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Research Article

Evaluation of Antioxidant Activity of *Vernonia amygdalina* Leaves and Its Flavonoid-Phenolic Content

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Abstract

Bitter leaf, *Vernonia amygdalina* (VA), is used in various alternative medicine in several countries, including Indonesia. This study aims to evaluate the antioxidant activity of VA leaves by three different *in vitro* methods. The antioxidant activity was evaluated by radical scavenging of 1,1-diphenyl-2-picryl-hydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and cupric reducing antioxidant capacity (CUPRAC). The antioxidant capacity by FRAP and CUPRAC methods were expressed as mg of quercetin equivalents (QE) per g extract (mgQE/g) and mg of ascorbic acid equivalents (AAE) per g extract (mgAAE/g), respectively. All methods were measured by UV-Visible spectrophotometry at maximum wavelength. The results exhibited that VA leaves ethanolic extract contains flavonoid-phenolic. The VA leaves ethanolic extract has an antioxidant activity by scavenging the DPPH method with the half inhibition concentration (IC_{50}) was 8,22 µg/mL. The antioxidant capacity of VA leaves ethanolic extract by FRAP and CUPRAC methods was 21,03 mgQE/g and 160,64 mgAAE/g extract, respectively. The VA leaves ethanolic extract has a powerful antioxidant potency which might correlate to the flavonoid-phenolic content in the extract.

Keywords: Antioxidant, DPPH, FRAP, CUPRAC, bitter leaf.

Evaluasi Aktivitas Antioksidan Daun *Vernonia amygdalina* dan Kandungan Flavonoid-Fenoliknya

Abstrak

Daun pahit, *Vernonia amygdalina* (VA) digunakan dalam berbagai pengobatan alternatif di beberapa negara termasuk di Indonesia. Penelitian ini bertujuan untuk mengevaluasi aktivitas antioksidan daun VA dengan tiga metode *in vitro* yang berbeda. Aktivitas antioksidan dievaluasi dengan pemulungan radikal 1,1-difenil-2-pikril-hidrazil (DPPH), *ferric reducing antioxidant power* (FRAP), cupric *reducing antioxidant capacity* (CUPRAC). Kapasitas antioksidan dengan metode FRAP dan CUPRAC masing-masing dinyatakan sebagai mg setara kuersetin (QE) per g ekstrak (mgQE/g) dan mg setara asam askorbat (AAE) per g ekstrak (mgAAE/g). Semua metode diukur dengan spektrofotometri *UV-Visible* pada panjang gelombang maksimum. Hasil penelitian menunjukkan bahwa ekstrak etanolik daun VA memiliki aktivitas antioksidan dengan metode *scavenging* DPPH dengan konsentrasi setengah penghambatan (IC_{50}) adalah 8,22 µg/mL. Kapasitas antioksidan ekstrak etanolik VA dengan metode FRAP dan CUPRAC masing-masing adalah 21,03 mgQE/g dan 160,64 mgAAE/g ekstrak. Ekstrak etanolik daun VA memiliki potensi antioksidan sangat kuat yang mungkin berkorelasi dengan kandungan flavonoid dan fenolik pada ekstrak.

Kata Kunci: Antioksidan, DPPH, FRAP, CUPRAC, daun pahit.

1. Introduction

Free radicals are molecules that carry one or more unpaired electrons and can exist independently[1]. Free radicals have an odd number of electrons, which are highly reactive and unstable. Free radicals can react quickly with other molecules by capturing electrons to become stable. Free radicals are balanced by taking electrons in nearby molecules. Meanwhile, molecules that are attacked become free radicals due to the loss of electrons and start a chain reaction that causes damage to cells[2].

Free radicals can be generated from internal and external factors. Body metabolism is one of the internal factors, while external factors include cigarette smoke, ultraviolet radiation, radical-triggering substances in food, and other pollutants. Radical reactions that occur continuously in the body, if not stopped, will result in various degenerative diseases. Diseases caused are chronic, such as heart attacks, cancer, cataracts, premature aging, and decreased kidney function[2]. To prevent these diseases, antioxidants are needed.

Antioxidants are balancing compounds that are beneficial to health in overcoming excess oxidants. Previous studies have described several radical scavenging methods to determine antioxidant potential[3]. One of the plants that people on the African continent believe to have medicinal properties is a bitter leaf (*Vernonia amygdalina*)[4]. Thus, this leaf is also known in Indonesia as the African leaf. African leaves contain flavonoid and phenolic compounds, often associated with their potential as antioxidants[5]. Chemical components in plants are closely related to where the plant grows. Therefore, this study aims to examine the antioxidant activity of bitter leaves growing in Makassar City.

The antioxidant potential of the bitter leaf has been tested using three methods; 1,1-diphenyl-2-picryl-hydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and cupric reducing antioxidant capacity (CUPRAC). The analytical instrument used in these antioxidant assays is UV-Visible spectrophotometry. We found that the *V. amygdalina* leaves (VAL) extract has an antioxidant potency and could be developed for further study.

2. Methods

2.1. Tools

In this study, we used analytical instruments such as glassware (Pyrex®), micropipette, oven (Memmert®), pH meter (Jenco®), centrifuge (OneMed®), UV-Visible spectrophotometer (Apel®), weighing scale analytical (KERN®), and vortex (IKA® Vortex Genius 3).

2.2. Materials

All chemicals were of analytical grade and purchased commercially. Quercetin, ascorbic acid, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), ferric reducing antioxidant power (FRAP) reagent, cupric reducing antioxidant capacity (CUPRAC) reagent, and other chemicals were purchased from Merck Co. (Darmstadt, Germany). Millipore-Q50 Ultrapure water system (Sartorius) is used to produce deionized water. The stock solution of quercetin and ascorbic acid (1000 µg/mL) was prepared by dissolving 10 mg into 10 mL ethanol. Calibration standards used standard solutions of quercetin and ascorbic acid, respectively. The bitter leaves (Vernonia amygdalina) were obtained in Makassar. The Division of Botany, Laboratory of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Muslim Indonesia, Makassar-Indonesia, confirmed the sample.

2.3. Procedure

2.3.1. Extraction

The VAL was washed with water and then dried by air-dried. The dried samples were sorted and then cut into small pieces and powdered. A total of 50 g of sample was macerated with 500 mL of 96% ethanol at room temperature for three days. The liquid extract was filtered and concentrated under reduced pressure to obtain a thick extract. The thick extract was stored in the vacuum desiccator to obtain dry extract, and calculated the yield by the following formula:

$$\% \text{ yield} = \frac{\text{total weight of extract}}{\text{total weight of sample}} \text{ x } 100\ \%$$

2.3.2. Flavonoid-Phenolic Analysis

The 96% ethanol was mixed with up to 5 mg of VAL extract. The mixture was heated and filtered to provide a stock solution. To the VAL solution, magnesium, 1 mL concentrated HCl, and 1 mL amyl alcohol were added. The formation of yellow color indicates the presence of flavonoids in the sample[5]. To the VAL extract solution, 1% $FeCl_3$ was added. The formation of a green-to-black color indicates the presence of phenolic in the sample[5].

2.3.3. Determination of antioxidant activity by DPPH assay

As described previously, the antioxidant activity was investigated by scavenging the DPPH radical with slight modifications[6]. The 1000 µg/mL quercetin was diluted to prepare concentration series (0,2; 0,4; 0,6; 0,8; 1,0 µg/mL). Each 0.5 mL of quercetin was mixed with 3,5 mL of DPPH-solution (0,4 mM) and 2 mL methanol in a lockable glass envelope, transferred in a disposable polystyrene cuvette, and the UV-Visible spectrum measured after exactly 30 min of reaction time in the 37 °C. A blank sample was prepared similarly, but only with methanol 2 mL and DPPH-solution 1 mL. Every sample was prepared and measured three times before being analyzed. Because the DPPH is photosensitive, materials are kept away from light until they are analyzed[7]. With the equation, the inhibition of the DPPH radical was estimated.

Inhibition is the ratio between the decrease of the absorbance in the sample and the initial absorbance of the blank DPPH solution at 515 nm by UV-Visible spectrophotometry. The same procedure was done for the VAL extract (2; 4; 6; 8; and 10 µg/mL).

The percentages of DPPH radical inhibition as a function of effect and extracted fractions were calculated using the following equation: ACO is the absorbance of the control at t = 0 min, and AAT is the absorbance of the samples at t = 30 min.

$$\% \text{ Inhibition} = \frac{A_{CO} - A_{AT}}{A_{CO}} \text{ x } 100\%$$

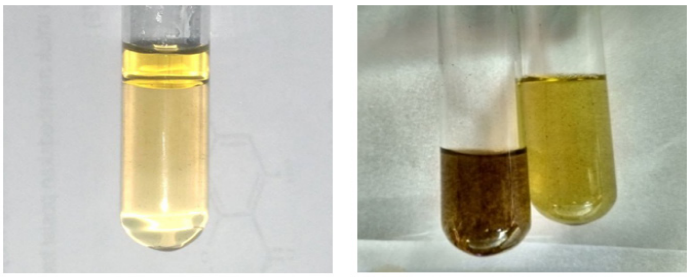
2.3.4. Determination of antioxidant activity by FRAP assay

The FRAP method investigated the antioxidant activity as described previously with slight modifications[8]. Briefly, quercetin (10; 20; 30; 40; 50 µg/mL) was used as a reference standard to obtain a calibration curve. Each concentration of quercetin (1 mL) was mixed with phosphate buffer pH 6,6 (1 mL), potassium ferricyanide ($K_3Fe(CN)_6$, 1 mL), and incubated for 20 min at 50 °C. To the mixture, trichloroacetic acid (TCA, 1 mL), deionized water (1 mL), and ferric chloride ($FeCl_3$, 1 mL) were added, respectively. The UV-Visible spectrophotometric method monitored the absorbances at the maximum wavelength at 702 nm. The same procedure was carried out on the VAL extract ethanol (1000 µg/mL) triplicate. The absorbance of quercetin was used to construct the linear regression, y = a + bx. The linear regression equation was then plotted by replacing the y variable with the absorbance of VAL extract, respectively, to obtain the x value as the initial concentration for the following calculation. The antioxidant activity was expressed as mg of quercetin equivalents (QE) per g extract (mgQE/g).

$$\text{Antioxidant capacity (mgQE/g)} = \frac{\text{Volume of extract (mL)} \; x \; \text{Initial concentration (x)}}{\text{Weight of extract (mg)}}$$

2.3.5. Determination of antioxidant capacity by CUPRAC assay

The antioxidant capacity was investigated by CUPRAC method as described previously with slight modifications[8]. Briefly, ascorbic acid (16; 20; 30; 40; 50 µg/mL) was used as a reference standard to obtain a calibration curve. Each concentration of ascorbic acid (1 mL) was mixed with 0,01 M cupric chloride ($CuCl_2$, 1 mL), neocuproine ($7,5 \times 10^{-3}$ M, 1 mL), ammonium acetate buffer pH 7,1 ($NH_4CH_3CO_2$, 1 mL), 96% ethanol (1 mL), and the volume was made up with deionized water to 4,1 mL. The mixture was left for 30 minutes, and the solution's absorbance was measured by the UV-Visible spectrophotometric method at the maximum wavelength at 450 nm. The same procedure was carried out on the VAL extract (100 µg/



a b

Figure 1. The color reaction of VAL extract by specific reagent indicating (a) flavonoid and (b) phenolic content

mL) triplicate. The absorbance of ascorbic acid was used to construct the linear regression, y = a + bx. The linear regression equation was then plotted by replacing the y variable with the absorbance of VAL extract, respectively, to obtain the x value as initial concentration to be used in the following calculation. The antioxidant capacity was expressed as mg of ascorbic acid equivalents (AAE) per g extract (mgAAE/g).

$$\text{Antioxidant capacity (mgAAE/g)} = \frac{\text{Volume of extract (mL)} \; x \; \text{Initial concentration (x)}}{\text{Weight of extract (mg)}}$$

3. Result

Flavonoid-Phenolic Analysis

The results of the qualitative evaluation in Figure 1 showed that the VAL extract contained flavonoid and phenolic compounds based on the color reaction shown in the extract. Determination of antioxidant activity by DPPH assay, FRAP assay, and CUPRAC assay shown in the table 1,2, and 3.

4. Discussion

4.1. Determination of antioxidant activity by DPPH assay

Determination of the antioxidant activity of flavonoid-phenolic compounds in plants is generally carried out through a radical scavenging mechanism. The DPPH radical is the most commonly used indicator to see the ability of compounds to reduce free radicals. This *in vitro* method is often used for antioxidant activity because it is simple, fast, and sensitive[9,10]. This method only requires DPPH without adding substrate because free radicals are available directly to touch the substrate. The interaction between the compound and the DPPH radical can be observed through the color change from purple to yellow. The color change indicates that DPPH has been reduced by donating hydrogen or electrons from antioxidant compounds. This color change was measured using a UV-Visible spectrophotometer[6].

The obtained absorbance data then calculated the inhibition percentage, converted into IC_{50}. Table 1 shows that the IC_{50} of the extract was 8,22 µg/mL, while that of quercetin was 1,83 µg/mL. Although the IC_{50} of quercetin was lower than the VAL extract, both IC_{50} quercetin and VAL

Table 1. Antioxidant activity of VAL extract by DPPH method

	Concentrations (µg/mL)	Absorbance (515 nm)	Inhibition (%)	IC_{50}(µg/mL)
Quercetin	0.2	0.43	29.35	1.83
	0.4	0.42	31.18	
	0.6	0.40	33.99	
	0.8	0.38	36.48	
	1.0	0.36	39.64	
VAL extract	2	0.54	10.91	8.22
	4	0.47	21.98	
	6	0.39	34.21	
	8	0.29	50.58	
	10	0.24	60.99	

Table 2. The antioxidant capacity of VAL extract by FRAP method

	Concentrations (µg/mL)	Absorbance (702 nm)	Linearity regression	Antioxidant capacity (mgQE/g)	Average (mgQE/g)
Quercetin	10	0.22	y = 0.0229x – 0.005	-	-
	20	0.36	(R2 = 0.995; r =		
	30	0.47	0.997)		
	40	0.59			
	50	0.68			
VAL extract	1000	0.34	-	20.09	21.03
	1000	0.36		22.00	
	1000	0.35		21.00	

extract were in the same category of powerful antioxidants. Categories as antioxidants based on the DPPH test are divided into; very strong, strong, medium, and weak with IC_{50} <50, 50-100, 100-250, and 250-500 g/mL, respectively[11].

4.2. Determination of antioxidant capacity by FRAP assay

The polyphenol content of the extract correlated with antioxidant activity[12,13]. To determine the ability of the extract to reduce free radicals, the *in vitro* or *in vivo* evaluation is needed. The FRAP method's principle of antioxidant testing is to reduce Fe^{3+} complexes to Fe^{2+} [14]. Free radicals are compounds or molecules containing one or more unpaired electrons in their outermost orbital. The presence of unpaired electrons causes these compounds to be very reactive in attacking and binding electrons surrounding them, including cell membrane macromolecules, DNA, and proteins. Free radicals are formed through normal cellular metabolic processes and as a result of responses to external factors such as air pollution and ultraviolet light[15].

The absorbance measurement of quercetin was used as a comparison to construct the linear regression equation, as shown in Table 2. The linearity data shows that the correlation coefficient was close to the optimal value, which means that the relationship between concentration and absorbance was in line. In addition, this data also confirms the accuracy of the spectrophotometer used and the accuracy of the preparation of the quercetin standard solution. The calculation of antioxidant capacity, as shown in Table 2, exhibited that the VAL has an antioxidant capacity which equivalent to quercetin.

4.3. Determination of antioxidant capacity by CUPRAC assay

The CUPRAC method is a simple and versatile antioxidant activity test for various polyphenols, including phenolic acids, hydroxycinnamic acids, flavonoids, carotenoids, anthocyanins, and as for thiols, synthetic antioxidants, vitamins C and E. The chromogenic oxidizing reagent used for the CUPRAC assay is bis(neocuproine) the copper(II) cation (Cu(II)-Nc) which acts as an electron-transfer agent, and the CUPRAC chromophore[3,16]. In the CUPRAC method, this reagent works at pH 7; the oxidation reaction is completed at room temperature within 30 min. Slow-acting antioxidants may require slightly higher temperature incubation to complete their oxidation with CUPRAC reagent. Cu(I)-chelate absorbance is formed due to a redox reaction with reducing polyphenols. Vitamin C was measured at 450 nm (for the Cu(I)-Nc spectrum obtained by reacting various reference concentrations with CUPRAC reagent). The yellow-orange color is due to the formation of Cu(I)-Nc chelates. The CUPRAC reagent reacts with n-electron reducing antioxidants, shown by the following reaction[3].

The principle of the CUPRAC test is to use copper(II) bis(neocuproine) as a chromogenic reagent reacting with an n-electron reductant antioxidant. The reagent $Cu(Nc)^{22+}$ (blue) is reduced to $Cu(Nc)^{2+}$ (yellow). The principle of this method is based on a simple oxidation-reduction reaction between antioxidants and free radicals, which

Table 3. The antioxidant capacity of VAL extract by CUPRAC method

	Concentrations (µg/mL)	Absorbance (450 nm)	Linearity regression	Antioxidant capacity (mgAAE/g)	Average (mgAAE/g)
Ascorbic acid	16	0.29	y = 0.021x – 0.037 (R2 = 0.992; r = 0.995)	-	-
	20	0.42			
	30	0.58			
	40	0.77			
	50	1.04			
VAL extract	100	0.30	-	160.48	160.64
	100	0.30		160.48	
	100	0.30		160.92	

can be measured by reducing cupric ions (Cu^{2+}) to cuprous (Cu^{+}) employing electron donors by antioxidants[17-19].

The absorbance of ascorbic acid constructed the linear regression equation, as shown in Table 3. The linearity data exhibited that the correlation coefficient was close to the optimal value, which means that the relationship between concentration and absorbance was in line. The antioxidant capacity of VAL extract is shown in Table 3.

Antioxidant evaluation using three different methods aims to confirm the antioxidant ability of VAL based on different antioxidant mechanisms. The results indicate that VAL extract has antioxidant abilities based on each method used, confirmed the previous study[20]. The three methods have different principles concerning antioxidant activity, so it might be difficult to compare each other. However, based on the data obtained, the IC_{50} comparison between the extract and the reference in the DPPH method shows similar values. Thus, the DPPH method is much more suitable for determining the antioxidant activity of VAL extract. This antioxidant ability is closely related to the chemical content of flavonoids and phenolics from VAL extract.

5. Conclusion

The VAL ethanolic extract has a powerful antioxidant potency based on the IC_{50} of the extract by the DPPH method. In addition, the antioxidant capacity of VAL ethanolic extract exhibited high potency by FRAP and CUPRAC methods, respectively. The antioxidant activity of VAL ethanolic extract might be related to the flavonoid-phenolic content in the extract. Therefore, the VAL extract could be developed as an antioxidant agent by assessing the antioxidant activity *in vivo* for further study.

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