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Antibacterial and antioxidant potential of endophytic fungi isolated from mangroves

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ABSTRACT

The exploration of endophytes as a possible alternative for plant secondary metabolites is still an emerging field. Studies focused on the pharmacological importance of endophytes have revealed them to be an abundant and dependable source of novel bioactive compounds. Medicinal plants and plants that populate distinct biotopes have been considered as promising plant sources for the isolation of endophytes with high bioactivities. The present study examined anti bacterial and antioxidant potential of endophytic fungi isolated from two mangrove plants. Of the seven endophytic fungi isolated, four were found to exhibit anti bacterial activity. Among these three isolates also showed anti oxidant activity. Highly potential fungus which showed antibacterial activities at the lowest amount tested and antioxidant activity comparable to that of ascorbic acid was characterized as Fusarium proliferatum.

Key words: Endophytes, Mangroves, Bioactive compounds, Antibacterial activity, Anti oxidant activity

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INTRODUCTION

Nature has all the time provided the world with broad and structurally diverse array of compounds that play a major role in meeting the global demand for new pharmacologically active substances. Plants in particular have historically been viewed as the main source of novel bioactive compounds. However, plant availability is a limiting factor in the commercial success of some natural products. At times, a large quantity of plant is required to produce sufficient amounts of the bioactive compounds for clinical use. This has raised concerns like environmental degradation, loss of biodiversity and threat to endangered species [1]. Following the isolation of taxol producing endophyte *Taxomyces andreanae* from the bark of the tree *Taxus brevifolia* [2], many research studies are now focusing on the role of microorganisms living inside the plants in producing bioactive compounds.

Endophyte is a kind of microbe which resides in the intracellular or intercellular area of healthy plant tissues without causing any obvious symptoms or distinct negative effects in their plant hosts [3]. Endophytes, hidden within healthy host plant are a poorly investigated group of microorganisms representing an abundant and dependable source of novel bioactive compounds with huge potential. There are many reports and studies on the biological activities of endophytes like anti microbial, anti neoplastic, antioxidant, antiviral, anti diabetic, immunosuppressive, anti thrombotic, anti-inflammatory, anti Alzheimer's activity etc [4].

The rationale for selecting promising plant sources for the isolation of endophytes gives particular interest on plants which themselves are used as medicinal plants and plants that populate distinct biotopes and have to cope with extreme living conditions; for example, inhabitants of rainforests or mangrove forests [5]. There are many reports on the use of mangroves in folklore medicine in the treatment of many ailments [6]. This is further supported by recent investigations on bioactivities of common mangroves [7-10]. Therefore with the hope that in this unique

ecosystem the chance to find endophytes with high bioactivities is most probable, the present study explored endophytic fungi in mangrove ecosystem to evaluate their pharmacological potential.

MATERIALS AND METHODS

Plant sample collection & surface sterilization

Leaves and stems of 2 mangrove species *Rhizophora mucronata* and *Excoecaria agallocha* were collected from Ayiramthengu mangrove forest in southwest coast of Kerala. Plants were taxonomically authenticated based on the records maintained at Aquaculture Development Authority of Kerala, Ayiramthengu. Plant parts were washed in running tap water and were surface sterilized by dipping in 70% ethanol for 3 min, 4% sodium hypochlorite for 3 min, followed by rinsing in sterile distilled water for 2 min. The surface sterilization procedure was validated by imprinting the surface sterilized plant part onto nutrient media and was maintained as control.

Isolation, mass culture and crude extract preparation of fungal endophytes

The surface sterilized plant parts were cut into small pieces of about 1cm and were inoculated onto potato dextrose agar and incubated for 5 -7 days at 28^0C . Fungal endophytes growing out from the cut ends were isolated and sub cultured. Pure cultures were prepared from isolates with different morphology. These isolates were mass cultured in 100 ml potato dextrose broth at 28^0C for 7-10 days in a rotary shaker. Mycelia were separated by filtering using whatman No.1 filter paper. The supernatants were then extracted thrice with equal volumes of ethyl acetate. The ethyl acetate fractions were evaporated to dryness using rotary evaporator. The dried extracts were weighed, dissolved in DMSO (100mg/ml) and were stored at -4^0C until use. The crude extracts were checked for their antibacterial and antioxidant activities.

Antibacterial activity

Antibacterial activities of the isolated endophytic fungi were checked against selected human pathogens *(Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus).* For primary screening, agar plugs were cut from the plate culture of each fungal endophytes and were placed on Muller Hinton agar plate swabbed with respective pathogens. After incubation endophytes showing zone of inhibition at least against one pathogen were selected. Crude extracts of these isolates were further checked for their antibacterial activities using well diffusion method [11]. An initial volume of 25ul crude extract was transferred to wells bored on Muller Hinton Agar plates swabbed with different pathogens. After an incubation period of 24 hrs the diameter of zone of inhibition was noted. Those crude extracts which could not produce zone of inhibition at 25 ul were tested for their activity by increasing the volume to 50ul and 100ul. DMSO was used as negative control and tetracycline was used as positive control.

Anti oxidant activity

Anti oxidant activity exhibited by the isolates was measured in terms of hydrogen-donating or radical scavenging ability, using DPPH method [12]. Ascorbic acid was used as a reference standard. The percentage inhibition of DPPH radicals by the samples was calculated according to formula

Percentage inhibition= [{Abs control - Abs sample}/ Abs control] x 100

Identification of potential isolate

Highly potential fungal endophyte was first characterized by morphological observation and spore staining [13]. For molecular identification, DNA was isolated from the culture and was used in PCR to amplify the ITS region using ITS1 and ITS4 primers [14]. The ~450 bp amplicon was gel eluted and the product was sequenced by Sanger's method of DNA sequencing [15]. The sequencing results were assembled and compared with NCBI data base.

RESULTS AND DISCUSSION

Total 7 fungal endophytes were isolated from the leaves and stems of 2 mangroves under study. Since no microbial growth occurred in the control, the surface sterilization was considered complete and isolated fungi were considered as endophytes. Isolates with different colony morphology were numbered based on the name of the plant and part from which they were isolated.

Antibacterial activity

Of the seven isolates, only four showed zone of inhibition in primary screening. Crude extracts of all these four isolates were able to show antibacterial activity against multiple pathogens in well diffusion study (Table 1). Isolate RM-S-01 was found to produce zone of inhibition at a minimum amount of 25ul, against all the tested pathogens

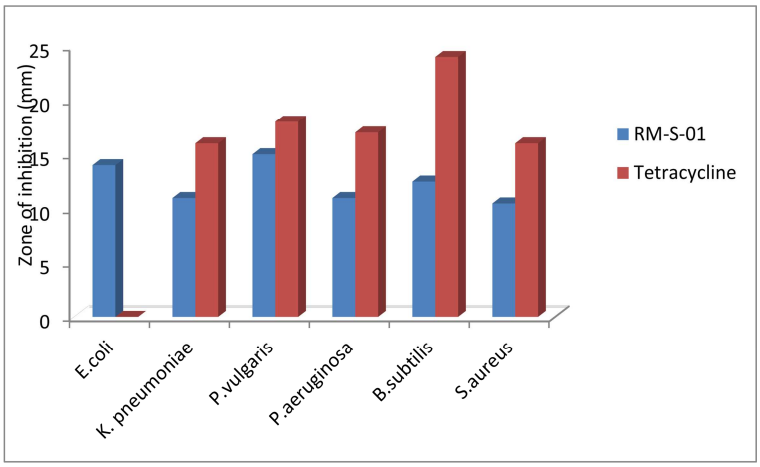
(Table 1&Figure 1). Some isolates could not produce zone of inhibition against certain pathogens even at the highest amount used in the present study.

Table 1: Zone of inhibition shown by crude extracts of endophytic fungal isolates against test pathogens

Isolate	*E.coli*	*K.pneumoniae*	*P.vulgaris*	*P.aeruginosa*	*B. subtilis*	*S. aureus*
RM.L.01	8mm**	10mm**	15mm*	0	10mm*	10mm**
RM.S.01	14mm*	11mm *	15mm *	11mm *	12.5mm*	10.5mm *
RM.S.02	0	9mm**	0	12mm**	10mm**	0
EA.L.02	20mm*	18mm***	20mm**	26mm***	20mm**	20mm**

*Volume of extract required for inhibition- *25ul, **50ul, ***100ul*

Figure 1: Comparison of zone of inhibition of crude extract of RM-S-01 and Tetracycline (30mcg) against test pathogens

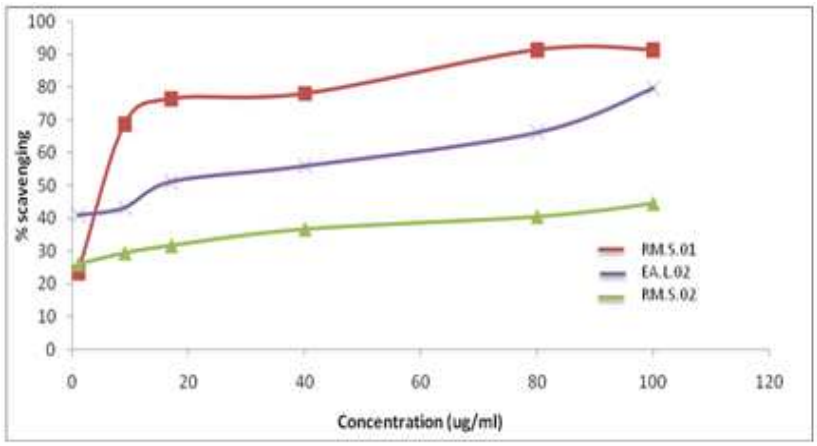


The increase in bacterial resistance towards the existing antibiotics has prompted the search of new alternatives from natural sources. In the present study crude extracts of four fungal endophytes have shown potential antimicrobial activity comparable to standard antibiotics. The crude extract of the fungus RM-S-01 isolated from the stem of *Rhizophora mucronata* was able to show strong antibacterial activity against all the six pathogens tested in the study. Similar studies conducted on different endophytic fungi from Nethravathi Mangrove forest, Vellar estuary and Sarawak Mangroves Forest Reserve [16-18] also have proven the antibacterial potential of mangrove endophytic fungi.

Antioxidant activity

Ethyl acetate extracts of only 3 fungal isolates showed scavenging activity against DPPH radicals at the concentrations tested in the present study (Figure:2).

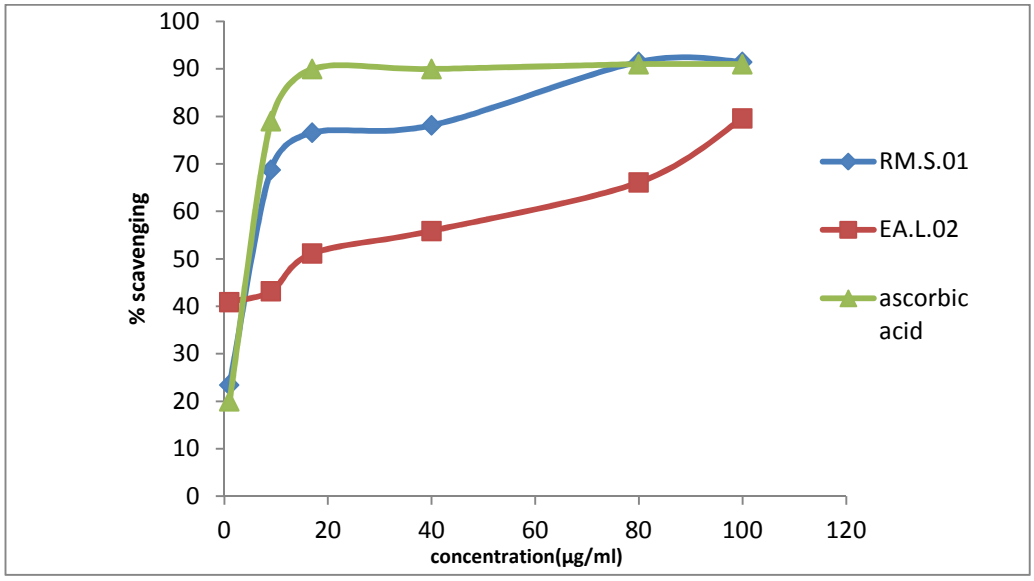
Figure 2: DPPH radical scavenging activity of crude extracts of endophytic fungi with antioxidant property



When compared with the DPPH scavenging activity of standard antioxidant Ascorbic acid , the fungal extract RM-S-01 exhibited EC50 at a concentration of 5 ug/ml comparable to EC50 of ascorbic acid (Figure: 3). Fungal extract EA-L-02 showed EC50 at a concentration of 16.5ug/ml. The fungus RM-S-02 could not give EC50 even at a

concentration of 100ug/ml. Hence endophytic fungal extracts RM-S-01 and EA-L-02 can be considered as potentially good antioxidants.

Figure: 3 Comparison of DPPH scavenging activity (EC50) of endophytic fungi showing high antioxidant activity and standard antioxidant



Antioxidants act as radical scavengers, and inhibit lipid peroxidation and other free radical mediated processes. Thus they are able to protect the human body from several diseases attributed to the reaction of radicals such as aging, cancer, neuro degenerative disorders, atherosclerosis and inflammations. Use of synthetic antioxidants has been reported to involve toxic side effects thus necessitating the search for natural antioxidants and free radical scavengers [19]. DPPH assay is well accepted in natural product antioxidant studies. As antioxidant donate proton to this radical the absorption decreases. The antioxidant effect is proportional to the DPPH free radical conversion to DPPH by anti-oxidant compound [20].

In our study crude extracts of three fungi were found to show DPPH free radical scavenging activity. The crude extract of isolate RM-S-01 exhibited EC50 value similar to that of standard antioxidant. Although the other extracts exhibited lower antioxidant activity compared to the standard anti oxidant at the concentrations tested in the study, it can be explained by the fact that crude extracts are the mixture of various compounds, some compounds can inhibit the potency of the active compound [21]. Endophytic fungi from mangroves viz, *Phomopsis amygdale*, *Trichoderma sp* and *Alternaria sp* have been reported to show high antioxidant activities against various free radicals which go in line with the result of the present study [22,23,18]. Further investigation is needed to discover the unidentified antioxidant constituents in the extracts of endophytic fungal isolates.

Identification of potential isolate

The fungal endophyte RM-S-01, whose crude extract showed antibacterial activity against all tested pathogens at the lowest amount and antioxidant activity comparable to that of ascorbic acid was selected as highly potential among the endophytes. The fungus produced white to light pinkish floccose aerial mycelium when grown on potato dextrose agar. Conidial spores were observed on spore staining [13]. Molecular analysis of the fungus showed a 98% match with *Fusarium proliferatum* (accession number HM245296.1).

There are reports of endophytic *F. proliferatum* isolated from *Dysoxylum binectariferum* and *Syzygium cordatum* [24,25] showing cytotoxic properties, which along with our antibacterial and antioxidant studies emphasize the need of further exploration of endophytes for new sources of bioactive metabolites.

CONCLUSION

Our study demonstrates mangroves as an ideal source material for isolation of endophytes with pharmacological activities. It is worth mentioning that the mangrove species which harbor the endophytes of the present study have been widely used in folklore medicine. Endophytes in these plants hold great potential in producing bioactive compounds. Further research like fractionation and purification of the active compounds in the crude extract and their structure elucidation will reveal the unknown compounds in our isolates.

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