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ANTIOXIDANT ACTIVITY OF ETHYL ACETATE EXTRACT OF NUTMEG *(Myristica fragrans* Houtt) STEM BARK

Ilham Maulana, Binawati Ginting*, Nurdin and Saiful Fakri

Department of Chemistry, Faculty of Mathematics and Natural Sciences, Syiah Kuala University, Banda Aceh, 23111, Indonesia
*Corresponding E-mail: binawati@unsyiah.ac.id

Abstract. Antioxidant activity test for the extract of ethyl acetate nutmeg stem bark (*Myristica fragrans* Houtt) was carried out using *2,2-diphenyl-1picrylhydrazil* (DPPH). The test results of antioxidant activity of ethyl acetate nutmeg stem bark extract with DPPH method at a concentration of 25 ppm, 50 ppm and 100 ppm obtained IC_{50} value = 68.14 ppm with IC_{50} value of vitamin C (as positive control) 3.657 ppm. The results of column chromatographic separation of ethyl acetate nutmeg stem bark extract obtained 5 combined fractions namely MFEK 1, MFEK 2, MFEK 3, MFEK 4 and MFEK 5. The test results of the fraction antioxidant activity in a good category were the MFEK 1 fraction. The MFEK 2 fraction to MFEK 5 showed an antioxidant activity lower than compared to the ethyl acetate extract. This shows that the ethyl acetate nutmeg stem bark extract has a better inhibitory activity, because there are several active compounds contained in the extract which can inhibit free radicals, so the IC_{50} value of the extract is better than the fractions.

Keywords: Nutmeg stem bark (*Myristica fragrans*Houtt), Ethyl Acetate Extract, DPPH, Antioksidant Activity

I. INTRODUCTION

Antioxidant activity was one of the targets of the study in this research that functioned as a natural ingredient in medicines. Public attention about the side effects of synthetic antioxidants that make natural antioxidants an alternative. The use of synthetic antioxidants is limited because the results of research have been conducted to show that synthetic antioxidants such as BHT (Butylated Hydroxy Toluene) can be toxic to experimental animals and carcinogenic [1]. Nutmeg is one of the valuable herbs that have been used for centuries throughout the world. Apart from its use in food and beverage additives, nutmeg has also been used in traditional medicine for stomach and kidney disorders. Nutmeg is also a rich source of essential oils, triterpenes, and various types of phenolic compounds. In addition to antioxidants, antimicrobial and central nervous system nutmeg effects have also been widely reported. Bioactivity The content of secondary metabolites of nutmeg plants, especially parts of fruit, has been widely investigated, as reported in the ethanol extract of nutmeg seeds, where this extract shows anticancer activity [2], anti-inflammatory and inhibitory activity of NO (Nitric Oxide) production [3]. NO inhibition activity was also shown by neolignan compounds from the chloroform extract [4] and methanol extract [5], whereas the methanol extract of pala mace contains neolignan compounds which show anticancer activity [6]. Bioactivity of other parts of nutmeg plants such as leaves, stems and roots still has not been studied optimally because the use of nutmeg plants is still focused on the fruit only. Several studies on secondary metabolites in nutmeg leaves have been reported, among others Helen *et al*., 2012 [7] reported that nutmeg leaves oil contains 20 types of compound components with the main composition namely β-pinene 22.69%, and β-tujen 13,93% showed antimicrobial and anticancer activity. Phytochemical test of methanol nutmeg leaves extract have been reported to contain alkaloid compounds, flavonoids, terpenoids and tannins, while ethyl acetate extracts contain flavonoids and have antifungal activity against *Candida albicans* [8]. The ethyl acetate nutmeg leaves extract also reported has antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, while the methanol extract of nutmeg leaves had a smaller activity [9]. In addition, efficacy of nutmeg plants is used for antioxidant activity as reported by Ref. [10],[11],[12],[13], [14],[15],[16] and [17]. Antioxidants are compounds that can inhibit

oxidative damage to biological macromolecules due to oxidation of various free radicals or commonly called *Reactive Oxygen Species* (ROS), where the main character of antioxidant compounds is their ability to capture and stabilize free radicals [18]. Tested antioxidant and anticancer activity on MCF-7 cells from nutmeg stem bark, the results of the study reported that there was a strong antioxidant activity with an IC_{50} value of 63.755 ppm and an anticancer activity of IC_{50} 22.62 ppm [19]. Based on the description above we will test the antioxidant activity of the nutmeg stem bark extract (*Myristica fragrans* Hout). Nutmeg stem bark samples were taken from Paya Peulumat, East Labuhan Haji sub-district, South Aceh Regency.

II. METHODOLOGY

The equipment used in this study are, blender, erlenmeyer, measuring cup, measuring flask, beaker, separating funnel, analytical scale, test tube, tube rack, maceration device, vacuum device evaporator, oven, distillation equipment, drop pipette, micro pipette. For instruments used in this research is UV visible (Shimadzu UV-160A Model). Materials used in this study are: ethyl acetate, technical methanol, Liberman-Bourchard reagent (glacial acetic acid-H_2SO_4(P)), Mayer reagent (potassium tetraiodo mercuric), Dragendorf reagent ($Bi(NO_3)_3$) and Wagner reagent (I_2 in Ki). Thematerials used for the antioxidant test are methanol p.a, DPPH powder and vitamin C.

Plant Material

Sample used in this study are nutmeg stem bark (*Myristica fragrans* Hout) obtained from Paya Peulumat Village, East Labuhan Haji sub-district, South Aceh district.

Extraction of Nutmeg Stem Bark

Peeled nutmeg stem bark was mashed, weighed as much as 5 kg then macerated with methanol solvent for 24 h, then filtrate and residue were filtered, then the filtrate obtained was evaporated using a rotary evaporator and then methanol extract and solvent were filtered. The methanol extract was partitioned with *n*-hexane to obtain methanol and *n*-hexane layers. The methanol layer was evaporated again using a rotary evaporator to obtain methanol and residual extracts. The methanol extract obtained was extracted with ethyl acetate and then filtered again to produce methanol extract and ethyl acetate extract. The ethyl acetate extract obtained was dissolved in methanol and added HCl 2N then heated for 1 hour, then the mixture was partitioned with chloroform, the chloroform fraction was evaporated to obtain total phenolic. After that, the total phenolic obtained was partially isolated and purified and partially tested for its antioxidant activity.

Antioxidant Test

Preparation of DPPH 0,4 mM Solution

DPPH powder (BM 394.32 g/mol) was weighed as much as 7.9 mg, then dissolved with methanol in a 50 mL measuring flask, closed then homogenized. The solution is stored in a dark bottle and always made a new one for each will be used.

Preparation of variations nutmeg extract and Vitamin C

To make variations in the concentration of methanol nutmeg leaves extract, a mother liquor of 500 ppm is made by dissolving each extract as much as 5 mg into ethanol until the volume reaches 10 mL. Furthermore, from the mother liquor, variations in solution concentrations of 25 ppm, 50 ppm and 100 ppm were made. Variations in the concentration of test antioxidant activity in the extract are shown in Table 1

Table 1 Various concentrations of test antioxidant activity to extracts

Consentration (ppm)	Main solution taken (µL)	DPPH solution (mL)	Information
25	250	1	Volume be apponinted to 5 mL
50	500	1	
100	1000	1	

As a comparison, the antioxidant activity of vitamin C was tested, because vitamin C is a compound that has a very high antioxidant activity. The main vitamin C solution is made by dissolving 3 mg of vitamin C dissolved with methanol until the volume is exactly 5 mL. Then diluted to 3 ppm, 6 ppm, 9 ppm, 12 ppm and 15 ppm. Then it was homogenized with vortex mixer and incubated for 30 min at 37°C, its absorption was measured at a wavelength of 517 nm. Various concentrations of antioxidant activity in vitamin C are shown in Table 2.

Table 2 Various concentration of the antioxidant activity test in vitamin C

Concen-tration (ppm)	Solvent used (µ L)	DPPH solution (mL)	Information
3	25	1	Volume be apponinted to 5 mL
6	50	1	
9	75	1	
12	100	1	
15	125	1	

Preparation of Blank solution

1 mL of 0.4 mM DPPH solution and adjusted its volume to 5 mL with methanol in a test tube (which is covered with aluminum foil), then homogenized with a vortex mixer and incubated for 30 min at 37°C. Then the absorption was measured at a wavelength of 517 nm using a UV-Vis instrument.

Antioxidant test of ethyl acetate extract of nutmeg stem bark and Vitamin C

Ethyl acetate extract of nutmeg stem bark at concentration of 25 ppm 250 µL, concentration of 50 ppm 500 µL and concentration of 100 ppm 1000 µL, each added with DPPH solution of 0.4 mM as 1 mL and the volume was adjusted to 5 mL with methanol and container covered with aluminum foil. Subsequently homogenized using a vortex mixer and incubated for 30 min at 37°C. Then read the absorption a λ = 517 nm [20].

Calculation Method of IC_{50} Value

IC_{50} value is the antioxidant concentration in ppm (µg/mL) which can inhibit 50% of free radicals. The IC_{50} value is obtained from the intersection of the line between 50% of the inhibition and the axis of concentration, then entered in the equation Y = a + bX where Y = 50 and the value of X indicates IC_{50}. The percentage of inhibition is calculated using the following formula:

$$Inhibition = \frac{blank\ absorption}{sample\ absorption}\ x\ 100 \quad [1]$$

III. RESULTS AND DISCUSSION

Extraction of nutmeg stem bark

Extraction of secondary metabolites of nutmeg stem bark is carried out gradually. The maceration process of nutmeg stem bark samples was carried out by soaking for 24 h using methanol solvent. Soaking is carried out until a clear filtrate is obtained. Methanol filtrate was concentrated by evaporation using a rotary evaporator to obtain methanol extract. The concentrated methanol extract obtained was partitioned with petroleum ether to remove chlorophyll and fat. The methanol layer which has been separated by a petroleum ether layer is partitioned with n-hexane so that *n*-hexane extract is obtained. The methanol layer that has been evaporated is extracted with ethyl acetate to obtain insoluble parts and soluble parts with ethyl acetate. The part which is dissolved with ethyl acetate then tested for phytochemical content and isolated and tested for its antioxidant activity.

Table 3 The results of extraction nutmeg stem bark *(M. Fragrans).*

Solvent Extract	Sample Weight (g)	Weight Of Extract (g)	Yield (%)
Methanol	500.8	60.52	12.08
Ethyl Acetate		11.25	2.25
n-Hexane		28.31	5.65

(% Yield of the extract = (extract weight/sample weight) × 100)

Isolation results obtained were separated from phenolic compounds from the ethyl acetate extract of nutmeg stem bark by using gravity column chromatography with its stationary phase using silica gel G-60 GF_{254} and for the mobile phase using eluent n-hexane and ethyl acetate in elution gradient. Then each fraction obtained was analyzed using Thin Layer Chromatography (TLC). When the analysis process uses TLC fractions that have the same stain pattern combined. TLC chromatogram from a combination of fractions there are combined 5 fractions called Myristica Fragrans Ethyl Acetate Stem Skin (MFEK) 1-5.

Phytochemical test

Content of secondary metabolites in the sample was phytochemically tested to ethyl acetate extract of nutmeg stem bark. The tests carried out were tests of alkaloids, terpenoids, steroids, saponins and flavonoids. The presence of alkaloids can be tested with three reagents namely Dragendorf, Mayer and Wagner. The alkaloids if reacted by Dragendorf will form a red color. Mayer will produce white deposits, Wagner will produce chocolate deposits. The phytochemical test of steroid and terpenoid compounds can be tested using Liebermann-Burchard reagent. The change in color to red after reacting with Liebermann-Burchard reagent shows the presence of terpenoids and changes in color to green or blue indicate the presence of steroid compounds. Ethyl acetate extract from nutmeg stem bark showed that it contained flavonoids and phenolic compounds which were tested by means of residues extracted with ethanol then added HCl and Mg powder to produce a pink or purple color which showed the presence of flavonoids [21]. The results of the phytochemical test of fresh samples and ethyl acetate extracts of nutmeg stem bark are shown in Table 4. The table shows the results of phytochemical test on fresh samples of nutmeg stem bark contain secondary metabolites of alkaloid, terpenoid, phenolic and flavonoid groups, whereas ethyl acetate extract of nutmeg stem bark contains flavonoids and phenolic compounds.

Table 4 The results of nutmeg bark phytochemical test

Type metabolites secondary	Fresh samples bark	Ethyl acetate stem bark extract
Alkaloids	-	-
-Dragendorf	+	--
-Wagner	+	--
-Meyer	+	--
Steroids	-	--
Terpenoid	+	--
Flavonoids	+	+
Saponin	-	--
Phenolic	+	+
Alkaloids	-	-

Description: (+) shows positive results and (-) negative results

Antioxidant activity test of the ethyl acetate extract of nutmeg stem bark

The testing of antioxidant activity on ethyl acetate extract of nutmeg stem bark using DPPH. The antioxidant activity test was carried out by varying the extract concentration by 25 ppm, 50 ppm and 100 ppm. As a positive control, vitamin C is used with variations in concentrations of 3 ppm, 6 ppm, 9 ppm, 12 ppm and 15 ppm. Antioxidant activity can be determined based on IC_{50} (Inhibition Concentration) value.

Table 5 The test results of the antioxidant activity of ethyl acetate extract of nutmeg stem bark

Sample	Abs. Control (DPPH)	Concen-tration (ppm)	I (%)	IC_{50} (ppm)
Extract stem bark	0.927	25	0.645	68.14
		50	0.553	
		100	0.321	
MFEK 1	0.927	25	42.50	47.39
		50	52.54	
		100	64.19	
MFEK 2	0.927	25	20.82	107.55
		50	34.74	
		100	46.39	
MFEK 3	0.927	25	28.37	77.81
		50	42.83	
		100	57.17	
MFEK 4	0.927	25	27.29	70.71
		50	38.30	
		100	65.37	
MFEK 5	0.927	25	15.21	116.96
		50	27.72	
		100	43.15	

Note : Abs = Absorbance
I= Inhibition

The test results of the antioxidant activity of ethyl acetate extract of nutmeg stem bark are shown in Table 5 and the correlation of the % inhibition curve to the concentration of ethyl acetate extract of nutmeg bark is shown in Figure 1. Based on Table 5 it is known that IC_{50} values of the five fractions have varied value categories, from good to unfavorable categories. The range of IC_{50} value categories is based on those reported by [22].

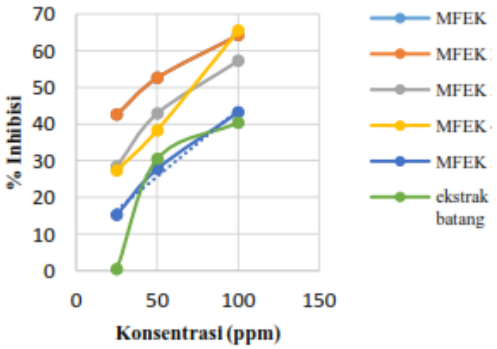

Figure 1 The correlation of % inhibition vs the concentration of ethyl acetate extract of nutmeg stem bark and its fractions

The Fraction category with good antioxidant activity is the MFEK 1 fraction with IC_{50} value of 47.39 ppm, while for the antioxidant activity that not good is in the MFEK 4 fraction, MFEK 3, MFEK 2 and MFEK 5 with IC_{50} values of 70, respectively. 71 ppm, 77.81 ppm, 107.55 ppm and 116.96 ppm. Based on IC_{50} values, each combined fraction of MFEK 2 to MFEK 5 is greater than the test results of antioxidant activity in MFEK extract which has an IC_{50} value of 68.14 ppm. This shows that the MFEK extract has a better inhibitory activity presumably due to the presence of several active compounds contained in the extract that can synergize in inhibiting free radicals, so that the IC_{50} value of the extract is better than the obtained fraction. The following can be seen in Figure 1, the correlation curve of the % inhibition on the concentration of ethyl acetate extract of nutmeg stem bark and its fractions.

Tabel 6 Faction Regression Equation of MFEK 1 to MFEK 5

Sample	Regression Equations	IC50 (ppm)
MFEK 1	y = 0.2811x + 36.677	47.39
MFEK 2	y = 0.3255x + 14.995	107.55
MFEK 3	y = 0.3702x + 21.197	77.81
MFEK 4	y = 0.5126x + 13.754	70.71
MFEK 5	y = 0.3634x + 7.4973	116.96

The IC_{50} value indicates the smallest concentration of a compound can inhibit 50% of free radicals. The IC_{50} value also shows the strength of a compound as an antioxidant. Based on Table 5 and Figure 1 shows the antioxidant activity of the nutmeg stem bark ethyl acetate extract was categorized at a good level with IC_{50} = 68.14 ppm, while the fractions were categorized at an unfavorable level except

for the MFEK 1 fraction with IC_{50} value = 47.39 ppm.

The combined fraction of MFEK 1 to MFEK 5 was tested to determine the content of phenolic compounds. The MFEK 3 fraction is known to contain phenolic compounds as evidenced by the formation of blackish blue stains after dipping with $FeCl_3$. The MFEK 3 fraction combination was re-purified with column recromatography obtained by 3 combined fractions, namely MFEK 3.1, MFEK 3.2 and MFEK 3.3. Based on the results of column chromatography in the MFEK 3.2 fraction, TLC chromatogram analysis showed a separate stain pattern and the potential to be purified, but the stain pattern of MFEK 3.2 fraction was seen not singly with the presence of tail stains so it needed to be recrystallized. The results of recrystallization of the MFEK 3.2 fraction resulted in pure isolates analyzed by KLT with a single stain pattern with Rf 0.42. Figure 2 shows the TLC chromatogram of pure isolate recrystallized from MFEK fraction 3.2

Figure 2 (a) Chromatogram of pure isolate TLC seen at UV □=254 nm (b) Pure isolate after dipping with $FeCl_3$ formed phenolic blue (+) stain

Based on the TLC chromatogram analysis, the pure isolates obtained were phenolic compounds, this was evidenced by the formation of blackish blue stains after dipping with FeCl3. Pure isolates were then tested for antioxidant activity with IC_{50} values = 41.79 ppm. Antioxidant activity of pure isolates MFEK 3.2 is stronger than the fraction.

CONCLUSION

Ethyl acetate extract of nutmeg stem bark has a strong antioxidant activity with IC_{50} values of 64.14 ppm. Phytochemical test result of fresh samples of nutmeg stem bark contains secondary metabolites of alkaloids, terpenoids, and flavonoids, while ethyl acetate extracts of nutmeg stem bark contain flavonoids. MFEK fraction has a better inhibitory activity presumably because the presence of several active compounds in the fraction can synergize in inhibiting free radicals, so the IC_{50} value of the extract is stronger than the extract obtained. MFEK 3.2 pure isolates have antioxidant activity that stronger than fractions with IC_{50} value = 41.79 ppm.

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