Antioxidant Assay and Total Flavonoid Determination of Ethanolic Extract of Walnut (*Canarium indicum* L.) Leaves and Its Fractions

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ABSTRACT

Antioxidants are compounds that inhibit free radicals. Many plants have antioxidant activity because they contain antioxidant compounds such as flavonoids. This study aimed to evaluate the antioxidant activity, phytochemical contents, total flavonoid content and to establish the relationship of total flavonoid content and antioxidant activity of extract and fractions of walnut (*Canarium indicum* L.) leaves. Antioxidant activity of ethanolic extract, n-hexane fraction, ethyl acetate fraction, and ethanolic-water fraction of walnut leaves was examined by using the DPPH method and determination of total flavonoid content was determined spectrophotometrically. The results showed that antioxidant activity of ethanolic extract, n-hexane fraction, ethyl acetate fraction, and ethanolic-water fraction based were 25.294 ± 0.055; 175.245 ± 0.4999; 20.135 ± 0.009; and 28.806 ± 0.0424 µg/ml, respectively. The phytochemical content of ethanol extract of walnut leaves are saponins, flavonoids, tannins, and polyphenols. The total flavonoid content of ethanolic extract, n-hexane fraction, ethyl acetate fraction and ethanolic-water fraction were 2.624 ± 0.012; 0.499 ± 0.023; 3.846 ± 0.006; and 1.596 ± 0.006 gram quercetin equivalent/gram extract, respectively. Correlation between antioxidant activity and total flavonoid content revealed that 63.2 % of antioxidant activity was influenced by the presence of flavonoid compounds.

Keywords: *Canarium indicum* L. leaves, extract, fractions, antioxidants, total flavonoid.

INTRODUCTION

Free radicals are molecules or fragments of molecules containing one or more unpaired electrons in their atomic orbitals. These free radicals are dangerous because they are very reactive, looking for their electron pairs to achieve stability (Valko *et al*., 2007). Free radicals can cause oxidative stress, which plays a significant role in the development of chronic and degenerative diseases such as cancer, arthritis, aging, autoimmune damage, cardiovascular and neurodegenerative diseases (Pham-Huy *et al*., 2008).

Free radicals that damage the body can be neutralized by antioxidant compounds. Antioxidants are compounds that can inhibit free radicals in the body. These antioxidant compounds will give one or more electrons to free radicals to become normal molecules again and stop the damage caused (Sasikumar *et al*., 2009). The human body does not have excess antioxidant reserves, so if there is excessive free radical exposure, the body needs exogenous antioxidants (Hariyatmi, 2004). Sources of antioxidants can be either synthetic or natural antioxidants, but antioxidants from synthetic materials such as BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene) can increase the risk of carcinogenesis (Whysner *et al*., 1994). So we need to look for natural antioxidants that are safer to develop, one of them are originated from plants.

Plants contain compounds that have antioxidant activity, one of which is a flavonoid compound. Flavonoids are a group of polyphenol compounds known to have antioxidant, antimicrobial, antiallergic, antiviral, anti-inflammatory and vasodilator properties (Pietta, 2000). One of the plants that have potential as an antioxidant is a walnut (*Canarium indicum* L.). Walnuts grow widely in eastern Indonesia, such as North Sulawesi, Maluku, and Seram Island (Thomson and Evans, 2006). Previous studies revealed that the leaves of several species of the genus *Canarium* L. have potent antioxidant activity, so it is possible that walnut leaves also have the potential to have antioxidant activity because the closer the kinship, the more phytochemical compounds are similar, including compounds that have antioxidant activity (McNair, 1935).

This study was aimed to determine the antioxidant activity, phytochemical content, total flavonoid content, and the relationship of antioxidant activity with the total flavonoid levels on extracts and fractions from *Canarium indicum* L. leaves. It is expected to obtain potential natural antiradical sources that are useful to prevent degenerative diseases which are related to the oxidation process.

METHODS

Preparation of Ethanol Extract
The walnut leaves used were old enough, taken from the Meru Betiri Jember National Park collection, Young and dried leaves were not used. 200 g of dried walnut leaf powder was digested in 2,000 ml of 96% ethanol for 1 hour at 45 ºC. The filtrate obtained was concentrated with a rotary evaporator at 45 ºC to obtain concentrated ethanol extract.

Fractionation
15 g of thick extract was dissolved in 100 ml of ethanol pa. 100 ml of warm distilled water was added to that extract solution at 45 ºC then put into a separating funnel. 200 ml n-hexane was added, shaken vigorously and allowed to stand immediately. The ethanol-water phase was extracted again with n-hexane twice. Then 200 ml ethyl acetate was added to the ethanol-water phase, shaken slowly and allowed to stand until finished, then opened. Each fraction was evaporated with rotary evaporator, thus, n-hexane fraction, ethyl acetate fraction and ethanol-water fraction were obtained.

Antioxidant Activity Test
The antioxidant activity assay was done using the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate)method from the Molyneux (Molyneux, 2003) with modifications carried out as follows:

DPPH Solution Preparation
Weighed as much as 10 mg DPPH and dissolved with ethanol in a 10 ml flask so that solution has a concentration of 1000 µg/ml. Then pipetted 2 ml and dissolved to 50 ml of ethanol to obtain a concentration of 0.004%.

Maximum DPPH Waves Determination
DPPH 0.004% solution was determined by the absorption spectrum using a UV spectrophotometer at wavelengths of 400 nm to 800 nm then the maximum wavelength was determined.

Control Solutions Preparation
Ethanol p.a pipetted 200 µL then put into vials and added 800 µL DPPH solution then shaken until homogeneous and absorbance measured at its maximum wavelength.

Operating Time Determination
DPPH solution was added to the extract solution and vitamin C solution (4: 1), shaken until homogeneous, then absorbance was observed at the maximum wavelength of DPPH, with intervals of 5 minutes to obtain a stable absorbance that is no visible decrease in absorbance until 60 minutes (1 hour).

Extract and Fraction Solutions Preparation
A total of 25 mg of each extract and fraction of walnut leaves was dissolved with 10 ml of ethanol in a 10 ml volumetric flask so the concentration of 2500 µg/ml was obtained. Then dilution was carried out to obtain concentrations of 10, 20, 30 and 40 µg/ml for the ethanol extract test sample, ethyl acetate fraction and ethanol-water fraction. Then-hexane fraction solution was made with a concentration of 75, 100, 125, 150 µg/ml. Vitamin C solutions with 3, 5, 7 and 9 µg/ml concentrations were used as a comparison.

Antioxidant Activity Measurement
DPPH 0.004% solution was pipetted 800 µL plus 200 µL of each extract and fraction test solution. The mixture was allowed to stand for the obtained operating time, then its absorbance was measured at the maximum wavelength of DPPH. The blanko used was an extract solution with 1: 4 ethanol (200 µL extract: 800 µL ethanol). DPPH solution with 4: 1 ethanol (800 µL DPPH: 200 µL ethanol) was used as a control. As a comparison, vitamin C concentration of 3, 5, 7, and 9 µg/ml was used with the same treatment as the test solution. DPPH reduction (%) was calculated using the formula:

$$\frac{Control\ absorbance - Sample\ absorbance}{Control\ absorbance} \text{ x } 100\%$$

The linear regression uses the sample concentration (µg/ml) VS the percentage of inhibition (%). Antioxidant activity is expressed by 50% inhibition concentration (IC_{50}), i.e., the sample concentration that can reduce 50% of DPPH radical. The IC_{50} value was obtained from the linear regression.

Phytochemical Screening
Alkaloids Identification
0.3 grams of the extract was added 5 ml of HCl 2 N, heated on water bath for 2-3 minutes while stirring. After chilling added 0.3 grams of NaCl, stirring evenly then filtered out. The filtrate obtained was added with 5 ml of HCl 2 N and divided into three parts called IA, IB and IC solutions. Mayer reagent added an IA solution, IB solution was added with Wagner reagent and IC solution was used as a blanko. The presence of turbidity or sediment indicates the presence of alkaloids (Departemen Kesehatan Republik Indonesia, 1995).

Saponin Identification
0.3 grams of the extract was put into a test tube, then added 10 ml of distilled water, shaken vigorously for 30 seconds. The positive froth test contains saponins if there is a stable froth for more than 30 minutes with a height of 3 cm above the liquid surface (Departemen Kesehatan Republik Indonesia, 1995).

Flavonoids Identification
2 mg of thick extract was dissolved in 1 ml of ethanol (95%) P. Then 0.1 grams of magnesium P powder was added and 10 drops of concentrated hydrochloric acid were added. Red-orange to purple-red indicates a flavonoid. The orange-yellow color indicates the presence of flavones, chalcones, and aurons (Departemen Kesehatan Republik Indonesia, 1995).

Polyphenols and Tannins Identification
0.3 grams of the extract was added to 10 ml of hot distilled water, stirred and left to room temperature, then added 3-4 drops of 10% NaCl, stirred and filtered. The filtrate is divided into three parts of ± 4 ml each and is called the IA, IB and IC solution (Departemen Kesehatan Republik Indonesia, 1995).

Total Flavonoids Determination

Determination of total flavonoid levels was carried out by spectrophotometry using aluminum chloride reagents according to the procedure of Chang *et al*. (2002). As much as 0.5 ml of 200 µg/ml extract solution dissolved in 1.5 mL ethanol. Then it is added 0.1 ml $AlCl_3$10%, 0.1 mL 1M potassium acetate and 2.8 mL distilled water. Left to stand for 30 minutes and absorbance was measured at 415 wavelengths nm, blanko was made the same as the test solution by replacing 10% $AlCl_3$ with distilled water. Calculations are made from an average of the twice measurement and flavonoid content is expressed by comparison of standard quercetin standards (Chang *et al*., 2002).

Data Analysis

All analyses were carried out twice replication. Antioxidant activity was indicated by the IC_{50} value obtained from the regression equation of the test solution's concentration with a percent reduction of DPPH. The total flavonoid value was obtained from the substitution of the absorbance value of the sample in the quercetin standard curve regression equation expressed by grams of quercetin equivalent to each gram of sample extract. Correlation between total flavonoid levels and antioxidant activity was obtained using a regression equation between total flavonoids with IC_{50} values.

RESULTS AND DISCUSSION

Antioxidant Activity Test

The antioxidant activity test was carried out using the DPPH method. The principle of the DPPH method is that the antioxidant compound will donate its hydrogen atom to the DPPH radical so that it will cause DPPH to be a non-radical reduced form characterized by the fading of its purple color which is characterized by a decrease in absorption from DPPH at its maximum wavelength. The IC_{50} value is used as a test parameter for antioxidant activity, the smaller the value, the greater the damping power (Molyneux, 2003).

The antioxidant activity test begins with making visible light spectra of DPPH 0.004% solution, which is known to have a maximum wavelength at 516 nm and 30 minutes operating time, so the test uses wavelength at 516 nm and an incubation time of 30 minutes. Based on the results of the antioxidant activity test of extracts and walnut fraction and comparison of vitamin C obtained a relationship between the concentration of the test solution and the percent reduction of DPPH as shown in Figure 1.

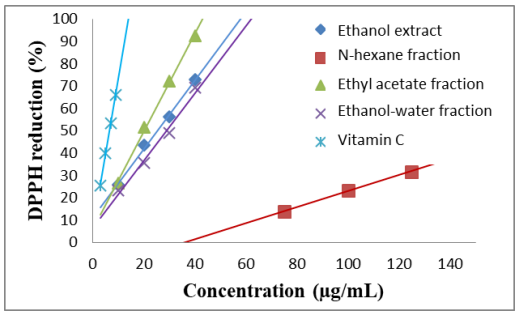

Figure 1. Relationship of concentration test with% average DPPH reduction (n = 2)

IC_{50} values of walnut leaf extracts and fractions, as well as a comparison of vitamin C, obtained from the regression equation of the concentration of the test solution with percent reduction of DPPH can be seen in Table 1.

According to Blois (Blois, 1958), the level of antioxidant strength is very firm (IC_{50}<50 µg/ml), strong (IC_{50} 50-100 µg/ml), moderate (IC_{50} 101-150 µg/ml), weak (IC_{50}> 150 µg/ml).

Table 1. IC50 values of extract and fraction average walnut leaves

Sample	IC_{50} (µg/ml) (average ± SD)
Ethanol extract	25,294 ± 0,055
n-hexane fraction	175,245 ± 0,499
Ethyl acetate fraction	20,135 ± 0,009
Ethanol-water fraction	28,806 ± 0,042
Vitamin c	6,564 ± 0,016

Total Flavonoids

Total flavonoid levels expressed by grams of quercetin equivalent to each gram of extract using the standard curve equation y = 0.006x - 0.054 (r^2 = 0.986). The results of determining the total flavonoid extract and fraction of walnut leaves can be seen in Table 2.

Table 2. Total flavonoid of walnut leaves extract and fraction

Sample	Value (g QE/g extract) (average ± SD)
Ethanol extract	2,624 ±0,012
n-hexane fraction	0,499 ± 0,023
Ethyl acetate fraction	3,846 ± 0,006
Ethanol-water raction	1,596 ± 0,006

Based on the results of the determination of total flavonoid levels, it is known that ethyl acetate fraction has the highest total flavonoid levels, followed by ethanol extract, ethanol-water fraction and n-hexane fraction have the lowest total flavonoid levels. It is thought that compounds that act as antioxidants in walnut leaves are flavonoids that are semi-polar and therefore tend to be present in ethyl acetate, such as catechins and proanthocyanidins (Robinson, 1995). The number of hydroxyl groups and types of substituents in the structure of flavonoids that affect their solubility, the more hydroxyl groups that have caused the greater the solubility of water in water, and the less hydroxyl groups and the more substituents that are less polar such as methoxy groups cause decreasing solubility of flavonoids (Akowuah *et al*., 2005).

Phytochemical Screening

Phytochemical test results of walnut leaf ethanol extract can be seen in Table 3.

Relationship of Total Flavonoid Levels to the Antioxidant Activity of Walnut Leaves

The relationship of total flavonoid levels to the antioxidant activity of walnut leaves was determined using linear regression, as shown in Figure 2.

Table 3. Phytochemical screening results of ethanol extract of walnut leaves.

Compound	Reagen	Result	Info
Alkaloid	Mayer, Wagner	No sediment	-
Steroid	Concentrated sulfuric acid	No red ring on the surface	-
Saponin	Froth test	Forms a stable foam >30 minutes	+
Flavonoid	Mg powder & HCl	Color changes to red-purple	+
Tanin	NaCl 10%	Forms a white sediment	+
Polyphenol	FeCl3	Color changes to blackish green	+

Notes: (-) = not detected.
(+) = detected

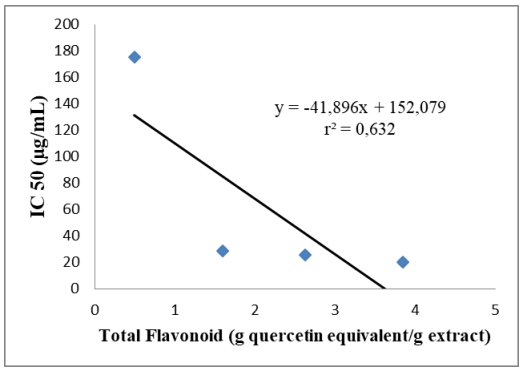

Figure 2. Linear correlation of total flavonoids (x) with antioxidant activity (y) walnut leaves.

Based on the total linear flavonoid correlation results with the antioxidant activity of walnut leaves, the value of $r^2 = 0.632$. This shows 63.2% of the antioxidant activity of the walnut extracts and fractions due to the contribution of flavonoid compounds. It can also be concluded that the antioxidant activity of walnut leaf extracts and fractions is not only due to the presence of flavonoid compounds. Antioxidant activity can also be derived from the presence of secondary antioxidant metabolites such as volatile oils, carotenoids, and vitamins so that 36.8% of antioxidant activity produced by extracts and walnut fractions is influenced by the presence of compounds other than flavonoids (Javanmardi *et al*., 2003).

CONCLUSION

Ethanol extract of walnut leaves contains flavonoid compounds, polyphenols, tannins, and saponins. It does not contain alkaloid compounds and steroids.

Correlation of total flavonoid levels with antioxidant activity of extracts and walnut fraction was known that 63.2% antioxidant activity of extracts and walnut fractions due to the contribution of flavonoid compounds. Further research is needed to purify the ethyl acetate fraction from ethanol extracts of walnut leaves to determine pure compounds that act as antioxidants.

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