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Research Article

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In vitro antioxidant activity and phytochemical screening of *Aquilaria malaccensis* leaf extracts

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ABSTRACT

The present study investigated water extraction (WE) and methanol extraction (ME) of A. malaccensis dried leaves (DL) and fresh leaves (FL) for its in vitro antioxidant activity that may contribute to their pharmacological effects. Total phenolic content (TPC) of this plant was determined by Folin-Ciaocalteu assay, while antioxidant potential were evaluated by TAC, CUPRAC and DPPH method. Tests for steroids, triterpenoids, flavonoids, saponins, tannins and alkaloids were positive in both water and methanol extracts. WEDL showed the highest inhibition of the DPPH radical (48.07 ± 0.68 %) at concentration 1000 ug/ml and IC_{50} value was found to be 1.091 mg/ml, relative to ascorbic acid, having an IC_{50} of 0.219 mg/ml. It also showed the highest CUPRAC value (3.32 ± 0.01μg/ml) as well as the highest TPC and TAC (181.11 ± 0.61 and 398.74 ± 0.66 gallic acid equivalent (GAE) mg/g) at a concentration of 1000 μg/ml) as compared to the other studied extracts. In conclusion, the results of this study clearly indicated that the extracts of A. malaccensis possess significant antioxidant activities and could be used as a potential source of natural antioxidant agents that may be due to the presence of phytochemicals.

Key words: *A. malaccensis*, antioxidant activity, total phenolics, DPPH, phytochemical

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INTRODUCTION

Aquilaria spp. are the most valuable and highly fragrant forest products locally known as agarwood, aloeswood, eaglewood, gaharu, kalamabak or oudh depending on the region [1]. Several genera that might be a source of agarwood production from the family Thymelaeaceae might be endangered due to the deterioration of their natural resources include *Aquilaria, Gonystulus*, and *Gyrinops* having been listed in Appendix II of the Convention on Internal Trade in Endangered Species of Wild Fauna and Flora [2]. In Malaysia and Indonesia, the resin produced by *Aquilaria* species generally called as a gaharu and *A. malaccensis* known as a main genus to produce agarwood or gaharu. Agarwood has many uses such as perfumes, incense and medicines. The main markets for these products are in South and East Asia and the Middle East [3].

The identification of the botanical and ecological aspects of this species has been widely identified from a chemical constituent point of view that can be explain its uses as an important and excellent source of pharmaceutical products [4]. Many parts of this plant, including the leaves, skin, seeds, wood and roots are valuable in medicinal

properties. It is highly sought after for its resin and essential oils while less has been focused on the health beneficial effects of other parts of the plant despite the various ethnopharmacological evidences. These include antioxidant activities, analgesic, antipyretic, anti-inflammatory [5,6], antihyperglycemic [7], and antimicrobial for various medicinal purposes.

Antioxidant have been able to destroy a single oxygen molecule and neutralize chemically active products of metabolism in order to protecting oxidative damage to cells, which cause several diseases such as cancer, ageing [8] and diabetes [9]. Most researchers commonly use different methods for measuring antioxidant capacity of the compound extract. The antioxidant capacity and flavoring properties of many plant extracts is related to the presence of phenolic compounds such as phenolic acids, polyphenols and flavonoids [10]. However, there is a little known about the potential phenolic compounds and antioxidant activity of herbs on human health [11]. Even though more research works has been carried out on this planet, including woods and leaves, but there is no enough scientific data available on the antioxidant activities of various extracts of the dried and fresh leaves. Thus, we have carried out the research work on antioxidant activities by using total antioxidant activity (TAC), radical scavenging assays such as 2,2-diphenyl-1-picrylhydrazy (DPPH) and cupric reducing antioxidant capacity (CUPRAC) assay. The present study was also investigating the total phenolic content and phytochemical screening by using standard methods.

EXPERIMENTAL SECTION

Plant Collection and identification

The *A. malacenssis* leaves were collected at Ladang Karas, Merchang in Terengganu. Plants were identified with the herbarium of Institute of Bioscience UPM, voucher specimen No. SK 2422/14. The fresh leaves were cleaned and dried in oven at 40^{o}C (not exceeding 50^{o}C) [12]. These were hereafter referred to as dried form. The leaves were cut into small slices (~ 0.5-1.5 cm) and powdered using a dry grinder.

Sample Preparation

Methanol extract: About 50 g of each ground samples were separately weighed and soaked in 1000 ml of methanol for 72 hours at room temperature. The supernatant was filtered using Whatman No. 1 filter paper. Then, the filtrates were removed under reduced pressure at 40 °C by using a rotary evaporator until it was dried.

Water extract: From grounded powder, a 50 g of each samples were soaked with distilled water at a ratio of 1:1 and boiled for 30 min [13]. Then, it was filtered by a Whatman No.1 filter paper and freeze dried using a vacuum freeze dried for 72 hours to remove solvent.

The extracts were labelled as water extraction of dried leaves (WEDL), water extraction of fresh leaves (WEFL), methanol extraction of dried leaves (MEDL) and methanol extraction of fresh leaves (MEFL). The process was repeated triplicates for each different samples. The extracts were placed in a glass bottle and then stored at −4 °C prior analysis.

Phytochemical analysis

The extracts were tested by the Libermann Burchard, Salkowski, Shinoda, Foam, Ferric chloride and Mayer's tests determine the presence of steroids, triterpenoids, flavonoids, saponins, tannins and alkaloids respectively.

Determination of total phenol content

Samples were measured for total phenolic content according to the previous method [13]. Briefly, an aliquot of 12.5 µl of each plant was mixed with 250 µl of 2% sodium carbonate solution in 96-well microplate and allowed to react for 5 min at room temperature. Then 12.5 µl of diluted Folin-Ciocalteu phenol reagent (1:1 with water) and allowed to stand for 30 min at room temperature before the absorbance of the reaction mixture was read at 650 nm using a spectrophotometer. Calibration was achieved with an aqueous garlic acid solution. The TPC of the extracts was expressed as mg gallic acid equivalent (GAE) per gram of each plant on dry basis and all determinations were performed in triplicates.

Antioxidant assay

Total antioxidant activity (TAC)

The antioxidant capacity of the extracts was evaluated by the phosphomolybdenum assay [13]. About 0.1 ml of sample solution was mixed separately with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate

and 4 mM ammonium molybdate). Then, the reaction mixture was incubated at 95°C for 90 min. The formation of the green phosphate complex by the sample analyte showed the reduction of Mo (VI) to Mo (V) at acidic pH. By using a spectrophotometer, the mixture was measured at at 695 nm against blank after cooling at room temperature. The results of antioxidant activity were reported as the number of equivalents of ascorbic acid in micrograms per gram of extract.

2,2-diphenyl-1-picrylhydrazy (DPPH) radical scavenging activity

The scavenging activity of the extracts was determined using DPPH-scavenging assay. Briefly, 1 ml of each extract was allowed to react with l ml of 0.2 mM DPPH in 95% methanol. The solution was then incubated in dark at room temperature for 30 min and the absorbance (A) was measured at 517 nm using a spectrophotometer. All samples were performed in triplicates. Ascorbic acid was used as a positive control. The scavenging activity was calculated as a percentage of DPPH decolouration relative to a negative control using the following equation:

Free-radical scavenging activity (%) = A (blank) - A (extract) / A (blank) x 100

Cupric reducing antioxidant capacity (CUPRAC)

The capacity to reduce cupric ions was determined using the CUPRAC assay. The extracts were mixed with 50 µl samples extracted, 50 µl of $CuCl_2$ solution (1.0×10^{-2}M), 50 µl of neocuproine alcoholic solution (7.5×10^{-3}M) and 50 µl NH_4Ac buffer solution. Absorbance against a reagent blank was measured at 450 nm after 30 minutes.

Statistical Analysis

The data obtained in this study are means ± confidence interval of three replicate determinations. Statistical comparisons employed Tukey's test, with $P<0.05$ considered statistically significant. Concentrations yielding 50% inhibition (IC_{50}) value were calculated by interpolation from linear regression analysis. All statistical analyses used SPSS software.

RESULTS AND DISCUSSION

The preliminary phytochemical screening tests for the crude extracts of *A. malacenssis* leaves revealed the presence of alkaloids, terpenoids, flavonoids, steroids, saponins and tannins (Table 1). Some of the secondary metabolites from a single or combined with others in plant extracts are liable for the antioxidant activity [14]. Some phytochemicals have antioxidant activity where it provides protection against damage and the risk of developing chronic disease can be substantially reduced [15].

Table 1: Phytochemical screening of methanol and water extracts of *A. malaccensis*

	WEDL	WEFL	MEDL	MEFL
Steroids	+	+	+	+
Terpernoids	+	+	+	+
Flavonoids	+	+	+	+
Saponins	+	+	+	+
Tannins	+	+	+	+
Alkaloids	+	+	+	+

+ = Present

** WEDL; water extraction of dried leaves, WEFL; water extraction of fresh leaves, MEDL; methanol extraction of dried leaves, MEFL; methanol extraction of fresh leaves.*

The total phenol content is reported in Table 2 as gallic acid equivalents by reference to a standard curve (Y= 0.002x - 0.045 and r^2=0. 996). Among the samples studied in 100 to 1000 µg/ml, WEDL has the highest total phenolic content (181.11 ± 0.61 mg GAE/g) followed by MEDL<MEFL<WEFL. The phenolic contents in these extracts showing the leaf extracts contain the main active compound groups and acts as a type of antioxidants. In previous studies of *Aquilaria* spp., the presence of various polyphenol compounds was reported [16, 17, 18]. Boiling water extracted more polyphenols from dried plant that led to higher total phenols being extracted compared to the methanol extract. For fresh leaf extract, methanol being volatile and organic solvent which able to degrade the cell, whereas in water they are degraded polyphenols in this extract [19].

Table 2: Total phenolic content of methanol and water extracts of *A. malaccensis*

Sample	Total Phenolic Content GAE (mg/g)					
	Concentration (ug/ml)					
	100	200	400	600	800	1000
WEDL	33.87 ± 0.61[a]	55.89 ± 0.36[a]	72.75 ± 1.01[a]	107.18 ± 0.94[a]	142.33 ± 0.47[a]	181.11 ± 0.61[a]
WEFL	19.13 ± 0.94[b]	34.87 ± 1.08[c]	56.22 ± 0.77[d]	85.38 ± 1.89[b]	108.51 ± 0.64[b]	129.79 ± 2.08[b]
MEDL	31.72 ± 1.03[a]	42.66 ± 1.33[b]	68.07 ± 1.69[a]	102.84 ± 0.29[c]	139.98 ± 1.40[a]	176.17 ± 1.16[a]
MEFL	27.71 ± 0.20[c]	39.95 ± 0.56[b]	60.30 ± 1.44[b]	94.74 ± 0.72[c]	138.35 ± 0.40[a]	158.26 ± 0.32[c]

Each value in the tables above were performed in triplicates and represented as Mean ±SEM (n=3). Means with different superscripts in a column are significantly different (P < 0.05).

Meanwhile, the total antioxidant activities of different extracts of this plant are recorded in Table 3. From the results, the total antioxidant activity is the highest in dried plants. Hence, the TAC activity of both extracts was found to be increasing continuously as the concentration increases. The highest TAC activity was observed in WEDL (for 0.1 mg/ml, 155.22; for 0.2 mg/ml, 180.74; for 0.4 mg/ml, 239.57; for 0.8mg/ml, 276.55; for 0.8mg/ml, 353.09 and for 1mg/ml, 398.74 µg/mg equivalent of ascorbic acid). The increase in antioxidant activity may be described by the increase of solvent polarity. Since, high phenol content present in WEDL was significantly correlated with the highest antioxidant compared to others.

Table 3: Total antioxidant activity of methanol and water extracts of *A. malaccensis*

Sample	Total Antioxidant Capacity GAE (mg/g)					
	Concentration (ug/ml)					
	100	200	400	600	800	1000
WEDL	155.22± 0.77[a]	180.74 ± 0.60[a]	239.57 ± 0.23[a]	276.55 ± 0.45[a]	353.09 ± 0.84[a]	398.74 ± 0.66[a]
WEFL	93.87 ± 0.23[c]	102.98 ± 0.74[c]	116.05 ± 0.47[c]	151.76 ± 0.81[c]	172.17 ± 0.39[c]	187.04 ± 0.79[c]
MEDL	138.04 ± 1.20[b]	168.81 ± 0.53[b]	210.56 ± 0.30[b]	244.17 ± 0.74[b]	279.24 ± 0.65[b]	325.48 ± 0.98[b]
MEFL	97.64 ± 0.60[d]	98.33 ± 0.69[d]	123.57 ± 0.28[d]	149.86 ± 0.65[d]	188.56 ± 0.57[d]	230.11 ± 0.32[d]

Each value in the tables above were performed in triplicates and represented as Mean ±SEM (n=3). Means with different superscripts in a column are significantly different (P < 0.05).

In addition, the chromogenic oxidizing reagent of the developed CUPRAC, i.e., bis-(neocuproine) copper (II) chloride (Cu (II)-Nc) reacts with polyphenolic antioxidants and oxidized them into the corresponding quinones and Cu(II)-Nc. Table 4 shows the dose-response curves for the reducing powers of different solvent extracts comparable with that of ascorbic acid (the positive control). The result showed the reducing power of the leaf extract also increased with increasing the concentrations. Therefore, it is highly probable that these extracts may presence of reductones [21]. Compounds by providing reduced power to act as electron donors that may act as primary and secondary antioxidants [22].

Table 4: The Reducing power of methanol and water extracts of *A. malaccensis*

Sample	Cupric Reducing Antioxidant Capacity (nm)					
	Concentration (ug/ml)					
	100	200	400	600	800	1000
AA	1.84 ± 0.04[a]	2.00 ± 0.09[a]	2.36 ± 0.05[a]	2.69 ± 0.06[a]	3.06 ± 0.02[a]	3.51 ± 0.08[a]
WEDL	0.63 ± 0.01[b]	1.08 ± 0.01[b]	1.74 ± 0.04[b]	2.25 ± 0.01[b]	2.92 ± 0.04[a]	3.32 ± 0.01[a]
WEFL	0.35 ± 0.01[d]	0.63 ± 0.03[c]	1.01 ± 0.01[c]	1.51 ± 0.02[d]	1.92 ± 0.10[c]	2.29 ± 0.03[c]
MEDL	0.54 ± 0.01[c]	1.00 ± 0.01[b]	1.67 ± 0.10[b]	2.03 ± 0.02[c]	2.35 ± 0.01[b]	2.86 ± 0.05[b]
MEFL	0.48 ± 0.01[c]	1.01 ± 0.02[b]	1.61 ± 0.11[b]	1.98 ± 0.01[c]	2.25 ± 0.02[b]	2.68 ± 0.04[b]

Each value in the tables above were performed in triplicates and represented as Mean ±SEM (n=3). Means with different superscripts in a column are significantly different (P < 0.05).

Besides, antioxidant activity was assessed by determining the percentage of inhibition of DPPH. The quality of the antioxidants in the extracts was determined by the IC_{50} values which represent the concentration of the sample need to scavenge 50% of the DPPH free radicals. Ascorbic acid was used as a standard for the present investigation. The water and the methanol of dried leaves showed very similar values percentage inhibition of DPPH at a concentration of 1000 µg/ml which is 48.07 ± 0.68 % and 40.76 ± 0.55 % (Table 5). The IC_{50} of extracts is presented as in Table 6. It showed that the lower the IC_{50} value indicated the higher scavenging potential. Their antioxidant activity is affected by phenolic compound on DPPH which able to donate hydrogen atoms to form stable [20].

Table 5: The scavenging effect and IC_{50} activity of methanol and water extracts of *A. malaccensis*

Sample	Percentage activity (%) Concentration (ug/ml)						IC_{50} (ug/mL)
	100	200	400	600	800	1000	
AA	46.29 ± 0.54^{a}	47.32 ± 0.17^{a}	48.30 ± 0.44^{a}	50.78 ± 0.50^{a}	56.05 ± 0.12^{a}	65.05 ± 0.16^{a}	219
WEDL	13.94 ± 0.34^{c}	14.45 ± 0.29^{c}	15.92 ± 0.41^{b}	18.67 ± 0.06^{b}	29.87 ± 0.44^{c}	48.07 ± 0.68^{c}	1091
WEFL	12.38 ± 0.16^{b}	12.84 ± 0.19^{b}	13.60 ± 0.14^{b}	14.85 ± 0.09^{b}	16.36 ± 0.16^{b}	22.77 ± 0.44^{b}	1326
MEDL	10.77 ± 0.46^{c}	12.01 ± 0.35^{c}	14.62 ± 0.58^{b}	17.89 ± 0.47^{b}	24.58 ± 0.15^{e}	40.76 ± 0.55^{e}	1938
MEFL	11.08 ± 0.13^{d}	12.67 ± 0.29^{c}	14.56 ± 0.25^{c}	17.47 ± 0.31^{c}	19.67 ± 0.10^{d}	32.28 ± 0.25^{d}	3793

Each value in the tables above were performed in triplicates and represented as Mean ±SEM (n=3). Means with different superscripts in a column are significantly different (P < 0.05).

Overall, boiling water extraction of dried *A. malaccensis* resulted in higher levels of TPC and activities of antioxidants mainly due to the fact that boiling water could completely activate the degradative enzymes as against the methanol solvent. So, the number of compounds with free hydroxyl groups was increased by exposure the plant materials at high temperature during extraction process [23]. Nevertheless, methanol extract was still found to yield lower recovery of TPC and their antioxidant properties than boiled water in dried plant materials, where polyphenol oxidases have been inactivated. But, methanolic extracts of fresh sample revealed higher activity of both TPC and antioxidant activities compared to boiled water extracts may be due to the methanol was able to denatured the polyphenols oxidases [24].

CONCLUSION

The antioxidant activity correlated with active compounds phytochemicals. Water and methanol extract of dried and fresh leaves showed that it may account for its antioxidant properties in *A. malaccensis*. This study on in vitro antioxidant activities on *A. malaccensis* has the beneficial input of the agarwood plantation as well as pharmaceutical industry to stimulates the use of agarwood leaves as a new value added neutraceutical products. Further, the screening of phenolic compound and the antioxidant activities were found to be very useful tools to provide in depth the characteristic of phenolics that are present in agarwood leaves.

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