Antioxidant Activity Evaluation of Agarwood *Aquilaria malaccensis* Lamk. Leaves Extract Using DPPH, FRAP and ABTS Assays

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ABSTRACT
Indonesia has been known to have large forest areas and rich in biodiversity, which could be the source of primary and secondary metabolites. Along with the technological development and research in medicine, agarwood, which was initially used only for topical body treatments, room fragrances, and religious ceremonies, is started to be investigated for herbal medicines. This study aimed to perform metabolite profiling with spectrophotometry and evaluate the antioxidant activity in agarwood *Aquilaria malaccensis* Lamk using three assays: DPPH, FRAP and ABTS. The mature leaves used in this study were selected by their though leaf structure and dark green color. The leaves were dried and powdered before being extracted by soxhlet with three different solvents polarity i.e. ethanol, ethyl acetate, and *n*-hexane. Metabolite profiling was done by scanning the extracts from 200-800 nm. Antioxidant activities were analyzed using 2,2-diphenyl-1-picryhydrazil (DPPH), ethyl benzotiazolin sulfonate (ABTS) and *Ferric Reducing Antioxidant Power* (FRAP). The result showed that ethanol has the highest yield of extract, followed by ethyl acetate then *n*-hexane with 10.7%, 8.07%, and 5.23%, respectively. Ethanol contained the highest flavonoid compound compared to the others and has the highest antioxidant activity with IC_{50} of 37.22 µg/mL using DPPH assay, 66.33 µg/mL using ABTS and 220.188 ± 1.66 µmol TE/g sample using FRAP assays. Therefore, it can be concluded that ethanol extract of *Aquilaria malaccensis* Lamk. has the highest antioxidant activity and the richest flavonoid compounds compared to the others.

Keywords: *Agarwood, Antioxidant, Aquilaria malaccensis Lamk. Metabolite profiles*

1. INTRODUCTION

Agarwood is one of non-timber forest product that has the usability potential to be developed. Along with the development in science and research, agarwood, which was initially used strictly only for body and room fragrance, and community religious ceremonies, started to be inspected and developed its usability for herbal medicines. According to Nugraha et al. [1], agarwood can treat various diseases, including stress, asthma, rheumatic, inflammation of kidneys, stomachache, and an antibiotic for tuberculosis, treatment of tumors and cancer.

Mega and Swastini [2] stated that agarwood contains anti-free radicals that prevent natural and artificial free radical reactions, also metabolic products in the body. Supported by Hendra et al. [3], it stated that the methanol extract of *Aquilaria malaccensis* Lamk.'s old leaves produces high antioxidant activity with phenols and flavonoids acting as antioxidant compounds. These results were obtained by the test using diphenyl-picryl-hydrazil (DPPH) as the free radical compound. The DPPH assay is the most assays used for antioxidant activity due to its low cost and robustness. However, this assay was reported effective for flavonoid content but not for the other phenolic compounds. This causes a

weakness in assessing the antioxidant activity of samples containing non-flavonoid-derived antioxidants [4].

Aside from DPPH assays, Griffin and Bhagooli [5] mentioned that other assays could be used to determine antioxidant capacity, such as ferric-reducing-antioxidant-potential (FRAP). FRAP is considered a simpler and cheaper assay than other available ones, and it could measure total antioxidant activity. In addition to FRAP, another antioxidant assay (ABTS) has been reported using azino-bis-etilbenzotiazoline sulfonate acid as free radicals to evaluate the antioxidant activity of samples in the aqueous phase [6]. These different assays suggest that the evaluation of the antioxidant activity of an herbal cannot be carried out only with a single assay.

The objective of this study was to evaluate the antioxidant activities of agarwood *A. malaccensis* leaves extracts (from three solvents that differed in polarity; *n*-hexane, ethyl acetate, and ethanol) using three assays DPPH, FRAP, and ABTS and correlate it with the metabolite profile measured using UV/VIS spectrophotometry. Thus, this study provides more information on the development of *A. malaccensis* leaves as an herbal source for natural antioxidants.

2. MATERIAL AND METHODS

2.1. Material

The leaves of agarwood *A. malaccensis* were collected from Karanganyar, Center of Java, Indonesia. The leaves had a dark green color with a tough texture.

2.2. Preparation and Extraction of Agarwood Leaves

After being washed with water, the leaves were wiped with dry tissue and air-dried at room temperature, continued drying using an oven at 50°C until reaching its constant weight. The leaves were powdered with a blender afterward. An amount of 10 g of the leaves powder was extracted with 150 mL of each solvent, which are *n*-hexane (pa. Merck), ethyl acetate (pa. Merck), and ethanol (pa. Merck) using a soxhlet extractor. The crude extracts were evaporated to dryness by continuously being subjected to fans for 2 – 4 days. The dark brown sticky extracts were weighed and stored at 4 °C for further use.

2.3. UV/Vis Scanning

Each crude extract was dissolved in its solvent and then diluted to 125 µg/mL. The sample solution was scanned with UV/Vis Spectrophotometer (Genesys) with a wavelength range of 200 – 800 nm and by 5 nm interval. The absorbances were recorded and analyzed further for extract fingerprinting.

2.4. DPPH Test

Antioxidant activity by DPPH test was conducted according to the methods of Handayani et. al. [7] with modification on the sample concentration and the mixed solution's proportion, which is 1 mL of DPPH added on 1 mL of sample. 1 mL of 0.1 mM DPPH in methanol was added to 1 mL of the extract solution and then mixed with Vortex (Genie-2) until homogeneous. The samples were incubated at room temperature for 30 minutes in the dark. A blank solution was prepared with 1 mL of methanol added with 1 mL of 0.1 mM DPPH. The absorbance of the sample was measured at a wavelength of 517 nm with a UV/VIS Thermo Spectrophotometer (Genesys). Ascorbic acid was used as a positive control. The antioxidant activity was determined by subtracting the absorbance of the blank by the absorbance of the sample, then divided by the absorbance of the blank.

2.5 ABTS Test

Antioxidant activity by ABTS test was conducted according to Setiawan et al. [8] with modification on the usage of $K_2S_2O_8$ 3,5 mg, which dissolved in 5 mL of aquabidest, 5 mL of ABTS reagent and mixed with 3.5 mg of $K_2S_2O_8$ solution. Thus 15 mL of the final volume was reached. The mixture was poured into a dark bottle containing 5 mL of ABTS and then 5 mL of aquabidest was added. The reagent was incubated at room temperature for approximately 12 – 16 hours. The volume was made up with aquabidest up to 15 mL after incubation. An amount of 67 µL of the sample and 133 µL of ABTS was placed into 96 microplates well then incubated for 15 minutes in the dark. A blank solution was prepared with 67 µL of aquabidest added by 133 µL ABTS. The absorbance of the sample was measured at the wavelength of 495 nm with ELx800 Absorbance Microplate Reader (BioTek). Trolox was used as a positive control. The antioxidant activity was determined by subtracting the absorbance of the blank by the absorbance of the sample, divided by the absorbance of the blank.

2.6. FRAP Test

Antioxidant activity by FRAP test was conducted according to Selawa et al. [9] with modification on the wavelength used due to optimization with microplate reader used in this test. FRAP reagent was made with 2.5 mL of TPTZ, 2.5 mL of FeCl, and 25 mL of acetate buffer were dissolved and made up with aquabidest 100 mL in exact. $FeSO_4.7H_2O$ and trolox were used as standard. The sample was dissolved with its solvent at 500 µg/mL. The sample and standard were poured into a microplate reader

as much as 50 µL, and then 150 µL of FRAP reagent was added. The absorbance of the sample and standard was measured at a wavelength of 495 nm with a microplate reader.

2.7 Data Analysis

Triplicates were used for the entire test. UV/Vis scanning data were analyzed with Metaboanalyst online software. DPPH and ABTS test data were analyzed as IC_{50} by calculating its regression aquations in Microsoft Excel 2016. FRAP test data was attempted to be included in the absorbance range of the standard curve $FeSO_4.7H_2O$ and trolox concentration series and expressed in units of µmol TE/g sample.

3. RESULTS AND DISCUSSION

3.1. Agarwood Leaves Extraction

Soxhletation was chosen as the extraction method in this study because of its advantages which minimized damage on compounds in the extract due to the low boiling point of the solvent, and with soxhletation, more compounds can be extracted only with a small amount of solvent due to the circulation of immersion took more place and faster [10]. The type of solvents used on extraction affected the amount of extracts obtained. Based on the study by Hendra et al. [3], the polar solvent is a better solvent for extraction of *A. malaccensis* because the yield extract was the highest than other solvents such as chloroform, which is a non polar solvent.

Table 1. Yields of agarwood leaves extraction using three solvents

Extracts	Weight (g)		Yield (%)
	Dry simplicia	Thick extract	
n-Hexane	30	1.57	5.23
Ethyl acetate	30	2.42	8.07
Ethanol	30	3.21	10.7

According to Kristanti [11], the polarity of solvents used for extraction determines the type of pigment extracted. This statement fits well with this study conducted, where the ethanol extract gave dark green color, ethyl acetate extract is slightly lighter than ethanol's, and *n*-hexane gave brownish-green color. Based on a study of Putra et al. [12], the yield value shows how many components of the sample were extracted by the solvent used. From Table 1, we conclude that *A. malaccensis* have more polar phytoconstituents than non-polar, which can be interpreted as well that polar solvents are better at extracting compounds in *A. malaccensis*. These results' give the same result as Hendra et al. [3], which reported that methanol extract gave the highest yield compared to chloroform and water extracts.

3.2. UV/Vis Scanning

UV/Vis scanning was used to characterize the phytoconstituents of the extracts. This is based on the fact that each metabolite has a specific maximum absorbance on a particular wavelength. Thus, each extract may have specific UV/Vis absorbance spectra. According to De Caro [13], this method can be used if the wavelength reference to detect the compound is already known.

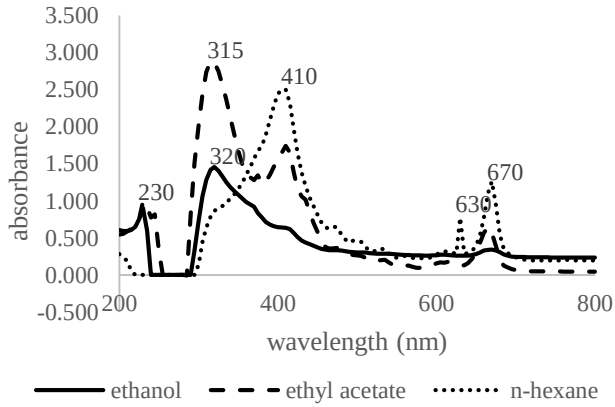

Figure 1. UV/Vis scanning spectrogram of ethanol, ethyl acetate and *n*-hexane extract of Aquilaria malaccensis leaves extracts.

Figure 1 shows that all of the extracts have different metabolites. This statement is concluded by the difference of each extracts spectrogram's peak. Based on data by Markham [14], flavones, flavonols (3-OH substituted), isoflavones, flavanones, and flavanonols were detected in the ethanol extract at the peak of 320 nm. We also detected almost the same compounds in ethyl acetate except for flavonols (3-OH substituted). Meanwhile, in *n*-hexane extract, only auron was detected. A study by Bunghez et al. [15] mentioned that quinone was detected at a 400 – 412 nm wavelength from methanol extract of basil and thyme. At a wavelength of 220 – 280 nm and 330 – 420 nm in cloves, cinnamon, rosemary and sage, phenolic acids and their derivatives were detected. Oregano has the highest total flavonoids at a wavelength of about 330 nm, while at peak 630 nm and 670 nm, according to Zain et al. [16] is known not to be a range of antioxidant compounds, but rather the wavelength of porphyrin emission.

The data absorbance dataset was analyzed further with PCA (Principal Component Analysis) using Metaboanalyst software. The score plot of the extracts showed that three extracts are well separated from each other (Figure 2).

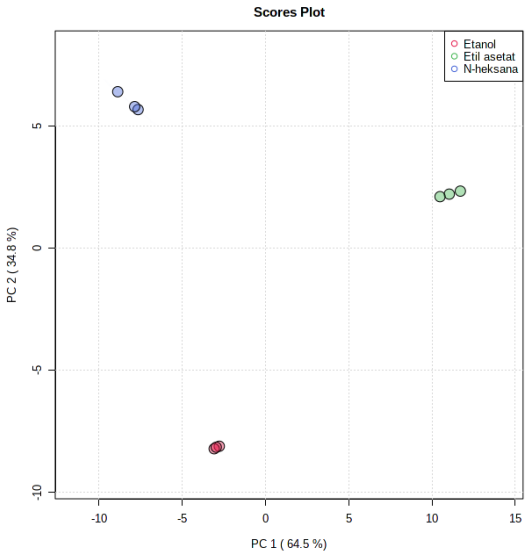

Figure 2. PCA score plot of PCA analysis of UV/Vis scanning of *Aquilaria malaccensis* leaves extracts i.e. ethanol, ethyl acetate and *n*-hexane.

Figure 2 shows that each extract was in a different quadrant (Q). Quadrant was made up of two Principal Components (PC), which are new variables those form linearity and are used to differentiate each sample. The values of total PC of a score plot described the variances involved in the model. Good score plots are those that have a sum of Q2 PC at least 50%. In this study, the sum of Q2 from PC1 and PC2 was 99.3%, predicting that the score plot is a good model. From the score plots shown in Figure 2, all the extracts were separated well, where the ethyl acetate extract lied on the positive side of PC1 and PC2, the ethanol extract lied on the negative side PC1 and PC2, while the *n*-hexane extract lied on the negative side of PC1 and the positive side of PC2. Judging from PC1, ethyl acetate showed the most significant difference or the most different among all the extracts meanwhile ethanol and *n*-hexane plotted on the negative side.

To determine which absorbance at the specific wavelength that important for each extract, one can check the loading plot (data not shown). From the loading plot, extreme variables for each extract were selected and checked. Ethanol extract is characterized by 545 nm, ethyl acetate by 355 nm, while *n*-hexane is 485 nm. Based on the study of Lin and Harnly [17], at the wavelength range of 350 – 352 nm may correspond for several phenolic compounds including quercetin, isorhamnetin, rutin, luoteolin, while at peak 358 nm indicated the presence of myricetin. At wavelength 520 nm, the presence of polymeric pigments and anthocyanins was indicated, which were relatively high [18]. Based on a database by Taniguchi and Lindsey [19], 480 nm- 485 nm is known to be the wavelength range for two dyes, azo dyes and cyanine dyes.

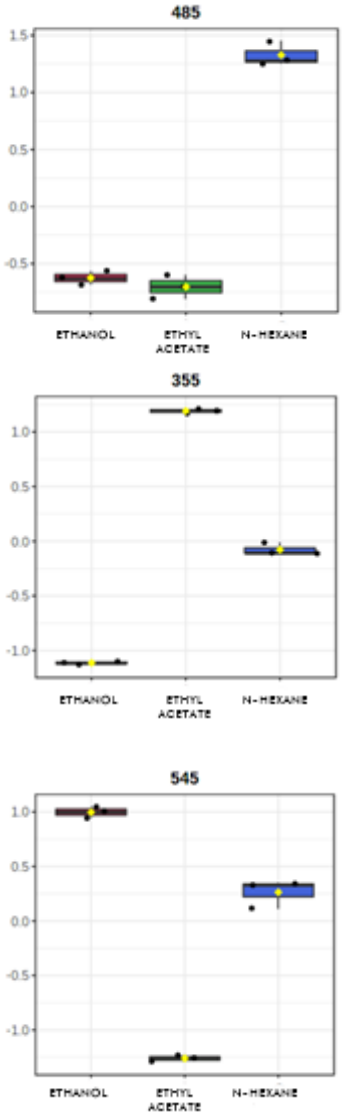

Figure 3. The specific wavelength that contributes to the separation of different leaves extract of *Aquilaria malaccensis.*

3.3. Antioxidant Activity of Agarwood Leaves

3.3.1. Antioxidant activity measured with DPPH test

The result showed that ethanol extract of agarwood had the highest antioxidant activity compared to ethyl acetate and *n*-hexane extracts. As Molyneux [20] stated, the increased sample concentration used, the more free radical of DPPH was reduced by antioxidant compounds contained in the sample due to the presence of H donor of sample to DPPH molecule. This result is also in line with the study conducted by Rashid et al. [21], where the ethanol extract had higher antioxidants activity than *n*-hexane. (Figure 4). This statement is also supported by

the yield of ethanol which had the highest percentage among other extracts.

The ethanol extract of *A. malaccensis* leaves showed the highest antioxidant activity with an IC50 value of 37.22 µg/mL compared to the others. Both ethyl acetate and *n*-hexane extracts had IC_{50} > 150, based on the grouping by Jun et al. [22]. This meant that the antioxidant activity that occurred could be categorized as moderate (Table 2.). Previous research by Batubara et al. [23] stated that glycosides, steroids/triterpenoids, flavonoids and tannin were found in ethanol extract, where these compounds are associated with antioxidant activity in plants.

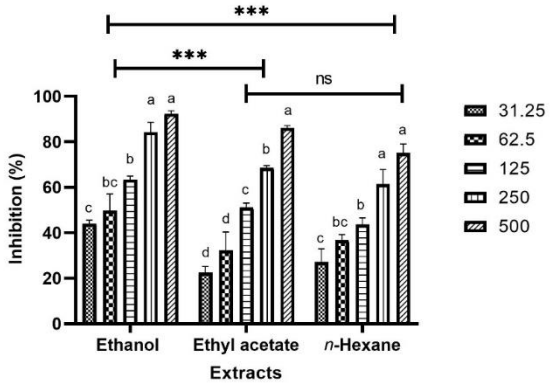

Figure 4. Free radical scavenging activity of *Aquilaria malaccensis* Lamk leaves extracts with DPPH assay. *** = $p < 0.0001$ significant different between extracts. Ns: not significantly different. The different letters above histogram showed differences within each extract analyzed using ANOVA $p< 0.05$.

Table 2. IC_{50} value of agarwood leaves extracts and vitamin C with DPPH test

Sample	Extract	IC_{50} (µg/mL)	Antioxidant activity category
Vitamin C	-	15.04	very strong
Agarwood leaves	*N*-hexane	206.25	moderate
	Ethyl acetate	177.41	moderate
	Ethanol	37.22*	very strong

*= extract with lowest IC_{50} value

3.3.2. Antioxidant activity measured with ABTS assay

Similar to the result of the DPPH assay, ethanol extract of *A. malaccensis* leaves also had the highest antioxidant activity among the three extracts used. The indicator of the ABTS test was the fading of blue color in the ABTS reagent, which indicated protons were received from the sample. So the free radical compounds in the reagent became stable [24]. ABTS assay has never been performed on *A. malaccensis*. However, a study conducted by Wang et al. [25] stated that polar extract of Agarwood *A. crassna* leaves gave a positive response to ABTS reagent.

Ethanol extract from Agarwood leaves showed IC_{50} value of 66.33 µg/mL (Table 3.). This value was the lowest compared to the other two extracts. The % inhibition of ethanol is also significantly higher than the other extracts (Figure 4.). IC_{50} is inversely proportional to antioxidant activity, so the lower IC_{50} value, the higher antioxidant's activity. These results were following the result obtained by the DPPH assay where the ethanol extract belongs to the strong antioxidant activity, but it can be said that the antioxidant compounds in the *A. malaccensis* are more responsive when tested with DPPH, it can be seen from the IC_{50} value obtained by DPPH test compared to ABTS test. DPPH and ABTS basically have the sample principle, which is the capacity of free radical absorbance produced by specific organic molecules. Flavonoids are included in the intermediate antioxidants, acting as hydrophilic and lipophilic antioxidants [26]. The IC_{50} value of ABTS assay is supposedly lower than DPPH due to the limited ability of DPPH where it correlated positively only on the activity of lipophilic compounds while ABTS positively correlates with oxygen radical absorbance capacity (ORAC), phenol content, and flavonoid content. This result possibly happened because of the high sensitivity of the ABTS reagent, it depends on the incubation time and the correlation between the number of samples and ABTS concentration, so if there's a slight error in the workmanship, it would change the final result [27, 28].

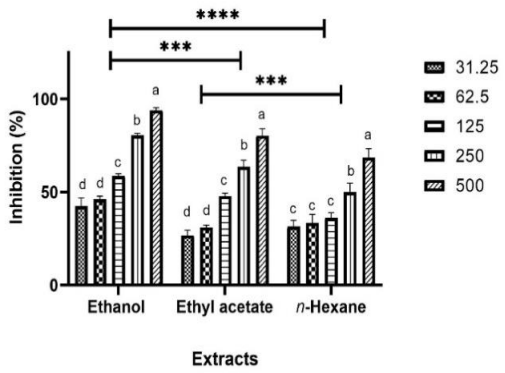

Figure 5. Free radical scavenging activity of *Aquilaria malaccensis* Lamk leaves extracts measured using ABTS assay. *** = $p < 0.0001$ significant different between extracts. The different letters above the histogram showed differences within each extract analyzed using ANOVA $p< 0.05$.

Table 3. IC_{50} value of agarwood leaves extracts and trolox with ABTS test

Sample	Extract	IC_{50} (µg/mL)	Antioxidant Activity Category
Trolox	-	18.01	very strong
Agarwood leaves	*n*-Hexane	267.86	low
	Etil acetate	194.46	moderate
	Ethanol	66.33*	strong

*= extract with lowest IC_{50} value

3.3.3. Antioxidant activity measured with FRAP assay

In this test, standard solutions were used to obtain standard curves, which were $FeSO_4.7H_2O$ and trolox. Ethanol extract showed the highest antioxidant activity compared to other extracts. These results are following the research by Triyasmono et al. [29], wherein the ethanol extract of agarwood leaves species *A. malaccensis* showed the highest antioxidant activity (Table 4.).

Table 4. Antioxidant activity of *Aquilaria malaccensis* Lamk leaves extracts with FRAP assay

Sample	Average Fe^{2+} Value in ET	Concentration (g/L)	Antioxidant Activity (µmol TE/g sample)
n-Hexane	0.247	0,5	0.494 ± 0.30
Ethyl acetate	55.170	0,5	110.341± 2.92
Ethanol	110.094	0,5	220.188±1.66

Based on this study, it can be concluded that the highest antioxidant activity found in the ethanol extract with value 220.188 ± 1.66 µmol TE/g sample, and the lowest antioxidant activity was in *n*-hexane with 0.494 ± 0.3 µmol TE/g sample. These results are comparable with the results of the DPPH and ABTS tests, but only the FRAP test wasn't indicated by the IC_{50} value. FRAP test is more sensitive to compounds that can reduce Fe and due to its electron transfer-based mechanism. FRAP test cannot detect antioxidant compounds based on hydrogen transfer, such as ABTS and DPPH. Thus, FRAP test is only used as complementary data [27]. Based on the UV/Vis scanning result, ethanol extract has the highest content of phenolic compounds, especially flavonoids. This may explain the positive correlation and the highest antioxidant activity of ethanol extract. A study by Arif et al. [30] showed a similar result; this is because of FRAP test's mechanism is the reduced of reagent due to the presence of an electron donor. In the ethyl acetate extract, the value of antioxidant activity was only half of ethanol extract. This is caused by the polarity of ethyl acetate, which is categorized as semi-polar. While in *n*-hexane, the antioxidant value was minimal (almost close to zero) due to its non-polarity. Moreover, in the spectrogram peak on UV/Vis scanning data, the *n*-hexane extract did not contain any phenolic compound.

Ethanol, ethyl acetate, and *n*-hexane extracts of *A. malaccensis* showed an antioxidant activity when evaluated by three different type assays i.e., DPPH, ABTS and FRAP. The ethanol extract had the highest antioxidant activity for all assays. This high antioxidant activity of the ethanol extract may correlate with the phenolic contents, as it is shown in the spectrogram of UV/Vis scanning.

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