

SHORT COMMUNICATION

ANTIMICROBIAL PONTENTIAL OF FUNGAL ENDOPHYTES OBTAINED FROM *Avicennia Marina* AND *Excoecaria Agallocha* IN NEGOMBO

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ABSTRACT

Mangroves are halophytic plants that are typically found along the intra-tidal regions. The coastal ecosystem of mangroves in Sri Lanka hold one of the most unique mangrove species that are yet to be studied. These ecosystems provide shelter to various organisms including humans. Fungal endophytes present in these plants have many medicinal properties that can be utilized in the development of drugs. Antibiotic resistance is a major concern in the pharmaceutical industry, hence alternative plant-based drugs with fewer costs and side effects show great potential. In the current investigation, the main objective was to test for antibacterial properties of fungal endophytes isolates from two mangroves: *Avicennia marina* and *Excoecaria agallocha* against *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922). In both mangrove species, three endophytic species were identified based on their morphological characteristics: *Aspergillus niger*, *Penicillium viticola* and *Aspergillus versicolor*. Only *P. viticola* was obtained from *E. agallocha*. Based on the results obtained from antibiotic susceptibility tests, *A. niger* (stem) was the most active and showed the best bacteriostatic and bactericidal activity against both *S. aureus* and *E. coli*. *P. viticola* produced viable results (mean zone of inhibition of 21 mm against *S. aureus*) but were not as productive as *A. niger*. This study indicated that these endophytic fungi held medicinal properties that can be further evaluated

Keywords: *Mangroves, Fungal endophytes, Negombo, E. agallocha, A. marina*

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1. INTRODUCTION

Fungal endophytes are well known for their competence in inhabiting many plant species [1]. This association is seen as a benefit due to its ability to protect the host from certain environmental stresses and pathogens [2]. According to Himes-Cornell, Grose and Pendleton [3], mangroves are commonly termed as ‘blue forest ecosystem’ which interest

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many due to their immense capability in providing several organisms with a home, it also plays a part in regulating the climate by trapping carbon dioxide [3]. Furthermore, along with being stressors of weather conditions, mangroves are vital for human communities in providing materials such as wood and timber, food and even shelter [19, 20].

Antibiotic resistance has become a major public health concern due to the emergence of new diseases, increased population, lack of good sanitation and disposal [4]. Since, modern medicine utilizes antibiotics frequently, an alternative is required to produce efficacious results. Subramani, Narayanasamy and Feussner [5], suggest how plant-based drugs show potential in replacing currently used synthetic drugs. However, even though drugs with a plant origin show promising results, at present only few antimicrobial drugs are in use. Fungal endophytes isolated from mangroves show pharmaceutical properties that are ideal for drug production [6]. Phytocompounds and their metabolites identified in these endophytes show promise in inhibiting a range of microorganisms and other diseases such as cancer.

Mangroves require a specific amplitude (tidal) to survive, hence in Sri Lanka majority of its mangrove species are seen close to lagoons (Chillaw, Negombo, Thondaimanar) and river mouth [7,21]. Despite Sri Lanka harboring several species, *A. marina*, *Rhizophora mucornata*, *E. agallocha*, *Bruguiera gymnorhiza* are some of the widely available species. Namazi *et al*., [8], explains how a study done on *A. marina*, shows the presence of various phytochemicals with the capabilities of eliciting antiviral, antifungal and antibacterial properties.

Moreover, *A. marina* was traditionally used in Persia for the treatment of HIV, cold sores, ulcers and rheumatic diseases. Further, several parts obtained from *E. agallocha* proved to be an effective treatment for diabetes, epilepsy, cancer, leprosy and various infectious diseases [22]. Latex extracted from these shrubs can cause temporary blindness and hence these species are widely known as 'the blind your eye mangrove' [9]. The valuable properties of these species are enough to overlook the slight inconvenience it causes. With this knowledge, the objective of this study was to analyse the antimicrobial properties of endophytic fungi colonizing *A. marina and E. agallocha* via well diffusion and MIC/MBC tests.

2. MATERIALS AND METHODS

2.1 Sample collection and sterilisation

Samples (*A. marina* and *E. agallocha*) were collected from NARA (National Aquatic Resources Research and Development Agency) in Negombo, Sri Lanka. Stem, leaves and roots were stored in zip-lock bags in the refrigerator until use after a thorough wash and air drying. Each of the samples was cut into 5cm pieces and sterilised using 70% ethanol and 5% sodium hypochlorite, followed by a proper rinsing using sterile distilled water before air-drying.

2.2 Identification of fungi and subculturing

Potato dextrose agar (PDA) was prepared, to which pieces of the sample were placed and incubated (ThermoScientific BB15 6856) at 27°C for 4-5 days. After incubation, fungal endophytes were identified from the grown fungal colonies using lactophenol cotton blue (LPCB) and a light microscope (Labomed). A sub-culture was made to obtain pure fungal colonies, using PDA by plating each fungal endophyte separately. Potato dextrose broth was used for the second sub-culturing and was incubated at room temperature in a roller shaker (KJMR – II) for a week.

2.3 Preparation of crude from endophytic fungi

Sub cultured fungal endophytic broth was centrifuged at 3060 rpm. Filter paper (Whatmann qualitative filter paper, Sigma-Aldrich) was then used to filter out any mycelia and fungal blocks present, after which 10ml of ethyl acetate (EtOAc) was added and left to incubate overnight. EtOAc fractions and the broth (subculture) were separated and completely dried in a fume cupboard (Biobase). DMSO was then added to dilute the sample for further testing process.

2.4 Antibiotic susceptibility test (ABST):

Well diffusion assay: Evaporated broth and EtOAc fractions (with concentrations of 10 mg/ml) were dissolved in 1% DMSO (1000 µL). *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) suspension were prepared using 0.5M Mc Farland's solution. Well diffusion was conducted with Müeller Hinton agar (MHA), swabbed with *S. aureus* and *E. coli*. 15µL of both (crude) and their respective EtOAc were added into the wells.

Autoclaved distilled water was used as a negative control while diluted liquid gentamycin was used as the positive control (3 replicates were made).

Minimum Inhibitory concentration (MIC) and Minimum Bactericidal concentration (MBC): For the MIC test, serial dilution was carried out to obtain 6 concentrations of the crude (0.5 ml crude sample and 3.5ml distilled water in each of the tubes). Gentamycin and distilled water were used as positive and negative controls, respectively. 20 µl of test organisms were used and incubated 27°C for 24 hours. MBC was conducted by using Nutrient agar to spread MIC (using sterile glass spreader) and dilutions lower than MIC and left to incubate.

2.5 Statistical analysis

SPSS version 21 was used as statistical software to analyse the correlation between the results.

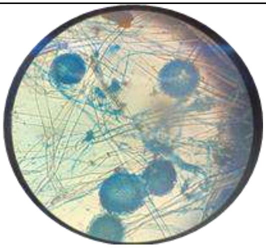
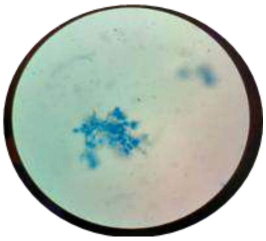
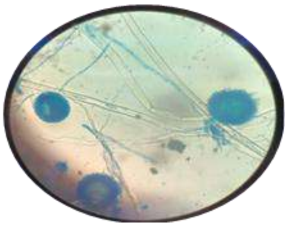
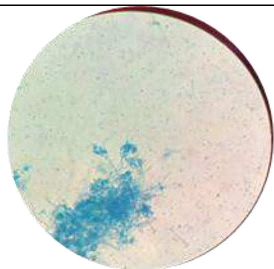
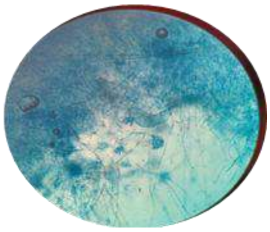
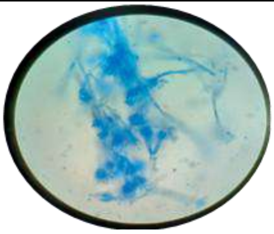
3. RESULTS AND DISCUSSION

3.1 Fungal identication

Table 1 depicts the type of fungal endophyte observed in each sample. Two different *Aspergillus* species (*A. niger* and *A. versicolor*) were identified in *A. marina* (in leaves, stem and roots) while *P. viticola* was the only type of *penicillium* species identified in the leaves and roots of *A. marina* and only in the roots of *E. agallocha*. The main structural difference that helped distinguish between the two endophytic fungi were the elongated conidiospore and the brush-like conidia around a vesicle which is absent in the *Penicillium* species where the conidiospore are branched and septate-like [10]. Phylogenic examination needs to be done for more accurate characterisation. Based on a study conducted by Khalil *et al.*, [11] in which leaves of *A. marina* from Saudi Arabia coastline were evaluated, based on the results obtained, *Chaetomium* species were isolated the most, while no *Penicillium* species were isolated, which is unlike the present investigation.

Comparatively fungal endophytes isolated from *E. agallocha* were very scarce, with only results from the root sample (*P. viticola*). The study reported by Chaeprasert *et al.*, [12], yield better results while extracting fungal endophytes from various mangrove species. This may be due

Table 1: Fungal endophytes observed

Endophytic fungi (host)	**Colony morphology**	**Micro-morphology**
A. niger A. marina –stem)		
P. viticola (*A. marina* – leaves)		
A. niger (*A. marina*-leaves)		
P. viticola (*A. marina*- roots)		
A. versicolor (*A. marina*-roots)		
P. viticola (*E. agallocha*-roots)		

to the long incubation period (4 weeks) they may induce a better fungal growth. Moreover, the broth used for the sub-culturing prevents any bacterial growth due to it acidic environment thus only culturing fungi. Ethyl acetate along with centrifugation promotes the release of phytochemicals, along the solvent also minimising cell toxicity [13].

3.2 Well diffusion

Both EtOAc and broth fractions were used to test for antimicrobial properties to have a comprehensive understanding as to which has absorbed more metabolites that help hinder growth [14].The graph seen in figure 2 indicates that broth sample of *Penicillium viticola* from the roots of *A. marina* has the highest mean zone of inhibition (MZI) against *S. aureus* (21 mm) whereas *Aspergillus niger* from the leaves of *A. marina* seem to make the largest MZI against *E. coli*. However, comparatively fewer zones were seen in EtOAc extracts (figure 2), *A. niger* has an MZI of 23 mm against *E. coli* marking it as the largest zone in the entire study. Even though the broth extract of *Aspergillus versicolor* had no effect against *E. coli*, its EtOAc fraction seems to have the largest MZI against the same (12mm). According to the investigation conducted by Prihanto, Firdaus and Nurdiani [15], *Penicillium* seems to have a wider antibacterial activity by being effective against both *S. aureus* and *E. coli*. Statistical analysis concluded that MZI results were signification due to p≤ 0.05.

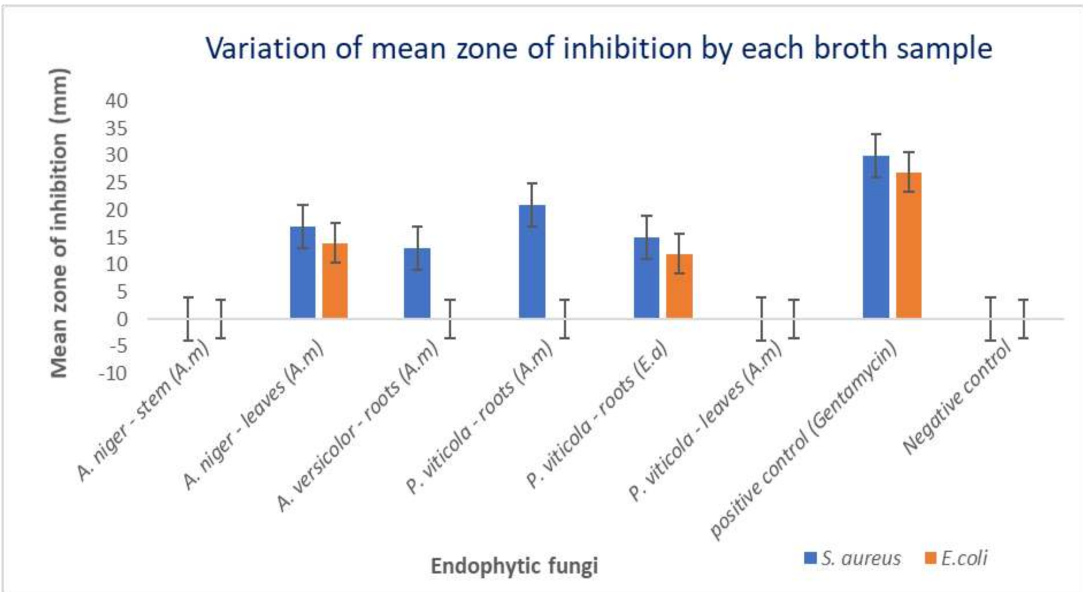


Figure 1: Mean inhibition zones of different endophytic fungi in broth (*A.m- Avicennia marina* and *E.a- Excoecaria agallocha*)

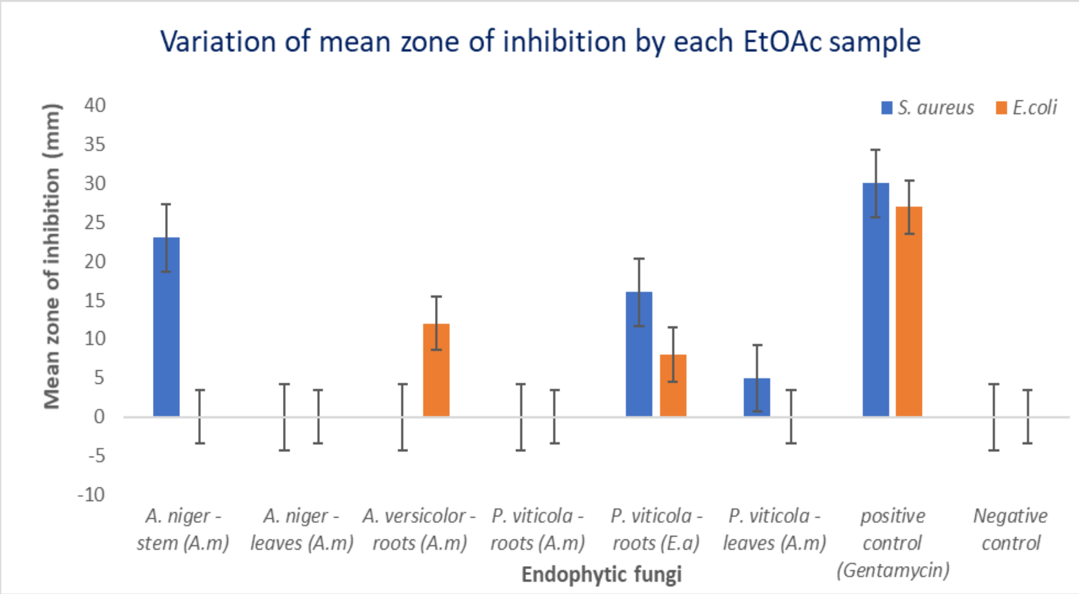


Figure 2: Mean inhibition zones of different endophytic fungi in EtOAc (*A.m- Avicennia marina* and *E.a- Excoecaria agallocha*)

3.3 MIC and MBC

Serial dilution results showed no turbidity in the first three tubes (dilution of 10^{-1}, 10^{-2}, 10^{-3}) implying that the bacteria were completely inhibited, further confirmation should be performed using spectrophotometer should be performed. The lack of inhibition seen in the last three tubes was due to the diluted crude, [17]. Each of the endophytes completely inhibited and killed test organisms at different concentrations. *P. viticola* has the lowest MBC against *E. coli* (0.625 mg/mL) and *S. aureus* (1.25 mg/mL) stating that it was the most potent bactericidal activity against both test organisms, while *A. versicolor* has a similar effect as *P. viticola* against *S. aureus*. *A. niger* had the highest MBC (5 mg/ml) indicating the lowest potency against *S. aureus*. In a similar investigation Hamzah *et al* [18] used a microdilution method that proved that crude obtained from fungal endophytes could stop the bacterial growth at a small concentration. Further studies can be conducted to analyse which metabolite types are responsible for these antimicrobial activities and used to produce plant-based medications

CONCLUSION

Results obtained from this study helped reinforce the theory that, the metabolites obtained from endophytic fungi are capable of inhibiting or completely hindering the growth of test bacterium. *Avicennia marina* seemed to have yielded better results among the two mangrove samples with at least one endophyte discovered in all its parts (roots, stem and leaves). Broth samples were able to absorb the metabolites better and produce viable results in comparison to ethyl acetate samples. *P. viticola* seems to be highly effective in suppressing the growth and causing bactericidal effects against both test species whereas, *Aspergillus versicolor* is able to completely hinder the growth of *S. aureus*. *A. niger* from both mangrove species shows the lowest bactericidal activity against both *E. coli and S. aureus*.

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