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RESEARCH ARTICLE

Hydrodistillation and Soxhlet extraction of Agarwood leaf extract from *Aquilaria malaccensis*

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Abstract

Agarwood (*A. malaccensis*) is a valuable tree; highly sought after the resin and its essential oil. It also possesses bioactive compounds that are beneficial for health. However, less is focused on the agarwood leaf despite the recorded use in traditional medicine. As such, this present work focused on the screening of operational parameters of hydrodistillation and Soxhlet extraction of agarwood leaf extract (ALEX) with subsequent phytochemical and gas chromatography mass spectrometry (GCMS) analyses. Hydrodistillation failed to obtain any essential oil. Therefore, only Soxhlet extraction was further studied based on Plackett-Burman design generated using Design Expert software, version 6. The parameters studied were solvent type and volume, time, leaf type and particle size. Based on the ALEX yield obtained, solvent type was found to be the most significant parameters followed by solvent volume, particle size and time. Meanwhile leaf type was found to be the least influential parameter. Ethanol gave higher yield as compared to hexane. Run 2 gave the highest ALEX yield (121.34 ± 31.6 mg/g) and Run 4 gave the lowest yield (20.38 ± 4.1 mg/g). Based on phytochemical analysis, ALEX possess phenol, flavonoid, alkaloid, saponin and steroid compounds . GCMS analysis has identified a total of 50 compounds from ALEX in 12 experiments. Hexadecanoic acid was found to be the major compound in run 2 (highest yield) and phytol was found to be the major compound in run 4 (lowest yield). To this end, ALEX was succesfully obtained through Soxhlet extraction and the significant parameters can be further studied to achive optimal yield.

***Keywords*:** Agarwood, hydrodistillation, operational parameters, phytochemicals, Soxhlet

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INTRODUCTION

Aquilaria malaccensis, a species of *Aquilaria* is an agarwood tree normally found in Asian countries such as Malaysia, Indonesia and Thailand. It is called by many names in different countries such as, eaglewood, aloeswood, gaharu (Malaysia), oud, jin-koh (Japan) and cheng xian (China) (Khalil *et al*., 2013; Hashim *et al.*, 2016). It grows in a low-land tropical forest with height of up to 40 m and diameter of 60 cm.. It is known to be the most valuable and expensive tree in the world where the resin is mainly used as ingredients in making perfume as well as for religious and medicinal purposes (Harbone, 2001). Many studies have been conducted on agarwood tree, but least has been done on its leaf whilst other parts of the tree were reported to have health beneficial compounds (Samadi *et al.*, 2016). Recently, agarwood leaf has received increased attention by researchers particularly to investigate the chemical constituents present in the leaf. A few recent studies were conducted on the leaves including; the extraction and identification of bioactive compound of agarwood leaves (Lee *et al*., 2016), characterization of methanolic extract of agarwood leaves (Khalil *et al.*, 2013), anti-corrosion behavior of *A. crassna* leaves (Helen *et al.*, 2014), antioxidant activity of *A. malaccensis* leaves (Huda *et al.*, 2009), anti-inflammatory activity of *A. malaccensis* leaves (Manar Eissa *et al*., 2018) as well as characteriation and anticancer effects of agarwood leaf essential oil (Zainurin *et al*., 2018),

There are several methods that can be used to extract agarwood leaf extract such as hydrodistillation, solvent extraction, supercritical fluid carbon dioxide extraction (SFE), and maceration (Khalil *et al.*, 2013; Samadi *et al.*, 2016). This study focused only on hydrodistillation and Soxhlet extraction. Hydrodistillation was selected because it is one of the common process to extract essential oil, safe to be operated and environmentally friendly. Meanwhile, Soxhlet extraction was chosen due to its good performance rendering it to be a standard model and serve as reference to other methods (Castro *et al.*, 2010; Siddiqui *et al.*, 2016). In this study, the screening of operational parameters was done based on Plackett-Burman design generated by Design Expert software. According to this design, the effect of each parameter is quantitatively determined by finding the difference between the measurement's average value of the high-level factor from the one from low-level factor. The factors selected for hydrodistillation were temperature, solid to liquid ratio, time, particle size, leaf type and soaking effect. Meanwhile, five selected factors for Soxhlet extraction were type of solvent, volume of solvent, time, particle size and leaf type. Twelve experimental runs were conducted to study the effect of these parameters on agarwood leaf extract (ALEX) yield. The second part of this study was to determine the phytochemicals of ALEX by phytochemical tests and GCMS analysis.

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EXPERIMENTAL

Materials

A. malaccensis leaves were collected from a local farm in Selangor, Malaysia. The leaves were identified and the species validated by Faculty of Forestry, Universiti Putra Malaysia. A sample was deposited to its herbarium. The leaves were collected from two types of tree, inoculated and non-inoculated (3 years period of inoculation). Both trees are eight years old.

Sample preparation

The leaves were sent to The Cucurbit Company Sdn. Bhd. (Shah Alam, Selangor) to be washed, dried in the oven at 50˚C and ground according to the required size (5 mm and 10 mm). After receiving the ground leaves, they were kept in schott bottles, covered with aluminum foil to prevent from light exposure.

Extraction of ALEX by hydrodistilation

A pilot tudy on hydrodistillation process was conducted as shown in Table 1. The parameters were selected based on literature.

Extraction of ALEX by Soxhlet

Plackett-Burman design generated using Design Expert software, version 6 was used to screen the important operational parameters of Soxhlet extraction that may affect yield of ALEX. Five parameters were studied (solvent type, solvent volume, time, leaf type and particle size) on ALEX yield as response. Number of 12 experimental runs were generated as depicted in Table 3. The main effect of each parameter was quantitatively measured by calculating the difference between the average of the measurements (yield) made at high (+1) and low (-1) level. The yield, Y of ALEX was calculated based on Eq. 1:

$$Y\ (mg/g) = [\ W_E\ (g) / W_L\ (g)\ ] \times 1000 \quad (1)$$

where W_E = weight of extract; W_L = weight of ground leaves.

Meanwhile, the main effect of each parameter from experimental runs were calculated based on Eq. 2:

$$\text{Main effect} = (\ \Sigma\text{Yield from high level factor (+1) /No. of experiment}) - (\ \Sigma\text{Yield from low level factor (-1) /No. of experiment}) \quad (2)$$

Preliminary phytochemical analysis of agarwood leaf extract (ALEX)

ALEX was tested to determine the presence of phenol, flavonoid, alkaloid, saponin and steroid based on the standard procedures described by (Ghani 1998; Harbone, 2001; Sofowora, 2005). These analyses were conducted in ALEX with the highest and lowest yield.

Phenol test (Sodium hydroxide test)

5 mg of extract was dissolved in 10 ml distilled water, and filtered. The filtrate was added with few drops of 10% ferric chloride. Formation of greenish black color indicated the presence of phenols.

Flavonoid test (Sodium hydroxide test)

2 mg of extract was added to 1 ml of distilled water, mixed and filtered. The filtrate was added with few drops of sodium hydroxide solution. Formation of intense yellowish color confirmed the presence of flavonoid which turned to original color of the extract upon addition of diluted sulfuric acid (5%).

Table 1 Operational condition used for pilot study on hydrodistillation of ALEX.

Run	Temperature (˚C)	Solid to liquid ratio (g/ml)	Time (h)	Leaf type	Leaf size	Soaking effect	Dried vs. fresh leaf
1	Not measured	1:10	3	Non-inoculated	Fine	Non-soaked	Dried
2	100˚C	5:10	3	Non-inoculated	Fine	Non-soaked	Dried
3	100˚C	1:5	3	Non-inoculated	Fine	Non-soaked	Dried
4	100˚C	1.20	2	Inoculated	Coarse	Non-soaked	Dried
5	100˚C	1:20	2	Non-inoculated	Coarse	Non-soaked	Fresh
6	80˚C (First 5 h), 100˚C (next 3 h)	1:5	8	Inoculated	Coarse	Non-soaked	Dried
7	100˚C	1:10	2.5	Non-inoculated	Coarse	Soaked	Fresh
8	100˚C	1:20	2.5	Non-inoculated	Coarse	Soaked	Fresh
9	100˚C	1:20	3	Inoculated	Fine	Non-soaked	Dried

Table 2 Analytical conditions for GCMS analysis based on Hashim *et al.,* (2014) with few modifications.

Program		Condition			
		Rate °C/min	Value °C	Hold time min	Run time min
Oven Program	Initial		80	2	2
	Ramp	10	250	10	29
Carrier gas			Helium		
Gas flow			2 ml/min		
Split Ratio			1:50		
Injection Volume			1 µL		
Mode			Split		
Interface temperature			250°C		
Electron impact (emission current)			70 eV		
Scan range			32 to 500 amu		

Alkaloid test (Dragendorff test)

25 mg of extract was dissolved in dilute sulfuric acid (5%) in ethanol. The mixture was heated for 5 to 10 minutes and filtered. The filtrate was treated with few drops of Dragendorff's reagent and orange color formed indicated the presence of alkaloid.

Saponin test (froth test)

0.25 g of the extract was dissolved in 10 ml of distilled water in a 50 ml schott bottle. The mixture was then shaken vigorously and formation of stable froth (foam) that remain unchanged for more than 10 minutes indicated the presence of saponin.

Steroid test (Liebermann-Burchardt tets)

2 to 3 mg of the extract was heated with 2 ml of acetic acid in water bath and cooled at room temperature. Few drops of concentrated sulfuric acid were slowly added to the mixture. A dark green color formed indicated the presence of steroid.

Gas chromatography-mass spectrometry (GCMS) analysis on agarwood leaf extract (ALEX)

Sample for GCMS analysis was prepared for each extract from the 12 experimental runs. Amount of 0.075 to 0.150 g of the extract was dissolved with 1 ml of respective solvent (ethanol/n-hexane) used for that particular extraction. The mixture was stirred well and 200 µl of the mixture was diluted with 1.8 ml of respective solvent (ethanol/n-hexane). After that, the diluted extract was filtered, and transferred to GCMS vials for analysis.

GCMS analysis was conducted using gas chromatography system; Agilent 7890A (Agilent Technologies) coupled with Agilent 5975C quadrupole mass spectrometer and autosampler. The column used was Hewlett Packard HP-5MS silica capillary column (30 mm x 25 mm x 25 µm film thickness). The analytical conditions for GCMS test are listed in Table 2. The detected peaks from GCMS were based on the total ion chromatography (TIC) and mass chromatograms were further identified using National Institute of Standards and Technology (NIST) 2008 mass spectral library.

RESULTS AND DISCUSSION

Pilot study on hydrodistillation of ALEX

Nine experimental runs listed in Table 1 was conducted, however ALEX was not succesfully extracted. This may be due to several possible reasons. First, essential oil content in agarwood leaf may have been very little as such it requires a large amount of leaves and larger system to extract it. Second, the essential oil is a heat sensitive compound and easy to evaporate which might have caused their loss during drying process. Third, the apparatus used was not optimal and a more sophisticated apparatus system such as Clevenger apparatus may be more suitable (Samadi *et al.*, 2016). Subsequently, the essential oil would have been dissolved in water. A solvent with low polarity index such as n-hexane and n-pentane could be used to prevent the oil from dissolving in water by adding it on top of the receiver flask.

Screening of Soxhlet operational parameter on ALEX

The yield of the ALEX obtained from all experiments are presented in Figure 1 and Table 3. Run 2 (ethanol) gave the highest yield (121.34 ± 31.6 mg/g) while, Run 4 (hexane) gave the lowest yield (20.38 ± 4.1 mg/g). Figure 1 also shows that all extractions using ethanol gave higher yield compared to hexane. Meanwhile, the main effects of each parameter are shown in Figure 2. The result showed that type of solvent was the most significant parameter with negative main effect of -76.23. Meanwhile, leaf type was the least significant parameters with the main effect of -1.38 which is near zero. Type of solvent, volume of solvent, time and particle size were considered as significant parameters and recommended to be used in further studies.

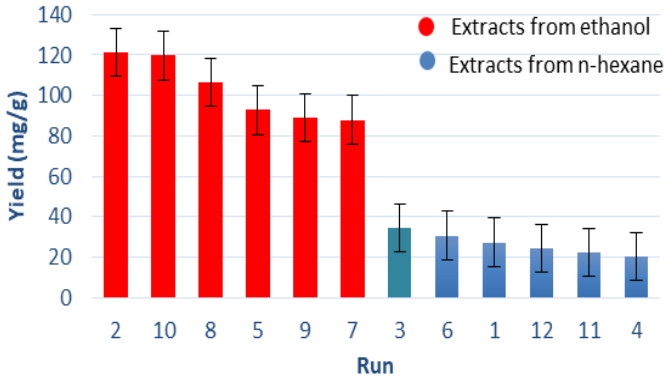

Fig. 1 Yield of ALEX from twelve experiments conducted using ethanol and n-hexane.

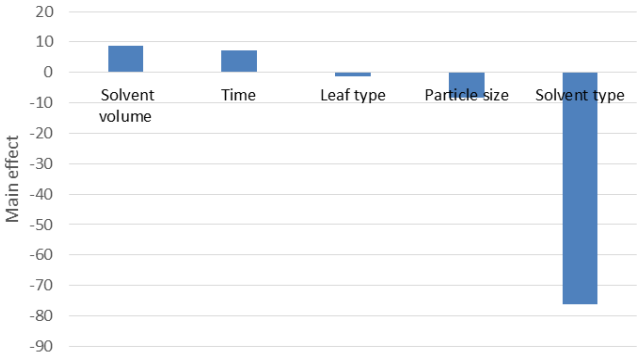

Fig. 2 Main effect of the operational parameters of soxhlet extraction on the yield of ALEX based on the experimental results using Plackett-Burman design.

Table 3 Experimental runs for soxhlet extraction of ALEX generated based on Plackett-Burman design with different parameters together with their levels [low (-1); high (+1)] and yields from each experiment.

Run	Solvent	Volume (mL)	Time (h)	Leaf	Particle (leaf) Size (mm)	Yield (mg/g)
1	Hexane (+1)	180 (-1)	9 (+1)	Inoculated (+1)	5 (-1)	27.40 ± 3.8
2	Ethanol (-1)	300 (+1)	9 (+1)	Inoculated (+1)	5 (-1)	121.34 ± 31.6
3	Hexane (+1)	300 (+1)	6 (-1)	Inoculated (+1)	5 (-1)	34..38 ± 1.0
4	Hexane (+1)	180 (-1)	6 (-1)	Non-inoculated (-1)	10 (+1)	20.38 ± 4.1
5	Ethanol (-1)	300 (+1)	6 (-1)	Non-inoculated (-1)	5 (-1)	92.78 ± 6.7
6	Hexane (+1)	180 (-1)	9 (+1)	Non-inoculated (-1)	5 (-1)	30.74 ± 1.2
7	Ethanol (-1)	180 (-1)	6 (-1)	Inoculated (+1)	10 (+1)	87.94 ± 3.4
8	Ethanol (-1)	180 (-1)	6 (-1)	Non-inoculated (-1)	5 (-1)	106.51 ± 6.4
9	Ethanol (-1)	180 (-1)	9 (+1)	Inoculated (+1)	10 (+1)	88.84 ± 2.1
10	Ethanol (-1)	300 (+1)	9 (+1)	Non-inoculated (-1)	10 (+1)	119.66 ± 2.8
11	Hexane (+1)	300 (+1)	9 (+1)	Non-inoculated (-1)	10 (+1)	22.44 ± 0.9
12	Hexane (+1)	300 (+1)	6 (-1)	Inoculated (+1)	10 (+1)	24.33 ± 0.4
Effect	-76.23	8.85	7.35	-1.38	-8.26	

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Phytochemical analysis

Qualitative phytochemical tests were conducted on samples with the highest and lowest yield. Both extracts are positive for all phytochemicals tested. The summary of the analyses is shown in Table 4.

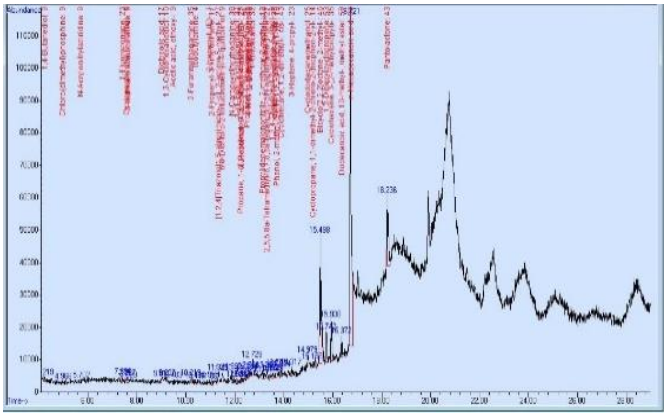
Table 4 Phytochemical constituents of ALEX. Samples were from Run 2 (highest yield) and Run 4 (lowest yield). Note: (+) indicates positive result.

Phytochemical	Test	Observation	Result	
			Run 2	Run 4
Phenols	Ferric chloride test	Greenish-black color was formed	+	+
Flavonoids	Sodium hydroxide test	Yellow color was formed upon addition of sodium hydroxide which turned to its original color after addition of dilute sulfuric acid.	+	+
Saponins	Froth test	A stable froth was formed.	+	+
Alkaloids	Dragendorff test	Orange color was formed	+	+
Steroids	Liebermann-Burchard test	A dark green color was formed	+	+

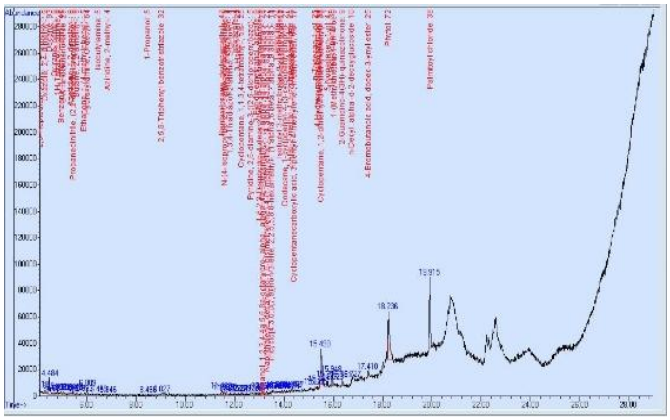
Gas chromatography-mass spectrometry analysis of ALEX

Extract from total of 12 experimental runs gave an overall of 50 compounds. Hexadecanoic acid and its methyl ester derivative, phytol, and Tetramethyl-2-hexadecen-1-ol were found to be the major compounds of the extracts. The GCMS analysis was studied in more depth for ALEX with the highest (Run 2) and lowest (Run 4) yield and part of the compounds identified are summarized in Table 5. There were total of 10 and 12 compounds identified from extract with the highest and lowest yield respectively.

Hexadecenoic acid (47.747%) and phytol (26.929%) were found to be the major compounds in ALEX with the maximum (Run 2) and minimum (Run 4) yields respectively. Phytol was detected in both extracts. Besides, based on the comparison with previous study (Yufeng *et al.*, 2007; Khalil *et al.*, 2013; Lee *et al.*, 2016), the compounds identified were similar in which hexadecenoic acid and phytol were part of the major compound from agarwood leaf. Fig. 3 shows the chromatograms for Run 2 (maximum yield) and Run 4 (minimum yield).



(a)



(b)

Fig. 3 Chromatograms of ALEX from: (a) Run 2 (highest yield) (b) Run 4 (lowest yield).

Table 5 Major compounds of ALEX present in Run 2 (highest yield) and Run 4 (lowest yield) identified by GCMS analysis.

No	Compounds	ALEX from run 2 (maximum yield)		ALEX from run 4 (minimum yield)	
		Retention time (min)	Area (%)	Retention time (min)	Area (%)
1	Phytol	18.236	9.860	18.236	26.929
2	n-Hexadecanoic acid	16.721	47.747	NA	NA
3	Diethylmalonic acid, monochloride, hexadecyl ester	NA	NA	19.915	23.287
4	Trichloroacetic acid, undec-10-enyl ester	15.498	16.049	NA	NA
5	Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-, [1R (1.alpha.,2.beta.,5.alpha.)]-	NA	NA	15.498	13.558
6	Decane	NA	NA	4.484	5.578
7	6-Octen-1-ol, 3,7-dimethyl-, propanoate	15.938	4.964	NA	NA
8	9-Octadecynoic acid, methyl ester	15.742	3.061	NA	NA
9	Spiro[2.3]hexan-5-one, 4,4-diethyl-	NA	NA	15.758	2.850
10	Citronellyl isobutyrate	NA	NA	15.948	2.628

NA: not available.

CONCLUSION

In this study, extraction of agarwood leaf extract (ALEX) was perfomed by hydrodistillation and Soxhlet extraction to screen the operational parameters that would affect these processes based on the yield obtained. However, hydrodistillation failed to give any extract hence, the screening proces was done only on Soxhlet extraction. The result showed that type of solvent was the most significant parameter and leaf type was the least one affecting the extract yield. Based on main effect calculated, type of solvent, volume of solvent, time and particle size were considered as important parameters and further study on these parameters are recommended. Based on phytochemical analysis, ALEX showed positive results in the presence of phenol, flavonoid, alkaloid, saponin and steroid. Meanwhile, the GCMS

analysis revealed the total of 50 compounds from all extracts with hexadecanoic acid and phytol were detected as the major constituents in the experimental runs 2 and 4 respectively which yielded maximum and minimum ALEX.

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