DETERMINATION OF THE ANTIBACTERIAL PROPERTIES OF ENDOPHYTIC FUNGAL CRUDE EXTRACTS ISOLATED FROM *Acrostichum aureum*

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INTRODUCTION

Antibiotics that are synthesized by microorganisms prevent infection by other microorganisms. The ancient use of soil and mildewed bread as antibiotics have gained tremendous development to the currently available agents over the years following the introduction of Salvarsan, the first commercially introduced antibiotic by Paul Ehrlich in 1930 (Thomas, 2012). Antibiotic-resistant pathogenic strains have emerged due to their misuse and expanded use as therapeutics and in poultry. Hence, scientists are attempting to generate antimicrobial agents from natural products such as mangrove fungal endophytes to overcome antibiotic resistance and the adverse effects of synthetic agents (Ribeiro *et al*., 2018).

Bacterial or fungal endophytes reside in the internal plant tissues asymptomatically and maintain mutualistic, symbiotic, antagonistic or pathogenic associations with the host. Mangrove plants provide habitats for aquatic organisms and endophytic microbes. Unlike the true mangrove species, associate mangroves grow in terrestrial environments with fewer adaptations like viviparity and modified root systems. Unfavourable, high saline, temperate and windy habitats inhabited by the mangroves favour the synthesis of metabolites by the fungal endophytes (Bibi *et al*., 2019). The antimicrobial phytochemicals are synthesized as a defence mechanism and intracellular signal to protect their host from plant pathogens, which has gained attention in the research field. The largest mangrove patch in Sri Lanka is found surrounding the Puttalam-Kalpitiya lagoon. Negombo lagoon is the largest patch in the Western province hence, it was chosen for sample collection (Chandrasekara *et al*., 2016). *Acrostichum aureum*, an associate mangrove fern of *Pteridaceae* family is the plant of choice in this study. The fern grows abundantly in dry, unmaintained grounds upto 4 metres in height. The stem bears multiple, long leaves with blunt ends (Thomas, 2012). Pharmaceutical industries have utilized ferns to generate medicinal products for skin, gut and respiratory syndromes. Rhizomes of *Acrostichum aureum* have been used to treat wounds, ulcers, worms, cough, elephantiasis, urine tract infection and fever in traditional Malaysian, Micronesian and Chinese medicinal practices. Ancient Bangladeshian, Columbian and Costa Rican nations have used its leaf extracts for peptic ulcers, boils and as emollients and medicinal baths. *Acrostichum aureum* samples have also been reported to be anti-oxidative, anti-tyrosine and cytotoxic against cervical, colon, gastric and breast cancers (Badhsheeba and Vadivel, 2020).

The main objective of the study is to detect the antibacterial properties of endophytic fungal crude extracts isolated from *Acrostichum aureum* by analyzing their bactericidal and bacteriostatic nature. The study was conducted to obtain more data as only limited literature is available on the chosen subject.

METHODOLOGY

Sample collection and preparation:

An adequate quantity of fresh, disease-free leaves, roots and stems of *Acrostichum aureum* were collected 48 hours before the experiment from the National Aquatic Resources Research and Development Agency in Negombo, Sri Lanka. The samples were washed, dried under ambient conditions and stored in the refrigerator in zip-lock bags until use. Each sample was cut into 5 centimetres (cm) long pieces using a sterile blade. Surface sterilization was carried out by

immersing the samples subsequently in 70% ethanol, 1% sodium hypochlorite and 70% ethanol for 30 seconds, 2 minutes and 30 seconds, respectively. The sterilized samples were rinsed thoroughly with distilled water and dried under ambient conditions.

Fungal identification and isolation of pure fungal colonies:

Pieces of 5 cm^2 of the dried samples were plated on potato dextrose agar (PDA) plates and incubated at 27.5 ^{O}C for five days. Streptomycin was introduced to the media to avoid the generation of bacterial colonies. A thin hyphal layer of a morphologically distinct fungal colony was placed on a drop of lactophenol cotton blue (LPCB) stain on a sterile microscopic slide. As a mounting and staining agent, the components of LPCB stain are involved in killing and preserving the fungal structures, staining the cell wall and preventing specimen drying (Xie *et al*., 2007). The prepared specimen was viewed under a light microscope. Microscopic examination was performed again following the sub-culturing of distinct colonies on fresh PDA plates. The fungal species were identified by comparing the obtained microscopic images with previous literature and Commonwealth Mycological Institute (CMI) descriptions. The identified colonies were subcultured into fresh potato dextrose broth (PDB) and incubated at room temperature in a rotary mixer for seven days. Constant shaking of the PDB cultures increases aeration and mycelial breakage thus promotes fungal growth and subsequent phytochemical synthesis.

Endophytic fungal crude extract preparation:

The cultures were initially vortexed and then centrifuged for 10 minutes at 3000 rotations per minute (rpm) to ensure the efficient release of phytochemicals by breakage of mycelia. The extracts were filtered using a cheesecloth and Whatman Number 1 filter paper to obtain clear filtrates. Ethyl acetate was chosen for solvent extraction as it can dissolve both polar and non-polar components. The extracted filtrates and mycelia were incubated separately with 20 ml of ethyl acetate at room temperature for two days using a rotary mixer. Following the incubation, only the solvent layers of the respective samples were pipetted out and pooled in sterile Petri-plates. The solvent was completely evaporated using a fume hood and dry oven to make sure that no antimicrobial activity was exerted by the solvent itself. The crude extracts were dissolved in 1% Dimethyl Sulfoxide and stored at -4 ^{O}C (Maria, Sridhar and Raviraja, 2005).

Well diffusion assay:

Staphylococcus aureus (ATCC Number: 25923) and *Escherichia coli* (ATCC Number: 25922) cell suspensions were prepared using 0.5 Mc Farland. Each crude extract was deposited into the respective wells in the Muëller Hinton Agar (MHA) plates that were swabbed with the respective test organism. MHA provides better diffusion of crude extracts. Gentamicin disk and autoclaved distilled water were used as the positive and negative controls, respectively. The prepared plates were incubated at 37 ^{O}C for 24 hours (Denkova *et al*., 2017). The well diffusion assay was performed in triplicates.

MIC and MBC Tests:

Six different concentrations of the crude extracts were prepared by serial dilution technique. Liquid gentamicin and autoclaved distilled water were used as the positive and negative controls, respectively. Each tube was inoculated with 20 µl of the respective cell suspension and incubated at 37 ^{O}C for 24 hours. MIC and the dilutions lower than MIC were spread onto nutrient agar (NA) medium and incubated at 37 ^{O}C for 24 hours (Owuama, 2017). The mean zones of inhibition (MZI) were compared by an Analysis of Variance (ANOVA) test using Statistical Package for the Social Sciences version 25 (SPSS).

RESULTS AND DISCUSSION

The fungal species were cultured on a slightly acidic, carbohydrate-rich PDA medium and incubated at the optimum temperature as both culture media and culture conditions have been proven to impact the fungal growth and the subsequent phytochemical synthesis (Badhsheeba and Vadivel, 2020).

Table-01 tabulates the types of endophytic fungal species that were identified. *Aspergillus fumigatus* and *Aspergillus ochraceus* were identified from the roots and stems, respectively. *Aspergillus fumigatus* was identified morphologically using the whitish-yellow, droplets-like colonies as in figure- 01 and by the microscopic observation of phialides encompassing two-thirds of the uniseriate, columnar conidial heads (Figure-02) as reported by Nyongesa, Okoth and Ayugi in 2015. *Aspergillus ochraceus* was identified by the morphological arrangement of yellow, submerged colonies with pale brown reverse sides and the microscopic view of the biseriate arrangement of phialides on the conidial heads (Ellis *et al*., 2007) as in figures-03 and 04, respectively. The fungal endophyte isolated from leaves was identified as *Penicillium chrysogenum* (Figure-05) based on the microscopic observation of brush-shaped conidiophores bearing conidia (Figure-06). These findings partially correlated with the previous isolation of *Aspergillus*, *Alternaria*, *Acremonium*, *Fusarium* and *Penicillium* species by other studies.

Antibiotic susceptibility tests (ABSTs) detect the antimicrobial potential of the tested agent. Well diffusion provides relevance to the inhibitory nature of the agent as zones of inhibition around each well, to which the agent is deposited. Minimum Inhibitory Concentration (MIC) is the minimum concentration at which the test organism is inhibited. Minimum Bactericidal Concentration (MBC) is the minimum concentration at which the organism is killed (Kalili *et al*., 2012). The crude extract that was deposited in the well, diffuses through the media and creates zones of inhibition by hindering the fungal growth. Prominent zones of inhibition were produced by the gentamicin disk, in both *Escherichia coli* and *Staphylococcus aureus* plates and no inhibition was exhibited by the negative control. According to figure-07, the highest inhibition was executed by *Penicillium chrysogenum*, which produced an MZI of 1.55 cm and 1.01 cm against *Staphylococcus aureus* and *Escherichia coli*, respectively. *Aspergillus ochraceus* (MZI- 1 cm against *Staphylococcus aureus* and 1.05 cm against *Escherichia coli*) exhibited a more potent activity than *Aspergillus fumigatus*. *Aspergillus fumigatus* was inhibitory only against *Escherichia coli* which is contradictory as gram-positive bacteria are more susceptible to antibiotics than gram-negatives. The gram-negative bacteria are more resistant due to the presence of an additional protective layer that retracts the invading antibiotics while the peptidoglycan cell wall of gram-positive bacteria absorbs the antibiotics (Ribeiro *et al*., 2018). However, a similar contradiction was reported from crude extracts of *Acrostichum aureum* used by Numbere and Maduike (2021). The non-inhibitory action can be due to bacterial resistance against the fungal crude extract. The obtained well diffusion results correlated with those from Maria and colleagues (2005) who reported the inhibition of both *Staphylococcus aureus* and *Escherichia coli* by ethyl acetate crude extracts of *Acrostichum aureum*. However, no published evidence supports the inhibition of the test organisms by *Aspergillus ochraceus*.

MIC was performed to detect the inhibitory nature of the fungal extracts. A lack of turbidity was observed in the dilutions of 10^{-1}, 10^{-2} and 10^{-3} by comparing to the positive control. Hence, the concentration corresponding to the dilution of 10^{-3} was considered the MIC as it indicated a complete inhibition of the fungal growth at the lowest concentration. More turbid samples were observed with the reduction in crude extract concentration as the inhibitory strength of the fungal extract decreases with decreasing concentration. MIC and concentrations higher than the MIC were chosen for the MBC test. MBC was performed to detect the bactericidal nature of the crude extract. The results (Table-02) were categorized into less than 30, 30-300 and more than 300 according to the colony-forming units (CFUs) observed. The obtained MBCs were different or similar to that of MIC, thus indicating that the same fungal crude extract can be bacteriostatic or bactericidal at the same concentration or different concentrations. All the tested fungal crude

extracts were bactericidal toward both *Escherichia coli* and *Staphylococcus aureus*. The MBC of *Aspergillus fumigatus* against both *Staphylococcus aureus* and *Escherichia coli* is the concentration of the 10^{-1} dilution. The concentration of the dilution 10^{-3} is the MBC of *Aspergillus ochraceus* against both *Escherichia coli* and *Staphylococcus aureus*, which was similar to its MIC. The MBC of *Penicillium chrysogenum* is the concentration of the dilution 10^{-2} against both *Escherichia coli* and *Staphylococcus aureus*.

CONCLUSIONS/ RECOMMENDATIONS

Recent researches focus on the development of medicinal products from natural agents due to their low toxicity, low cost and increased effectiveness. Hence, this study was designed to determine the antimicrobial activity of endophytic fungal crude extracts isolated from *Acrostichum aureum*, a mangrove plant. *Aspergillus fumigatus*, *Aspergillus ochraceus* and *Penicillium chrysogenum* were identified from the plant samples. According to the well diffusion results, all the generated fungal crude extracts were inhibitory against both *Escherichia coli* and *Staphylococcus aureus* except, *Aspergillus fumigatus*, which was active only against *Escherichia coli*. However, the MIC and MBC results indicated that all three fungal crude extracts were potent against both test organisms. Hence, based on the results of this study, it can be concluded that ethyl acetic crude extracts of endophytic fungi isolated from *Acrostichum aureum* can be used to develop antibiotics targeting antibiotic-resistant pathogens. The current study can be further expanded with the incorporation of molecular identification techniques for fungal identification and the use of more concentrated crude extracts. Different culture media, culture conditions and solvents can be tested to maximize the yield. Assays can also be designed to test the anti-oxidative and enzymatic actions of *Acrostichum aureum*.

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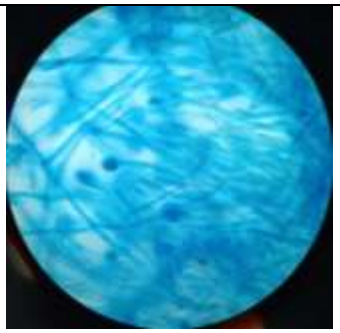
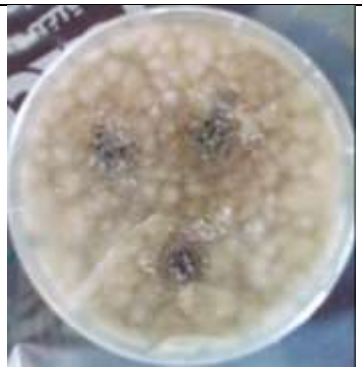
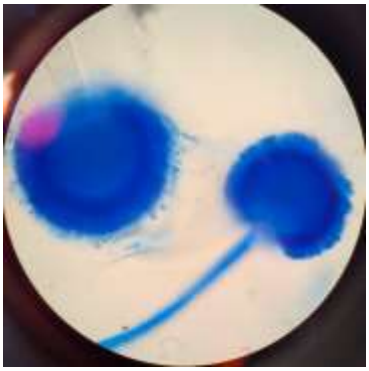
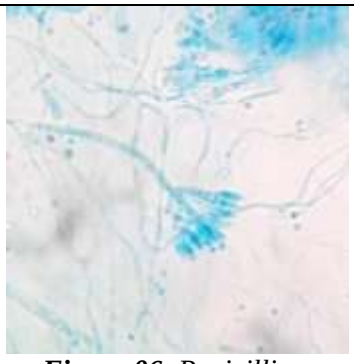
ACKNOWLEDGMENTS

The authors would like to thank the staff of the National Aquatic Resources Research and Development Agency, Negombo, Sri Lanka for providing the samples and the School of Science, Business Management School for the resources and the workspace.

Table 01: Table of the respective fungal colonies and their microscopic images.

Sample name	Morphology of the culture plates	Microscopic images of the identified fungal species
Acrostichum aureum roots	 ***Figure 01***: *Aspergillus fumigatus* colonies.	 ***Figure 02***: *Aspergillus fumigatus*
Acrostichum aureum stem	 ***Figure 03***: *Aspergillus ochraceus* colonies.	 ***Figure 04***: *Aspergillus ochraceus*
Acrostichum aureum leaves	 ***Figure 05***: *Penicillium chrysogenum* colonies.	 ***Figure 06***: *Penicillium chrysogenum*

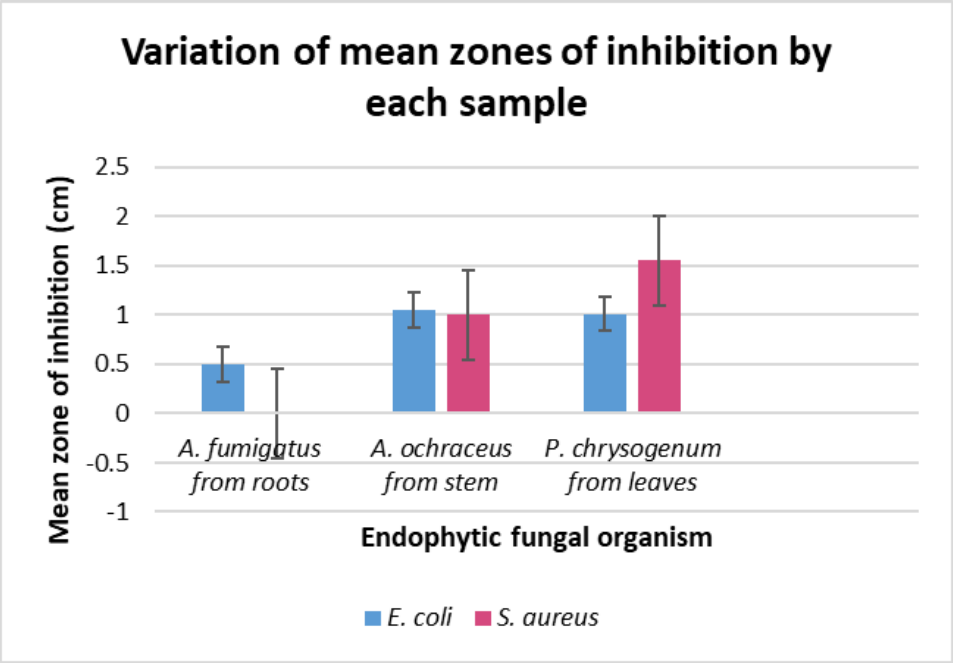


Figure 07: Graphical variation of the MZI by the respective fungal species (IBM SPSS Statistics for Windows, 2017).

Table 02: MBC results. MBC results are presented in the form of number of CFUs generated by the test organisms corresponding to each dilution.

Fungal endophyte	MBC results against *Escherichia coli*			MBC results against *Staphylococcus aureus*		
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
Aspergillus fumigatus	0	1	> 300	0	> 300	> 300
Aspergillus ochraceus	> 300	0	0	1	< 30	0
Pencillium chrysogenum	< 30	0	> 300	> 300	0	< 30