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Agarwood Leaf Essential Oil Characterization and Effects on MCF-7 Breast Cancer Cells

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***Abstract*— Breast cancer continues to remain as the leading cause of cancer mortality among women worldwide. Agents for prevention and cure for breast cancer are contiuously being researched. İn particular, agarwood essential oil from resin infiltrated heartwood has been reported to have substantial evidences of medicinal benefits. Nevertheless, there is very limited report on agarwood leaf essential oil (ALEO). Hence, this present study was conducted to evaluate the physicochemical properties, chemical constituents and anticancer activity of ALEO. ALEO was observed to be of pale-yellow colour with sweet smell. Other physicochemical properties include refractive index of 1.44, specific gravity of 0.886, saponification value of 131.88 mg KOH/g, acid value of 2.80 mg KOH/g and iodine value of 105.07 gI_2/100g. The profiling of chemical constituents using gas chromatography-mass spectrometry (GCMS) revealed 19 compounds. Hexadecanoic acid was the major compound (64.41%). The biomarkers of agarwood; azulene (0.619%) and guaiol (0.2997%) were also detected. ALEO was tested for anticancer activity against MCF-7 cancer cells using WST-8 assay. ALEO showed the IC_{50} value of 31% (v/v) against MCF-7 cells after 36 hours of treatment. In conclusion, this study provides information on ALEO physicochemical properties and chemical constituents that can be used as benchmark for quality assurance as well as proof that ALEO holds a potential as anticancer agent.**

***Keywords*— Agarwood leaf; breast cancer; essential oil; GCMS; MCF-7.**

I. INTRODUCTION

Based on Global Cancer Statistics conducted by the International Agency for Research on Cancer (IARC), out of 8.2 million cancer-related deaths recorded in 2012, it was reported that more than 500,000 deaths were due to breast cancer [1], [2]. World Health Organization (2015) defined cancer as the rapid growth of abnormal cells (single cell that transforms from a normal cell into tumour cell) which have the tendency to develop and spread at any part of the body [3]. For many years, breast cancer has been listed as the top killer among women which accounted for 15% of all cancer deaths among females and 25% of the total cancer cases [1]-[3].

Breast cancer commonly occurs in the breast tissue with the major sign of the presence of lump in the breast. In addition, breast cancer begins in the cells from the lining of milk ducts and the lobules which are responsible to supply milk to the ducts. Ductal and lobular carcinoma refer to the cancers that develop from the ducts and lobules respectively [4]. The risk factors that may trigger breast cancer include hormonal and reproductive factors, overweight, physical activity and alcohol consumption [3], [4]. However, it was reported that the mortality from breast cancer is continuously reducing due to screening and early detection as well as the enhancement of the adjuvant therapy. Nevertheless, breast cancer continues to remain as the leading cause of cancer mortality for women whereby one in every eight women has the potential to be diagnosed with breast cancer in her lifetime [5], [6].

Despite the emergence of modern anticancer therapy, it is estimated that more than 60% of clinically approved anticancer drugs are derivatives of the medicinal plant due to its efficacy and safety as well as being eco-friendly and low-cost as compared to the conventional treatments methods [2]. Hence, natural compounds from plant species including from agarwood species have been continously being explored and

investigated as the alternative for anticancer drugs. Agarwood which is also known as gaharu, Oudh, Jin-koh and chen-xiang, is treasured for its dark-aromatic resin which is formed either naturally or artificially in the heartwood of *Aquilaria* species [7], [8]. It is one of the most valuable woods in the world with high demand and price due to its myriad applications in three main areas: perfume, incense and medicine [7].

Interestingly, other than the prized fragrant wood, agarwood tree also offers many different types of raw material which can be exploited. In particular, agarwood leaves which generally have a length of 5 to 11 cm and diameter of 2 to 4 cm with an elliptical blades shape; have been commercialized into products such as agarwood tea and essential oils [9]. Recently, research focus has been shifted towards the agarwood leaf due to its wide range of functional groups with diverse chemical constituents which may correlated with their pharmacological activities [9]. Previous studies reported that agarwood essential oil from the resin infiltrated heartwood exhibited anticancer activity [10]-[12]. Nevertheless, study on agarwood leaf essential oil (ALEO) in terms of its quality assessment, chemical constituents and biological effects paticularly anticancer activity are limited. It is therefore, the interest of this study to investigate the potential anticancer effects of ALEO which may be positively associated with its physicochemical properties and chemical constituents.

II. Material and Method

A. Raw Material, Cell Line and Culture Medium

50 ml of agarwood leaf essential oil (ALEO) was purchased from Best Formula Industries (EssentialOils.com.my). ALEO was extracted using cold press method as stated by the manufacturer. However, the species of agarwood used was not specified. Michigan Cancer Foundation (MCF-7) breast cancer cell line was purchased from ATCC. The cell line was cultured and maintained in Dulbecco's modification of Eagle's medium (DMEM) with high glucose and L-glutamine (Gibco®) and 10% (v/v) fetal bovine serum (FBS) at 37°C and 5% CO_2.

B. Experimental Methods

1) Physicochemical Analysis: Several physicochemical properties of ALEO were investigated. The colour and clarity were visually observed while the characteristic odour was analysed by sniffing [13]. The refractive index of ALEO was determined by using ATAGO PAL-α (0-85%) handheld refractometers based on the manufacturer's instructions and the Brix values were converted to refractive index based on the International Scale (1936) of Refractive Indices of Sucrose Solutions at 20°C [14], [15]. The specific gravity of ALEO was measured by using DA-130N (Kyoto Electronics, Japan) portable density/specific gravity meter (densimeter) based on manufacturer's instructions. Saponification value was determined by standard procedure based on Paudyal et al. (2012) [16]. The acid value was determined based on Barkatullah et al. (2012) [17] and AOAC (2000) [18]. Subsequently, the iodine value was determined based on Paudyal et al. (2012) [16].

2) GCMS Profiling of ALEO Chemical Constituents: The volatile constituents of the triplicate ALEO samples were analysed by using gas chromatography system (GCMS); Agilent 7890A (Agilent Technologies) coupled with Agilent 5975C quadrupole mass spectrometer and autosampler. Hewlett Packard (HP-5MS) ultra inert silica capillary column (30 m x 0.25 mm; 0.25 μm) was used. The analytical conditions for GCMS analysis were based on Hashim et al. (2014) [19]. The detected peaks and mass chromatograms were further used to identify total ion chromatography (TIC) in accordance to the National Institute of Standards and Technology (NIST) 2008 mass spectral library.

3) Investigation of ALEO In-Vitro Anticancer Activity: The procedure for this section was based on the protocol of WST-8 assay provided by Nacalai Tesque, Inc. [20]. Accutase was added to the culture flask to detach the confluent cells. Cell counting was performed in order to determine the seeding number for the 96-well tissue culture plate. The cell suspension with 5000 cells/well was prepared using the culture medium. 100 μl of the cell suspension was added to each well of a 96-well plate. The medium was pre-incubated in CO_2 incubator at 37°C for 24 hours. Then, 10 μl of the ALEO prepared sample was added into each well of the plate. The medium was incubated for 48 hours at 37°C in CO_2 incubator. Next, 10 μl of WST-8 reagent was added into each well. Then, the medium was incubated for 1 hour at 37°C in CO_2 incubator. The experiments were conducted in triplicate and the measurement of the absorbance was conducted at 450 nm by the microplate reader. IC_{50} value was derived from curve-fitting methods. The percentage viability of the cells was calculated using formula below [21]:

$$\text{Percentage of cell viability (\%)} = \frac{\text{Mean absorbance sample}}{\text{Mean absorbance control}} \times 100 \quad (1)$$

III. Results and Discussion

A. Physicochemical Analysis

The commercial importance of oils generally depends on qualitative parameters such as physicochemical characteristics which give the baseline data to evaluate its suitability for consumption and use. The practical importance of the essential oil in daily life can be explored by studying various physicochemical properties of the oils. The common physicochemical properties of essential oil include organoleptic properties (colour and odour), refractive index, specific gravity, saponification value, acid value and iodine value. These properties are significantly influencing the quality of oil and therefore are normally used as quality control parameters [17].

From the physicochemical tests of this present study, ALEO had a pale-yellow colour with sweet strong smell. The refractive index of ALEO was 1.440 while its specific gravity was 0.886. Refractive index describes the propagation of light through a medium measured by a refractometer. It is also used to differentiate water from other solvents as well as to determine the purity level of the

essential oil [22]. Generally, the value of refractive index is more than one for light passing from less dense medium (air) into a denser medium (oil) [23]. The presence of high amount of water content in oil results in small refractive index because light is easily refracted in water as compared to oil due to the difference in density of the liquids. Therefore, the essential oil with large refractive index has better purity and quality than the essential oil with small refractive index since it indicates less amount of water present in the essential oil. On the other hand, specific gravity of oil refers to the ratio of the weight of a given volume of the oil to the weight of an equal volume of water at room temperature. Specific gravity value of essential oil is usually less than one [17]. In comparison, the agarwood essential oil has higher specific gravity of 0.952 [24] as compared to ALEO (0.886). Technically, agarwood or the dark aromatic resin is formed within the heartwood of the agarwood species. Thus, the oil extracted from the heartwood is more concentrated and denser which results in a higher specific gravity as compared to the oil extracted from the leaves [7].

Saponification value of ALEO was 131.88 mg KOH/g. Saponification value is defined as the index of average molecular mass of the fatty acids in the essential oil. High saponification value indicates high molecular mass of fatty acids in the oil [17]. Based on GCMS analysis, there were seven fatty acids compounds identified which accounted for 69.64% of the total oil content. Thus, high percentage area showed that ALEO has high molecular weight of fatty acids that resulted in a high saponification value. Saponification value also suggests the suitability of the oil for soap production [17]. This is because soap is the potassium or sodium salts of fatty acids prepared by saponification process [25]. Hence, it suggests that ALEO can be potentially used for making soap.

Acid value is defined as the mass of potassium hydroxide in milligram which is required to neutralize one gram of chemical substance [22]. For this present study, the calculated acid value of ALEO was 2.805 mg KOH/g which is within the limit since the maximum level of acid value of oil extracted from plant-based sources by cold pressed is 4.0 mg KOH/g [26]. The low amount of acid value indicates slower deterioration and rancidity of oil. Rancidity will cause oil to have foul smell while deterioration will lead to chemical degradation; affecting the stability and volatile property of the essential oil which will then lead to lower aromatic quality and therapeutic value of essential oil [27], [28].

Subsequently, iodine value measures the number of grams of iodine consumed by 100 g of oil or fat [25]. The measured iodine value of ALEO was 105.073 gI_2/100g. The oil with low iodine value is less likely to be oxidised [25]. Thus, it suggests that ALEO has high level of oxidative deterioration as compared to olive oil since the maximum level of iodine value of virgin olive oil is 75 to 94 gI_2/100g [26]. Oxidation of the essential oil may disrupt chemical composition leading to loss of efficacy or it can become hazardous [29]. The measured physicochemical properties were compared with published results of olive oil (the most common base oils obtained from plant) and agarwood oil from infiltrated heartwood [24], [26], [30]; and summarized in Table 1.

TABLE I
PHYSICOCHEMICAL PROPERTIES OF AGARWOOD LEAF ESSENTIAL OIL (ALEO) IN COMPARISON WITH OLIVE OIL AND AGARWOOD OIL (FROM RESIN INFILTRATED HEARTWOOD); NA: NOT AVAILABLE.

Physicochemical property	**Olive oil[26]**	**ALEO**	**Agarwood oil**
Colour	Yellow to green	Pale yellow	Dark brown to dark yellow [24]
Odour	NA	Sweet smell	Sweet smell [24]
Refractive index	1.468-1.471	1.440	1.520 [24]
Specific gravity	0.910-0.916	0.886	0.952 [24]
Saponification value (mg KOH/g)	184-196	131.88	195 [24]
Acid value (mg KOH/g)	6.6 (Max)	2.80	8.6 [30]
Iodine value (gI_2/100g)	75-94	105.07	186 [24]

B. GCMS analysis of ALEO Chemical Constituents

Profiling of chemical constituents in plant species is an important step to complement investigation of medicinal effects of the plants. Chemical constituents identified from plants are potential source of drug templates [31]. The GCMS analysis revealed 19 compounds in ALEO. Isopropyl palmitate (hexadecanoic acid) which is a fatty acid was the most abundant component detected which accounted for 64.41% of overall oil content followed by 2-Propanol, 1,1'-oxybis- (8.4572%) and 1-Propanol, 2-(2-hydroxypropoxy)- (7.1958%). The relative contents expressed in the form of percentage of all compounds found in ALEO analyzed by GCMS are listed in Table 2 and categorized based on their functional groups. Meanwhile, Fig. 1 and Fig. 2 show an exemplary chromatogram of ALEO and the presence of azulene and guaiol with the peaks labeled (i) and (ii) respectively.

Previous study reported that hexadecanoic acid was found to be the major compound of *Aquilaria sinensis* leaf essential oil (48.86%) [32] as well as the major compound (49.47% of total essential oil) in healthy agarwood (*Aquilaria sinensis* (Lour.) Gilg) which contributes to its aromatic smell [33]. It was also reported that isopropyl myristate (tetradecanoic acid), isopropyl palmitate (hexadecanoic acid) and isopropyl stearate (octadecanoic acid) were present in the oils extracted from naturally infected and healthy *Aquilaria agallocha* Roxb. [34]. Moreover, fatty acids such as n-hexadecanoic acid, 9-octadecenoic acid and (E), tetradecanoic acid are reported to posses antitumour activity [31]. Meanwhile, Dahham *et al*. who studied on the essential oil from the stem bark of *Aquilaria crassna*, stated that the presence of sesquiterpene compounds were reported to be the main active compounds in agarwood species and were believed to be the active principles of the plant [11]. Further, azulene (0.6555%) and guaiol (0.2997%); identified at minute 11.133 and 12.239 respectively were believed to be the biomarker of agarwood species with guaiol being one of the six compounds that could be indicated as the reference compound to determine the quality of agarwood [33], [34]. Overall, compounds

identified in ALEO are consistent with agarwood biomarkers reported elsewhere suggesting the authenticity of the oil.

TABLE II
CLASSIFICATION OF THE IDENTIFIED CHEMICAL CONSTITUENTS OF ALEO BASED ON FUNCTIONAL GROUPS

Functional group	**Compound**	**Area (%)**
Monoterpene	Santolina epoxide	0.477
Sesquiterpene	Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-,[1S-(1. alpha.,7.alpha.,8a.beta.)]-	0.619
Alkane	Naphthalene, 2-butyldecahydro-	0.471
	trans, cis-2-Ethylbicyclo[4.4.0]decane	0.336
Alcohols	2-Propanol, 1,1'-oxybis-	8.457
	1-Propanol, 2-(2-hydroxypropoxy)-	5.406
	2-Butanol, 3,3'-oxybis-	1.453
	7.alpha.-Ethyl-8.beta.-hydroxy-2,6-dimethylbicyclo[4.4.0]dec-1-ene	0.940
	Guaiol (sesquiterpene alcohol)	0.300
	Patchouli alcohol (sesquiterpene alcohol)	2.356
Aldehydes	3-(4-Isopropylphenyl)-2-methylpropionaldehyde	0.656
Esters	Allyl phenoxyacetate	1.670
	Prop-2-ynyl (E)-2-methylbut-2-enoate	0.430
	Benzyl benzoate	0.762
	Isopropyl myristate (Tetradecanoic acid)	0.951
	i-Propyl 14-methyl-pentadecanoate	1.038
	Isopropyl palmitate (Hexadecanoic acid)	64.406
	Isopropyl stearate (Octadecanoic acid)	0.384
Ethers	Benzenamine, 3-ethoxy-	0.643

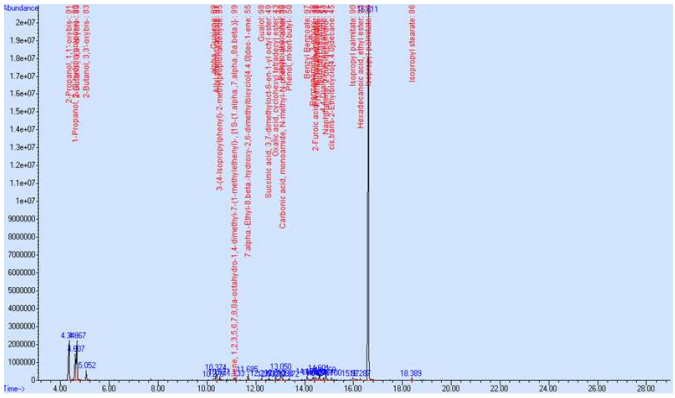

Fig. 1 Exemplary chromatogram of ALEO obtained using GCMS Agilent 7890A equipped with MSD quadrupole detector 5975 with capillary column of HP-5MS

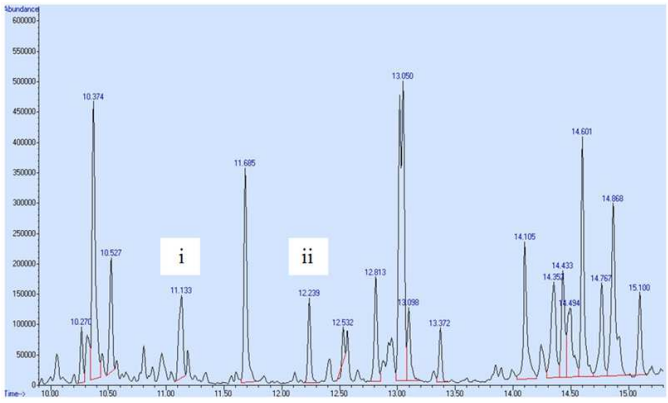

Fig. 2 The presence of the agarwood biomarkers labelled: (i) Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-,[1S-(1.alpha.,7.alpha.,8a.beta.)]- at minute 11.133 and (ii) Guaiol at min 12.239

C. In-vitro Anticancer Activity of ALEO

The determination of inhibitory concentration 50% (IC_{50}) value was performed by using WST-8 assay. Fig. 3 shows the MCF-7 cells viability after being treated with ALEO for 36 hours. Based on the fitted curve as depicted in Fig. 3, it can be observed that 31% (v/v) of ALEO caused 50% cell inhibition suggesting that ALEO has anticancer activity.

Dahham et al. (2016) revealed that the presence of monoterpenes, sesquiterpenes and aromatic compounds in essential oil from the stem bark of *Aquilaria crassna*, might have potent anticancer activities towards several types of cancers [12]. In the study, the essential oil exhibited significant antiproliferative activity against pancreatic cancer cells (MIA PaCa-2) which might be elucidated by the presence of phenolic and aromatic compounds such as azulene, β-caryophyllene, 2-Naphtalenemethanol, benzenedicarboxylic acid, cyclodecene and octamethyl [12]. Sesquiterpenes have been reported to be responsible for antitumor activity including apoptotic activity against human promyelotic leukemia (HL-60) [35]. In another study, the presence of sesquiterpene compounds in myrrh essential oil was able to inhibit the anticancer effects against MCF-7 cells [10]. On the other hand, α-humulene, caryophyllene oxide, tetradecanoic acid, n-hexadecanoic acid, isoledene and 9-octadecenoic acid were reported to have antitumor properties [31].

To this end, the anticancer effects of ALEO against the MCF-7 breast cancer cells could be justified by the presence of sesquiterpene, aromatic compounds and fatty acids.

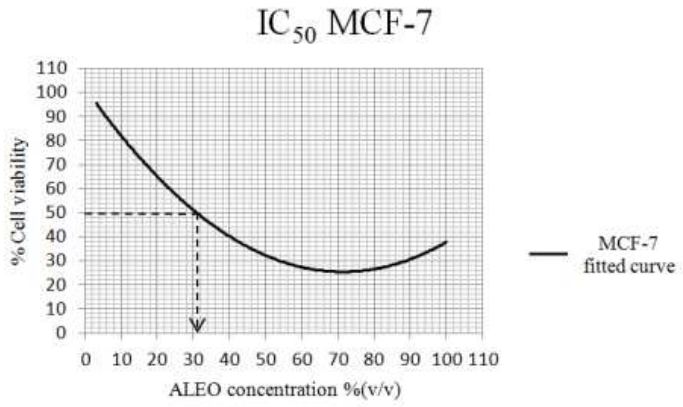

Fig. 3 Dose-response analysis of ALEO towards MCF-7 cells using WST-8 assay in 96-well format. Agarwood leaf essential oil (ALEO) concentration at 31% (v/v) was able to inhibit 50% of cell viability after 36h of treatment. Experiments were conducted in triplicates.

IV. Conclusions

In conclusion, ALEO shows anticancer effects towards MCF-7 breast cancer cells. The anticancer effects shown could be explained by the presence of sesquiterpene, aromatic compounds and fatty acids. In terms of physicochemical properties, the values were found to be comparable and within the standard range with other essential oils. The presence of guaiol and azulene (biomarker) indicate that this commercial oil was extracted from agarwood plant species justifying the authenticity of the oil.

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