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PHYTOCHEMICAL AND ANTI OXIDANT SCREENING OF *AQUILARIA MALACCENSIS* LEAVES.

PENYARINGAN FITOKIMIA DAN ANTIOKSIDAN DAUN AQUILARIA MALACCENSIS

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Abstract

Aquilaria malaccensis is an endangered economic plant used for production of agar wood worldwide. The sequential maceration extraction methods utilizing solvents with different polarities namely hexane, ethyl acetate and methanol yielded the corresponding crude extract. The aqueous and methanol extracts along with dry powder of leaf of the plant was screened for the presence of phytochemicals. They were also tested for antioxidant activities. The result indicates the presence of alkaloids, flavanoids, triterpenoids, steroids and tannins. The phytochemical screening suggests that flavanoids present in this species might provide a great value of antioxidant activity. Preliminary screenings of the free radical scavenging activity on the extracts of the plants with 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) were tested and showed positive result. Quarcetine was used as reference standard. The extracts exhibited strong antioxidant activity radical scavenging activity with IC50 value of 8.0 × 10^2 µg/ml, 1.6 × 10^2 µg/ml, 1.4 × 10^2 µg/ml, 30.0 µg/ml and 3.33 µg/ ml for hexane, DCM, ethyl acetate, methanol and quercetine respectively. Determination on antioxidant activity of each crude extract showed that methanol crude extract had the highest IC_{50} value than ethyl acetate, dichloromethane and hexane crude extract. This means that methanol possess the highest inhibition of DPPH radical scavenging activity compared to the other crudes but still lower than Quercetin (standard). Phytochemical analysis on the hexane extract of Aquilaria malaccensis has been conducted. Several chromatographic method has been employed to the hexane extract of the leaves which led to the isolation of three compounds namely Stigmasterol, ß-sitosterol and 3-fridelanol The present study has proved the usefulness of agarwood tree for medicinal purposes and its potential as a source of useful drugs.

Key words: *Aquilaria malaccensis*, phytochemicals, antioxidant activity.

INTRODUCTION

Aquilaria malaccensis is one of 15 tree species in the Indomalesian genus *Aquilaria*, family Thymelaeaceae. It is a large evergreen tree growing up to 40 m tall and 1.5-2.5 m in diameter, found typically in mixed forest habitat at altitudes between 0 and 1000 m above sea level. The species has a wide distribution, being found in Bangladesh, Bhutan, India, Indonesia, Malaysia, Myanmar, the Philippines, Singapore and Thailand. *A. malaccensis* and other species in the genus *Aquilaria* sometimes produce resin-impregnated heartwood that is fragrant and highly valuable. There are many names for this resinous wood, including agar, agarwood, aloe(s)wood, eaglewood, gaharu and kalamabak. This wood is in high demand for medicine, incense and perfume across Asia and the Middle East.

Studies revealed that agarwood has remarkable anticancer activity (Gunasekera et al. 1981). The benzene extracts of the plant have central nervous system antidepression activities (Okugawa et al. 1993). Rise in demand for agarwood resulted in irrational cutting of the tree trunk for extraction of the chemical. This has resulted in the tree becoming endangered. Although thorough phytochemical research has been carried out on the trunk and resin of agarwood, little is known about the chemical constituents of agarwood leaves. There is no detail systematic documentation of presence and type of phytochemicals in the leaves of the agarwood tree. Hence the present study aimed at an

evaluation of the presence of different phytochemicals along with antibacterial activity of methanol soluble, water soluble extracts and dry powder obtained from leaf of *Aquilaria malaccensis*.

EXPERIMENTAL

Plant material

The leaves of *Aquilaria malaccensis* were harvested from the forest within the Malaysian Nuclear Agency in Bangi on July 2006. The voucher specimen (SK148/06) was deposited at the herbarium of Laboratory of Natural Products, Malaysian Nuclear Agency.

Extraction and Fractionation

The fresh plant sample (8.844 Kg) were air-dried under the shade before cutting into smaller pieces. The dried material (3 Kg) was ground to powder, and then macerated/ percolated in methanol (10L). The extract was filtered and evaporated under reduced pressure to give 426.58g of MeOH extract. Methanol (150ml) and water (300ml) and was added to the methanol extract (400g) resulting an aqueous solution. The aqueous solution was subjected to liquid-liquid partitioning with hexane (5 x 450ml) which on evaporation yielded a hexane fraction (36.12g). This was followed by successive series of extraction with dichloromethane (6 x 450ml), ethyl acetate (5 x 300ml) and butanol (8 x 300ml). Each of the solvent was then removed under reduced pressure to give 11.68g of CH_2Cl_2, 8.77g of EtOAc, 76.17 g of BuOH, and 293.84 g of water fractions. The dry crude extracts were purified by column chromatography. These afforded to three compounds.

Phytochemical Screening

Phytochemical test was done to determine the presence of the secondary metabolites in *Aquilaria malaccensis* leaves. Four crude extracts from different polarities was subjected into Alkaloid Test (Mayer), Triterpenoid/Steroid Test (Liebermann-Burchard), Saponin Test and Flavonoid Test.

Antioxidant Screening

The samples are tested against two antioxidant bioassays :

i. Xanthine/Xanthine Oxidase Superoxide Scavenging System - The assay evaluates the scavenging activity of the sample on superoxide free-radical anions.

ii. DPPH Radical Scavenging Assay - The assay evaluates the reducing activity of the sample that determines its antioxidant potential (AOP)

Phytochemical Analysis

The UV spectra was measured with a UV-1240 SHIMADZU spectrophotometer using $CHCl_3$ as solvent while UV shift reagents were prepared based on the methods described by Mabry *et al.* [3] and Markham [4]. ^1H and ^{13}C-NMR spectra were recorded on a Varian INNOVA500 (U.S.), in $CDCl_3$ and chemical shifts are given on a δ (ppm) scale with TMS as internal standard. MS were recorded using Trace PolarisQ Gas Chromatography.

RESULTS

Phytochemical Screening

Methanol and ethyl acetate crudes show positive result for both alkaloid and saponin test with constant foam for at least 2 hours (+3). While no changes are observed on dichloromethane and hexane crude extract for both tests. As for flavanoid test only dichloromethane gave a negative results. Triterpenoids are found in methanol extract while other extracts gave positive indication for the presence of steroids.

Table 1. Summary of the phytochemical screening tests on crude extracts of *A. malaccensis*

Crude Extract	Alkaloid Test	Triterpenoid / Steroid Test	Saponin Test	Flavonoid Test
Hexane	-	+S	-	+
Dichloromethane	-	+S	-	+
Ethyl Acetate	+	+S	+	-
Methanol	+	+T	+	+

Indicator:

- : Negative result
+ : Positive result
+S : Positive results for steroid
+T : Positive results for triterpenoid

A. Antioxidant Screening

This screening was done to determine whether there are any antioxidant properties in the crude. Figure 1 shows the percentage of inhibition H-donor activity of Quercetin as measured using DPPH assay with different concentration and figure 2 shows the percentage of inhibition H-donor activity of hexane, dichloromethane, ethyl acetate and methanol crude extract as measured using DPPH assay with different concentration.

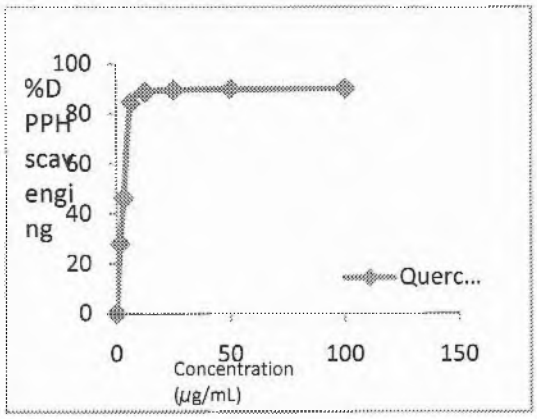


Figure 1. Percentage of inhibition H-donor activity of Quercetin as measured using DPPH assay with different concentration

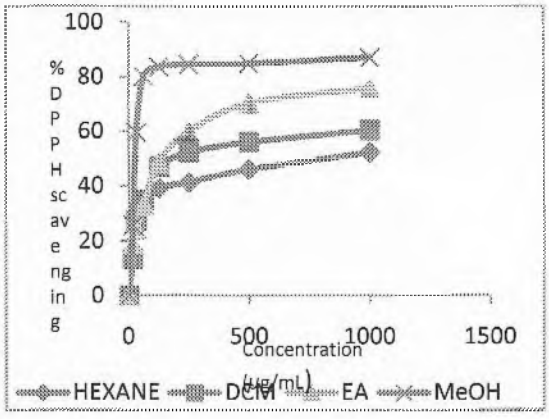


Figure 2. Percentage of inhibition H-donor activity of hexane, dichloromethane, ethyl acetate and methanol crude extract as measured using DPPH assay with different concentration

DPPH assay clearly shows the highest potential of antioxidant properties in each crude extracts of *A. malaccensis* especially the methanol crude extract. The polar fraction shows a high potential antioxidant properties.

Table 2. Percentage of DPPH Free Radical Scavenging Activity of the crude extracts of *A. malaccensis*

Sample Description	DPPH Radical Scavenging (%)		Superoxide Scavenging (%)	
Standard	Ascorbic acid (AA) 0.005mg/ml		Superoxide Dismutase (SOD) ($6x10^{-3}$ U/ml)	
Sample Concentration	0.25mg/ml		0.25mg/ml	
Hex Fraction	37.9 ± 1.90	L	64.4 ± 4.00	M
DCM Fract	33.8 ± 2.85	L	53.0 ± 1.75	M
EA Fract	37.3 ± 1.60	L	68.2 ± 0.25	M
Buthanol Fract	96.2 ± 1.55	H	89.9 ± 0.35	H

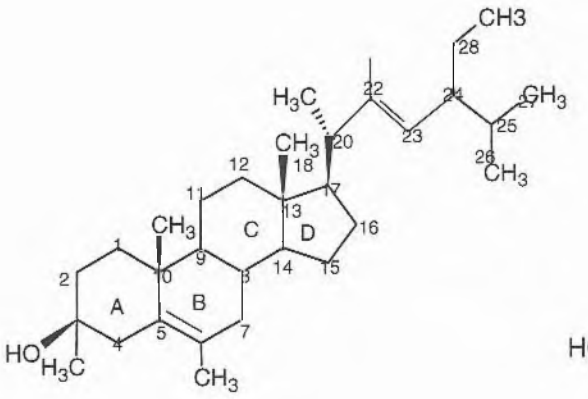
Indicator:

H – High M – Moderate L – Low

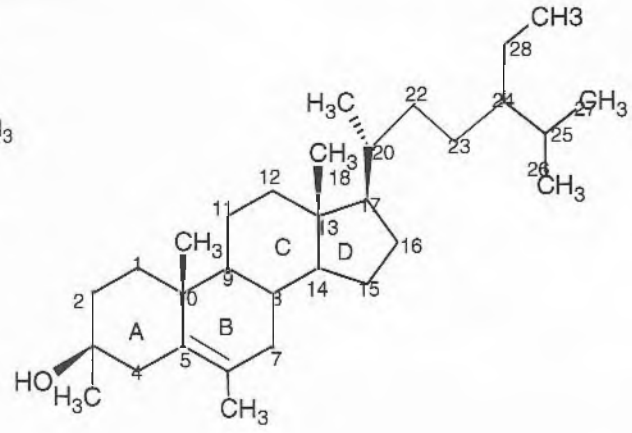
The buthanol crude possessed the highest antioxidant activity than DCM, EA and Hexane crude extract. Buthanol extracts were the most effective DPPH and Superoxide scavengers.

B. Phytochemical Analysis

The hexane extract from the leaves of *A. malaccensis* was subjected to column chromatography using silica gel to afford several fractions. Purifications were carried out on selected fractions by repeated column chromatography yielding three pure compounds **1**, **2** and **3**. The compounds were identified by spectroscopic methods, i.e. UV, MS and NMR, and as well as by comparison with literature data



1



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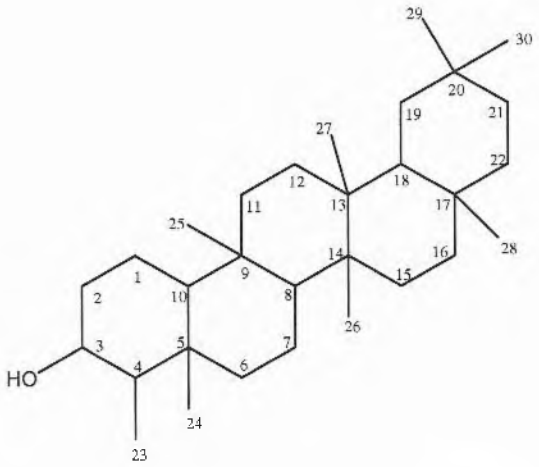

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Compound 1 and 2 were obtained from n-hexane-EtOAc, (1:1). Further purification by a small column and preparative TLC (Silica gel 60 F_{254}) yielded compound **1** which was detected on TLC as a dark pink band at R_f 0.82 after spraying with 10% H_2SO_4 when using hexane – ethyl acetate (1:1) as the mobile phase. Compound **1** was isolated as white needles (12.0mg) TLC: R_f 0.82 (n-hexane-EtOAc, 1:1); UV (MeOH) λ_{max} nm: 188; ^{1}H-NMR ($CDCL_3$, 400 MHz) : 5.35 (d, *J*=5.1 Hz; H-6), 5.13 (dd, *J*=8.78, 8.8 Hz; H-22), 5.03 (dd, *J*=8.8, 8.8 Hz; H-23) 3.52 (m, H-3), 2.00 (m, H-4), 1.04 (s, H-21), 1.02 (s, H-19), 0.85 (t, *J*=307 Hz; H-29), 0.83 (d, *J*=1.8Hz, H-26), 0.81 (s, H-27), 0.70 (s, H-18); ^{13}C-NMR ($CDCl_3$, 100 MHz) : 140.74 (C1), 138.30 (C22), 129.3 (C23), 121.69 (C6), 71.78 (C3), 56.76 (C14), 56.05 (C17), 51.23 (C9), 50.10 (C25), 42.30 (C13), 39.75 (C4), 37.78 (C12), 37.25 (C1), 36.50 (C10), 36.10 (C-20), 31.9 (C7), 31.60 (C8), 28.9 (C25), 28.2 (C2), 26.08 (C16), 24.3 (C15), 21.2 (C26,28), 21.10 (C27), 21.06 (C11), 19.39 (C19), 19.0 (C21), 12.20 (C29), 12.04 (C18).

^{1}H-NMR data for compound **1** indicated distinctive peaks at δ 3.52 (m, H-3), δ 5.35 (d, *J*=5.1 Hz, H-6), and two double-doublet peaks at δ 5.13 (*J*=8.8, 8.8 Hz, H-22) and δ 5.03 (*J*=8.8, 8.8 Hz, H-23). In addition the ^{13}C-NMR spectrum showed the presence of six methyl groups at δ 0.70-1.04 (c-18, C-19, C-21, C-26, C-27 and C-29). Thus compound **1** was concluded to be stigmasterol due to the similarity of its spectroscopic data with the literature [6].

Compound **2**: Colorless needles, mp 133–135°C; ^{1}H NMR (500 MHz, $CDCl_3$) δ5.39 (1H, *m*, H-6), 3.56 (1H, *m*, H-3), 1.05 (3H, *s*, Me-19), 0.96 (3H, *d*, *J* = 6.5 Hz, Me-21), 0.89 (3H, *t*, *J* = 7.4 Hz, Me-29), 0.87 (3H, *d*, *J* = 6.7 Hz, Me-26), 0.85 (3H, *d*, *J* = 6.7 Hz, Me-27), 0.72 (3H, *s*, Me-18); ^{13}C NMR (125 MHz, $CDCl_3$, based on DEPT, HMQC, HMBC experiments); EIMS *m/z* 414 $[M]^+$ (39), 396 (100), 381 (21), 329 (17), 303 (19), 273 (11), 255 (24), 213 (20), 161 (18), 145 (22), 107 (22), 95 (23), 55 (21).

Compound **2** was obtained as white crystalline solid with melting point 119°C. The ^{1}H NMR spectrum showed six singlet peaks at δ 0.76, 0.86, 0.90, 0.92, 1.00 and 1.06 corresponding to six methyl groups of β-sitosterol. Carbon C-O, carbon olefinic and carbon quaternary locations can be determined by comparing the obtained data to the ^{13}C-NMR spectrum data that was reported [5]. Chemical shifts on δ140.8 and δ121.8 showed the presence of C olefinic C-5 and C-6. The peak at δ71.9 was assigned to the oxymethine proton C-3 [5]. The signals at δ36.6 and 42.4 were consistent with the position of the quaternary carbon, C – 10 and C – 13, respectively. According to the ^{1}H, ^{13}C, 2D NMR (H, H-COSY, HMQC, HMBC) experiments and also MS spectra and by comparing these spectroscopic data with those reported in the literatures, this compound was assigned to be ß-sitosterol [5].

Compound **3** was obtained as yellow amorphous solid; m.p. 248-249 °C (lit. 246-247 °C); $FeCl_3$, Mg/HCl: positive; UV λmax (MeOH) 258, 357 nm; (+ NaOH) 271, 330, 403 nm; (+ $AlCl_3$) 273, 403 nm; (+$AlCl_3$/HCl) 270, 359, 400 nm; IR ν_{max} (KBr) cm^{-1}: 3430 (-OH), 1652 (C=O). 1608 (C=C, aromatic), 1504 and 1068; ESIMS m/z 435 $(M+H)^+$, 303 $[(M+H)]^+$.

^{1}H-NMR (500 MHz, CD_3OD) δ 6.18 (d, J = 2.2, H-6), 6.37 (d, J =2.2, H-8), 7.51 (d, J = 2.1, H-2'), 6.88 (d, J = 8.3, H-5'), 7.47 (dd, J = 8.3, 2.1, H-6'), 5.46 (d, J = 7.6, H-1''). ^{13} C-NMR (125 MHz, CD_3OD) δ: 72.92(C-3), 61.585(C-10), 53.427(C-4), 49.407(C-8), 43.055(C-18), 41.959(C-6), 39.905(C-13), 39.512(C-22), 38.604(C-9), 38.065(C-14), 37.336(C-5), 36.313(C-2), 35.786(C-16), 35.572(C-19), 35.424(C-11), 35.259(C-30), 33.045(C-15), 32.564(C-21), 32.320(C-29), 32.022(C-28), 30.896(C-12), 30.255(C-17), 28.407(C-20), 20.348(C-26), 18.878(C-25), 17.779(C-7), 16.626(C-24), 16.023(C-1), 11.850(C-23).

The molecular formula of compound **3** was deduced as $C_{30}H_{52}O$ by the El-mass spectrum at *m/z* 428. The IR spectrum of compound 3 showed absorption for hydroxyl at band 3430. ^{1}HNMR spectrum exhibited eight methyl groups (δ: 0.868, 0.939, 0.954, 0.975,1.000, 1.005, 1.016 and 1.180). A methane proton which assigned to H-3 resonated at more downfield than other methane group cause by deshielding effect from adjacent hydroxyl group. ^{13}CNMR spectrum showed signals corresponding to 30 carbons, resolved into 8 methyl, 11 methylene, 5 methine and 6 quaternary carbons. Long-range proton-carbon correlations were observed by HMBC experiment especially to determine the quaternary carbons. From HMBC spectrum, the correlations of H-18/C-13, H-4/C-10, H-10/C-8, H-8/C-10, and H-3/C-5 were observed. All these data supported the structure of compound **3** as 3-fiedelanol.

CONCLUSION

The phytochemical screening suggests that flavanoids present in this species might provide a great value of antioxidant activity. All the crude extracts exhibited a positive DPPH free radical scavenging activities. The IC_{50} values of DPPH free radical scavenging activity was in decreasing order: Buthanol > DCM > EA > Hexane

This means that buthanol possess the highest inhibition of DPPH radical scavenging activity compared to the other crudes but still lower than Quercetin (standard). The present study has proved the usefulness of agarwood tree for medicinal purposes and its potential as a source of useful drugs.

Three components isolated from hexane extract were identified as stigmasterol **1**, β-sitosterol **2** and 3-friedelanol **3**. All the three components were identified based on their GC-MS, ^{1}H and ^{13}C-NMR data and also by comparison with the literature [6,5.].

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