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Antimicrobial activity of the lichens *Parmotrema andium* and *Dirinaria applanata*

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ABSTRACT

The objective of this research is to investigate the *in vitro* antimicrobial activity of two species of lichen isolated from the Similipal Biosphere Reserve (SBR), *Parmotrema andium* and *Dirinaria applanata*. Two test species were extracted with the solvents, methanol and acetone, and the extracts were tested against four human pathogenic bacteria and fungi. The antimicrobial activity was carried out by agar well diffusion method along with determination of minimum inhibitory concentration (MIC) by broth microdilution method. The study results revealed that the solvent extracts of both the lichen species showed highest antimicrobial activity against *Vibrio cholerae* with larger zone of inhibition (20 ± 0.85 mm). The antibacterial activity was found to be more promising than the antifungal activity. Among the lichen species, *D. applanata* was found to have better antimicrobial activity as compared to *P. andium* as evidenced from the size of zone of inhibition. In terms of antimicrobial properties, solvent extracts of lichens were more active, with MICs ranging from 62.5 to 500 µg/ml.

1. INTRODUCTION

Lichen is a symbiotic organism that consists of a fungus (mycobiont) and a photosynthetic partner (photobiont). Lichens are complex ecosystems that include numerous related fungi, bacteria, and other microscopic species and are permanently associated with the lichen thallus [1]. Drug therapies useful against a variety of microbial pathogens have already been ideantified from several vascular plants, fungi, prokaryotes, marine organisms etc., yet there still remains a vast potential reservoir of lichens secondary metabolites. Lichens are well known for producing various compounds such as dibenzofuranes, depsides, depsidones, etc. with a special structure. The majority of these compounds displayed different biological activities [2]. The antibiotic potential of lichen secondary compounds was first investigated by Burkholder et al. [3]. Since then more than 1,000 secondary compounds have been identified [4], only a few have been tested against bacteria and identified as potential antibiotics. Different biochemical effects of lichens and their secondary metabolites are known, such as: antiviral, antibiotic, anti-tumor, anti-allergic, anti-herbivoral activity which inhibit plant growth and the function of different enzymes [5,6]. Lichen extracts have a distinct antimicrobial activity along with their components [7]. It is well known that resistance to many antibiotics is very well established among microorganisms. Lichens consist of biologically active substances that are special and varied, primarily with antimicrobial activities. Due to the marked antimicrobial activity of secondary metabolites, lichens are highly regarded by researchers as essential new sources of bioactive substances [8]. The extensive use of antibiotics has several causes of antibiotic resistance and encouraged the spread of microorganisms that are multiple resistant, creating a major medical problem in the treatment of infectious diseases. Thus, new sources of novel bioactive compounds were identified for the purpose, such as medicinal herbs, fungi, and lichens [9]. In medicine, lichens are very important because microorganisms' resistance to many common antibiotics posses serious threats to human health and creates a major medical problem in the treatment of infectious diseases [10]. On searching the literature, it was noted that there is a rich diversity of lichen species found in the study site, Similipal Biosphere Reserve (SBR) [11] but no such work has been carried

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out on bioactivity of several lichens species found in this region. Therefore, the *in vitro* antimicrobial activity of the methanol and acetone extract of the lichens *Parmotrema andium* and *Dirinaria applanata* has been taken up in this investigation.

2. MATERIALS AND METHODS

2.1. Collection of Lichens

Two species of lichens (*P. andium* and *D. applanata*) were collected from SBR and presented in Figure 1. The Similipal massif lies between 21°28′ to 22°08′ N latitude and 86°04′ to 86°37′ E longitudes in the Mayurbhanj district of Odisha. It is scattered from dry deciduous to moist green forests with a wide variety of rainfall and edaphic differences, which is ideal for harboring many species of flora and fauna. The species were preserved in the Department of Biotechnology, Maharaja Sriram Chandra Bhanja Deo University, Baripada, Odisha, India. Identification of lichens was done using standard methods [12,13].

2.2. Preparation of Lichen Extracts

The dried lichen material was well grounded to uniform powder using a sterile mortar and pestle. Then, at room temperature for 72 hours, 0.5 g of dried powder lichen material was soaked in 10 ml of suitable solvent (methanol and acetone). Extracts were subsequently filtered through a Whatman No.1 filter paper and under reduced pressure the filtrate was evaporated. The extracts were then tested for their antimicrobial activity.

2.3. Phytochemical Activity

The phytochemical evaluation was carried out on both the solvent extracts of *P. andium* and *D. applanata* using standard protocol for screening the presence of alkaloids, glycosides, phenolic compounds, flavonoids, saponins, tannins, steroids, anthocyanin, quinones, and resins compounds [14].

2.4. Antimicrobial Activity

2.4.1. Microorganisms

Two Gram-positive (*Staphylococcus aureus* MTCC-96*, Bacillus subtilis* MTCC-441) and two Gram-negative (*Vibrio cholerae* MTCC-3906*, Escherichia coli* MTCC-443) and four strains of fungus (*Candida albicans* MTCC 183, *Aspergillus niger* MTCC-1344*, Penicillium verrucosum* MTCC-1758*, Fusarium oxysporum* MTCC-284) were used for the experimental purpose. These microorganisms were obtained from IMTECH Chandigarh and maintained in Department of Biotechnology, Maharaja Sriram Chandra Bhanja Deo University, Baripada, Odisha, India.

2.4.2. Agar well diffusion assay

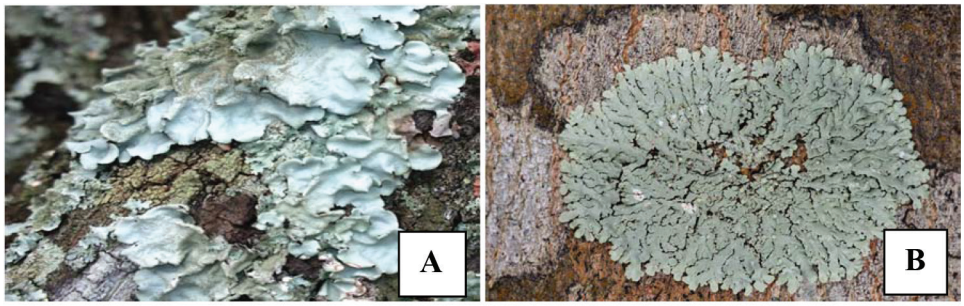
The antimicrobial activity was assayed by agar well diffusion [15] on Mueller–Hinton agar (MHA) for test bacteria and potato dextrose agar (PDA) for fungi. The 20 ml of sterilized MHA/PDA was poured into sterile petriplates, after solidification, 20 µl of test pathogens (10^6 Colony Forming Unit (CFU)/ml) were uniformly swabbed on the respective plates. In the inoculated plates, 6 mm diameter wells were bored using a sterile cork borer and 50 µl of lichen extracts dissolved in Dimethyl sulfoxide (DMSO) at 1 µg/µl concentration were loaded into the respective wells and incubated at 37°C for 24 hours for bacteria and 28°C for 48–72 hours for fungi. After the incubation period, the zone of inhibition formed around the wells were measured and expressed in millimeter (mm). As a positive control for antibacterial and antifungal activity, ampicillin and clotrimazole of 0.1 µg/µl were used, respectively. The studies have been carried out in triplicates.

2.4.3. Minimum inhibitory concentration (MIC)

For the determination of MIC, the standard 96 well plates with Mueller–Hinton broth (MHB) were used. The various concentration of lichen extracts (1,000, 500, 250, 125, 62.5, 31.25, and 15.62 µg/ml) were prepared using two-fold serial dilution along with MHB and pathogen as control. Approximately 50 µg of the test bacterial and fungal inoculum with a concentration of 10^6 CFU/ml were added [16]. The samples were analyzed with microplate reader (Biorad, iMark-11457) after 24 and 48 hours for Bacteria and Fungus, respectively. The minimum inhibition concentration was defined as the lowest concentration of the extract in the broth medium that inhibits the growth of the pathogens being studied [17].

2.5. Statistical Analysis

The result obtained from the experiment was statistically analyzed. All the experiments were carried out in triplicate and the results



P. andium *D. applanata*

Figure 1: (A–B) Lichen species collected from SBR.

were expressed as the mean values with standard deviations (±SD).The data were subjected to analysis of variance (one way) and the means were compared by the least significant difference test, where $p = 0.05$ was treated significant.

3. RESULTS

3.1. Phytochemical Analysis

The study results revealed the presence of various phytoconstituents as presented in Table 1. The alkaloids, phenols, flavonoids, and saponins was predominant and found in both the solvent extracts of two test species of lichens. The presence of phytoingradients was maximum in methanol extract in *D. applanata* as compared to acetone extracts whereas the lichen species *P. andium* did not show such promising result. The findings thus indicated that the solvent extract of both the lichen species, *D. applanata* possess more number of phytochemicals than *P. andium*.

3.2. Antimicrobial Activity

Four common human pathogenic bacteria (two Gram +ve and two Gram -ve) and fungus were used for antimicrobial study. Differential antimicrobial activity was obtained in the solvent extracts of two lichen species tested against the human pathogens (Table 2). The methanol extract of *D. applanata* exhibited significant antibacterial activity showing larger zone of inhibition i.e., 20 ± 0.85 mm against *V. cholerae*, whereas *P. andium* showed moderate antibacterial activity to all the test pathogens. Furthermore, antifungal activity was more prominent in *D. applanata* as marked with 19 ± 0.75 mm zone of inhibition against *C. albicans*. The study results were compared with the standard antibiotics for Bacteria (ampicillin) and for Fungi (clotrimazole) and DMSO was used as negative control.

3.3. Minimum Inhibitory Concentration (MIC)

The antimicrobial activity against the test pathogens of both lichen species was determined by the MIC values and shown in Table 3. The investigated lichen extracts revealed the antimicrobial activity against the test pathogens. Depending on the solvents used for extraction, their concentration and the pathogens used, there is variation in MIC values. The MIC for the various components was in a range of 62.5–500 µg/ml among the test bacteria and fungus. The notable antimicrobial effect was obtained in methanol extract of *D. applanata* and the MIC value was also significantly lower against gram-negative bacteria than Gram-positive. Among the fungal pathogens *C. albicans* depicted better MIC value.

4. DISCUSSION

The human experience of medicine has been revolutionized by the discovery of various antibiotics. However, due to the rapid use of antibiotics discovered so far, multidrug-resistant pathogens have

Table 1: Phytochemical analysis of *P. andium* and *D. applanata*.

Phytochemicals	*P. andium*		*D. applanata*	
	Methanol	Acetone	Methanol	Acetone
Alkaloid	+	+	+	+
Glycoside	−	−	−	−
Tannin	−	−	+	−
Flavonoid	+	+	+	+
Steroid	−	−	−	−
Saponin	+	−	+	+
Resin	−	−	+	−
Quinones	−	−	−	−
Phenol	+	-	+	+

+ present; − absent.

Table 3: MIC of methanol and acetone extract of *P. andium* and *D. applanata*.

Pathogens	MIC (µg/ml)			
	P. andium		*D. applanata*	
	Methanol	Acetone	Methanol	Acetone
S. aureus (MTCC-96)	250	500	125	250
B. subtilis (MTCC-441)	250	500	125	500
V. cholerae (MTCC-3906)	125	250	62.5	125
E. coli (MTCC-443)	125	250	62.5	250
A. niger (MTCC-1344)	250	500	125	250
C. albicans (MTCC-183)	250	500	62.5	125
P. verrucosum (MTCC-1758)	125	250	250	500
F. oxysporum (MTCC-284)	125	250	125	250

Table 2: Antimicrobial activity (zone of inhibition, mm) of *P. andium* and *D. applanata*.

Pathogens	Diameter of inhibition zone (mm)				
	P. andium		*D. applanata*		Antibiotics[a]
	Methanol	Acetone	Methanol	Acetone	
S. aureus (MTCC-96)	13 ± 0.74	12 ± 0.83	19 ± 0.81	17 ± 0.79	28 ± 0.82
B. subtilis (MTCC-441)	15 ± 0.81	14 ± 0.82	17 ± 0.83	14 ± 0.78	28 ± 0.81
V. cholerae (MTCC-3906)	17 ± 0.83	12 ± 0.78	20 ± 0.85	14 ± 0.81	29 ± 0.83
E. coli (MTCC-443)	15 ± 0.79	14 ± 0.79	16 ± 0.81	15 ± 0.77	25 ± 0.81
A. niger (MTCC-1344)	14 ± 0.74	13 ± 0.71	15 ± 0.78	14 ± 0.81	28 ± 0.75
C. albicans (MTCC-183)	17 ± 0.75	15 ± 0.73	19 ± 0.75	14 ± 0.82	30 ± 0.74
P. verrucosum (MTCC-1758)	16 ± 0.74	12 ± 0.74	17 ± 0.76	15 ± 0.83	29 ± 0.72
F. oxysporum (MTCC-284)	14 ± 0.73	13 ± 0.72	17 ± 0.73	15 ± 0.79	28 ± 0.73

Values are expressed as mean ± SD in triplicates.
[a]Ampicillin and clotrimazole.

continuously emerged. Scientists around the world are currently paying attention to lichen secondary metabolites because of their promising efficacy over commonly used compounds [18]. In pursuit of new antimicrobial agents, some lichen compounds have been tested for antimicrobial activity [19–21]. In this study, *in vitro*, antimicrobial activity of methanol and acetone extract from the lichens *D. applanata* and *P. andium* were examined. In our result, the lichen compounds showed very strong antimicrobial activity and the antibacterial activity was observed stronger than antifungal activity. The results also demonstrated that both the extracts have significant antibacterial effects against Gram-negative bacteria. The high sensibility of Gram-negative bacteria might be interpreted by the fact that the structures of the cell envelope are different between both Gram- negative and Gram-positive bacteria. The former has an outer membrane formed by an inner phospholipid layer surmounted by lipopolysaccharide macromolecules which prevent the diffusion of hydrophobic compounds. The outer membrane along with thin and viscous cell wall of Gram-negative bacteria can be easily permeable [22]. In our study, the strength of antimicrobial activity varied among the two extracts, and better activity was observed in methanol extracts. Presumably, methanol was the most efficient solvent for the extraction of phenolic and flavonoid compounds which were responsible for antimicrobial activity [23]. Enzyme inhibition by oxidized compounds, probably by reaction with sulfhydryl groups or by more unspecific interactions with protein groups, are the mechanisms thought to be responsible for phenolic toxicity to microorganisms [24]. Flavonoids are phenolic structures containing a carbonyl group and flavonol [25] is obtained by the addition of a 3-hydroxyl group. Flavonoids are also phenolic compounds that are hydroxylated but occur as a unit of C6-C3 connected to an aromatic ring. *In-vitro* antimicrobial substances against a wide variety of microorganisms have been found to be effective. This activity is likely to be due to their ability to complex with extracellular and soluble proteins and to complex with cell walls of bacteria [26], often resulting in protein inactivation and loss of function. Furthermore, more lipophilic flavonoids may also disrupt microbial membranes [27]. For that reason, the potential range of flavonoids as antimicrobial compound is of interest. The activity prediction conducted in this study provided that among both the lichen species, the antimicrobial activity of the solvent extracts of *D. applanata* was more prominent than *P. andium*, against all the test pathogens. This outcome was consistent with another published work showing that the antimicrobial activity of the extracts of acetone, methanol, petroleum ether, and diethyl ether of three species of foliose lichen *Dirinaria picta*, *Dirinaria Papillulifera*, and *D. applanata* were significant against human pathogenic bacteria and fungi using well-diffusion method [28,29]. Further reports revealed that the methanol, acetone, hexane, dichloromethane extracts of *P. praesorediosum* showed inhibitory activity against some bacterial and fungal pathogens [30,31].

5. CONCLUSION

The current study sheds light on the antimicrobial properties of extracts from *P. andium* and *D. applanata* growing in SBR. The results reported here pointed out that the two lichen extracts possess differential antimicrobial activities. However, complementary studies should be conducted to identify the major metabolites that are responsible for this biological activity and their mechanism of action. The possibility for the use of lichens in the treatment of different diseases caused by pathogens is indicated in the current results. Thus, lichens tend to be a strong and safe natural antimicrobial agent on the basis of these findings and are also useful for managing different diseases of humans, animals, and plants.

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7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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