

Original Research Article

Anticancer potentials of secondary metabolites from endophytes of *Barringtonia acutangula* and its molecular characterization

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ABSTRACT

KEYWORDS

Endophytic;
Barringtonia acutangula;
anticancer assay,
Genomic DNA;
PCR
Standardization;
BLAST of the
endophytic fungus.

Two species of Endophytic fungi (EFB01 and EFB02) were isolated from leaves of *Barringtonia acutangula*, a medicinal plant and secondary metabolite extraction was performed using organic solvents. The extracts were screened for anticancer assay against Human Colon Cancer cell lines HT29. Extract obtained from endophytic fungus, EFB01 showed highest cytotoxicity. Endophytic fungus, EFB01 was selected for molecular identification. Genomic DNA was extracted using CTAB (Cetyl trimethyl ammonium bromide) buffer. The total genomic DNA concentration was estimated by absorbance at 260 nm. PCR standardization was carried out using ITS (Internal transcribed spacer) primers for endophytic fungus, EFB01. Using sequence analysis of ITS region and BLAST (Basic local alignment search tool) the endophytic fungus, EFB01 was identified as *Colletotrichum gloeosporioides*.

Introduction

Fungi are plant-like organisms that lack chlorophyll. An endophytic fungi is a fungal microorganism, which spends the whole or part of its life cycle colonizing inter and /or intra-cellularly inside the healthy tissues of the host plants, typically causing no apparent symptoms of diseases. Endophytes are now considered as an important component of biodiversity. The distribution of endophytic mycoflora differs with the host. Some Endophytes are widespread and others are highly specific to single host in single environment. These endophytes

protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites (Azevedo *et al.*, 2000; Carroll and Carroll, 1978; Strobel, 2003). The endophytic fungi play important physiological and ecological roles in their host life. In recent years, there has been evidence that the production of secondary metabolites by an endophyte is not random, but seems to be correlated with its ecological niche. The metabolic interactions of endophytes with its host may favor the synthesis of biologically active secondary metabolites.

Plant endophytic fungi are novel source of bioactive secondary metabolites with potential application in agriculture, medicine and food industry. Some of the interesting compounds produced by endophytic microbes are taxol, cryptocin, cryptocandin, jesterone, oocydin, isopestacin, the psuedomycins and ambuic acid.

Endophytes can be identified in several ways, usually through amplifying and sequencing a small piece of DNA. Some endophytes can be cultured from a small piece of their host plant in an appropriate growth medium. *Colletotrichum* is a genus of fungi that are obligate symbionts to plants in the form of endophytes. The amount of taxol produced by this endophytic fungus, *Colletotrichum gloeosporioides* (strain JGC-9) was about 163.4 µg/L (Gangadevi and Muthumary, 2008). This taxol has Strong cytotoxic activity towards BT 220, H116, Int 407, HL 251 and HLK 210 human cancer cells in vitro, tested by Apoptotic assay. Medicinal plants are known to harbour endophytic fungi that are believed to be associated with the production of pharmaceutical products (Zhang *et al.*, 2006). In the present study, *Barringtonia acutangula*, an important medicinal plant, was investigated for the presence of endophytic community.

Barringtonia acutangula (L.) Gaertn. is a small to medium evergreen tree, with spreading crown. Leaves about 12.5 cm long, cuneate-elliptic, minutely denticulate-crenate. Flowers small, fragrant, dark scarlet, in slender pendulous many-flowered racemes, up to 30 cm long. Fruit 2.5-3.7 cm long, bluntly quadrangular, broadest in the middle (Fig.1).

Given the high incidence of bioactive extracts and the fact that most of the isolated fungi could not be identified due to lack of spore formation, the results suggested that

Thai medicinal plants can provide a wide variety of endophytes that might be a potential source of novel bioactive compounds (Suthep Wiyakrutta *et al.*, 2004).

Fig.1 *Barringtonia acutangula* leaves



Twenty three endophytic fungi from leaf, stem and root of *Ocimum basilicum* (Tulshi) were isolated and purified. One of the fungi (2L-5) isolated from the leaves was fermented in a large scale and extracted with ethyl acetate. The fungal extract was found to be active against two bacteria, *Bacillus cereus* and *Staphylococcus aureus* (Aminul Haque *et al.*, 2005).

Using specific PCR primers and probes we identified the presence of several fungal groups and species in the air samples. Specific detections through probe hybridization to PCR products amplified with universal or group-specific fungal primers have promising applications in the examination of air samples for environmental monitoring.

Application of PCR and probe hybridization techniques in detection of air borne fungal spores in environmental samples. (Zhang *et al.*, 2006). We have designed two taxon –

selective primers for the internal transcribed spacer (ITS) region in the nuclear ribosomal repeat unit.

The primers, ITS1-F and ITS4-B, were intended to be specific to fungi and basidiomycetes, the universal primers preferentially amplify the fungal ITS region. The Present study was initiated to isolate endophytic fungus from *Barringtonia acutangula* and their medicinal and molecular studies performed.

Materials and Methods

Collection of *barringtonia* leaves

Healthy leaves from *Barringtonia acutangula* tree were collected from the river bed in Kovilanchery, Chennai. Rinse the leaves with distilled water until the surface is cleaned thoroughly. Keep the leaves over the tissue paper for drying. After the leaves are dried, the leaves are cut over the midrib region. The alternative midrib pieces are taken to avoid colonization of same organism.

Isolation of endophytic fungi

The surface sterilized leaves were trimmed using sterile blade and inoculated into plates containing sterile Potato Dextrose Agar medium. The plates were incubated at 28°C for 7 days and observed for growth of fungus from leaves.

Extraction of secondary metabolites

Minimal media was prepared and autoclaved. Endophytic Fungus EFB01, EFB02 was inoculated into minimal media. Flasks were then incubated at 28°C for 10 – 14 days in shaker. After incubation, extraction was done with different solvents

(Chloroform, Ethyl acetate). First the fungus grown in the media is removed by filtering the medium using filter paper. Then the solvents are added to the media in the ratio 1:1 in separating funnel and left for 15 minutes. The organic phase was collected and kept for drying at 37°C. The dry weight of the extract was determined.

Cell line study using animal cell culture

Culturing of animal cells in *in vitro* condition is called animal cell culture. This is done to know the drug response, gene expression, antibody production (MOAbs), cytotoxic analysis, propagation of viruses, recombinant proteins, antitumour evaluation/screening. Media is the major constituent factor in the culturing of animal cells. For the growth of the cell in invitro condition, media is required. Media is now commercially available with all the nutrients present in the required volume. Widely used media are Dulbecco's Minimal Essential Medium (DMEM) and Rosewell Park Memorial Institute (RPMI 1640) medium.

RPMI 1640 Media Preparation

16.4gms of the RPMI 1640 medium was suspended in 900ml tissue culture grade water with constant, gentle stirring until the powder is completely dissolved. It may be necessary to lower the pH to 4.0 with 1N HCl to completely dissolve the powder, after it has dissolved completely; the pH can be raised to 7.2 with 1N NaOH prior to the addition of sodium bicarbonate. 2.0gms of sodium bicarbonate powder were added for 26.67ml, therefore 7.5% sodium bicarbonate for 1 litre of medium and stir until dissolved. The pH was adjusted to 0.2-0.3 pH units below the desired pH 1N

HCl or 1N NaOH since the pH tends to rise during filtration. The final volume was made up to 1000ml with tissue culture grade water. The medium was sterilised immediately by filtering through a sterile membrane filter with a porosity of 0.22 micron or less using positive pressure rather than vacuum to minimize the loss of carbon dioxide. The sterile supplements were aseptically added as required and the desired amount of sterile medium was dispensed into sterile containers. The liquid medium is stored at 2-8°C and in dark till use.

Maintenance of cell line

For maintaining the cell line, sub culturing was done. This was done to facilitate the cell growth by removing the cells from the medium and introducing them into a new fresh medium. Mostly, enzymatic methods were used for cell maintenance. The enzyme used here was TPVG.

Cell culture flasks were selected by confluency by observing under microscope. Growth medium was removed from the flasks and the cells were incubated at 37°C after adding the enzyme. Initially all cells get detached from the surface. The cells were suspended in 5ml of the (RPMI 1640 with 10% FBS) medium. The suspension was aspirated few times to break cell clumps. Cell line, date of seeding and passage number was marked on the bottom. The cell suspension was transferred to fresh T-Flasks. 5ml of cell suspension was added to each well.

Seeding of HT 29 cell line

The cell lines were seeded in 2 kinds of plate, 24titre plate and 96titre plate. 24 titre plate consists of 420µl of RPMI 1640 and 80µl of cell suspension and 96 titre plate

consists of 80µl of medium and 20µl of cell suspension.

Both the plates are allowed to grow till the formation of monolayer. Then these plates are used for DNA fragmentation and MTT assay (Methyl thiazol tetrazolium assay).

MTT assay

This method is based on the ability of live but not dead cells to reduce a yellow tetrazolium dye to a purple formazan product. In brief, approximately 5×10^3 cells/well (cell line) were seeded into 96 well plate, 100µl of RPMI 1640 medium was added and incubated at 37°C. After 24hours, the medium was discarded and fresh medium was added with different concentration of extract (Chloroform, Ethyl acetate) such as 125, 250, 500 (100-2000µg/ml). The plates were incubated for 1-3 days at 37°C in a CO₂ incubator. After respective incubation period, medium was discarded and 100µl fresh medium was added with 10µl of MTT (5mg/ml). After incubation at 37°C in a CO₂ incubator for 4 hours, the medium was discarded and 200µl of DMSO (Dimethyl sulfoxide) was added to dissolve the formazon crystals. Then the absorbance was read in a spectrophotometer at 630nm and cell survival was calculated by the following formula.

$$\begin{aligned} \text{Viability \%} &= \text{Test OD} / \text{Control OD} \times 100 \\ \text{Cytotoxicity \%} &= 100 - \text{viability\%} \end{aligned}$$

Molecular characterization of endophytic fungus by genomic DNA isolation

Add 10 ml (for a 0.5 g pad) of CTAB extraction buffer gently mix to wet the entire powdered pad. Place in 65° C water bath for 30 min. Cool, add equal volume of CHCl₃: IAA (24:1). Mix, centrifuge for 10 min at full speed. Transfer aqueous

supernatant to a new tube. Add an equal volume of isopropanol. High molecular weight DNA should spool out upon mixing. spool out the DNA with a glass rod or hook, pour out the remaining supernatant. Rinse the spooled DNA with 70% ethanol. Air dry, add 1- 5 ml TE containing 20 µg/ml RNase suspend the samples, place in 65° C bath, or allow pellets to re suspend overnight at 4°C .

Analysis of DNA by Agarose gel electrophoresis

Agarose was weighed and transferred to a conical flask. 50 ml of 1X TAE was added and Agarose was melted to a clear solution by heating. It was allowed to cool until it reached bearable temperature. 2.5µl of ethidium bromide stock solution was added. A gel casting tray was placed in a leveling table and the melted agarose was poured. After the gel solidified, the comb was taken out carefully. The casted gel was placed in an electrophoresis tank and 1X TAE buffer was added until the gel was completely submerged. DNA sample was mixed with the gel loading buffer and loaded into the well. The samples were then electrophoresed at 50V until the gel loading buffer reached 2/3rd of the gel. This gel was then viewed under UV Transilluminator.

Quantification of DNA by spectrophotometric method

The spectrophotometer was calibrated and the UV lamp was turned on. The wavelength was set at 260nm and 280nm. Absorbance at 260 and 280 nm was set at zero with TE buffer or sterile water as blank. 3µl of the DNA was taken in a quartz cuvette and made up to 3ml with TE buffer or sterile water. Absorbance of the sample at 260 and 280 nm was noted.

Polymerase chain reaction

50- 100ng of DNA is used for molecular identification. The PCR reaction is performed for 20µl . The PCR tubes were placed in thermocycler and the samples were amplified in thermocycler. Amplified samples were then electrophoresed on 1.5% agarose gel. EFB01 Fungus was taken for molecular identification as it showed very high significant anticancer activity (Table.1,2).

Table.1 PCR reaction conditions

Initial denaturation	94°C – 3min
Denaturation	94°C – 1min
Annealing	51°C – 1min
Extension	72°C – 1min 20sec
Final extension	72°C – 7min
Hold	4°C
Total number of cycles	32

Analysis of PCR product by agarose gel electrophoresis

Agarose was weighed and transferred to a conical flask. 50 ml of 1X TAE was added and Agarose was melted to a clear solution by heating. It was allowed to cool until it reached bearable temperature. 2.5µl of ethidium bromide stock solution was added. A gel casting tray was placed in a leveling table and the melted agarose was poured. After the gel solidified, the comb was taken out carefully. The casted gel was placed in an electrophoresis tank and 1X TAE buffer was added until the gel was completely submerged. DNA sample was mixed with the gel loading buffer and loaded into the well. The samples were then electrophoresed at 50V until the gel loading buffer reached 2/3rd of the gel. This gel was then viewed under UV Transilluminator. The PCR product was subjected to DNA sequencing and the results were analysed using BLAST tool.

Table.2 Reaction set up for ITS amplification - standardization

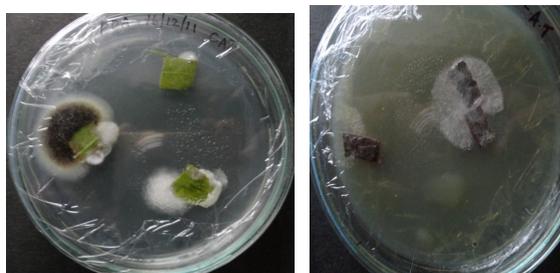
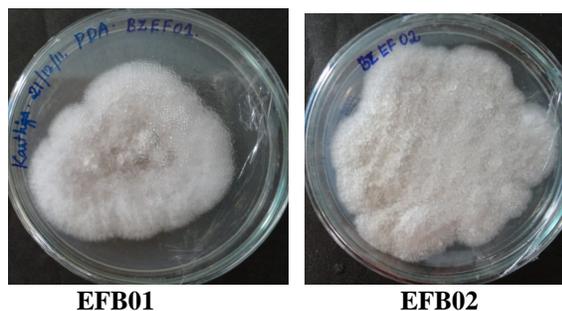
Contents	Stock Conc	Final Conc	Final Volume
Mili Q Water	-	-	10.8µl
Taq Buffer (with MgCl ₂)	10x (20mM MgCl ₂)	1x (2.0mM MgCl ₂)	2µl
dNTPs	2mM	0.2mM	2µl
ITS-F	3µM	0.3µM	2µl
ITS-R	3µM	0.3µM	2µl
Template DNA	-	100 ng/µl	1µl
Taq Polymerase	5U/µl	1 U	0.2µl

Results

In the present study, *Barringtonia acutangula*, an important medicinal plant, was investigated for the presence of endophytic fungi. Healthy leaves from *Barringtonia acutangula* tree were collected from the river bed in Kovilanchery, Chennai and the leaves are washed with distilled water. The leaves are surface sterilized using chemicals aseptically. The midrib pieces of leaves are selected for inoculation.

Potato Dextrose Agar media was used for isolation of fungi. The surface sterilized leaves were inoculated on autoclaved Potato Dextrose Agar media on sterile petridish and incubated at 28° C for 7 days. After 7 days fungal growth is seen on the surface of the leaves in petriplates (Fig.2). The fungi would start appearing as thin and slender mycelia. About two species of endophytic fungi were isolated. The two species of fungi were subcultured into a plate containing sterile Potato Dextrose Agar media and it is incubated at 37°C for 4 – 5 days (Fig.3).

In the present study endophytic fungus of *Barringtonia acutangula* has been selected for extraction of secondary metabolites. Secondary metabolite extraction was performed by inoculating two endophytic fungi (EFB01 & EFB02) in Minimal media and incubating the flask for 10-14 days in shaker. The media was separated from the fungal mycelia by filtering the media using filter paper. The extraction was carried out by adding solvents (chloroform and ethyl acetate) to the media in the ratio 1:1 in separating funnel and left for 15 minutes. Organic phase was collected and kept for air drying at room temperature. The extracts were labelled as: EFB01-CHCl₃, EFB01-EA, EFB02-CHCl₃ & EFB02-EA.

Fig. 2 Isolation of Endophytic fungi from *Barringtonia acutangula* leaves**Fig.3** Pure isolates of endophytic fungus from *Barringtonia acutangula* leaves

Anti-cancer assay of secondary metabolic product of fungi

The cytotoxic effect of fungal endophyte isolated from *Barringtonia acutangula* was tested by the MTT assay, which showed the effect of its secondary metabolites on the cell viability in HT29, human colon cancer cell line. The cells

treated with the fungal extracts of concentration ranging between 125µg/ml and 500µg/ml showed a significant decrease in the cell viability (Table.3,4). The fungal extract from EFB01 showed 52% cyto-toxicity while that of EFB02 exhibited 40% cyto-toxicity which is nominal when compared to the metabolite from EFB01.

Table. 3 Anti-cancer assay of secondary metabolic product of fungi (EFB01)

Activity	EFB01			Control	PC
	500µg	250µg	125µg		
Concentration	500µg	250µg	125µg		
Viability	47.16024	52.28495	49.27239	100	25.7644
Cytotoxicity	52.8398	47.7151	50.7276	0	74.2356

Table. 4 Anti-cancer assay of secondary metabolic product of fungi (EFB02)

Activity	EFB02			Control	PC
	500µg	250µg	125µg		
Concentration	500µg	250µg	125µg		
Viability	59.16156	60.41667	62.3265	100	25.7644
Cytotoxicity	40.8384	39.5833	37.6735	0	74.2356

Table.5 DNA quantification by UV spectrophotometry Method

Sample	A ₂₆₀	A ₂₈₀	Concentration of DNA (ng/µl)	Purity ratio =A ₂₆₀ /A ₂₈₀
EFB01	0.40	0.23	20000	1.739
EFB02	0.34	0.21	17000	1.619

Molecular characterization of endophytic fungi

Genomic DNA isolation was carried out using CTAB buffer. The isolated DNA was analysed on 0.7% Agarose gel. The method employed for the genomic DNA isolation resulted in obtaining high quality DNA (Fig. 4). The purity of DNA was estimated using DNA quantification by spectrophotometric method (Table. 5). The purity of DNA isolated from EFB01 was about 1.73. The ratio between 1.7 and 1.9 is indicative of fairly pure DNA preparation. PCR standardization was done using ITS Primers for EFB01 fungus. Annealing temperature was standardized at 51°C. The PCR product was subjected to DNA sequencing (Fig.5, 6).

Sequence analysis was done and the results were analysed using BLAST. BLAST analysis of the sequences determined the identical sequences from the database. Majority of the hits from the BLAST search were from a fungus *Colletotrichum gloeosporioides*. Thus the organism was found to be *Colletotrichum gloeosporioides* (Fig. 7-10).

Fig. 4 Agarose gel electrophoresis showing Genomic DNA



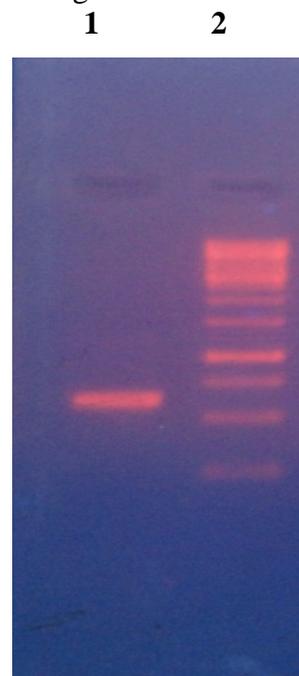
Lane 1 – Genomic DNA from EFB01
Lane 2 – Genomic DNA from EFB02

Fig.5 Polymerase Chain Reaction for ITS - Standardisation at 51°C



Lane 1: 1kb DNA Ladder,
Lane 2: PCR Product for ITS region-EFB01

Fig. 6 Polymerase Chain Reaction – ITS Region (Standardized at 51°C) for Sequencing



Lane 1-PCR Product of ITS region of EFB01 (600 bp),
Lane 2-1Kb DNA Ladder

Fig.7 Sequencing of endophytic fungi *C.gloeosporioides*

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TTCAAGGGTCCTCCCGCTTTGGGTGGAACCAGCGGAGGGATCATTACTGAGTTTACGGCTCTAC
AACCCCTTTGTGAACATACCTATAACTGTTGCTTCGGCGGGTAGGGTCTCCGTGACCCTCCCGGC
CTCCCGCCCCGGGGCGGGTCGGCGCCCCGCCGAGGATAACCAAACCTCTGATTTAACGACGTTT
CTTCTGAGTGGTACAAGCAAATAATCAAAACTTTTAAACAACGGATCTCTTGGTTCTGGCATCGA
TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCT
TTGAACGCACATTGCGCCCCGAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCC
TCAAGCTCTGCTTGGTGTGGGGCCCTACAGCTGATGTAGGCCCTCAAAGGTAGTGGCGGACCC
TCCCGGAGCCTCCTTTGCGTAGTAACTTTACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCG
TAAAACCCCCCAATTTCCAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAG
CATATCAATAAGCAAGGAAA
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Fig. 8 Graphical representation of sequencing of endophytic fungi *C.gloeosporioides*

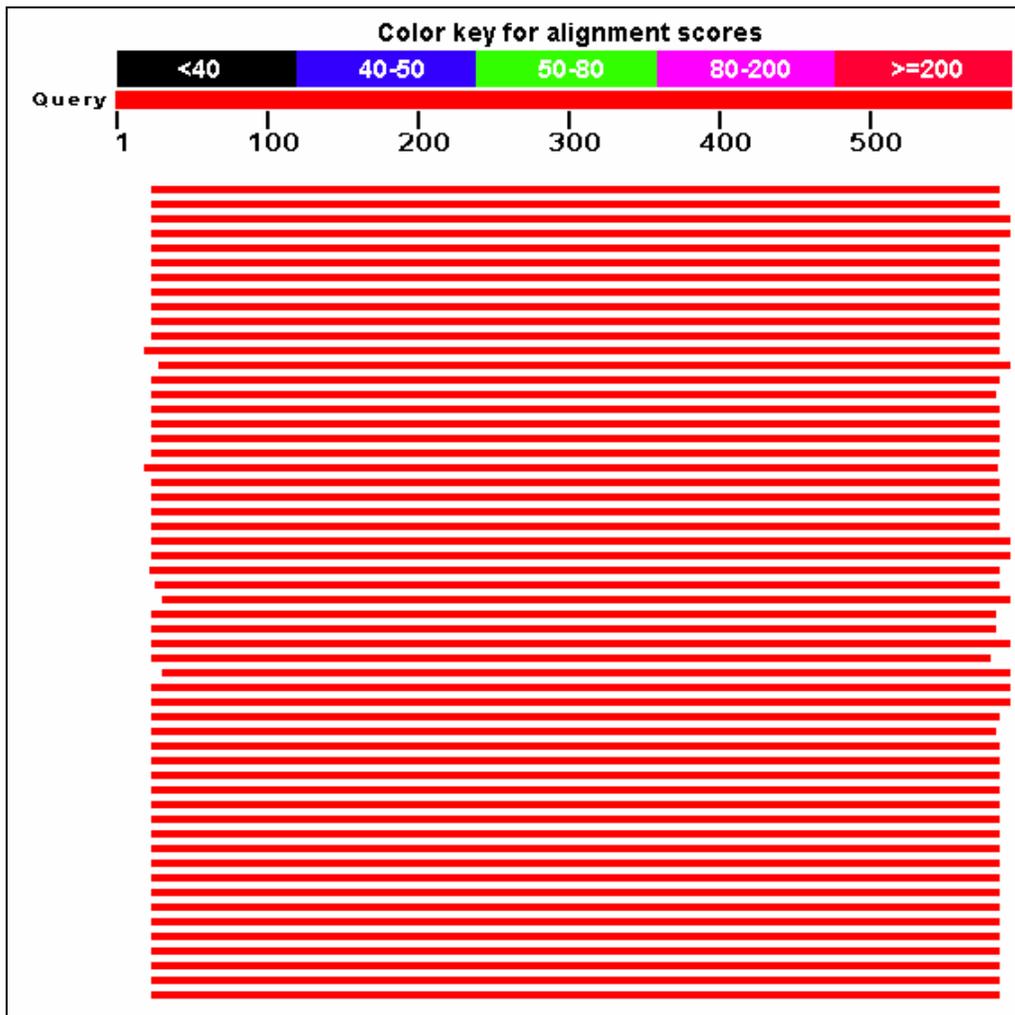


Fig.9 Tabular representation of sequencing of endophytic fungi *C.gloeosporioides*

Sequences producing significant alignments:						
Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AY266373.1	Colletotrichum gloeosporioides strain CMUBS1 internal trans	1003	1003	94%	0.0	99%
AY266379.1	Colletotrichum gloeosporioides strain ZE0041 internal transc	1003	1003	94%	0.0	99%
EU552111.1	Colletotrichum gloeosporioides culture-collection CBS:12268	1002	1002	95%	0.0	99%
AJ301908.1	Colletotrichum gloeosporioides 18S rRNA gene, 5.8S rRNA ge	1002	1002	95%	0.0	99%
HM537032.1	Fungal endophyte sp. g24 18S ribosomal RNA gene, partial :	1000	1000	94%	0.0	99%
GU066671.1	Colletotrichum gloeosporioides isolate 104AM/T 18S ribosom	1000	1000	94%	0.0	99%
FJ972604.1	Colletotrichum siamense isolate BML16 18S ribosomal RNA ge	1000	1000	94%	0.0	99%
AY266394.1	Colletotrichum gloeosporioides strain PDC113 internal transc	1000	1000	94%	0.0	99%
HM575266.1	Colletotrichum gloeosporioides isolate CM0705 18S ribosoma	996	996	94%	0.0	99%
AY266378.1	Colletotrichum gloeosporioides strain CMUBE1814 internal tr	996	996	94%	0.0	99%
HM537025.1	Fungal endophyte sp. g11 18S ribosomal RNA gene, partial :	996	996	94%	0.0	99%
GQ352482.1	Glomerella sp. 150GM/L 18S ribosomal RNA gene, partial sec	996	996	95%	0.0	99%
AB439815.1	Colletotrichum gloeosporioides genes for 18S rRNA, ITS1, 5.	996	996	94%	0.0	99%
AF272779.1	Colletotrichum gloeosporioides isolate APL7 internal transcri	996	996	94%	0.0	99%
HM131514.1	Colletotrichum sp. LC924 internal transcribed spacer 1, part	994	994	94%	0.0	99%
GU066652.1	Colletotrichum gloeosporioides isolate 79CA/S 18S ribosoma	994	994	94%	0.0	99%
GQ865569.1	Colletotrichum gloeosporioides strain CCGHN01 18S ribosom.	994	994	94%	0.0	99%
GQ424104.1	Colletotrichum gloeosporioides strain CCGHN03 18S ribosom.	994	994	94%	0.0	99%
GQ424105.1	Colletotrichum gloeosporioides strain CCGHN01 18S ribosom.	994	994	94%	0.0	99%
GQ352476.1	Glomerella sp. 116GM/L 18S ribosomal RNA gene, partial sec	994	994	95%	0.0	99%
FJ823432.1	Colletotrichum gloeosporioides isolate sw1 18S ribosomal RN	994	994	94%	0.0	99%
EU671076.1	Glomerella cingulata isolate A2 18S ribosomal RNA gene, par	994	994	94%	0.0	99%
AY266399.1	Colletotrichum musae strain PDC147 internal transcribed spa	994	994	94%	0.0	99%
AY714052.1	Colletotrichum gloeosporioides 18S ribosomal RNA gene, par	994	994	94%	0.0	99%
FN566873.1	Colletotrichum gloeosporioides 18S rRNA gene (partial), ITS	993	993	95%	0.0	99%
FN566872.1	Colletotrichum gloeosporioides 18S rRNA gene (partial), ITS	993	993	95%	0.0	99%
JF730186.1	Colletotrichum sp. GM 18S ribosomal RNA gene, partial sequ	993	993	94%	0.0	99%
HM535385.1	Colletotrichum sp. J-4 18S ribosomal RNA gene, partial sequ	993	993	94%	0.0	99%

Fig.10 Alignment sequencing of endophytic fungi *C.gloeosporioides*

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>gb|AY266373.1| Colletotrichum gloeosporioides strain CMUBS1 internal transcribed
spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete
sequence; and internal transcribed spacer 2, partial
sequence
Length=599
Score = 1003 bits (1112), Expect = 0.0
Identities = 560/561 (99%), Gaps = 1/561 (0%)
Strand=Plus/Plus

Query 26 GAACCAGCGGAGGGATCATTACTGAGTTTACGGCTCTACAACCCCTTTGTGAACATACCTA 85
Sbjct 34 GAACCAGCGGAGGGATCATTACTGAGTTTACG-CTCTACAACCCCTTTGTGAACATACCTA 92

Query 86 TAACTGTTGCTTCGGCGGGTAGGGTCTCCGTGACCCCTCCCGGCCCTCCCGCCCCGGGCGG 145
Sbjct 93 TAACTGTTGCTTCGGCGGGTAGGGTCTCCGTGACCCCTCCCGGCCCTCCCGCCCCGGGCGG 152

Query 146 GTCGGCGCCCGCCGGAGGATAACCAAACCTCTGATTTAACGACGTTTCTTCTGAGTGGTAC 205
Sbjct 153 GTCGGCGCCCGCCGGAGGATAACCAAACCTCTGATTTAACGACGTTTCTTCTGAGTGGTAC 212

Query 206 AAGCAAATAATCAAACCTTTTAAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC 265
Sbjct 213 AAGCAAATAATCAAACCTTTTAAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC 272

Query 266 AGCGAAATGCGATAAGTAATGTGAATTGCAGAAATTCAGTGAATCATCGAATCTTTGAACG 325
Sbjct 273 AGCGAAATGCGATAAGTAATGTGAATTGCAGAAATTCAGTGAATCATCGAATCTTTGAACG 332

Query 326 CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCCGAGCGTCATTTCAACCCTCA 385
Sbjct 333 CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCCGAGCGTCATTTCAACCCTCA 392

Query 386 AGCTCTGCTTGGTGTGGGGCCCTACAGCTGATGTAGGCCCTCAAAGGTAGTGGCGGACC 445
Sbjct 393 AGCTCTGCTTGGTGTGGGGCCCTACAGCTGATGTAGGCCCTCAAAGGTAGTGGCGGACC 452

Query 446 CTCCTGGAGCCCTCCTTTGCGTAGTAACCTTTACGTCTCGCACTGGGATCCGGAGGACTCT 505
Sbjct 453 CTCCTGGAGCCCTCCTTTGCGTAGTAACCTTTACGTCTCGCACTGGGATCCGGAGGACTCT 512

Query 506 TGCCGTAATAAACCCCAATTTTCCAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTG 565
Sbjct 513 TGCCGTAATAAACCCCAATTTTCCAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTG 572

Query 566 AACTTAAGCATATCAATAAGC 586
Sbjct 573 AACTTAAGCATATCAATAAGC 593
    
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Discussion

Fungi are plant-like organisms that lack chlorophyll. An endophytic fungi is a fungal microorganism, which spends the whole or part of its life cycle colonizing inter and /or intra-cellularly inside the healthy tissues of the host plants, typically causing no apparent symptoms of diseases. Endophytic microorganisms are found virtually in every plant on earth. These organisms reside in the living tissues of the host plant and participate in a variety of relationships ranging from symbiotic to pathogenic. Endophytic fungi tend to live with the plant over its entire life.

Medicinal plants harbour endophytic mycoflora (Zhang *et al.*, 2006). Therefore, it is important to explore endophytic mycoflora in the medicinal plants. In the present study, *Barringtonia acutangula*, an important medicinal plant, was investigated for the presence of endophytic fungi. Healthy leaves from *Barringtonia acutangula* tree were collected and washed with distilled water. The leaves are surface sterilized using chemicals aseptically. The midrib pieces of leaves are selected for inoculation. Potato Dextrose Agar media was used for isolation of fungi. The surface sterilized leaves were inoculated on autoclaved Potato Dextrose Agar media on sterile petridish and incubated at 28° C for 7 days. After 7 days fungal growth is seen on the surface of the leaves in petriplates. About two species of endophytic fungi were isolated. The two species of fungi were subcultured in sterile Potato Dextrose Agar media for further study.

Secondary metabolites are naturally derived metabolites and/or by-products from microorganisms, plants, or animals. Endophytes are the source for novel bioactive secondary metabolites. In recent

years, there has been evidence that the production of secondary metabolites by an endophyte is not random, but seems to be correlated with his ecological niche. The metabolic interactions of endophytes with its host may favor the synthesis of biologically active secondary metabolites. Some of the secondary metabolites produced by endophytic fungi are toxic to humans and animals and yet others can modify the growth and metabolism of plants.

Fungal endophytes of grasses produce a variety of ergot alkaloids, loline alkaloids, lolitrem, growth hormone and paramine alkaloids both *in vitro* and *in vivo*. (Clay and Schardl, 2002). In *Claviceps*, ergot alkaloids are confined to sclerotium. In contrast, the alkaloids can be isolated from whole parts of the infected plant. The alkaloid concentrations were higher in young than old leaves and in leaf sheaths, then in leaf blades in case of plants infected with endophytes. Bhagat *et al* (2012) for the first time demonstrated that endophytic fungus of grass grown in synthetic medium produced precursors of auxin *in vitro*. Cryptocin, a tetramic acid, produced by *Cryptosporiopsis quercina*. This unusual compound possesses potent activity against *Pyricularia oryzae* Cavara and a number other plant pathogenic fungi (Li and Strobel, 2001).

Cancer occurs when cells become abnormal and keep dividing and forming new cells without any control or order. If cells divide when new ones are not needed, they form a mass of excess tissue, called a tumor. Tumors can be benign (not cancer) or malignant (cancer). Both internal and external factors play some role in the promotion and initiation of carcinogenesis. Through high throughput screening (HTS) and bioassay-guided isolation, bioactive compound sclerotiorin has been isolated

from an endophytic fungus *Cephalotheca faveolata* (Periyasamy Giridharan *et al.*, 2012). Sclerotiorin was found to be potent anti-proliferative against different cancer cells. An endophytic fungus *Colletotrichum gloeosporioides* (strain JGC-9) was isolated from *Justicia gendarussa*, a medicinal plant and screened for taxol production (Gangadevi and Muthumary, 2008). This fungal taxol isolated from the organic extract of this fungal culture also had strong cytotoxic activity towards BT 220, H116, Int 407, HL 251 and HLK 210 human cancer cells in vitro, tested by Apoptotic assay.

In the present study the anticancer activity of extracts against human colon cancer cell lines, HT29 was tested by using MTT assay. The extracts showed a high significant activity against the cancer cells. The fungal extract from EFB01 showed 52% cytotoxicity and that of EFB02 exhibited 40% cytotoxicity. EFB01 showed the highest cytotoxicity, hence it was chosen for molecular characterization. Therefore, any research on endophyte-plant symbiosis, such as in this study is of value, especially taking into account the positive biological activity as anti-tumour agents. Effective extracts could provide potential leads towards the development of novel and eco friendly biologically agents. Thus, biological controls to prevent diseases offer an attractive alternative to disease management without the negative impact of chemical control. This study reinforced the assumption that endophytes of medicinal plants play an important role in the search for anti-tumoral compounds. The observations from our study encourage further investigation on these plants.

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