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Antibacterial activity of crude extract from *Aspergillus niger* isolated from the stilt roots of *Rhizophora apiculata* along South Andaman coast, India

Madhusudan Thorati and JK Mishra

Abstract
Endophytic fungi are known repositories of secondary metabolites. They also serve as protectors and growth enhancers to their respective plant hosts. The present study was aimed at studying the antibacterial activity of the extract of endophytic fungus *Aspergillus niger* isolated from the stilt roots of *Rhizophora apiculata*. The ethyl acetate extract of the fungus species was assayed for bioactivity against five human pathogenic bacterial strains *Staphylococcus aureus (*MTCC96), *Micrococcus luteus (*MTCC106*), Pseudomonas aeruginosa (*MTCC326*)* and *Enterococcus faecalis (*MTCC439*), Proteus mirabilis (*MTCC1429) by disc diffusion method. Sensitivity of the strains varied from *P. mirabilis*, which was most sensitive followed by *P. aeruginosa, M. leuteus, S. aureus and E. faecalis* in order. It suggested that both gram positive and negative bacterial strains responded to the effect of crude extract exhibiting the activity zone with a range of 23mm to 15mm at 50µl and 100µl concentrations respectively. Phytochemical analysis of the crude extract revealed the presence of terpenoids, saponins and proteins, suggesting that these biomolecules have potential to serve as antibacterial compounds.

Keywords: Mangrove, Stilt roots, Endophytic fungus, Antibacterial Activity.

Introduction
Mangroves plants grow in saline coastal habitats and possess specialised roots and form a network like system in its habitat. The network in combination with the soils of the mangrove habitat in the intertidal zone forms a kind of environment that attracts rich microbial communities [1]. But mostly the endophytes reside in the internal tissues of host and in case of mangroves, they exist in roots [2]. Fungi are ubiquitous eukaryotic, heterotrophic organisms and accounts for about one million species among the higher plants [3]. In case of mangroves more than 200 species of endophytic fungi have been isolated and identified and these endophytes were proven to be a well-established source for structurally diverse and biologically active secondary metabolites [4, 5]. It is also suggested that the mangrove roots host a plethora of endophytic fungi [6]. In recent time these fungi have got the attention of scientists due to their beneficial effects as protectors and enhancing the growing capacity of the host plant [7]. As reported by Strobel [8] endophytic fungi serve as a major source of metabolites with higher biological activity. It is also well documented that the anti-cancer compound Taxol was first obtained from *Taxus brevifolia* is also being produced by endophytic fungi *Taxomyces andreanae* [9].
Another immunosuppressant drug cyclosporine isolated from the fungus *Tolypocladium inflatum* served as a candidate compound for the design of compound like Debio-025, which is a potential antiviral drug [10]. Similarly statin, one of the metabolite isolated from both the fungi *Penicillium citrinum* and *Aspergillus terrus* are used in the treatment of cardiovascular diseases [10, 11]. Also fungal metabolites like strobilurines isolated from *Strobilurus* sp. served as a lead compound for the development of synthetic fungicide such as trifloxystrobin [12]. Approximately 140 new natural products have been isolated from endophytic fungi during 1987 – 2000, and from 2000 to 2006 a similar number were also characterised [13, 14]. It was reported that Cyclopeptide 1962A was isolated from an unidentified fungus associated with the mangrove *Kandelia candel* having cytotoxic activity against human breast cancer [15].
It is well understood that the bacterial resistance is the major concern for the search of new antibiotics and it should be from novel natural resources and it is believed that search for novel compounds can be directed towards the plants growing in unique environment or endemic locations as they are presumed to harbour novel microbes that may produce unique metabolites[16]. The objective of this study was to find probable antibacterial compounds from the endophytic fungi associated with the roots of pristine mangrove ecosystem of South

Andaman Coast, Andaman and Nicobar Islands, India.

Materials and Methods

Isolation of fungal strain

Sixty stilt roots from ten individual mangrove plant of *Rhizophor aapiculata* were collected from Chidiyatapu(Lat. $11^0.30´$N Long. $92^0.41'$ E) along the coast of South Andaman, during low tide and brought to the laboratory in sterile polythene bags and processed within few hours of collection to reduce the chance of contamination. Samples were washed with sterile water in order to remove the adherent sediment particles, and roots were then rinsed with sterile sea and distilled water (1:1) for 3 minutes. Surface sterilization was done by sequential rinse of the roots in 95% ethanol (30 sec) 5% sodium hypochlorite (5 min), 95% ethanol (for 30s) and sterile sea water (Fisher *et al*., 1986). The roots were cut in to 2cm pieces each with sterile razor and placed on potato dextrose agar medium supplemented with antibiotic (Gentamicin 80 mg) aseptically. The plates were wrapped with paraffin and placed in incubator at 27 °C for 1 week. The plates were observed at regular intervals for fungal growth, and pure culture was obtained by transferring the fungal hyphal tips into sterile PDA plates aseptically.

Identification of Fungus

For the identification of the fungus the hyphal tips from pure culture were transferred to a sterile slide and stained with lactophenol cotton blue solution and observed under stereomicroscope (Olympus Bx41). The fungus was morphologically identified as *Aspergillus niger* according to the keys provide by Thom and Raper [17] and Watanabe [18].

Molecular Characterisation.

The total genomic DNA was isolated based on the method prescribed by [19]. The ITS regions of 5.8S rDNA region (ITS1 &ITS4) were amplified by employing ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'TCCTCCGCTTATTGATATGC-3') primers. The polymerase chain reaction product was sequenced in a sequencing firm and the data was submitted to gene bank for accession number.

Frequency of Occurrence

The frequency of occurrence denotes number of samples in which particular fungi was found against the total number of samples collected. Frequency of *A. niger* was calculated based on the formula drafted by Sarma and Hyde [20].

$$\text{Frequency of occurrence} = \frac{\text{Number of samples in which particular fungus recorded}}{\text{Total number of samples examined}} \times 100$$

Fungal fermentation and extraction

To extract the bioactive molecules, fungus was grown on PDA plates for 3-4 days and fresh mycelial plugs were inoculated into three 500ml Erlenmeyer flask containing 200 ml of potato dextrose broth and incubated for 3 weeks at 26±1 °c under static conditions [21]. In order to obtain mycelial free broth the culture was vacuum filtered. The filtrate was extracted with equal volumes of ethyl acetate (EtOAc) until it turned colourless and evaporated to dryness under reduced pressure at 45 °C using rotary vacuum evaporator (BuchiRotovapour II) leaving behind pale brown coloured powder. The mycelial mat was plotted in Whatmans filter paper No.1 to remove the moisture if any and crushed with the help of sterile mortar and pestle with 50ml ethyl acetate and subjected to sonication for 4-5 hours and centrifuged at 10000rpm for 30 minutes in order to obtain intracellular metabolites. The supernatant left brown coloured powder when subjected to dryness in incubator at 45 °c. Crude extracts from filtrate and mycelium were combined, weighed (125mg) and stored at -40 °C for further studies.

Column Chromatography

Silica gel (60-120) mesh size with methanol was used in packing the column acting as stationary phase. Crude extract was loaded on silica bed and methanol was pipetted into column gently avoiding the breakage of silica bed. The fraction was collected in sterile glass beaker and ran through the column several until yellow coloured fraction obtained.

Phytochemical Screening of the Extract

The phytochemical analysis of the crude extract was done for compounds such as Terpenoids, sterols, saponins, phenols and proteins [22].

Test for Terpenoids

To 5 ml of the crude extract 2ml of chloroform was added and shaken gently, 3ml of concentrated sulphuric acid was added to the above solution mixture and observed for the formation of reddish brown ring at the interface. Formation of reddish brown ring indicates the presence of terpenoids.

Test for saponins – Froth test

To 4ml of distilled water 1ml of the crude extract was added and shaken vigorously and observed for formation foam. Formation of foam indicates the presence of saponins.

Test for phenols – Ferric chloride test

To 2ml of crude extract 4-5 drops of ferric chloride solution was added, formation of bluish black colour indicates the presence of sterols.

Test for sterols – Salkowskis test

To 1.5ml of crude extract chloroform was added and mixed thoroughly, to the filtrate few drops of concentrates sulphuric acid was added. Formation of golden yellow colour indicates the presence of phytosterols.

Test for proteins - Xanthoproteic test

To 2ml of crude extract 3ml of concentrated Nitric acid was added and observed. Formation of dark yellow colour indicates the presence of proteins.

Antibacterial Assay

Pathogenic bacterial strains such as *Staphylococcus aureus (*MTCC96), *Micrococcus luteus (*MTCC106*), Pseudomonas aeruginosa (*MTCC326*), Enterococcus faecalis (*MTCC439*)* and *Proteus mirabilis (*MTCC1429*)* were subjected to antibacterial assay by using the crude extract following standard disc diffusion method [23]. Test organisms with inoculum size of 10^6 -10^7 colony forming units (cfu) were streaked on the surface of Muller-Hinton Agar plates. Two different concentrations of crude extract (50µl and 100 µl)

were pipette into sterile disc (10mm Diameter, Himedia), dried and placed on the surface of inoculated plates. Gentamycin sulphate (10mcg/dics, Himedia) was used as positive inhibitory control and ethyl acetate was used as negative inhibitory control. Plates were sealed with paraffin and incubated at 35 °C for 48 hours.

Results

Molecular identification

The sequenced nucleotide of rDNA ITS region was 596 bp, its blast in NCBI resulted the present fungal strain as *Aspergillus niger*. The gene bank accession number of the present species is KY963648. A rooted molecular phylogenetic tree was constructed by neighbour-joining analysis [24] of the aligned sequences of ITS regions (Figure.1). From the phylogenetic study, the topology of mangrove endophyte *A. niger* was identical to *A. niger* (accession numbers KM458779, KP748369, DQ206869, HQ285532, AJ853742) with 99.1 and 98.9% bootstrap values respectively.

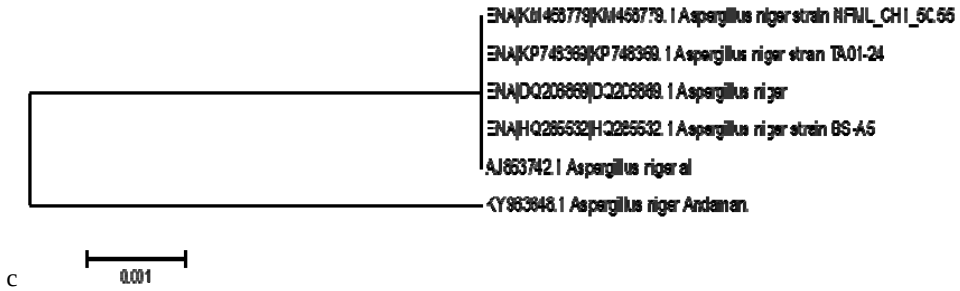

Fig 1: Neighbour-joining tree, showing the phylogenetic positions of the isolates and closest matching taxa based on ITS region of 5.8s gene sequence data. Bootstrap values (1000 replications).

Frequency of Occurrence

A total of sixty (60) stilt roots from 10 pants of *Rhizophora apiculata* were collected and processed for isolation of endophytic fungi. Out of 60 samples *A. niger* was isolated from 44 samples and remained as the prominent and frequently occurred fungus with 73.3%.

Phytochemical analysis

The phytochemical analysis of extract was carried out to test the presence of certain compounds like terpenoids, sterols, saponins, phenols and proteins, only three compounds terpenoids, sterols and proteins turned positive which depicted in Table -1

Table 1: Phytochemical Analysis of extract. Antibacterial activity

Sl. No	Test	Result
1.	Terpeniod	+ ve
2.	Saponin	-ve
3.	Phenols	-ve
4.	Phytosterols	+ ve
5.	Proteins	+ve

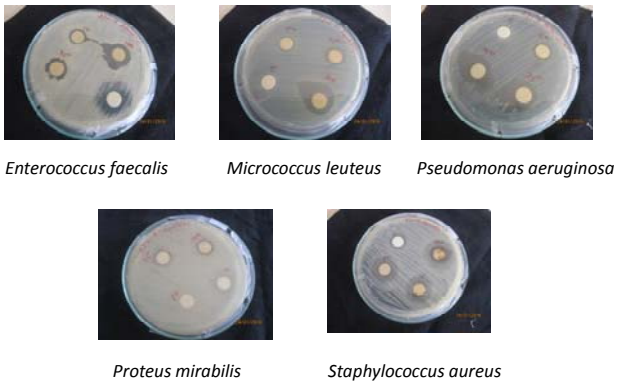

Fig 2: Inhibitory activity of crude extracts of *Aspergillus niger* against five pathogenic bacterial strains.

Antibacterial assay revealed the potentiality of endophytic fungi *A. niger* crude extract, containing significant pathogen inhibiting compounds. Inhibitory activity of crude extract was visualised as clear zones around sterile discs (Figure– 2). Both Gram positive and Gram negative bacterial strains were found sensitive to the effect of crude extract (Table -2). Significant activity was observed against *P. mirabilis* and good activity against other pathogens. *E. faecalis* was found moderately sensitive.

Table 2: Antibacterial activity of crude extract against pathogens.

Bacterial strains		Inhibition zone (in mm)	
Sl. No	Gram Positive Bacteria	Crude Extract 125mg/ml (50µl)	Crude Extract 125mg/ml (100µl)
1.	*Staphylococcus aureus*(MTCC96)	15	19
2.	*Micrococcus leuteus*(MTC106)	18	20
3.	*Enterococcus faecalis*(MTC439)	16	18
	Gram Negative Bacteria		
4.	*Pseudomonas aeruginosa* (MTC326)	16	21
5.	*Proteus mirabilis*(MTC1429)	17	23

Discussion

In the present study sixty stilt roots were collected from *Rhizophora apiculata* and a total of five different fungi species were isolated. But the fungus with significant reproductive structures was selected for antibacterial study by its crude extract. Based on the morphological and analysis of ITS regions (ITS1 & ITS4) the fungal strain was identified as *Aspergillus niger*. Significant studies on frequency of occurrence of marine fungi have been carried out by [25, 26, 27], but reports on frequency of endophytic fungi are scarce. The attempt in the present study was made to record the *A. niger* frequency which accounted73.3%. The phytochemical analysis revealed the presence of terpenoids, phytosterols and proteins in the sample.

Antibacterial activity in the present investigation revealed that crude extract has hindered both gram positive and gram negative bacteria. Maria [28] isolated four species of endophytic fungi from mangrove roots and carried out similar study on *Pseudomaonas aeruginosa* (NCIM 2063), *Staphylococcus aureus* (NCIM 2079) by using ethyl acetate as solvent for extraction of secondary metabolites that resulted lesser inhibition zone compared with present study. The ethyl acetate crude extract showed broad spectrum of antibacterial activity by exhibiting inhibitory zones in the range of 15-18mm at 50µl concentration and 18-23mm at 100µl concentration. The activity zones proportionately increased with increase in the concentration of crude extract. *P. mirabilis (*MTCC1429) was found to be the most sensitive strain with 23mm inhibitory zone at highest concentration of the crude extract, while *E. faecalis* (MTCC439) was less sensitive with activity zone of 18mm. The present study indicates the secondary metabolites of the extract can be act as source of wide spectrum antibiotics.

Conclusion

The present study of mangrove endophytic fungi *A. niger* suggests that endophytes from marine environments have potential to be a productive source for the discovery of useful drugs for better pharmaceuticals.

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References

1. Kathiresan K, Bingham BL. Biology of mangrove and mangrove ecosysytems. B Advances in Marine Biology. 2001; 40:81-251
2. Bacon CW, JF white. Microbial Endophytes. Marcel DekkerInc, New York, NY, 2000.
3. Arnold AE, EA Herre. Mycologia. 2003; 95(3):388-398.
4. Li My, Xiao Q Pan JY, Wu J. Natural Products from semi-mangrove flora: source, Chemistry and bioactivities. Natural Products Reports. 2009; 26(2):281-298.
5. Ka-Lai Pang, Lillan LP, Lillan LP, Vrijmoed, TelkKhiang Goh, Norumolplaingam *et al*. Fungal endophytes associated with *Kandelia candel* (Rhizophoraceae) in Mai Po Nature Reserve, Hong Kong. Botanica Marina. 2008; 51:171-178.
6. Ananda K, Sridhar KR. Diversity of endophytic fungi in the roots of mangroves species on the west coast of India. Canadian Journal of Microbiology. 2002; 48:871-878.
7. Dreyfuss MM, Chapela IH. (Fungi as producers of secondary metabolites. In Gullo, V. P (ed) Discovery of Natural Products with Therapeutic Potential. Butterworth-Heinemann: Boaton, 1994, 49-79.
8. Strobel GA. Rain forest endophytes and bioactive products, Crit. Rev. Biotechnology. 2002; 22:315-333.
9. Stierle A, Strobel G, Stierle D. Taxol and Taxane production by Taxmycesandreance, an endophytefunguspf pacific yew. Science. 1993; 260:214-216.
10. Marks, Butler S. The Role of Natural Products Chemistyry in Drug discovery, Journal of Natural Products. 2004; 67(12):41-2153.
11. Paul M Dewick. Medicinal Natural Products. 2nd Edition John Willey ans sons Ltd England, 2000.
12. H Balba. Review ofstrobilurin fungicide chemicals. Journal of Environmental science health. Cyclophilin Inhibitor Debio-25 shows potent Anti-Hapatitis C Effect in patients Coinfected with Hepatitis C and Human Immuno defeciency Virus, Hepatology. 2007: 42(4):441-451, 47:817-826.
13. Tan RX, WX Zou. Endophytes; a rich source of functional metabolites. Nature Products Reports. 2001; 18:448-459.
14. Zhang HW. Biology and chemistry of endophytes. Natural products Reports. 2006; 23.5:753-771.
15. Huang H, She Z, Lin Y, Vrijmoed LLP, Lin W. Cyclic peptides from an endophytic fungus obtained from a mangrove leaf (*Kandeliacandel*). Journal of Natural Products. 2007; 70:1696-1699.
16. Strobel, Bryn Daisy. Bioprospecting for Microbial Endophytes and their Natural products. Microbiology and Molecular Biology Reviews. 2003; 67:491-502.
17. Thom C, Raper KB. A Manual of Aspergilli. Part 1 Waverly press, USA, 1945.
18. Wantabe T. Pictorial Atlas of Soil and Seed Fungi Morphologies of Cultured Fungi and Key to Species. 2nd ed. CRC press USA, 2002.
19. Aljanabi SM, Martinez I Universal and rapid salt extraction of high quality genomic DNA for PCR based technique, Nucleic Acids Research. 1997; 25:4492-4493.
20. Sarma VV, KD Hyde. A review on frequently occurring fungi in mangroves. Fungal Diversity. 2001; 8:1-34.
21. Pongpaichit S, Rungjindamani N, Rukachaisirikul V, Sakayarjo J. Antimicrobial activity in cultures of endophytic fungi isolated from *Graciniaspecies*. FEMS Immunology Medical Microbiology. 2006; 48:367-372.
22. Meena J, Anchana Devi. Isolation, Characterisation and Antimicrobial Activity of Pigment Isolated from *Monascus sp*. Current Research in Microbiology and Biotechnology. 2015; 3:713-717.
23. Bauer AW, Kirby WMM, Sherris JC, Truck M. Antibiotics susceptibility test by a standardized single disc method. American Journal of Clinical Pathology. 1966; 45:493-496.
24. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution. 1980; 16:111-20.
25. Hyde KD, EGB Jones. Marine mangrove fungi. Marine Ecology. 1988; 9:15-33.
26. Jones EGB, TK Tan. Observations on manglicolous fungi from Malaysia. Transactions of British Mycological Society. 1987; 89:390-392.
27. Gayatri R, Nambiar, K Raveendran. Mangilicoulos Marine Fungi of Kerala (South India). Botanical Research Institute. 2009(c); 2(3):206-210.
28. Maria G. Sridhar KR, Raviraja NS. Antimicrobial and enzyme activity of endophytic fungi from south west coast of India. Journal of Agriculture Technology. 2005; 1:67-80.