Full Length Article

Gas Chromatography-Mass Spectrometry Analysis of Agarwood Extracts from Mature and Juvenile *Aquilaria malaccensis*

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Abstract

Chemical composition of crude extracts from infected woods of *Aquilaria malaccensis* were compared to that of healthy wood and commercial agarwood. Infected woods were collected six months after drilling of wild mature trees or after fungal inoculation into the stem of 4-year-old trees. Agarwood substances were extracted in methanol and were subjected to GC-MS analyses. The major compounds were chromone derivative, aromatic compounds, sesquiterpenes, monoterpenes, sterols and fatty acid methyl ester. Aromatic compounds constituted of aldehyde, phenol, ether and ketone groups. In the agarwood extract of the juvenile fungal-elicited tree but not in the healthy wood, some major compounds found were 2-(2-phenylethyl) chromone derivative, 4-phenyl-2-butanone, (1S,4S,7R)-1,4-dimethyl-7-(prop-1-en-2-yl)-1,2,3,4,5,6,7,8-octahydroazulene [guaiene], 1,1,4,7-tetramethyl-2,3,4,5,6,7,7a,7b-octahydro-1aH-cyclopropa[h]azulen-4a-ol [palustrol], and 4-(4-methoxyphenyl) butan-2-one [anisylacetone]. These were also found from agarwood of different grades and agarwood collected from the wild mature tree, in addition to agarospirol, alloaromadendre oxide (2), α-elemol, γ-eudesmol, and guaiol. This work demonstrated that in young *A. malaccensis* trees, fungi may be associated to the formation of important agarwood compounds and can be detected as early as six months after inoculation. © 2014 Friends Science Publishers

Keywords: Agarwood extracts; Aromatic compound; Chromone; *Gaharu*; GC-MS; Monoterpene; Sesquiterpene; Sterol

Introduction

Aquilaria malaccensis is the main producer of agarwood in Malaysia. The species can be found abundantly in the Peninsular but not in the eastern part of Malaysia. Agarwood is regarded as the most valuable resinous fragrant wood in the world because of its high price in the market, which can reach up to US$100,000 per kilogram for superior quality (Naef, 2011). High demand for agarwood in the international market has seriously affected the natural resources of all *Aquilaria* species and as a result, they were listed as endangered species in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (CITES, 2010].

General understanding of agarwood formation normally associates the phenomenon with wounding and fungal infections. Studies have shown that a diverse group of fungi are commonly found in wounded wood of *A. malaccensis* (Tamuli *et al*., 2000; Mohamed *et al*. 2010). The resin is often regarded as a pathological product produced by fungal invasion on the host around affected areas as tree defense reaction (Oldfield *et al*. 1998; Van Beek and Phillips, 1999). Agarwood contains aromatic terpenes, with sesquiterpenes as the main active compounds,

and chromones as another major contributor to the unique fragrance of agarwood (Naef, 2011). In order to meet the demand for agarwood, cultivation of *Aquilaria* trees in huge acreages are now being practiced in many countries. Artificial inoculation techniques using diverse methods such as deliberate wounding coupled with introduction of inducing-agents such as chemicals and microbes have been applied (Pojanagaroon and Kaewrak, 2005; Zhang *et al.*, 2010).

Different induction methods resulted in different agarwood qualities. Some reported that essential oils originated from agarwood induced by nail in setting and holing of *Aquilaria* stem contain high number of major sesquiterpenes and aromatic groups, while those induced by trunk breaking contain high amount of fatty acids (Lin *et al*., 2010). Chemicals such as sodium chloride has been shown to stimulate agarwood of similar quality to wild agarwood in *A. sinensis* (Chen *et al.*, 2011). However, artificial induction using microbes yielded less promising results. Chemical constituents of essential oils from *A. agallocha* infected with the fungi *Chaetomium globosum* and *Fusarium oxysporum* for 30 days, had similar profiles to that of healthy trees and trees that were mechanically wounded with screws (Tamuli *et al*., 2005; Bhuiyan *et al*.,

2009). Among the chemical constituents found, fatty acids predominantly exist in both healthy and inoculated woods, while 10-epi-γ-eudesmol was identified in naturally infected wood (Tamuli *et al.*, 2005). Aristolene, a major compound in agarwood was detected in healthy (3.85%) and screw-wounded trees (5.36%) (Bhuiyan *et al.*, 2009), while caryophyllene oxide, an oxygenated sesquiterpene was identified in healthy (11.25%) and naturally infected *A. sinensis* (33%) (Lin *et al.*, 2010). Here, the chemical constituents in the extracts of fungal-elicited young wood and naturally infected mature wood from *A. malaccensis*, were compared to the chemical profiles of commercial agarwood, all of Malaysian origin. For extraction, methanol was used and the crude extracts were subjected to GC-MS analyses.

Materials and Methods

Plant Material

Fungal-elicited wood (I) was harvested from 4-year-old trees grown in the nursery after 6 months of artificial inoculation. Naturally infected wood (W) was collected after 6 months of wounding from a wild growing mother tree, while healthy wood (H) was collected from unwounded parts of the same tree. The wound was made 6 cm into the xylem using an electric drill with a diameter of 16 mm. The commercial agarwood (Super A, SA) was purchased from an international agarwood dealer in Kuala Lumpur and the grade was determined by the dealer. Fresh wood samples were air-dried and powdered followed by Soxhlet extraction for 6 hours using methanol as the solvent. After extraction, the solvent was removed by means of a rotary evaporator under reduced pressure to yield the extracted compound.

Analysis of the Extracts

Extracts were analyzed on a Shimadzu GC-2010 equipped with a SGE BPX 5 fused silica capillary column, 30 m×0.25 mm, 0.25 μm film thickness. The injection port and detected temperatures were set at 230°C and 250°C, respectively. Samples were injected by splitting and the split ratio was 1:50. The carrier gas was He at a flow rate of 1.0 mL/min. Oven temperature was kept at 50°C for 3 min, increasing to 320°C at a rate of 5°C/min and holding for 20 min, then increasing to 340°C at a rate of 15°C/min and holding for 5 min. Injector temperature was 280°C, while the detector temperature was 340°C. GC/MS analyses were carried out on a Shidmazu QP2010 Plus gas chromatography, EI electron impact ion source, 70 eV, mass range 40-700 m/z, with similar condition as described in GC programs. Identification of the chemical components was based on the comparison of the calculation of their retention indices and authentic mass spectra data with the existing National Institute of Standards and Technology (NIST) 2008 library

(U.S. Department of Commerce, Gaithersburg, MD) and literature.

Analysis of Chromone Derivative

Agarwood extracts from the SA and W samples were analyzed to determine the identity of the chromone derivative. Proton, COSY (correlation spectroscopy), ROESY (rotating frame Overhauser effect spectroscopy), 1H-13C HSQC (heteronuclear single quantum coherence) and HMBC (heteronuclear multi-bond correlation) NMR (Nuclear Magnetic Resonance) experiments were recorded in d_4-methanol on a Bruker DRX 600 spectrometer.

Results

Table 1 shows the results of the identified compounds in all the wood samples. A total of 39 compounds were identified from the four samples, with the major constituents being derivatives of 2-(2-phenylethyl) chromone, aromatic compounds, sesquiterpenes, monoterpenes, sterol compounds and fatty acid methyl ester. Besides chromone, the dominant compounds were 4-phenyl-2-butanone (4.5%), palustrol (4.0%), benzaldehyde (1.9%), and benzenepropanoic acid methyl ester (0.9%). Compounds detected included major aromatic compounds (group of aldehyde, phenol, ether, ketone) in addition to oxygenated sesquiterpenes, monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, fatty acid methyl esters, and sterols.

    This investigation revealed that chemical composition of the fungal-elicited young wood were very similar to that of high quality commercial agarwood and naturally infected agarwood from wild *A. malaccensis*. They were rich in important fragrant compounds such as benzaldehyde, 4-phenyl-2-butanone, benzenepropanoic acid, methyl ester, guaiene, palustrol, anisylacetone, 8-napthol,1-(benzyloxy)- and 2-(2-phenylethyl) chromone derivative. The chromone derivative was found at high percentages (between 17.6% to 18.8%) in all agarwood samples, except in the healthy wood (Table 1). This indicates that the chromone derivative is a major constituent of Malaysian agarwood. We further analyzed the compound to verify its identity using NMR (Fig. 1). The strongest signals observed in the NMR spectra of the SA and the W wood samples were characteristics of a 2-(2-phenylethyl) chromone derivative (Shimada *et al.*, 1982).

    Indeed, two coupled multiplets, at δ_H 2.98 ppm (δ_C 32.9 ppm) and δ_H 2.89 ppm (δ_C 35.5 ppm) were assigned respectively to the H-7' and the H-8' of the CH_2CH_2 fragment. On the one hand, H-7' notably gives HMBC correlation with the phenyl C-1' quaternary carbon at δ_C 140.7 ppm and NOE (nuclear Overhauser effect) correlations with the H-2'/H-6' phenyl protons at δ_H 7.18 ppm (δ_C 128.6) and with the H-3'/H-5' phenyl protons at δ_H 7.21 ppm (δ_C 128.7 ppm). The latters appear to be coupled to the phenyl H-4' proton at δ_H 7.13 ppm (δ_C 126.8 ppm).

Table 1: Chemical constituents of various agarwood extracts from Malaysian agarwood and from infected mature and juvenile Aquilaria malaccensis

Compounds	RI[a]	Relative Peak Area (%)			
		SA[b]	W[c]	I[d]	H[e]
2-Propenoic acid,2-methyl-,ethyl ester	752	-	-	-	0,3
2(3H)-Furanone,dihydro-	825	-	-	-	0,3
Benzaldehyde	982	0,6	0,6	0,3	0,4
3(2H)-Furanone,4-hydroxy-2,5-dimethyl-	1022	-	-	-	0,7
2-Methoxyphenol [*Guaiacol*]	1090	-	-	-	1,3
4-Phenyl-2-butanone [*Benzylacetone*]	1228	1,0	2,2	1,3	-
Benzenepropanoic acid, methyl ester	1259	0,2	0,5	0,2	-
1,3-Dimethoxy-2-hydroxybenzene [*Syringol*]	1279	-	-	0,3	2,0
2-Methoxyhydroquione	1311	-	-	-	0,3
Benzenepropanoic acid	1349	-	-	0,6	-
4-Hydroxy-3-methoxybenzaldehyde [*Vanillin*]	1360	-	-	-	0,4
2H-1-Benzopyran-2-one,3,4-dihydro-	1392	-	-	0,3	0,9
Phenol,2-methoxy-4-propyl-	1402	-	-	-	0,8
1H-Cyclopropa[a]naphthalene,1a,2,4,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, (1aR,7R,7aR,7bS)-[(-)-Aristolene]	1403	2,0	2,7	-	-
4-(4-methoxyphenyl) butan-2-one [*Anisylacetone*]	1417	0,3	-	0,3	-
Alloaromadendrene oxide-(2)	1462	0,3	0,5	-	-
Caryophyllene oxide	1507	0,3	-	-	-
α-Elemol	1522	1,6	1,1	-	-
(1S,4S,7R)-1,4-Dimethyl-7-(prop-1-en-2-yl)-1,2,3,4,5,6,7,8-octahydroazulene [*Guaiene*]	1523	0,3	-	0,7	-
1,1,4,7-tetramethyl-2,3,4,5,6,7,7a,7b-octahydro-1aH-cyclopropa[h]azulen-4a-ol [*Palustrol*]	1530	1,4	1,5	1,1	-
2-propanone,1-(4-hydroxy-3-methoxyphenyl)-	1538	-	-	-	1,1
gamma.Gurjunenepoxide-(2)	1558	1,0	-	-	-
2,6,6,9-Tetramethyl-1,4-8-cycloundecatriene [*Humulene*]	1579	0,3	-	-	-
Agarospirol	1598	0,3	0,7	-	-
Guaiol	1614	0,9	1,4	-	-
Benzopheone	1603	-	-	0,5	3,0
γ-Eudesmol	1626	3,1	3,8	-	-
2-Butanone,4-(4-hydroxyl-3-methoxyphenyl)-	1638	-	-	-	0,3
Phenol, 4-(3-hydroxy-1-propenyl)-2-methoxy-	1653	-	-	-	6,6
6-Isopropenyl-4,8a-dimethyl-1,2,3,4,5,6,7,8,8a-octahydro-napthalen-2-ol	1690	-	1,3	-	-
3,7-Cyclodecadiene-1-methanol,alpha.,alpha., 4,8-tetramethyl-,[s-(Z-Z)]	1694	-	1,8	-	-
Benzenepropanoic acid,2,5-dimethoxy	1727	-	-	3,3	6,3
Hexadecanoic acid,methyl ester	1878	-	-	0,2	-
9-Octadecenoic acid, methyl ester,(E)-	2085	-	-	0,4	-
8-Napthol,1-(benzyloxy)-	2314	0,2	-	0,6	-
2-(2-phenylethyl) chromone derivative	2389	17,6	18,8	18,5	-
Stigmastanol	2720	-	-	0,3	0,4
Stigmast-5-en-3-ol,(3.beta.)	2731	-	-	-	1,8
Stigmasterol	2739	-	-	0,9	0,5

[a]retention indices using a BPX 5 column

[b]SA- super A grade agarwood chip, [c]W-wood collected from a wild mother tree after 6 months of wounding, [d]I-wood from juvenile tree grown in the nursery after 6 months of artificial inoculation, and [e]H-healthy wood from the same mother tree

'-' indicates not detected

On the other hand, H-8' gives a NOE correlation with the distinctive singlet of the H-3 chromone moiety at δ_H 6.08 ppm as well as HMBC correlations with C-3 (δ_C 113.3 ppm) and the C-2 quaternary carbon at δ_C 140.7 ppm. Then, H-3 gives an HMBC correlation with C-10 at 120.9 ppm. Unfortunately, the quality of the NMR spectra did not allow us to characterize further the second cycle (C-5 to C-8) of the chromone moiety. A precise and unambiguous identification of this 2-(2-phenylethyl) chromone derivatives would require the purification of the compounds but we did not pursue this matter as it is not within the scope of this present study. Besides, agarwood extracts are known to contain many derivatives of 2-(2-phenylethyl) chromone, with a total of thirty-nine different ones have been identified in various agarwood qualities (Naef, 2011). At least four 2-(2-phenylethyl)-4H-chromen-4-one derivatives have been reported from *A. malacensis* and an additional new derivative has been described recently from methanol extracts (Wu *et al.*, 2012).

Discussion

The compounds reported here, specifically benzaldehyde, benzenepropanoic acid and anisylacetone were also reported by Lin *et al.* (2010). In addition, they also found a chromone, 8-methoxy-2-(2-phenylethyl)-4H-1-benzopyran-4-one in the ether extract of infected wood from *A. sinensis* after six months and one year of inoculation with the fungus *Melanotus flavolivens*. These findings show promising results for microbe induction method unlike in previous experiments (Tamuli *et al.*, 2005; Bhuiyan *et al.*, 2009).
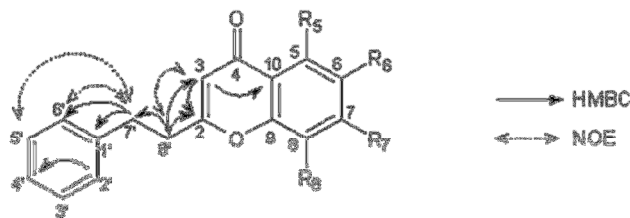
Fig. 1: Selected heteronuclear multi-bond correlation (HMBC) and nuclear Overhauser effect (NOE) correlation of the 2-(2-phenylethyl) chromone derivative from methanol extracts of agarwood samples

Chromones are reported to be responsible for the warm, balsamic, sweet and long-lasting odour when agarwood is burnt (Naef, 2011).

Interestingly, the fungal-elicited wood shared some similarities with healthy wood in the aromatic and sterol compounds. Among them are benzopheone, benzenepropanoic acid, 2,5-dimethoxy, 2H-1-benzopyran-2-one,3,4-dihydro- 1-3 dimethoxy [syringol], stigmasterol, and stigmastanol. Several terpene types such as aristolene, agarospiral, alloaromadendrene oxide-(2) and guaiol were not identified from the young infected wood (Table 1), indicating that these major compounds contribute to the unique odor formed in high quality agarwood (SA and W), while gamma.gurjunenepoxide-(2) was found exclusively in the SA agarwood. Both stigmasterol and stigmastanol are important pharmaceutical intermediates since they are known phytosterols, which have potent anti-inflammatory effects and anti-catabolic properties (Gabay *et al.*, 2010).

When looking at available data from Malaysian agarwood, 4-phenyl-2-butanone and agarospirol were identified consistently in *A. malaccensis* agarwood samples (Nor Azah *et al.*, 2008; Saiful and Mashitah, 2010; and this present study). Two compounds, Jinkohol II and Kusunol were not detected in our samples, which had originated from *A. malaccensis* populations in the states of Malacca and Terengganu. They are also absent in agarwood samples from Kelantan, Pahang and Terengganu states, but are present from Selangor population (Nor Azah *et al.*, 2008). This indicates that environment and genetic factors may have roles in causing the varying aromatic compounds. Some major terpenes that are present in Malaysian agarwood are jinkoh-eremol, caryophellene oxide, eudesmol, and α-guaiene (Saiful and Mashitah, 2010).

Chromone, although a major compound in agarwood, has not been reported from solvent-extracted *A. malaccensis* agarwood from Malaysia (Nor Azah *et al.*, 2008; Saiful and Mashitah, 2010), but has been reported from *A. malaccensis* of Laos origin (Wu *et al.*, 2012). Here, we report the presence of 2-(2-phenylethyl) chromone derivatives in all Malaysian agarwood tested (including grades A, B, C, and D; data not shown), and at high percentages (between 17% to 19%). This compound was not detected in non-infected wood. Other important compounds such as β-agarofuran, α-

agarofuran and 10-epi-γ-eudesmol were not detected in this study, perhaps, because these were methanol extracts and not hydrodistilled (Tamuli *et al.*, 2005; Nor Azah *et al.*, 2008; Saiful and Mashitah, 2010; Chen *et al.*, 2011; Pripdeevech *et al.*, 2011). Different extraction methods employed could result in different compounds.

In conclusion, the analysis of the extracts obtained from the six-month fungal-elicited wood has high similarity to that of naturally infected and commercial agarwood. This suggests that agarwood could be produced artificially by fungi inoculation method. Indeed, the role of fungi in forming agarwood compounds need to be studied further.

Acknowledgments

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