

# *In vitro* Antimicrobial Activities of Endophytic Fungi Isolates from Medicinal Tree - *Melia azedarach* L.

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**Abstract** Endophytes, found ubiquitous in all plant species in the world, contribute to their host plants by producing plethora of substances that provide protection and ultimately survival value to the plant. Many researchers have proven that endophyte is a new and potential source of novel natural products for exploitation in modern medicine, agriculture and industry. So far, a great number of novel natural products possessing antimicrobial activities have been isolated from endophytes. These are endophytic microorganisms that inhabit the intercellular spaces of plant tissues and are often responsible for antimicrobial production. The aim of the present study was to screen for antimicrobial activity in two endophytic filamentous fungus *Aspergillus flavus* and *Trichoderma koningii* Oudem isolated from surface sterilized leaves and root of *Melia azedarach* L. and exhibited significant inhibitory activity on the test microorganism by using agar diffusion method. The strongest antimicrobial activity was exhibited by *Aspergillus flavus* against *Staphylococcus aureus* where as also in *Trichoderma koningii* Oudem, with  $20.2 \pm 0.6$  and  $18.05 \pm 0.8$  inhibition, respectively. The two endophytic fungi isolated from leaves and roots tissues of *Melia azedarach* L. as well as their antimicrobial activity detected in the crude fungal extracts cultures were being reported for the first time.

**Keywords** Endophytic Fungi, *Melia Azedarach* L. Antimicrobial Activity

## 1. Introduction

Microbial endophyte is a microbial colony which resident inside of healthy plant tissues. Endophytic microbes have been recognizing as a chemical producer with a broad range biological activity. World health problems caused by drug-resistant bacteria and fungi are increasing. An intensive search for newer and effective antimicrobial agents is needed. Endophytic fungi have been recognized as useful sources of bioactive secondary metabolites[1],[2]. A recent complete study has indicated that 51% of biologically active substances isolated from endophytic fungi were previously unknown[1]. Many endophytic fungi have the ability to produce antimicrobial substances[4-11]. The mis-usage of antibiotics since the “Golden Age of Antibiotics” in 1950s had caused the threats of antibiotic resistant “super bugs”. Hence, today, many academic institutions are investing necessary resources to search for new natural products with significant pharmacological activity. Majority of the organisms containing new metabolites are disappearing rapidly and screening of the natural products has become costly and laborious process. Endophytes, which occupy a unique biotope with global estimation up to one million

species, are a great choice to avoid replication in the study of natural products[3]. *Melia azedarach* L. (Meliaceae) commonly known as Mahanimba, Ramyaka, Dreka, Karmuka, Keshamushti, Persian lilac and White cedar. *M. azedarach* L. is a native of West Asia and now naturalized throughout the warm countries. In India, it grows wild in the Sub - Himalayan tract up to 1800 m. The various parts of the tree are reputed to have the same therapeutic values as those of neem tree (Vaidyaratnam PSV, 1994). However, increasing use of *Melia azedarach* L. in recent years for making traditional medicine. Since the endophytes can be found in nearly all living plant species, a scientific basis in plant selection is necessary for the study of endophytes, to isolate microorganism with pharmaceutical potential. Previous history of the plant's extensive use in traditional medicines can be criteria for plant selection[3]. As part of our continuing efforts towards finding new antimicrobial compounds, *Melia azedarach* L., commonly known as Bakhan in India, was used in this study. The photochemistry and pharmacological studies conducted on this medicinal herb have successfully isolated several natural products including diterpenes, monoterpenes, triterpenes, saponins, flavonoids hexoses, organic acids, rosmarinic acid, chromene and myoinositol[12][13]. However, therapeutic effect of this medicinal herb is due to the polyphenols. Numerous reports are available on the pharmaceutical potentials of *Melia azedarach* L. wide range of biological activities including diuretic activity, antihypertensive,

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anti-inflammatory, antibacterial, antifungal, hepto-protective, antioxidant, antitumor, anti-allergic, and antimalarial activity[12][14].

However the medicinal values of their endophytes have not been investigated. The objective of this study was to isolate endophytic fungi from *Melia azedarach* L. and to study the antimicrobial activity of the isolates on various pathogenic microorganisms.

## 2. Method and Materials

### 2.1. Plant Sample Collection

Mature healthy plant leaves and roots were collected by sampling from different parts of the tree growing in the University Nursery at Rajasthan University, Jaipur. All samples were stored in sterile polythene bags and samples were used to isolate endophytic fungi within 12 hours of collection.

### 2.2. Isolation of Endophytic Fungi

#### 2.2.1. From Leaves

Discs of leaves (0.5 cm diameter) were cut using a sterile scalpel. Samples were washed thoroughly in running tap water. Surface of the plant tissues was treated by following the methodology of Murali *et al.*[15]. Plant tissues were immersed in 75% ethanol for 1 min and in an aqueous solution of sodium hypochlorite (2.5% available chlorine) for 15 min, followed by washing with 70% ethanol for 5 sec. The tissues were then rinsed in sterile distilled water and allowed to surface-dry under sterile conditions. The surface-sterilized samples were placed on Petri dishes containing Potato Dextrose Agar (PDA) supplemented with streptomycin (100 µg/ml) to inhibit bacterial growth and incubated at room temperature at around 25°C.

#### 2.2.2. From Roots

Be present concerned to isolate endophytic fungi only from within roots that appeared healthy according to the definition of Hooker *et al.*[16]. They observed discoloration and darkening of roots as they approached mortality. Roots were surface sterilized to kill mycelium and spores on the root surface without eliminating mycelium growing in the outer cortex. Paler roots of healthy appearance were washed in tap water to roughly remove soil particle from the root tissue than cut away as close to the shoot base as possible, and surface sterilized in a 72% ethanol solution for 30 s, transferred to a solution of 10% sodium hypochlorite for 10–15 min, and rinsed four times in sterile distilled water for four and five times. The roots were then dried in a stream of sterile air (to reduce bacterial growth). The sterilized fine roots were cut into 0.3 to 0.5 cm segments, plated on modified medium PDA containing 1 ml 10% chloramphenicol in ethanol, 400 ml 0.5% dichloran, 10 g sucrose, 10 ml 1% Triton X-100 and 990 ml sterile distilled

water. Dichlorans (a dye)[17] and Triton (a nonionic detergent)[18]. The processes reduce the radial growth rate of fungal mycelia and prevent rapidly growing species from over-growing and obscuring the slower growing species. Eight root segments were placed on each plate, and for each quadrat a total of 15 plates were inoculated. Plates were incubated in diffuse daylight at room temperature (20–25°C). Root pieces exhibiting fast growing rapidly sporulating fungi were removed, while slower growing fungi select and were sub cultured. After 3 weeks the number of colonies per plate was recorded, colonies re-isolated where necessary, and each identified to species level where possible.

### 2.3. Identification of Isolated Endophytic Fungi

Cultural and morphological characters of the fungus for studying the cultural and morphological characters, the fungus was grown on PDA. Cultural characters such as color and nature of the growth of the colony were determined by visual observation. Morphological characteristics of the fungus like mycelia, conidiophores and conidia were microscopically studied under the light microscope. Mycelia, conidiophores and conidia produced by the fungus in the culture were examined under the microscope.

For the present investigation only two endophytic fungi (*Trichoderma koningii* Oudem and *Aspergillus flavus*) were used study of antimicrobial activity among all identified fungi.

### 2.4. Fermentation and Extraction

Each of the endophytic fungi isolated from different plant part as leaves and root of *Melia azedarach* L. In the first stage, these fungi were grown in submerged culture and in the second stage they were grown as stationary culture. These fungi were grown on PDA at 25°C for 7 days. Seven day-old cultures on PDA of the endophytic fungi were inoculated into Malt Extract Both (MEB) in Erlenmeyer flasks 250 ml, followed by stationary condition and incubated for 30 days at 25°C. After 3 weeks of incubation the culture was harvested and passed through four layers of muslin cloth to separate the mycelia mat from the culture filtrate. The fermentation broth of each fungal endophyte was filtered, and the filtrate was extracted three times with methanol at room temperature. Evaporation of the extracted solution was done in a rotary evaporator. The broth culture was filtered to separate the filtrate and mycelia. The filtrate was used for preliminary testing of antimicrobial activity.

### 2.5. Microorganisms Used

Clinical laboratory bacterial isolates of *Staphylococcus aureus* (Gram +ve) and *Pseudomonas aeruginosa* (Gram -ve) and fungal isolates viz. *Penicillium chrysogenum* and *Fusarium oxysporum* were collected from the stock cultures of Microbiology Laboratory, SMS Medical College, Jaipur India.

### 2.6. Antimicrobial Assay

The antibacterial screening of methanolic extracts of fungi were carried out by determining the zone of inhibition using agar diffusion method [19][20]. The strains of microorganisms obtained were inoculated in conical flask containing 100 ml of nutrient broth. These conical flasks were incubated at 37°C for 24 hours. The extracts were dissolved in methanol, which was already tested for antibacterial activity against all test microorganisms and found to have no antimicrobial activity. The extracts were made as 50 mg/ml concentration solutions and finally sterilized by filtration using 0.45 µm Millipore filters. The sterile discs (6 mm in diameter) were impregnated with 2.5 µl of above extract solution and placed in inoculated agar. These plates were firstly placed at low temperature (approximately 28°C) for one hour and consent to the maximum diffusion of compounds from the test discs to the agar plates and later incubated at 37°C for 24 hour in case of bacteria and 48 hours for fungi, after which the zone of inhibition could be easily observed. Three replicates of each test extracts were examined and the mean values were then recorded. The inhibition zones produced by the endophytic fungi extracts.

### 3. Results and Discussion

Total 35 endophytic fungi isolates and identified from each of the leaves and root surface sterilized tissue of *Melia azedarach* L. (Table-1) In this process total 970 leaves and root samples (leaves and root segments) were use. In which one species of *Aspergillus flavus* (Figure 1; A) and one species of *Trichoderma koningii* Oudem (Figure 1; C) were belonging to class Ascomycetes and sorderiomycetes, respectively. Preliminary results on *Melia azedarach* L. leaves and root tissue isolation show only 2 classes of fungi which produced sterile mycelium (Figure 1; B-D) (Table -2). The fungal colony is identified on the based colony morphology and examination of spores and fruiting bodies. The identification under light microscopes showed presence *Aspergillus flavus* and *Trichoderma koningii* Oudem are the dominant mycoflora from the leaves and roots of *Melia azedarach* L. These both fungi were used for their antimicrobial activity on some pathogenic microbes (*S. aureus*, *P. aeruginosa*, *P. chrysogenum* and *F. oxysporum*) by agar diffusion method. This is the first report on the endophytic fungi (*Aspergillus flavus* and *Trichoderma koningii* Oudem) associated with *Melia azedarach* and their crude fungal extract shows antimicrobial activity.

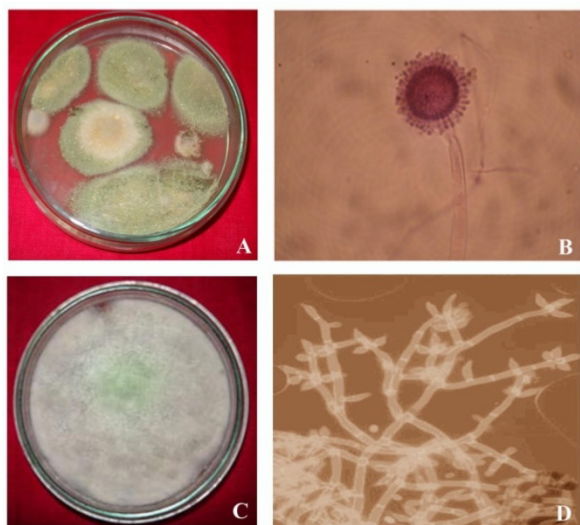
**Table 1.** Occurrence of fungal species in leaves and root tissue of *Melia azedarach* L.

| S. No | Class of isolated endophytic fungi | Name of isolated endophytic fungi   | Endophytic fungi isolated from | Frequency of colonization | Number of isolated endophytic fungi. | *Identification No. of isolated endophytic fungi |
|-------|------------------------------------|-------------------------------------|--------------------------------|---------------------------|--------------------------------------|--|
| 1.    | Ascomycetes                        | <i>Aspergillus flavus</i>           | Leaves                         | 16.6%                     | 5                                    | 8352   |
| 2.    | Sordariomycets                     | <i>Trichoderma koningii</i> . Oudem | Root                           | 14.2%                     | 7                                    | 4302   |

\*ID number from ITCC identification / culture supply Services, Division of plant pathology, Indian Agricultural Research Institute, New Delhi

**Table 2.** Morphological description of endophytic fungi isolated from root tissue of *Melia azedarach* L.

| Plant Organ | Taxon           | Fungi                             | Morphological description   |
|-------------|-----------------|-----------------------------------|---|
| Leaves      | Ascomycetes     | <i>Aspergillus flavus</i>         | <b>Colonies:</b> light greenish-yellow<br><b>Reverse side colonies:</b><br><b>primary stage-</b> yellowish,<br><b>Mature stage-</b> brownish. Conidiophores arose from submerged hyphae.<br><b>Conidiophores walls:</b> pitted, rough and uncolored.<br><b>Conidial head:</b> hemispherical<br><b>Conidia:</b> pyriform and globose.<br><b>Conidia size:</b> 3-4  |
| Root        | sordariomycetes | <i>Trichoderma koningii</i> Oudem | <b>Colonies:</b><br><b>primary stage-</b> transparent<br><b>Mature stage-</b> green or yellow less frequently white.<br><b>Conidiophore:</b> verticillately branched and that bear conidia in a drop of clear green liquid at the tip of each phialide.<br><b>Conidial head:</b> spherical green.<br><b>Conidia:</b> broadly ellipsoidal, smooth walled and green in mass.<br><b>Conidia size:</b> 3-5 x 2-4 µm |

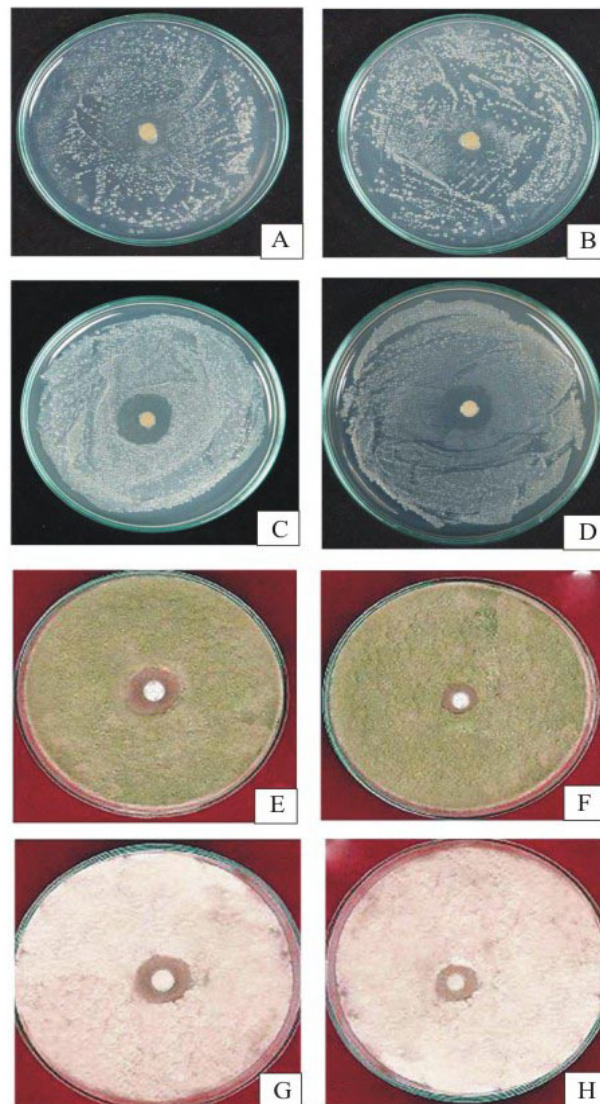


**Figure 1.** A: Cultivation of *Aspergillus flavus* from leaves of *M. azedarach* Linn. B: Microscopic figure of *Aspergillus flavus*. Mature conidiophores bearing sterigmata and chain of conidia in *Aspergillus flavus*. C: Cultivation of *Trichoderma koningii* Oudem from root of *M. azedarach* Linn. D: Microscopic figure of *Trichoderma koningii* Oudem. Conidiophores, Phialides and intercalary phialide in *Trichoderma koningii* Oudem

The results of the antifungal and antibacterial activity of methanolic extracts of endophytic fungi isolated from *Melia azedarach* L. are presented in (Table-3). Results showed that all the test samples used in the study depicted antimicrobial activity against one or more species of the tested organisms. Both methanol extract of *Aspergillus flavus* and *Trichoderma koningii* Oudem showed higher activity against *Staphylococcus aureus* with  $20.2 \pm 0.6$  mm and  $18.05 \pm 0.8$  mm inhibition zone, respectively (Figure 2; A & B). However, the methanolic *Trichoderma koningii* Oudem extract of (IZ =  $14.36 \pm 0.7$ ) (Figure 2; C) inhibited the growth of *P. aeruginosa* effectively and *Aspergillus flavus* extract (IZ =  $10.22 \pm 0.8$  mm) (Figure 2; D) was found to be less effective against *P. aeruginosa* as compared to all microbes.

All the extracts obtained from endophytic fungi of *M. azedarach* demonstrated potent fungicidal activity against *Penicillium chrysogenum* and *Fusarium oxysporum*. *Aspergillus flavus* extracts (IZ =  $16.4 \pm 0.7$  mm) (Figure 2; E) exhibited greater activity than *Trichoderma koningii* Oudem (IZ =  $10.02 \pm 0.4$  mm) (Figure 2; F) against *Penicillium chrysogenum*. Whereas in *Fusarium oxysporum*, *Aspergillus flavus* extract showed higher (IZ =  $17.05 \pm 0.2$ )

(Figure 2; G) activity than *Trichoderma koningii* Oudem (IZ =  $12.08 \pm 0.9$ ) (Figure 2; H).



**Figure 2.** A: Methanolic extract of *Aspergillus flavus* against *Pseudomonas aeruginosa*. B: Methanolic extract of *Trichoderma koningii* Oudem against *Pseudomonas aeruginosa*. C: Methanolic extract of *Aspergillus flavus* against *Staphylococcus aureus*. D: Methanolic extract of *Trichoderma koningii* Oudem against *Staphylococcus aureus*. E: Methanolic extract of *Aspergillus flavus* against *Penicillium chrysogenum*. F: Methanolic extract of *Trichoderma koningii* Oudem against *Penicillium chrysogenum*. G: Methanolic extract of *Aspergillus flavus* against *Fusarium oxysporum*. H: Methanolic extract of *Trichoderma koningii* Oudem against *Fusarium oxysporum*

**Table 3.** antimicrobial activity of *Aspergillus flavus* and *Trichoderma koningii* extracts

| Zone of inhibition (mm)           |                              |                               |                                |                           |
|-----------------------------------|------------------------------|-------------------------------|--------------------------------|---------------------------|
| Microbes                          | <i>Staphylococcus aureus</i> | <i>Pseudomonas aeruginosa</i> | <i>Penicillium chrysogenum</i> | <i>Fusarium oxysporum</i> |
| Extracts                          |                              |                               |                                |                           |
| <i>Aspergillus flavus</i>         | $20.2 \pm 0.6$               | $10.22 \pm 0.8$               | $16.4 \pm 0.7$                 | $17.05 \pm 0.2$           |
| <i>Trichoderma koningii</i> Oudem | $18.05 \pm 0.8$              | $14.36 \pm 0.7$               | $10.02 \pm 0.4$                | $12.08 \pm 0.9$           |

Values are mean inhibition zone (mm)  $\pm$  S.D of three replicates

In case of other species of *Trichoderma* like *Trichoderma reesei* and *Trichoderma viride* showed potent antibacterial activity against the bacterial strains of *E. coli*, *S. agalactiae*, *S. aureus*, *S. pneumoniae*, *S. pyogenes*, *E. faecalis*, *K. Pneumoniae*, *S. Sonnei*, *P. mirabilis* and *P. aeruginosae* have been reported[21]. Similarly, other species of *Aspergillus* (*Aspergillus niger*) exhibited moderate antimicrobial activity against the bacterial stains (*K. pneumoniae*, *E. coli*, *S. aureus*, *P. aeruginosa*, *P. fluorescens*, *C. michiganensis*, *X. oryzae*, *X. axanopodi*) and fungal stains (*A. flavus*, *A. niger*, *A. nidulans*, *A. flaviceps*, *A. carthami*, *A. helianthi*, *C. cartham*, *F. solani*, *F. oxysporum*, *F. verticilloides*, *N. oryzae*)[22].

The agar diffusion method which was used in the present study is one of the most commonly employed methods among those that evaluate antimicrobial activity. The antimicrobial properties of endophytic fungi have been investigated by a number of researchers on some plants, including *Melia azedarach* L.[23], *Mirabilis jalapa* L.[24], *Camptotheca acuminata*[25], *Quercus variabilis*[26] etc.

The test organisms used in this study are associated with various forms of human infections i.e. *Pseudomonas aeruginosa* causes Septicemia and can infect the gall bladder, surgical wounds, skin lesions especially in debilitate and immunodeficient patients. *S. aureus* can cause a range of illnesses from minor skin infections, such as pimples, impetigo, boils (furuncles), cellulitis folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as Pneumonia, Meningitis, Osteomyelitis, Endocarditis, Toxic shock syndrome (TSS), Bacteremia, and Sepsis. *Penicillium chrysogenum* causes a variety of manifestations of infection such as invasive Aspergillosis, involving several organ systems (particularly Pulmonary disease), Pulmonary Aspergilloma and Allergic Bronchopulmonary Aspergillosis etc.

## 4. Conclusions

In conclusion, results from the current studies indicate that endophytic fungi demonstrated a broad spectrum of activities against bacteria (Gram+ve and Gram-ve) and fungi and support its use as a potent antibacterial and antifungal agent.

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