Received: 14 January 2024 *Accepted: 25 July 2024*

Lichen-associated Fungi Inhabiting from a Mangrove Ecosystem in Sri Lanka: A Novel Source of Antibacterial Agents

[1]Happitiya, H. A. D. N. N.,[1,4]Nanayakkara, C. M., [1]Ariyawansa, K. G. S. U., [1]Ediriweera, S. S., [2]Wijayawardene, N. N., [3]Jayasinghe, R. P. P. K., [5]Don-Qin Dai, & [2]Karunarathna, S. C.

nipuninhappitiya@gmail.com, chandi@pts.cmb.ac.lk, sameera@pts.cmb.ac.lk, surani@pts.cmb.ac.lk, nalinwijayawardene@yahoo.com, prabathj@nara.ac.lk, cici_1101@126.com, samanthakarunarathna@gmail.com

[1]Department of Plant Sciences, University of Colombo. Colombo 03, Sri Lanka.

[2]Center for Yunnan Plateau Biological Resources Protection and Utilization, Qujing Normal University, P.R. China.

[3]National Aquatic Resources Research and Development Agency, Colombo 15, Sri Lanka

[4]Sri Lanka Institute of Biotechnology, Homagama, Sri Lanka.

[5]College of Biological Resources and Food Engineering, Qujing Normal University P.R. China.

Abstract

The global threat of antimicrobial resistance has spurred interest in discovering innovative antimicrobial agents from diverse sources. Amid the rise of new diseases, the quest for novel drug leads has intensified. This study explores the antibacterial potential of lichen-associated fungi in mangrove ecosystems, using NARA Regional Research Centre in Kalpitiya, Sri Lanka as the study site. Lichen-associated fungi were isolated from collected lichens and the antibacterial activities of the isolates were tested using two gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* (ATCC 11778) and two gram-negative bacteria: *Pseudomonas aeruginosa* (ATCC 25853) and *Escherichia coli* (ATCC 25922). Putative fungal isolates were primarily screened using agar plug diffusion assay and ethyl acetate extracts of 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, 3.43, 4.2, 4.5 cm of zone diameter at 100 mg/mL, and 1.95, 3.08, 3.7, 4.3 cm of zone diameter at 50 mg/mL against *P. aeruginosa, S. aureus, B. cereus*, and *E. coli* respectively. All nine fungal isolates showed promising antibacterial 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: Broad-spectrum antibacterial activity, Bioactive secondary metabolites lichen, Mangrove, *Trichosporon faecale.*

Introduction

The use of antibiotics is becoming limited due to emergence of antimicrobial resistance (AMR), new diseases and re-emergence of old diseases. AMR is one of the most important global health concerns. As per WHO, AMR was the direct cause of 1.27 million global deaths in 2019. Therefore, search for novel alternatives to combat antibiotic-resistant microbes is a prime target of current medical research. Natural products have been in the pipeline as therapeutic alternatives 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 (Weerasinghe 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 acts as a barrier to the entry of unwanted organisms. Hence lichens rely mainly on chemical defence mechanisms to ward off the invading microorganisms. Secondary metabolites synthesized by lichen-associated fungi assist the antimicrobial mechanisms of the lichen to protect itself against pathogens. Relatively very few studies have been undertaken on the 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. As a preliminary study, the National Institute of Aquatic Research Development Authority (NARA) Regional Research Centre was selected as the study site.

Materials and Methods

Collection 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 samples were stored in acid-free paper bags and processed within one week of collection. Part of the lichens were air-dried, herbarium samples were prepared and deposited at the herbarium of the Department of Plant Sciences, University of Colombo.

Identification of the lichen samples

Initially, the macroscopic features like thallus form, colour, texture, and branching pattern were recorded and photographs were taken. Then chemical spot tests: K (A 10% solution of Potassium hydroxide), C (Undiluted commercial bleaching/Calcium hypochlorite), KC (K test immediately after the C test), and I (A solution of 0.5% potassium iodide) were performed by testing on lichen both cortical and medulla areas.

As a visualization method of lichen substances micro-crystallization was done using GAW (H2O: glycerol: ethanol with 1:1:1) and GE (acetic acid: glycerol with 1:3) reagents. Finally, microscopic structures on lichen surfaces were observed using light microscope with a surface light source, and microscopic photographs were taken, and the nature of reproductive structures were observed in each lichen sample. Identification was done using standard keys online and consulting Prof. RGU Jayalal of Samaragamuwa University of Sri Lanka.

Isolation and morphological characterization 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 to remove any surface contaminations. The thalli pieces were blotted dry with sterile filter paper and 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). A total of 121 fungi were isolated and they were coded for convenience. Lichen-associated fungi were characterized by using colony morphology and micromorphology.

Agar plug diffusion assay

Fungal isolates were subjected to preliminary screening for antibacterial activity 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 the 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 the test microorganisms. After incubation, inhibition zone diameter (IZD) was measured along two perpendicular axes.

Extraction of secondary metabolites

Based on the viability, growth rates, and preliminary screening results, 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.

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, IZD was measured along two perpendicular axes.

Statistical analysis

The data were presented as the mean ± standard error of the mean (SEM). Mean zone diameters were analyzed by ANOVA and independent sample t-test ($p < 0.05$) using IBM SPSS Statistics 26.

Molecular identification of lichen-associated fungi

Molecular identification was done for the isolates with higher antibacterial activities (LIF 0803, LIF 0809, LIF 0809) 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 (Brahmanage et al., 2023). Consensus sequences were then uploaded to the BLAST search engine to get related sequences (Galinato et al., 2021).

Results and Discussion

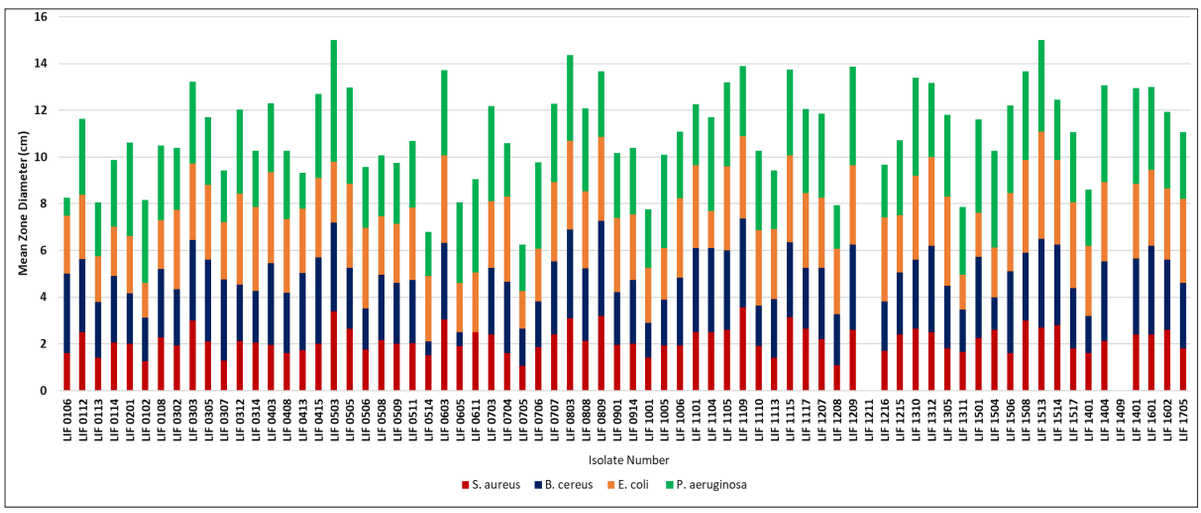
Lichen thallus is a microhabitat for numerous microbes. Due to the absence of protective barriers some parasitic intruder microbes may cause extensive damage, resulting in localized necrotic patches or partial death of the thallus. Therefore, the first line of defense for lichens is chemical in nature (Nash, 2008). Lichen-associated fungi are known to synthesize secondary metabolites with antimicrobial properties (Mathushika & Nanayakkara, 2021).

Agar plug diffusion assay

In the current study, 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 antibacterial activity in agar plug diffusion assay against all four test bacteria and resulting zone diameters 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.

Antibacterial potential of lichen-associated fungi against *Staphylococcus aureus, Bacillus cereus, Escherichia coli* and *Pseudomonas aeruginosa* as evidenced by agar plug diffusion assay.



Agar well diffusion assay

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, LIF 0503, LIF 0505, LIF 0803, and LIF 0809 were yielded from *Roccella* spp. 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 an agar well diffusion assay (Figure 3).

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).

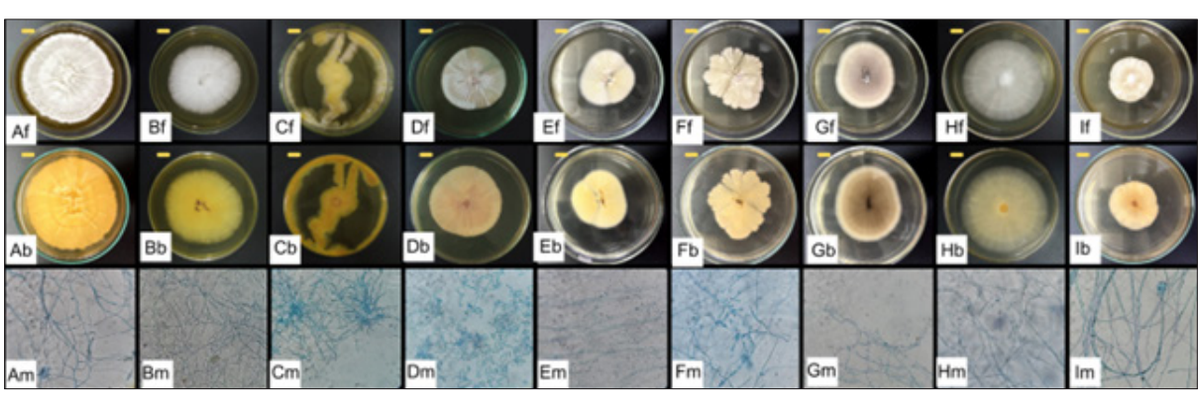
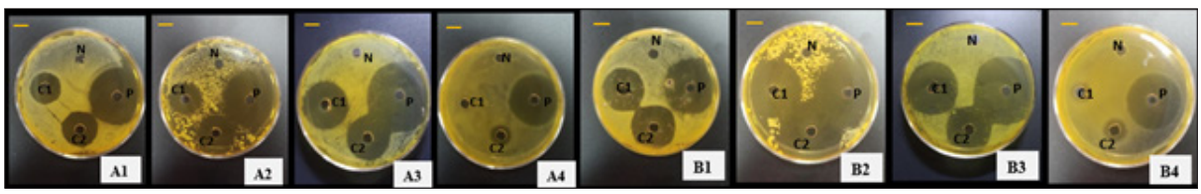


Figure 3.

Agar well diffusion assay (A) LIF 1513 (B) LIF 0803 (1) *Staphylococcus aureus* (2) *Bacillus cereus* (3) *Escherichia 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 antibacterial activity against three bacterial species at both concentrations (Figure 4 and 5).

Figure 4.

Antibacterial activity of nine fungal crude extracts against Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus cereus, Escherichia coli at the concentration 100 mg/mL

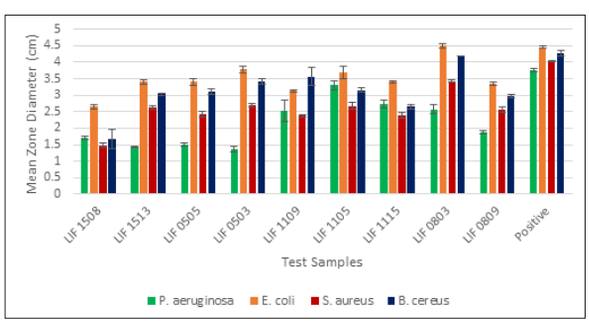
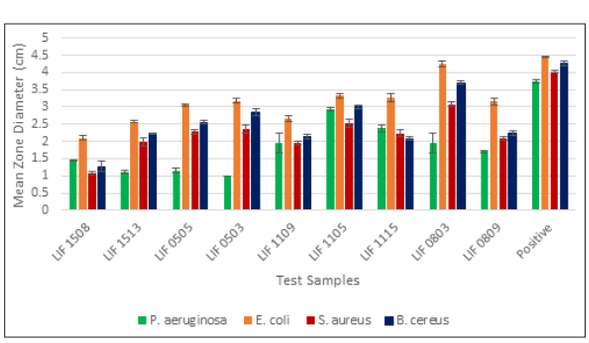


Figure 5.

Antibacterial activity of nine fungal crude extracts against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* at the concentration 50 mg/mL



These mean 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 activity for *Pseudomonas aeruginosa*. Although the antibacterial activity of LIF 0803 against *Escherichia coli* at 100 mg/mL is greater than positive control it was significantly not different ($P>0.05$). Crude extracts are not in pure forms. Therefore, this observation has high significance since the activity was shown by a crude extract. This indicates powerful antibacterial activity, and the purified form may hold greater promise in drug discovery. However, sometimes these potent antibacterial activities may result from the synergism among the compounds in the crude extract. Therefore, after the purification of one compound, there is a feasibility of losing the activity. Of a total of nine isolates, only three isolates (Table 1), LIF 0803, LIF

0809, and LIF 1109 were sent for sequencing due to fund limitations.

Table 1.

Antibacterial effect of LIF 0803, LIF 0809 and LIF 1109 as measured by inhibition zone diameters.

Isolate No.	Mean zone diameter ± SEM (cm)			
	Pseudomonas aeruginosa		*Escherichia coli*	
	100 mg/mL	50 mg/mL	100 mg/mL	100 mg/mL
LIF0803	2.58 ± 0.29^{c}	1.95 ± 0.59^{d}	4.5 ± 0.14^{ab}	4.5 ± 0.14^{ab}
LIF0809	1.88 ± 0.1^{d}	1.7 ± 0.08^{de}	3.35 ± 0.13^{ab}	3.35 ± 0.13^{abc}
LIF1109	2.53 ± 0.62^{c}	1.95 ± 0.55^{d}	3.13 ± 0.05^{ab}	3.13 ± 0.05^{bc}
Isolate No.	*Staphylococcus aureus*		*Bacillus cereus*	
	100 mg/mL	50 mg/mL	100 mg/mL	50 mg/mL
LIF0803	3.43 ± 0.05^{b}	3.08 ± 0.13^{b}	4.2 ± 0^{a}	3.7 ± 0.12^{b}
LIF0809	2.55 ± 0.17^{cde}	2.08 ± 0.1^{efg}	2.98 ± 0.13^{cde}	2.25 ± 0.13^{ef}
LIF1109	2.38 ± 0.1^{e}	1.95 ± 0.13^{g}	3.57 ± 0.86^{de}	2.15 ± 0.13^{ef}

Note: The same letters are not significantly different from each other at 5% level.

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).

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

LIF 0809 is mostly related to *Trichosporon faecale* culture CBS:4828 than LIF 0803 as per the blast results. Also, the antibacterial 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 (Mehta et al., 2021). 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 are 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 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 ligninases (Gómez et al., 2016) has 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 mostly because of the synergistic activities of the compounds present in the crude (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. Hence, further purification of such compounds is warranted.

Conclusions, Recommendations and Suggestions

Conclusions

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.

Recommendations

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 potential 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.

Suggestions

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.

Acknowledgment

Authors thank Prof. Udeni Jayalal and Mr. Hasanka Dangalla, for their immense support in lichen identification. Sample collection was supported by (NARA) Regional Research Centre, Kalpitiya, Sri Lanka, and the study was supported by the Department of Plant Sciences, University of Colombo.

References

Brahmanage, R. S., Wijayawardene, N. N., Nanayakkara, C. M., Muthumala, C. K., Wijesundara, S., Dai, D. Q., & Ariyawansa, K. G. S. U. (2023). Eucalyptus leaf spot disease caused by Coniella eucalyptorum in Sri Lanka. *Phytotaxa*, *630*(1), 40–50. https://doi.org/10.11646/PHYTOTAXA.630.1.3.

Demain, A. L., & Sanchez, S. (2009). Microbial drug discovery: 80 years of progress. *The Journal of Antibiotics*, 62(1), 5–16. https://doi.org/10.1038/JA.2008.16.

Galinato, M. G. M., Bungihan, M. E., Santiago, K. A. A., Sangvichien, E., & dela Cruz, T. E. E. (2021). Antioxidant activities of fungi inhabiting Ramalina peruviana: insights on the role of endolichenic fungi in the lichen symbiosis. *Current Research in Environmental and Applied Mycology*, 11(1), 119–136. https://doi.org/10.5943/CREAM/11/1/10.

Girmenia, C., Pagano, L., Martino, B., D'Antonio, D., Fanci, R., Specchia, G., Melillo, L., Buelli, M., Pizzarelli, G., Venditti, M., & Martino, P. (2005). Invasive infections caused by Trichosporon species and Geotrichum capitatum in patients with hematological malignancies: A retrospective multicenter study from Italy and review of the literature. In Journal of Clinical Microbiology (Vol. 43, Issue 4, pp. 1818–1828). https://doi.org/10.1128/JCM.43.4.1818-1828.2005.

Kaszycki, P., Czechowska, K., Petryszak, P., Międzobrodzki, J., Pawlik, B., & Kołoczek, H. (2006). Methylotrophic extremophilic yeast Trichosporon sp.: a soil-derived isolate with potential applications in environmental biotechnology *. www.actabp.pl.

Marcellano, J. P., Collanto, A. S., & Fuentes, R. G. (2017a). Antibacterial activity of endophytic fungi isolated from the bark of cinnamomum mercadoi.

Pharmacognosy Journal, 9(3), 405–409. https://doi.org/10.5530/PJ.2017.3.69.

Marcellano, J. P., Collanto, A. S., & Fuentes, R. G. (2017b). Antibacterial activity of endophytic fungi isolated from the bark of cinnamomum mercadoi. Pharmacognosy Journal, 9(3), 405–409. https://doi.org/10.5530/PJ.2017.3.69.

Mathushika, J., & Nanayakkara, C. (2021). Lichens as potential antimicrobial agents: A comprehensive review. *International Journal of Pharmaceutical Research and Applications*, *6*, 564–580. https://doi.org/10.35629/7781-0604564580.

Mehta, V., Nayyar, C., Gulati, N., Singla, N., Rai, S., & Chandar, J. (2021). *A Comprehensive Review of Trichosporon spp.: An Invasive and Emerging Fungus*. https://doi.org/10.7759/cureus.17345.

Middelhoven, W. J., Scorzetti, G., & Fell, J. W. (2004). Systematics of the anamorphic basidiomycetous yeast genus Trichosporon Behrend with the description of five novel species: Trichosporon vadense, T. smithiae, T. dehoogii, T. scrabaeorum and T. gamsii. International Journal of Systematic and Evolutionary Microbiology, 54(3), 975–986. https://doi.org/10.1099/ijs.0.02859-0.

Nash, T. H. (2008). Lichen Biology. Lichen Biology, Second Edition, 1–486. https://doi.org/10.1017/CBO9780511790478.

Padhi, S., & Tayung, K. (2015). In vitro antimicrobial potentials of endolichenic fungi isolated from thalli of Parmelia lichen against some human pathogens. Beni-Suef University Journal of Basic and Applied Sciences, 4(4), 299–306. https://doi.org/10.1016/j.bjbas.2015.11.006.

Ranković, B. (2015). Lichen secondary metabolites: Bioactive properties and pharmaceutical potential. Lichen Secondary Metabolites: Bioactive Properties and Pharmaceutical Potential, 1–202. https://doi.org/10.1007/978-3-319-13374-4/COVER.

Weerasinghe, R. H., Maduranga, K., Attanayake, R. N., Shevkar, C., Kate, A. S., Weerakoon, G., Kalia, K., & Paranagama, P. A. (2021). Bioactive Properties and Metabolite Profiles of Endolichenic Fungi in Mangrove Ecosystem of Negombo Lagoon, Sri Lanka. https://doi.org/10.1177/1934578X211048652.