ISOLATION OF ENDOPHYTIC FUNGI FROM LEAVES OF 
AZadirachta Indica AND PRELIMINARY SCREENING FOR 
ANTIMICROBIAL ACTIVITY

Pranay Jain* and Tarun Kumar

Department of Biotechnology Engineering, University Institute of Engineering and 
Technology, Kurukshetra University, Kurukshetra, Haryana.

ABSTRACT
Endophytes are microorganisms that reside asymptotically in the 
tissues of higher plants and promising source of novel organic natural 
metabolites exhibiting a variety of biological activities. The Objective 
of present study was to isolate endophytic fungal strains from the 
leaves of Azadirachta indica and screen the isolated strains for 
antibacterial and antifungal metabolites production. The leaves were 
sterilized, inoculated in the potato dextrose agar medium and incubated 
at 28±2°C for 7days. A total of 4 strains TK1, TK2, TK3, and TK4 
were isolated and used for further studies. The strains were cultivated 
on potato dextrose broth in 500 ml Erlenmeyer flask containing 200 ml 
medium and incubated in BOD shaking incubator for 10-12 days at 
28±2°C with periodic shaking at 150 rpm. Resulting broth was 
separated from the mycelium and extracted 2-3 times with ethyl acetate 
to obtain crude extracts. Agar well diffusion method was used to 
evaluate the antimicrobial potential of the fungal isolates. Out of these four strains TK3 
identified as Aspergillus spp. showed antimicrobial activity against five Gram positive 
bacteria (Streptococcus pyogenes, Streptococcus mutans, Staphylococcus aureus, Bacillus 
subtilis, and Bacillus megaterium), two Gram negative bacteria (Escherichia coli and 
Pseudomonas fluorescens) and five fungal pathogens (Candida tropicalis, Candida glabrata, 
Candida albicans, Alternaria solani and Fusarium graminearum). Zone of growth inhibition 
against test pathogens ranged between 16-36 mm. The results showed the ability of the 
isolated fungal strain TK3 capable of producing antimicrobial compounds and helped in 
justifying the traditional use of Azadirachta indica against human pathogenic bacteria.
KEYWORDS: Asymptomatic, Antifungal, Antibacterial, Endophytes, *Azadirachta indica*.

INTRODUCTION
The term endophyte (from Greek endon, within; phyton, plant) (Schulz and Boyle, 2005), was first used to referred to any organism found within tissues of living plants (Arnold, 2008). All vascular plants harbor endophytic organisms in all parts like leaves, stem, bark, fruit, and roots (Zhang et al., 2006). The opportunity to find new antimicrobial products from interesting endophytes among myriads of plants in different niches and ecosystem is great. Endophytes are a source of large number of bioactive secondary metabolites with unique structure including alkaloids, benzopyranones, flavonoids, phenolicacids, quinines, steroids, terpenoids, tetralones, xanthones and other (Tan and Zou, 2001). Such bioactive metabolites find wide ranging application as agrochemical, antibiotics, immunosuppressant, autoparasitic, antioxidant and anticancer agents (Gunatilika, 2006). Many studies have emphasized endophytes from medicinal plants, since its isolation until their application in different areas (Bernardi et al., 2010; Garcia et al., 2012; Gazis and Chaverri, 2010; Orlandelli et al., 2012; Rhoden et al., 2012). A survey reveals that the number of novel chemical structures produced by endophytes (51%) is significantly higher than the soil fungus (38%), suggesting that these frequently overlooked endophytes are the novel source of bioactive secondary metabolites (Khan et al., 2012).

*Azadirachta indica* is native to India and also known as neem. It belongs to the family meliaceae. All parts of this plant show an array of negative effects on insects including ovipositor deterrent, anti-feedant, and other inhibitory activities (Butterworth et al., 1972; Koul et al, 1990). More than 100 compounds have been isolated from various parts of the neem tree (Siddiqui et al., 1988; Ley et al., 1993) and most of the active principles (Limnoids) belong to the group of tetranortriterpinoids especially „Azadirachtin” and its analogs (Kaushik et al., 2002). Several reports in the recent years show that the endophytic fungi from this host produce several bioactive compounds (Wu et al., 2008; Li et al., 2007; Kharwar et al., 2009; Wu et al., 2009).

In lieu of the above justification, the current study was carried out to isolate the fungal endophytes from the leaves of *Azadirachta indica* and screen the strains for their antimicrobial activity against human pathogenic microorganisms.
MATERIAL AND METHOD

Collection of Plant Sample
Healthy and full-grown leaves free from insect, disease and mechanical damage were collected from various locations in Kurukshetra, Haryana, India. Leaves were excised with the help of a sterile scalpel. The leaves samples were collected into a small sterilized air tight polythene zip bags and preserved at 4°C in refrigerator until processed for isolation.

Surface Sterilization of Leaf Sample
The collected explants were washed with running tap water for 3-4 times to remove all the particulates like dust and debris adhered on the surface. The samples were surface sterilized by immersing into 70% ethyl alcohol for 60 seconds, treated with 4% sodium hypochlorite solution for 3-4 min, and then again rinsed with 70% ethyl alcohol for 1 min. After plant material is surface sterilized with the above reagents, it was rinsed thoroughly with sterile water 3-4 times (Strobel et al., 2003).

Inoculation of Leaf Sample
The surface sterilized leaves were excised into small pieces (5×5 mm) with the help of sterile scalpel and inoculated into the petri plates containing Potato dextrose agar medium (PDA). For suppressing the bacterial growth, medium was supplemented with an antibacterial agent e.g. 100μg/ml of streptomycin and incubated at 28 ± 2 °C for 7 days. The plates were frequently observed for fungal growth. On the basis of morphological characteristics fungal mycelia growing out of the sample plates were selected, subcultured and maintained in PDA plates (Strobel et al., 2003). Pure fungal isolates were also preserved in PDA slant tubes for long term preservation.

Cultivation of Fungal Strains
The fungal cultures were grown in Potato dextrose broths for the production of fungal metabolites. Liquid potato dextrose medium (200 ml) was autoclaved in 500 ml Erlenmeyer flask and inoculated with 2-3 discs (6mm in diameter) of isolated pure fungal strain growing on PDA. Flasks were incubated for 10-12 days in BOD shaking incubator for 15 days at 28±2°C with periodic shaking at 150 rpm (Tayung et al., 2010).
Extraction of Fungal Metabolites

After fermentation the broth was separated from the mycelium with the help of cheesecloth. Then this filtrate was extracted 2-3 times with ethyl acetate (1:1) in a separating funnel by vigorous mixing for 30-40 min. The organic phase (Ethyl acetate) containing the fungal metabolites is separated and evaporated in a rotary vacuum evaporator for obtaining the crude extracts (Tayung et al., 2010).

Procurement and Maintenance of Test Pathogens

Human pathogenic bacterial and fungal microorganisms were purchased from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. The tested pathogens include five Gram positive bacteria *Streptococcus pyogenes* (MTCC 1924), *Streptococcus mutans* (MTCC 497), *Staphylococcus aureus* (MTCC 7443), *Bacillus subtilis* (MTCC 121), and *Bacillus megaterium* (MTCC 428); two Gram negative bacteria *Escherichia coli* (MTCC 40) and *Pseudomonas fluorescens* (MTCC 1748); five fungal pathogens *Candida tropicalis* (MTCC 3421), *Candida glabrata* (MTCC3814), *Candida albicans* (MTCC 227), *Alternaria solani* (MTCC 10690) and *Fusarium graminearum* (MTCC 2089). All pathogenic microorganisms were preserved in slants of brain heart infusion agar at 4°C in the refrigerator for future use.

Standardization of Inoculum

Pathogenic microorganisms were grown for 24 h, in LBB medium and adjusted at a concentration adjusted according to 0.5 McFarland standards. McFarland standards are used to adjust the turbidity of bacterial suspensions to obtain a required population of cells in broth medium. McFarland standards were prepared by mixing 0.05 ml of 1.175% barium chloride dihydrate (BaCl₂•2H₂O), with 9.95 ml of 1% sulfuric acid (H₂SO₄) together. The reaction between these two compounds resulted in barium sulfate precipitate, which causes turbidity in the solution. The standard could be compared visually to a suspension of bacteria in sterile saline or nutrient broth (Andrews, 2001).

Determination of Antimicrobial Activity

The antimicrobial activity of the fungal isolates was determined by agar well diffusion method (Khan et al., 1988). The standardized inoculum (100μl) was spread with the help of sterilized cotton swabs on the surface of Muller Hinton Agar plates. On each MHA plate two wells of about 6.0 mm were aseptically punched with the help of sterile cock borer. One of these wells was filled with 100μl of crude extract dissolved in DMSO. Another well was used
for filling DMSO as a negative control. The plates were be incubated at 37°C for 24 h and the zone of inhibition was measured and compared with the control. Experiment was performed in triplicates in each case for validating the results (Pranay Jain and Priyanka Sharma, 2014).

The antimicrobial action was assessed by the diameter (mm) of growth inhibition zones and compared with commercially available antibacterial and antifungal agents like erythromycin and fluconazole (Tayung et al., 2010).

Identification of Fungal Isolate
The isolated endophytic fungus was further stained with lactophenol cotton blue for its identification. The prepared slides with observed under trinocular microscope. Detailed morphological study was carried out. Morphological identification of fungal pathogen was based on the morphology of the fungal culture colony or hyphae, the characteristics of the spores and reproductive structures (Barnett and Hunter, 1998).

RESULTS
Antimicrobial Activity
Out of the four fungal strains isolated and screened for their antimicrobial activity only TK3 identified as an *Aspergillus* sp. was found to be active against all 12 test pathogenic microorganisms. The organic ethyl acetate extracts of TK3 have shown tremendous activity in the form of large zone of growth inhibition. The measured diameter of the zone of growth inhibition against different pathogens and their comparison with the commercially available antibiotics is shown in table 1. The fungal metabolites were found to be most effective against *Streptococcus aureus*, *Bacillus megaterium*, *Escherichia coli*, *Pseudomonas fluorescens*, *Candida glabrata* and *Fusarium graminearum* as shown in figure 1.
Table 1. Antimicrobial activity of metabolites produced by isolated endophytic fungal strain TK3 (Aspergillus spp.).

<table>
<thead>
<tr>
<th>Test Pathogens</th>
<th>Zone of Growth Inhibition (in mm)</th>
<th>Fungal strain TK3</th>
<th>Erythromycin (15 mcg disc) (Antibacterial antibiotic) Positive control</th>
<th>Fluconazole (10 mcg disc) (Antifungal antibiotic) Positive control</th>
<th>DMSO Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram Positive Bacteria</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>19±0.35</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus mutans</td>
<td>22±1.30</td>
<td>10±0.5</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus aureus</td>
<td>32±1.18</td>
<td>12±1</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Bacillus subtilis</td>
<td>30±0.5</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td>Bacillus megaterium</td>
<td>34±.86</td>
<td>10±1.1</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
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<tr>
<td>Gram Negative Bacteria</td>
<td></td>
<td></td>
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<tr>
<td>Escherichia coli</td>
<td>36±1.50</td>
<td>17±0</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
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<tr>
<td>Pseudomonas fluorescens</td>
<td>31±0.66</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
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<tr>
<td>Fungal Pathogens</td>
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<td>Candida tropicalis</td>
<td>25±0.48</td>
<td>ND</td>
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<tr>
<td>Candida glabrata</td>
<td>30±1.80</td>
<td>ND</td>
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<tr>
<td>Candida albicans</td>
<td>25±0.50</td>
<td>ND</td>
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<tr>
<td>Alternaria solani</td>
<td>16±0.68</td>
<td>ND</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Fusarium graminearum</td>
<td>35±0.42</td>
<td>ND</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

All data were the means obtained from tests carried out in triplicates ± S.D of three replicates.

Fig. 1 Antimicrobial activity of the ethyl acetate extracts (organic) of TK3 (Aspergillus spp.) against pathogenic microorganisms A (Fusarium graminearum), B (Staphylococcus aureus), C (Pseudomonas fluorescens), D Bacillus megaterium, E (Escherichia coli) and F (Candida albicans).
DISCUSSION

In recent times a number of new and interesting bioactive metabolites such as antibiotics, antiviral, anticancer and antioxidant compounds having a potential of pharmaceutical, industrial and agricultural application have been isolated and characterized from fungal endophytes. Many researchers have demonstrated that the endophytes isolated from medicinal plants are excellent producers of strong fungicidal, bactericidal and cytotoxic metabolites.

In a study Bharathidasan and Panneerselvam (2011) have investigated *Avicennia marina* for endophytic mycoflora as a possible source of bioactive secondary metabolites. A total 10 fungal species were isolated out of which three were identified as *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus* species. Khan et al (2007) have carried out bioprospecting bioprospecting of fungal endophytes from *Calotropis procera*, a widely used medicinal plant in Indian sub-continent. In their study they have isolated a total of 8 fungal species out of which three were found to be *Aspergillus* species.

Xuan (2014) have isolated a fungal strain *Trichoderma* species from the fruit of *Azadirachta indica* collected in Yuanjiang Country, Yunnan Province, P. R. China. It was classified as a *Trichoderma* species by its morphological characteristics and ITS rDNA sequence analysis. Kusari and Spiteller (2011) here reported the production of the natural insecticides, azadirachtin A and B, by an endophytic fungus *Eupenicillium parvum*, isolated from the Indian neem plant (*Azadirachta indica*). This manuscript lends further evidence on the capability of endophytes to produce host plant secondary metabolites, although further research is necessary for the biotechnological application of the endophytic fungus.

In present study a medicinal plant of Indian origin *Azadirachta indica* was selected to isolate endophytic fungus producing antimicrobial metabolite. Isolated fungal strain TK3 was identified as *Aspergillus* species on the basis of detailed study of fungal culture colony or hyphae, characteristics of the spores and reproductive structures (Barnett and Hunter, 1998). Ethyl acetate extracts of fungal strain have shown antibacterial and antifungal activity against all test pathogens. On comparing antimicrobial activity of endophytic crude extract with the commercially available antibiotics e.g. erythromycin and fluconazole, it was found that the metabolites of TK3 were more effective. Fluconazole did not show any activity against test pathogens. Erythromycin have shown relatively small zone of growth inhibition ranging between 10-17 mm only. On the other hand metabolites of TK3 have shown zone ranging
from 16-36 mm. *Fusarium graminearum* (35 mm), *Staphylococcus aureus* (32 mm), *Pseudomonas fluorescens* (31 mm), *Bacillus megaterium* (34 mm), *Escherichia coli* (36 mm) and *Candida albicans* (25 mm) were found to be most susceptible to metabolites of isolated fungal strain.

**CONCLUSION**

In the present study, four fungal strains were isolated from the leaves of *Azadirachta indica* out of which TK3 showed a considerable amount of antimicrobial activity. Moreover, future pharmacological studies on isolation and identification, safety and efficacy can be applied for the fungal extract aiming their pharmaceutical application.

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