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Research Article

Antimicrobial Activity of *Leptogium burnetiae*, *Ramalina hossei*, *Roccella montagnei* and *Heterodermia diademata*

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Abstract

Lichens are self-supporting symbiotic association comprising of a photosynthetic partner (Photobiont) and a fungal partner (Mycobiont). Lichens have traditional importance and are known to exhibit a range of bioactivities. The present study was carried out to screen the antimicrobial efficacy of extracts of four macrolichens *viz*., *Leptogium burnetiae* C.W. Dodge, *Ramalina hossei* H. Magn and G. Awasthi, *Roccella montagnei* Bel. Em. D.D. Awasthi and *Heterodermia diademata* (Taylor) D.D. Awasthi collected at different regions of Karnataka, India. The shade dried and powdered lichen materials were extracted using methanol. Antibacterial activity of lichen extracts was tested against clinical isolates of burn, dental caries and urinary tract infections by Agar well diffusion assay. Antifungal activity was tested against two reference yeast strains and *Colletotrichum capsici* (isolate from anthracnose of chilli) by Agar well diffusion assay and Poisoned food technique respectively. The extracts of selected lichens were effective in inhibiting bacteria and fungi. The clinical isolates of bacteria were inhibited by lichen extracts dose dependently. In case of urinary tract isolates, Gram positive bacteria have shown more susceptibility to lichen extracts when compared to Gram negative bacteria. Among yeasts, *Cryptococcus neoformans* was susceptible to higher extent than *Candida albicans* to lichen extracts. The mycelial growth of *C. capsici* was markedly lesser in plates poisoned with lichen extracts. Overall, extract of *R. hossei* was more effective against clinical isolates and *C. capsici* while extract of *H. diademata* was more effective against yeast strains. In conclusion, the selected lichens were effective in inhibiting bacteria and fungi and can be used to control diseases caused by pathogenic microorganisms. The inhibitory effect of lichens could be ascribed to the presence of secondary metabolites. The lichens can be used to develop novel therapeutic agents active against pathogens.

1. INTRODUCTION

Lichens are symbiotic organisms composed of Photobiont (algae or blue-green algae) and Mycobiont (fungi) together forming an independent physiological unit. The photobiont provides the lichen with nutrients by carrying out photosynthesis, and the mycobiont helps in absorption of water and nutrients from surroundings and also protect the photobiont. Hence, lichens are self-supporting and grow on rocks, roofs, tree trunks etc. Lichens occur in different growth forms namely crustose, foliose and fruticose. They do not have specialized organs such as roots, leaves etc., and this permits them to survive in harshest environmental conditions. They inhabit in almost every possible ecological niche ranging from artic to tropical regions and from the plains to the highest mountains. They are considered as the primary colonizers of terrestrial ecosystem. Lichens represent one of the dominant organisms and together with mosses, lichen covers 10% of terrestrial habitats. Lichens are most popular as indicators of air pollution. Lichens are considered as valuable resources of medicine, food, fodder, perfume, spices and dyes. Lichens are consumed by people in North America, Europe, Asia and Africa. Lichens are generally considered as famine foods and are eaten only in times of their needs. Many lichen species are used as spice and flavoring agents in the preparation of certain kinds of foods. Lichens have been traditionally used to treat various ailments such as dyspepsia, bleeding piles, diabetes, bronchitis, pulmonary tuberculosis, spermatorrhoea, bleeding piles, leprosy etc. throughout the world. They produce characteristic secondary metabolites (lichen substances) which seldom occur in other organisms. Majority of these metabolites are produced by fungal partner. These compounds are helpful in the lichen taxonomy. The lichen metabolites have diverse biological activities such as antimicrobial, antioxidant, enzyme inhibitory, cytotoxic, antiherbivore, phytotoxic, analgesic, wound healing, antitermite, antiinflammatory and others. There are >20000 lichens described all over the world so far and 10% of them are found in India. In India, rich lichen diversity can be seen in Himalayas and Western Ghats[1-6]. Literatures are available on the biological activities of macrolichens of Karnataka, in particular lichens from Western Ghats of Karnataka. The macrolichens of Karnataka have been shown to exhibit bioactivities such as antimicrobial, antioxidant, enzyme inhibitory, anthelmintic, cytotoxic and insecticidal activity[2,4,6-12]. The present study aimed at screening antimicrobial activity of four corticolous macrolichens namely *Leptogium burnetiae*, *Ramalina hossei*, *Roccella montagnei* and *Heterodermia diademata* collected from different regions of Karnataka, India.

2. MATERIALS AND METHODS

2.1. Collection and identification of lichens

Four macrolichens of this study were collected from different places of Shivamogga district, Karnataka, India. Details on the type, family and collection place is shown in Table 1. The collected lichens were identified based on morphological, anatomical and chemical tests. Color reactions were done on the cortex and medulla by using three reagents namely 10% potassium hydroxide (K), Steiner's stable paraphenylenediamine solution (P) and calcium hypochlorite solution (C). Thin layer chromatography (TLC) was performed to identify characteristic secondary metabolites using solvent system A (Benzene:1,4-Dioxane:Acetic acid in the ratio 90:25:4)[13,14,15].

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Table 1: Macrolichens of the present study

Lichen	Form	Habitat	Family	Place of collection
R. hossei	Fruticose	Corticolous	Ramalinaceae	Umblebailu
L. burnetiae	Foliose	Corticolous	Collemataceae	Haniya
H.diademata	Foliose	Corticolous	Physciaceae	Shikaripura
R. montagnei	Fruticose	Corticolous	Roccellaceae	Aynur

2.2. Extraction

The lichen thalli were shade dried and powdered. 25g of each lichen material was transferred into separate conical flaks containing 100ml methanol (HiMedia, Mumbai). The flasks were left for 48 hours with occasional stirring. The contents of the flaks were filtered through sterile Whatman No. 1 filter paper, concentrated and stored in desiccator[16].

2.3. Antibacterial activity of lichen extracts

Agar well diffusion assay was performed to screen antibacterial potential of lichen extracts against a total of 8 clinical isolates of bacteria which included two isolates of *Streptococcus mutans* (Sm-01 and Sm-02) from dental caries, two isolates of *Staphylococcus aureus* (Sa-01 and Sa-02) from burn specimens and four drug resistant isolates *viz*., *S. aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Klebsiella pneumoniae* from urinary tract infection. The test bacteria were inoculated into sterile Nutrient broth (HiMedia, Mumbai) tubes and incubated at 37°C for 24 hours. The broth cultures were then swabbed uniformly on sterile Nutrient agar (HiMedia, Mumbai) plates using sterile cotton swabs. Using a sterile cork borer, wells of 6mm were punched in the inoculated plates. 100µl of lichen extracts (10 and 20mg/ml of Dimethyl sulfoxide [DMSO]), Chloramphenicol (reference antibiotic, 1mg/ml of sterile water) and DMSO (25%, in sterile water) were transferred into respective wells. The plates were incubated in upright position at 37°C for 24 hours and the zones of inhibition formed were measured using a ruler[16].

2.4. Anti-yeast activity of lichen extracts

In order to screen anti-yeast activity of lichen extracts, we performed Agar well diffusion assay. The test fungi *viz*., *Candida albicans* NCIM-3466 and *Cryptococcus neoformans* NCIM-3378 were inoculated into sterile Sabouraud's dextrose broth (HiMedia, Mumbai) tubes and incubated at 37°C for 48 hours. Using sterile cotton swabs, the broth cultures were swab inoculated on sterile Sabouraud's dextrose agar (HiMedia, Mumbai) plates. Wells of 6mm were punched in the inoculated plates using a sterile cork borer. 100µl of lichen extracts (10 and 20mg/ml of DMSO), Fluconazole (reference antibiotic, 1mg/ml of sterile water) and DMSO (25%, in sterile water) were transferred into respective wells. The plates were incubated in upright position for 48 hours at 37°C and the zones of inhibition formed were measured using a ruler[16].

2.5. Anti-mold activity of lichen extracts

Poisoned food technique was employed to determine the efficacy of lichen extracts to inhibit mycelial growth of *Colletotrichum capsici* isolated from anthracnose of chilli. Potato dextrose agar (HiMedia, Mumbai) was prepared, poisoned with lichen extracts (1mg extract/ml of medium), sterilized by autoclaving and dispensed into sterile plates. The control (without extract) and poisoned plates were inoculated with the test fungus by point inoculation method. The plates were incubated at 28°C for 5 days. After incubation, the colony diameters (CD) were measured in mutual perpendicular directions using a ruler. The antifungal activity of lichen extracts was recorded in terms of inhibition of mycelial growth (%) and was calculated using the formula:

Inhibition of mycelial growth (%) = (C – T / C) x 100, where C is CD in control plate and T is CD in poisoned plates[17].

3. RESULTS

3.1. Secondary metabolites in selected lichens

The secondary metabolites detected in selected lichens are depicted in Table 2. Secondary metabolites were not detected in *L. burnetiae*. Erythrin and Roccellic acid were present in *R. montagnei*. Usnic acid and Sekikaic acid were detected in *R. hossei* and Zeorin was present in *H. diademata*.

Table 2: Secondary metabolites detected in selected lichens

Lichen	Secondary metabolites
R.montagnei	Erythrin, Roccellic acid
H.diademata	Zeorin
R.hossei	Usnic acid, Sekikaic acid
L.burnetiae	None detected

3.2. Inhibitory activity of lichen extracts against *S. aureus* isolates

Table 3 depicts the antibacterial effect of lichen extracts against *S. aureus* isolates from burn. The extracts were effective in inhibiting the isolates in a dose dependent manner. Isolate Sa-01 was inhibited to higher extent by extract of *H. diademata* followed by *R. hossei* and *L. burnetiae*. Extract of *R. montagnei* was ineffective against Sa-01. In case of inhibition of isolates Sa-02, extract of *R. hossei* was more effective followed by *H.diademata*, *R. montagnei* and *L. burnetiae*. The reference antibiotic exhibited high inhibitory activity than lichen extracts. DMSO was not found to inhibit isolates.

Table 3: Inhibition of *S. aureus* isolates by lichen extracts

Treatment	Concentration	Zone of inhibition in cm (Mean±S.D)	
		***Sa*-01**	***Sa*-02**
R.montagnei	10mg/ml	0.0±0.0	1.8±0.1
	20 mg/ml	0.0±0.0	2.3±0.0
H.diademata	10mg/ml	2.5±0.1	2.0±0.1
	20 mg/ml	2.8±0.1	2.1±0.1
R.hossei	10mg/ml	2.1±0.1	2.3±0.1
	20 mg/ml	2.4±0.0	2.6±0.2
L.burnetiae	10mg/ml	1.5±0.1	1.6±0.0
	20 mg/ml	1.7±0.1	1.7±0.1
Chloramphenicol	1mg/ml	2.6±0.2	2.4±0.1
DMSO	25%	0.0±0.0	0.0±0.0

3.3. Inhibitory activity of lichen extracts against *S. mutans* isolates

The result of inhibitory effect of lichen extracts against *S. mutans* isolates is shown in Table 4. All lichen extracts were effective against both the isolates and the effect was concentration dependent. Both the isolates were inhibited to higher extent by extract of *H.diademata* and *R. hossei* than that of other two extracts.

Table 4: Inhibition of *S. mutans* isolates by lichen extracts

Treatment	Concentration	Zone of inhibition in cm (Mean±S.D)	
		***Sm*-01**	***Sm*-02**
R.montagnei	10mg/ml	1.3±0.0	1.3±0.1
	20 mg/ml	1.6±0.0	1.5±0.0
H.diademata	10mg/ml	1.8±0.0	1.6±0.0
	20 mg/ml	2.0±0.0	1.8±0.1
R.hossei	10mg/ml	1.7±0.1	1.6±0.0
	20 mg/ml	1.9±0.2	1.9±0.1
L.burnetiae	10mg/ml	1.3±0.1	1.2±0.1
	20 mg/ml	1.7±0.1	1.5±0.1
Chloramphenicol	1mg/ml	2.8±0.2	3.2±0.2
DMSO	25%	0.0±0.0	0.0±0.0

3.4. Inhibitory activity of lichen extracts against urinary tract isolates

The lichen extracts exhibited varied inhibitory potential against urinary tract isolates and the result is shown in Table 5. The extracts displayed dose dependent inhibitory activity against bacteria. Overall, the extracts were effective against Gram positive bacteria to higher extent than Gram negative bacteria. Among Gram positive bacteria, *E. faecalis* was inhibited to higher extent by extract of *R. montagnei*, *H.diademata* and *R. hossei* whereas *L. burnetiae* was more effective against *S. aureus*. In case of Gram negative bacteria, *E. coli* was found more susceptible than *K. pneumoniae* to lichen extracts. Overall, *K. pneumoniae* was least inhibited by lichen extracts among urinary tract bacteria. Reference

antibiotic caused high inhibition of test bacteria than lichen extracts. There was no inhibition of bacteria by DMSO.

Table 5: Inhibition of uropathogenic bacteria by lichen extracts

Treatment	Concentration	Zone of inhibition in cm (Mean±S.D)			
		S. aureus	*E. faecalis*	*E. coli*	*K. pneumoniae*
R.montagnei	10mg/ml	1.7±0.1	1.8±0.1	1.1±0.0	0.0±0.0
	20 mg/ml	1.9±0.1	2.1±0.1	1.3±0.1	0.8±0.0
H.diademata	10mg/ml	1.8±0.1	1.8±0.0	1.4±0.0	0.0±0.0
	20 mg/ml	1.9±0.1	1.9±0.1	1.6±0.1	0.0±0.0
R.hossei	10mg/ml	1.4±0.0	2.1±0.1	2.8±0.2	0.0±0.0
	20 mg/ml	1.6±0.0	2.3±0.1	3.2±0.2	0.8±0.0
L.burnetiae	10mg/ml	1.4±0.0	1.2±0.0	0.0±0.0	0.0±0.0
	20 mg/ml	1.8±0.0	1.6±0.0	1.0±0.0	0.8±0.0
Chloramphenicol	1mg/ml	3.3±0.1	3.0±0.2	2.2±0.2	1.8±0.1
DMSO	25%	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

3.5. Anti-yeast activity of lichen extracts

Table 6 depicts the result of anti-yeast activity of extracts of selected lichens. The extracts were found to exhibit dose dependent inhibitory activity against *C. albicans* and *C. neoformans*. Among yeasts, marked inhibitory activity was shown by lichen extracts against *C. neoformans*. Extracts of *R. montagnei* and *H. diademata* inhibited *C. albicans* to a negligible extent only at 20mg/ml concentration. *R. hossei* and *L. burnetiae* were not effective against *C. albicans*. In case of *C. neoformans*, marked antifungal effect was shown by extract of *H.diademata*. Other lichen extracts displayed weak inhibitory activity. Reference antibiotic caused higher inhibition of test fungi. DMSO did not cause inhibition of fungi.

Table 6: Inhibition of *C. albicans* and *C. neoformans* by lichen extracts

Treatment	Concentration	Zone of inhibition in cm (Mean±S.D)	
		C. albicans	*C. neoformans*
R.montagnei	10mg/ml	0.0±0.0	0.8±0.0
	20 mg/ml	0.8±0.0	1.1±0.0
H.diademata	10mg/ml	0.0±0.0	2.0±0.2
	20 mg/ml	0.8±0.0	2.2±0.1
R.hossei	10mg/ml	0.0±0.0	0.0±0.0
	20 mg/ml	0.0±0.0	0.8±0.0
L.burnetiae	10mg/ml	0.0±0.0	0.0±0.0
	20 mg/ml	0.0±0.0	0.8±0.0
Fluconazole	1mg/ml	3.2±0.1	2.2±0.0
DMSO	25%	0.0±0.0	0.0±0.0

3.6. Inhibitory potential of lichen extracts against *C. capsici*

The result of antifungal activity of lichen extracts in terms of reduction in colony diameter of *C. capsici* is shown in Table 7 and Figure 1. The poisoning of medium with lichen extracts resulted in considerable reduction in the mycelial growth of *C. capsici* when compared to colony diameter on control plates. Among lichen extracts, marked antifungal effect was observed in case of *R. hossei* (which caused >90% inhibition of mycelial growth) followed by *L. burnetiae*, *H. diademata* and *R. montagnei*.

Table 7: Colony diameter of *C. capsici* on control and poisoned plates

Treatment	CD in cm (Mean±S.D)
Control	3.5±0.2
R.montagnei	2.2±0.1
H.diademata	1.6±0.1
R.hossei	0.2±0.0
L.burnetiae	1.1±0.1

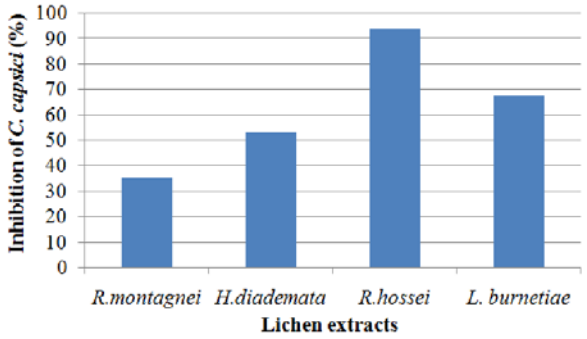

Figure 1: Inhibition of *C. capsici* (%) by lichen extracts

4. DISCUSSION

Throughout history, infectious agents have threatened mankind and caused millions of deaths. The diseases caused by infectious agents such as bacteria, fungi and viruses were drastically reduced after the discovery and subsequent use of antibiotics. The antibiotic therapy saved countless lives. However, these miracle drugs were challenged by the development of resistance in pathogens. Bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus faecalis* and *Mycobacterium tuberculosis* are among the most important drug resistant pathogens. The antimicrobial resistance leads to ineffectiveness of treatment and increased morbidity, mortality and health care expenditure. These pathogens also have the ability to acquire and transmit resistance which made the antibiotic resistance a global issue. High cost, side effects of antibiotics and development of resistance in pathogens against antibiotics stimulated research on finding antimicrobials from natural sources[4,18,19,20,46-52].

It has been shown that the lichens are among the promising sources of chemotherapeutic agents with activity against a range of pathogenic organisms including clinical and drug resistant strains[12,21-27, 53-59]. In the present study, we subjected the extracts of four macrolichens for inhibitory activity against clinical isolates of *S. aureus*, *S. mutans* and urinary tract pathogens. The lichen extracts were found effective against clinical isolates in a dose dependent manner. Among uropathogens, Gram positive bacteria have shown high susceptibility to lichen extracts than Gram negative bacteria. Similar results were observed in earlier studies on lichens[27,28,29,60,61]. The lesser susceptibility of Gram negative bacteria to lichen extracts could be ascribed to their cell wall structure i.e., the presence of outer membrane in Gram negative bacteria acts as an additional barrier for the entry of substances into the cells[30,31].

Several fungi are known to cause dreadful diseases in man. Among these, *Candida albicans* and *Cryptococcus neoformans* are frequently encountered fungal pathogens in HIV-infected patients and those receiving immunosuppressive treatment for cancer, organ transplantation, and other serious medical conditions. *C. albicans* is a common etiological agent of bloodstream infections and accounts for majority of nosocomial fungal infections. *C. neoformans* is encapsulated yeast responsible for causing fatal infection in both immunocompetent and immunocompromised patients. Like bacteria, these fungi have also gained resistance against most commonly used antimycotic drugs such as Fluconazole, Itraconazole, Amphotericin B etc[32,33]. Hence, search for alternatives is of utmost importance. In the present study, we determined the inhibitory effect of extracts of selected lichens against *C. albicans* and *C. neoformans*. It is observed that the extracts exhibited dose dependent inhibitory activity. Overall, *C. neoformans* displayed higher susceptibility than *C. albicans*. Similar result was observed in an earlier study of Pavithra *et al.*[4] where extract of *Usnea pictoides* inhibited *C. neoformans* to higher extent when compared to *C. albicans*.

Chilli belonging to the genus *Capsicum* (Solanaceae) is an herbaceous, annual, dicotyledonous flowering plant grown in tropical and subtropical regions. It is an important commercial crop grown worldwide for consumption, nutritional and economy purposes. It is a spice (ripe and dried form) as well as vegetable (green fruit). India is the largest producer of chilli in terms of international trade. Chilli is nutritionally rich as it contains steam-volatile oils, fatty oils, capsaicinoids, carotenoids, vitamins, protein,

fibre and minerals. The chilli cultivation is influenced by a number of diseases caused by fungi, bacteria and viruses which result in marked reduction in productivity. Among various diseases of chilli, Anthracnose (both pre-harvest and post-harvest) is most important. It causes yield loss (up to 50%) and deterioration of fruit quality. The symptoms of Anthracnose on chilli fruit include sunken necrotic tissues with concentric rings of acervuli. Several species of *Colletotrichum* such as *C. capsici, C. acutatum, C. gloeosporioides, C. coccodes* and *C. dematium* are implicated in causing anthracnose. Among these, *C. capsici* is the most important pathogen. Fungicides such as mancozeb, captan, bavistin, thiram, copper oxychloride, cosan, benlate and ziram are used to control anthracnose disease. However, the resistance against these fungicides has been noticed in most fungal pathogens including *C. capsici*. Thus, search for alternative disease control strategies are of immense interest. Natural products have been extensively studied for the control of phytopathogenic fungi as they are cost effective and have potential efficacy with no or negligible side effects[17,34-39,62,63]. It has been shown that lichens and their metabolites exhibit inhibitory activity against phytopathogenic fungi[40-44]. In the present study, the extracts of selected macrolichens exhibited marked inhibitory effect against *C. capsici* isolated from anthracnose of chilli. Among lichens, highest and least inhibitory effect was displayed by *R. hossei* and *R. montagnei* respectively. In a previous study, we reported antifungal activity of three *Parmotrema* species against mycelial growth of *C. capsici*. *P. tinctorum* was found to inhibit the fungus to a higher extent[45,64-67].

5. CONCLUSION

The extracts of selected lichens were shown to display marked inhibitory effect against clinical isolates of dental caries, burn and urinary tract infections, reference yeasts and *C. capsici*. These lichens can be used to control diseases caused by these pathogens. The inhibitory efficacy of lichens could be ascribed to the presence of secondary metabolites. These lichens appear as promising candidates for the development of therapeutic agents.

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