

Title	Isolation, characterization and antimicrobial activities of endophytic fungi from leaves, stems and inner barks of <i>Azadirachta indica</i> A. Juss.
All Authors	Aye Thandar Lwin and Khin Khin Soe
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Abstract	<p>The isolation, characterization and antimicrobial activities of endophytic fungi from leaves, stems and inner barks of <i>Azadirachta indica</i> A. Juss. (Neem) were studied. The plants were collected from University of Mandalay Campus. This study was carried out at Microbiology Laboratory of Botany Department, University of Mandalay from December 2017 to July 2018. Potato Dextrose Agar (PDA) medium was used for the isolation of endophytic fungi. Nine endophytic fungi were isolated and classified from leaves, stems and inner barks of <i>Azadirachta indica</i> A. Juss. Among them, ATL 1, ATL 2, ATL 3, ATL 4 were isolated from leaves. ATL 5, ATL 6 were isolated from stems. ATL 7, ATL 8, ATL 9 were isolated from inner barks. The isolated fungal strains were confirmed ATL 1 as <i>Colletotrichum</i> sp., ATL 2 as <i>Curvularia</i> sp., ATL 3 as <i>Pestalotiopsis</i> sp., ATL 4 as <i>Nigrospora</i> sp., ATL 5 as <i>Aspergillus</i> sp., ATL 6 as <i>Trichoderma</i> sp., ATL 7 as <i>Fusarium</i> sp., ATL 8 as <i>Penicillium</i> sp., ATL 9 as <i>Aspergillus</i> sp. Four isolated fungal strains: ATL 1 - ATL 4 from leaves of <i>Azadirachta indica</i> A. Juss. (Neem) were tested for antimicrobial activities at Biotechnology Research Department, Kyaukse. Those isolated fungal strains showed the antimicrobial activities against <i>Staphylococcus aureus</i>, <i>Bacillus cereus</i>, <i>Escherichia coli</i> and <i>Candida albicans</i>. The isolated fungal strains; ATL 2 (<i>Curvularia</i> sp.), ATL 3 (<i>Pestalotiopsis</i> sp.) and ATL 4 (<i>Nigrospora</i> sp.) showed the inhibition zones (25 mm) against <i>Bacillus cereus</i> and ATL 1 (<i>Colletotrichum</i> sp.), ATL 2 (<i>Curvularia</i> sp.), ATL 3 (<i>Pestalotiopsis</i> sp.) and ATL 4 (<i>Nigrospora</i> sp.) showed the inhibition zones (18 mm) against <i>Escherichia coli</i>. Those isolated endophytic fungi from leaves of Neem plants will be a good source of bioactive and antimicrobial activities against the pathogenic bacteria in agriculture and medicines.</p>
Keywords	<i>Azadirachta indica</i> A. Juss., Endophytic fungi, Antimicrobial
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ISOLATION, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITIES OF ENDOPHYTIC FUNGI FROM LEAVES, STEMS AND INNER BARKS OF *AZADIRACHTA INDICA* A. JUSS.

Aye Thandar Lwin¹ and Khin Khin Soe²

Abstract

The isolation, characterization and antimicrobial activities of endophytic fungi from leaves, stems and inner barks of *Azadirachta indica* A. Juss. (Neem) were studied. The plants were collected from University of Mandalay Campus. This study was carried out at Microbiology Laboratory of Botany Department, University of Mandalay from December 2017 to July 2018. Potato Dextrose Agar (PDA) medium was used for the isolation of endophytic fungi. Nine endophytic fungi were isolated and classified from leaves, stems and inner barks of *Azadirachta indica* A. Juss. Among them, ATL 1, ATL 2, ATL 3, ATL 4 were isolated from leaves. ATL 5, ATL 6 were isolated from stems. ATL 7, ATL 8, ATL 9 were isolated from inner barks. The isolated fungal strains were confirmed ATL 1 as *Colletotrichum* sp., ATL 2 as *Curvularia* sp., ATL 3 as *Pestalotiopsis* sp., ATL 4 as *Nigrospora* sp., ATL 5 as *Aspergillus* sp., ATL 6 as *Trichoderma* sp., ATL 7 as *Fusarium* sp., ATL 8 as *Penicillium* sp., ATL 9 as *Aspergillus* sp. Four isolated fungal strains: ATL 1 - ATL 4 from leaves of *Azadirachta indica* A. Juss. (Neem) were tested for antimicrobial activities at Biotechnology Research Department, Kyaukse. Those isolated fungal strains showed the antimicrobial activities against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Candida albicans*. The isolated fungal strains; ATL 2 (*Curvularia* sp.), ATL 3 (*Pestalotiopsis* sp.) and ATL 4 (*Nigrospora* sp.) showed the inhibition zones (25 mm) against *Bacillus cereus* and ATL 1 (*Colletotrichum* sp.), ATL 2 (*Curvularia* sp.), ATL 3 (*Pestalotiopsis* sp.) and ATL 4 (*Nigrospora* sp.) showed the inhibition zones (18 mm) against *Escherichia coli*. Those isolated endophytic fungi from leaves of Neem plants will be a good source of bioactive and antimicrobial activities against the pathogenic bacteria in agriculture and medicines.

Keywords: *Azadirachta indica* A. Juss., Endophytic fungi, Antimicrobial

¹MSc Student, Department of Botany, University of Mandalay

²Lecturer, Department of Botany, University of Mandalay

Introduction

Azadirachta indica A. Juss. (syn. *Melia Indica* Brandis, *Melia azadirachta* Linn) is an indigenous medicinal plant in India and Africa. Neem is an evergreen tree growing in tropical to subtropical regions belongs to the family Meliaceae (Tenguria & Khan 2011).

Azadirachta indica A. Juss. (neem) has been extensively studied for its pesticidal and medical properties in Asiatic countries. Despite almost every part of the tree having a bitter taste, parts such as leaves, bark, flower, fruit, seed and root have advantages in medical treatment and industrial product (Tenguria & Khan 2011).

Endophytes are microbes that colonize the living internal tissues of plants without causing any immediate disease symptoms or overt negative effects. These fungal endophytes which lives with the living tissues of higher plants without producing any apparent symptoms. Endophytes show a protective role against insect herbivory and many are potential producers of novel antimicrobial secondary metabolites (Taware *et al.* 2017).

Endophytic fungi are a good source of antibiotics. Natural products from endophytic microbes gave been observed to inhabit of kill a wide variety of harmful disease causing agents but not limited to phytopathogens, as well as bacteria, fungi, viruses and protozoan that affect humans and animals. Endophytic fungi are also capable to produce antimicrobial metabolites. The endophytic fungus isolated from an Indian medicinal plant having an antimicrobial activity against several bacteria and fungi including *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Salmonella enteric* and *Escherichia coli* and fungal organisms *Aspergillus niger* and *Candida albicans* (Sandhu 2014).

The aims and objectives of this research are to isolate the endophytic fungi from the leaves, stems and inner barks of *Azadirachta indica* A. Juss., to study the morphology of isolated fungi and to examine the antimicrobial activities.

Materials and Methods

The leaves, stems and inner barks of *Azadirachta indica* A. Juss. were collected from Mandalay University Campus during December 2017 to July 2018.

Preparation of Potato Dextrose Agar (PDA) Medium

Potato dextrose agar (PDA)	3.9 g
Distilled water	100 ml
pH	6.5

After autoclaving streptomycin (0.1 g) was added to the medium.

Isolation and Identification of Endophytic Fungi

The healthy leaves, stems and inner barks of *Azadirachta indica* A. Juss. were studied for isolation of endophytic fungi. The isolation of endophytic fungi was done by the methods of (Strobel *et al.* 2003 as cited in Jain & Kurma 2015). The morphological and microscopical characters were observed by the method of Barnett (1955).

Antimicrobial Activity for Endophytic Fungi

The screening of endophytic fungi was carried out Microbiology Laboratory of Botany Department, University of Mandalay. The isolated fungi were grown on PDA medium and were inoculated into seed medium and incubated for 3 days at 25°C. Seed medium (10 ml) was transferred to the fermentation medium (Ando & Inaba 2004). The fermentation was carried out for 4 days. After the end of fermentation 50 µl fermentation broth was used to check the antimicrobial activity against test organisms by well diffusion method. The agar well diffusion method was used for antimicrobial activity evaluation by modifying the method described by Schlegel & Zaborosch 1993). Antimicrobial activity was carried out at the Biotechnology Research Department, Kyaukse. One Gram-negative bacterium (*Escherichia coli*), three Gram-positive bacteria (*Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus cereus*) and one fungal (*Candida albicans*) were used as the tested microorganisms for this experiment.

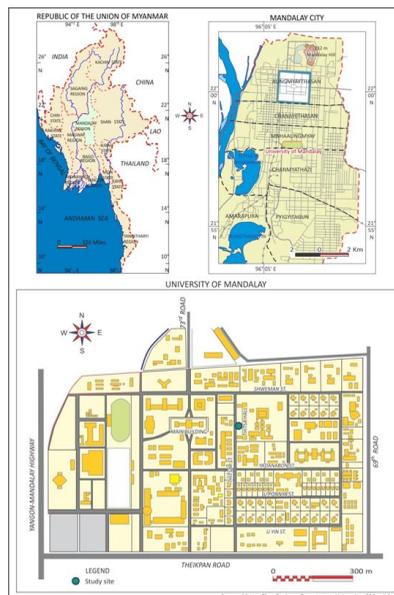


Fig. 1 Location map of sample collection site

Results

In the present study, morphological characters of *Azadirachta indica* A. Juss. were described. The total nine strains of endophytic fungi were isolated from leaves, stems and inner barks of *Azadirachta indica* A. Juss. The macroscopical and microscopical characteristics of endophytic fungi were described in Table 1.

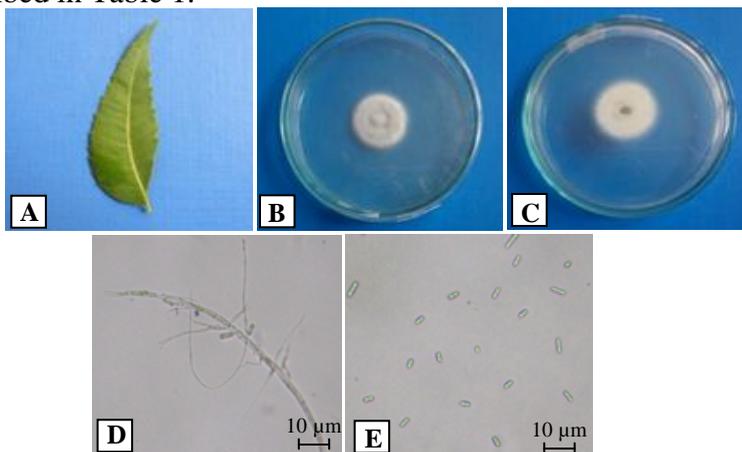


Figure 2. A. Leaves sample of *Azadirachta indica* A. Juss.
 B. Surface colony characters of *Colletotrichum* sp. (ATL 1) 2 days
 C. Reverse colony characters of *Colletotrichum* sp. (ATL 1) 2 days
 D. Photomicrograph of *Colletotrichum* sp. (ATL 1)
 E. Photomicrograph of conidia of *Colletotrichum* sp. (ATL 1)

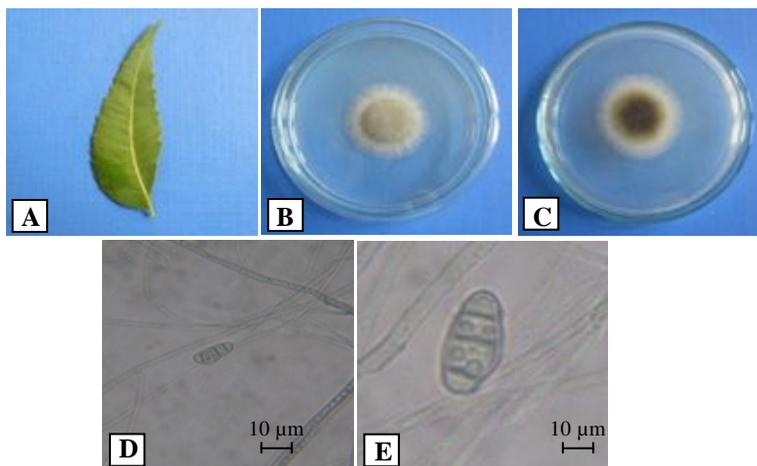


Figure 3. A. Leaves sample of *Azadirachta indica* A. Juss.
 B. Surface colony characters of *Curvularia* sp. (ATL 2) 2 days
 C. Reverse colony characters of *Curvularia* sp. (ATL 2) 2 days
 D. Photomicrograph of *Curvularia* sp. (ATL 2)
 E. Photomicrograph of conidia of *Curvularia* sp. (ATL 2)

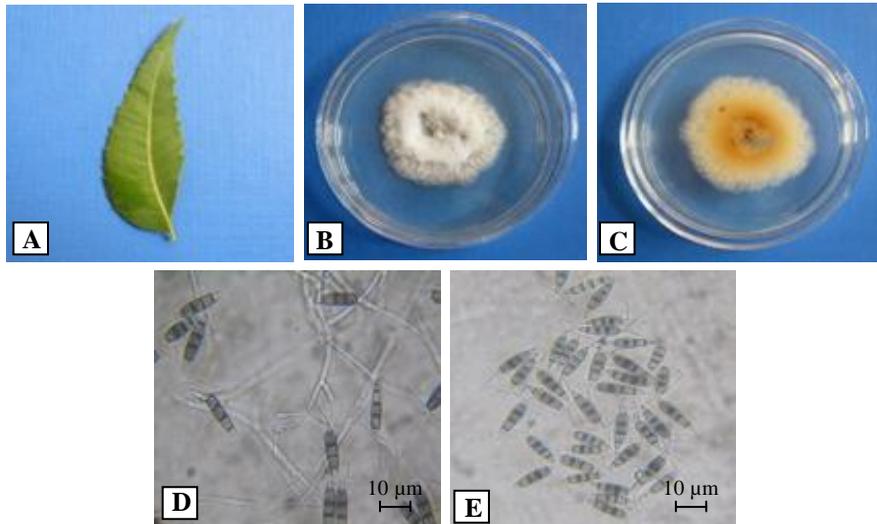


Figure 4. A. Leaves sample of *Azadirachta indica* A. Juss.
 B. Surface colony characters of *Pestalotiopsis* sp. (ATL 3) 4 days
 C. Reverse colony characters of *Pestalotiopsis* sp. (ATL 3) 4 days
 D. Photomicrograph of *Pestalotiopsis* sp. (ATL 3)
 E. Photomicrograph of conidia of *Pestalotiopsis* sp. (ATL 3)

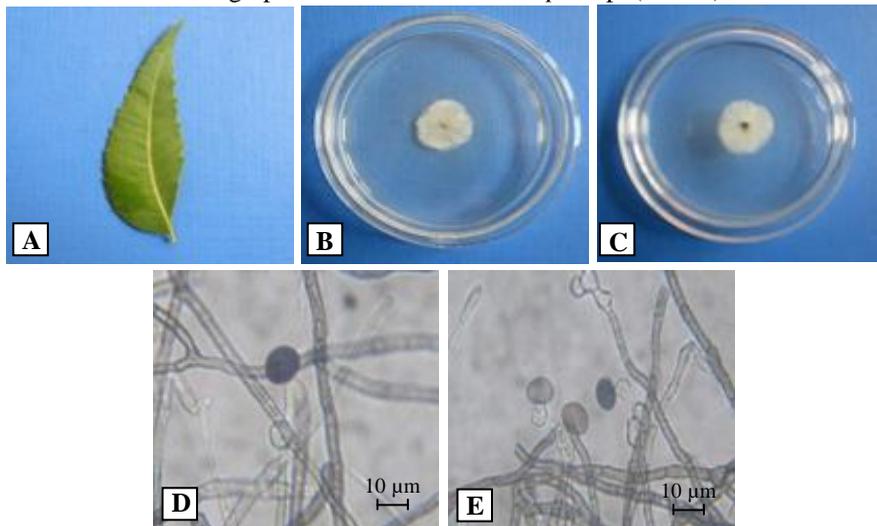


Figure 5. A. Leaves sample of *Azadirachta indica* A. Juss.
 B. Surface colony characters of *Nigrospora* sp. (ATL 4) 2 days
 C. Reverse colony characters of *Nigrospora* sp. (ATL 4) 2 days
 D. Photomicrograph of *Nigrospora* sp. (ATL 4)
 E. Photomicrograph of conidia of *Nigrospora* sp. (ATL 4)

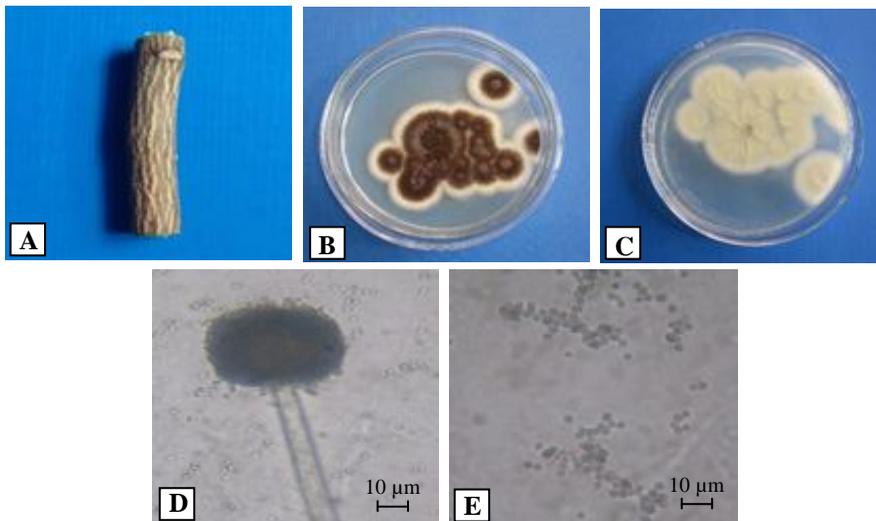


Figure 6. A. Stem sample of *Azadirachta indica* A. Juss.
 B. Surface colony characters of *Aspergillus* sp. (ATL 5) 2 days
 C. Reverse colony characters of *Aspergillus* sp. (ATL 5) 2 days
 D. Photomicrograph of *Aspergillus* sp. (ATL 5)
 E. Photomicrograph of conidia of *Aspergillus* sp. (ATL 5)

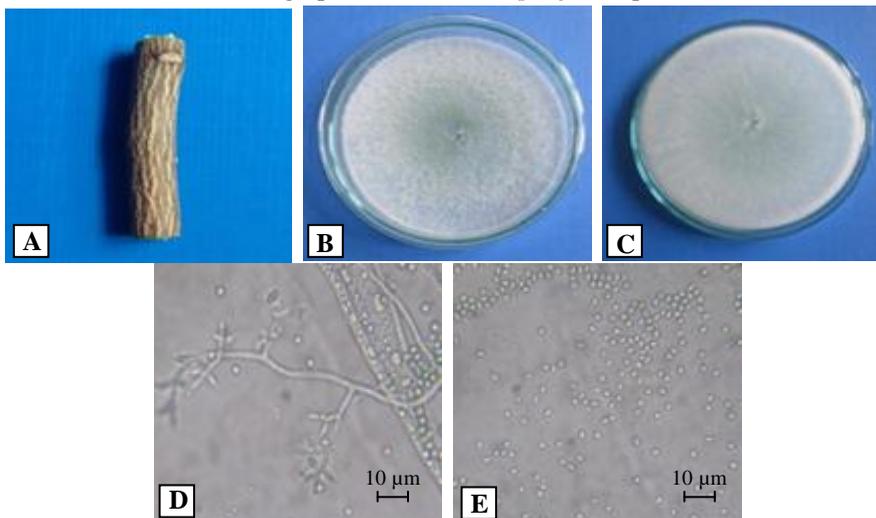


Figure 7. A. Stem sample of *Azadirachta indica* A. Juss.
 B. Surface colony characters of *Trichoderma* sp. (ATL 6) 2 days
 C. Reverse colony characters of *Trichoderma* sp. (ATL 6) 2 days
 D. Photomicrograph of *Trichoderma* sp. (ATL 6)
 E. Photomicrograph of conidia of *Trichoderma* sp. (ATL 6)

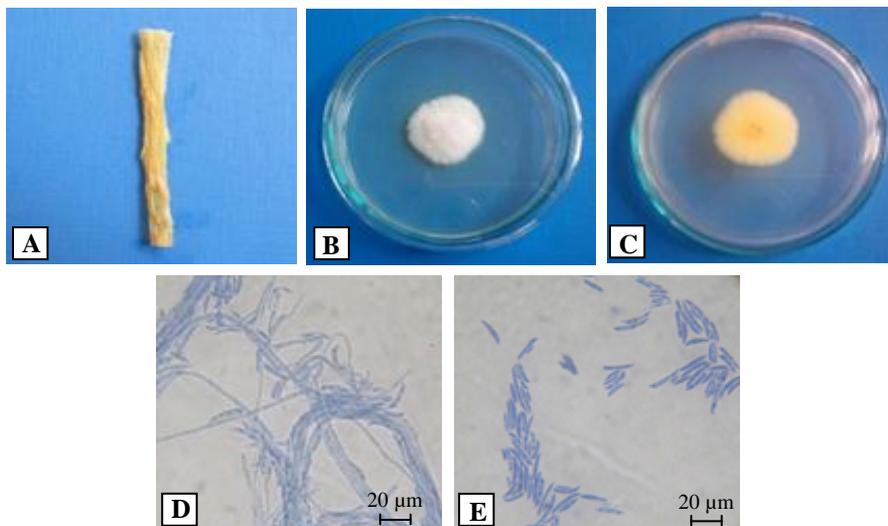


Figure 8. A. Inner bark sample of *Azadirachta indica* A. Juss.
 B. Surface colony characters of *Fusarium* sp. (ATL 7) 2 days
 C. Reverse colony characters of *Fusarium* sp. (ATL 7) 2 days
 D. Photomicrograph of *Fusarium* sp. (ATL 7)
 E. Photomicrograph of conidia of *Fusarium* sp. (ATL 7)

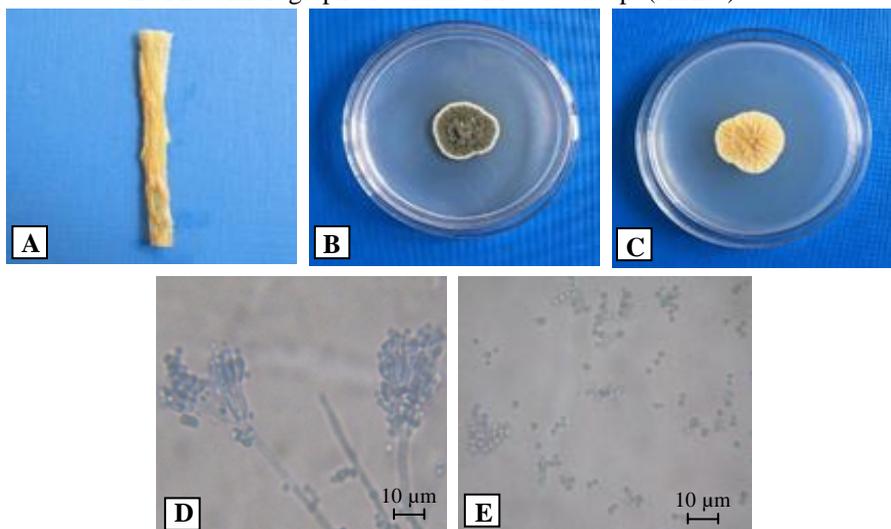


Figure 9. A. Inner bark sample of *Azadirachta indica* A. Juss.
 B. Surface colony characters of *Penicillium* sp. (ATL 8) 4 days
 C. Reverse colony characters of *Penicillium* sp. (ATL 8) 4 days
 D. Photomicrograph of *Penicillium* sp. (ATL 8)
 E. Photomicrograph of conidia of *Penicillium* sp. (ATL 8)

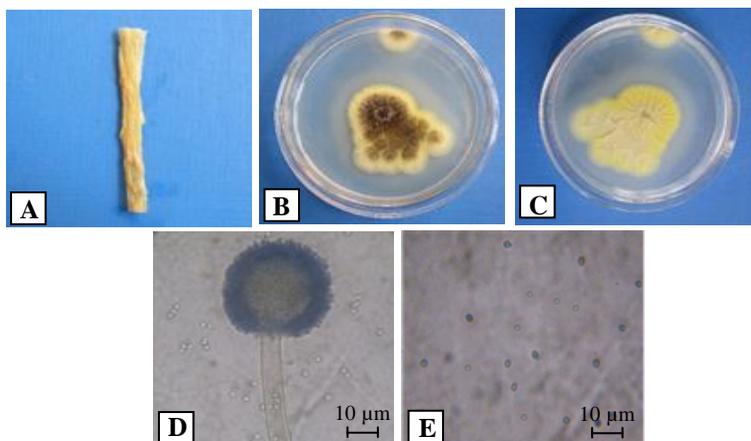


Figure 10. A. Inner bark sample of *Azadirachta indica* A. Juss.
 B. Surface colony characters of *Aspergillus* sp. (ATL 9) 2 days
 C. Reverse colony characters of *Aspergillus* sp. (ATL 9) 2 days
 D. Photomicrograph of *Aspergillus* sp. (ATL 9)
 E. Photomicrograph of conidia of *Aspergillus* sp. (ATL 9)

Table 1 Characteristics of endophytic fungi isolated from leaves, stems and inner barks of *Azadirachta indica* A. Juss.

Isolated strains	Endophytic Fungi	Macroscopical characters	Microscopical characters	Sources
ATL 1	<i>Colletotrichum</i> sp.	White at first, become grayish over time, reverse creamy	Hyphae septate, conidia 1-celled, ovoid to oblong	Leaves
ATL 2	<i>Curvularia</i> sp.	Gray in central, white in peripheral, reverse dark-green	Hyphae septate, conidia fusiform, curved in shape, black in colour	Leaves
ATL 3	<i>Pestalotiopsis</i> sp.	White, reverse pale orange	Hyphae septate, conidia fusiform, hyaline pili	Leaves
ATL 4	<i>Nigrospora</i> sp.	White initially, turns to black, reverse white initially, become black	Hyphae septate, conidia 1-celled, globose to somewhat flattened, black in colour, conidiophores simple	Leaves
ATL 5	<i>Aspergillus</i> sp.	Black in central, white in peripheral, reverse white	Hyphae septate, conidia 1-celled, spherical, green in colour, conidiophores upright, simple	Stem
ATL 6	<i>Trichoderma</i> sp.	White initial, become greenish, reverse whitish-green	Hyphae septate, conidia 1-celled, spherical, green in colour	Stem
ATL 7	<i>Fusarium</i> sp.	White, woolly in texture, reverse yellow	Hyphae septate, macroconidia septate, canoe-shaped	Inner bark
ATL 8	<i>Penicillium</i> sp.	Gray, reverse pale yellow	Hyphae septate, conidia 1-celled, spherical, green in colour, conidiophores arise from the mycelium	Inner bark
ATL 9	<i>Aspergillus</i> sp.	Black in central, peripheral in greenish-yellow, reverse yellow	Hyphae septate, conidia 1-celled, globose, green in colour, conidiophores upright	Inner bark

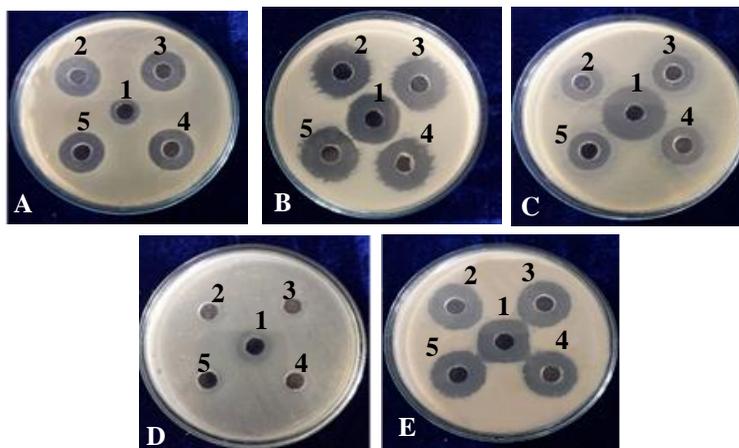


Figure 11. Antimicrobial Activity of Isolated Endophytic Fungi

A. Test on *Staphylococcus aureus*

B. Test on *Bacillus cereus*

C. Test on *Escherichia coli*

D. Test on *Enterococcus faecalis*

E. Test on *Candida albicans*

1. Tetracycline hydrochloride (control)
2. *Colletotrichum* sp. (ATL 1)
3. *Curvularia* sp. (ATL 2)
4. *Pestalotiopsis* sp. (ATL 3)
5. *Nigrospora* sp. (ATL 4)

Table 2 Antimicrobial activity test for endophytic fungi

	Diameter of Inhibition Zone (mm)				
	Pathogenic Bacteria				
Endophytic Fungi	<i>Staphylococcus aureus</i>	<i>Bacillus cereus</i>	<i>Escherichia coli</i>	<i>Enterococcus faecalis</i>	<i>Candida albicans</i>
<i>Colletotrichum</i> sp.	19	24	18	-	22
<i>Curvularia</i> sp.	20	25	18	-	22
<i>Pestalotiopsis</i> sp.	20	25	18	-	22
<i>Nigrospora</i> sp.	19	25	18	-	22
Tetracycline hydrochloride	12	23	27	14	22

Discussion and Conclusion

In the present study, the endophytic fungi were isolated from three plant parts of *Azadirachta indica* A. Juss. (Neem). There are leaves, stems and inner barks. Nine strains of endophytic fungi, namely *Colletotrichum* sp., *Curvularia* sp., *Pestalotiopsis* sp., *Nigrospora* sp. were isolated from leaves, *Aspergillus* sp., *Trichoderma* sp. were isolated from stems and *Fusarium* sp., *Penicillium* sp., *Aspergillus* sp. were isolated from inner barks of *Azadirachta indica* A. Juss.

In the present study, the macroscopical characters of ATL 1 fungal strain conidia were hyaline, 1-celled, ovoid, cylindrical with rounded ends and smooth wall. Barnett (1955) stated that conidia were hyaline 1-celled and ovoid. Those characters were agreed with the statement of Barnett (1955). Therefore, ATL 1 fungal strain can be confirmed as *Colletotrichum* sp.

The macroscopic characters of ATL 2, ATL 3 and ATL 7 fungal strain conidia were 3- to 5- celled, fusiform, curved in shape. Barnett (1955) stated that conidia were hyaline, 3- to 5- celled and fusiform. Those characters were agreed with the statement of Barnett (1955). Therefore, ATL 2 fungal strain can be confirmed as *Curvularia* sp., ATL 3 as *Pestalotiopsis* sp., and ATL 7 as *Fusarium* sp.

The macroscopic characters of ATL 4 fungal strain conidia were hyaline vesicle at the end of conidiophores, 1-celled, globose to somewhat flattened and black. Barnett (1955) stated that conidia were hyaline, 1-celled, globose to somewhat flattened. Those characters were agreed with the statement of Barnett (1955). Therefore, ATL 5 fungal strain can be confirmed as *Nigrospora* sp.

The macroscopical characters of ATL 5, ATL 6, ATL 8 and ATL 9 fungal strain conidia 1-celled, spherical and green in colour. Barnett (1955) stated that conidia were hyaline, 1-celled, globose or ovoid and green in colour. Those characters were agreed with the statement of Barnett (1955). Therefore, ATL 5 and ATL 9 fungal strain can be confirmed as *Aspergillus* sp., ATL 6 as *Trichoderma* sp., ATL 8 as *Penicillium* sp.

In the present study, *Colletotrichum* sp. (ATL 1), *Curvularia* sp. (ATL 2), *Pestalotiopsis* sp. (ATL 3), *Nigrospora* sp. (ATL 4) showed the antimicrobial activities against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Candida albicans*, however, did not show the antimicrobial activities against *Enterococcus faecalis*. Srivastava & Anandrao (2015) stated endophytic fungi of *Colletotrichum* sp., *Curvularia* sp., *Pestalotiopsis* sp., *Fusarium* sp., *Trichoderma* sp., *Nigrospora* sp., *Penicillium* sp., *Aspergillus*

sp. against *Xanthomonas vesicatoria* (10 mm), *Pseudomonas syringae* (10 mm), *Bacillus cereus* (20 mm) and *Bacillus subtilis* (15 mm). These findings were agreed with Srivastava & Anandrao (2015).

According to the study, the antimicrobial activities of endophytic fungi, *Curvularia* sp. (ATL 2), *Pestalotiopsis* sp. (ATL 3) and *Nigrospora* sp. (ATL 4) isolated from leaves of *Azadirachta indica* A. Juss. showed the inhibition zones (25 mm) against *Bacillus cereus*. In contrast, ATL 1 (*Colletotrichum* sp.) ATL 2 (*Curvularia* sp.), ATL 3 (*Pestalotiopsis* sp.) and ATL 4 (*Nigrospora* sp.) showed the inhibition zones (18 mm) against *Escherichia coli*.

Therefore, it is noted that the endophytic fungi can be isolated from leaves, stems and inner barks of *Azadirachta indica* A. Juss. Four isolated fungal strains: ATL 1 - ATL 4 from leaves of *Azadirachta indica* A. Juss. (Neem) may be regarded as a good source of antimicrobial activities against the pathogenic bacteria. This study will be beneficial for the production of endophytic fungi from *Azadirachta indica* A. Juss. (Neem) to provide further purposes of agricultural products and natural products for medicines.

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