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Original Article

# BIOACTIVE POTENTIAL OF ENDOPHYTIC FUNGI *ASPERGILLUS FLAVUS* (SS03) AGAINST CLINICAL ISOLATES

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**ABSTRACT**

**Objective:** This study was to evaluate the antimicrobial potential of endophytic fungi isolated from the medicinal plant *Moringa oleifera* Lam. collected from the Omalur region, Salem district.

**Methods:** The endophytic fungi were isolated from stem, leaves, flowers and calyx of *Moringa oleifera* by surface sterilization method. The samples were surface sterilized by immersing it in 70% ethanol for 5 seconds followed by 4% sodium hypochlorite for 90 seconds and then a final rinsing in sterile distilled water. Then fungal biomass was extracted for intracellular metabolites by using ethyl acetate as solvent. The crude extract was filtered, and the filtrate was dried under vacuum at 40 °C. The filtrate was analyzed for antimicrobial activity. The fungi which showed the maximum activity was identified and the metabolite present in the ethyl acetate extract was characterized and identified by GC-MS and NMR analysis.

**Results:** The predominant endophytic fungi isolated belongs to the genera of *Aspergillus spp, Aspergillus flavus, Aspergillus versicolor, Aspergillus niger, Aspergillus ochraceus, Aspergillus terreus* and dematiaceous fungi namely *Bipolaris spp.* From this *Aspergillus flavus* showed the highest zone of inhibition was observed against *Staphylococcus aureus* and *Bacillus* 22 mm and strain of *Candida tropicalis* 19 mm. The efficiency of the bioactive compound was identified by GC-MS and NMR analysis and found to be Fenaclon, (R)(-) 14 methyl-8-hexadecyn-1-ol, Trans-β-farnesene (E)-β-farnesene, 9-Octcadecene,1,1, Dimethoxy

**Conclusion:** This study results indicate that the bioactive metabolites produce the endophytic fungi *Aspergillus flavus* could be promising source as antimicrobial agents.

**Keywords:** *Moringa oleifera*, *Aspergillus flavus,* Antimicrobial Activity, FT-IR, GC-MS, NMR


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## INTRODUCTION

Endophytes are symbiotic organisms that inhabit the interior of the plants without causing an apparent harm to the host [1]. All vascular plants harbor endophytic organisms which protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites. These endophytic fungi play an important physiological and ecological role in their host life [2]. The distribution of endophytic mycoflora differs with the host. Medicinal plants are known to harbor endophytic fungi that are believed to be associated with the production of pharmaceutical products [3]. A variety of relationships exist between fungal endophytes and their host plants, ranging from symbiotic to antagonistic or opportunistic pathogenic [4]. These endophytic fungi produce several compounds possessing anticancer, antidiabetic, insecticidal and immune-suppressive compounds [5]. They produce biologically active metabolites especially xanthonoids, bioflavonoid, triterpenoids, polyisoprenylated benzophenone, lactones and phenolic acids [6].

The emergence of multi-drug resistant pathogens is increasing a global challenge to both human and veterinary medicine. There is a need to develop novel antimicrobial agents to minimize the threat of antimicrobial resistance and exhibits high effectiveness, low toxicity and negligible environmental impacts [7, 8]. *Moringa oleifera* is a medicinal plant belonging to monogeneric family Moringaceae. Almost all the parts of this plant have been used for the various ailments in the indigenous medicine [9]. This study was conducted to investigate the antimicrobial potential of endophytic fungi associated with *Moringa oleifera* against pathogenic microbes.

## MATERIALS AND METHODS

### Source of endophytic fungi

The stem, leaves, flowers and calyx of *Moringa oleifera* (Murungai) were sampled for the investigation of the endophytic fungal communities. Healthy and mature plants were carefully chosen for sampling from Omalur region, Salem district. The plant was randomly collected from different sites brought to the laboratory in sterile bags and processed immediately to reduce the chances of contamination.

### Isolation and identification of endophytic fungi

Surface sterilization was done by immersing the plant parts in 4% sodium hypochlorite solution for 90 sec followed by 70% ethanol treatment for 5 sec, thoroughly washed with sterile distilled water and blot dried between sterile filter paper [10]. The surface sterilized segments were placed onto Petri dishes containing Sabouraud dextrose agar (SDA) medium. Six segments were placed for each plate. The plates were continuously monitored for growth. The endophytic fungi were identified according to their macroscopic (front and reverse side of fungal colonies) and microscopic characteristics such as the morphology of fruiting structures and spore morphology.

### Fermentation and extraction

The isolated endophytic fungi (SS03) were grown on PDA at 25 ° C for5 d. Two or three pieces of the grown culture was cut from the plate and were inoculated onto 1000 ml Erlenmeyer flask containing 300 ml Malt Extract broth for 15 d at 25 °C, 120rpm. The broth culture was filtered to separate the filtrate and the mycelia. The filtrate was screened antimicrobial activity. Filtrates including all those showing antimicrobial activity were extracted three times with ethyl acetate. After evaporation of the combined ethyl acetate extracts, the brown color residue was obtained. The crude extract was dissolved in DMSO to obtain different concentrations [11].

### Antimicrobial activity

The bacterial strains used in this study were *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa* and yeast strains which

are dental isolates of *Candida* spp. All the strains were maintained on agar slants without any contamination. The antimicrobial evaluation of the culture filtrate was determined by agar well diffusion method [12]. The Mueller-Hinton agar (MHA) plates were inoculated with an overnight culture of bacterial and fungal suspension, by evenly spreading with sterile cotton swabs. The agar wells were prepared by scooping out the media with a sterile cork borer (7 mm in diameter). The wells were then filled with different concentration 20 µl, 40 µl and 60 µl of the *Aspergillus flavus* extract (SS03) dissolved in DMSO. The plates were then incubated at 37 ℃ for 24h and the zone of inhibition was recorded and compared with the control.

**GC-MS analysis and NMR analysis**

The powdered residue of extracted bioactive compound was analyzed using a Shimadzu gas chromatography apparatus (Model-TRACE ULTRA VER: 5.0) using a MS DSQ II capillary column (30m) equipped with QP MS detector (EI, 70 ev) with helium as a gas at a flow rate of 1 ml/minute. The components were identified by computer search, followed by matching the mass spectral data with those held in the database library. The compound name, molecular formula, molecular weights were ascertained and tabulated. The ethyl acetate endophytic fungi fraction subjected to GC-MS analysis was performed at SRM University, Chennai, Tamilnadu, India. The interpretation on mass spectrum GC-MS performed based on the database of National institute standard and technology having more than 62000 patterns. The spectrum of the unknown compound was compared with the spectrum of the known compound in the NIST library. The compound name, molecular formula, molecular weights were ascertained and tabulated and nuclear magnetic resonance analysis was also performed.

**RESULTS**

In this study, about 72 segments (18 segments of each part respectively) of the medicinal plant were screened for the isolation of the endophytic fungi. A total of 15 endophytic fungi was isolated and identified from 72 segments of the medicinal plant. The stem segments showed a maximum repository for the endophytic fungi than the leaf segments. Endophytic fungi names were *Aspergillus flavus, Aspergillus versicolor, Aspergillus niger, Aspergillus ochraceus, Aspergillus terreus* and *Bipolaris* spp.

All the 15 endophytic fungi culture filtrate was checked that antimicrobial activity against clinical pathogens. The endophytic fungi culture filtrate of *Aspergillus flavus* (SS03) showed maximum activity against the tested pathogens. The 60 µl concentration showed that highest activities were *Bacillus subtilis* and *Candida tropicalis* (table 1). *Aspergillus flavus* (SS03) proved as a good bioactive compound against these clinical isolates tested. These strains were further taken for extraction with ethyl acetate to obtain a fine residue of the bioactive compound which was further dissolved in DMSO and subjected to the spectral analyses (GC-MS, and NMR).

In GCMS analysis, 8 compounds from the ethyl acetate fraction fungal strain (SS03) were identified based on the molecular formula, molecular weight, and peak value. The unknown compounds were compared with the NIST library. The compounds identified were 3A, α, 4β, 7A, (β)-octahydro-4-methoxy-3A, 7A; Benzene-nonyl; Fenaclon; 5, 14 dimethyl-2, 10-dioxa-13-methyl; (R)(-)14 methyl-8-hexadecyn-1-ol; Trans-β-farnesene (E)-β-farnesene; 9-Octcadecene, 1, 1, Dimethoxy; 1-formyl-2, 2, dimethyl-3-trans-(3-methyl-2-buten-1-yl) (Table2). In Nuclear Magnetic Analysis, aromatic and hydroxylic compounds were identified in the crude fungal extract (table 3). Fenaclon, (R) (-) 14 methyl-8-hexadecyn-1-ol, Trans-β-farnesene (E)-β-farnesene, 9-Octcadecene, 1,1, Dimethoxy, aromatic and hydroxylic compound were present in the filtrate. Both techniques peak value showed that (fig. 1and2). So these compounds were provided the antibacterial activity, antimicrobial activity and various pharmacological activities.

**Table 1: Antibiogram pattern of ethyl acetate extract of endophytic fungus *Aspergillus flavus* (SS03)**

| S. No. | Clinical isolates | Inhibition zone of aspergillus flavus culture filtrate mm±SD | |
|---|---|---|---|
| | | | Positive control |
| 1 | *S. aureus* | 25.5±0.5 | 21.13±0.12 |
| 2 | *B. subtilis* | 29.56±0.2 | 20.33±0.57 |
| 3 | *P. aeruginosa* | 20.66±0.57 | 21.13±0.12 |
| 4 | *Candida albicans* | 18.0±1.0 | - |
| 5 | *Candida tropicalis* | 26.22±0.20 | 21.13±0.12 |
| 6 | *Candida krusei* | 22.0±0.57 | 19.0±1.0 |

Values are means of three replicates±Standard Deviation itive control Gentamicin-20mcg/Disc and Co-Trimoxazole-25mcg/Disc

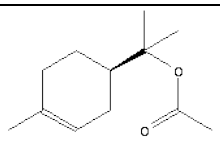
**Table 2: Compounds identified in the ethyl acetate fungal crude extract (SS03) by GC-MS**

| Retention time | Compound name | Molecular formula | Molecular weight |
|---|---|---|---|
| 2.080 | 3A, α, 4β,7A,(β)-octahydro-4-methoxy-3A, 7A | $C_{12}H_{20}O_2$ | 196 |
| 2.400 | Benzene-nonyl | $C_{15}H_{24}$ | 204 |
| 13.192 | Fenaclon | $C_{11}H_{14}ONCl$ | 184 |
| 14.822 | 5, 14 dimethyl-2, 10-dioxa-13-methyl | $C_{15}H_{18}O_3$ | 246 |
| 16.712 | (R)(-)14 methyl-8-hexadecyn-1-ol | $C_{17}H_{32}O$ | 252 |
| 18.713 | Trans-β-farnesene $$(E)-β-farnesene | $C_{15}H_{24}$ | 204 |
| 25.562 | 9-Octadecene, 1, 1, Di-methoxy | $C_{20}H_4O_2$ | 312 |
| 26.842 | 1-formyl-2, 2, dimethyl-3-trans-(3-methyl-2-buten-1-yl) | $C_{15}H_{24}O$ | 246 |

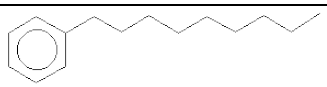
**Table 3: Compounds identified in the ethyl acetate fungal crude extract (SS03) by NMR**

| δ Value ppm | Class | Type of proton |
|---|---|---|
| 1.04 | Hydroxylic | R-OH |
| 6.15 | Aromatic | Ar-H |
| 2.05 | Iodides | CH-I |
| 1.56 | Tertiary | $R_3CH$ |
| 0.52 | Primary | $RCH_3$ |
| 0.13 | Alcohols | CH-OH |
| 1.33 | Secondary | $R_2CH_2$ |

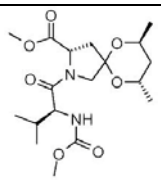
**Structural elucidation of fungal crude extracts compound**



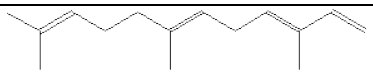
$C_{12}H_{20}O_2$



$C_{15}H_{24}$



$C_{11}H_{14}O\,NCl$



$C_{15}H_{24}$

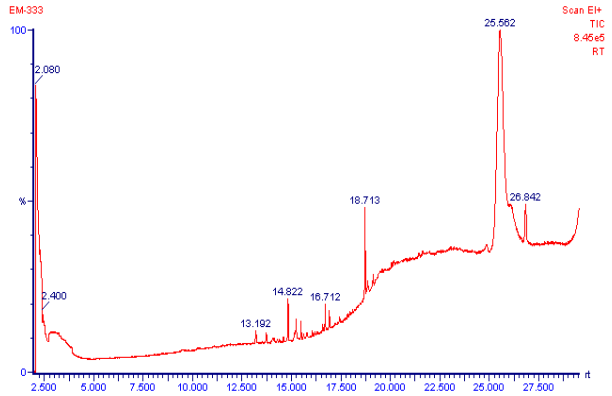



**Fig. 1: GC-MS chromatogram of the ethyl acetate fungal crude extract**

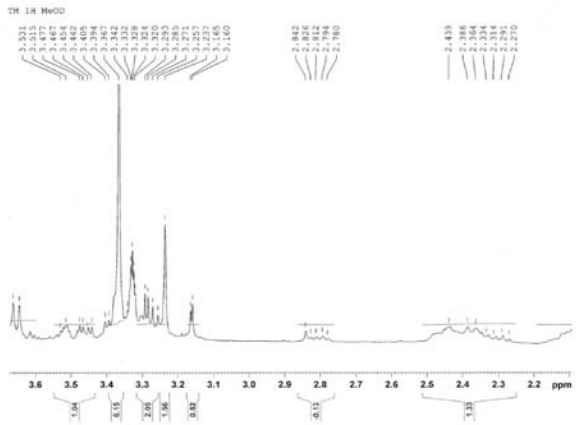



**Fig. 2: $^1$H NMR analysis of crude fungal extract (SS03)**

## DISCUSSION

Medicinal plants and herbal medicines catapulted into a patent war zone as highly valuable commodities through modern technologies. Many developed and developing countries were actively engaged with medicinal plants for therapeutically precious and biologically active Phytochemical [13]. This study also showed such a trend of the diversity of endophytic fungi which was apparent with the leaves, stem, flowers and calyx parts of medicinal plant *Moringa oleifera* collected from the Omalur region. Out of 72 segments screened, the predominant fungal genus isolated was *Aspergillus* spp. These results were similar to the studies of [14] isolated that *Alternaria* spp., *Aspergillus* spp., *Bipolaris* spp., *Exosphiala* spp., *Nigrospora* spp., and *Penicillium* spp. in *Moringa oleifera* and the study of (5) which reported *Aspergillus* spp., as the predominant in the leaves, stem and roots of *M. oleifera.* The extracted fungal extract showed inhibitory activity against all gram-positive and yeast strains. This study coincides with the study of ([15] observed that *Aspergillus* spp. and *Pestalotiopsis* spp. which inhibited gram-positive bacteria and *Candida albicans*, while inhibition by *Aspergillus* sp. was confined only to bacterial isolates. [7] Demonstrated that the *Aspergillus candidus* crude extract showed a most sensitive zone of inhibition against *Candida albicans* and *Streptococcus pyrogens*. The ethyl acetate extract fraction of *Aspergillus flavus* (SS03) bioactive compounds were subjected to GC-MS and NMR analysis peaks showed characteristics stretching frequency which indicated the presence of functional groups of aromatic compounds. These groups were similar to the study of [16] who reported the bioactivity of *Phomopsis* spp. in a medicinal plant *Allamanda cathartica*. In this study, the phenols, primary, secondary and tertiary alcohols, iodides, fluorides, carboxylic acids, hydroxylic acids, and aldehydes were found to be predominant while the([11] found phenols as the major constituents which play a major role in biological activities. As the endophytic fungal extracts showed a diverse group of bioactive compounds, the crude extracts should be further purified and other *in vivo* studies have to be hypothesized to rule out the emerging drug-resistant pathogens through the modern technologies in pharmaceutics.

## CONCLUSION

It can be concluded that endophytic fungi are a novel source of the bioactive compound. The discovery and production of secondary metabolites from endophytic fungi have begun as an exciting field in biotechnology. The endophytic fungi *Aspergillus flavus* are promises to be of antimicrobial compounds. The diversity of these medicinal plants has to be conserved in the future years for the benefit of the mankind.

## CONFLICT OF INTERESTS

Declared none

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