Antibacterial Activities of Lichen-associated Fungi in Mangrove Ecosystems in Sri Lanka as Potent Candidates for Novel Antibiotic Agents

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ABSTRACT

Antimicrobial resistance is a global threat to humans, prompting an increasing interest in exploring and developing novel antimicrobial substances derived from diverse sources. Together with the emergence of new diseases the search for novel drug leads has intensified. Less explored microbial habitats have become prime targets in mining for novel antimicrobial molecules. Secondary metabolites synthesized by lichen-associated fungi are good potential targets in this regard. Hence, this study was carried out to explore the antibacterial potential of lichen-associated fungi in mangrove ecosystems by taking National Aquatic Resources Research and Development Agency (NARA) Regional Research Centre, Kalpitiya, Puttalam District, Sri Lanka as the study site. Lichen-associated fungi were isolated from collected lichens by plating out surface sterilized lichen thalli pieces. Antibacterial activities of the isolates were tested using two gram-positive bacteria: *Staphylococcus aureus* and *Bacillus cereus* and two gram-negative bacteria: *Pseudomonas aeruginosa* and *Escherichia coli*. In this study, 72 putative fungal isolates

were primarily screened for their antibacterial activity using agar plug diffusion assay and ethyl acetate crude fungal extracts of nine fungal isolates with marked activity were secondarily screened using the well diffusion assay in triplicate. Isolate LIF 0803 identified as *Trichosporon faecale* showed the most outstanding antibacterial activities as 2.58 ± 0.29, 3.43 ± 0.05, 4.2 ± 0, 4.5 ± 0.14 cm of zone diameter at 100 mg/mL and 1.95 ± 0.59, 3.08 ± 0.13, 3.7 ± 0.12, 4.3 ± 0.19 cm of zone diameter at 50 mg/mL against *P. aeruginosa, S. aureus, B. cereus*, and *E. coli*. All nine fungal isolates showed promising antimicrobial activity against both gram positive and negative bacteria. Therefore, this study showed that lichen-associated fungi in mangrove ecosystems have potent antibacterial activities. Hence, bioassay guided fractionation of active compounds from lichen-associated fungi and structure elucidation are warranted.

Keywords**:** Antibacterial agents; Broad spectrum antibiotics, Mangrove ecosystem; Secondary metabolites; *Trichosporon faecale*

1. INTRODUCTION

Today acquiring antibacterial resistance by pathogenic microorganisms against available antibiotic drugs is becoming a global health issue. Therefore, search for novel alternatives to combat antibiotic-resistant microbes is a prime target of current medical research. Natural products are proposed as a therapeutic alternative to conventional antimicrobial treatments (Ranković, 2015). Among them, about 50–60% is produced by plants whereas only 5% have a microbial origin (Demain & Sanchez, 2009). The untapped potential of microbial diversity holds the promise of discovering previously unknown and valuable metabolites that could have significant therapeutic uses. As a result, research is directed to investigate a range of unexplored and elusive microorganisms across different environments, to uncover novel metabolites (Padhi & Tayung, 2015).

Lichens are symbiotic associations of algae or cyanobacteria (photobiont) and filamentous fungi (mycobiont). Apart from the mycobiont, other fungi that reside on lichens are referred to as lichen-associated fungi (Galinato et al., 2021). Lichens associated with the mangrove ecosystems are known to have more habitat stress than lichens found in other terrestrial ecosystems due to the harsh environmental conditions. To endure and survive under those stressful conditions while protecting the photobiont, lichen-associated fungi in the mangrove ecosystem exhibit a broad spectrum of bioactivities (Maduranga et al., 2021). As a result, various secondary metabolites having numerous potential applications, are produced by these fungi to aid their survival mechanisms. Among them, antibiotic properties of lichen-associated fungi are of special interest to scientists. Lichens do not possess a clearly defined epidermal layer or any other physical protection that act as a barrier for the entry of unwanted organisms. Hence lichens rely mainly on chemical defence mechanisms to ward off the invading microorganisms. Secondary metabolites synthesised by lichen associated fungi, assist antimicrobial mechanisms of the lichen to protect itself against pathogens. Relatively very few studies have been undertaken on antibacterial potential of lichen-associated fungi in mangrove ecosystems. The main objective of this present study was to evaluate the antibacterial potential of lichen-associated fungi in mangrove ecosystems in Sri Lanka.

2. MATERIALS AND METHODS

2.1. COLLECTION AND IDENTIFICATION OF THE LICHEN SAMPLES

The healthy-looking thalli were collected into sterile plastic collection bags from the National Institute of Aquatic Research Development Authority (NARA) Regional Research Centre (8.25° or 8° 15’ North latitude, 79.7707° or 79° 46’ 15” East longitude) site in Kalpitiya, Sri Lanka. The sample were

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stored in acid-free paper bags and processed within one week of collection.

2.2. ISOLATION AND IDENTIFICATION OF LICHEN-ASSOCIATED FUNGI

Healthy lichen thalli were cleaned with running tap water to eliminate contaminating solid particles. Segments were cut and dipped in 70% ethanol for 10 s, followed by 0.05% Clorox® for 5 min and then washed in sterilized distilled water three times. The thalli pieces were blotted dry with sterile filter paper sand placed on water agar plates. Once the fungal hyphae grew out from the thallus segments, putative fungi were isolated by transferring hyphal tips to fresh potato dextrose agar media (PDA). Lichen-associated fungi were identified using morphological and molecular identification methods. Molecular identification was done by DNA barcoding through Internal Transcribed Spacer (ITS) sequencing of the extracted DNA which was amplified by polymerase chain reaction (PCR) using ITS1 and ITS4 primer pair. Consensus sequences were then uploaded to the BLAST search engine to get related sequences(Galinato et al., 2021).

2.3. AGAR PLUG DIFFUSION ASSAY

Fungal isolates were subjected to preliminary screening through agar plug diffusion method (Marcellano et al., 2017a) against four test bacteria: *Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* (ATCC 11778) as gram positive indicators and *Pseudomonas aeruginosa* (ATCC 25853) and *Escherichia coli* (ATCC 25922) as gram-negative indicators. Test organisms were inoculated into 0.85% saline water and the turbidity was adjusted to be on par with 0.5 McFarland turbidity standard. A volume of 100 µL of the respective bacterial suspension was spread on a Petri dish containing Mueller Hinton agar (MHA) and kept aside for 30 min for the absorption of water in the suspension. Then, agar plugs (≈8 mm diameter) were cut from the actively growing areas of a seven-day old fungal colony and were transferred to the MHA plate containing the test bacteria. These plates were sealed and kept in a refrigerator at 4 °C for one hour to aid the diffusion of metabolites. The plates were then incubated at 37 ˚C for 24 hours to enable the growth of test microorganisms. After incubation, inhibition zone diameter was measured along two perpendicular axes.

2.4. EXTRACTION OF SECONDARY METABOLITES

Based on the preliminary screening selected nine fungal isolates with notable antibacterial activities were cultured on an appropriate number of plates with modified Malt Extract Agar (malt extract; 20.0 g, glucose; 20.0 g, peptone; 5.0 g, trace amounts of $CuSO_4.5H_2O$ and $ZnSO_4.7H_2O$, agar; 15.0 g per 1 L). After 3-4 weeks of incubation, each fungus together with the medium was cut into small pieces and extracted into 70 mL of ethyl acetate (EtOAc) under sonication and filtered. The filtrate was evaporated to dryness under reduced pressure (BUCHI-R- 200 rotary evaporator). The crude extract was redissolved in 10% sterile DMSO to obtain 100 mg mL^{-1}, and 50 mg mL^{-1} concentrations.

2.5. AGAR WELL DIFFUSION ASSAY

Fungal crude extracts were semi quantitatively analyzed using agar well diffusion assay against the four bacterial species mentioned above. Four wells with a diameter of 8 mm were punched in a plate seeded with respective bacteria and 20 µL of two crude extracts at 100 mg mL^{-1}, and 50 mg mL^{-1}, concentrations were separately introduced into a well. A volume of 20 µL of 1mg mL^{-1} Ciprofloxacin and 10 % sterile DMSO were used as positive control and negative controls, respectively. Three physiological replicates were done for each fungal extract. After incubation, inhibition zone diameter was measured along two perpendicular axes.

3. RESULTS AND DISCUSSION

Lichen thallus is a micro habitat for numerous microbes. Some parasitic intruder microbes may cause extensive damage, resulting in localized necrotic patches or partial death of the thallus. Therefore, lichen-associated fungi must synthesize secondary metabolites with antimicrobial properties (Nash, 2008). A total of 121 fungal isolates were obtained from 17 lichen samples collected from NARA Regional Research Centre, Kalpitiya. Out of 72 fungal isolates tested, 69 fungal isolates showed antimicrobial activity in agar plug diffusion assay against all four test bacteria and created zone diameter ranging from 0.6 to 5.2 cm (Figure 1). This demonstrates the presence of antibacterial activities of variable strengths in fungal secondary metabolites.

Figure 1- Antifungal activity of lichen associated fungi against *Staphylococcus aureus*, *Bacillus cereus*, *E coli* and *Pseudomonas aeruginosa* as evidenced by agar plug diffusion assay

Figure 2- Colony morphology and microscopic characteristics of nine lichen-associated fungal isolates with the best antibacterial activities. A: LIF 0503 B: LIF 0505 C: LIF 0803 D: LIF 0809 E: LIF 1105 F: LIF 1109 G: LIF 1115 H: LIF 1508 I: LIF 1513, f: Front, b: Below, m: microscopic view (Scale: 1.0 cm)

The nine isolates (Figure 2) with relatively high antibacterial activities ranging from 2.6 to 5.2 cm of zone diameter, among the seventy-two isolates were selected for the secondary screening. These fungal isolates were yielded from four lichen samples as LIF 0503, LIF 0505, LIF 0803, and LIF 0809 from *Roccella* sp. and LIF 1109, LIF 1105, LIF 1115, LIF 1508, and LIF 1513 from two different crustose lichens. The ethyl acetate extract of those nine isolates was subjected to agar well diffusion assay (Figure 4).

Figure 4- Agar well diffusion assay (A) LIF 1513 (B) LIF 0803 (1) *Staphylococcus aureus* (2) *Bacillus sp* (3) *E coli* (4) *Pseudomonas aeruginosa* N: Negative control P: Positive control C1: 50 mg/mL C2: 100 mg/mL (Scale: 1 cm)

The crude metabolite extracts showed promising antimicrobial activity against three bacterial species at both concentrations (Figure 3). These zone diameter values given by all extracts except that of LIF 0803 at 100 mg/mL were significantly different ($P<0.05$) from the positive control. Except LIF 1508 and LIF 1513, unclear inhibition zones were observed against *Pseudomonas aeruginosa* for all other isolates. Out of those nine isolates, LIF 0803 showed the most remarkable activity against all four test pathogens as indicated by the highest mean zone diameter against *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli* and the third highest results for *Pseudomonas aeruginosa*. Although antibacterial activity of LIF 0803 against *Escherichia coli* at 100 mg/mL is greater than positive control it was significantly not different ($P>0.05$).

Figure 3- Antimicrobial activity of two concentrations (100 mg/mL, 50 mg/mL) of nine fungal crude extracts against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* A: 100 mg/mL; B: 50 mg/mL

Of a total of nine isolates, only three isolates showing higher activities (Table 1), LIF 0803, LIF 0809 and LIF 1109 were sent for sequencing due to fund limitations. By referring to blast results, both LIF 0803, and LIF 0809 were identified as *Trichosporon faecale*, and LIF 1109 was identified as *Phaeoacremonium scolyti* (Table 2). LIF 0809 is mostly related to *Trichosporon faecale* culture CBS:4828 than LIF 0803 as per the blast results. Also, the antimicrobial activities and morphological features of those two isolates were slightly different. Hence, employing genus-specific primers is advisable for more conclusive identification. *Trichosporon* species is an anamorphic basidiomycetous non-candida yeast. It has been recorded in a wide

range of habitats: soil, sediments, wastewaters, decaying wood and most importantly in clinical samples (Middelhoven et al., 2004). Although species of *Trichosporon* causes fungemia in humans and hence considered as a health threat (Girmenia et al., 2005), the genus has drawn industrial interest based on its remarkable activity of xenobiotic bioremediation. It produces a battery of enzymes that can degrade a range of substrates such as aromatic compounds, complex nitrogenous compounds, etc. (Kaszycki et al., 2006). Production of antibacterial compounds has been reported for the first time to the best of our knowledge. Further this fungus has not been reported as a lichen associated fungus so far. *Phaeoacremonium* spp. are a group of filamentous fungi that are commonly found in soil, wood, and plant material and commonly isolated from stems and branches of diseased woody hosts, and humans with phaeohyphomycosis. Their ability to produce a range of enzymes, including cellulases, xylanases, and lignases (Gómez et al., 2016) have a high value in industries like biomass degradation, biofuel production, and bioremediation of pollutants. It can be assumed that the lichens may be recruiting fungi having antimicrobial activities as a protective measure against microbial invasions. However, its potent antibacterial activity warrants further investigations leading to isolation and structure elucidation of active compounds.

Crude extracts often show better activity against gram-positive bacteria because of their structural differences (Marcellano et al., 2017b). In this study, the crude extracts were found to be effective against both gram-positive and gram-negative bacteria which is not a very common phenomenon. It can be assumed that either one broad spectrum metabolite or several metabolites of narrow spectrum could be involved in this activity.

Table 1: Antimicrobial effect of LIF 0803, LIF 0809 and LIF 1109 as measured by inhibition zone diameters

Isolate No.	Zone diameter (cm)							
	Pseudomonas aeruginosa		*Staphylococcus aureus*		*Bacillis* sp.		*E. coli*	
	100 mg/mL	50 mg/mL	100 mg/mL	50 mg/mL	100 mg/mL	50 mg/mL	100 mg/mL	50 mg/mL
LIF0803	2.58 ± 0.29	1.95 ± 0.59	3.43 ± 0.05	3.08 ± 0.13	4.2 ± 0	3.7 ± 0.12	4.5 ± 0.14	4.3 ± 0.19
LIF0809	1.88 ± 0.1	1.7 ± 0.08	2.55 ± 0.17	2.08 ± 0.1	2.98 ± 0.13	2.25 ± 0.13	3.35 ± 0.13	3.15 ± 0.17
LIF1109	2.53 ± 0.62	1.95 ± 0.55	2.38 ± 0.1	1.95 ± 0.13	3.57 ± 0.86	2.15 ± 0.13	3.13 ± 0.05	2.65 ± 0.17

Table 2: BLASTn results for obtained for ITS sequences of LIF 0803, LIF 0809 and LIF 1109

	BLASTn outcome against NCBI database				**Nearest match in NCBI database**	**Accession No.**
	E value	**Score**	**% Identity**	**Query cover**		
LIF 0803	0.0	1020	99.82%	100%	*Trichosporon faecale*	KY105736.1
LIF 0809	0.0	1029	99.82%	100%	*Trichosporon faecale*	KY105736.1
LIF 1109	0.0	1096	98.56%	99%	*Phaeoacremonium scolyti*	KC166687.1

Crude extracts are complex mixtures of secondary metabolites and antibacterial agents (bioactive compounds) exist in low concentrations in the crude extracts. Therefore, isolation of the active compound through bioassay guided fractionation would be a better mechanism to assess the antibacterial potential of the secondary metabolites produced by these fungi. By perusing the results obtained, it can be predicted that the pure forms of the compounds would have more therapeutic potentials to strengthen the existing antibiotics to defeat "super-pathogens". Yet, careful investigations and assessments are needed since the natural compounds tend to have synergistic activities, which might be lost if the individual compounds are isolated.

4. CONCLUSION

This study showed that the less explored groups of lichen-associated fungi in mangrove ecosystems have potent antibacterial activities against both gram-positive and gram-negative bacteria. The remarkable antibacterial activities of *Trichosporon faecale* suggest further studies to isolate the bioactive compounds from the crude extract and evaluate the availability to use as a novel antibacterial agent in therapeutic applications in the future.

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