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CHEMICAL CONSTITUENTS AND TOXICITY EFFECTS OF LEAVES FROM SEVERAL AGARWOOD TREE SPECIES (*AQUILARIA*)

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While chemical constituents in agarwood produced by *Aquilaria* sp. are widely recognised for its fragrance and pharmaceutical properties, not much is known about the potential of the leaf or its toxicity effects to human. As an alternate source for income generation, the green leaves are commonly processed into food products such as tea, capitalising on its potential of medicinal properties. This study investigated hydrodistilled and solvent-extracted leaf extracts from three commercially available *Aquilaria* species: *Aquilaria crassna, Aquilaria malaccensis* and *Aquilaria sinensis*, using GC-FID and GC-MS. A total of 115 compounds were obtained from the essential oils, and hexane and methanol extracts. Among the major compounds are hexadecanoic acid and squalene, which possess antifungal and antioxidant properties, respectively. Effects of the essential oil and crude extract were tested against human's peripheral blood mononuclear cells (PBMCs) using MTT assay for cytotoxicity and comet assay for genotoxicity. Only the methanol extract of *A. malaccensis* had cytotoxic effect (IC_{50} = 24.5 mg mL^{-1} and LD_{50} = 4537 mg kg^{-1}), which can be classified as slightly hazardous (Class III). This extract also caused DNA fragmentation with comet-like appearance ($p < 0.05$) in PBMCs, implying that it has genotoxic effect as well. The results provided evidence that leaf extracts from certain *Aquilaria* species could be slightly hazardous to human. Therefore, additional research is needed to ensure its safety for human consumption.

Keywords: *Aquilaria crassna, Aquilaria malaccensis, Aquilaria sinensis,* cytotoxicity, genotoxicity

INTRODUCTION

The genus *Aquilaria* (Thymelaeaceae) is an evergreen tropical woody tree and is widely distributed in the Indomalesia region. *Aquilaria* tree is the major source of agarwood, a highly esteemed fragrant resin that is formed when the tree is infected by a disease or insect, or simply wounded. Agarwood is often used in making incense, perfume and medicine, or consumed in religious ceremony and as ornamental (Lee & Mohamed 2016). The high demand for agarwood in the market causes the natural *Aquilaria* stands in the wild to be heavily exploited. The declining number of *Aquilaria* trees in the wild, due to illegal harvesting and random logging, has caused the genus to be included in Appendix II lisitng of the Convention on International Trade in Endangered Species (CITES) (CITES 2011). To promote agarwood's sustainability, *Aquilaria* tree cultivation is currently enhanced in countries such as Cambodia, China, Indonesia, Laos, Malaysia, Sri Lanka, Thailand and Vietnam (Subasinghe et al. 2012).

Due to the long periods devoted for tree growth and induction, many farmers choose to support their living by looking into other options such as using leaf materials for food products, which can give some economic profit while waiting for their agarwood to mature (Zhou et al. 2008, Pranakhon et al. 2011). The earliest scientific documentation on utilisation of *A. sinensis* leaves as tea was recorded in 2007 by a group of Chinese researchers, assessing the toxicology safety of agarwood tea (also known as 'Chenxiang' tea) sold in the Hainan market (Wu et al. 2007). Besides tea, the use of *Aquilaria* leaves has been diversified, sold as raw ingredients in making coffee, biscuits and ice creams, and used as essence in ointment production.

The identification of bioactive compounds in *Aquilaria* leaves are useful in finding new compounds that may be potent remedial agents. For example, *A. sinensis* leaves have been used as ingredients in traditional medicine for the treatment of trauma-related illnesses such as fractures and bruises (Zhou et al. 2008). The *A. crassna* leaves have been used as supplements to combat various health conditions such as high blood pressure, constipation, headache and diabetes, and to treat digestive ailment and as a mild sedative (Kakino et al. 2010, Pranakhon et al. 2011). Generally, *Aquilaria* leaves reportedly contain various chemical compounds (Adam et al. 2017) that are related to beneficial pharmacological properties such as anti-arthritic (Rahman et al. 2016), anti-inflammatory (Wang et al. 2008, Zhou et al. 2008, Qi et al. 2009, Feng et al. 2011), anticancer (Nie et al. 2009, Chen et al. 2014, Zhong et al. 2014), antitumor (Wei & Bin 2011), antioxidant (Han & Li 2012, Duan et al. 2015, Simatupang et al. 2015), antibacterial (Khalil et al. 2013), antifungal (Chen et al. 2014), antidiabetic (Feng et al. 2011, Pranakhon et al. 2011, Said et al. 2016) lipid-lowering (Wu et al. 2012), laxative (Kakino et al. 2010, Li et al. 2013), acetylcholinesterase (AChE) inhibitory (Bahrani et al. 2014) and hepatoprotective (Alam et al. 2017).

The increasing demand for natural products has led to scientific interest in their biological effects. Therefore, additional research are needed to examine their toxicity effects and to provide regulation for their safe consumption. Some of the active ingredients in the extracts probably have incredibly high toxic dose, which could be harmful to humans (Jain et al. 2013). In this work, chemical compounds in the essential oils and crude extracts of *Aquilaria* sp. leaves were identified and their cytotoxicity and genotoxicity on human cells were determined.

MATERIALS AND METHODS

Plant materials

The mature leaves of *A. crassna*, *A. malaccensis* and *A. sinensis*, age five to seven years were collected from an *Aquilaria* Germplasm at the Forest Research Institute of Malaysia (FRIM), Kepong, Selangor, Malaysia, on 21st of March 2016. Voucher specimens were prepared and deposited at the Herbarium of the Faculty of Forestry, Universiti Putra Malaysia, Serdang, Selangor, Malaysia, for references. At the laboratory, the leaves were rinsed using tap water and left to dry for seven days at room temperature. The dried leaves were then pulverised to powder using a grinder and stored for extraction.

Extraction techniques

Hydrodistillation

50 g of powdered sample was placed in an Erlenmeyer flask containing 650 ml deionised water and the essential oil was extracted by water distillation using a Clevenger-type apparatus. A few drops of hexane was added into the collecting chamber to trap the essential oil. Heat was supplied to the heating mantle (50 °C) and the distillation process was allowed to proceed for 3 hours (Paterson 1982). The essential oils were collected, dried over anhydrous sodium sulphate to remove excess water, and stored in vials that were refrigerated until further analysis. The percentage yield of essential oil was calculated according to equation 1 (Kasim et al. 2014).

$$\text{Percentage of essential oils (\%)} = \frac{\text{Weight of oil} \times 100}{\text{Weight of sample}}$$

Solvent extraction

20 g of powdered samples were soaked separately in 200 ml of solvents (methanol and hexane) and left for 72 hours at room temperature under dark conditions. The sample was filtered using Whatman No. 1 filter paper and the solvent was evaporated under reduced pressure using a rotary evaporator at 60 °C and 100 rpm. The

crude extract was kept at -20 °C until further use. Similarly, the yield percentage of the extract was calculated on a dry weight basis, as shown in equation 1.

Gas chromatography-flame ionisation detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS) analyses

An Agilent 7890 gas chromatograph coupled to a 5973 quadruple mass spectrometer equipped with DB-1 capillary column (30 m × 250 µm, film thickness 0.25 µm) and a selective mass detector was used for GC-MS and GC-FID detection to identify the chemical compounds. An electron ionisation system was set with an ionisation energy of 70 eV. The injector was set at 230 °C and the detector at 250 °C. The oven temperature program was set as follow: initial temperature at 60 °C, hold for min, increased by 3 °C per min until 240 °C, and finally hold for 5 min. The flow rate of carrier gas (helium) was maintained at 1.3 ml per min. Compounds were identified by comparing the retention indices (RIs) and mass spectra with literature data and National Institute of Standards Technology (NIST) libraries. Retention indices were calculated using a homologous series of n-alkanes (C7–C20) (Tajuddin & Yusoff 2010).

Preparation of working solutions for toxicity experiments

The dried filtrate of essential oils and crude extracts were dissolved completely in dimethyl sulphoxide (DMSO) to give the desired stock solutions for cytotoxicity and genotoxicity tests. The samples were diluted in 10-fold serial dilutions in sterile water for five levels as working concentrations for cytotoxicity treatments.

Isolation and preparation of human peripheral blood mononuclear cells (PBMCs)

Anonymous blood samples were received from a blood bank collected in heparinised collection tubes. The PBMCs were isolated from the blood using Ficoll-Paque Plus. Freshly isolated PBMCs with viability of at least 98% were diluted in RPMI-1640 medium with 2.05 mM L-glutamine and 25 mM HEPES, supplemented with 10% fetal bovine serum (FBS), 5 µg ml^{-1} phytohemagglutinin (PHA), 100 µg ml^{-1} streptomycin and 100 µg ml^{-1} penicillin. The cells were counted using a haemocytometer under the light microscope.

In vitro assay for cytotoxicity using 3-(4,5-dimethylthiazl-2-yl)-2,5 diphenyltetrazolium bromide (MTT assay)

MTT assay was performed according to the method described by Mosmann (1983) to identify toxicity posed by *Aquilaria* leaf extracts at cellular levels. The cell suspension (PBMCs) was prepared at a concentration of 1×10^6 cells ml^{-1} in Roswell Park Memorial Institute (RPMI) medium. An aliquot of 125 µl of the prepared cells were seeded in a well of a 96-well plate, followed with addition of 12.5 µl of the leaf extract, and left to incubate for 4 hours in a humidified incubator at 37 °C and 5% CO_2. Each treatment was conducted in triplicates. Untreated cells (without leaf extract) were used as negative controls. After incubation, the plate was centrifuged at 1500 rpm for 10 min and the medium was removed by pipetting. A 10 µl of 5 mg ml^{-1} MTT solution was added to each well. The plate was wrapped with an aluminum foil and incubated for 4 hours at 37 °C. This was to allow cellular reduction of MTT, which forms a violet formazan crystal through the action of mitochondrial succinate dehydrogenase activity of the viable cells. The formazan crystal was solubilised by adding 100 µl of DMSO to each well and left in the dark for 2–4 hours, after which it was quantified by reading the absorbance at 570 nm using a fluorescence microplate reader. For blanks, wells containing just the RPMI medium and MTT without cells were used. All values were expressed as average ± SD. Percentage of cell viability is calculated using the following equation to reveal the cytotoxicity of the plant essential oils and extracts.

$$\text{Cell viability (\%)} = \frac{\text{Average viable of treated cells} \times 100 \text{ Average viable}}{\text{Negative control cells}}$$

The % of cell viability was plotted against the concentration tested. The concentration of essential oils and extracts that produced 50% inhibition concentration (IC_{50}) or cell death was calculated from the viability curve. The IC_{50} values were used in calculating the lethal dose (LD_{50}) to proclaim sample's hazardous category as regulated by the World Health Organization (WHO 2004).

In vitro assay for genotoxicity testing using the comet assay

The cell suspension was prepared at a concentration of 6×10^5 cells ml^{-1} in RPMI medium. The comet assay was performed according to the method described by Singh et al. (1988), with slight modification. A total of 500 µl of the cells and 50 µl of leaf extract were added in a 1.5 ml microcentrifuge tube and gently mixed. The tube was incubated at 37 °C for 4 hours. The negative control cells were incubated in medium only, but the positive control cells were exposed to UV light for 30 min. A total of 100 µl of the cell mixture was added into 1 ml of RPMI medium containing 5 mg of low melting agarose (LMA). The cell mixture was dropped onto a glass slide, pre-coated with 1% normal melting point agarose, covered with a cover slip (22 mm × 50 mm), and stored at 4 °C for 10 min. The cover slip was removed and the slide submerged in a lysis solution (8 M NaCl, 0.6 M EDTA pH 8, 0.2 M tris base, 0.1% triton X-100) for 1 hour. The slide was then soaked in an electrophoresis buffer (6 mM EDTA pH 10, 0.75 M NaOH) for 40 min, and electrophoresis was performed for 25 min at 26 V/300 mA at 4 °C. After electrophoresis, the slide was immediately neutralised three times in 0.4 M Tris buffer (pH 7.5) for 5 min each. The slide was then stained in 1 µg ml^{-1} ethidium bromide (40 µl per slide) overnight at 4 °C in the dark. The images of the cells were viewed using an image analysis system (Isis) attached to a fluorescence microscope, equipped with 560 nm excitation filter, 590 nm barrier filter and a CCD video camera. At least 150 cells were examined for each experiment. To quantify the level of DNA damage, the extent of DNA migration was defined using Olive Tail Moment (OTM), which shows the relative amount of DNA in the tail of the comet, multiplied by the median migration distance using Comet Assay Software Project (CASP) software. All values were expressed as median ± SD. The nonparametric Mann-Whitney test was used for statistical analysis of the comet assay results; $p < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Yield percentage and chemical constituents of *Aquilaria* sp. leaf

The yield of essential oils and crude extracts was based on the weight of dried and ground plant materials (Table 1). Essential oil yields were lower than methanol and hexane extracts in all the three species, and between crude extracts, methanol produced higher yield than hexane. Among essential oils, *A. crassna* gave the highest yield percentage (0.06%) compared with *A. malaccensis* (0.04%) and *A. sinensis* (0.02%). Meanwhile, in hexane extracts, both *A. malaccensis* and *A. sinensis* gave the same yield percentage (0.4%) which was higher than *A. crassna* (0.1%). In methanol extracts, *A. malaccensis* gave the highest yield percentage (12.9%) compared with *A. sinensis* (10.2%) and *A. crassna* (7.2%) (Table 1).

The study identified higher numbers of chemical compounds in essential oils (Table 2). In addition, the number of chemical compounds in the methanol extract was higher, compared to hexane extract, despite originating from the same amount of leaves (Table 3 and 4). This is probably due to the polarity of the solvents and good solubility for phenolic compounds from plant materials. Chemical compounds are more likely to dissolve in methanol, a polar solvent compared to the non-polar hexane. Solvents with high polarities are more efficient at extracting phenolic compounds from plant leaves than less polar solvents (Roby et al. 2013).

Table 1 Percentage yield of essential oils and crude extracts from the leaves of several *Aquilaria* sp.

Aquilaria sp.	Percentage of yield (%)		
	Essential oils	Crude extracts	
		Hexane	Methanol
Aquilaria crassna	0.06	0.1	7.2
Aquilaria malaccensis	0.04	0.4	12.9
Aquilaria sinensis	0.02	0.4	10.2

Table 2 Chemical compounds detected in essential oils hydrodistilled from the leaves of several *Aquilaria* sp.

No.	Compounds	RI	Percentage area (%)			Identification
			Aquilaria crassna	*Aquilaria malaccensis*	*Aquilaria sinensis*	
1	Benzaldehyde	935	-	0.1	-	RI
2	α-Pinene	940	-	0.2	-	RI
3	Octenol	966	-	0.7	-	RI
4	α-Copaene	1369	0.3	-	-	RI
5	β-Elemene	1388	0.4	-	-	RI
6	Caryophyllene	1421	0.9	-	-	RI, MS
7	α-Guaiene	1440	1.8	-	-	RI, MS
8	α-Humulene	1457	-	0.5	-	RI
9	β-Agarofuran	1474	0.3	-	-	RI
10	Germacrene D	1480	0.3	-	-	RI
11	γ-Maaliene	1482	0.4	-	-	MS
12	*β*-Guaiene	1483	1.6	-	-	RI
13	*β*-Selinene	1486	0.5	-	-	RI
14	*γ*-Muurolene	1488	0.7	-	-	MS
15	α-Muurolene	1496	0.6	-	-	RI
16	γ-Guaiene	1499	0.2	-	-	RI
17	*α*-Selinene	1501	1.0	-	-	RI, MS
18	*δ*-Cadinene	1516	1.1	0.3	-	RI, MS
19	δ-Elemol	1530	0.3	-	-	RI
20	β-Elemol	1535	0.1	-	-	RI
21	Germacrene B	1554	0.9	0.6	0.3	RI
22	*nor*-Ketoagarofuran	1555	2.3	0.6	0.3	RI
23	Nerolidol	1564	1.3	0.7	0.3	RI
24	Spathulenol	1566	0.3	-	0.4	MS
25	Epoxybulnesene	1572	0.5	-	-	RI
26	Isoaromadendrene epoxide	1590	0.8	-	-	MS
27	Caryophyllene oxide	1600	5.3	1.9	-	RI, MS
28	1,5-epoxy-nor-Ketoguaiene	1614	0.2	-	-	RI
29	10-epi-γ-Eudesmol	1619	0.5	-	0.3	RI
30	epi-α-Cadinol	1640	0.3	-	-	RI
31	α-Cadinol	1641	0.4	0.5	-	RI
32	Jinkoh-eremol	1643	0.4	-	-	RI
33	α-Eudesmol	1652	0.7	-	-	RI
34	Bulnesol	1664	0.2	-	0.3	RI
35	Dehydrojinkoh-eremol	1673	0.1	-	-	RI
36	epi-α-Bisabolol	1678	0.2	-	-	RI
37	α-Bisabolol	1683	0.2	-	-	RI
38	Selina-3,11-dien-9-one	1687	0.3	5.9	3.0	RI
39	Pentadecanal	1691	0.4	-	-	RI
40	Rotundone	1703	0.2	-	-	RI
41	cis-Farnesol	1707	0.7	-	-	MS
42	Selina-3,11-dien-14-ol	1750	-	2.5	-	RI
43	Guaia-1(10),11-dien-9-one	1752	-	-	3.6	RI
44	Selina-4,11-dien-14-al	1758	3.4	-	-	RI
45	cis-3-Heptadecene	1762	0.91	-	-	MS
46	2-Hexadecanol	1774	4.6	-	-	MS
47	Hexadecanal	1794	-	7.6	-	MS
48	Guaia-1(10),11-dien-15-al	1806	0.5	-	-	RI
49	Karanone	1812	0.6	-	0.9	RI
50	oxo-Agarospirol	1822	1.6	0.5	0.8	RI
51	Eudesmol	1880	-	0.5	0.7	RI
52	2-Hydroxyguaia-1(10),11-dien-15-oic acid	1932	0.4	-	-	RI
53	n-Hexadecanoic acid	1950	32.0	69.3	43.0	RI, MS
54	trans-3-Eicosene	2000	-	-	3.9	MS
55	Phytol	2105	15.8	0.6	-	MS
56	Linoleic acid	2109	-	3.2	-	MS
57	cis- 9-Octadecenal	2358	-	5.4	-	MS
58	Squalene	2663	23.7	0.7	11.9	MS

RI = indicates the retention indices which were calculated against C_8–C_{20} n-alkanes on the DB-1 column, MS = comparison of the MS with those of the NIST library (> 90% matching from the library)

Table 3 Chemical compounds detected in hexane extracts from the leaves of several *Aquilaria* sp.

No	Compounds	RI	Percentage area (%)			Identification
			Aquilaria crassna	*Aquilaria malaccensis*	*Aquilaria sinensis*	
1	β-Cubebene	1390	-	-	0.1	MS
2	α-Cadinol	1689		-	0.2	RI
3	Rotundone	1703	0.3	0.3	0.1	RI
4	Selina-3,11-dien-9-one	1721	-	0.2	-	RI
5	Selina-3,11-dien-14-ol	1750	0.3	0.3	0.4	RI
6	3-Methyloctadecane	1873	0.5	-	-	MS
7	n-Hexadecanoic acid	1950	2.6	2.7	1.2	RI, MS
8	2-Methylnonadecane	1966	1.1	-	-	MS
9	Squalene	2663	5.3	2.9	34.2	RI, MS

RI = indicates the retention indices which were calculated against C_8–C_{20} n-alkanes on the DB-1 column, MS = comparison of the MS with those of the NIST library (> 90% matching from the library)

There were no differences in the identities of chemical compounds from essential oils and crude extracts when using GC-FID or GC-MS approaches. However, the number of compounds identified by GC-FID was higher than GC-MS. The GC-FID and GC-MS analysis of the essential oils resulted in the identification of 58 different compounds (Table 2). Nine compounds were identified in the hexane extracts (Table 3). Squalene in *A. sinensis* had the highest percentage (34.2%) compared to *A. crassna* (5.3%) and *A. malaccensis* (2.9%). Meanwhile, 48 compounds were identified in the methanol extracts (Table 4). In addition, squalene amount was found higher in the methanol extract of *A. sinensis* (56.9%) compared to *A. crassna* (23.0%) and *A. malaccensis* (25.6%). Vitamin E (3.0%) and β-sitosterol (0.6%) were only found in the methanol extracts of *A. sinensis*. The findings correlated with Kang et al. (2014) that when *A. sinensis* leaves were macerated in methanol and ethanol solvents, sterols were present. Meanwhile, γ-sitosterol was only found in methanol extracts of *A. crassna*.

The leaf compounds of *Aquilaria* sp. can be divided into groups of monoterpene, diterpene, triterpene, sesquiterpene, fatty acids, steroids and others. Chemical compounds identified in both essential oils and crude extracts of *Aquilaria* sp. leaves were mainly from the group of terpenes with sesquiterpenes being the dominant member. Most compounds belonging to this class has significant medicinal and remedial values leading to potential applications in treating numerous health problems (Reddy & Couvreur 2009).

Major compounds of the essential oils were n-hexadecanoic acid (48.1%), phytol (15.8%), squalene (12.1%), hexadecanal (7.6%), caryophyllene oxide (7.2%), octadecenal (5.4%), guaia-1(10),11-dien-9-one (3.6%), selina-4,11-dien-14-al (3.4%), selina-3,11-dien-9-one (3.1%) and selina-3,11-dien-14-ol (2.5%) (Table 2). Main compounds of the hexane extracts were squalene (14.1%), n-hexadecanoic acid (2.2%), and phytol was not identified (Table 3). Meanwhile, squalene (35.2%), n-hexadecanoic acid (13.1%), octadecadienal (9.9%), phytol (3.8%) and vitamin E (3.0%) were the main compounds in the methanol extracts followed by β-maaliene (2.4%) and oleic acid (1.9%) (Table 4). The n-hexadecanoic acid and squalene were always present in essential oils and crude extracts of the leaves.

For instance, the hexadecanoic acid and linoleic acid identified in *A. malaccensis* essential oil, have potential as antifungal agent against some plant pathogenic fungi (Abubacker & Deepalakshmi 2003). Squalene has the ability to reduce the development of numerous tumors and possess antioxidant properties in plant species such as *Amaranthus caudatus* and *Olea europaea* (Huang et al. 2009, Reddy & Couvreur 2009). Experimental studies in rodents demonstrated that squalene can prevent chemically induced skin tumorigenesis and works as an oxygen-scavenging agent in inhibiting lipid peroxidation on human's skin surface (Smith 2000, Huang et al. 2009). Phytol, a significant compound found in *A. crassna* essential oils and methanolic extracts of the three species, possess anti-inflammatory,

Table 4 Chemical compounds detected in methanol extracts from the leaves of several *Aquilaria* sp.

No	Compounds	RI	Percentage area (%)			Identification
			Aquilaria crassna	*Aquilaria malaccensis*	*Aquilaria sinensis*	
1	Nonane	906	0.1	0.1	0.1	RI
2	Octenol	966	0.5	0.3	1.0	RI
3	Sabinene	970	-	0.3	0.1	RI
4	Myrcene	986	2.0	1.5	1.6	RI
5	2-Hydroxy-benzaldehyde	1003	0.3	0.6	0.6	RI
6	α-Tolualdehyde	1011	0.1	0.1	0.3	RI
7	Limonene	1025	0.7	0.7	0.5	RI
8	(E-2-Butenylbenzene	1060	0.2	-	-	
9	Acetophenone	1066	0.2	0.1	0.3	RI
10	Nonanal	1085	0.3	0.2	0.2	RI
11	Linalool	1087	0.2	0.2	0.1	RI
12	Octyl acetate	1196	1.6	-	-	RI
13	4-Phenyl-2-butanone	1210	-	-	1.6	RI
14	2-Isopropyl-5-methylphenol	1283	-	0.1	-	RI
15	2-(2-Furfuryl)-5-methylpyrazine	1346	0.5	0.4	0.3	RI
16	2-Iodoethyl octanoate	1583	2.7	-	-	RI
17	Pyranone	1406	-	-	3.3	MS
18	β-Maaliene	1414	-	2.4	-	RI
19	7-Tetradecyne	1416	-	-	0.4	MS
20	β-Selinene	1419	-	0.1	0.1	MS
21	(1-Butylpentyl)-benzene	1437	0.4	0.4	-	RI
22	γ-Gurjunene	1472	0.5	0.1	0.5	RI
23	β-Agarofuran	1474	0.1	-	0.1	RI
24	α-Muurolene	1496	1.9	-	0.9	RI
25	γ-Guaiene	1499	0.1	0.1	0.2	RI
26	α-Elemol	1530	0.1	-	0.2	RI
27	10-epi-γ-Eudesmol	1619	-	-	0.2	RI
28	epi-α-Cadinol	1640	-	0.2	-	RI
29	Jinkoh-eremol	1643	0.2	0.1	0.1	RI
30	Kusunol	1650	0.1	0.1	-	RI
31	α-Eudesmol	1652	-	-	0.2	RI
32	Bulnesol	1664	0.6	-	0.6	RI
33	Selina-3,11-dien-9-one	1721	0.4	0.3	0.3	RI
34	Selina-4,11-dien-14-oic acid	1728	0.3	-	-	RI
35	Selina-3,11-dien-9-al	1735	-	-	0.1	RI
36	Selina-3,11-dien-14-ol	1750	-	0.4	0.6	RI
37	Sinenofuranol	1776	0.1	0.1	-	RI
38	oxo-Agarospirol	1822	1.3	1.2	-	RI
39	n-Hexadecanoic acid	1950	15.5	14.6	9.3	RI, MS
40	1,4-Eicosadiene	1985	0.8	-	-	MS
41	Eicosene	1991	-	-	0.5	MS
42	Phytol	2111	2.71	7.09	1.46	RI, MS
43	Oleic acid	2116	-	1.9	-	MS
44	Z-9,17-Octadecadienal	2297	16.41	-	3.36	MS
45	Squalene	2663	23.0	25.6	56.9	RI, MS
46	Vitamin E/α-Tocopherol	3112	-	-	3.0	MS
47	β-Sitosterol	3220	-	-	0.6	MS
48	γ-Sitosterol	3224	1.2	-	-	MS

RI = indicates the retention indices which were calculated against C_8-C_{20} n-alkanes on the DB-1 column, MS = comparison of the MS with those of the NIST library (> 90% matching from the library)

antiallergic, antinociceptive effect in rat models, antimicrobial, as well as antioxidant activities against free radicals created *in vitro* (Pejin et al. 2014, Santos et al. 2013).

Some chemical compounds identified in the leaves, such as benzaldehyde, caryophyllene, n-hexadecanoic acid, phytol, squalene, eudesmol and oxo-agarospirol were also found in other parts of *Aquilaria* trees such as the stem and agarwood itself. Previous studies have shown that these compounds are also detected in agarwood oils (Tajuddin & Yusoff 2010). For instance, the compounds present in the methanol extract of agarwood oils such as benzaldehyde (1.9%), caryophyllene (0.3%), hexadecanoic acid (0.2%) and oxo-agarospirol (1.0%) were also reported. Besides, (-)-guaia-1(10),11-dien-15-al, (-)-selina-3,11-dien,9-one and (+)-selina-3,11-dien,9-ol that is detected in the highest quality of agarwood oil was also present in the essential oils of *Aquilaria* leaves (Ismail et al. 2014).

Cytotoxic effect of *Aquilaria* leaf

Essential oils (Table 5, Figure 1a, d, g) and hexane extracts (Table 5, Figure 1b, e, h) from the three species did not significantly affect the viability of the cells. The viability of the cells was above 50%; no cytotoxic effect was observed in these samples. However, for methanol extracts of *A. crassna* and *A. sinensis*, the percentage of the cell viability at the highest concentration tested was above 100% (Table 5, Figure 1c, i). This could be explained by possible interference in the MTT assay due to the presence of phenolic compounds in the methanolic extracts. Some of the chemical compounds such as kaempferol, resveratrol, ascorbic acid, N-acetylcysteine and vitamin E in the extracts may reduce MTT salt to blue formazan by cell-independent chemical reaction (Bruggisser et al. 2002, Wang et al. 2010). The compounds may also change the activity of the succinate dehydrogenase or interrupt with the MTT assay directly, when they are found abundantly. The assay using the second highest concentration for methanol extracts of *A. crassna* (2 mg ml^{-1}) and *A. sinensis* (3 mg ml^{-1}) did not show any significant effects on the viability of the cells. Among all the extracts tested, only *A. malaccensis* leaf methanol extract showed 50% cell inhibition (IC_{50} = 24.5 mg ml^{-1}) (Table 5, Figure 1f). At this concentration and above, the extract could exert cell toxicity. The lethal dose (LD_{50}) value, a statistical estimation of the number of toxicant (mg) per body weight (kg) required to induce the death of 50% of a large population of test animals was 4537 mg kg^{-1}. This level reflects that more than 2000 mg kg^{-1} of the plant extract is needed to be consumed orally to achieve 50% cell death, while more than 4000 mg kg^{-1} is needed in dermal application to reach similar negative effect (WHO 2004). Following the WHO's (2004) regulation, the tested leaf extract belongs to Class III (slightly hazardous) of toxic chemicals.

Genotoxic effect of *Aquilaria* leaf

Comet assay, a gel electrophoresis-based method was used to measure the DNA damages in human PBMCs. In this assay, DNA strand breaks were represented by median of Olive Tail Moment (OTM) in the comet's tail, relative to total amount of DNA (Table 6). The OTM is the product of the tail length and percentage of DNA in the tail, which indicates the level of DNA damages in human PBMCs (Olive & Banáth 2006).

The PBMCs treated with essential oils and hexane extracts from the three species displayed spherical shape of nucleoids with no long tail of DNA streaming out (Figure 2a). Similar results were obtained for methanol extracts of *A. crassna* and *A. sinensis* at concentrations 2 mg ml^{-1} and 3 mg ml^{-1}, respectively. There was no induced DNA damage in the PBMCs, compared to untreated cells ($p > 0.05$) (Figure 2b). However, the methanol extract of *A. malaccensis* leaf (24.5 mg ml^{-1}) showed DNA fragmentation with comet-like appearance ($p < 0.05$) (Figure 2c) similar to UV-induced damage control (Figure 2d).

Comet assay is known as a significant tool for measuring DNA damage and repair in genetic toxicology (Wood et al. 2010). The concentrations used in a comet assay were selected based on the cytotoxicity test results. If the extracts do not exhibit cytotoxicity, the maximum concentration was employed instead. Through this assay, only the leaf methanol extract of *A. malaccensis* (24.5 mg ml^{-1}) had toxicity effects on human PBMCs. The treated cell appeared in such a comet-like structure, in which the cell had a long tail of DNA streaming out from the nucleoid, with a gradual increase of strand breaks. The damage

Table 5 Cytotoxicity of leaf essential oils from different *Aquilaria* sp. against human PBMCs

Aquilaria sp.	Type of extracts	Maximum extract concentration (mg ml^{-1})	Range of cell viability (%)	IC_{50} value (mg ml^{-1})
Aquilaria crassna	EO	10	50.9 ± 0.1 – 73.2 ± 0.1	-
	HE	0.4	83.5 ± 0.2 – 87.1 ± 0.2	-
	ME	20	173.3 ± 0.3 – 86.8 ± 0.2	-
Aquilaria malaccensis	EO	7	69.8 ± 0.6 – 96.6 ± 0.6	-
	HE	2	75.1 ± 0.2 – 88.8 ± 0.2	-
	ME	30	43.5 ± 0.1 – 76.3 ± 0.1	24.5
Aquilaria sinensis	EO	3	68.6 ± 0.1 – 81.9 ± 0.1	-
	HE	2	66.4 ± 0.2 – 79.1 ± 0.2	-
	ME	30	138.2 ± 0.3 – 75.5 ± 0.2	-

Values are mean in percentage of cell viability ± SD of three replicates, EO = essential oils, HE = hexane, ME = methanol, – = no IC_{50} value calculated as the percentage of cell viability is above 50%

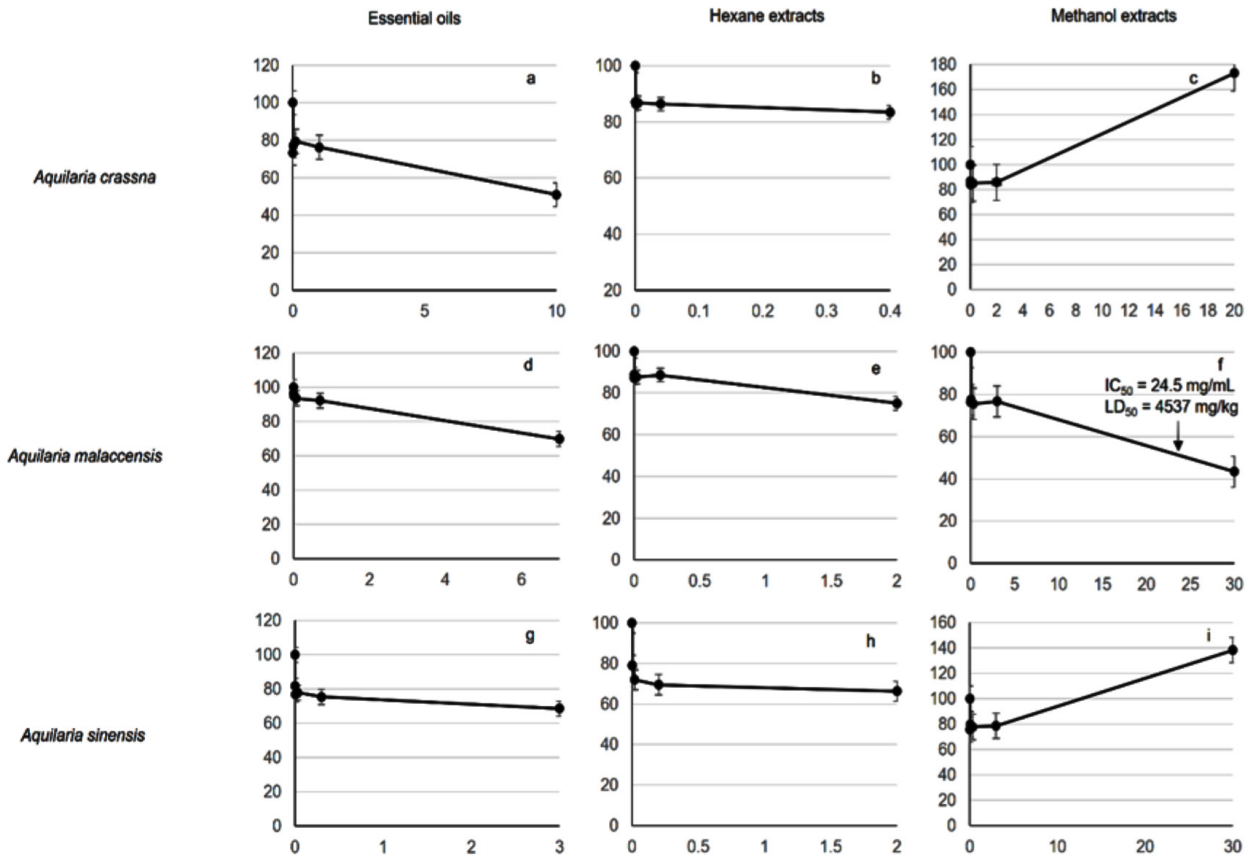

Figure 1 Cytotoxic effects of *Aquilaria* species on the viability of human PBMCs; leaf essential oils and extracts were prepared in 10-fold serial dilutions; x-axis = indicates extracts concentrations (mg ml^{-1}), y-axis = indicates percentage of cells viability, IC_{50} = indicates the concentration at 50% of cell reduction and lethal dose, LD_{50} = specify the predicted dose that can be consumed according to WHO category of toxic chemicals

might cause disturbances in DNA transcription and replication processes leading to cell death or aging (Azqueta & Collins 2013).

The present study showed that methanol extracts of *A. malaccensis* had both cytotoxic and genotoxic effects, probably due to the high concentration used. The result correlated to Sinha et al. (2014) in which essential oils such as palmarosa, citronella, lemon grass, vetiver, citral and geraniol stimulate cytotoxicity and genotoxicity in human lymphocytes at higher concentrations. However, at low concentrations,

Table 6 The level of DNA damage expressed as Olive Tail Moment (OTM) in PBMCs after treatment with essential oils and extracts from the leaves of several *Aquilaria* sp.

Aquilaria sp.	Type of extracts	Maximum extract concentration (mg ml^{-1})	Parameter	
			Olive Tail Moment (OTM)	p-value
Aquilaria crassna	EO	10	0.20	0.16
	HE	0.4	0.41	0.42
	ME	2	0.42	0.34
Aquilaria malaccensis	EO	7	0.54	0.06
	HE	2	0.35	0.48
	ME	30	5.70	< 0.0001
Aquilaria sinensis	EO	3	0.32	0.42
	HE	2	0.54	0.23
	ME	3	0.56	0.23
Positive control			8.56	< 0.0001
Negative control			0.36	-

$p < 0.05$ = significance difference in comparison to negative control, EO = essential oils, HE = hexane extracts, ME = methanol extracts

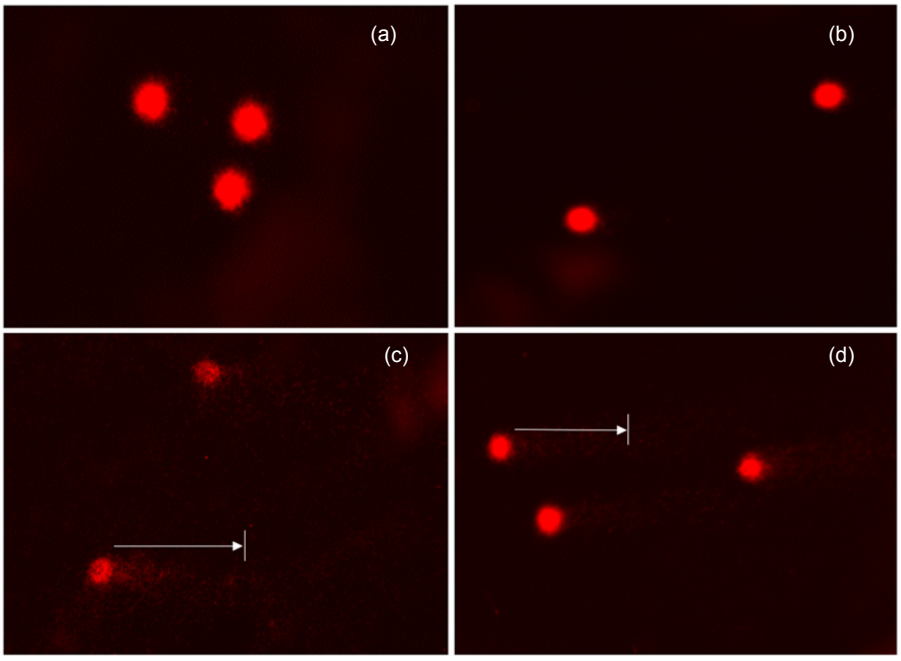

Figure 2 Comet assay images of PBMCs; human PBMCs treated with hexane extract showed no cytotoxic effect (a), appeared as round whole cells similar to healthy untreated cells (b), cells treated with leaf methanol extract of *Aquilaria malaccensis* had damaged DNA seen as Olive Tail Moment (OTM) (arrows) (c), comparable to UV-damaged cells, which served as positive control (d); images were captured using a fluorescence microscope, equipped with 560 nm excitation filter, 590 nm barrier filter and a CCD video camera PCO at 200 x magnification; scale on each figure a, b, c, d = 5 mm

these oils were found to be safe for human consumption. Hence, the leaf extracts from *Aquilaria* sp. need to be consumed carefully, regardless of its numerous health benefits, because of the side effects that may be harmful to human body if taken in a high dose (Jain et al. 2013).

CONCLUSIONS

The major compounds detected in both essential oils and extracts of *A. crassna, A. malaccensis* and *A. sinensis* leaves were mostly sesquiterpenes, which were hexadecanoic acid and squalene. Essential

oils and crude extracts of *Aquilaria* leaves were relatively safe to consume without major toxicity concern, but should be wisely taken in a dose-dependent manner. The present study provided a benchmark in setting up indicators for measuring toxicity threats from the use of *Aquilaria* leaves, which is becoming a popular ingredient in preparing food and drinks. However, additional research work is needed to ensure its safety for human consumption.

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