Isolation of Endophytic Fungi From *Aquilaria sinensis* (Lour.) Gilg.

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Abstract

Endophytic fungi were isolated from leaves, bark and wood of *Aquilaria sinensis* (Lour.) Gilg. The plant samples were collected from Agarwood plantation situated at Sein Kanout Mya Kanout Mountain in the eastern part of Mandalay region. Potato Dextrose Agar (PDA) medium was used for culturing the endophytic fungi. The resulted endophytic fungi were *Aspergillus* sp. (CM 01), *Penicillium* sp. (CM 02), *Cladosporium* sp. (CM 03), *Nigrospora* sp. (CM 04) and *Phoma* sp. (CM 05) isolated from leaves, *Aspergillus* sp. (CM 06) isolated from bark and *Cladosporium* sp. (CM 07), *Aspergillus* sp. (CM 08) and *Penicillium* sp. (CM 09) isolated from wood of *Aquilaria sinensis* (Lour.) Gilg.

Keywords: *Aquilaria sinensis*, Agarwood, Endophytic fungi.

Introduction

The genus *Aquilaria* belongs to Thymelaeaceae family. *Aquilaria* species is an aromatic plant that is commonly known as "Gaharu wood'' in South East Asia. It is found mostly in Malaysia, Indonesia, India, Singapore, Bangladesh, Myanmar, Philippine, and Thailand (Ibrahim *et al*. 2011). *Aquilaria malaccensis* Lam. has been listed in the CITES Red Data Book at Appendix II since 1997 (Kalaya Lu 2005).

In Myanmar, Kachin State of eastern Himalaya tract is considered to be the centre of origin of *Aquilaria* due to high population density and high degree of species diversity. Nine species of *Aquilaria* were found in Kachin State namely *Aquilaria subintegra* Ding Hou, *Aquilaria crassna* Pierre ex lamk., *Aquilaria beccariana* Tiegh., *Aquilaria hirta* Ridl., *Aquilaria microcapa* Baill., *Aquilaria rostrata* Ridl., *Aquilaria sinensis* (Lour.) Gilg., *Aquilaria agalocha* Roxb. and *Aquilaria malaccensis* Lam. Among them *Aquilaria agalocha* Roxb. *and Aquilaria malaccensis* Lam. were recorded in Myanmar (Kalaya Lu 2005).

Aquilaria can be found in upper Sagaing Division (Hkantee Phaung Pyin), Chin State, Northern Shan State, Paung Long region (Central Myanmar) Kaw Lin, Win Tho, Pinlaw Bu (Sagaing Division), Than doung (Ka Yin State) and Taninthary Division (Kalaya Lu 2005). Recently, commercial scale of Agarwood has been cultivated throughout Myanmar. However, in Mandalay, manageable scale of Agarwood has initially been cultivated since 2010; mostly were at the Sein Kanout Mya Kanout mountain range at the altitude of 1777-1800 meter above sea level; plantations have been increasing year by year.

Although Mandalay is not a habitat of Agarwood, cultivators tried to acclimatize the Agarwood around the environment of Mandalay. Now, Agarwood are well adapted to microclimate condition of Mandalay, especially at Sein Kanout Mya Kanout mountain ranges area. Most of the Agarwood species were *Aquilaria crassna* Pierre ex Lamk., *Aquilaria sinensis* (Lour.) Gilg. and *Aquilaria agalocha* Roxb.; their source were nursery centre of Myitkyina; founded by Agarwood Association of Kachin State. Most of the growers situated at Sein Kanout Mya Kanout were cultivated under licence, which was issued by Ministry of Environmental Conservation and Forestry. Majority of certificated plantation was identified as *Aquilaria sinensis* (Lour.) Gilg.

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Aquilaria sinensis (Lour.) Gilg. is the main plant species for the production of agarwood used as traditional Chinese drugs since 16th century (Gong and Guo 2009). Agarwood cannot be generated in normal wood tissues, but might be formed when the *Aquilaria* plants are injured by insects, physical cuts, bacterial infections, or chemical stimulation (Chen *et al.* 2012). Agarwood has significant anticancer activities, analgesic and anti-inflammatory activities, and anti-depression activities (Cui *et al*. 2011).

Plants appear to be a reservoir of untold numbers of endophytic organisms (Kameshwari *et al*. 2015). Endophyte includes all organisms which colonize the living internal tissues of their hosts without producing symptoms of disease. Common endophytes include a variety of bacteria, fungi and actinomycetes (Premjanu and Jayanthy 2012).

Endophytic fungi are unexplored group of organisms that has enormous potentials for new pharmaceutical substances. They play an essential role to provide protection to their host against attack by other pathogens and environmental factors (Selvi and Balagengatharathilagam 2014). These endophytic fungi were involved in defense mechanism and other essential secondary metabolites are produced and were able to induce enhance the production of plant secondary metabolites which are source of very important chemical constituents for plant defense as well as pharmaceutical industry (Kameshwari *et al*. 2015). Medicinal plants and their endophytes are important resources for discovery of natural products (Selvi and Balagengatharathilagam 2014). Li *et al.* (2014) stated that Agarwood ('Chenxiang' in Chinese) is a resinous wood formed in the heartwood of *Aquilaria* trees (Thymelaeaceae) in response to injury by cutting, drilling, burning, or incursion of moths, microorganisms, etc., which has been used for medicinal, religious, and ceremonial purposes for a long time. The main active compounds in Agarwood are sesquiterpenes and 2-(2-phenylethyl) chromone derivatives.

Their research led to the identification of new and known eudesmane-type sesquiterpene from the endophytic fungus *Nigrospora oryzae* A8 obtained from *Aquilaria sinensis* (Lour.) Gilg. which also regarded as a biosynthetic precursor of common skeletons of sesquiterpenes from Agarwood. This research demonstrated that the endophytic fungi from *A. sinensis* might play a role in the formation of Agarwood. Therefore, it is important to study the endophytic fungal communities isolated from *Aquilaria* trees.

The aim and objectives of this research are to isolate the endophytic fungi from the leaves, bark and wood of *Aquilaria sinensis* (Lour.) Gilg. and to identify the endophytic fungi by using macroscopical and microscopical characteristics.

Materials and Methods

Collection of Plant Samples

The plant samples of *Aquilaria sinensis* (Lour.) Gilg. were collected from Agarwood plantation situated at Sein Kanout Mya Kanout Mountain in the eastern part of Mandalay in November 2015. Plant identification was made from fresh samples referred to Dassanayake (1981).

Isolation of Endophytic Fungi

The leaves, bark and wood of *Aquilaria sinensis* (Lour.) Gilg. were utilized for isolation of endophytic fungi. The isolation of endophytic fungi was done by the methods of Shan *et al*. (2000). The morphological and microscopical characters were observed by the methods of Barnett (1955) and Dube (1990). The fungal spores were measured according to the method of Kokate (2000).

Results

In present study, morphological characters of *Aquilaria sinensis* (Lour.) Gilg. were described. Nine strains of endophytic fungi were isolated from the leaves, bark and wood of *Aquilaria sinensis* (Lour.) Gilg. These isolated strains were namely as CM 01, CM 02, CM 03, CM 04, CM 05, CM 06, CM 07, CM 08 and CM 09. The characteristics of endophytic fungi isolated were presented in Table 1 and Figure 2-10.

Table 1 Characteristics of Endophytic Fungi Isolated from *Aqualaria senensis* (Lour.) Gilg.

Endophytic Fungi	Source	Macroscopical Characters		Microscopical Characters
		Surface	Reverse	
Aspergillus sp.	Leaf	Light yellow to orange yellow, flaky texture	Yellowish brown	Hyphae septate, conidiophores upright, vesicle globose, conidia spherical.
Penicillium sp	Leaf	Bluish green with white edge to dark green	Pale-yellow	Conidiophores short smooth, penicilli monoverticillate, phialides ampulliform, conidia one-celled, spherical
Cladosporium sp.	Leaf	Olive-green to olivaceous brown, velvety	Black	Conidiophores septate, erected and branched variously near the upper or middle portion; conidia one or two-celled, spherical, ellipsoidal to cylindrical
Nigrospora sp.	Leaf	White wooly at first, become grey to black	Black	Condiophores short, simple or branched, conidia black, one-celled globose to somewhat flattened
Phoma sp.	Leaf	Little white to gray	Brown	Pycinidia almost spherical, dark brown, condia are released from the pycinidia, conidia unicellular, oblong to oval, hyaline
Aspergillus sp.	Bark	Yellowish green with white mycelia at the edge	Yellow	Hyphae septate; conidiphores upright, simple, hyaline, smooth, conidial heads hemisperical to subglobase; conidia globose
Cladosporium sp.	Wood	Grayish brown, compact and folded	Brown	Hyphae septate; conidiophores dark, branched, septate; conidia one or two-celled, sometimes three-celled, ovoid to cylindrical
Aspergillus sp.	Wood	White to brown	Yellow	Hyphae septate, conidiophores upright, vesicle subglobose, conidia spherical, one-celled
Penicillium sp.	Wood	Bluish green with white margin	Yellow	Conidiophores arose from the mycelium, penicilli monoverticillate, conidia one-celled, spherical

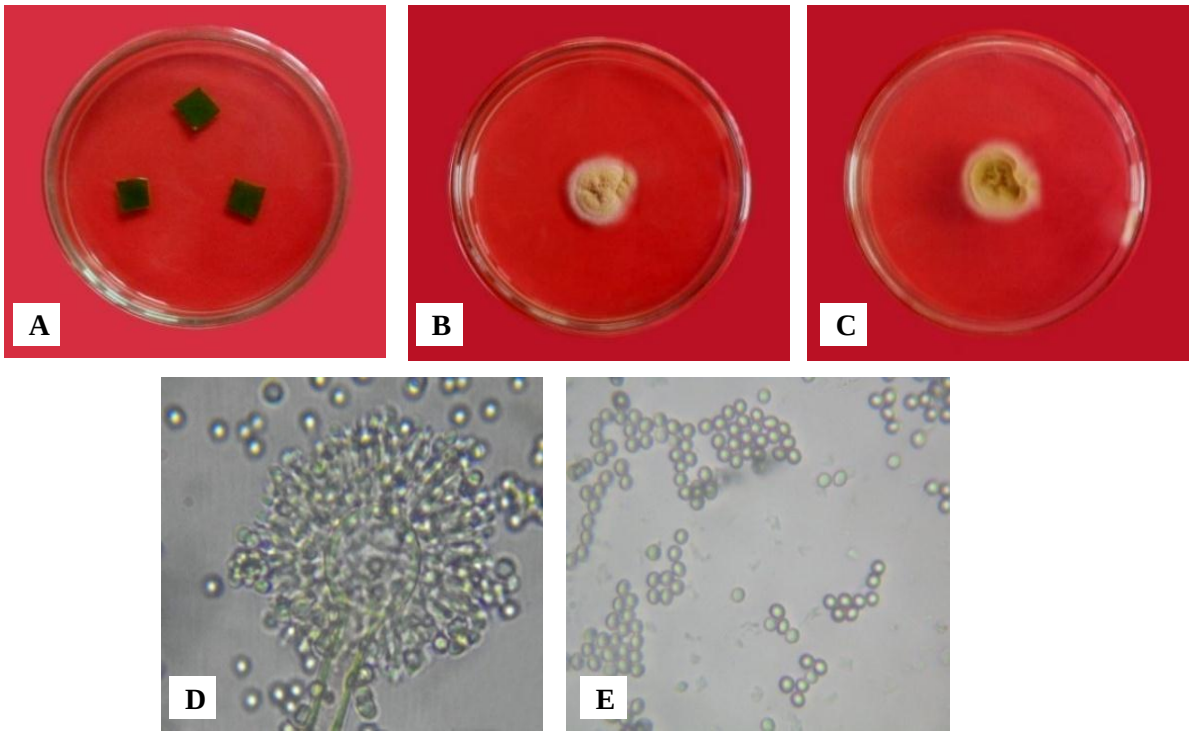

Figure 1. A. Pieces of leaf cultured on PDA medium
B. Surface colony characters of CM 01 (4 days)
C. Reverse colony characters of CM 01 (4 days)
D. Photomicrograph of conidial head of *Aspergillus* sp. (CM 01)
E. Photomicrograph of conidia of *Aspergillus* sp

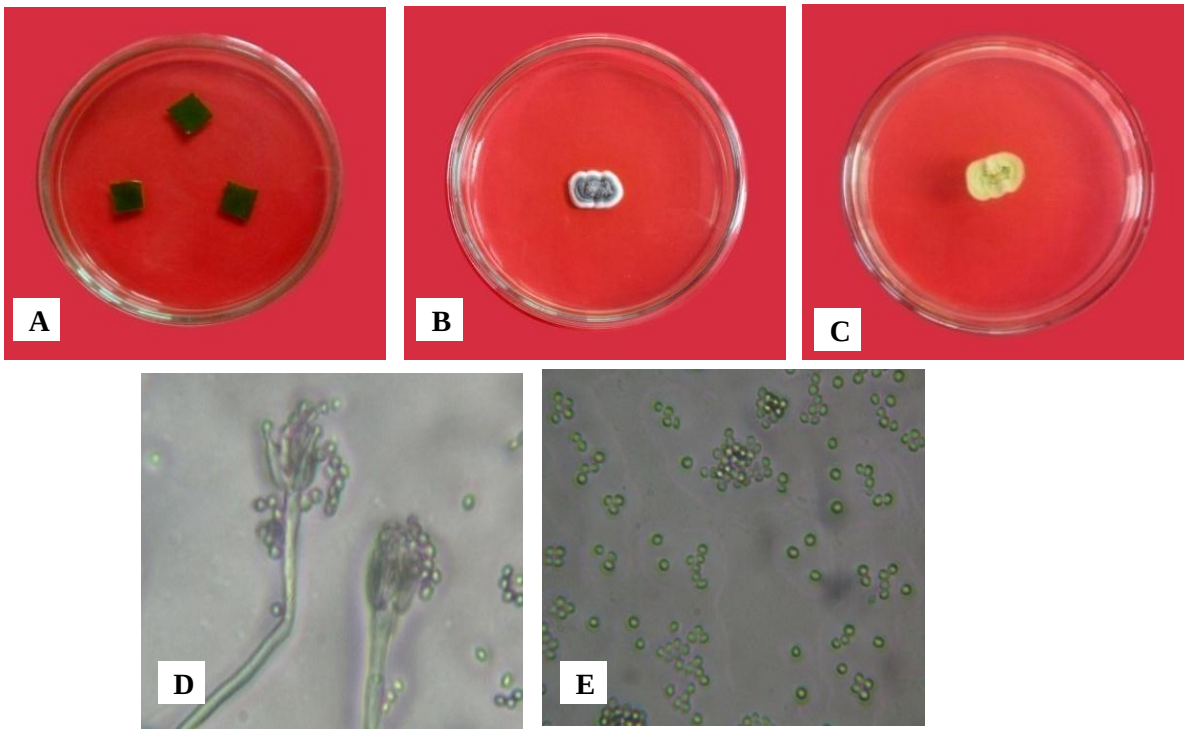

Figure 2. A. Pieces of leaf cultured on PDA medium
B. Surface colony characters of CM 02 (4 days)
C. Reverse colony characters of CM 02 (4 days)
D. Photomicrograph of *Penicillium* sp. (CM 02)
E. Photomicrograph of conidia of *Penicillium* sp.

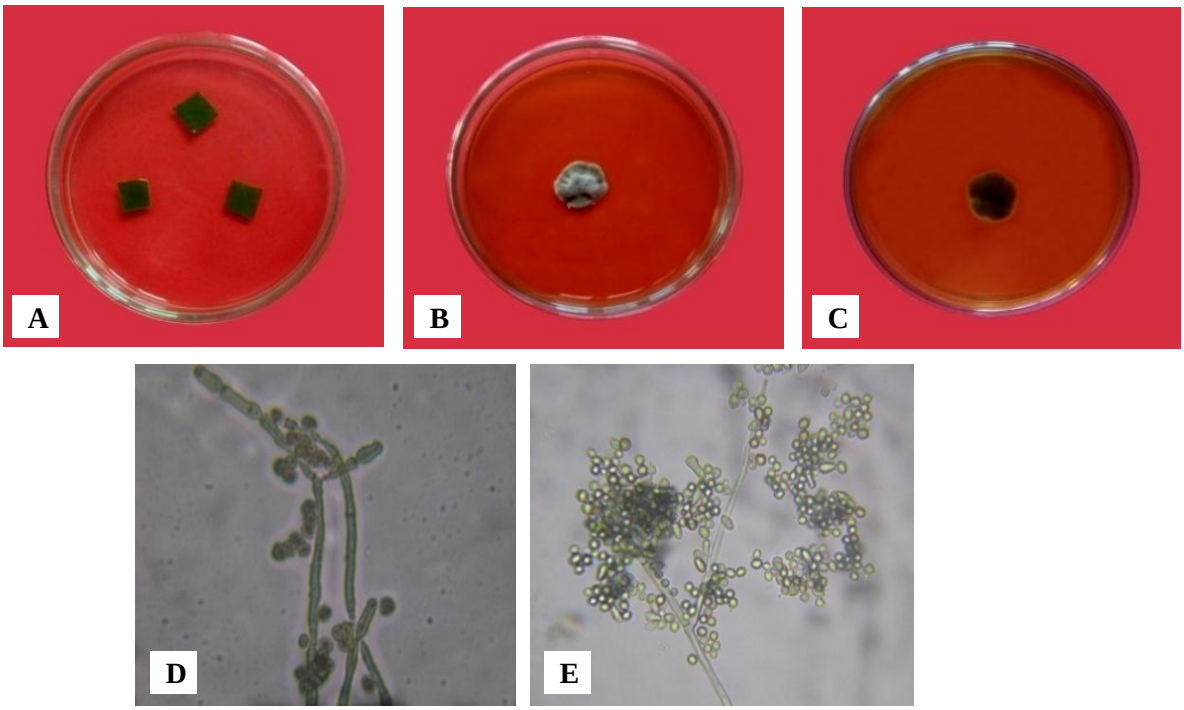

Figure 3. A. Pieces of leaf cultured on PDA medium
B. Surface colony characters of CM 03 (5 days)
C. Reverse colony characters of CM 03 (5 days)
D. Photomicrograph of *Cladosporium* sp. (CM 03)
E. Photomicrograph of conidia of *Cladosporium* sp.

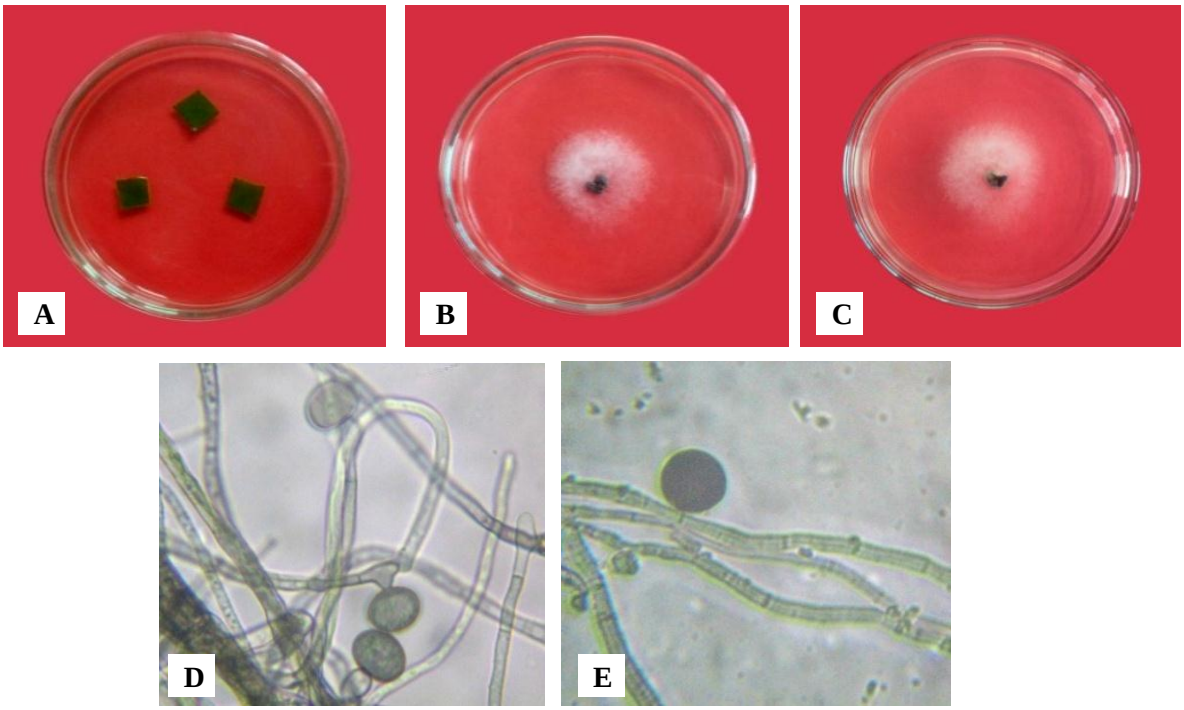

Figure 4. A. Pieces of leaf cultured on PDA medium
B. Surface colony characters of CM 04 (4 days)
C. Reverse colony characters of CM 04 (4 days)
D. Photomicrograph of *Nigrospora* sp. (CM 04)
E. Photomicrograph of conidia of *Nigrospora* sp.

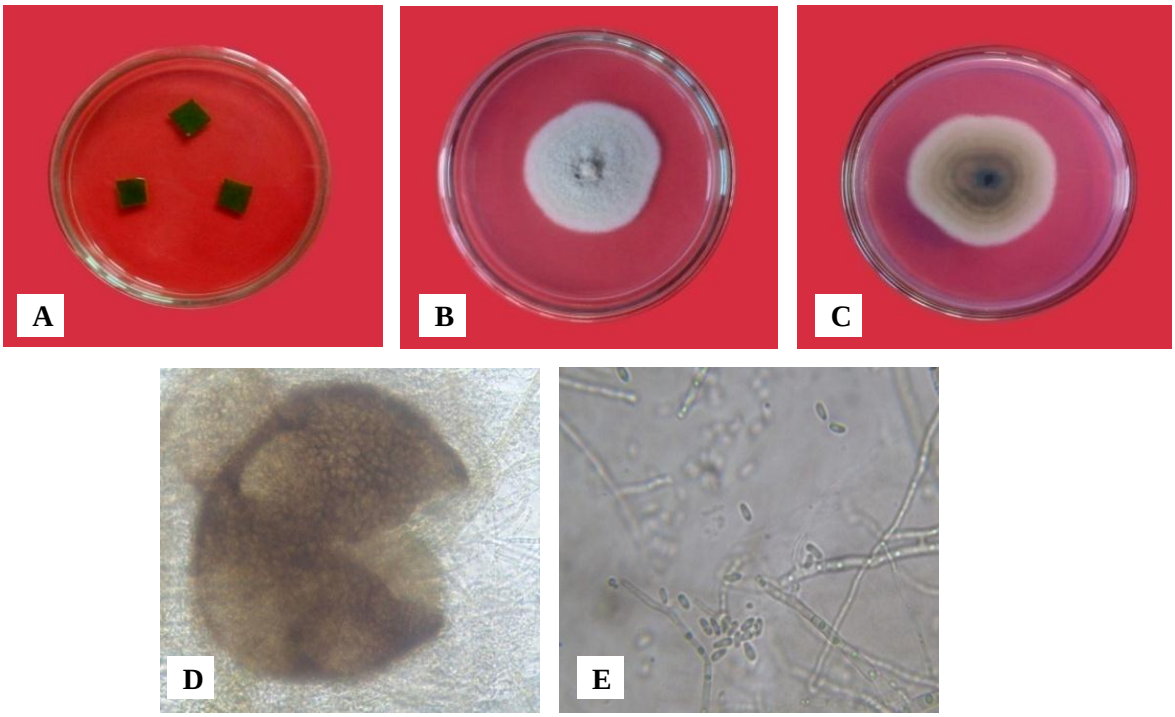

Figure 5. A. Pieces of leaf cultured on PDA medium
B. Surface colony characters of CM 05 (5 days)
C. Reverse colony characters of CM 05 (5 days)
D. Photomicrograph of pycinidium of *Phoma* sp. (CM 05)
E. Photomicrograph of conidia of *Phoma* sp.

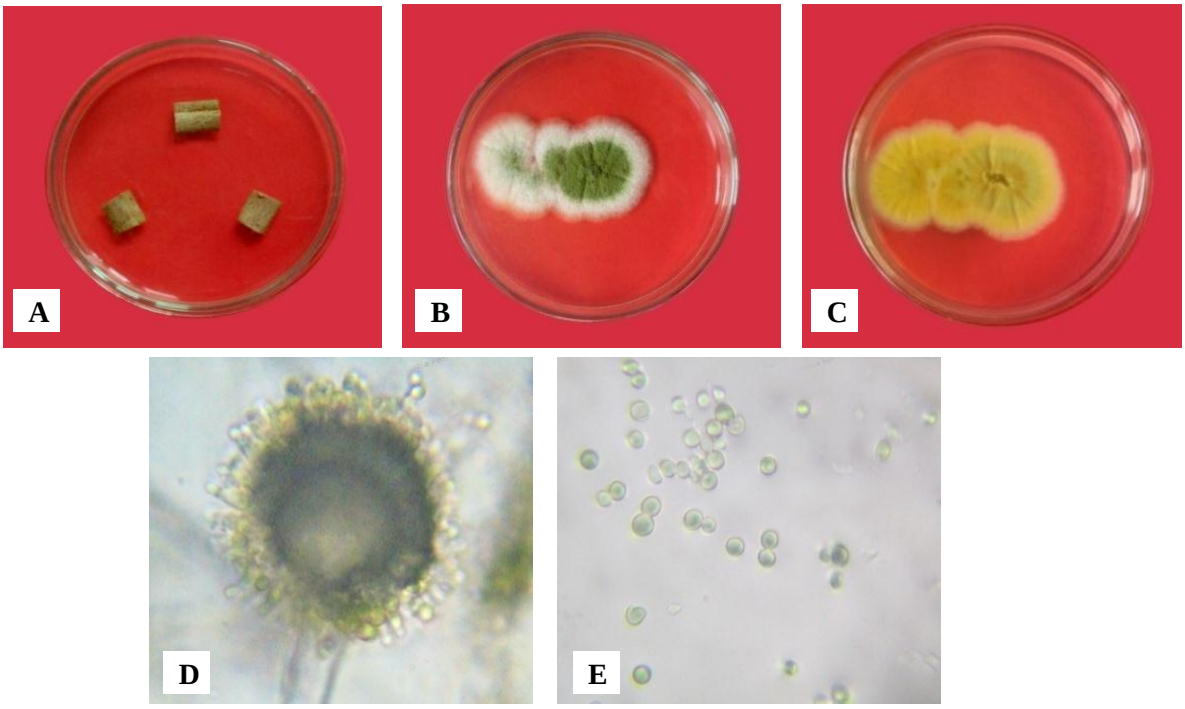

Figure 6. A. Pieces of bark cultured on PDA medium
B. Surface colony characters of CM 06 (4 days)
C. Reverse colony characters of CM 06 (4 days)
D. Photomicrograph of conidial head of *Aspergillus* sp. (CM 06)
E. Photomicrograph of conidia of *Aspergillus* sp.

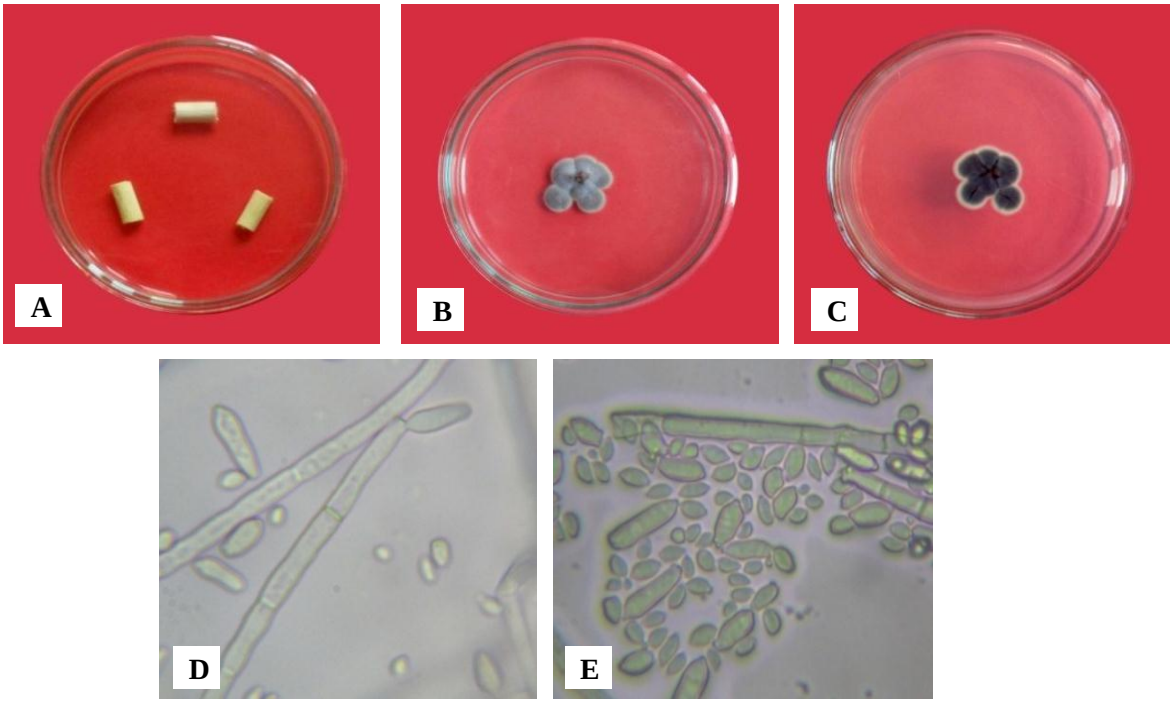

Figure 7. A. Pieces of wood cultured on PDA medium
B. Surface colony characters of CM 07 (4 days)
C. Reverse colony characters of CM 07 (4 days)
D. Photomicrograph of *Cladosporium* sp. (CM 07)
E. Photomicrograph of conidia of *Cladosporium* sp.

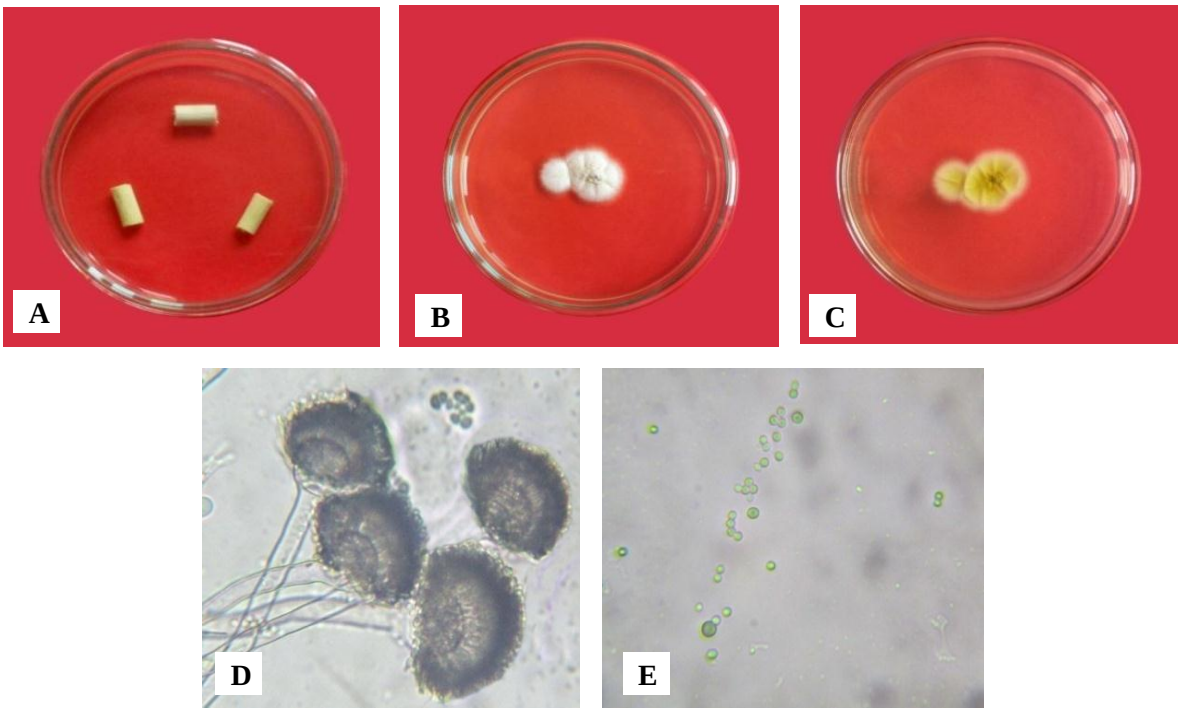

Figure 8. A. Pieces of wood cultured on PDA medium
B. Surface colony characters of CM 08 (4 days)
C. Reverse colony characters of CM 08 (4 days)
D. Photomicrograph of *Aspergillus* sp. (CM 08)
E. Photomicrograph of conidia of *Aspergillus* sp.

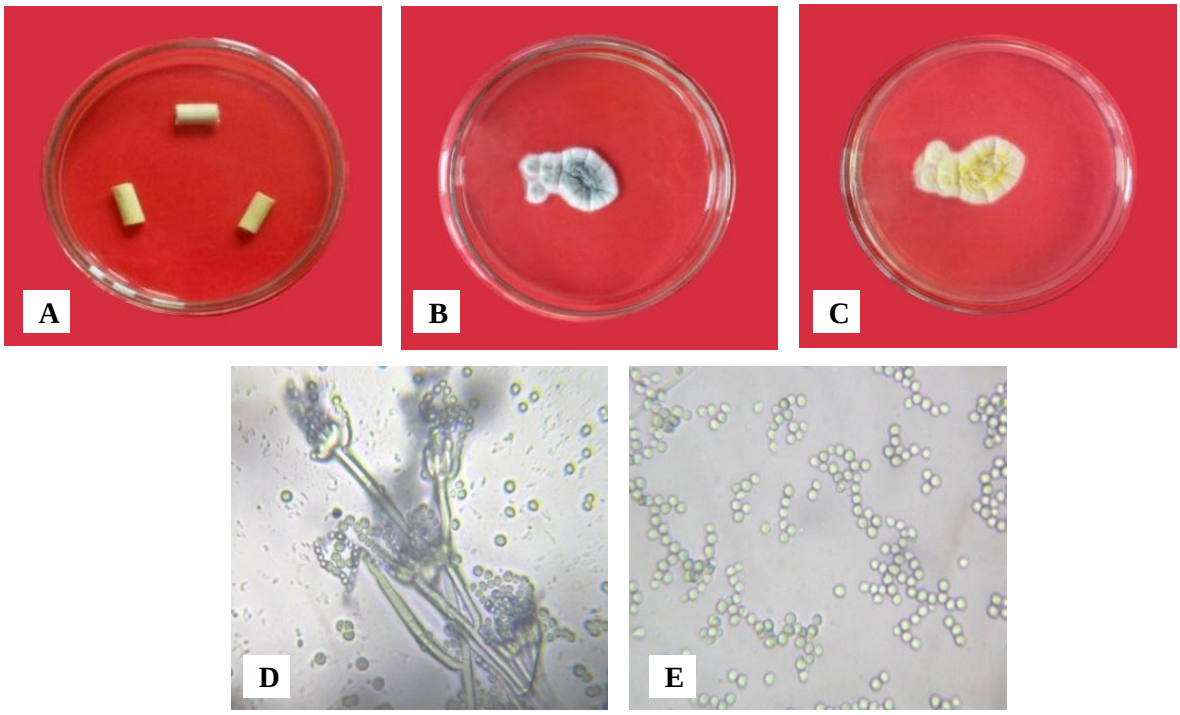

Figure 9. A. Pieces of wood cultured on PDA medium
B. Surface colony characters of CM 09 (4 days)
C. Reverse colony characters of CM 09 (4 days)
D. Photomicrograph of *Penicillium* sp. (CM 09)
E. Photomicrograph of conidia of *Penicillium* sp.

Disscusion and Conclusion

In the present investigation, endophytic fungi were isolated from three source parts of *Aquilaria sinensis* (Lour.) Gilg. plant such as leaves, bark and wood. Direct isolation method was applied in this investigation. The present study resulted nine strains of endophytic fungi, namely *Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., *Nigrospora* sp. and *Phoma* sp. from leaves, *Aspergillus* sp. from bark and *Cladosporium* sp., *Aspergillus* sp. and *Penicillium* sp. from wood. In this study, Potato Dextrose Agar (PDA) medium was used for the isolation of endophytic fungi from *Aquilaria sinensis* (Lour.) Gilg. Optimal temperature for isolation of the strains being 25˚C and pH was in the range of 6.5-7.0.

In the present study, the microscopical characters of CM 01, CM 06 and CM 08 showed that hyphae were septate; conidiophores upright; vesicle globose; conidia one-celled, spherical. Barnett (1955) reported that the conidiophores of *Aspergillus* upright, simple, terminating in a globose to elliptical swelling, bearing phialides at the apex, conidia one-celled, spherical. The characters of CM 01, CM 06 and CM 08 were agreed with Barnett (1955). Therefore, CM 01, CM 06 and CM 08 were *Aspergillus* sp.

The macroscopical characters of CM 02 and CM 09 showed that colonies were bluish-green in the center with white edge and became dark green on PDA medium at 25ºC. The reverse side of colony was usually pale-yellow. The optimal pH range was from 6.5 to 7.0. The microscopical characters of CM 02 and CM 09 showed that conidiophores arose from the mycelium. The phialides were ampulliform which beared chains of conidia. Conidia were one-celled, mostly spherical. Barnett (1955) stated that conidiophores of *Penicillium* are arising

from the mycelium, conidia hyaline or brightly colored in mass, one-celled, mostly spherical. These characters of CM 02 and CM 09 in this study were agreed with the statement of Barnett (1955). Therefore, CM 02 and CM 09 were *Penicillium* sp.

Laurence (2013) reported that the endophytic fungi *Mucor* sp., *Beauveria bassiana*, *Trichoderma* sp., *Phoma tracheiphila*, *Microsporium* sp., *Penicillium* sp., *Aspergillus flavus*, *Fusarium oxysporum* and *Colletotrichum gleosporides* were isolated from *Aquilaria* spp.

Khan *et al*. (2007) mentioned the biodiversity of the endophytic fungi isolated from *Calotropsis procera* (Ait.) R. Br. The endophytic fungi isolated from stems and leaves of *Calotropsis procera* include *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus* sp., *Penicillium sublateritium*, *Phoma chrysanthemicola*, *Phoma herdericola*, *Phoma* sp. and *Candidan albicans*.

In this study, three strains of *Aspergillus* sp. from leaves, bark and wood and two strains of *Penicillium* sp. from leaves and wood of *Aquilaria sinensis* (Lour.) Gilg. were observed. Therefore, *Aspergillus* sp. and *Penicillium* sp. can be classified as endophytic fungi which were agreed with the statement of Laurence (2013) and Khan *et al*. (2007).

Ogorek *et al*. (2012) reported that the characters of genus *Cladosporium* are conidiophores almost erected, branched, and floccose; conidia 1- or 2-celled, sometimes 3-celled, variable in shape and size, globose and ovate when one-celled, then usually with a cross-wall. In this study, the macroscopical and microscopical characters of CM 03 showed that the colony was olive-green to olivaceous-brown, velvety on PDA medium at 25˚C and the reverse side of colony was black. The optimal pH range was 6.5-7.0. The conidiophores were septate, erected and branched variously near the upper or middle portion; conidia one or two-celled, spherical, ellipsoidal to cylindrical. These characters of CM 03 were agreed with the statement of Ogorek *et al*. (2012). Therefore, CM 03 was *Cladosporium* sp.

Gong and Guo (2009) mentioned that endophytic fungi for screening of antimicrobial activities were isolated from the different parts of *Dracaena cambodiana* and *Aquilaria sinensis*. *Myceliasterilia* sp., *Fusarium* sp. 4, *Cladosporium edgeworthrae*, *Fusarium* sp. 1 and *Glomerularia* sp. were the most dominant endophytes in *A. sinensis*. In this study, CM 03 or *Cladosporium* sp. was isolated from the leaf of *Aquilaria sinensis* (Lour.) Gilg. Therefore CM 03 or *Cladosporium* sp. can be identified as endophytic fungus which agreed with the statement of Gong and Guo (2009).

Macroscopical characters of CM 04 showed that colony was initially white wooly and turning into grey with black areas and turns to black eventually from both front and reverse on the PDA medium at 25˚C. The optimal pH range was 6.5-7.0. The microscopical characters of CM 04 showed that the conidiophores were short, simple, or branched. The conidia were black, one-celled, globose to somewhat flattened, hyaline vesicle at the end of conidiophores. Dube (1990) and Barnett (1955) stated that the characters of genus *Nigrospora* are conidiophores typically small, ampulliform (flask-shaped) forming a single conidium at the tip. The characters of CM 04 were agreed with the statement of Dube (1990) and Barnett (1955). Therefore, CM 04 was *Nigrospora* sp.

Li *et al*. (2014) stated that the endophytic fungal strain *Nigrospora oryzae* A8 was isolated from the root and the Agarwood part of *Aquilaria sinensis.* In this reserach, CM 04 or *Nigrospora* sp. was isolated from the leaves of *Aquilaria sinensis* (Lour.) Gilg. Therefore, CM 04 or *Nigrospora* sp. was agreed as endophytic fungus with the statement of Li *et al*. (2014).

Barnett (1955) stated that the characters of genus *Phoma* are pycinidia dark, lenticular to globose; conidia one-celled, hyaline, ovate to elongate. The macroscopical and

microscopical characters of CM 05 colony was very little white to gray mycelium on PDA medium at 25˚C. The reverse side of the colony was brown. The optimal pH range was 6.5-7.0. Pycinidia were almost spherical, dark brown. Conidia are released from the pycinidia. Conidia were unicellular, oblong to oval, hyaline. These characters of CM 05 were agreed with the statement of Barnett (1955). Therefore, CM 05 was *Phoma* sp.

Cui *et al*. (2011) stated that antitumor and antimicrobial activities of 28 kinds of endophytic fungi isolated from medicinal parts of *Aquilaria sinensis* (Lour.) Gilg. These endophytic fungi include *Cladosporium tenuissimum, Phoma herbarum* and *Phoma medicaginis*. In the present study, CM 05 or *Phoma* sp. was observed from the leaves of *Aquilaria sinensis* (Lour.) Gilg. Therefore, CM 05 or *Phoma* sp. was agreed with the statement of endophytic fungi by Cui *et al*. (2011).

Strain CM 07 showed that the macroscopical and microscopical characters of colony were grayish brown, compact and folded on PDA medium at 25ºC. The reverse side of colony is brown. The optimal pH range was 6.5-7.0. The hyphae were septate. The conidiophores were dark, branched, septate; conidia one or two-celled, sometimes three-celled, ovoid to cylindrical. Barnett (1955) and Ogorek *et al*. (2012) stated that the conidiophores of *Cladosporium* were dark, branched; conidia one or two-celled, ovoid to cylindrical. These characters of CM 07 in this study were agreed with the statement of Barnett (1955) and Ogorek *et al*. (2012). Therefore, CM 07 was *Cladosporium* sp.

Gong and Guo (2009) mentioned that endophytic fungi for screening the antimicrobial activities were isolated from the different parts of *Dracaena cambodiana* and *Aquilaria sinensis. Myceliasterilia* sp., *Fusarium* sp. 4, *Cladosporium edgeworthrae, Fusarium* sp. 1 and *Glomerularia* sp. were the most dominant endophytes in *A. sinensis*. In this study, CM 07 or *Cladosporium* sp. was isolated from the wood of *Aquilaria sinensis* (Lour.) Gilg. Therefore CM 07 or *Cladosporium* sp. can be identified as endophytic fungus which agreed with the statement of Gong and Guo (2009).

In the present investigation, *Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., *Nigrospora* sp., *Phoma* sp. from leaves, *Aspergillus* sp. from bark and *Cladosporium* sp., *Aspergillus* sp. and *Penicillium* sp. from wood of *Aquilaria sinensis* (Lour.) Gilg. have been found as the endophytic fungi.

Cui *et al*. (2011) stated that plants may serve as a repository of untold numbers of organisms known as endophytes. Most endophytes are capable of synthesizing bioactive compounds that may provide plants with a defense against pathogens, and some of these compounds have proven useful for novel drug discovery.

In a microbe-plant relationship, endophytes contribute substances that possess various types of bioactivity, such as antibacterial, antifungal, antibiotic, antitumor, antioxidant, anti-inflammatory, etc (Selvi and Balagengatharathilagam 2014).

It is known that endophytic fungi existing in plant are the important potential source of antimicrobial substance. They can be involved in a symbiotic association with their host plant. Some literature suggested that Agarwood are pathological products formed as the results of against fungal infection (Gong and Guo 2009). It can be considered that there may exist special endophytic fungi. Therefore, it is important to study the endophytic fungi isolated from *Aquilaria sinensis* (Lour.) Gilg.

In recent research work, endophytic fungus *Nigrospora* sp. (CM 03) was isolated from the leaf of *Aquilaria sinensis* (Lour.) Gilg. Li *et al.* (2014) stated that their research led to the identification of a new and known eudesmane-type sesquiterpene from the endophytic fungus

Nigrospora oryzae A8 obtained from *Aquilaria sinensis* (Lour.) Gilg. which also regarded as a biosynthetic precursor of common skeletons of sesquiterpenes from Agarwood. Therefore, present research finding such as isolation of *Nigrospora* sp. possibly plays important role in formation of Agarwood and it can be considered as trigging agent for future studies.

In conclusion, that endophytic fungi *Nigrospora* sp. are promising source of natural bioactive secondary metabolites and with great potential for future study.

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