SHORT COMMUNICATION

DETERMINATION OF THE ANTIBACTERIAL PROPERTIES OF CRUDE EXTRACTS OF ENDOPHYTIC FUNGI ISOLATED FROM *Acrostichum aureum* AND *Sonneratia alba*

*K. Rajan, F. A. Rahman, D. M. S. U. Dissanayaka**

School of Science, Business Management School, Sri Lanka

ABSTRACT

Current issues associated with drug resistance and the adverse effects of synthetic agents have prompted researchers to focus on natural, medicinal agents such as mangroves and their associated fungal endophytes. The current study was developed to determine the antibacterial activities of crude extracts of endophytic fungi isolated from leaves, roots and stems of *Acrostichum aureum*, Karan koku in Sinhala and *Sonneratia alba*, Kirala in Sinhala. The endophytic fungal isolates were identified as *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus fumigatus*, *Aspergillus terreus*, *Penicillium citrium* and *Penicillium resticulosum* and the respective crude extracts were generated using ethyl acetate. The crude extracts were subjected to antibiotic sensitivity tests (ABSTs) against two test organisms: *Staphylococcus aureus* (ATCC ®: 25923) and *Escherichia coli* (ATCC ®: 25922). The highest inhibitory action was exerted by *Penicillium resticulosum* against both the test organisms (*E. coli*: 15±0.05 mm; *S. aureus* 22±0.05 mm) which was the most abundantly identified endophyte in this study. The Minimum Inhibitory Concentration of the crude extracts was detected to be 1. 25 mg/ ml. However, the highest bactericidal action was noted from *Penicillium citrinum*. It is evident that the fungal endophytes isolated from mangrove plants possess antibacterial properties that can be used as a natural source for the generation of antimicrobial therapeutics.

Keywords: *Antibacterial, Mangrove fungal endophytes, Acrostichum aureum, Sonneratia alba*

DOI. *http://doi.org/10.4038/jsc.v13i2.49*

1. INTRODUCTION

Mangroves are trees or shrubs that thrive under the extreme saline, temperate and anaerobic conditions of coastal regions, abundantly in Brazil and Asian countries. Plant endophytes, succulent leaves and aerial or buttress roots are important adaptations of mangroves [1]. An area of 160.12 km^2 in Sri Lanka is occupied by mangroves, where the

Corresponding author e-mail: *udeshinis@gmail.com*

largest mangrove breeding ground is located in the Puttalam-Kalpitiya lagoon. Negombo, the largest mangrove patch in the Western Province is rich in Rhizophoraceae, Avicenniaceae, *Acanthus ilicifolius* and *Acrostichum aureum* species [2]. Scientists have studied the use of mangroves to treat diabetes, cancer, digestive problems, infection and snake bites [3]. However, the extensive harvest of mangrove plants for these studies led to the deforestation of already endangered mangroves. Hence, endophytes have been detected as the alternative solution which synthesizes the same types of secondary metabolites as their hosts [4].

Plant endophytes inhabit the internal plant tissues and associate with the host without causing any visible infections. They maintain saprophytic, parasitic, mutualistic or commensalism associations with the host to obtain nutrients and recompense them by synthesizing phytochemicals that render protection from pathogens or environmental stress, promote plant growth and enable nutrient cycling [5]. Fungal endophytes are an omnipresent community which generates many medicinally important secondary metabolites such as flavonoids, quinones, phenols and steroids [6]. Various trials have been initiated to generate natural antimicrobial products to overcome antibiotic resistance and the side effects of synthetic antibiotics. Approximately, half (48.9%) of all pharmacological studies that have been conducted from 1990 to 2019 were based on the antimicrobial nature of mangrove fungal endophytes [7].

Acrostichum aureum (*A. aureum*) and *Sonneratia alba* (*S. alba*), the two mangroves chosen for this study are extensively used in Asian folklore medicinal practices to cure cough, fever and parasitic infections. Rhizome and leaf extracts of *A. aureum* have been proven to heal wounds, peptic ulcers, boils and elephantiasis and are used as moisturizers and medicinal baths. Studies have detected its antimicrobial, anti-oxidative, anti-cancer and anti-tyrosine activities [8]. Various parts of *S. alba* have been used for skin ailments, inflammation, contused injuries and diarrhoea. The leaves and bark of *S. alba* have proven analgesic, tranquillizing and anti-oxidative properties [9]. Therefore, the primary focus of this research is to detect the antibacterial nature of the mangrove fungal endophytes via antibiotic sensitivity tests (ABSTs). Since only limited literary evidence is currently available, this study also provides awareness of the antimicrobial nature of the less acknowledged mangrove fungal endophytes.

2. MATERIALS AND METHODOLOGY

2.1 Sample collection

Healthy, undamaged roots, leaves and stems of *A. aureum* and *S. alba* were collected from the National Aquatic Resources Research and Development Agency, Negombo, Sri Lanka. The samples were initially washed with running water and air-dried to remove soil particles for transport in zip-lock bags. Surface sterilization was carried out within 24 hours of sample collection to remove the surface contaminants according to the guidelines provided in a study by Buatong and colleagues [10].

2.2 Isolation and identification of pure fungal cultures

Smaller segments of the sterilized samples were plated on Streptomycin-infused Potato Dextrose Agar (PDA) and incubated for five days at 27.5 °C. The nutrient media and optimum incubation conditions were selected based on the literary analysis. Fungal identification was performed only based on their colony morphology and by the observation of lactophenol cotton blue (LPCB) stained microscopic structures due to the limitation of resources for a phylogenetic analysis [11, 12]. Pure isolates were obtained by sub-culturing the identified colonies on PDA and Potato Dextrose Broth (PDB), subsequently. The fungi were sub-cultured in PDB as it provides increased access to nutrients than the growth on the surface of PDA. The PDB cultures were subjected to constant agitation while being incubated in the rotary mixer for a week to increase the aeration and promote the fungal growth and the subsequent phytochemical secretion [4].

2.3 Preparation of fungal crude extracts

The PDB cultures were centrifuged at 3000 rpm and filtered through Whatman Number 01 filter paper and Cheesecloth, subsequently. The filtrates and the respective mycelia were extracted in ethyl acetate (CAS No. 141-78-6) for two days and subjected to vigorous centrifugation and vortexing to enable enhanced release of the metabolites by cell breakage [13]. The solvent layers were separated and completely evaporated to ensure that no antimicrobial activity was executed by the solvent. The dried crude extracts were dissolved in 1% Dimethyl Sulfoxide (CAS No. 67-68-5) to a final concentration of 10 mg/ml and stored at -4 °C.

2.4 Performance of antibiotic sensitivity tests

ABSTs were performed according to the Clinical Laboratory Standards Institute M100 guidelines [14] in duplicates. Sterile 1% Dimethyl Sulfoxide and Gentamicin were used as the negative control and positive control, respectively for well diffusion and in determining Minimum Inhibitory Concentration (MIC). *Staphylococcus aureus* (*S. aureus* ATCC ®: 25923) and *Escherichia coli* (*E. coli* ATCC ®: 25922) cell suspensions were prepared using the 0.5 Mc Farland standard. Well diffusion assay was performed by depositing the crude extracts of 10 mg/ ml into the respective well on the Muëller Hinton Agar (MHA) plates that were swabbed with the respective test organism and incubating them at 37 °C for 24 hours. MIC was performed using six serial dilutions of crude extracts with a concentration of 5 mg/ ml, 2.50 mg/ ml, 1.25 mg/ ml, 0.62 mg/ ml, 0.31 mg/ ml and 0.16 mg/ ml, respectively. Each dilution was inoculated with the respective cell suspension and incubated at 37 °C for 24 hours. Aliquots from the tubes containing MIC and the concentrations higher than MIC were spread onto nutrient agar medium for the determination of Minimum Bactericidal Concentration (MBC).

2.5 Data analysis

The One-Way Analysis of Variance (ANOVA) test was performed using the Statistical Package for the Social Sciences (SPSS) version 25.

3. RESULTS AND DISCUSSION

The microscopic analysis identified overall six different types of endophytic fungi of phylum *Ascomycota* from both the plants with a majority from *S. alba*. Figure 1 represents the types of fungal species identified from each mangrove plant. However, previous analyses have isolated *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium* and *Trichoderma* from both of these plants [7, 15]. Table 1 tabulates the number of isolates from each plant and the respective parts.

Aspergillus fumigatus (*A. fumigatus*), *Aspergillus ochraceus* (*A. ochraceus*) and *Penicillium resticulosum* (*P. resticulosum*) were isolated from the roots, stem and leaves of *A. aureum*, respectively. *Penicillium citrinum* (*P. citrinum*) and *Aspergillus niger* (*A. niger*) were identified from *S. alba* roots while *P. resticulosum* was detected from both the stem and leaves. Leaves also harboured *Aspergillus terreus* (*A. terreus*).

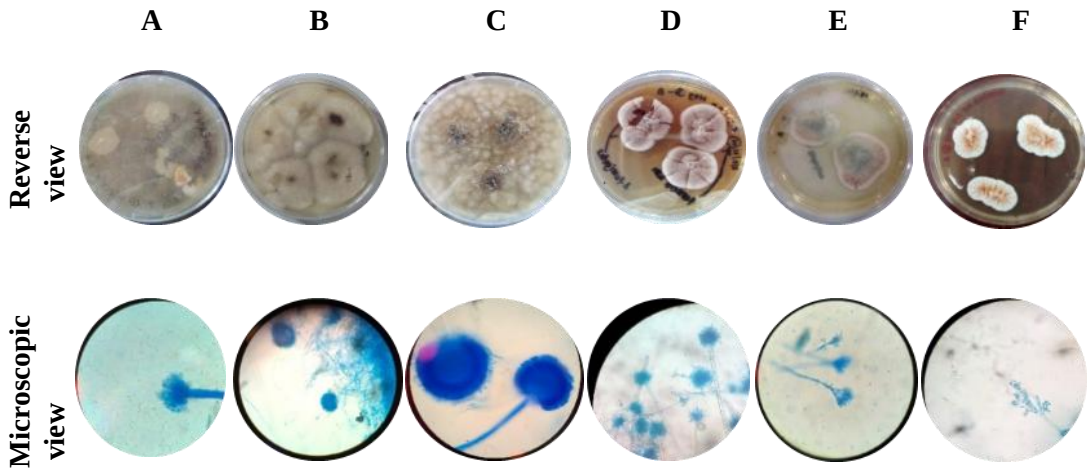

Figure 1: Different types of the identified fungal endophytes. A: Aspergillus fumigatus; B: Aspergillus niger; C: Aspergillus ochraceus; D: Aspergillus terreus; E: Penicillium citrinum; F: Penicillium resticulosum

Table 1: Table of the number of fungal isolates from each mangrove sample

Plants	Total number of isolates	Number of isolates from roots	Number of isolates from stem	Number of isolates from leaves
A. aureum	3	1	1	1
S. alba	5	2	1	2

As indicated in Table 1, *S. alba* was rich in fungal endophytes because their presence also helps this salt-intolerant, true mangrove species to tolerate the saline water during high tide contrary to the associate mangrove, *A. aureum* which requires fewer adaptations in the terrestrial environment [16]. Abundant colonization was observed in the nutrient-rich roots and leaves to prevent possible pathogenic colonization. The difference in the fungal colonization pattern among the plants and different parts and with those of the previous research is due to the influence of the plant age, nutrient contents and the habitats [17]. However, the current fungal identification in the study must be improved using molecular and phylogenetic techniques. Figures 2A and 2B represent the mean zones of inhibition (MZI) generated by the well diffusion assay. According to the data analysis, significant ($p \leq 0.05$) MZI were generated by the samples of *A. aureum* while those of *S. alba* were non-significant ($p \geq 0.05$).

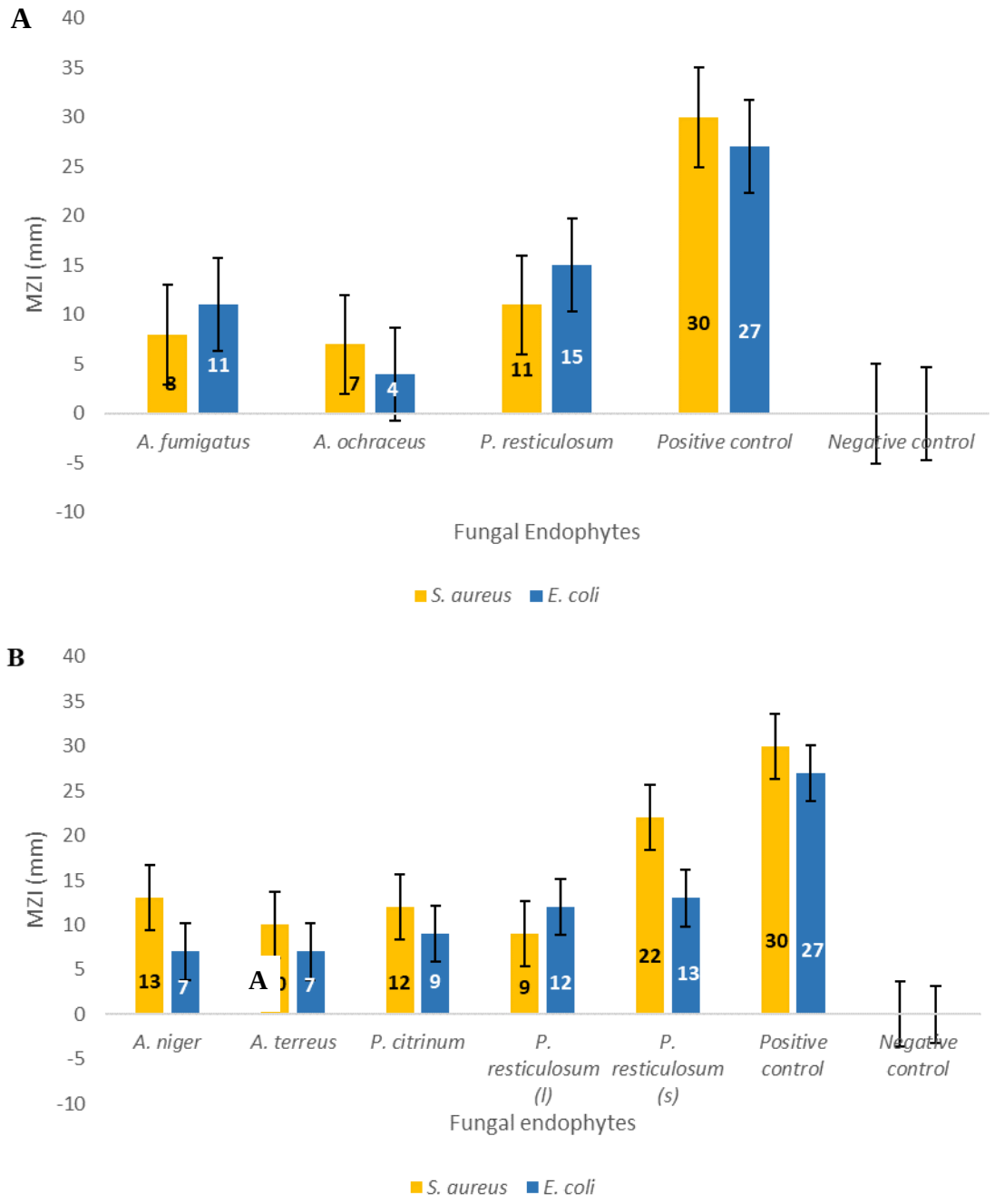

Figure 2: Variation of MZI among the fungal endophytes isolated from A. aureum (A) and S. alba (B); l: leaves; s: stem [18].

Inhibition of the test organism by the antimicrobial metabolites of a particular fungal endophyte was observed as a clear zone around the respective well. The results of the well diffusion assay were generated in the form of MZI±standard deviation. Relatable inhibitory actions of *Aspergillus* and *Penicillium* species from the chosen plants were reported by Maria [19]; Kalyanasundaram [20]; Harizon [9] and their respective co-

scientists. An overall highest inhibition was exhibited by *P. resticulosum*. *P. resticulosum* of *A. aureum* exhibited the highest inhibitory action against *E. coli* (15±0.05 mm) while that identified from *S. alba* stem was the highest against *S. aureus* (22±0.05 mm). An overall lowest inhibitory action was recorded from *A. ochraceus* (*E. coli*: 4±0.05 mm; *S. aureus*: 7±0.01 mm). No previous researches report the inhibition of *E. coli* by *A. ochraceus*. According to the graphical comparison (Figure 2), fungal endophytes isolated from *S. alba* exhibited stronger inhibitory action against the test organisms.

Generally, gram-positive bacteria are more susceptible to antimicrobial substances than gram-negatives. An additional protective layer of the gram-negative bacteria blocks the entry of antibiotics and protects the cell [21]. However, discrepant results were exhibited by *A. fumiagatus* and *P. resticulosum* of *A. aureum* and *S. alba* leaves in this study as they produced wider zones of inhibition for *E. coli*, a gram-negative bacterium. Numbere and Maduike [8] reported a similar contradictory, greater inhibition against *E. coli* from *A. aureum* fungal crude extracts. The study was ensured contamination-free due to the lack of inhibition by the negative control. The positive control produced zones of inhibition in both *E. coli* (27.30±0.80 mm) and *S. aureus* (30.00±0.88 mm) cultures.

Clear suspensions were observed at a concentration of 1.25 mg/ ml, which was chosen as the MIC, the minimum concentration at which the growth of the test organisms was inhibited. MIC test in the study could have been made more accurate using the spectrometric measurement of the turbidity in MIC samples. The minimum concentration at which zero Colony Forming Units were generated was chosen as the MBC of each sample, which is represented in Figure 3. All of the fungal crude extracts isolated in this study were bactericidal against both the test organisms.

According to the MBC test results in Figure 3, the crude extract of *P. citrinum* was the most potent bactericidal agent as it generated zero colonies of both the test organisms at the lowest concentration (1.25 mg/ ml). The crude extracts of *A. niger* and *A. ochraceus* were also bactericidal against *S. aureus* at a concentration of 1.25 mg/ml. *A. fumigatus* and *A. terreus* were bactericidal at a concentration of 5 mg/ ml against both the test organisms which exhibited their lowest potency.

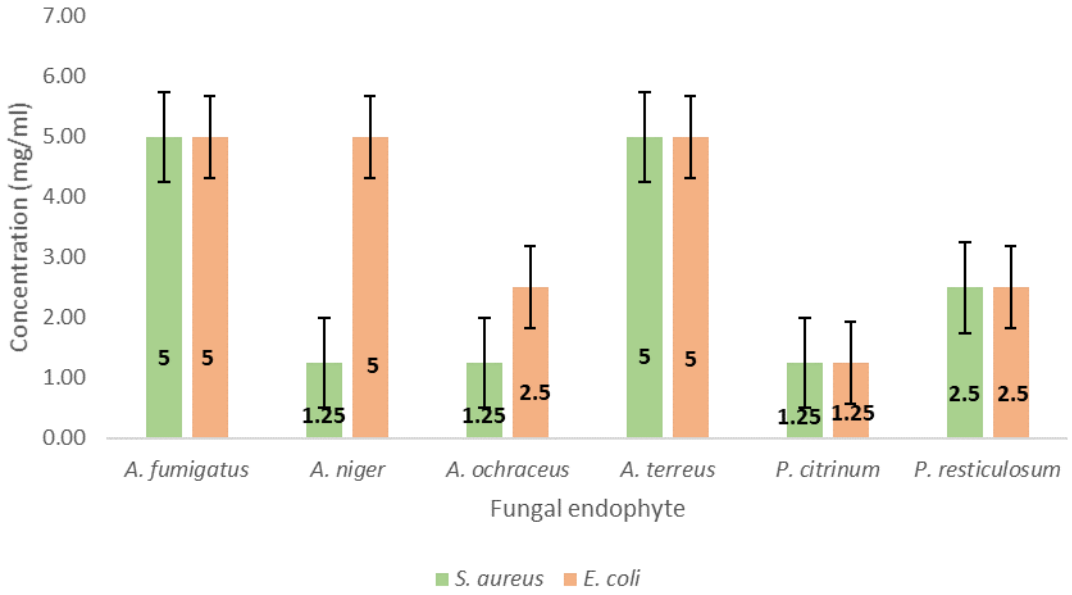

Figure 3: Variation of MBC among different fungal endophytes [18].

The difference between the well diffusion and MBC results is because the fungal endophyte that was bacteriostatic might not be bactericidal at the same concentration.

The antagonistic activity of the crude extracts against *E. coli* and *S. aureus* in this study depicts their potential broad-spectrum activity against the gram-positive and gram-negative bacteria. However, the synthesis of antimicrobial phytochemicals by the mangrove fungal endophytes is low in quantity and requires special, harsh conditions. Further studies can be conducted on the types of metabolites and the mechanisms responsible for this antimicrobial nature for use in manufacturing drugs against antibiotic-resistant, pathogenic strains. Tests can also be conducted by altering culture conditions, type of solvents, extraction techniques and test organisms to determine an appropriate, target-specific concentration of these agents to avoid adverse, off-target effects. This study can be expanded by designing assays to test the cytotoxic, enzymatic and antiviral activities of the studied species.

CONCLUSION

The ABST results suggest that the fungal crude extracts isolated from *A. aureum* and *S. alba* have useful antimicrobial properties. The *P. resticulosum* crude extract isolated from *A. aureum* leaves was most inhibitory against *E. coli*. The most potent inhibitory isolate against *S. aureus* was *P. resticulosum* which was isolated from *S. alba* stem.

These well diffusion results suggest the possible use of these crude extracts in generating bacteriostatic antibiotics. The most potent bactericidal agent was *P. citrinum* isolated from the roots of *S. alba*. Therefore, the fungal endophytes isolated from *S. alba* and *A. aureum* can be used to treat infections caused by *E. coli* and *S. aureus*.

ACKNOWLEDGEMENT

We extend our gratitude to the staff of the National Aquatic Resources Research and Development Agency, Negombo, Sri Lanka for providing the mangrove samples and the School of Science, Business Management School for providing the laboratory space and the resources.

REFERENCE

[1] Kuzhalvaymani, K., Elizabethjacquline, L. and Subha, T.S. (2020). Endophytic fungi from mangroves -Review, *International Journal of Pharmaceutical Sciences and Research*, 11 (9), pp. 4134-4143. *IJPSR*. Available at: 10.13040/IJPSR.0975-8232.11(9).4134-43

[2] Ravimannan, N. and Sepali, E. (2020). Antibacterial activity of endophytic fungi isolated from mangroves of Jaffna Peninsula, Sri Lanka, *African Journal of Plant Science*, **14 (6)**, pp. 243-247. *Academic Journals*. Available at: https://doi.org/10.5897/AJPS2020.1987

[3] Abeysinghe, P.D. (2010). Antibacterial activity of some medicinal mangroves against antibiotic resistant pathogenic bacteria, *Indian Journal of Pharmaceutical Sciences*, **72 (2)**, pp. 167–172. *PubMed Central*. Available at: https://doi.org/10.4103%2F0250-474X.65019

[4] Rossiana, N., Miranti, M. and Rahmawati, R. (2016). Antibacterial activities of endophytic fungi from mangrove plants *Rhizophora apiculata* L. and *Bruguiera gymnorrhiza* (L.) Lamk. on *Salmonella typhi*, *Towards the Sustainable Use of Biodiversity in a Changing Environment: From Basic to Applied Research*, **1744 (020040)**, pp. 1-7. *AIP Conference Proceedings*. Available at: 10.1063/1.4953514

[5] Yu, H., Zhang, L., Li, L., Zheng, C., Guo, L., Li, W., Sun, P. and Qin, L. (2010). Recent developments and future prospects of antimicrobial metabolites produced by endophytes, *Microbiological Research*, **165 (2010)**, pp. 437-449. *ScienceDirect*. Available at: 10.1016/j.micres.2009.11.009

[6] Ribeiro, S.F.L., Garcia, A.C., dos Santos, H.E.D., Montoya, Q.V., Rodrigues, A., de Oliveira, J.M. and de Oliveira, C.M. (2018). Antimicrobial activity of crude extracts of endophytic fungi from *Oryctanthus alveolatus* (Kunth) Kuijt (Mistletoe), *African Journal*

of Microbiology Research, **12 (11)**, pp. 263-268. *AcademicJournals*. Available at: 10.5897/AJMR2017.8772

[7] Bibi, S.N., Gokhan, Z., Rajesh, J. and Mahomoodally, M.F. (2019). Fungal endophytes associated with mangroves- Chemistry and biopharmaceutical potential, *South African Journal of Botany*, **134 (50)**, pp. 187-212. *ScienceDirect*. Available at: https://doi.org/10.1016/j.sajb.2019.12.016

[8] Numbere, A.O. and Maduike, E.M. (2021). Investigation of the anti-bacterial properties of mangrove fern, *Acrostichum aureum* in the Niger Delta, Nigeria, *African Journal of Biotechnology*, **20 (4)**, pp. 142-149. *AcademicJournals*. Available at: 10.5897/AJB2021.17307

[9] Harizon., Pujiastuti, B., Kurnia, D., Sumiarsa, D., Shiono, Y. and Supratman, U. (2015). Antibacterial Triterpenoids from the Bark of *Sonneratia alba* (Lythraceae), *Natural Product Communications*, **10 (2)**, pp. 277-280. Available at: 10.1177/1934578X1501000215

[10] Buatong, J., Phongpaichit, S., Rukachaisirikul, V. and Sakayaroj, J. (2011). Antimicrobial activity of crude extracts from mangrove fungal endophytes, *World J Microbiol Biotechnol*, **27 (2011)**, pp. 3005-3008. *Springer*. Available at: http://dx.doi.org/10.1007/s11274-011-0765-8

[11] Thorati, M., Mishra, J.K. and Kumar, S. (2016). Isolation, identification of endophytic fungi from mangrove roots along the coast of South Andaman sea, Andaman and Nicobar Islands, India, *Journal of Marine Biology & Oceanography*, **5 (2)**, pp. 1-5. *ResearchGate*. Available at: 10.4172/2324-8661.1000157

[12] Barnett, H.L. and Hunter, B.B. (1998). *Illustrated genera of imperfect fungi*. 4th edn. Saint Paul, Minnesota, USA: Amer Phytopathological Society.

[13] Thomas, T. (2012). *In vitro* evaluation of antibacterial activity of *Acrostichum aureum* Linn., *Indian Journal of Natural Products and Resources*, **3 (1)**, pp. 135-138 *ResearchGate*.

[14] Clinical and Laboratory Standards Institute (CLSI). (2020). *Performance standards for Antimicrobial Susceptibility Testing*. 30th edn. CLSI Supplement M100.

[15] Handayani, D., Rivai, H., Mulyana, R., Suharti, N., Rasyid, R. and Hertiani, T. (2018). Antimicrobial and cytotoxic activities of endophytic fungi isolated from mangrove plant *Sonneratia alba* Sm, *Journal of Applied Pharmaceutical Science*, **8 (2)**, pp. 49-53 *JAPSonline*. Available at: 10.7324/JAPS.2018.8207

[16] Milon, M.A., Muhit, M.A., Goshwami, D., Masud, M.M. and Begum, B. (2012). Antioxidant, Cytotoxic and antimicrobial activity of *Sonneratia alba* bark, *International Journal of Pharmaceutical Sciences and Research*, **3 (7)**, pp. 2233-2237 *IJPSR*. Available at: http://dx.doi.org/10.13040/IJPSR.0975-8232.3(7).2233-37

[17] Lee, N.L.Y., Huang, D., Quek, Z.B.R., Lee, J.N. and Wainwright, B.J. (2020). Distinct fungal communities associated with different organs of the mangrove *Sonneratia alba* in the Malay Peninsula, *IMA Fungus*, **11 (17)**, pp. 1-9 *BMC*. Available at: https://doi.org/10.1186/s43008-020-00042-y

[18] IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.

[19] Maria, G.L., Sridhar, K.R. and Raviraja, N.S. (2005). Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India, *Journal of Agricultural Technology*, **1**, pp. 67-80. *ResearchGate*.

[20] Kalyanasundaram, I., Nagamuthu, J. and Muthukumaraswamy, S. (2015). Antimicrobial activity of endophytic fungi isolated and identified from salt marsh plant in Vellar estuary, *Journal of Microbiology and Antimicrobials*, **7 (2)**, pp. 13-20 *academicJournals*. Available at: http://www.academicjournals.org/JMA

[21] Sheeba, H., Ali, M.S. and Anuradha, V. (2019). Bioactive compounds and antimicrobial activity of fungal crude extract from medicinal plants, *Journal of Pharmaceutical Sciences and Research*, **11 (5)**, pp. 1826-1833 *ResearchGate*.