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Isolation and antibacterial activity of endophytic fungi from *Madhuca longifolia* Bark

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Abstract

The present inspection on isolation and antibacterial activity analysis of endophytic fungi from *Madhuca longifolia bark* and to evaluate its antimicrobial activities. Endophytic fungi were isolated from the leaf segments from *Madhuca longifolia bark* using PDA medium and fruiting structures were introduced in four different culture media and identified by macroscopic and microscopic methods. The fungi were grown in PD broth for 21 days and extracted with ethyl acetate. The crude extract was collected and used for antimicrobial analysis. Twenty endophytic fungi were isolated from *Madhuca longifolia bark* and were identified by macroscopic and microscopic methods as members of *Colletotrichum* sp., *Alternaria* sps, *Pestalotiopsis* sps *Diaporthea* sp., *Phomopsis* sp., *Mycosperellacea* sp., *Fusarium* sp., *Pleosporales* sp. and *Pseudocercospora* sp. Extracts prepared from the fungal isolates were screened for the antimicrobial properties against *Bacillus subtilis, Staphylococcus aureus, Salmonella typhi* and *Vibrio cholera.* Very interestingly, three endophytic fungal isolates *Colletotrichum* sps, *Alternaria* sps, *Pestalotiopsis* sps were found to have highest activity against the pathogens screened. The study proves the promising natural product biosynthetic potential of fungi associated with *Madhuca longifolia* bark.

Keywords: Antimicrobial activity, colletotrichum, endophytic fungi, *Madhuca longifolia,* bark

Introduction

Fungi are known to have the ability to produce a wide variety of natural products, including potent toxins and life-saving drugs. The emerging and increasing threat towards health originated from various biological and chemical agents generates an increasing demand for novel natural products with superior biological activity. One of the most attractive groups of organisms for the novel natural products is fungi. They are well known for the presence of chemical scaffolds with amazing structural diversity [1]. So the identification of fungi from novel sources and characterization of their metabolites is a promising approach. Most fungi have wealth of genes coding for far more natural products than they actually produce. This is of great significance for fungi associated with plants, where some of them even to have the ability to produce the same compound produced by the host plant [2]. Considering these amazing features, studies on endophytic fungi from medicinal plants is very important.

Fungal endophytes are recognized as important depository of novel secondary metabolites, some of which have beneficial biological activities [3]. Bioactive compounds produced by endophytic fungi broadly include alkaloids, steroids, terpenoids, isocoumarins, quinones, flavonoids, phenylpropanoids, lignans, peptides, phenolics, aliphatics, and volatile organic compounds [4]. But most interestingly there are several examples for endophytic fungi producing plant specific compounds. The endophytic fungus *Taxomyces andreanae* associated with *Taxus brevifolia* was shown to have the ability to form the anticancer drug taxol similar to host plant [5]. The anti-leukemia agent vincristine was reported to be synthesized by an endophytic fungus, *Mycelia sterilia* from leaves of *Catharanthus roseus* [6].

Endophytic fungal metabolites have shown to have specific properties also. The Anti trypanosomal activity of *Diaporthe phaseolorum* recovered from *Viguiera arenaria* [7] and antimalarial compound Pulluarin produced by *Aureobasidium pullulans* from leaf of *Caulophyllum* sp. [8] are examples for this. *Pestalotiopsis microspora*, an endophytic fungi associated with endangered tree *Torreya taxifolia* is known to have the ability to produce cytotoxic torreyanic acid [9]. Peptide antifungal-anticancer leucinostatins are shown to be produced by endophytic *Acremonium* sp. isolated from *Taxus baccata* [10]. Asperfumin, a bioactive metabolite produced by endophytic fungi *Aspergillus fumigatus*, has shown to inhibit *Candida albicans* [11].

As the broad applications of endophytic fungi are just begun to explore, the studies on tremendous bioactive metabolites expected from them will be very important. Enormous potential of fungal metabolites and increased demand for novel bioactive compounds signifies the exploration of endophytic fungi from *Madhuca longifolia*.

Madhuca longifolia, which belongs to the family *Sapotaceae* is known to have traditional use for the treatment of leprosy, diabetic and skin diseases [12]. In the current study, endophytic fungi were isolated and identified from *Madhuca longifolia* bark and the isolates were screened for their antimicrobial properties.

Materials and Methods

Isolation of endophytic fungi

Healthy and mature *Madhuca longifolia* bark collected from local farms were used as source material for the isolation of fungi. Surface sterilization procedure for the isolation of endophytic fungi was carried out as described by Aravind *et al* [13] with minor modifications. Plant samples were washed under running tap water for 10 minutes followed by immersion in 70% EtOH for 1 minute and in NaOCl (2.5% available chlorine) for 10 minutes. This was then drained and immersed in 70% EtOH again for 30 sec. Finally, the samples were rinsed with sterile distilled water several times and the final wash was plated on to media as control. Plant samples were then cut aseptically into 1 cm long segments. The cut surface of the segments were grown on petridishes containing Potato Dextrose Agar media amended with pencillum antibiotics. The control and inoculated plates were incubated at 28 °C for 5 days and observed for the fungal growth. The fungal isolates obtained were further purified on PDA medium. The isolates were initially subjected to staining and microscopic observation and were further identified by molecular methods.

Identification of endophytic fungi

The fungi were identified on the basis of morphological characteristics according to Domsch [14] and Aggarwal and Hasija [15].

Antibacterial Activity

Activity of the crude extracts of endophytic fungi prepared as explained above was tested against *Bacillus subtilis, Staphylococcus aureus, Salmonella typhi,* and *Vibrio cholerae*. Antimicrobial activity was determined by using well diffusion method. The turbidity of the broth cultures of test organisms adjusted to 0.5 McFerland standard were inoculated on to Muller Hinton Agar plates using sterile cotton swab. About 6 mm size wells were made and 45µl of crude extract was added into it and kept for incubation at 37 °C for 24 hours. Extract taken from uninoculated potato dextrose broth was used as was used as control. Antimicrobial activity was analysed based on the zone of inhibition formed [16].

Results

Isolation of endophytic fungi

After several rounds of standardization of surface sterilization procedure, the isolation resulted in the purification of 18 endophytic fungi *Colletotrichum* sps, *Alternaria* sps, *Pestalotiopsis* sps from *Madhuca longifolia* (Table 1). The absence of growth in the control plate ensured the proper surface sterilization of the used plant tissue and confirmed the isolated microbes as endophytes. The isolates were initially distinguished by the difference in colony characters and further by morphological features using staining techniques. The isolates with distinct characters were selected, purified and sub-cultured for maintenance as pure culture on PDA slants for further studies.

Table 1: Endophytic fungi isolated from Bark of *Madhuca longifolia*

S. No	Plant part	Endophytic fungi
1.	Bark	*Alternaria* sps
2.	Bark	*Colletotrichum* sps
3.	Bark	*Diaporthea* sps
4.	Bark	*Phomopsis* sps
5.	Bark	*Mycosperellacea* sp
6.	Bark	*Fusarium* sps
7.	Bark	*Pestolopsis* sps

Morphological Identification of Endophytic fungi

The colonies appearing on petriplates were sub-cultured into the tube containing potato dextrose agar medium for identification. Fungi were again cultured from slant to petriplates containing potato dextrose agar medium without antibiotic (Tetracycline) for 7 days. Morphological identification was done according to the standard taxonomic key included colony diameter, texture, color and the dimensions and morphology of hyphae and conidia.

Screening for Antibacterial activity

Screening of the antibacterial activity of the isolates was conducted using the agar well diffusion method against both gram positive and gram negative bacteria. The crude extracts of seven *Phomopsis* sps, *Colletotrichum* sps, *Alternaria* sps, *Pestalotiopsis* sps, *Mycosperellacea* sps, *Diaporthea* sps, *Pseudocercospora* sps, *Fusarium* sps and *Pleosporales* sps endophytic fungal isolates showed different level of antibacterial activity against the test organisms like *Bacillus subtilis, Staphylococcus aureus, Salmonella typhi* and *Vibrio cholerae* and is summerised as Table 2. Most of the fungal extracts were active against *Staphylococcus aureus* and *Bacillus subtilis,* however among the isolated strains *Alternaria* sps, showed highest zone of inhibition against *Bacillus subtilis* and *Staphylococcus aureus* where the inhibition was in the range 12 mm. The crude extract of *Pestalotiopsis* sps was active against *Staphylococcus aureus, Vibrio cholera* and *Salmonella typhi* with 18 mm of zone of inhibition. At the same time, *Colletotrichum* sps. showed activity against *Staphylococcus aureus, Vibrio cholera* and *Bacillus subtilis,* with zone of inhibition about 15 mm (Table 2.b).

Table 2: Antibacterial activity of endophytic fungal isolates against pathogenic bacteria strain

S. No	Name of endophytic fungi	Zone of Inhibition (mm)			
		Bacillus subtilis	***Staphylococcus aureus***	***Salmonella typhi***	***Vibrio cholera***
1	*Alternaria* sps	10	5.0	-	10.0
2	*Colletotrichum* sps	-	7	11	-
3	*Pestalotiopsis* sps	5	4	-	6

Discussion

Endophytic fungi are one of the most unexplored groups of organisms in terms of its biosynthetic potential [17]. As endophytic microorganisms have the potential even to produce compounds that are same or similar to that of their host plants, their studies from medicinal plants is very important. Plants of *Taxus* sp. was the only source of taxol production before the demonstration of *Taxomyces andreanae* for taxol production (18). An another example for taxol production is by endophytic fungi *Pestalotiopsis microspora* associated with *Taxus wallacbiana*, [19]. Now many endophytic fungi are known to have the potential to produce taxol which itself is representation of enormous biosynthetic potential of endophytic fungi. This also confirms endophytic fungi from medicinal plants as untapped source for drug discovery. Since *Madhuca longifolia* bark is having various medicinal properties, endophytic fungi associated with them can have much application.

Even though much more species of endophytic fungi can be expected from the plant, the conditions and media used in the current study might have favoured the growth of the species obtained. Among these isolates, *Colletotrichum* sp. has been reported as common endophytes of *Taxus mairei* and other plants [19]. Identification of species of *Colletotrichum sp.* and *Phomopsis* sp. as endophyte of *Piper hispidum* which belongs to the same family of the plant selected for the study is supportive to the results obtained in the study. Very interestingly, *Colletotrichum gloeosporoides* associated endophytically with *Justicisa gendarussa* was shown to have the ability to produce not only taxol but also industrially important enzymes like α amylase and glucoamylase [20, 21].

Fungi of the genus *Pestalotiopsis*, occurring on a wide range of substrata, are broadly distributed in the world [22]. Endophytic species of *Pestalotiopsis*, commonly isolated from tropical plants, are considered as main members of the *Pestalotiopsis* community in nature, which have been commonly isolated particularly from tropical higher plants [23]. Molecular studies have shown a conspicuous monophyletic character that *Pestalotiopsis* possess relatively fusiform conidia formed within compact acervuli and the conidia are usually 5-celled with 3 coloured median cells and colourless end cells, and with two to more apical appendages arising from the apical cell [24]. Many important secondary metabolites that are potential leads for treatment of human diseases and control of plant diseases, such as acetogenins, antioxidants, immunosuppressants, and anticancer agents, *etc*., have been identified from this genus [25]. Thus, the focus of this part of introduction is to summarize the known secondary metabolites from fungi of *Pestalotiopsis* species and their bioactivities. These can be grouped into eleven types, including alkaloids, chromenones, chromones, coumarins, lactones, peptides, phenol, phenolic acids, polyketides, quinones, and terpenoids, *etc*

Alternaria is common saprobe found on many plants and other substrata worldwide, including pine needles [26]. Julia Kjer *et al.* [27] isolated two new 10-oxo-10*H*-phenaleno [1,2,3-dechromene]-2-carboxylic acids, xanalteric acids I and II, and 11 known secondary metabolites were obtained from extracts of the endophytic fungus *Alternaria* sp., isolated from the mangrove plant *Sonneratia alba* collected in China. The two new compounds xanalteric acids I an II exhibited weak antibiotic activity against multidrug-resistant *Staphylococcus aureus*. Altenusin had displayed broad antimicrobial activity against several additional multidrug-resistant bacterial and fungal strains. Musetti *et al* [28] reported that three dipeptides, belonging to the family of diketopiperazines (DKPs) were extracted from broth culture of the grapevine endophyte *Alternaria* species and were tested against *Plasmopara viticola* on leaves of grapevine plants grown in greenhouse. Betania Barros Cota *et al.* [29] reported that Altenusin, a biphenyl isolated from the endophytic fungus *Alternaria* sp., inhibited trypanothione reductase from Trypanosoma cruzi. Bioactive natural products of different classes, such as alkaloids, steroids, terpenoids, isocoumarins, uinines, phenylpropanoids, lignans and phenolic acids, have already been isolated from endophytic fungi [30]. Moreover, altersetin purified from an endophytic *Alternaria* sp. displayed potent activity against pathogenic Gram-positive bacteria [31].

In the present study, the crude extracts of endophytic fungi were found to have activity against clinical pathogens *Bacillus subtilis, Staphylococcus aureus, Salmonella typhi* and *Vibrio cholerae*. Among this, most of the fungi were active against *Staphylococcus aureus.* Previous reports of Shu *et al* [32] and Chaves *et al* [33] show the ability of endophytic fungi to produce metabolites with antimicrobial activity. Endophytic fungi from *Terminalia brownie* showed significant activity against *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeroginosa, E.coli and Candida albicans* [34]. These reports and our results strongly support the view that the endophytic fungi isolated from medicinal plants are promising sources of antimicrobial agents [35, 36]. The results of the bioactivity suggest the presence of diverse metabolites in the fungal isolates obtained. These microbes with their metabolite richness and diversity clearly indicate promising applications of endophytic fungus obtained in the study. Even these fungi can be a novel source for the production of compounds which can have a diverse implication. The present investigation is a report on occurrence of endophytic fungi with antibacterial metabolites in bark of *Madhuca longifolia.* Currently we are working on the characterization of the biologically active metabolites from the isolated endophytic fungi.

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