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Microscopy and Chemical Composition of Healthy and Resinous Wood from the Agarwood-Producing Species, *Aqualaria Beccariana*

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Abstract: *Aquilaria beccariana* is a vulnerable Malaysian agarwood-producing species due to illegal harvesting and indiscriminate deforestation. Despite its current conservation status, the chemical profiling of this valuable species is seemingly non-existent. The current study aimed to evaluate the morphological characteristics of *A. beccariana* wood and identify the volatile chemical compounds of its wood and essential oil. The field emission scanning electron microscope (FESEM) was used to study the wood morphology, while gas chromatography with flame ionization detection and gas chromatography coupled with mass spectrometry was used. The FESEM analysis revealed that vessel pits were distinct in the healthy wood samples but hardly visible in the resinous wood sample. The monoterpene, sesquiterpenes, and sesquiterpenoid were detected, whereby 35 constituents were from the resinous wood sample, which consisted of 15.29% sesquiterpenes and 50.68% sesquiterpenoid. The major compounds were kessane, α-longipinene, α-curcumene, eudesmol, and epi-α-bisabolol. Approximately 32 compounds were identified in the essential oil sample, comprising 36.69% of sesquiterpenes and 49.58% of sesquiterpenoids. The principal compounds were 7-epi-γ-eudesmol, γ-cadinene, allo-aromadendrene, kessane, and nor-ketoagarofuran. This study provides valuable information on the volatile chemical compound profiles of *A. beccariana*; thus, it would further contribute to the search for potential chemical markers for species detection and agarwood classification efforts.

Keywords: *Aquilaria*; agarwood; headspace volatile; essential oil; chemical composition; sesquiterpene.

1. Introduction

Aquilaria beccariana Tiegh (Thymelaeaceae) is one of the five species of *Aquilaria* that is naturally available in Malaysia and has been identified to produce the fragrant resin known as agarwood or gaharu [1]. The locals and aborigines also know the tree as Karas Batu and Candan Gajah [2]. In Malaysia, natural populations of *A. beccariana* can be found in the selected regions in Johor but are widely distributed in Sabah and Sarawak [1].

The formation of agarwood on *Aquilaria* trees is triggered by wounding treatments that activate the tree's defense mechanism, secreting the fragrance resin to impede further invasion of microorganism attacks through its wound [3]. The agarwood is harvested, while some are extracted for its essential oil, a valuable ingredient for the production of incense, perfumery, and traditional medicine [4,5]. Agarwood has been an ingredient of Traditional Chinese Medicine for treating several diseases and possesses different pharmacological properties [6,7]. Agarwood hydrosol is also believed to have chemical properties similar to essential oils and antioxidant activity [8]. Agarwood has also been recently studied as a bio-diesel source [9,10]. As the formation of agarwood in the wild is rare, the high demand for this natural resource has led to illegal harvesting and indiscriminate felling of these trees in search of agarwood in the wild [11]. On the other hand, rapid development in human lifestyle has also led to extensive forest deforestation to give way to agricultural land expansion and urban development [12]. Such phenomena have threatened the existence of this endangered species in the wild; thus, placed on the Red List of Threatened Species since 1998 by the International Union for Conservation of Nature (IUCN) [13].

High-quality agarwood is reputed to be able to float in water and give off a strong, distinctive perfume when burned. However, there is no standard to evaluate the quality of agarwood based on chemical components; rather, it is evaluated according to the individual's specific requirements. *A. beccariana* has received fewer reports on its taxonomic and chemical profiles than *Aquilaria malaccensis*, the most common *Aquilaria* species in Malaysia. Categorizing these natural products is difficult in Malaysia, despite several criteria for agarwood grading having been presented by the Malaysian Forestry Department [14]. This is due to the difference in chemical makeup between agarwood and its essential oil. Last but not least, sesquiterpene and 2-(2-phenylethyl)-chromone are regarded to be two crucial kinds of compounds present in high-quality agarwood [5].

The complexity of herbs and extracts raises significant classification and quality issues that increase the need for appropriate analytical methods for their identification and standardization. Characterization and proper quality assurance are important steps in ensuring the quality of plant-derived natural products. [15]. Because adulterants can exist in trace amounts, their analysis necessitates the use of high-efficiency methods such as microscopy (for molds, insect parts, or particles) and chromatography, which is sometimes combined with immunoaffinity extractions, spectroscopy (MS, NMR, FTIR, ICP-AE, ICP-MS, ESR), and DNA-based methods [16]. Metabolomics profiling, also known as fingerprinting, is a method for determining the overall chemical composition of plant-derived meals subjected to various environments [17]. Metabolomics primarily uses hyphenated technologies for volatile component profiling, such as solid-phase microextraction (SPME) followed by gas chromatography (GC) combined with mass spectrometry (MS) [18].

The chemical compounds in the agarwood of *A. malaccensis* can be characterized using both these analytical tools [19]. Additionally, the chemical profile of *A. malaccensis* using the GC×GC/TOFMS has successfully identified a number of sesquiterpene hydrocarbons, contributing to establishing a universal standard to classify the aromatic products from *Aquilaria* species [20]. However, there are limited studies on the chemical composition of agarwood of *A. beccariana* and its essential oil. Thus, we aimed to observe the surface condition of the healthy wood and resinous agarwood of *A. beccariana* using the Field Emission Scanning Electron Microscope (FESEM) technique, as well as to characterize the chemical profiles of *A. beccariana*, both its healthy wood and agarwood and its essential oil,

using Gas Chromatography-Mass Spectrometer (GC-MS) and Gas Chromatography with Flame Ionization Detection (GC-FID) method. Both methods are highly useful for identifying fingerprint compounds in volatile oils [21]. We envisioned that the findings of this study would also contribute to developing a chemical-based standard for *A. beccariana*, which will be helpful in species identification and quality control.

2. Materials and Methods

2.1. Plant material and sample preparation.

Aquilaria beccariana wood samples were obtained from a natural population in Rompin, Pahang, Malaysia (Latitude: 2°48'59.99" N; Longitude: 103°28'59.99" E). The collection took place in April 2017. The Bio Aromatic Research Centre, Universiti Malaysia Pahang, kept the voucher specimens (BARCE01-BARCE03). The healthy, fresh, and resinous woods were oven-dried for seven days at 40°C. The dry wood was cut into small pieces and milled (1.0 mm powder size). A tiny section of the wood with a maximum diameter of 3 cm× 3 cm ×3 cm was used for FESEM examination.

2.2. Microscopic morphology analysis.

Electron microscopic analysis of dried healthy and resinous woods was performed in a JSM-7500F FESEM (JEOL, Japan), and images were saved. Analysis was done using high voltage (HV) power of 15 kV and high vacuum mode.

2.3. Essential oil extraction.

About 20 g of resinous agarwood powder was immersed in 200 mL of distilled water in a volatile oil distilling device 12 hours before hydrodistillation (Pyrex, France). Anhydrous sodium sulfate was used to remove the water content from the oil after it had been extracted in hexane. Hexane was removed from the solution using nitrogen gas, and it was then kept at 4°C in amber glass vials for future investigation.

2.4. Solid-phase micro-extraction headspace extraction.

About 0.2 g of the powdered healthy and resinous wood was put into separate clear glass vials containing 4 mL polytetrafluoroethylene (PTFE)/silicone septum. For the purpose of adsorption in volatile headspace, the samples were subjected to solid-phase micro-extraction headspace extraction (SPME) fiber (a 50/30 m DVB/CAR/PDMS - divinylbenzene/carboxy/polydimethylsiloxane) at 40°C for 30 min. For thermal desorption at 240°C, the fiber was kept in the GC glass liner for three minutes.

2.5. Gas chromatography analyses.

In order to perform chemical profiling of wood and essential oil, an Agilent 7890A GC system (Agilent Technologies, USA) equipped with a flame ionization detector (GC-FID) and Agilent 7890B coupled with a 5977A quadrupole mass spectrometer detector (GC-MS) (Agilent Technologies, USA) was used. Purified helium was used to transport the volatile substances at a flow rate of 1.2 mL per minute via the DB-1ms capillary column, which has a 30 m length, 0.25 mm inner diameter, and 0.25 m film thickness. The ionization energy for the GCMS system was fixed at 70 eV. For wood samples, the oven's temperature was programmed

to rise by 3°C per minute from 60 to 230 °C (with a 3 min hold), while the input and detector temperatures were set at 230 °C. The input and detector temperatures were set at 250°C, and the oven programming was set from 80°C to 250°C (with a 3 min hold) at 3°C/min. By comparing the compounds' retention indices to data available in the National Institute of Standard and Technology (NIST) database, the compounds' compositions were identified [22].

3. Results and Discussion

3.1. Microscopic observation.

Micro-anatomy is the primary set of pharmacognostic methods for evaluating any fresh or raw samples of different plant parts [23]. Qualitative analysis of the surface structures obtained through the FESEM technique displayed variations in morphological appearance between healthy wood and agarwood of *A. beccariana*. While the simple vessel pit structure in the healthy wood can be observed even at lower magnification power, i.e., 200× (Figure 1A), and was easily spotted under greater magnification power, i.e., 5000× (Figure 1B) and 10000× (Figure 1C); however, the surface of resinous wood experienced significant degradation and vessel pits were not seen around the resinous area (Figure 1 D-E). Precipitated products were observed concealing the vessel walls and pits on the surface of the non-resinous wood as seen under 10000× magnification power (Figure 1F).

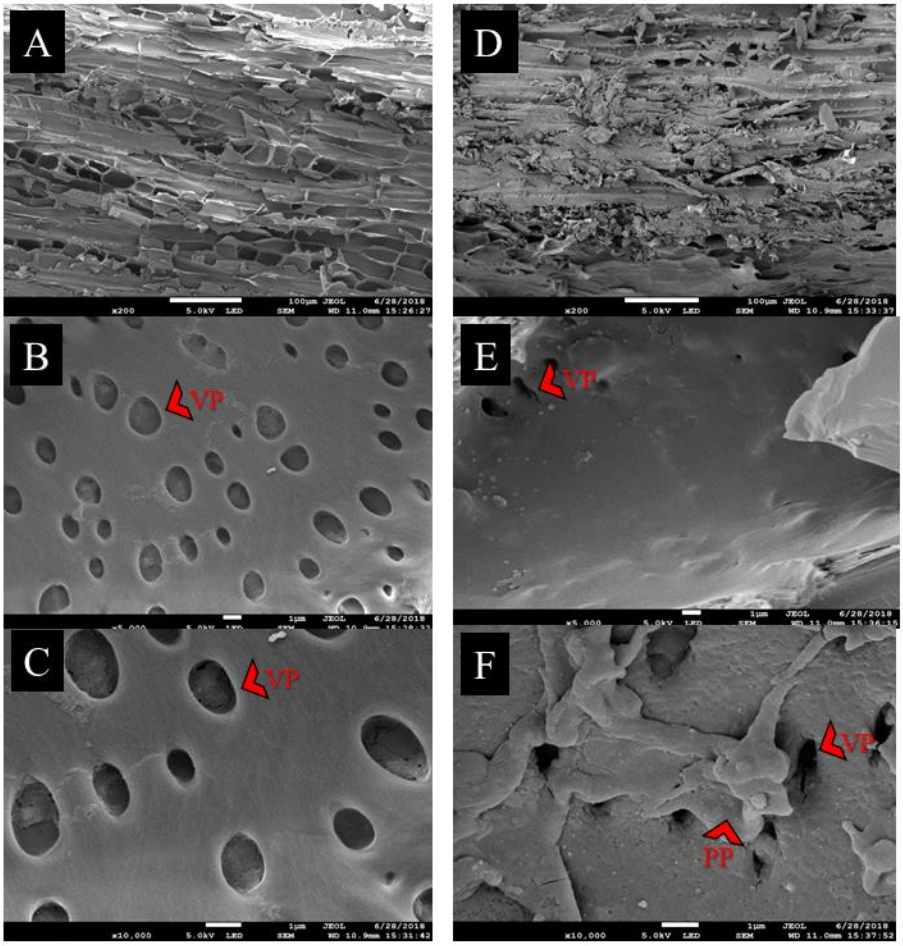

Figure 1. Field Emission Scanning Electron Microscope (FESEM) images of (A-C) healthy and (D-F) resinous wood (agarwood) of *Aquilaria beccariana* at the magnification rate of 200×, 5000×, and 10000×, respectively. Arrows indicate the vessel pits (VP) and the presence of precipitated products (PP) on the wood surface.

Based on the literature, members of *Aquilaria* shared similar wood anatomy features [24]. Precipitated products found on the surface of the resinous wood of *A. beccariana* are the source of aroma for agarwood, which is regarded as a barrier to separate the decayed or wounded wood from newly formed cells. The precipitated products were speculated to be produced in the parenchyma cells that contain included phloem [25]. Light, brownish, resinous substances were observed in the parenchyma cells when the starch began to decrease, which further penetrated the ray and axial parenchyma cells of the outermost sapwood. These brownish substances were related to the resin gaharu [26]. Similar to *A. malaccensis*, resin accumulation is found to take place in the xylem of *A. beccariana* (Figure 1F), while the resin boundary was suggested to play a major role in the compartmentalization process around the defect area as part of the plant defense system [27].

3.2. Chemical profiling.

A total of 52 monoterpene, sesquiterpenes, and sesquiterpenoids were detected and identified from the three different samples used in this study, including the healthy wood, resinous wood, and the essential oil of *A. beccariana* (Table 1). Across the ten compounds found in the healthy wood sample, epoxybulnesene was recorded specifically in the healthy wood sample, while four and one compounds were found to co-exist in the resinous wood sample and the essential oil, respectively. The other four, including α-caryophyllene, α-gurjunene, γ-gurjunene, and kessane, were detected in all three samples. The percentages of the compounds sesquiterpenes and sesquiterpenoid present in the healthy wood samples were 1.41% and 2.39%, respectively. Kessane was identified as the main constituent for sesquiterpenoid in the healthy wood sample, which consisted of 1.17% of all sesquiterpene and sesquiterpenoid obtained.

About 0.63%, 15.29%, and 50.68% of the resinous wood sample were monoterpenes, sesquiterpenes, and sesquiterpenoids. Among the 35 monoterpene, sesquiterpenes, and sesquiterpenoids detected in the resinous wood sample, one monoterpene (4-phenyl-2-butanone), one sesquiterpene (δ-guaiene), and 13 sesquiterpenoids were found unique. At least 12 of the compounds detected in the resinous wood sample were also detected in the essential oil but not present in the healthy wood. Like the healthy wood, kessane was the greatest in volume compared to other sesquiterpenes and sesquiterpenoids, with approximately 32.83% of all sesquiterpene sesquiterpenoids obtained.

The chemical analysis of the essential oil resulted in 36.69% and 49.58% of the compounds being from the sesquiterpene and sesquiterpenoid families, respectively. Among the 32 compounds in which eight sesquiterpenes and seven sesquiterpenoids were unique to the essential oil sample, γ-cadinene recorded the highest percentage area (14.26%) among the sesquiterpene compounds and nor-ketoagarofuran (14.62%) among the sesquiterpenoid compounds. The essential oil sample also included significant amounts of kessane (8.21%), allo-aromadendrene (6.34%), and 7-epi-eudesmol (6.22%). The effort to profile the chemical compounds present in agarwood contributes to identifying markers useful for classifying and quality control purposes [28-30]. Moreover, the quantity and quality of agarwood are influenced by stress implied on the plant by either mechanical or microbial growth. The content of oil content and metabolites can be varied [31]. Gasc chromatography-mass spectrometry was successfully applied in the aroma-containing chemical classification of different varieties of Fennel [32].

In this study, we noticed that the resinous wood sample was rich in compounds of the terpene groups – monoterpenes, sesquiterpenes, and sesquiterpenoids. Compounds derived from sesquiterpenoids are the major contributor to the chemical profile of agarwood [33]. It is proposed that the quality of the agarwood is a positive correlation to the percentage of certain aromatic compounds present in the sample, e.g., aromadendrene [34], which was only detected in the essential oil sample of *A. beccariana*; while others reported that chemical compounds such as 4-phenyl-2-butanone, α-guaiene, α-bulnesene, β-agarofuran, agarospirol, dehydrojinkoh-eremol, jinkoh-eremol, kusunol, nor-ketoagarofuran, and selina-3,11-dien-9-one, are potential chemical markers useful in agarwood classification [21,35]. Most of these proposed chemical markers were detected in the resinous wood and/or essential oil samples of *A. beccariana*, except for two compounds, agarospirol, and selina-3,11-dien-one, which were also present in the healthy wood sample; dehydrojinkoh-eremol was not detected in this study. A recent study showed that an inducement technology produced agarwood resin in resin in *A. beccariana*, which contained two sesquiterpenes, namely agarofuran, and agarospirol, which is in agreement with our results. Batubara *et al*. studied the chemical compositions of agarwood leaves of *A. beccariana by* GC-MS technique and identified 22.61% neophytadiene 11.13 % n-hexadecanoic acid, and 11.39% hexadecanoic acid, ethyl ester from natural-grown agarwood while from the cultivated agarwood 1,2,3,4-Cyclopentanetetrol, Oxacycloheptadec-8-en-2-one and 1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0] nonane (8.08%, 5.97% and 5% respectively [36]. Hexadecanoic acid methyl ester and neophytadiene are also from the agarwood leaves of *A. malaccensis* Lamk [37]. The nature of agarwood formation is sophisticated due to many factors that could pose challenges in its quality determination. When looking into the differences in chemical composition in agarwood and its essential oil, it might be crucial to consider several factors, including the origin of the species, the stimulation method for agarwood formation, and the type of oil extraction method used [5, 38]. In a recent study, Yao *et al*., (2022) [39] effectively developed an FT-NIR, GC-MS, and UHPLC-Q-Exactive Orbitrap/MS approach based on phytochemical profiling to discriminate between wild and farmed agarwood. Nedeltcheva-Antonova *et al*. used similar analytical approaches for a comprehensive chemical profiling of different industrial samples of Bulgarian lavender using gas chromatography-mass spectrometry (GC/MS) and gas chromatography with flame ionization detection (GC-FID), resulting in the detection of 111 compounds of 52.83-80.55% oxygenated monoterpenes and 7.80-15.21% sesquiterpenes [40].

Table 1. Volatile chemical composition of healthy and resinous wood and essential oil of *Aquilaria beccariana*.

Compounds	DB1 column	Relative peak area (%)a			Detection method
		Healthy wood	Resinous wood	Essential oil	
Monoterpene hydrocarbons (MH)					
4-phenyl-2-butanone	1216	-	0.63	-	FID
Total		0	0.63	0	
Sesquiterpene hydrocarbons (SH)					
α-copaene	1373	-	-	1.31	FID
α-gurjunene	1403	0.13	0.80	3.52	FID, MS
α-cedrene	1405	-	-	0.31	FID
Isocaryophyllene	1411	-	1.55	0.25	FID, MS
α-longipinene	1421	-	6.06	0.83	FID
β-gurjunene	1426	-	-	3.98	FID
β-caryophyllene	1434	0.39	0.78	-	FID
aromadendrene	1438	-	-	0.23	FID, MS
α-caryophyllene	1446	0.17	0.41	0.83	FID, MS

Compounds	DB1 column	Relative peak area (%)a			Detection method
		Healthy wood	Resinous wood	Essential oil	
γ-selinene	1453	-	-	1.91	FID
allo-aromadendrene	1461	-	0.40	6.34	FID, MS
α-curcumene	1470	-	4.66	1.29	FID
γ-gurjunene	1474	0.15	0.21	0.32	FID, MS
δ-guaiene	1492	-	0.09	-	FID, MS
γ-cadinene	1501	-	-	14.26	FID
δ-cadinene	1511	-	-	0.94	FID
α-calacorene	1528	0.57	0.33	-	FID
Dehydro-aromadendrene	1533	-	-	0.37	FID
Total		1.41	15.29	36.69	
Oxygenated Sesquiterpenes (OS)					
β-agarofuran	1476	-	0.51	2.13	FID
Dehydro- β-agarofuran	1489	-	0.14	3.39	FID, MS
Kessane	1515	1.17	32.83	8.21	FID, MS
Cashmeran	1473	-	-	4.28	FID
Elemol	1531	-	0.29	0.95	FID, MS
Cis-nerolidol	1553	-	-	0.88	FID
nor-ketoagarofuran	1566	-	0.19	14.62	FID
Epoxybulnesene	1575	0.24	-	-	FID
Caryophyllene oxide	1583	0.55	1.13	-	FID
7-epi-γ-Eudismol	1603	-	-	6.22	FID, MS
Guaiol	1609	-	1.19	-	FID
γ-eudesmol	1615	-	-	4.23	FID, MS
Agarospirol	1622	0.26	0.28	-	FID
β-eudesmol	1631	-	0.54	-	FID
epi-α-cadinol	1635	-	0.49	-	FID
α-eudesmol	1637	-	0.10	-	FID
Jinkoh-eremol	1646	-	0.81	0.25	FID
Kusunol	1654	-	0.52	0.40	FID
Bulnesol	1658	-	0.41	-	FID
epi-α-bisabolol	1682	-	1.78	-	FID
α-bisabolol	1691	-	0.72	-	FID
Selina-3,11-dien-9-one	1696	0.17	-	0.24	FID
Rotundone	1701	-	-	0.25	FID
Selina-3,11-dien-9-ol	1717	-	1.10	-	FID
Selina-4,11-dien-14-oic acid	1725	-	-	0.60	FID
Selina-3,11-dien-9-al	1740	-	0.44	0.23	FID
9,11-Eremophiladien-8-one	1744	-	1.62	2.21	FID
Guaia-1(10),11-dien-9-one	1756	-	0.40	-	FID
Selina-4,11-dien-14-al	1763	-	-	0.49	FID
Selina-3,11-dien-14-oic acid	1778	-	1.01	-	FID
Dihydrokaranone	1794	-	0.20	-	FID
Eudesmol	2319	-	3.79	-	FID
9-hydroxyselina-4,11-dien-14-oic acid	1958	-	0.19	-	FID
Total		2.39	50.68	49.58	

4. Conclusions

The findings obtained from this study could serve as helpful information on the chemical compounds present in the healthy and resinous wood of *A. beccariana*. In addition, they would further contribute to searching for potential chemical markers for species detection and agarwood classification efforts.

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Conflicts of Interest

The authors declare no conflict of interest.

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