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BIOASSAY GUIDED CHARACTERISATION OF ENDOMYCOPHYTES FROM *BARRINGTONIA ACUTANGULA* L. LEAVES

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ABSTRACT

Medicinal plants have been considered as a host of fungal endophytes with novel metabolites of pharmaceutical importance. Most endophytic fungi that live in higher plants are capable of producing secondary metabolites which are medicinally significant components whose activity against pathogens have been studied previously. Hence an effort was taken to isolate endophytic fungi from leaves of *Barringtonia acutangula* commonly called the Indian oak or Samudraphal, an evergreen tree with medicinal properties. Two endophytic fungi were successfully isolated and grown in culture plates followed by extraction of secondary metabolites. The antibacterial and antifungal activities of the secondary metabolites were checked. Molecular characterization was performed by PCR and sequencing of the ITS region, followed by BLAST and Phylogenetic analysis.The antimicrobial tests showed significant activities against bacteria like *Bacillus subtillis*, Methicillin resistant *Staphylococcus aureus* (MRSA) and fungi such as *Microsporumgypseum* and *Trichophytonrubrum*. The endophytic fungi were identified as *Colletotrichumgloeosporioides* (Accession no: JQ844304) and *Phomopsis* species (Accession no: KC168054). This research was aimed to identify bioactive Endophytic fungi that can serve as alternate sources of alkaloids that are usually produced by their hosts. Identification of such fungi would play a vital role in Pharmacology and Toxicology studies.

INTRODUCTION

A significant proportion of the population in many countries depend on traditional medicines for their health care needs (Lesney *et al.*, 2004; Okigbo and Mmeka 2006). *Barringtonia acutangula* commonly called the Indian oak or Samudraphall, is an evergreen tree with simple alternate phyllotaxy. It is known to possess medicinal properties and the leaf is believed to be used in ancient medicine as a cure for many ailments such as fever, diarrhoea and poisoning. This allows the plant to serve as a suitable subject for research to identify novel principles to treat diseases caused by common pathogens. Endophytes are microorganisms that live asymptomatically within plant tissues. They are presumably ubiquitous in the plant kingdom (Gao *et al.,* 2005*)*. Endophytic fungi such as *Acremonium sp.* are capable of producing various secondary metabolites (Jeamjitt *et al.*, 2006) and some fungi are even reported to exhibit antimicrobial activity (Gao *et al*., 2005). Previous research showed the antimicrobial activity of endophyticmycoflora isolated from *Mirabilis jalapa* (Devaraju *et al*., 2011) and fungal isolates from *R. pulcher* leaves showing activity against *Aspergillus niger*, *Penicillium sp*, *Alternaria* sp and *S. aureus* (Ebrahimi *et al*., 2010). Sometimes the secondary metabolites isolated from Endomycophytes are the same as those produced by their host plants hence making them alternative sources for production of important plant alkaloids (Devaraju *et al*., 2011).In addition, the identification and characterization of endophytic fungi hold the key to the possibilities of research on bio interaction between the host and fungi. Isolation of endophytic fungi from medicinal plants like *B. acutangula* can be very useful for future symbiotic study and pharmacology. Previous studies on *B.acutangula* showed that the ethanolic extracts of the leaf has anthelminthic activity (Babre *et al*., 2010; Padmavathi *et al.*, 2011) and its leaf extracts have also reportedly shown potent anti-cancer activity (Florida *et al*., 2012). Endophytic fungi have been isolated from the barks of *B.acutangula* (Nagaraja *et al*., 2010). Owing to the lack of extended research on the endomycophytes from leaf on *B.acutangula*, an attempt was made to isolate the various endophytic fungi and characterize them based on their medicinal properties. DNA bar coding has been described to act as a tool for identification. Firstly, molecular

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characterization has been used to classify pharmacologically significant organisms and study their bio-interactions (Jinbo *et al*., 2011). The Internal Transcribed Spacer (ITS) region was chosen due to its high variability (Korabecna, 2007) and the acceptance of ITS as the universal barcode marker for fungi (Schoch *et al*., 2012). Secondly, considering the relationship between *B.acutangula* and its endomycophytes, the question of whether these substances are produced by the plant itself or as a consequence of a mutualistic relationship still remain unanswered (Ebrahimi *et al.,* 2010). Therefore this work focuses on both the elucidation of medicinal properties exhibited by the metabolites of the endophytic fungi and its molecular characterization.

MATERIALS AND METHODS

Chemicals required: Laboline and Mueller Hinton Agar (MHA) was purchased from Hi Media, India and Ethyl Acetate, Chloroform, Dimethyl Sulphoxide (DMSO) were purchased from Merck (Germany).

Plant collection: The leaves of *Barringtonia acutangula* L. (Family: Lecythidaceae) were collected from Kovilanchery, Chennai in the summer (2012). The herbarium specimen (Voucher number BRT-P/004) was identified by Prof. Narasimhan of Botany Department, Madras Christian College, Chennai. Fresh leaves were separated from other parts, washed and cleaned for further use.

Microbial Cultures: *Bacillus subtillis* (MTCC 121), Methicillin resistant *Staphylococcus aureus* (MRSA) (MTCC 84), *Pseudomonas aeruginosa* (MTCC 424), *Microsporumgypseum* (MTCC 2819) and *Trichophytonrubrum* (MTCC 296) were obtained from Institute of Microbial Technology, Chandigarh.

Isolation of endophytic fungus: Endophytic fungi were isolated from the leaves of *B.acutangula*as previously described (Okuda *et al.*, 2005). The leaves were removed from the plant and the surface tissue was sterilized by washing it with Laboline for 15 minutes. This was followed by rinsing the leaves in sterile distilled water for sterility check. 0.1ml of the last wash of sterile water was used for spread plating in SDA-Sabouraud Dextrose Agar (Starch 5g, Peptone 5g, Dextrose 40g, Agar 2%, pH 5.5). The tissues were aseptically cut into small pieces, placed on to the prepared SDA plates and incubated at 26^{o}C for 7 days to allow for hyphal growth from within the plant tissue. The hyphal growth was transferred to fresh SDA plates and incubated at 30^{o}C for 7 days to allow growth of the fungus.

Extraction of Secondary Metabolites: An agar block containing fungi from each sub cultured plate was taken, inoculated into minimal media (Lactose 0.2g, $MgSO_4$ 0.2g, K_2HPO_4 0.5g, Glycine 0.09g, Tryptophan 0.4g, Distilled water 100ml)and incubated at 37^{o}C in shaker for 15 days. After incubation, the media were transferred to centrifuge tubes and centrifuged at 5000rpm for 10minutesat 17ºC. The supernatant containing the secondary metabolites was taken in separating funnel, Ethyl acetate and Chloroform was added in the ratio 1:1 to the separation. The mixture was shaken vigorously and left overnight. The organic phase was collected and the residual secondary metabolite after solvent evaporation using Rotary evaporator, was stored in glass vials.

Antibacterial assay: As described by Bauer *et al.,* 1966*,* Mueller Hinton Agar (MHA) was prepared according to the manufacturer's instructions (Hi Media, India). Molten media was dispensed into petri plates to a uniform thickness of 4mm, under strict aseptic conditions in laminar air flow chamber and the medium was allowed to solidify. For detection of anti-microbial activity 3 different bacterial cultures: *Bacillus subtilis*, *Pseudomonas aeruginosa* and Methicillin-resistant *Staphylococcus aureus* (MRSA**)** were used. MHA plates were swabbed with the cultures ($1x10^8$ cells/ml) individually in each plate using sterile cotton swabs. Agar wells were prepared by scooping out the media with sterile cork bores (7mm in diameter). The wells were then filled with 3mg/ml of the secondary metabolite dissolved in Di-phenyl Sulphoxide (DMSO). 5µl of DMSO and 4µl of Ciprofloxinwas used as negative and positive control respectively. The plates were incubated at 37^{o}C for 24 hours and zone of inhibition was recorded. The magnitude of antimicrobial action was assessed by the diameter (mm) of inhibition zones and compared with standard antibiotic Ciprofloxin. Inhibition zones around well of 6mm or more were defined as positive for biological activity. The minimum inhibitory concentration was determined by well diffusion method. By varying the concentration of the endophytic extracts, the zone of inhibition was measured and tabulated.

Antifungal assay

The antifungal activity was evaluated by agar tube dilution method as described by Blank and Rewbell, 1965.*Microsporumgypseum* and *Trichophytonrubrum* were obtained and Saboraud Dextrose agar (SDA) was used for growth of the fungi. To 10 ml of autoclaved media at 50ºC, 1mg of extract was added, mixed well and slant was prepared. Each tube was inoculated with the 4 mm diameter piece of respective fungus. Tubes were incubated at 28°C for 7 days and observed.

Fungal DNA isolation: The DNA was isolated from the fungus using CTAB method (Saghai-Maroof *et al.,* 1984). From the SDA plates a fungal mat was cut using a sterile nipper and ground into fine powder using liquid nitrogen in a mortar and pestle separately.10ml of CTAB buffer(0.1N HCl 1ml, 0.7M NaCl 7ml, 10mM EDTA 0.1ml and CTAB 0.1ml), 0.5ml of β-mercaptoethanol, 20 µl of proteinase K (20mg/ml) was added. The mixture was transferred to centrifuge tubes and incubated for 1 hour at 37^{o}C followed by 30 minutes at 65^{o}C. Equal volume of phenol: chloroform: iso-amylalcohol (25:24:1) was added to the mixture and centrifuged at 10000 rpm for 10 minutes at 4^{o}C. After performing chloroform washes and precipitation with 70% ethanol, the pellet was dissolved in 1x TE buffer and stored at -20ºC. Isolated DNA was run on 0.7% agarose gel and confirmed followed by quantification using spectrophotometry.

PCR: The PCR reactions for sequencing were carried out in a thermal cycler using universal primers to amplify the ITS region of the fungal genome. Amplification was performed in a 20µl reaction mixture containing 10.8µl milliQ water, 2µ PCR buffer, 2µl DNTPs, 2µl forward primer, 2µl reverse primer, 1µl diluted DNA(1µl DNA in 9µl milliQ water) and 0.2µl TaqPol. The thermal cycler program is as follows: Initial denaturation at 94ºC for 3 min followed by 32 cycles of

denaturation at 94ºC for 45s annealing at 51.5ºC for 1 min, extension at 72ºC for 1 min 20s and the final extension at 72ºC for 7 min. The amplified DNA was direct sequenced using the ITS primers in a 3730 DNA sequencing analyzer (ABI).

DNA sequence similarity: Sequence similarity searches were performed for the obtained fungal ITS sequences against the non-redundant database maintained by the National Center for Biotechnology Information using the nucleotide BLAST algorithm (http://www.ncbi.nlm.nih.gov).

Phylogenetic Analysis: The BLAST sequences were aligned using clustalw. Phylogenetic trees were generated by UPGMA method using MEGA4.0 (Tayung *et al*., 2011).

RESULTS AND DISCUSSION

Endophytic fungi were successfully isolated from *Barringtonia acutangula*. Pure isolates were maintained in Sabouraud dextrose agar (Fig 1). Two endophytic fungi (EFB01 and EFB02) were isolated. Secondary metabolites were extracted from EFB01 and EFB02 using ethyl acetate (EA) and chloroform. The extracts were labeled as: EFB01-CHCl3, EFB01-EA, EFB02-CHCl3 and EFB02-EA. The extracts were screened for antibacterial activity against *Pseudomonasaeruginosa, Bacillus subtillis* and *MRSA.* With the increasing prominence of multidrug resistant pathogens, drugs with potent inhibitory effects on these microbes have become imperative. Hence, Methicillin resistant *S.aureus* was included in the screening process. The extracts showed a high significant antibacterial activity against Gram positive bacteria (Table1). Hence Minimal Inhibitory Concentration studies were focused on *Bacillus subtilis* (Bauer *et al.,* 1966*)*. Minimal concentration studies showed that 15µg of EFB01 extract and 30µg of EFB02 extract showed zone of inhibition of 18-20mm and 18-25mm respectively (Table 2). The zone of inhibition was significant and a comparison with the standard drug was used to analyse the inhibitory potentials of the extracts. Similar activities were observed against the fungi *T.rubrum* and *M.gypseum* and the zone of inhibition was measured (Table 3.) Hence the strains EFB01 and EFB02 were selected for molecular characterization.

The isolated fungal DNA was analyzed on 0.7% Agarose gel yielding sharp high molecular bands and quality was verified using UV Spectrophotometer. PCR standardization was done using ITS Primers for EFB01 and EFB02 fungal DNA with varying concentration of template DNA and annealing temperature. The amplified PCR products were purified and sequenced using Sanger's sequencing method in a 3730 DNA sequencing analyzer (ABI). After carrying out a nucleotide BLAST the strain EFB01 was found to be *Colletotrichumgloeosporioides* (Accession no: JQ844304) with 94% query coverage. Strain EFB02 was found to be *Phomopsis* (Accession no: KC168054) species with 99% query coverage. Sequence analyses were performed based on the rRNA gene sequence (ITS1-5.8S-ITS2) using the UPGMA method to indicate the relationship of endophytic fungi phylotypes sequences to similar family members from Gen-Bank BLAST alignments (Fig. 5, Fig. 6) The phylogenetic analysis indicated the close relationship between *Phomopsis sp.* & *Diaporthelongicolla* and *C. gloeosporioides* & *C. siamense* respectively.

Table 1. Zone of inhibition (mm) of secondary metabolites from EFB01 and EFB02in Ethyl acetate and chloroform solvents

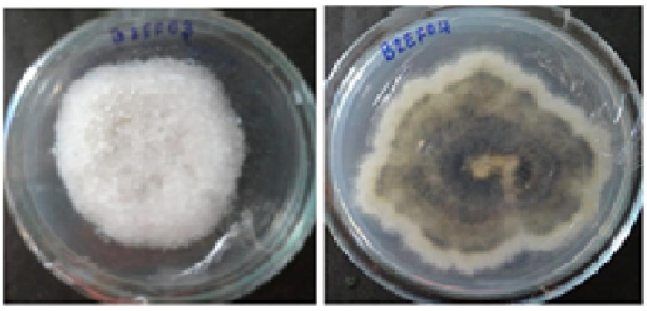
Sample	*Bacillus subtilis*	MRSA	*Pseudomonas aeruginosa*
EFB01-$CHCl_3$	28	32	-
EFB01- EA	36	45	-
EFB02-$CHCl_3$	27	31	-
Positive control	31	36	31
Negative control	-	-	-

Table 2. Minimal Inhibitory Concentration studies of secondary metabolites from EFB01 and EFB02 on *Bacillus subtilis*

Concentration	EFB01-$CHCl_3$ (mm)	EFB02-$CHCl_3$ (mm)	EFB01- EA (mm)	EFB02-EA (mm)
90µg	23	25	28	28
70 µg	21	23	27	28
50 µg	18	21	27	26
30 µg	19	18	23	25
15 µg	18	18	20	26
Positive control	31	32	31	31
Negative control	-	-	-	-

Table 3. Antifungal assay of secondary metabolites from EFB01 and EBF02 in ethyl acetate and chloroform solvents

Sample	Culture	Zone of inhibition (mm)
-ve control	*T. rubrum*	69
EFB02-EA	*T. rubrum*	78
EFB02-$CHCl_3$	*T. rubrum*	71
EFB01-EA	*T. rubrum*	95
EFB01-$CHCl_3$	*T. rubrum*	63
EFB01-$CHCl_3$	*M. gypseum*	95
EFB02-$CHCl_3$	*M. gypseum*	71
EFB01-EA	*M. gypseum*	85
EFB02-EA	*M. gypseum*	91
-ve control	*M. gypseum*	86



a) EFB01 b) EFB02

Fig.1. Pure Isolates of Endophytic fungi from *B. acutangula*

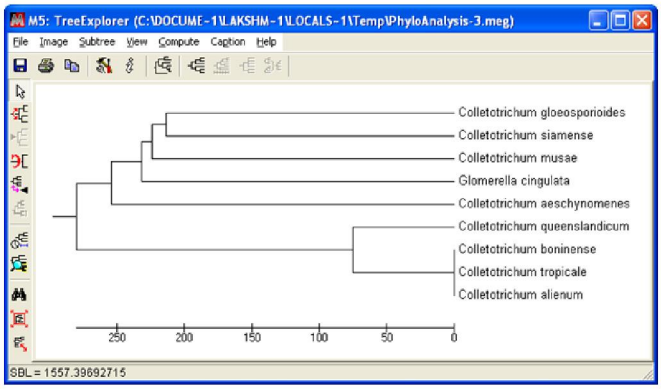


Fig. 2. Phylogenetic analysis of C.gloeosporioides with related species obtained by Gen- Bank BLAST alignment

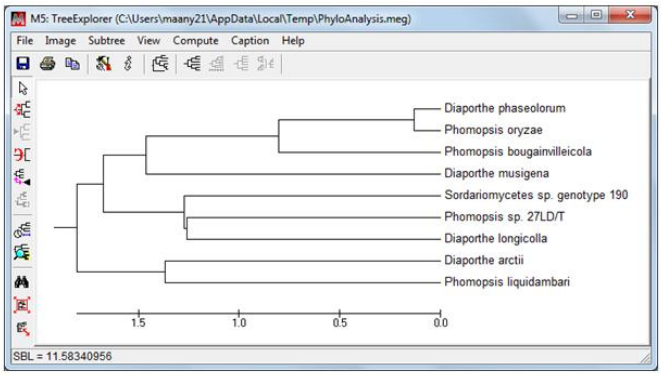


Fig. 4. Phylogenetic analysis of *Phomopsis* sp. with related species obtained by Gen- Bank by BLAST alignments

Conclusion

This work was mainly aimed at highlighting the use of endophytes in creation of novel pharmacologically active components and understanding the chemistry of bio-interactions between endophytic fungi and their hosts. This might not only allow the discovery and sustainable production of desirable natural products but also other mostly overlooked bioactive secondary metabolites (Kusari *et al.,* 2012).The fungi *Colletotrichumgloeosporioides* and *Phomopsis* sp were found to have significant activity against opportunistic pathogens like *P. aeruginosa, B. subtillis*, MRSA, *T. rubrum* and *M. gypseum*. The *Phomopsis* species of endophytic fungi isolated from *B. acutangula* is known to produce antibiotic Phomol earlier (Webber T., 1984). Also *C. gloeosporioides* had been screened for production of Taxol, a novel anticancer drug (Gangadevi *et al.,* 2008). Hence there is potential scope to isolate these fungi from *B.acutangula* and use them as nature's bioreactors for production of novel drugs. The isolation of endophytes was followed by their molecular characterization and phylogenetic analysis in view to study the endophytic diversity in medicinal plants and to continue our investigation of these endophytes.

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