

# CHARACTERISATION OF *AQUILARIA HIRTA* BASED ON MORPHOLOGY EVALUATION AND VOLATILE CHEMICAL COMPOUND

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Submitted August 2019; accepted January 2020

*Aquilaria hirta* is one of the agarwood tree species in Malaysia that produces a unique resinous wood and essential oil used in perfumery, medicinal, cosmetic and religious ceremonies. Currently, it has been listed as vulnerable (VU) and threatenad species in the IUCN Red List. This study aims to characterise the morphology of *A. hirta* via scanning electron microscopic analysis, and to identify the volatile chemical compounds of the wood and its essential oil using gas chromatography-flame ionisation detector and gas chromatography-mass spectrometry analyses. *Aquilaria hirta* was identified by the presence of hirsute on the abaxial side of leaves and midrib. The field emission scanning electron microscope analysis showed plant vessel pits can be seen in the healthy wood images compared with resinous wood images due to presence of resin and microorganisms. A total of 19 compounds were identified in resinous *A. hirta* wood consisting of 5.97% sesquiterpenes and 20.32% oxygenated sesquiterpenes. The major compounds are kessane,  $\gamma$ -cadinene,  $\alpha$ -caryophyllene,  $\beta$ -caryophyllene and caryophyllene oxide. There were 35 compounds found in *A. hirta* oil comprising 0.45% monoterpenes, 23.51% sesquiterpenes and 19.53% oxygenated sesquiterpenes. Major compounds detected were  $\gamma$ -cadinene, nor-ketoagarofuran, *allo*-aromadendrene,  $\gamma$ -gurjunene and  $\beta$ -gurjunene. This study provides a reference for the identification of *A. hirta* species based on morphology evaluation and volatile chemical compounds profile of the essential oil and wood.

Keywords: Agarwood, solid phase microextraction, sesquiterpene, sesquiterpenoid, monoterpenes

## INTRODUCTION

Agarwood is a resinous wood formed in *Aquilaria* species trees as a result of mechanical wounding followed by fungal infection. The wood and essential oil are being used in religious ceremonies, perfumery, medicinal and cosmetic industries. Since the past several years, exploitation of agarwood trees has been increasing due to the high demand from consumers. This has resulted in depletion of the natural resource, hence raising the price of agarwood. Since 2004, *Aquilaria* species have been listed in Appendix II of the Convention on the International Trade in Endangered Species (CITES 2017). *Aquilaria hirta* is one of the five *Aquilaria* species that produce fragrant resin in Malaysia (Faridah-Hanum et al. 2009). *Aquilaria hirta* is classified as vulnerable by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (IUCN 2015). Therefore, scientific conservation

effort must be conducted to characterise *A. hirta* tree, its essential oil, and the chip wood for creating a standard reference for this species. This plant is also considered as less explored agarwood species in Malaysia as very little reports on taxonomic and chemical profiles are available compared with *A. malaccensis* (Ng et al. 1997).

*Aquilaria hirta* could be identified by the presence of hirsute on the abaxial side of leaves and midrib. Species identification solely based on leaf morphology is challenging since the impact of different environmental condition such as climate change and soil composition lead to the changing of plant morphology (Lee et al. 2011). Thus, identification of *A. hirta* structures through scanning electron microscope is necessary for characterisation of physiological structures as well as the wood tissue of resinous and healthy part of the plant.

Agarwood and its essential oil have different quality or grades. Traditionally, physical appearances such as odour and colour have been used to grade their qualities. The method depends on human sense which is subjective, time-consuming, and has poor reproducibility and high labour expense. In Malaysia, some methods had been proposed for agarwood grading (Mazlan & Dahlan 2010, Nor Azah et al. 2013, MTIB 2014). Chemical compositions in agarwood and the oil are different among the grades. Therefore, there is a requirement for agarwood to be graded to its chemical profiles to ensure quality and originality. Gas chromatography–mass spectrometry (GC–MS) and gas chromatography–flame ionisation detector (GC–FID) are powerful methods to identify fingerprint compounds in volatile samples (Marriot et al. 2001). Some volatile marker compounds were identified using the methods for *A. malaccensis*, namely,  $\alpha$ -guaiene,  $\beta$ -agarofuran,  $\alpha$ -bulnesene, jinkoh-eremol, kusunol, selina-3,11-dien-9-one, oxo-agarospirol and guaiaa-1(10),11-dien-15, 2-olide (Tajuddin & Yusoff 2010). A total of 33 sesquiterpene hydrocarbons were identified using these methods as well as advanced analysis using GC $\times$ GC/TOFMS for *A. malaccensis* as an effort to establish a universal standard to classify the aromatic products from *Aquilaria* spp. (Tajuddin et al. 2016).

This study aims to characterise the morphology of *A. hirta* via scanning electron microscopic analysis, and to identify the volatile chemical compounds of the wood and its essential oil using GC–FID and GC–MS analyses.

## MATERIALS AND METHODS

### Plant materials

*Aquilaria hirta* wood and leaf samples were obtained from Rompin Forest, Pahang (02° 31.6' N, 103° 46.8' E, 29 m asl). The voucher specimen was kept at the Universiti Malaysia Pahang herbarium collection (BARCE03).

### Sample preparation

Both healthy and resinous woods were dried in an oven at 40 °C for 7 days. The dried wood was chopped and milled (1.0 mm powder size). Some woods and leaf samples were kept with maximum

dimensions of 3 cm  $\times$  3 cm  $\times$  3 cm and 3 cm  $\times$  3 cm respectively for scanning electron microscope analysis.

### Microscopic morphology analysis

The microstructures for fresh leaves were observed using scanning electron microscope equipped with electron detector. High resolution images were obtained with a magnification range from 10–500 $\times$ . Electron microscopy images of dried healthy and resinous woods were obtained in a field emission scanning electron microscope. High voltage power of 15 kV and high vacuum mode were used during the analysis.

### Extraction of essential oil

An amount of 20 g of resinous agarwood powder was immersed in 200 mL distilled water prior to hydrodistillation in a cleverger type apparatus for 12 hours. The oil was taken up in hexane and anhydrous sodium sulfate was added to remove water content. The solution was purged with nitrogen gas (N<sub>2</sub>) to remove hexane, then stored at 4 °C in glass amber vials prior to GC analysis.

### Solid phase microextraction (SPME)

Healthy and resinous agarwood powder (each 0.2 g) were transferred into different 4 mL clear glass vial with PTFE/silicone septum. The samples were exposed to SPME fibre, which was a 50/30  $\mu$ m DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethylsiloxane) at 40 °C for 30 min for volatile headspace adsorption. The fibre was then left for 3 min in the GC glass liner for thermal desorption at 240 °C.

### Gas chromatography analyses

Chemical profiling for wood and oil samples were conducted via GC system equipped with GC–FID and quadrupole mass spectrometer detector (GC–MS). The volatile compounds were carried by purified helium through DB-1ms capillary column (30 m in length  $\times$  0.25 mm inner diameter  $\times$  0.25  $\mu$ m film thickness) at a flow rate of 1.2 mL min<sup>-1</sup>. The GC–MS system was set with ionisation energy of 70 eV. Oven programming was set from 60 to 230 °C (3 min hold) at 3 °C min<sup>-1</sup> for wood samples with 230 °C used as inlet and detector temperatures. Oil

sample was run with oven programming of 80 to 250 °C (3 min hold) at 3 °C min<sup>-1</sup> and 250 °C as inlet and detector temperatures.

### Identification of chemical compounds

The components were identified by comparing their retention indices and mass spectra with published data by National Institute of Standards Technology (NIST). Compounds determination for GC–MS were based on > 90% similarity index of that NIST library. Kovats retention indices were calculated using a homologous series of n-alkanes (C<sub>7</sub>–C<sub>20</sub>) using equation 1 for GC–FID.

$$I = 100 \times \left[ n + (n - n) \frac{\log(t'_r \text{ unknown}) - \log(t'_r n)}{\log(t'_r N) - \log(t'_r n)} \right] \quad (1)$$

- I = Kovats retention index  
 n = Number of carbon atom in smaller n-alkane  
 N = Number of carbon atom in larger n-alkane  
 t'<sub>r</sub> = Retention time of unknown chemical compound  
 t'<sub>r</sub> n = Retention time of carbon atom in smaller n-alkane  
 t'<sub>r</sub> N = Retention time of carbon atom in larger n-alkane

## RESULTS AND DISCUSSION

In order to identify a species in *Aquilaria* genus, observation must also be made on the flowers and fruits. Unfortunately, the morphological characteristics of those plant parts were not available since samples were obtained outside breeding season unknowingly. According to the Anonymous (2015), the colour of the *A. hirta* flower appeared to be whitish to light yellow and the floral tube is cylindrical. It has small hairs on the surface and more pointed at the base compared with other *Aquilaria* species. The shape of the fruit capsule is oblanceolate acute and has a cuneate base, sharp apex, as well as a hairy texture. Being a timber, *Aquilaria* species bear flowers and fruits when they mature at 7–9 years of age. This means the botanical identification of agarwood can take years. Therefore, morphology evaluation using scanning electron microscopic analysis of herbarium sample of the leaf can be an

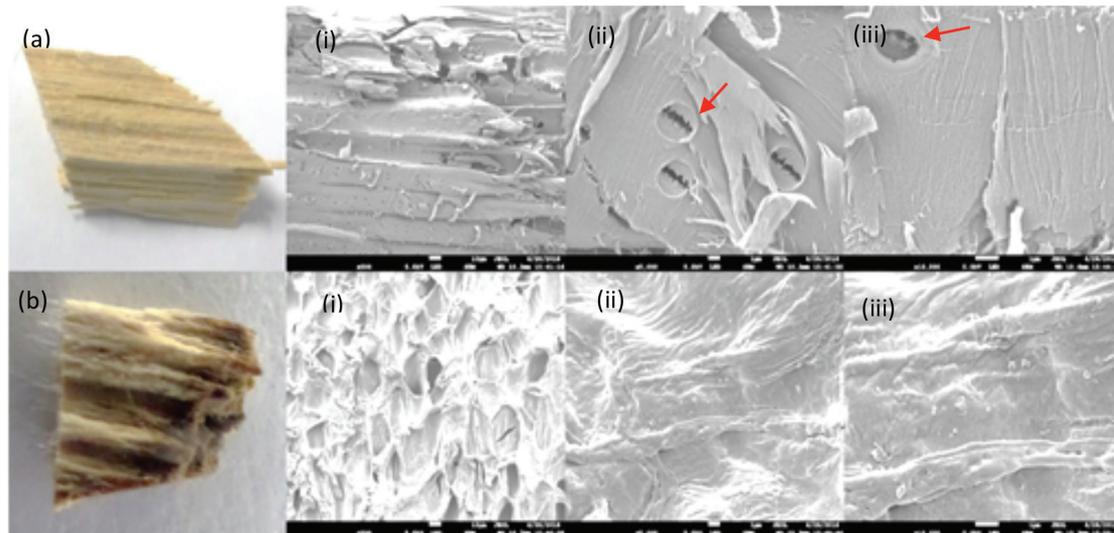
alternative for species identification of *Aquilaria* spp.

Healthy *A. hirta* wood has a pale beige colour while the affected wood is dark brown or black due to the increased mass and wood density from resin development as a result of the plant defense system (Tajuddin et al. 2016). Microscopic observation under light microscope revealed that phloem and parenchyma cells are important living cells for biosynthesis of agarwood in *Aquilaria* spp. as brownish substances were found in both structures (Mohamed et al. 2013).

Qualitative analysis of the structures of healthy and resinous part of *A. hirta* obtained using field emission scanning electron microscope displayed the presence of agarwood resin (Figure 1). The surface of infected or resinous wood showed extensive degradation (Figure 1b(i)) compared with healthy wood (Figure 1a(i)). Vessel pits (red arrow) can be seen in the healthy wood images (Fig 1a(ii–iii)) compared with resinous wood images (Fig 1b(ii–iii)) due to presence of resin and microorganisms. The penetration of microorganism into the wood matrix is the most probable cause of the observed structure in Figure 1b(i) (Gutarowska et al. 2015). Therefore, besides transporting water to adjacent tracheary elements in flowering plants, vessel pits can be used as a phylogenetic marker to identify *Aquilaria* spp. (Jansen et al. 2004).

Table 1 shows the chemical composition of volatile *A. hirta* wood and essential oil based on GC analysis. A total of 47 compounds were identified in the samples using GC–FID and GC–MS. A total of 19 compounds were found in the resinous part of *A. hirta* comprising 5.97% sesquiterpenes and 20.32% sesquiterpenoid. The identified sesquiterpenoid compounds were kessane, epoxybulnesene, caryophyllene oxide, 7-epi-γ-eudesmol epi-α-cadinol, α-bisabolol, selina-4,11-dien-14-oic acid and selina-3,11-dien-14-oic acid where kessane was the highest (15.39%). Meanwhile, healthy *A. hirta* wood contained 2.17% sesquiterpenes and 1.41% sesquiterpenoid and caryophyllene oxide was the main sesquiterpenoid constituent (0.71%).

There were 35 compounds found in *A. hirta* essential oil which contained 0.45% monoterpenes, 23.51% sesquiterpenes and 19.53% sesquiterpenoid. δ-Cadinene (11.19%) produced the highest percentage area among sesquiterpene compounds for *A. hirta* oil while the highest percentage area of sesquiterpenoid compounds was nor-ketoagarofuran (9.40%).



**Figure 1** Transverse section and microstructure images of *A. hirta* wood: (a) pale beige healthy wood and (b) brown resinous wood and their field emission scanning electron microscope images at (i) 500 $\times$ , (ii) 5000 $\times$  and (iii) 10000 $\times$

Major volatile compounds detected in the *A. hirta* essential oil were  $\gamma$ -cadinene (11.19%), nor-ketoagarofuran (9.40%), *allo*-aromadendrene (4.34%),  $\gamma$ -gurjunene (3.42%), and  $\beta$ -gurjunene (1.93%).

Identification of volatile chemical compounds is essential for grading and quality control purposes (Nor Azah et al. 2008, Pripdeevech et al. 2011, Tajuddin et al. 2013). In this study, we found that the agarwood samples were rich in terpene groups dominated by sesquiterpenoid, sesquiterpenes and monoterpenes. The quality of agarwood is increased with the increase in resin yield (Pasaribu et al. 2015). Compounds that are classified as marker compounds of agarwood essential oil include 4-phenyl-2-butanone,  $\alpha$ -guaiene,  $\beta$ -agarofuran,  $\alpha$ -bulnesene, nor-ketoagarofuran, agarospirol, jinkoh-eremol, kusunol, dehydrojinkoh-eremol and selina-3,11-dien-9-one (Ishihara et al. 1993, Tajuddin & Yusoff 2010). Interestingly, some of the marker compounds also present in both *A. hirta* wood and oil samples as listed in the Table 1. Major compounds detected in the essential oil extracted from the *in vitro* shoots of *A. hirta* are tetradecanal, hexadecanoic acid, methyl linoleate, linoleic acid, isophytol and phytol acetate (Hassan et al. 2011). In this study, the existence of the marker compounds identified by the previous researchers are verified for both *A. hirta* wood and oil samples.

The nature of agarwood formation is sophisticated due to many factors involved causes a challenge in determination of quality. There are several aspects that may result in different chemical constituents of agarwood and their essential oils including the origin of the species, the stimulation method of agarwood resin and method of extraction of agarwood oils (Naef 2011, Hashim et al. 2014). Deep and Tajuddin (2019) proposed that agarwood oil of specific origin should be classified separately based on their chemical compositions and the grading should be based on sesquiterpenoid contents in the pure essential oil. This study provided additional database for the agarwood identity and was designed to identify chemical compounds from the best sample from the wild only. The sample was collected from only one location in Endau–Rompin Forest and was considered as wild and had the best quality which meant it did not undergo inoculation process (Mohamed & Lee 2016). However, more sample collection will be carried out in future and statistical analysis should be included towards creating the standard reference.

## CONCLUSIONS

This plant was verified as *A. hirta* based on morphology evaluation of the leaf and wood via scanning electron microscopic analysis. Major

**Table 1** Chemical composition of volatile *Aquilaria hirta* wood and essential oil based on gas chromatography analysis

Chemical compound	Molecular formula	RI	Relative peak area (%)			Identification
			HW	RW	EO	
Aldehyde and ketone						
Benzaldehyde	C <sub>7</sub> H <sub>6</sub> O	939	0.20	0.85	-	FID, MS
4-phenyl-2-butanone	C <sub>10</sub> H <sub>12</sub> O	-	-	-	3.75	MS
Monoterpenoid						
Isoeugenol	C <sub>10</sub> H <sub>12</sub> O <sub>2</sub>	-	-	-	0.45	FID
Sesquiterpene						
$\alpha$ -Copaene	C <sub>15</sub> H <sub>24</sub>	1367	-	-	-	FID
$\alpha$ -Gurjunene	C <sub>15</sub> H <sub>24</sub>	1403	0.08	0.15	0.50	FID, MS
Isocaryophyllene	C <sub>15</sub> H <sub>24</sub>	1411	0.60	-	1.55	FID
$\alpha$ -Longipinene	C <sub>15</sub> H <sub>24</sub>	1419	-	-	0.59	FID
$\beta$ -Gurjunene	C <sub>15</sub> H <sub>24</sub>	1425	-	-	0.13	FID
$\beta$ -Caryophyllene	C <sub>15</sub> H <sub>24</sub>	1433	0.50	1.05	1.93	FID
Aromadendrene	C <sub>15</sub> H <sub>24</sub>	1436	-	-	0.56	FID, MS
$\alpha$ -Caryophyllene	C <sub>15</sub> H <sub>24</sub>	1445	0.27	1.38	1.55	FID
$\gamma$ -Selinene	C <sub>15</sub> H <sub>24</sub>	1455	-	-	0.79	FID
Alloaromadendrene	C <sub>15</sub> H <sub>24</sub>	1460	-	0.36	4.34	FID
$\alpha$ -Curcumene	C <sub>15</sub> H <sub>22</sub>	1467	-	0.51	0.14	FID
$\gamma$ -Gurjunene	C <sub>15</sub> H <sub>24</sub>	1473	0.13	0.20	3.42	FID, MS
$\delta$ -Guaiene	C <sub>15</sub> H <sub>24</sub>	1491	0.08	-	0.47	FID, MS
$\gamma$ -Cadinene	C <sub>15</sub> H <sub>24</sub>	1499	0.34	2.14	11.19	FID
$\delta$ -Cadinene	C <sub>15</sub> H <sub>24</sub>	1510	-	-	0.61	FID
$\alpha$ -Calacorene	C <sub>15</sub> H <sub>20</sub>	1525	0.17	-	-	FID
Dehydroaromadendrene	C <sub>15</sub> H <sub>24</sub>	1534	-	0.18	0.50	FID
Oxygenated sesquiterpene						
Dihydro- $\beta$ -agarofuran	C <sub>15</sub> H <sub>26</sub> O	1486	-	0.72	-	FID
Kessane	C <sub>15</sub> H <sub>26</sub> O	1516	-	15.39	1.64	FID, MS
Cis-nerolidol	C <sub>15</sub> H <sub>26</sub> O	1553	0.40	-	-	FID
nor-ketoagarofuran	C <sub>14</sub> H <sub>22</sub> O <sub>2</sub>	1563	-	-	0.68	FID
Epoxybulnesene	C <sub>15</sub> H <sub>24</sub> O	1578	-	0.62	9.40	FID
Caryophyllene oxide	C <sub>15</sub> H <sub>24</sub> O	1582	-	0.93	-	FID
7-epi- $\gamma$ -eudesmol	C <sub>15</sub> H <sub>26</sub> O	1598	0.71	0.41	-	FID
Guaiol	C <sub>15</sub> H <sub>26</sub> O	1608	0.11	-	-	FID
$\gamma$ -Eudesmol	C <sub>15</sub> H <sub>26</sub> O	1615	-	-	-	FID, MS
Agarospinol	C <sub>15</sub> H <sub>26</sub> O	1620	0.12	-	0.1	FID
$\beta$ -Eudesmol	C <sub>15</sub> H <sub>26</sub> O	1632	-	0.33	-	FID
epi- $\alpha$ -Cadinol	C <sub>15</sub> H <sub>26</sub> O	1635	-	0.88	0.14	FID
$\alpha$ -Eudesmol	C <sub>15</sub> H <sub>26</sub> O	1639	-	-	0.86	FID
Kusunol	C <sub>15</sub> H <sub>26</sub> O	1656	-	-	0.99	FID
Dehydrojinkoh-eremol	C <sub>15</sub> H <sub>24</sub> O	1673	-	0.19	-	FID
$\alpha$ -Bisabolol	C <sub>15</sub> H <sub>26</sub> O	1686	-	0.55	-	FID
Selina-3,11-dien-9-one	C <sub>15</sub> H <sub>22</sub> O	1695	0.07	-	0.48	FID
Rotundone	C <sub>15</sub> H <sub>22</sub> O	1700	-	-	0.74	FID
Selina-3,11-dien-9-ol	C <sub>15</sub> H <sub>24</sub> O	1715	-	-	0.58	FID
Selina-4,11-dien-14-oic acid	C <sub>15</sub> H <sub>22</sub> O <sub>2</sub>	1725	-	0.30	1.57	FID
Selina-3,11-dien-9-al	C <sub>15</sub> H <sub>22</sub> O	1734	-	-	0.86	FID
Selina-4,11-dien-14-al	C <sub>15</sub> H <sub>22</sub> O	1765	-	-	0.40	FID
Guaia-1(10),11-dien-15-oic acid	C <sub>15</sub> H <sub>22</sub> O <sub>2</sub>	1812	-	-	0.46	FID
Karanone	C <sub>15</sub> H <sub>20</sub> O	1821	-	-	0.28	FID
Oxo-agarospinol	C <sub>15</sub> H <sub>24</sub> O <sub>2</sub>	1836	-	-	0.35	FID
Carboxylic acid						
<i>n</i> -Hexadecanoic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	-	-	-	13.04	MS
trans-9-Octadecenoic acid	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>	-	-	-	11.20	MS
Total aldehyde and ketone			0.20	0.85	3.75	
Total monoterpenoid			-	-	0.45	
Total sesquiterpene			2.17	5.97	28.27	
Total sesquiterpenoid			1.41	20.32	19.84	
Total carboxylic acid			-	-	24.24	

RI = retention indices based on DB-1ms capillary column, HW = healthy wood, RW = resinous wood, EO = essential oil, FID = detection via GC-FID, MS = identification via GC-MS

volatile chemical compounds were successfully identified in both healthy and resinous wood as well as the essential oil by using GC–MS and GC–FID. Since *A. hirta* is classified as vulnerable and is the least explored Malaysia agarwood species, this study will provide a reference database for *A. hirta* species verification and quality analysis of the agarwood as well as the essential oil based on the volatile chemical compounds. In future, sustainable agarwood supply in the global market can be established by proper identification and quality control analysis to address the problem of fraud in the agarwood industry.

## ACKNOWLEDGEMENTS

We acknowledge the financial support awarded by the Ministry of Higher Education Malaysia (Fundamental Research Grant Scheme no. FRGS/1/2016/STG01/UMP/02/1) to Universiti Malaysia Pahang.

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