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ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF THREE *RAMALINA* SPECIES

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ABSTRACT

Lichens are an association of a photobiont (an alga or a cyanobacterium) and a mycobiont (a fungus). The lichen genus *Ramalina* is one of the cosmopolitan lichen genera and is characterized by fruticose thallus. In the present study, an antibacterial and antifungal activity of an extract of three *Ramalina* species (Ramalinaceae) viz. *R. hossei* Vain, *R. conduplicans* Vain and *R. pacifica* Asahina obtained by maceration process were investigated. The lichens were collected from different places of Shivamogga district, Karnataka, India and identified on the basis of morphological, anatomical and chemical tests. The antibacterial and antifungal activity of lichen extracts was carried out by Agar well diffusion and Poisoned food technique respectively. Overall, *B. cereus* and *E. coli* were inhibited to higher extent and least extent respectively by extracts of *Ramalina* species. *R. pacifica* and *R. hossei* inhibited bacteria to highest and least extent respectively. In the case of antifungal activity, marked and least inhibitory activity was shown by an extract of *R. hossei* and *R. pacifica* respectively. Among fungi, *Alternaria* sp. and *Fusarium* sp. were inhibited to highest and least extent respectively. The observed antimicrobial potential could be ascribed to the presence of secondary metabolites such as usnic acid, salazinic acid and sekikaic acid present in the *Ramalina* species.

Key words: Lichens, *Ramalina*, Antimicrobial, Agar well diffusion, Poisoned food technique

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INTRODUCTION

The development of resistance in bacteria has become one of the major global problems. Bacteria develop resistance against antibiotics in relatively shorter period of time making the therapy of diseases more difficult. Bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococci*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Mycobacterium tuberculosis* are among the many drug resistant bacteria. Unrestrained use of antibiotics and the ability of pathogenic bacteria to spread the resistance gene to susceptible strains by genetic means are resulting in gradual increase in resistance development rates. Besides, most of the antibiotics are costly and their use is often associated with certain adverse effects on the health of the individuals. Nowadays, the scientific community is focusing more on the search for alternatives for disease therapy. Natural products such as plants, microbes and lichens have been investigated and are found to be promising alternatives. Studies have shown the potential of lichens and their compounds to inhibit a wide range of pathogenic bacteria including antibiotic resistant strains[1-8].

Seed is the most important input for producing majority of food crops (about 90%) as these crops are mainly propagated by seeds. Several pathogenic fungi such as species of *Alternaria*, *Curvularia*, *Drechslera*, *Helminthosporium*, *Fusarium*, *Pyricularia* and *Rhizoctonia* are often found on seeds. The fungi are known to cause deterioration of seed quality, reduction in nutritive value, reduce the viability of seeds and reduction in seed germination and emergence. The use of synthetic fungicides is widely followed to manage the infections of plants caused by phytopathogenic fungi. These chemical agents are costly and their extensive use is associated with several drawbacks such as residual content in environment (leading to pollution), toxicity to humans and other non-target organisms and emergence of resistant strains of phytopathogenic fungi. Hence, there is a great need for searching alternatives for management of seed-borne and other pathogenic fungi. It is shown that natural products including lichens exhibit growth inhibitory activity against a wide range of phytopathogenic fungi[9-15].

Lichens are composite organisms comprised of an alga or a cyanobacterium (photobiont) and a fungus (mycobiont) found in an ecologically obligate and stable symbiosis. Lichens are non-vascular cryptogams found distributed in various geographical places of the world such as Antarctic regions, tropical and temperate forests, desserts and high mountains. Lichens occur in any one of the three major growth forms namely crustose, foliose and fruticose. In many parts of the world, lichens are used traditionally as medicine, spice, food and as a source for preparation of perfumes and dyes. Lichens are sensitive to pollution and are considered as the indicators of air pollution. Lichens are known to produce a number of low molecular weight compounds called lichen substances. These compounds are specific to lichens and are not produced by other organisms. Extracts and purified metabolites from lichens exhibit several bioactivities such as antimicrobial, anticancer, antiviral, antioxidant, enzyme inhibitory, anti-inflammatory, analgesic and antipyretic activity[16-26].

Ramalina Ach. (Ascomycetes: Lecanorales: Ramalinaceae) is one of the important, cosmopolitan and widely studied lichen genera and contains about 200 species. The genus was first described by Acharius. The species of *Ramalina* are characterized by a fruticose thallus which is attached to the substrate by a holdfast (basal). The thalli are heteromerous, pendent or erect, tufted or sparsed and dichotomously or irregularly branched. The branches may be circular, wide lobed or narrow strap shaped. The members of *Ramalina* are distributed in diverse habitats such as lowlands, highlands, rainforests and alpines. The species occur on various substrates like rocks, peaty soil, wood, and bark. In fresh conditions, the thalli are greenish-gray to yellowish-gray in color. The color changes to yellowish-brown to dark-brown on drying. The species of *Ramalina* contain pseudocyphellae. Usnic acid is one of the major metabolites found in the members of *Ramalina*[27-29]. Several *Ramalina* species are used traditionally as medicine, food and spice[23,30-33]. Various bioactivities such as antimicrobial, antioxidant, enzyme inhibitory, insecticidal, cytotoxic, anthelmintic and immunostimulatory activity are shown by some species of *Ramalina*[5,22,34-38]. The present study was performed to investigate antibacterial and antifungal activity of three *Ramalina* species viz. *R. hossei*, *R. conduplicans* and *R. pacifica*.

MATERIALS AND METHODS

Collection and identification of lichens

The lichens used in the present study were collected from different places of Shivamogga district, Karnataka, India during January-February 2017. The collected lichens were identified on the basis of morphological, anatomical and color tests. The color tests were carried out on cortex and medulla by using 10% potassium hydroxide (K), Steiner's stable para-phenylenediamine solution (P) and calcium hypochlorite solution (C). Thin layer chromatography (TLC) was used to detect the secondary metabolites present in lichens. Solvent system A (Benzene: 1, 4-Dioxane: Acetic acid) was used as developing solvent[27,39,40]. Details on the growth form, substratum and the place of collection of *Ramalina* are shown in Table 1. Characteristics of thallus and the result of color test and TLC of the selected *Ramalina* species is shown in Table 2.

Extraction

The dried and powdered lichen materials were extracted using methanol by maceration process. The lichen powders (10g) were left in methanol (100ml) for 48 hours in stoppered containers. The containers were stirred occasionally. The contents were filtered through Whatman filter paper and the filtrates were evaporated to dryness. The crude lichen extracts were stored in refrigerator[14,41].

Table 1: Growth form and place of collection of lichens

Lichen	Growth form and Substrate	Place of collection
Ramalina pacifica Asahina	Fruticose; *Areca catechu*	Shikaripura
Ramalina conduplicans Vain.	Fruticose; *Areca catechu*	Koodli
Ramalina hossei Vain.	Fruticose; *Gardenia gummifera*	Sagara

Table 2: Thallus features, Color test and TLC of *Ramalina* species

Lichen	Thallus characteristics	Color test	Secondary metabolites
R.conduplicans	Thallus corticolous, about 6-8cm long, branched (branches 2-3mm wide), erect to decumbent, greenish grey in color; upper side smooth and scarcely pseudocyphellate; lower side rugose, with raised, round to oblong, prominent pseudocyphellae; soredia absent; chondroid tissue uneven in thickness; medulla solid; apothecia about 2mm in diameter, ascospores straight or curved.	Cortex K-; Medulla K-, C-, KC -, Pd+ yellow	Usnic acid, Salazinic acid, Sekikaic acid
R.pacifica	Thallus corticolous, about 10cm long, pendulous, brownish grey in color, branched (branches 1-2mm wide), anastomosing, apically attenuate; striate, pseudocyphellae marginal; soralia laminal to marginal; chondroid tissue uniform, not cracked; medulla solid.	Medulla K-, C-	Usnic acid, Salazinic acid
R.hossei	Thallus corticolous, about 5-6cm long, tufted, erect, yellowish grey in color, branched (branches about 2mm wide), nervosa; marginal pseudocyphellae turn into soralia; soredia granular; chondroid tissue cracked; medulla solid; apothecia 1mm in diameter; ascospores straight or slightly curved.	Cortex K+ yellow; Medulla K-, C-	Usnic acid, Sekikaic acid

Antibacterial activity of *Ramalina* species

Agar well diffusion method was used to evaluate antibacterial potential of three *Ramalina* species. The test bacteria 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). The bacteria were procured from NCL, Pune, India. The pure cultures of test bacteria were maintained on nutrient agar slants under refrigeration condition. The test bacteria were seeded into sterile nutrient broth tubes and incubated overnight at 37^{o}C. The broth cultures of test bacteria were inoculated all over the surface of sterile nutrient agar plates by using sterile cotton swabs. Using sterilized cork borer, wells of 8mm diameter were punched in the inoculated plates. The wells were labeled and filled with 100µl of lichen extract (20mg/ml of DMSO), reference antibiotic (Chloramphenicol; 1mg/ml of sterile distilled water) and DMSO. The plates were incubated for 24 hours at 37^{o}C. The zones of inhibition were measures using a ruler[14,41].

Antifungal activity of *Ramalina* species

The test fungi used were *Alternaria* sp., *Curvularia* sp. and *Fusarium* sp. These fungi were isolated previously from sorghum seeds. The fungi were maintained on Potato dextrose agar slants under refrigerated condition. The antifungal activity of extract of *Ramalina* species was carried out by Poisoned food technique. The test fungi were inoculated into control (without extract) and poisoned Potato dextrose agar (0.5mg extract/ml of medium) plates aseptically. The plates were incubated for 5 days at room temperature followed by determining the size of colonies (diameter) in mutual perpendicular directions using a ruler. The antifungal effect of extracts was determined using the formula:

Inhibition of mycelial growth (%) = (Dc – Dt / Dc) x 100, where Dc and Dt refers to diameter of fungal colonies on control plates and poisoned plates respectively[14,41].

Statistical analysis

All experimental protocols were carried out in triplicates (n=3). The results are represented as Mean±Standard deviation (S.D).

RESULTS AND DISCUSSION

In the present study, we investigated the antibacterial potential of three *Ramalina* species by agar well diffusion assay. The extracts from selected lichens were shown to inhibit the growth of bacteria as evidenced by the presence of zones of inhibition around the wells. Among bacteria, *B. cereus* and *E. coli* exhibited highest and least susceptibility respectively to lichen extracts. Next to *B. cereus*, *P. aeruginosa* exhibited high susceptibility to lichen extracts. Reference antibiotic displayed high inhibition of test bacteria when compared to *Ramalina* species. The diluent DMSO did not cause inhibition of any test bacteria (Table 3).

Table 3: Antibacterial activity of *Ramalina* species

Treatment	Zone of inhibition in cm (Mean±S.D; n=3)			
	E. coli	***P. aeruginosa***	***B. subtilis***	***B. cereus***
R. pacifica	1.60±0.00	2.16±0.05	1.76±0.05	2.70±0.00
R. conduplicans	1.43±0.05	2.00±0.00	1.50±0.00	2.60±0.10
R. hossei	1.30±0.00	1.30±0.10	1.33±0.05	2.20±0.00
Antibiotic	2.66±0.05	2.90±0.00	3.40±0.00	3.66±0.05
DMSO	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Among lichens, *R. pacifica* exhibited marked antibacterial potential when compared to other two species of *Ramalina*. In a study, Hoskeri *et al*.[5] showed the potential of extract of *R. pacifica* to inhibit clinical isolates. In another study, Kekuda *et al*.[42] showed inhibitory activity of *R. pacifica* against reference bacteria. Next to *R. pacifica*, *R. conduplicans* was found to exhibit marked antibacterial activity against Gram positive and Gram negative bacteria. In an earlier study, Kambar *et al*.[14] showed concentration dependent inhibitory activity of solvent extracts of *R. conduplicans* against Gram positive and Gram negative bacteria. The study of Devi *et al*.[43] showed that methanol extract of *R. conduplicans* was effective in inhibiting bacteria to higher extent when compared to other solvent extracts. In the present study, the antibacterial activity observed against test bacteria was least in case of *R. hossei*. Extract of *R. hossei* caused marked inhibitory activity against *B. cereus* while inhibition of other test bacteria by extract was more or less similar. In a previous study, Vinayaka *et al*.[44] found marked antibacterial activity of methanol extract of *R. hossei* against test bacteria. In another study, Kekuda *et al*.[42] revealed potent antibacterial activity of extract of *R. hossei* against Gram positive and Gram negative bacteria. Recently, Kekuda and Vinayaka[8] showed the potential of *R. hossei*, *R. conduplicans* and *R. pacifica* to inhibit clinical isolates of *Streptococcus mutans*. Antibacterial activity of other species of *Ramalina* such as *R. farinacea*[6,45], *R. roesleri*[46], *R. canariensis*[47], *R. chondrina*[47], *R. fastigiata*[4,47], *R. fraxinea*[4,47], *R. pollinaria*[4], *R. polymorpha*[4], *R. capitata*[4], *R. menziesii*[48] have been investigated.

In the present study, we determined antifungal potential of three *Ramalina* species by Poisoned food technique. Extract of all three *Ramalina* species were effective in inhibiting test fungi but to a varied extent. Considerable reduction in the mycelial growth of test fungi was observed in poisoned potato dextrose agar plates. Antifungal potential of *Ramalina* species was in the order: *R. hossei* > *R. conduplicans* > *R. pacifica*. The susceptibility of test fungi to extracts was in the order: *Alternaria* sp. > *Curvularia* sp. > *Fusarium* sp (Table 4).

Table 4: Antifungal activity of *Ramalina* species

Treatment	Colony diameter in cm (Mean±S.D; n=3)		
	***Alternaria* sp.**	***Curvularia* sp.**	***Fusarium* sp.**
Control	5.10±0.00	4.70±0.10	3.46±0.05
R. hossei	0.66±0.05	1.80±0.10	2.20±0.00
R. pacifica	1.80±0.00	2.20±0.00	2.30±0.10
R. conduplicans	1.30±0.00	2.13±0.05	2.20±0.10

R. hossei inhibited *Alternaria* sp. to higher extent (87.05% inhibition) followed by *Curvularia* sp. (61.70% inhibition) and *Fusarium* sp (36.41% inhibition). In previous studies by Kambar *et al*.[49] and Kekuda *et al*.[42], the extracts of *R. hossei* were shown to exhibit marked antifungal activity against phytopathogenic fungi. In this study, *R. pacifica* was shown to cause inhibition of *Alternaria* sp. to higher extent (64.70% inhibition) followed by *Curvularia* sp. (53.19% inhibition) and *Fusarium* sp. (33.52% inhibition). In an earlier study, Kekuda *et al*.[42] showed potent antifungal activity of *R. pacifica* against a panel of phytopathogenic fungi with marked inhibitory activity against *Colletotrichum capsici*. It was observed in the present study that the extract of *R. conduplicans* inhibited *Alternaria* sp. to higher extent (74.50% inhibition) followed by *Curvularia* sp. (54.68% inhibition) and *Fusarium* sp (36.41% inhibition). In a similar study, Kambar *et al*.[14] showed antifungal effect of different solvent extracts of *R. conduplicans* against a panel of fungi from plant sources. In another study, Devi *et al*.[43] showed the potential of various solvent extracts of *R. conduplicans* to inhibit phytopathogenic fungi. Studies have shown the potential of other species of *Ramalina* to exhibit antifungal activity. In an earlier study, Shivanna and Garampalli[50] showed the potential of *R. farinacea* to inhibit *Fusarium oxysporum* and *F. solani*. The study of Goel *et al*.[12] showed the potential of *R. roesleri* to inhibit a range of phytopathogenic fungi including *Rhizoctonia bataticola*.

The lichens produce a number of secondary metabolites that seldom occur in other organisms. These compounds are called lichen substances and till now >1000 lichen secondary metabolites have been identified. Many of these compounds are low molecular weight phenolic compounds and are known to protect the lichens by functioning as sunscreen, anti-herbivore etc[21,26]. TLC is one of the techniques used to detect the secondary metabolites present in lichen extracts. In the present study, the thin layer chromatogram of the *Ramalina* species showed the presence of usnic acid in all species. Salazinic acid was present in *R. conduplicans* and *R. pacifica* while sekikaic acid was present in *R. hossei* and *R. conduplicans*. Antimicrobial activity of usnic acid[18,35,51], salazinic acid[52] and sekikaic acid[26,35,53] have been reported. The study of Cansaran *et al*.[4] showed a direct correlation between the amount of usnic acid and the antimicrobial activity observed.

CONCLUSIONS

The *Ramalina* species selected in this study were shown to exhibit marked antibacterial and antifungal activity. These lichens can be used to develop formulations or agents which can be used to treat infectious diseases and to manage seed-borne fungal diseases. The observed inhibitory activity of lichens could be ascribed to the presence of bioactive metabolites such as usnic acid, salazinic acid and sekikaic acid which have been detected by TLC.

CONFLICTS OF INTEREST

None declared

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