. (Perry *et al*., 1999; Kumar *et al*., 2011; Ul Haq *et al*., 2012; Pavithra *et al*., 2013; Shukla *et al*., 2014 and Vivek *et al*., 2014).

The members of the genus *Ramalina* Ach. are fruticose lichens belonging to the family Ramalinaceae, order Lecanorales, class Ascomycetes. The genus was first described by Acharius and comprises approximately 200 species. These lichens occur in diverse vegetation types and on diverse substrates such as rocks, wood, bark, peaty soil etc. The thallus is attached to the substrate by a basal holdfast. The thallus may be dichotomously or irregularly branched. The fresh thallus is usually gray, greenish-gray to yellowish gray and become yellowish brown to dark-brown on drying. Pseudocyphellae are found with a solid thallus (Fu *et al*., 2008; Lin, 2009). *R. conduplicans* is an edible lichen species being commonly used in central and southeastern Asian countries. In Yunnan province of southwestern China, it is used to prepare a traditional cold dish served at marriage banquets and in a stir-fried pork dish (Wang *et al*., 2001). The Rai and Limbu communities of East Nepal use *R. conduplicans* traditionally for preparation of food (Bhattarai *et al*., 1999). In many places of India, the lichen is used as a spice (Upreti *et al*., 2005). The proximate composition of *R. conduplicans* collected at Bhadra wildlife sanctuary, Karnataka has been reported (Vinayaka *et al*., 2009). Besides, *R. conduplicans* is shown to exhibit bioactivities such as antifungal (Wei *et al*., 2008), antioxidant (Luo *et al*., 2010; Vinayaka *et al*.,

2009), insecticidal (Kumar *et al*., 2010a), anthelmintic (Vinayaka *et al*., 2009) and amylase inhibitory activity (Vinayaka *et al*., 2013). The present study was conducted with an aim of determining antimicrobial activity of solvent extracts of *R. conduplicans*.

MATERIALS AND METHODS

Collection and Identification of Lichen

The fruticose lichen *R. conduplicans* found growing on barks of areca trees (corticolous) was collected at Hosalli, Shivamogga, Karnataka. The lichen was identified by morphological, anatomical and color tests. The color tests were done on cortex as well as medulla using 10% potassium hydroxide (K), Steiner's stable para-phenylenediamine solution (P) and calcium hypochlorite solution (C). Secondary metabolites were detected by thin layer chromatography using solvent system A (Benzene: 1,4-Dioxane:Acetic acid in the ratio 90:25:4) (Awasthi, 2000; Culberson and Kristinsson, 1970; Culberson, 1972).

Extraction

A known quantity (50g) of powdered lichen material was added to a clean flask and subjected to sequential extraction using solvents *viz*., petroleum ether, ethyl acetate and ethanol. The lichen material was left in each solvent for 48 hours and stirred occasionally. The contents were filtered through Whatman No. 1. The solvents were evaporated to dryness (Vinayaka *et al*., 2009).

Antibacterial Activity of Solvent Extracts of *R. conduplicans*

A panel of 15 bacteria (which included 6 reference bacteria (2 Gram positive and 4 Gram negative), 5 drug resistant uropathogens (2 Gram positive and 3 Gram negative), 2 isolates of *Staphylococcus aureus* from burn and 2 isolates of *Streptococcus mutans* from dental caries) were tested for their susceptibility to extracts of *R. conduplicans* by Agar well diffusion assay. The test bacteria were seeded into sterile Nutrient broth (HiMedia, Mumbai) tubes and incubated overnight at 37^{o}C. The broth cultures of test bacteria were swab inoculated on sterile Nutrient agar (HiMedia, Mumbai) plates. Using sterile cork borer, wells of 6mm were punched in the inoculated plates. 100µl of lichen extracts (10 and 20mg/ml of 25% dimethyl sulfoxide [DMSO; HiMedia, Mumbai]), reference antibiotic (Chloramphenicol, 1mg/ml of sterile distilled water) and DMSO (25%, in sterile distilled water) were added into labeled wells. The plates were incubated at 37^{o}C for 24 hours in upright position. Using a ruler, the zones of inhibition formed around the wells were measured (Kekuda *et al*., 2013).

Antifungal Activity of Solvent Extracts of *R. conduplicans*

Colletotrichum capsici (isolate from anthracnose of chilli), *Sclerotium rolfsii* (isolate from foot rot of fingermillet), and *Aspergillus flavus*, *Helminthosporium* sp., and *Alternaria* sp. (isolates from moldy grains of sorghum) were screened for their susceptibility to solvent extracts of *R. conduplicans* by Poisoned food technique (Dileep *et al*., 2013). The test fungi were inoculated at the centre of control (without solvent extract) and poisoned plates (1mg of solvent extract/ml of Potato dextrose agar). The plates were incubated at 28^{o}C for 5 days. The colony diameter of test fungi (on control and poisoned plates) was measured in mutual perpendicular directions using a ruler. The antifungal effect of solvent extracts in terms of reduction in mycelial growth of test fungi on poisoned plates was determined using the formula:

Inhibition of mycelial growth (%) = (C – T / C) x 100,

where C is the colony diameter on control plate and T is the colony diameter on poisoned plates.

RESULTS

Characteristics of *R. conduplicans*

Table 1 shows the morphological characteristics of the thallus and result of color tests and secondary metabolites detected by TLC.

Table 1: Characteristics of *R. conduplicans* of this study

Thallus	Fruticose thallus 3-5cm long, corticolous, pendulous, flattened, greenish grey, branched; upper side smooth, scarcely pseudocyphellate; lower side rugose, with raised, round, prominent pseudocyphellae; soredia and isidia absent, chondroid tissue present and uneven in thickness, distinctly cracked into hyphal bundles; medulla solid, white; pith filled
Colour test	Cortex K-; Medulla K-, C-, KC -, Pd+ yellow
Secondary metabolites	Usnic acid, Salazinic acid, Sekikaic acid

Colour and Yield of Solvent Extracts

The sequential extraction of the lichen resulted in high yield in case of ethyl acetate (2.37%) followed by ethanol (2.03%) and petroleum ether (0.45%). The color of ethyl acetate, petroleum ether and ethanol extracts obtained was brownish green, green and brown respectively.

Antibacterial Activity of Solvent Extracts against Reference Bacteria

The result of antibacterial activity of solvent extracts of *R. conduplicans* against reference bacteria is shown in Table 2. All solvent extracts displayed dose dependent inhibitory activity against reference bacteria. Among bacteria, high and least susceptibility was shown by *S. flexneri* and *S. typhi* respectively. *B. cereus* and *S. flexneri* inhibited to high extent among Gram positive and Gram negative bacteria respectively. Among solvent extracts, ethyl acetate extract was shown to be more potent and inhibited bacteria to high extent. Least inhibitory activity was shown by ethanol extract. Reference antibiotic inhibited bacteria to high extent when compared to solvent extracts. DMSO did not cause inhibition of any bacteria.

Antibacterial activity of Solvent Extracts against Urinary Tract Bacteria

The result of antibacterial activity of extracts of *R. conduplicans* against uropathogens is shown in Table 3. The inhibitory potential of solvent extracts was dose dependent. Among bacteria, high and least susceptibility was recorded in case of *E. faecalis* and *K. pneumoniae* respectively. *E. faecalis* and *P. aeruginosa* were inhibited to high extent among Gram positive and Gram negative bacteria respectively. Among solvent extracts, ethanol extract inhibited all test bacteria. Petroleum ether and ethyl acetate extract was not effective against *K. pneumoniae* and *E. coli* respectively. Reference antibiotic showed high inhibitory efficacy when compared to lichen extracts. DMSO did not cause inhibition of any bacteria.

Table 2: Antibacterial activity of *R. conduplicans* extracts against reference bacteria

Test Bacteria	Zone of Inhibition in cm						Antibiotic
	Ethyl acetate		Petroleum ether		Ethanol		
	20mg/ml	10mg/ml	20mg/ml	10mg/ml	20mg/ml	10mg/ml	
S. aureus	2.0	1.9	1.9	1.7	1.8	1.5	2.2
B. cereus	2.4	2.0	2.2	2.0	2.2	2.0	3.0
K. pneumoniae	2.5	2.4	2.4	2.2	2.0	1.8	2.7
E. aerogenes	2.5	2.3	2.3	2.1	2.0	1.8	2.9
S. typhi	1.9	1.6	1.6	1.0	1.4	1.2	2.6
S. flexneri	2.6	2.5	2.4	2.2	2.2	2.0	2.6

Table 3: Antibacterial activity of *R. conduplicans* against uropathogens

Test Bacteria	Zone of Inhibition in cm						Antibiotic
	Ethyl Acetate		Petroleum Ether		Ethanol		
	20mg/ml	10mg/ml	20mg/ml	10mg/ml	20mg/ml	10mg/ml	
S. aureus	1.5	1.3	1.3	1.1	1.1	1.1	2.6
E. faecalis	1.8	1.7	1.8	1.7	2.2	2.1	3.8
E. coli	0.0	0.0	1.3	0.8	1.1	0.8	1.7
K. pneumoniae	0.8	0.0	0.0	0.0	1.0	0.8	1.6
P. aeruginosa	1.8	1.6	1.7	1.5	1.7	1.6	2.8

Antibacterial Activity of Solvent Extracts against Burn Isolates

Antibacterial activity of solvent extracts of *R. conduplicans* against isolates of *S. aureus* (from burn) is depicted in Table 4. The extracts exhibited concentration dependent inhibitory activity. Among isolates, Sa-02 was inhibited to higher extent than Sa-01. Ethyl acetate extract was found to inhibit isolates to higher extent followed by petroleum ether and ethanol extracts. Reference antibiotic inhibited isolates to higher extent when compared to solvent extracts. DMSO was not effective in inhibiting isolates.

Table 4: Antibacterial activity of *R. conduplicans* against *S. aureus* isolates

Solvent Extract	Concentration (mg/ml)	Zone of Inhibition in cm	
		Sa-01	Sa-02
Ethyl acetate	10	2.0	2.3
	20	2.1	2.5
Petroleum ether	10	1.9	2.3
	20	2.0	2.3
Ethanol	10	1.6	2.0
	20	1.8	2.0
Antibiotic	1	2.8	2.6

Antibacterial Activity of Solvent Extracts against Dental Caries Isolates

Table 5 depicts the result of inhibitory efficacy of solvent extracts of *R. conduplicans* against *S. mutans* isolates. Extracts have shown the dose dependent antibacterial activity. Ethyl acetate extract inhibited isolate Sm-02 to high extent than isolate Sm-01. Other extracts inhibited isolates to more or less similar extent. Inhibitory activity of reference antibiotic was higher than that of solvent extracts. DMSO did not cause inhibition of isolates.

Table 5: Antibacterial activity of *R. conduplicans* against *S. mutans* isolates

Solvent Extract	Concentration (mg/ml)	Zone of Inhibition in cm	
		Sm-01	Sm-02
Ethyl acetate	10	1.7	2.0
	20	1.8	2.1
Petroleum ether	10	1.6	1.6
	20	1.7	1.7
Ethanol	10	1.6	1.5
	20	1.7	1.6
Antibiotic	1	3.7	3.5

Antifungal Activity of Solvent Extracts

The result of antifungal potential of solvent extracts of *R. conduplicans* is shown in the Table 6 and Figure 1. All solvent extracts inhibited test fungi to a varied extent (12 to 100% inhibition). Ethyl acetate extract inhibited *Alternaria* sp., *A. flavus* and *S. rolfsii* to higher extent when compared to other extracts. *C. capsici* was inhibited by petroleum ether extract to higher extent. Ethanol extract was more effective against *Helminthosporium* sp. when compared to other solvent extracts. Ethyl acetate extract completely suppressed the growth of *Alternaria* sp. Overall, marked antifungal effect was exhibited by Ethyl acetate extract.

Table 6: Antifungal effect of solvent extracts of *R. conduplicans*

Treatment	Colony Diameter in cm				
	C. capsici	***Helminthosporium* sp.**	***Alternaria* sp.**	***A.flavus***	***S. rolfsii***
Control	3.4	5.5	3.1	3.5	5.0
Ethyl acetate	1.7	3.7	0.0	1.7	0.5
Petroleum ether	1.1	4.2	2.2	2.3	4.4
Ethanol	1.2	2.9	1.4	2.0	1.8

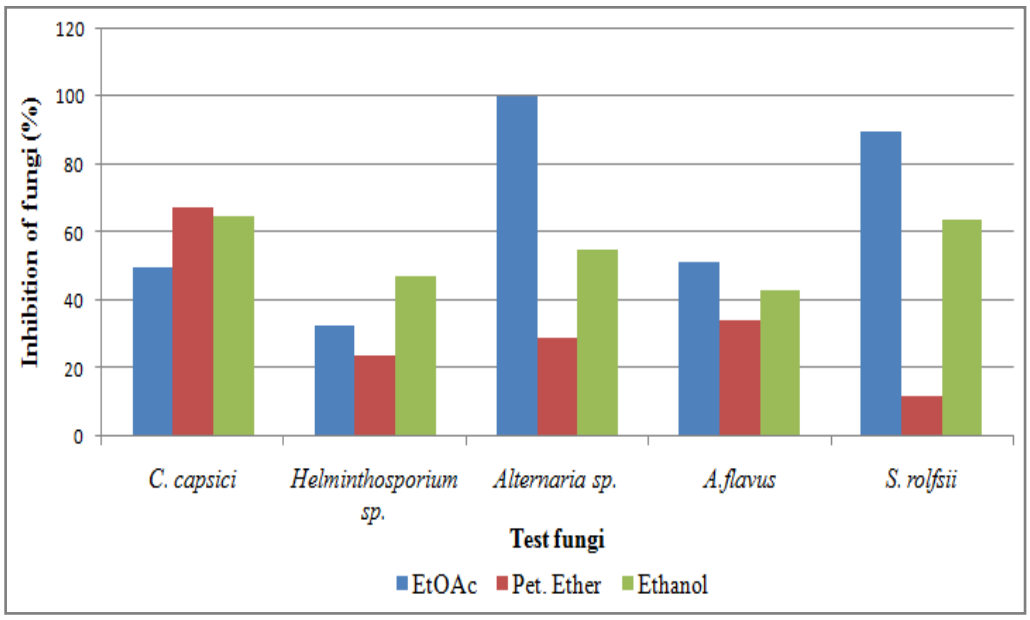

Figure 1: Inhibition of test fungi (%) by solvent extracts of *R. conduplicans* (EtOAc- ethyl acetae; Pet. Ether- petroleum ether)

DISCUSSION

Discovery of antibiotics is one of the major milestones in the field of chemotherapy. The use of these antibiotics prevented and controlled millions of deaths by life threatening infections caused by a range of pathogenic microorganisms. However, continuous and uncontrolled use of these wonder drugs resulted in the development of resistance in pathogens. Besides, long term use of such drugs often causes several side effects. This situation intensified search for finding lead compounds having inhibitory activity from natural origin. Lichens are known to be one of the best sources of bioactive agents with activity against a range of human pathogens. The lichen extracts and the bioactive principles from lichens are shown to exhibit inhibitory activity against wide variety of human pathogens including clinical strains (Smith and Coast, 2002; Verma *et al*., 2011; Javeria *et al*., 2013 and Kosanić *et al*., 2014). In the present study, we evaluated antibacterial activity of solvent extracts of *R. conduplicans* against reference bacterial strains and clinical isolates of burn, dental caries and urinary tract infections. It was observed that the extracts exhibited marked inhibitory effect against clinical isolates and the effect was dose dependent. The species of the lichen genus *Ramalina* were shown to exhibit inhibitory effect against bacteria including clinical isolates. The aqueous extract of *R. farinacea* was shown to exhibit inhibitory effect against *S. aureus*, *E. coli* and *B. subtilis* (Karagöz *et al*., 2009). The extract of *R. hossei* was shown to exhibit inhibitory activity against *S. aureus*, *E. coli* and *P. aeruginosa* (Kekuda *et al*., 2009). Hoskeri *et al*. (2010) reported inhibitory potential of solvent extracts of *R. pacifica* against clinical isolates of *P. aeruginosa*, *K. pneumoniae*, *Salmonella typhi, Salmonella paratyphi, E. coli* and *S. aureus*. The study of Santigo *et al*. (2010) revealed potent inhibitory effect of extracts of *R. dendriscoides* against Gram positive bacteria. Agboke and Esimone (2011) observed antibacterial activity of methanol extract of *R. farinacea* against clinical strains of *S. aureus*. It has been shown that acetone extract of *R. menziesii* exhibited marked activity against reference strains of bacteria and methicillin resistant *S. aureus* when compared to methanol extract (Shrestha and St. Clair, 2013).

Plants are vulnerable to infections caused by various pathogens such as bacteria, viruses and fungi. Among these, fungi are considered to be the major pathogens of plants causing huge number of diseases in agricultural and horticultural crops. The fungal diseases of plants results in drastic loss of yield and deterioration of its quality. The pre- and post-harvest crop losses are higher in developing countries. The fungal infection of plants leads to quality problems such as aspect, nutritional value, organoleptic characteristics, and limited shelf life. Besides, some fungi produce toxins in food commodities which cause adverse health effects on consumption. The management of fungal infections of agricultural commodities is mainly by the use of chemical fungicides. However, the use of these fungicides suffers from drawbacks such as resistance development in fungi, high cost and deleterious effects on non-target organisms including humans. Hence, search for alternative strategies for prevention and control of mycotic infections of plants is of immense interest. Natural products including lichens are shown to be promising alternates for synthetic fungicides (Park *et al*., 2008; Kumar *et al*., 2010b; Dellavalle *et al*., 2011; Shukla *et al*., 2011; Panea *et al*., 2013 and Vinayaka *et al*., 2014). In this study, we evaluated antifungal effect of solvent extracts of *R. conduplicans* against 5 fungi collected from different sources namely anthracnose of chilli, foot rot of finger millet and mouldly grains of sorghum. The extracts were effective against test fungi but to a varied extent. When compared to other extracts, Ethyl acetate extract was more effective against *Alternaria* sp., *A. flavus* and *S. rolfsii*. *C. capsici* and *Helminthosporium* sp. were inhibited to higher extent by petroleum ether and ethanol extracts respectively. Earlier studies have shown that *Ramalina* species exhibit antifungal activity against a range of fungi. In a previous study, Wei *et al*. (2008) reported antifungal activity of lichen forming fungi from *R. conduplicans* against *Colletotrichum acutatum*, causal agent of anthracnose of hot pepper. The solvent extracts of *R. hossei* were shown to exhibit varied inhibitory effect

against *Asperigllus niger* and *A. fumigatus* (Kumar *et al*., 2010b). Shukla *et al*. (2011) showed the antifungal effect of aqueous and acetone extracts of *Ramalina* sp. against phytopathogenic fungi. It has been shown that extracts of *R. roesleri* exhibit inhibitory activity against *Rhizoctonia bataticola* and other soil borne fungi (Goel *et al*., 2011).

CONCLUSIONS

The solvent extracts of *R. conduplicans* were shown to exhibit marked antimicrobial activity. The extracts exhibited inhibitory effect against reference strains of bacteria and clinical isolates from burn, dental caries and urinary tract infections. The extracts inhibited fungal isolates from anthracnose of chilli, foot rot of finger millet and mouldy grains of sorghum. The observed bioactivities could be ascribed to the presence of secondary metabolites in the lichen. The lichen *R. conduplicans* can be used in the treatment of bacterial infections and to manage fungal diseases of plants.

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