Bioprospecting for endophytic fungi and their metabolites from medicinal tree Aegle marmelos in Western Ghats, India.

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Abstract:

The increasing emergence of lead drugs for the resistance produced by the pathogenic strains and arrival of new diseases has initiated the need for searching novel metabolites with best anticancer and antimicrobial properties than the existing one. With this view, the investigation was conducted for the isolation, identification and biological evaluation of potential endophytic fungi of Aegle marmelos, a medicinal tree used for curing various disorders for more than three decades and it is a sacred tree in India. A total of 169 endophytic fungal strains obtained from sampling and among those 67 were pigmented strains. Upon antagonistic screening, five endophytic fungal strains exhibited antagonistic potentiality by inhibiting the pathogens. These five potent strains were characterized at molecular level by sequencing the
amplified ITS 1 and ITS 4 regions of rDNA and they were grouped under order Pleosporales, Eurotiales and Capnodiales. The metabolites from the respective strains were produced in fungal culturing media and extracted using polar solvents. Further the extracts of five endophytes manifested antimicrobial activity against tested clinical pathogens and *Alternaria alternata* (FC39BY), *Alternaria citrimacularis* (FC8ABr) and *Curvularia autraliensis* (FC2AP) exhibited significant antimicrobial profile against 9 of 12 tested pathogens, showing broad spectrum activity. The antioxidant levels of all the five endophytes revealed the highest activity at least concentrations and major activity was unveiled by the members of order Pleosporales FC2AP and FC8ABr. This research explains the value of endophytic fungal extracts and its significance of antimicrobial and antioxidant properties.

Keywords: Antioxidants, Antimicrobial, Endophytes, Molecular characterization, Metabolites.
1. Introduction

The role of fungal species in the ecosystems have been understood by little sampling and lack of characterization of fungal diversity. Over the past three decades the endophyte research has been popular by having knowledge of its ecology, life history and phylogeny. Although the term endophyte has been a controversial after the word has appeared and it has became synonymous with mutualism. Endophytes in woody plants are the poorly understood groups even though they are thought to be an important in plant populations and communities. They play a major role in physiology of plant tissues and protect the plants from pests, nematodes and insects and also provide resistance to the diseases caused by the environmental factors. These also help in nitrogen fixation and accelerate the plant growth. Endophytic fungal research was expanded in recent years from cataloguing species to examining the nature of the endophyte or plant interaction with particular emphasis on studying them residing in medicinal plants in a way to develop or discover novel compounds.

People in Indian subcontinent have a long history for the usage of medicinal plants for curing several diseases. Western Ghats of India are found to be one of the hotspots, comprises a diverse community of endophytic fungi residing in medicinal woody plants and it has been reported already. Endophytic fungi produce invaluable potential bioactive secondary metabolites which can be formulated as new drugs in biotechnological process and it can be a source for disease management. The secondary metabolites are the intermediates formed during the metabolism and they are found to be the potential drugs. This study focuses on potential bioactive metabolites from endophytic fungal isolates from medicinal plant *Aegle marmelos*. *Aegle marmelos* (L.) Correa (*A. marmelos*), is commonly known as bael and it belongs to the family Rutaceae. The parts of this plant such as root, bark, leaves and fruit have been widely used in indigenous
systems of Indian medicine like Ayurveda, Unanai and Siddha to treat various types of diseases like dysentery, diarrhoea and gastrointestinal problems. The plant also has antidiabetic, antibacterial and antifungal effects. Due to medicinal value of this host we have selected it to isolate and identify the metabolites from endophytic mycoflora from *A. marmelos* around Western Ghats region (India) and explore its bioactivities.

2. **Materials and methods**

2.1. **Plant sample collection**

Mature healthy, asymptomatic plant materials (bark, branches, leaves and root) were collected by sampling different parts of the trees of *A. marmelos* growing randomly in the Western Ghats region (Nilgiris cluster, Tamil Nadu, India). The sampling was performed on five trees of *A. marmelos* collected from foot hills of Vellingiri and Marudhamalai [Coimbatore, TamilNadu, India (11.0183° N, 76.9725° E)]. Bark samples were obtained by cutting tree bark at 150 cm above the ground level from a depth of 1–1.5 cm inwards with the help of sterile machete. Small discs of leaves (0.5 cm diameter) were cut using sterile pinch cutter. Root samples were obtained by digging the soil at least 1 m away around the main trunk and 2 ft in depth. Fifteen samples were taken from each tree, five each from root, inner bark, inner branches and leaves. From each sample 10 sub samples were prepared for further culturing to find out endophytic fungi. All the samples were collected in sterile polythene bags and brought to the laboratory in an icebox. Samples stored at 4°C were used to isolate endophytic fungi within 24 h of collection.

2.2. **Isolation of endophytic fungi**

The samples were rinsed gently in running tap water to remove dusts and debris. The samples were surface sterilized by modified method of Dobranic et al. [1]. The samples were
immersed in 70% ethanol for 5 s, followed by 4% sodium hypochlorite for 90 s and then rinsed in sterile distilled water for 10 s. This was done to remove epiphytic mycelia and bacteria adhered to the bark. The excess moisture was blotted in a sterile filter paper. The surface sterilized segments were taken for dissection using a sterile blade. The outer bark was removed and the inner cortex was cut into 1.0 cm x 0.5 cm pieces; leaves were pinched into 5mm diameter using pinch paper cutter and placed in petridishes containing PDA (Potato Dextrose Agar), MEA (Malt Extract Agar) and SDA (Sabouraud’s Dextrose Agar) media supplemented with streptomycin (250mg/L) and incubated at 28±2°C for 11-21 days in light/dark cycle. Tissues were observed for mycelial growth at an interval of 2 days and actively growing fungal mycelia was subcultured onto a fresh PDA/ SDA/ MEA media and stored in cryovials at -20°C.

2.3.Screening for potential strains:

Isolated pigmented endophytic fungal strains were screened for antagonism by cross streak method (dual culture technique). The four days old culture (pigmented isolates) was streaked on the centre of PDA, MEA and SDA media and incubated for 48 h at 28±2°C. The clinical pathogens (procured from PSG Hospitals, Coimbatore, TN, India) which are facultative anaerobic bacteria falls under Gram positive and Gram negative bacteria were *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Shigella* sp and *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhi* and *Proteus mirabilis* respectively were taken for antagonist assessment. Similarly, the fungal pathogens (Ascomycota) such as *Aspergillus niger* and *Candida albicans* were also taken for this study. All stated pathogens above were selected on the common basis of common infections (Nosocomial infections, Gastro Intestinal Infections and Urinary Tract Infections) caused in humans. The percentage of inhibition was calculated.
2.4. Identification and characterization of endophytic fungi:

The potential fungal isolates were identified up to the genus level by observing the presence of conidial mycelium, spore mass color, distinctive reverse colony color, diffusible pigment, sporophore and spore chain morphology. The strains were mounted on the sterile glass slides for staining with lactophenol cotton blue [2] and examined at 400x and 1000x light microscopy. The spore morphology (aerial hyphae, substrate mycelia and spore chain arrangement) was studied by growing the fungal strains in PDA, MEA and SDA on cover slips and viewed at 1000x in light microscopy after incubation for 4-7 days at 28±2°C. Molecular analysis was done using amplification of ITS1 and ITS4 fragments of rDNA. 18s rRNA or Ribosomal DNA sequence analysis was performed using specific PCR primers to amplify rDNA fragments of endophytes was used to validate the morphospecies of different groups of mycelia [3, 4].

2.5. Production and extraction of pigments

The pigmented strains were inoculated in different media such as PDB, SDB, MEB and Czapek Dox broth (CDB) to determine the maximum pigmentation inducing media. The flasks were kept in stationary phase and shaking condition at 100 rpm for 11 – 15 days at 28±2°C incubation in light and dark cycle. After incubation the total pigments were extracted from biomass and culture filtrate by solvent extraction method using polar and non polar solvents. The total pigments was evaluated using formula 1 \( \text{Abs}_T = \text{Abs}_{\text{intra}} + \text{Abs}_{\text{extra}} \) \[\text{Abs}_{\text{intra}} = \text{Abs}_{\text{extract}} \times D, \ D = 50 \ V, \ \frac{Y_p}{x} = \frac{\Delta \text{Abs}_T}{\Delta x} \] ( \text{Abs}_T : \text{Absorbance of extra plus intra cellular pigments (U); } \text{Abs}_{\text{extra}} \ \& \ \text{Abs}_{\text{intra}} : \text{Extra cellular } \& \text{intracellular absorbance (U); } \text{Abs}_{\text{extract}} : \text{Absorbance in the extract of cell disruption (U); } \ V : \text{Volume of the sample submitted to cell} \]
disruption for pigment extraction (mL); x : Cell concentration (g/L); Yp/x : Yield factor of pigments on cell growth (ULg$^{-1}$)]. The total pigment extract of each strain was concentrated in rotary vacuum evaporator and it was taken for further bioactivities studies.

2.6. Assessment of antimicrobial activity

2.6.1. Assessment of antibacterial profile

Antimicrobial assessment for the concentrated crude biomass pigment extract (BPE) and cell free culture filtrate pigment extract (CPE) of each potential isolates were determined by well diffusion method [5, 6] against clinical pathogens *S. aureus*, *S. epidermidis*, *E. faecalis*, *Shigella* sp, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *S. typhi* and *P. mirabilis* on MHA (Mueller Hinton Agar) media. 40µg of aliquots of extracts was filled in the respective wells. The diameter of inhibition zone was measured after 24h of incubation at 37°C for bacteria.

2.6.2. Assessment of antimycotic profile

This was determined by well diffusion technique using fungal clinical pathogens *A. niger* and *C. albicans* by swabbing on MHA plate and about 40µg of aliquots of extracts were filled in the respective wells. The plates were incubated for 48h at 28±2°C and then zone of inhibition was measured.

2.7. Analysis of antioxidant properties:

2.7.1. DPPH Radical Scavenging activity:

The antioxidants present in fungal crude pigment extracts were aliquotted into different concentrations (10 - 200µg) to determine extract’s ability for scavenging activity of 2, 2-diphenyl- 1- picrylhydrazyl (DPPH) radicals using the method of Yildirim et al. [7]. DPPH solution (1mM DPPH radical solution in 95% ethanol) was added to the crude pigment extracts
and made up to 1mL, vortexed well, and then incubated for 30 mins in dark hood at room temperature. After incubation, the samples were poured into microfuge tubes and centrifuged for 5 min at 13,500 rpm at RT. The absorbance of each sample at λ= 517nm was measured and 1mL of 95% EtOH/ MeOH was used as a control, and ascorbic acid were used as reference compounds. The antioxidant activity is given as percent (%) DPPH scavenging assay was calculated using the formula: 

\[
\left( \frac{\text{control absorbance} - \text{extract absorbance}}{\text{control absorbance}} \right) \times 100.
\]

2.7.2. Reducing power assay:

Total reducing power was determined as described by Oyaizu [8]. 1 ml of sample solution at different concentrations were mixed with 2.5 ml of phosphate buffer (0.2 mol/l, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50ºC for 20 min, 2.5 ml of 10% trichloroacetic acid (TCA) was added to the mixture and centrifuged at 3000g for 10min. The supernatant (5 ml) was mixed with 1 ml of ferric chloride (0.1%), and the absorbance was measured at 700 nm in a Spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing power.

2.7.3. Metal chelating activity:

The metal chelating activity was analyzed by the method of Dinis et al. [9] with slight modification. The reaction was performed in HEPES buffer (20mM) at pH 7.2. Various concentrations (10 – 200 µg) of samples were mixed with a solution of 12.5 µM ferrous sulphate solution. Addition of 75 µM ferrozine was to initiate the reaction and the mixture was shaken vigorously and incubated for 20 min at room temperature. After incubation the absorbance was measured at 562 nm. Ascorbic acid was used as the reference compound and the percentage
chelating capacity was calculated as; \( \% \) chelating activity = \( \left( \frac{A_0 - A_1}{A_0} \right) \times 100 \) where, \( A_0 \) = absorbance of the blank; \( A_1 \) = absorbance of the sample.

3. Results:

3.1. Isolation of endophytic fungi:

A total of 169 strains were isolated belonging to different taxa were obtained from 300 segments (fifteen from each root, inner stem, inner branch and leaves) of five trees and from that 67 were pigmented fungi. Of these 67 pigmented endophytic fungi 27 strains of \( A. \) marmelos were isolated from Marudhamalai hills and 40 strains were obtained from Vellingiri hills and this proved that this hill prevails to be diverse in nature. Each fungus was named in the order of fungus, sample location, sample number, strain number and strain mat color. The isolated strains belonged to Ascomycota, Basidiomycota and Dueteromycota.

3.2. Screening of isolated strains by antagonism:

Pigmented fungal isolates were screened for antagonistic property against facultative aerobic bacteria and among that 67 fungal isolates, maximum of 32 strains showed moderate activity. Major activities against maximum pathogens were observed in five fungal isolates (FC39BY, FC2AP, FC8ABr, FC75ABr and FC30AGr). The inhibition zone was measured in centimeters in terms of diameter.

3.3. Identification and characterization of the strains:

Five potential fungal strains were examined for morphological and molecular characteristics. The spore morphology of each isolate differed according to their phylum and class. The conidia of the isolates FC75ABr and FC8ABr were large, multicelled having ellipsoidal or V shaped sac which contained 4-8 ascospores which were similar to strain FC2AP
containing 4-6 ascopores in the sac and the sac was pale brown to mid brown in color. These three strains belonged to class Dothidiomycetes. FC39BY conidia were large multicelled obclavate and it had horizontal septa. The conidia of FC30AGr appeared as biseriate with philiades borne on brown septate. The characteristics of the five fungal strains were displayed in table 1 and figure 1.

The ITS rDNA region for the isolates were amplified, sequenced and compared with the sequence of organisms represented in NCBI database gene bank BLAST tool search and by using this we constructed the phylogenetic tree. The strains were Curvularia australiensis (FC2AP), Alternaria citrimacularis (FC8ABr), Alternaria alternata (FC39BY), Cladosporium cladosporidies (FC75ABr) and Aspergillus niger (FC30AGr). The sequences were submitted in gene bank and the accession number was obtained (Table 1).

3.4. Production and extraction of secondary metabolites:

The mass production of crude secondary metabolites from each isolated strains were carried out in selected media after summarizing the evaluation of maximum secondary metabolites production in different media (PDB, SDB, MEB and CDB) using formula 1. The foremost things noted for mass production was the cultivation time, water content which were the best factors for biosynthesis of metabolites in fermentation technique. During early stage of cultivation, the fungal strains produced white and short mycelium and then it developed into fungal mat. All the five strains did not utilize the CDB media components for their secondary metabolite production. On one hand FC8ABr and FC75ABr produced maximum metabolites in MEB and on the other hand PDB induced the maximum secondary metabolites production from FC39BY and FC30AGr. FC2AP was found to grow well and produced highest units of metabolites in SDB (Fig 2). The crude metabolites from each isolates were harvested and
extracted using different polar solvents. The crude secondary metabolites from FC2AP yielded 0.83UgL\(^{-1}\) in ethyl acetate whereas FC8ABr and FC30AGr produced 0.98 UgL\(^{-1}\) separately using methanol. About 0.7 UgL\(^{-1}\) and 0.3 UgL\(^{-1}\) of metabolites were yielded by FC39BY and FC75ABr by utilizing acetone and ethanol respectively.

**3.5. Assessment of antimicrobial activity:**

During resistance development in human pathogens, adverse effects of chemically synthesized drugs and emergence of new diseases needs an urgent demands for new natural antimicrobial agents which have no/ low impact to human health and environment. The results in figure 3 displayed the antimicrobial activity for the five potential strains against stated pathogens. FC2AP and FC8ABr extracts belonging to the order Pleosporales showed similar activity for all 12 pathogens whereas FC2AP exhibited higher activity against *C. albicans* and *S. epidermidis* and did not revealed any activity against *B. subtilis* and *P. mirabilis*. Crude metabolties extracts of FC8ABr, FC39BY and FC75ABr showed maximum activity against *S. aureus, K. pnuemoniae, P. aeruginosa, E. coli, Shigella sp and S. typhi*. FC30AGr extract inhibited the growth of *S. epidermidis* and *P. aeruginosa* by forming maximum zone. Among all these five strains FC2AP, FC8ABr and FC75ABr exhibited highest activity against maximum of ten pathogens.

**3.6. Analysis of antioxidant properties**

**3.6.1. DPPH radical scavenging assay:**

The free radical scavenging activities of fungal strains were determined by using ascorbic acid as standard. The absorbance of DPPH radical decreases by the antioxidant due to the scavening mechanism so the radical by hydrogen donation. It was noted by the color changing from purple to yellow. The strains FC39BY, FC8ABr, FC2AP, FC75ABr and FC30AGr showed
50% inhibition at a concentration of 174µg, 62 µg, 43 µg, 200 µg and 161µg respectively. This proved that the fungal extracts had the hydrogen donating capabilities at stipulated concentration and act as an antioxidant. Among the five extracts, FC2AP and FC8ABr were found to be acting as antioxidants at least IC₅₀ value (Fig 4).

3.6.2. Reducing power assay:

The reducing capacity of five different fungal extracts was determined by increasing in OD units (Fig 5). On comparing with five fungal crude metabolite extracts, FC2AP had highest reductive power ability even at least concentration. During the reaction, the antioxidant present in the fungal extracts convert Fe³⁺ to Fe²⁺and generally they were associated with the presence of reductones by breaking the free radical chain on donation of hydrogen.

3.6.3. Metal chelating assay:

The fungal extracts interfered with the formation of ferrous and ferrozine complex, indicating the presence of chelating activity by the antioxidants which leads to the capturing of ferrous ion before ferrozine can quantitatively form complexes with Fe²⁺. Therefore the levels of this Fe²⁺ in cells should be monitored and controlled carefully as the increased levels leads to cell damage. As all the five fungal extract were assessed to interrupt the complex formation at specific levels. The interruption activity at 50% of concentrations for FC39BY, FC8ABr, FC2AP, FC75ABr and FC30AGr were 156µg, 66 µg, 50 µg, 181 µg and 178µg respectively (Fig 6). So the extracts of these strains were well determined to be best antioxidants by the inhibition of red colored ferrozine complex at lower concentrations.

4. Discussion:
In searching for novel molecules the endophytic fungi represent an important genetic resource [10]. There is a greater opportunity to find new and interesting endophytic metabolites by considering the myriad of plants in the world [11]. Several investigations were made with the endophytic fungal isolation from medicinal plants but non invasive plants are overlooked for this study despite their uses in some traditional medicines and the uniqueness of their survival features. There are several reports solely on ecological and diversity role with biological elucidation. The research has been carried out on endophytic fungal isolation from the traditional medicinal tree (Aegle marmelos) which was a religious sacred tree in India. In this research, prominent sixty seven pigmented endophytic fungi were isolated which included the members of Ascomycotina and Deuteromycotina isolated from Western Ghats regions (Hotspot area). Reports of Gond et al. [12] exhibited that about 79 fungal strains were isolated from A. marmelos containing prevalent members of ascomycotina than deuteromycotina. The pigmented fungi were aimed for this research as they may contain more antioxidant properties than non pigmented strains through the fragmentary reports already stated. In this investigation, five potential fungal isolates were screened through antagonism and this revealed that the Western Ghats were more prevalent and rich with the diversity of bioactive metabolites/ compounds. The endophytic fungal strains that exhibited characteristic colony morphology and microscopic features such as hyphal and spore arrangements were putatively identified at genus level which was similar to the investigation of Larone [13]. The endophytes which could not be easily identified through this morphological features were subjected to sequencing of ITS-1 and ITS- 4 as molecular identification and the sequences were aligned with the databases from NCBI to obtain the species level and closely related species for the working strain. The results showed that the characterized fungal strains belonged to Ascomycetes.
Majority three species were dominated by class dothiodeomycetes and it included *C. australiensis* (FC2AP), *A. alternata*(FC39BY) and *A. citrimacularis* (FC8ABr). These isolates were very potent which was evident by the production of antimicrobial and antioxidant agents. There were differences between the fungal strains in the functional characteristics with respect to their ability in the production of pigmented secondary metabolites[14]. Our investigation implies that the members of Dothiodeomycetes especially the order Pleosporales showed the higher functional versatility and the fungi belonging to this group produce a wide range of biomolecules with bioactivity such as antimicrobial agents and the same was also reported in the research of Bhagat et al. [15]. The strains FC2AP and FC8ABr belonging to the order pleosporales exhibited highest antimicrobial activities against clinical pathogens. In order to induce the highest production of antimicrobial and antioxidant metabolites, the study had designed by employing different media and it was found that the metabolite production increased when amended with dextrose, this piece of work was also observed in the investigation of Padhi and Tayung [16] and they investigated the different media for increased metabolite production by the endophytic fungi. The metabolite production increased with the incubation time as it is a crucial parameters in metabolite production. Maximum metabolite production was observed to be 9-10 days in most of the fungi [17] but, in this study we observed the optimum incubation period range for all the five strains to be 11-21 days approximately. Maximum metabolites were produced by members in order Pleosporales and Eurotiales. A report from Strobel et al. [18] stated that the fungus *Gliocladium roseum* produced volatile antimicrobial metabolites after 18th day of incubation. Similarly in our investigation FC8ABr strain produced colored volatile metabolite after 18th day incubation and it showed the maximum inhibition against tested pathogens.
The metabolites are found to contain antioxidant properties and hereby antioxidant is defined as a biomolecule which donates hydrogen to the free radicals, causes damage to cells and tissues in humans and animals. In order to prevent this, it is essential for us to intake the antioxidant at specific levels in diet and hence, the microbial secondary metabolites are likely to have antioxidant properties where these can be modulated for medicinal purposes. The endophytic fungal extracts on antioxidant evaluation exhibited highest antioxidant activity even at least concentrations, especially isolates belonging to order Pleosporales unveiled highest antioxidant levels at lower concentration than other isolates and this is similar to the reports of Bhagat et al. [15]. The extracts were able to reduce the stable radical DPPH to the yellow colored diphenylpicrylhydrazine. The scavenging activity with DPPH radical was found to increase with the increasing concentrations. Extracts of *A. alternata* FC39BY manifested antioxidant activity at 50% which is similar to the earlier reports. Also these results coincide with the investigation of Silva et al. [19] that *A. alternata* extract represented the moderate antioxidant activity when compared to antimicrobial testing where the concentration of these two testing differed in activity. Earlier reports suggested that the members of order Pleosporales explored anticancer properties and in the same way our investigation also substantiated by the results obtained in antioxidant assessment for FC2AP and FC8ABr. As comparing DPPH radical scavenging activity with the metal chelating activity, the concentrations of fungal extracts were found to evince higher activity at lower concentrations for the second. It was reported that formation of r-bonds with the metal by chelating agents, which are found to be very effective because the redox potential was reduced as they are secondary antioxidants and thereby stabilizing the oxidized form of the metal ion [20]. Though the Cu$^{2+}$ and Fe$^{3+}$ are necessary for the organism’s transport, protection against oxidative stress, cell growth and development, they can also catalyze hydroxyl
radical formation via Fenton’s reaction [21]. Extracts of potent fungal isolates are antioxidants and they inhibit the free ions by chelating effects and thus helped in controlling the levels of Cu$^{2+}$ and Fe$^{2+}$ without causing any damages to the cell. With respect to this, isolates of Pleosporales (FC2AP and FC8ABr) had high chelating capacity which could easily trap the free radicals at minimal concentration.

5. Conclusion:

In conclusion, the endophytic fungal strains isolated from ethnomedicinal plants were explicating antioxidant components and scavenging effects at different levels. From the results obtained it is evident that the potent strains of this research have the prospective to be exploited as sources of novel bioactive metabolites, which may exhibit anticancer activity at lower concentrations as they revealed a high antioxidant activities. Further this research will focus on biomolecule purification and understanding the chemical nature of members in order Pleosporales for their anti cancer evaluation through in vivo studies.

6. References:


Cladosporium cladosporioides FC75ABr  Aspergillus niger FC30A Gr

Alternaria alternata FC39BY  Curvularia australiensis FC2AP

Alternaria citrimacularis FC8ABr
PIGMENT INDUCING MEDIA

In grams: GFM & UL/g for CPP

- GFM FC2A
- CPP FC2A
- GFM FC8A
- CPP FC8A
- GFM FC39BY
- CPP FC39BY
- GFM FC75ABr
- CPP FC75ABr
- GFM FC30AgR
- CPP FC30AgR

Strains & its production

Legend:
- PDB
- SDB
- CDB
- MEB
Figure legends

Fig. 1: Spore morphology of potential endophytic fungal strains from *Aegle marmelos*
The figure depicted was in 400× under light microscopy

Fig. 2: Pigment inducing media for five endophytic fungal strains from *Aegle marmelos*

Fig. 3: Antimicrobial activity of crude extracts from five endophytic fungal strains

Fig. 4: DPPH radical scavenging assay for five potential endophytic fungi from *Aegle marmelos*

Fig. 5: Reducing power assay for five potential endophytic fungi from *Aegle marmelos*

Fig. 6: Metal chelating assay for five potential endophytic fungi from *Aegle marmelos*
### Table 1: Characteristics of potential isolates

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Strain number</th>
<th>Related species</th>
<th>% similarity</th>
<th>Gene bank accession number</th>
<th>Morphological features</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FC2AP</td>
<td><em>Curvularia australiensis</em></td>
<td>94% with HG798740.1</td>
<td>KR363626</td>
<td>Pinkish gray colony on SDA; Conidia was ellipsoidal contained 4-6 ascospores which was pale brown to mid brown; conidia were clustered together on vertical septa; production of pinkish red pigments upon extraction with ethyl acetate.</td>
</tr>
<tr>
<td>2.</td>
<td>FC8ABr</td>
<td><em>Alternaria citrimacularis</em></td>
<td>100% with HG798724.1</td>
<td>KP863716</td>
<td>Brownish black colony on MEA; conidia was large, multicelled and V shaped sac contained 6-8 ascospores of mid brown to dark brown; conidia was single on each septae emerging at the sides of it; production of brownish black pigments upon extraction with methanol.</td>
</tr>
<tr>
<td>3.</td>
<td>FC39BY</td>
<td><em>Alternaria alternata</em></td>
<td>100% with HQ846574.1</td>
<td>KP863717</td>
<td>Yellowish brown colony on PDA; conidia was large, multicelled and ellipsoidal sac contained 4-6 ascospores of mid brown; conidia was obclavate and had horizontal and vertical septate; production of yellowish brown pigments upon extraction with acetone.</td>
</tr>
<tr>
<td>4.</td>
<td>FC75ABr</td>
<td><em>Cladosporium cladosporoides</em></td>
<td>99% with KM980007.1</td>
<td>KP863718</td>
<td>Reddish colony on MEA; conidia was large, multicelled, obclavate brown to black in color; had horizontal and vertical septa; production of red pigments upon extraction with ethanol.</td>
</tr>
<tr>
<td>5.</td>
<td>FC30AGr</td>
<td><em>Aspergillus niger</em></td>
<td>-</td>
<td>-</td>
<td>Greenish black powdery colony on PDA; conidial heads are large, globose, biserate dark brown to black in color; production of greenish black pigments upon extraction with methanol.</td>
</tr>
</tbody>
</table>
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