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Isolation of Fungi from Agarwood Timber and Chemical Constituents in Agarwood Oil

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ABSTRACT

Aquilaria crassna (agar wood) is an important economic plant that is traded at the global level due to its high value and demand. The extracted essential oils from agarwood are widely used as traditional medicines, incense in religious ceremonies and perfumes. The chemical constituents in agar wood oil were analyzed by gas chromatography mass spectrometry (GC-MS). In this study, methyl hept-2-ynoate was found to be a major component, coming in at 7.39 percent. In order to better understand the role of fungi in agarwood formation, fungi in agarwood timber from Chanthaburi Province were determined using a direct DNA sequencing with the specific primer of *18S rRNA*-based bioinformatic methods. When comparing DNA sequences in the NCBI database, fungi in agarwood, namely *Phoma herbarum* showed the highest percentage identity (96.06%). The potential of fungi isolated from agarwood timber will be evaluated for inducing agarwood production by the artificial inoculation method.

Keywords: fungi; agarwood essential oil; gas chromatography mass spectrometry; phylogenetic tree; bioinformatics

INTRODUCTION

Aquilaria crassna is a species of plant in the family Thymalaeaceae, which is mainly found in tropical rainforest areas, including Thailand (Kamonwannasit et al., 2013). This plant has also been known as one of the most common agarwood species. Aquilaria trees can produce agarwood within 20 months due to a self-defence mechanisms. Previous reports indicated that some fungi or their fermentation liquid can induce agarwood formation efficiently (Chen et al., 2017; Faizal et al., 2017). To some extent, agarwood resins are secreted by specialized structures in plants and resin formation and are produced when an internal injury and/or infection in the stems occurs to the plant (Tan et al., 2019). People of various ancient countries such as Egypt, China, Greece, India and Mesopotamia use agarwood essential oil or fragrant resins for various purposes including medicinal healing, incense in religious ceremonies, perfumes in the Arab countries, and ornamental materials (Chen et al., 2011; LÓpez-Sampson and Page, 2018). Also, it has been recognized as an expensive natural raw material since agarwood production is under threat of becoming endangered (Jayachandran et al., 2013; Kamonwannasit et al., 2013; Tan et al., 2019). The extracted essential oil (agarwood oil), can cost up to 1500 USD per tola (1 tola = 12 mL) for high quality or superior grade (agarwood oil) (Ismail et al.,2014; Wong et al., 2015). Many studies have also studied the differences of chemical composition in agarwood oil for determining the quality of agarwood oil (Wong et al., 2015).

In this study, we extracted and isolated an essential oil from agarwood by water-steam distillation and analyzed an essential oil using gas chromatograph-mass spectrometer (GC-MS) technique. Furthermore, fungi was isolated and studied the relationship of microorganisms in agarwood timber regarding responsibility of fungi in artificial infection.

MATERIAL AND METHODS

Plant Materials

Trees (*Aquilaria crassna* Pierre ex Lec), approximately 5 years old, were induced by unknown induction. All trees were grown in Kaeng Hang Maeo Sub-district, Chanthaburi, Province (13°0′30″N 101°54′18″E). Then, the agarwood was crushed and ground into a fine powder.

Extraction of essential oil

Ninety grams of the powdered sample were soaked in 900 ml water for 7 days. After that, the powder was extracted in methanol (25 ml) for 30 min by the ultrasonic method [117 Volts, 50/60 Hz, 8.1 A) 40kHz]. Conventional water distillation (hydrodistillation) method was carried out for 13 h in a simple distillation apparatus with a 1000 ml steam generator flask to extract the oil and the essential oil collected for GC-MS analysis.

Gas Chromatography-Mass Spectrometry Detection (GC-MS) analysis

The Essential oil was dissolved in dichloromethane (1: 50) and analyzed with gas chromatography-mass spectrometer (GC-MS) (7890B GC-MS, Agilent,USA) using an AT-WaxMS capillary column (30 m × 0.25mm ; film thickness 0.25 µm) and using Helium as the carrier gas with the constant flow rate of 1 ml/min. One µl of injection volume was used. The temperature of the injector part was 250°C and oven temperature programing was used. The initial oven temperature was maintained at 60°C for 1 minute, then increased to 250°C at 5°C/min and held for 10 min followed by temperature increase to 260°C at 10°C/min for 1 min. The mass spectroscopic system was operated in EI mode. Mass of the compounds was analyzed in the range of m/z 35–500 amu.

Fungal isolation

The powder (0.3 g) was soaked with 10 ml distilled water, then the mixture was incubated at 30°C for 48 h. One milliliter of suitable dilutions of the suspension was used to inoculate a set of three petri dishes each containing 15mL Potato Dextrose Agar (PDA) medium (HIMEDIA®, India). Plates were then incubated for 3 days at 30°C.

Colony PCR and Sequencing

The polymerase chain reaction was achieved in 50 µl of reaction mixture. DNA amplification was using colony PCR, which contain mycelium of fungus, 1 µl 0.5x MyTaq reaction buffer, 10 µl Mastermix (dNTP, $MgCl_2$, Buffer), 10 µM 1.25 µl primer forward and primer reward and adjusted with distilled water. Primer forward (5' GTA GTC ATA TGC TTG TCT C 3') and reverse (5' TCC GCA GGT TCA CCT ACG GA) 3') to *18 S rRNA* PCR partial amplification for fungi. The thermal cycling program was as follows: 1 min initial denaturation at 95 °C, followed by 40 cycles of 30 sec denaturation at 95 °C 30 sec primer annealing at 62 °C, 30 sec extension at 72 °C and a final

5 min extension at 72 °C. A negative control using water instead of sample was included in the amplification process. From each PCR reaction, PCR products were examined by agarose gel electrophoresis at 1.5% (w/v). And then, the amplified products were identified with ABI PRISM 377 DNA sequencer (Version 3.2) and ABI PRISM™ BigDye™ Termination kit.

Colony Identification of Microbes and Phylogenetic Evaluation

Sequencing results were individually inputted online into Nucleotide BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were aligned by ClustalX (1.81) software based on quality read values. The phylogenetic trees were mapped using Neighbor-Joining method by MEGA 7.0 software (Kumar et al., 2016).

RESULTS

GC–MS analysis and identification of compounds

Figure 1 presented the identification of essential oil via water-steam distillation of *A. crassna*. The extract was subjected to the GC-MS method. The GC-MS spectrum was identified for essential oil compounds from the Wiley 10 and NIST 14 libraries. The compounds are listed in Table 1. Some important constituents as 2(3H)-Naphthalenone,4,4a,5,6,7,8-hexahydro-4a,5-dimethyl-3-(1-methylethylidene)-,(4ar-cis)- (6.54%); 1H-Benzocycloheptene, 2,3,5,6,7,8,9,9a-octahydro-5,5,9-trimethyl-3-methylene-,(9S-trans)- (6.00%); 2-((2R,8R,8aS)-8,8a-Dimethyl-1,2,3,4,6,7,8,8a-octahydronaphthalen-2-yl)propan-2-ol (6.00%); Longipinane, (E)- (4.98%); beta.-Selinene (4.52%); 2-(6,10-DIMETHYLSPIRO[4.5] DEC-6-EN-2-YL)-2-PROPANOL (4.37%); 1-[1-(2-Benzyloxy-2-vinylbut-3-enyloxy)allyl]-4-methoxybenzene (3.21%); .alpha.-Cedrene oxide (2.86). These components are important substances in agarwood that is a high-demand product in the international market.

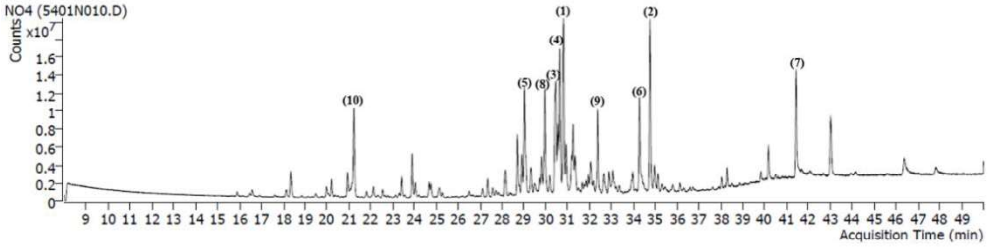


Figure 1. GC-MS chromatogram profiles of major components of agarwood oil of *Aquilaria crassna*.

Table 1. Some chemical compositions of agarwood oil of *Aquilaria crassna*

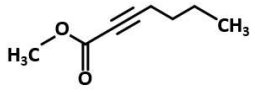
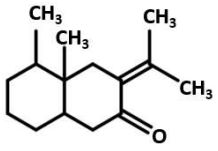
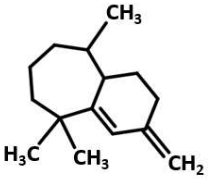
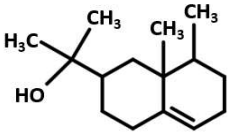
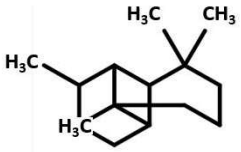
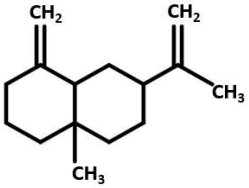
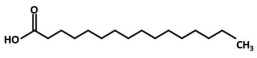
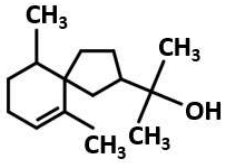
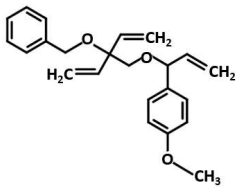
No.	Name of the components	For mula	Percentage of Total	Retention time (min)	Structures
1	Methyl hept-2-ynoate	$C_8H_{12}O_2$	7.39	30.81	
2	2(3H)-Naphthalenone, 4,4a,5,6,7,8-hexahydro-4a,5-dimethyl-3-(1-methylethylidene)-, (4ar-cis)-	$C_{15}H_{22}O$	6.54	34.76	
3	1H-Benzocycloheptene, 2,3,5,6,7,8,9,9a-octahydro-5,5,9-trimethyl-3-methylene-, (9S-trans)-	$C_{15}H_{24}$	6.00	30.42	
4	2-((2R,8R,8aS)-8,8a-Dimethyl-1,2,3,4,6,7,8,8a-octahydronaphthalen-2-yl) propan-2-ol	$C_{15}H_{26}O$	6.00	30.63	

Table 1. Some chemical compositions of agarwood oil of *Aquilaria crassna* (Cont.)

No.	Name of the components	For mula	Percentage of Total	Retention time (min)	Structures
5	Longipinane, (E)-	$C_{15}H_{26}$	4.98	29.02	
6	.beta.-Selinene	$C_{15}H_{24}$	4.52	34.28	
7	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	4.46	41.47	
8	2-(6,10-DIMETHYLSPIRO [4.5]DEC-6-EN-2-YL)-2-PROPANOL	$C_{15}H_{26}O$	4.37	29.96	
9	1-[1-(2-Benzyloxy-2-vinylbut-3-enyloxy) allyl]-4-methoxybenzene	$C_{23}H_{26}O_3$	3.21	32.37	

Phylogenetic Relationship of the *18S rRNA* gene

For sequence analysis, the deduced amino acid sequences of the 1,708 bp from fungi isolation of *Aquilaria crassna* was composed of 569 amino acid residues, respectively (Figure 2). By comparing the sequences with the NCBI database, the *18S rRNA* gene of microorganism in agarwood showed the highest percent identity with *Phoma herbarum* at 96. 06% . For the phylogenetic tree construction, the tree based on *18S rRNA* sequences were all found in an area of the tree where mutual distances between taxa were moderately different tree when compared with other species of *18S rRNA* region in NCBI's GenBank database, as shown in Figure 3.

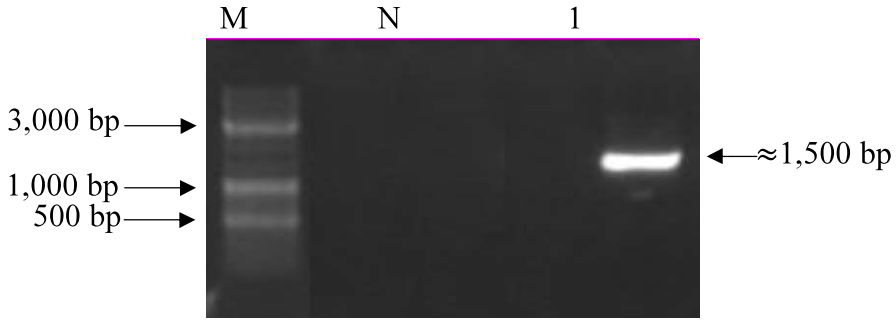


Figure 2. PCR-amplified *18S rRNA* products analyzed on a 1.5% agarose gel. Lane contain: (M) 100 bp DNA ladder; (N) Negative control; (1) PCR product of fungi isolation from *Aquilaria crassna*.

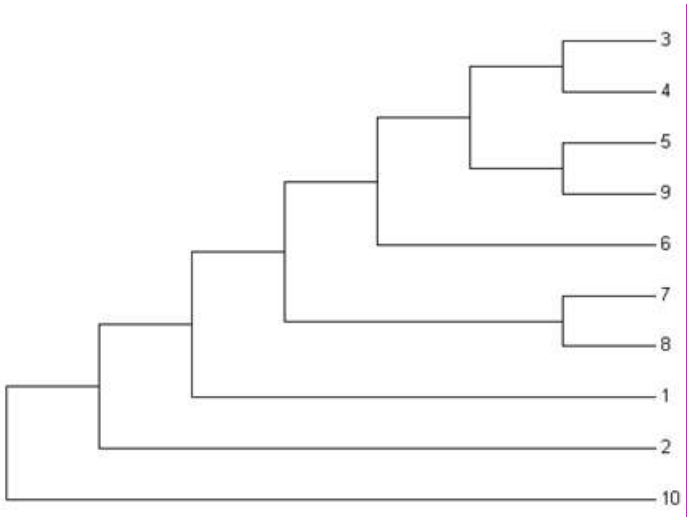


Figure 3. A neighbor- joining tree constructed from the percentage of sequence divergence between pairs of *18 S rRNA* sequences. The percent bootstrapping values (1,000 replicates) between branching groups are indicated 1; Fungal isolation from this study 2; *Phoma herbarum* (GU004251.1) 3; *Phaeosphaeriaceae* sp. (FJ744122.1) 4; *Phoma herbarum* (MG757513.1) 5; *Phoma* sp. (KF611891.1) 6; *Pleosporales* sp. (KM096358. 1) 7; *Didymella macrostoma* (KX519725. 1) 8; *Cochliobolus kusanoi* (JN941641. 1) 9; *Dothideomycetes* sp. (FJ517765.1) 10; *Drosophila melanogaster* (AH003249.2).

DISCUSSION

Agarwood is a well- known product that caused by stress in the wood or occurred from wounds in any way. Some researchers were interested in promoting agarwood formation with fungi. The results of this study provided the fungi in agarwood in *Aquilaria crassna* species is *Phoma herbarum*, which in *Dothideomycetes* Class. It is endophytic fungi that is also a versatile producer of many natural products with potential activities, but toxigenic pathogen to plants and animals, including humans under some occasions (Hamayun et al., 2009). Chen et al., 2017 reported the fungi isolated from an agarwood layer from *A. sinensis* tree which was found *Lasiodiplodia theobromae*. It has enable promoted agarwood formation and found

Dothideomycetes 81.82% from four different layers of the agarwood formation site of the trunk. Chhipa and Kaushik, 2017 demonstrated in the stem of *Aquilaria malaccensis*, Hypocreaceae was evaluated most dominant fungal family about 23.80% and indicated that *A. malaccensis* could be produced the Agarospirol by *Penicillium polonicum* (3.33%) within 3 months. Agarospirol is sesquiterpenes which sesquiterpenes and phenylethyl chromene were found the main fragrant compounds of agarwood (Chen et al., 2017). Of these, 7/10 were main compounds and good quality in agarwood by GC-MS analysis in this study. Essential oil contained 2(3H)-naphthalenone,4,4a,5,6,7,8-hexahydro-4a,5-dimethyl-3-(1-methylethylidene)-, (4ar-cis)- (6.54%); 1H-benzocycloheptene, 2,3,5,6,7,8,9,9a-octahydro-5,5,9-trimethyl-3-methylene-,(9S-trans)- (6.00%); 2-((2R,8R,8aS)-8,8a-dimethyl-1,2,3,4,6,7,8,8a-octahydronaphthalen-2-yl)propan-2-ol (6.00%); longipinane, (E)- (4.98%); beta.-selinene (4.52%); 2-(6,10-dimethylspro [4.5] dec-6-en-2-yl)-2-propanol (4.37%); 1-[1-(2-benzyloxy-2-vinylbut-3-enyloxy)allyl]-4-methoxybenzene (3.21%); .alpha.-cedrene oxide (2.86). Many reports are presented to the pathogenicity of endophytes could be an economical source of local community which can be created the oleoresin after infection in *Aquilaria* sp. tree as barrier to prevent further fungal encroachment (Chen et al., 2017; Tan et al., 2019). This research showed a marked difference in the oil compositions among the treatment with regards to their quality of *A. crassna*. From the knowledge in this research will lead to finding the appropriate fungi to stimulate the agarwood for producing high quality oil.

CONCLUSION

This study was carried out to find out the composition of essential oil through gas chromatography mass spectrometry (GC-MS). The results found the highest of methyl hept-2-ynoate (7.39%). In addition, we identified the fungi isolated from *Aquilaria crassna* based on *18S rRNA* sequences. It was found that the percentage identity of the *18S rRNA* gene of microorganism in agarwood is the highest possible with *Phoma herbarum* at 96.06%. The chemical constituents of essential compounds in the agarwood oil quality as well as role of the inducing agarwood production by the artificial inoculation method in *Aquilaria crassna* will be further elaborated in future studies.

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