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Screening some medicinal plants for endophytic fungi and their antibacterial activity against some human pathogens

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Abstract
The aim of the present study was to screen for antimicrobial activity in endophytic fungi isolated from surface sterilized leaves of some medicinal plants *viz., Anisomeles malabarica, Cardiospermum halicacabum, Aristolochia indica, Acacia mangium.* Totally 14 isolates were isolated from these plants. Preliminary analysis of fermentation broth of these isolates was tested for antimicrobial activity by agar diffusion method. Seven isolates displayed antimicrobial activity against at least one pathogenic bacterium. From these, one isolate from each plant was selected based on their morphotypes for further analysis. The isolates were identified using morphological/moleulcar methods (ITS) as *Alternaria alternata*, *Alternaria* sp., *Rhodotorula mucilaginosa* and *Phyllosticta citricarpa.* The crude ethyl acetate extract of *A. alternata* showed strongest antibacterial activity against the tested bacterial pathogens. These results indicate that *A. alternata* as a potential source of antimicrobial agent.

Keywords: endophytic fungi, its analysis, antimicrobial activity, medicinal plants

Introduction

In recent years, drug resistance in bacteria is increasing which cause severe health problem throughout the world. Hence, effective antimicrobial agents are in need. Endophytes are microorganisms that live within the healthy plant tissues and do not show any apparent adverse effect on the host [1] They are good sources of novel bioactive products, while they invade millions of unique biological niches that expand in a variety of unusual circumstances.[2] Natural bioactive products produced by endophytic fungi have unique structures [3] However, Endophytic fungi are poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in pharmaceutical industry [4, 5]. A comprehensive study has indicated that 51% of biologically active substances isolated from endophytic fungi were previously unknown [6, 7, 8] Endophytic fungi with antimicrobial activity of natural products can be used for industrial fermentation to produce natural active compounds for mass production at low cost, without pollution [9] In this study we have selected few medicinal plants *viz, Anisomeles malabarica* (Lamiaceae), *Cardiospermum halicacabum* (Sapindaceae), *Aristolochia indica* (Aristolochiaceae), *Acacia mangium* (Fabaceae) which are used in traditional, ayurvedic, siddha medicine for various treatment including rheumatism, cancer, wound healing, pneumonia etc. Some of these effects might be due to the presence of endophytic fungi harbored in these plants. Therefore, this study aimed to determine the endophytic fungal community present in the medicinal plants and their potential antimicrobial activities against some human pathogens. The potentially useful endophytic fungi were then identified based on the ITS sequence analysis.

2. Materials and Methods

2.1 Sources of Endophytic fungi

The leaf samples were collected from apparently healthy plants *viz., Aristolochia indica, Acacia mangium, Anisomeles malabarica, Cardiospermum halicacabum* from Dindigul district. All the samples were kept in sterile plastic bags and brought back to the laboratory for fungal isolation on the same day.

2.2 Isolation of endophytic fungi

The leaf samples were washed in mild detergent and thoroughly washed in running tap water for 10 - 15 min and air dried. The leaves were cut into small segments (2-3 cm) using sterile surgical blades. The segments were further surface sterilized by immersing in 0.5% (v/v) sodium hypochlorite solution for 3-5 minutes, followed by 70% (v/v) ethanol for 1 min. as reported previously. [10] Then they were rinsed in sterile distilled water thrice and allowed to surface dry using sterile blotting paper. The surface sterilized leaf segments were placed upside-down position in petriplate containing potato dextrose agar (PDA) supplemented with chloramphenicol (150mg/l). The plates were incubated at 25°C until the outgrowth of endophytic fungus was discovered. The hyphal tips that emerge from the cut end of leaf segments were transferred to PDA plates without antibiotics. Each fungal isolate was checked for purity and transferred to fresh medium. Stock cultures were stored at 4°C for further study.

2.3 Extraction of bioactive molecules

The endophytic fungal isolates were inoculated into 100 ml of Czapek's dox (CDB) broth and incubated at 25 ± 2°C with 150 rpm for 21 days. The fermentation broths were

separated through Whatman No.1 filter paper and the liquid filtrate was extracted with the same volume of ethyl acetate [11] The ethyl acetate fractions were evaporated to dryness *in vacuo* by a rotary evaporator and were used for antimicrobial assay.

2.4 Antimicrobial assay

The crude ethyl acetate extracts of the endophytic fungi were tested for their antibacterial activity using agar diffusion method against human pathogenic bacteria *Escherichia coli, Staphylococcus aureus, Salmonella typhi, Bacillus subtilis and Klebsiella pneumonia*. The bacteria were grown separately in nutrient broth medium overnight at 37ºC and 100µl of each bacterial culture was spread over the petriplate containing nutrient agar media. The crude extracts (50 µl) were added to sterile discs (6 mm diameter), placed over the agar plates. Streptomycin served as positive control and for negative control ethyl acetate was used. The plates were incubated at 37°C for 18 h, the inhibition zones (mm) were observed, measured. Experiments were repeated thrice with two replicates. Minimum inhibitory concentrations of crude ethyl acetate extract were determined by agar microdilution method. After incubation at appropriate conditions, the lowest concentration of extract that inhibited visible growth was recorded as minimum inhibitory concentration.

2.5 Identification of endophytic fungi

The isolated endophytic fungi were established by morphological and molecular approaches. The morphological identification was done based on the conidia, conidiophores, and mycelia using the microscope. The molecular identification was carried by internal transcribed spacer (ITS) amplification. The total genomic DNA of endophytic fungi was extracted following the method of Miller *et al*.[12] Fungal ITS rDNA regions were amplified by the universal ITS primers ITS1 (5′-TCCGTAGGT-GAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATT GATATGC-3') with the following parameters: 1 min initial denaturation at 94°C followed by 30 cycles of 30-s denaturation at 94°C, 1-min primer annealing at 57°C, 90-s extension at 72°C, and a final extension period of 10 min at 72°C.[13] The amplified products were sequenced at Eurofins genomics, Bangalore. The sequence analysis and comparison were performed with the nucleotide Basic Local Alignment Search Tool (BLAST) search for closest matched sequences in the GenBank (http://www.ncbi.nlm.nih.gov). The sequence was deposited in the GenBank and obtained the Accession Number. Multiple sequence alignment was performed using Clustal X and a phylogenetic tree was constructed by the Neighbor-joining method [14] using MEGA X Software [15].

2.6 Statistical analysis

All the data were subjected to one-way analysis of variance to determine the significance of individual differences in $p < 0.01$ and 0.05 levels. All statistical analysis was conducted using SPSS 16 software support.

3. Results and discussion

3.1 Isolation and identification of Endophytic fungi

A total of 14 endophytic fungi from A. *indica* (A1-4), *A. Mangium* (A5-6), *A. malabarica* (A7-11), *C. helicacabum* (C1-3) were isolated (Table 1). The preliminary analysis of the fermentation broth of these organisms showed antibacterial activity against at least one test bacterium (Table 1). From these, four isolates (one from each plant) were selected for antimicrobial assay based on their antibacterial activity and morphotypes. The isolated fungi was morphologically identified as *Alternaria alternata, Alternaria* sp. by Dr. Prameela Devi, IARI, new Delhi, India and assigned to the ITCC number (Table 2). Some of the endophytic fungi did not produce conidia or spores, and therefore only molecular method was used for identification and identified as *Rhodotorula mucilaginosa* and *Phyllosticta citricarpa.* The rDNA sequence analysis was used to re-confirm the fungi and the sequence was deposited in the GenBank and received Accession Numbers (Table 2).

3.2. Sequence analysis and phylogenetic tree construction

rDNA sequences of the endophytic fungi were compared with data available at NCBI. Clustal X software was used to perform Multiple Sequence Alignment which shows 100% sequence similarity towards selected organisms. The phylogenetic tree was constructed for these isolates based on genetic distance with the Neighbor-Joining method (Fig. 1). The bootstrap test (1000 replicates) shows the percentage of replicate trees linked to the taxa next to the branches. Ten nucleotide sequences were involved in the data analysis and the evolution profile was performed in MEGA X.

3.3 Antimicrobial assay

The increasing resistance of the microorganisms towards antibiotics has led to the serious health problem. This encourages researchers to study the new agents which can effectively inhibit the microbial growth [16] Endophytic fungi harboring medicinal plants are source of novel biologically active compounds. The natural products from endophytes are untapped source till now [17] *A. indica* and *A. mangium, A. malabarica, C. halicacabum* are well known medicinal plants whose leaves have been proven to have anti-inflammatory, antitumor, antimicrobial properties. Hence, these plants were used in the present study of a search for endophytic fungi able to produce antimicrobial substances. The result showed that the crude ethyl acetate extract obtained from the endophytic fungi inhibited both gram-positive and gram-negative bacteria. The highest antibacterial activity against selected bacteria was observed by *A. alternata* > *P. citricarpa* > *R. mucilaginosa* > *Alternaria* sp. (Table 3, Fig. 2). The inhibition by the extract of *A. alternata* was best against *E.coli*, followed by *S. typhi, S. aureus, B. subtilis* and *K. pneumonia* (Fig. 2). However, in all the cases the inhibition was higher than the positive control. The MIC of *A. alternata, A. mangium, R. mucilaginosa, P. citri* was found to be 150±25 µg, 275±10 µg, 225±25 µg, 350±12 µg/ml respectively.

The broad antibacterial activity of the bioactive metabolites indicates that the endophytic fungi *A. alternata* could be used as a potential agent to isolate novel antibacterial drug. This study is the first report about the antibacterial activity of endophytic fungi *A. alternata* residing inside the leaves of *A. indica*, which demonstrated the production of bioactive compounds with pharmaceutical potential. This

isolate could be a good candidate for further studies of their antibacterial activity.

Many novel bioactive compounds from endophytes have acknowledged as promising sources of antimicrobial products [18, 19, 20, 21]. Further investigation is on the way to identify the bioactive compounds.

In conclusion, endophytic fungi are promising sources of bioactive compounds with the potential application in a wide variety of therapeutic, agriculture and industries. This study shows the identification of endophytic fungi and their antibacterial activity. Among the isolates, *A. alternata* isolated from *A. indica* displayed strongest antibacterial activity against the tested bacterial pathogens. The maximum inhibition was observed for *E. coli* by the extract of *A. alternata*. The bioactive compound produced by this fungi need to be further characterized. Therefore, *A. alternata* can be used as a potential endophyte to explore bioactive compounds to use as novel antimicrobial drug.

Table 1: Preliminary Screening of fermentaticn broth for in vitro antibacterial activity of isolated endophytic fungi against pathogenic bacteria by agar plug diffusion assay.

Isolate	Ec	Test Org. KP	St	Sa	Bc
Al	+		-		
A2	+		-	+	
A3	+	+	+	+	+
A4	+	+	+		-
A5					+
A6	+	+	+		+
A7	+	-	-	+	
A8	+		+		
A9	+	+	+	+	+
A10	+		-		
All	-	+	-		
C1	-	+	-		
C2	-		+	+	-
C3	+	+	+	+	+

Isolates Al-4: isolated from Aristolochia indica, A5-6: from Acacia mangium A7-9: from Anisomeles malabarica, Cl-3: from Cardiospermum halicacabum Ec-E. coli, KP- Klebs fella pneumoniae, St-Salmonella typhi, Sa-Staphyllococcus aereus, Bc- Bacillus cereus

Table 2: Identification of endophytic fungi isclated from medicinal plants

Plant Source	Endophytic Fungi	Genbank Accession No.	ITCC No.	% Similarity
Aristolochia indica	Alternaria alternate	MK185010	9912.15	100
Acacia mangium	Alternaria sp.	MK185013	9835.15	100
Anisomeles malabarica	Rhodotorula mucilaginosa	MK185011	-	100
Cardiaspermum halicacabum	Phyllasticta citricarpa	MK185012	-	100

Table 3: Antibacterial activity of ethyl acetate extract of isolated endophytic fungi

Endophytic Fungi	Zone of Inhibition (mm)				
	Ec	KP	St	Sa	Bc
Alternaria alternata alternata	35±1	30±1	33±3	32±1	31±1
Alternaria sp.	22±2	25±2	20±2	25±2	20±2
Rhodotorula mucilaginosa	29±3	30±1	37±1	35±1	31±4
Phyllosticta citricarpa	32±1	28±4	29±1	30±4	30±3

Ec-E. Coli, KP-Klebsiella pneumoniae, St-Salmonella typhi, Sa-Staphyllococcus aereus, Bc- Bacillus cereus

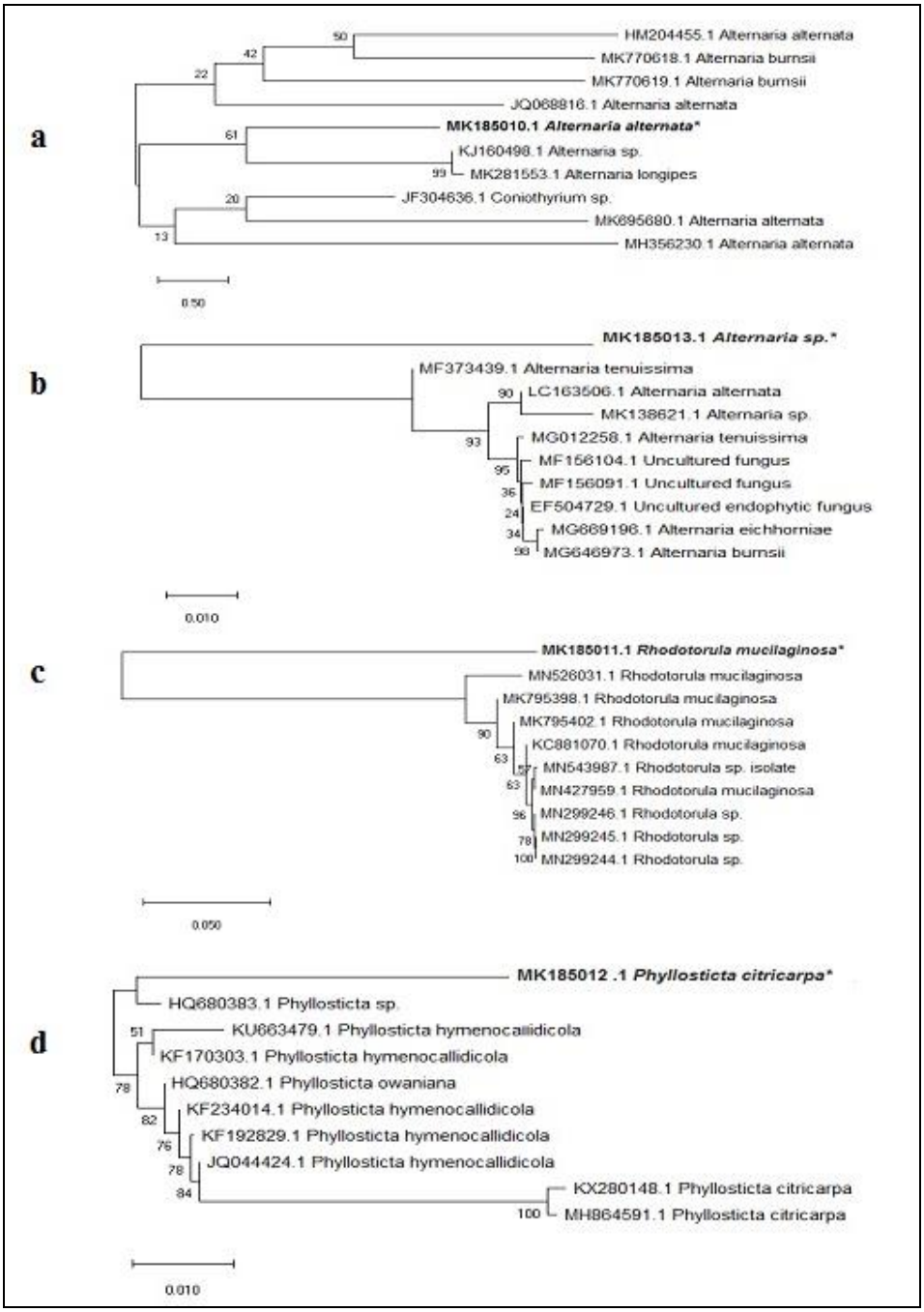

Fig 1: Phylogenetic tree (Neibour-joining) of the ITS sequence a) *A. alternata** b) *Alternaria sp** c) *R. Mucilaginosa** d) *P. Citricarpa**

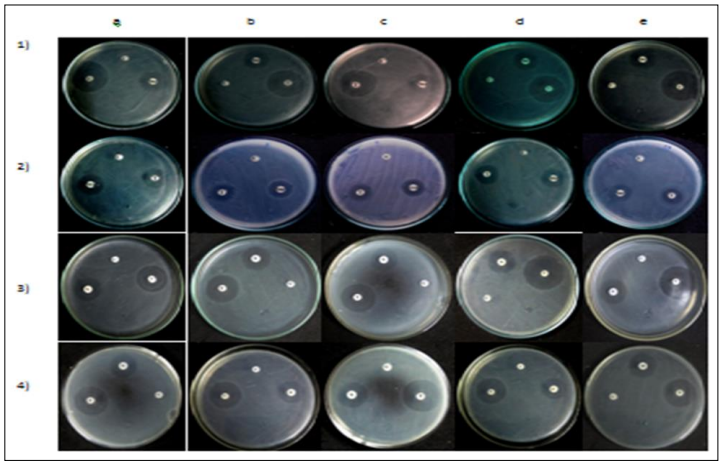

Fig 2: Antibacterial activity of ethyl acetate extract of: 1) *A. alternata 2*) *Alternaria sp* 3) *R. mucilaginosa 4*) *P. Citricarpa.* Test organisms: a) *E.coli* b) *K.pneumonia* c) *B.subtilis* d) *S.typhi* e) *S.aureus.* C- Negative control, S-Standard, T- Test sample.

Authors' Contribution

R. Anitha performed the experiment carried formal analysis and prepared the rough draft of the manuscript. M. Sathiyabama conceptualized, designed the experiment, carried formal analysis, interpretation and revised the manuscript.

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Conflict Of Interest

The Authors declare that there is no conflict of interest.

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