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Original Article

Analysis of Antioxidant Activity and Total Flavonoid Content of *Averrhoa bilimbi* L. Leaf Ethanol Extract Using the DPPH Method

Analisis Aktivitas Antioksidan dan Kandungan Flavonoid Total Ekstrak Etanol Daun Averrhoa bilimbi L. Menggunakan Metode DPPH

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Abstract

Starfruit (*Averrhoa bilimbi L.*) is not only a commonly consumed as a fruit but is also widely used in traditional medicine. The plant has long been utilized by the communities to help treat various ailments, including coughs and rectal inflammation. This study aimed to determine the total flavonoid content and antioxidant activity of the ethanol extracts of Averrhoa bilimbi leaves fractionated using n-hexane. The extraction process was carried out using the maceration method with 70% ethanol, followed by fractionation with 95% n-hexane to separate active compounds from the crude extract. The total flavonoid content in the n-hexane fraction of the ethanol extract was measured using a UV-Vis spectrophotometer at a wavelength of 229-230 nm, while antioxidant activity was evaluated using the DPPH method at 515 nm. The results showed that the total flavonoid content of the n-hexane fraction of the ethanol extract was 37.3 mgQE/g extract. The antioxidant activity test produced an IC50 value of 6.58 µg/mL, compared to a standard IC50 value of 2.45 µg/mL for Askorbat acid. These findings indicate that the n-hexane fraction of Averrhoa bilimbi leaves ethanol extract possesses very strong antioxidant properties.

Abstrak

Belimbing (Averrhoa bilimbi L.) tidak hanya umum dikonsumsi sebagai buah tetapi juga banyak digunakan dalam pengobatan tradisional. Tanaman ini telah lama dimanfaatkan oleh masyarakat untuk membantu mengobati berbagai penyakit, termasuk batuk dan radang dubur. Penelitian ini bertujuan untuk menentukan kandungan flavonoid total dan aktivitas antioksidan dari ekstrak etanol daun Averrhoa bilimbi yang difraksinasi menggunakan n-heksana. Proses ekstraksi dilakukan menggunakan metode maserasi dengan etanol 70%, diikuti dengan fraksinasi dengan n-heksana 95% untuk memisahkan senyawa aktif dari ekstrak kasar. Kandungan flavonoid total dalam fraksi n-heksana dari ekstrak etanol diukur menggunakan spektrofotometer UV-Vis pada panjang gelombang 229-230 nm, sedangkan aktivitas antioksidan dievaluasi menggunakan metode DPPH pada 515 nm. Hasil menunjukkan bahwa kandungan flavonoid total fraksi n-heksana dari ekstrak etanol adalah 37,3 mgQE/g ekstrak. Uji aktivitas antioksidan menghasilkan nilai IC50 sebesar 6,58 µg/mL, dibandingkan dengan nilai IC50 standar sebesar 2,45 µg/mL untuk asam Askorbat. Temuan ini menunjukkan bahwa fraksi n-heksana dari ekstrak etanol daun Averrhoa bilimbi memiliki sifat antioksidan yang sangat kuat.

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INTRODUCTION

One of the major causes of non-communicable diseases is oxidative stress, a condition characterized by an imbalance between free radicals and antioxidants in the body. Free radicals are naturally produced during metabolic processes. However, excessive or uncontrolled free radical formation may cause oxidative damage to cells and increase the risk of degenerative diseases such as atherosclerosis, heart failure, cancer, and Alzheimer's disease.

Various health problems, long predating formal healthcare and modern medicine, have been identified. Many people are increasingly using plants as traditional medicines, citing their reduced side effects compared to synthetic drugs and their ability to maintain the immune system. Free radicals in the human body originate from endogenous and exogenous sources. Endogenous sources include autoxidation, enzymatic oxidation, and respiratory burst processes, while exogenous sources include medications, radiation, cigarette smoke, and air pollutants.[14 15] Antioxidants play an important role in neutralizing free radicals and interrupting oxidation chain reactions, thereby protecting the body from oxidative damage.

Medicinal plants have long been used as traditional remedies because they are considered to have fewer side effects than synthetic drugs. One plant reported to possess antioxidant potential is *Averrhoa bilimbi* L. Previous studies demonstrated that ethanol extracts and fractions of *Averrhoa bilimbi* leaves exhibit hypoglycemic and hypolipidemic activities in diabetic rat models.[4] In addition, purified ethanol extracts have shown potential antihypertensive activity.[17]

Antioxidant activity can be evaluated using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method, which measures the ability of compounds to scavenge stable free radicals. [18] Reported that ethanol extract of *Averrhoa bilimbi* leaves exhibited very strong antioxidant activity with an IC_{50} value of 16.99 ± 0.12 µg/mL. Another study reported that 70% ethanol extract of *Averrhoa bilimbi* fruit showed strong antioxidant activity with an IC_{50} value of 74.625 ppm. [3]

Although several studies have reported the antioxidant activity of *Averrhoa bilimbi* leaves, most investigations have focused on crude ethanol extracts without further fractionation. Differences in extraction and fractionation methods may influence the composition of secondary metabolites and the biological activity of the resulting extracts. Limited information is available regarding the antioxidant activity and total flavonoid content of the n-hexane fraction of *Averrhoa bilimbi* leaves ethanol extract. Fractionation using n-hexane is important for separating compounds based on polarity and identifying fractions with potential bioactive properties. Therefore, this study aimed to determine the antioxidant activity based on IC_{50} values and the total flavonoid content of the n-hexane fraction of *Averrhoa bilimbi* leaves ethanol extract using the DPPH method.

METHODS

The type of research used is experimental research. To determine the total flavonoid levels and antioxidant activity of *Averrhoa bilimbi* leaves extract. This research was conducted at the Health Chemistry Laboratory, Faculty of Health, Nahdlatul Ulama University Surabaya. The research population was *Averrhoa bilimbi* plants obtained in Sekaran District, Lamongan, and the samples used were *Averrhoa bilimbi* leaves that were neither too old nor too young; there were no yellow spots, indicating that the leaves were not old. The plant samples have been identified/determined based on their scientific names at BRIN Cibinong Bogor, *Averrhoa bilimbi* leaves (*Averrhoa bilimbi* L), family *Oxalidacea*.

Tools used: in this research were a beaker glass, measuring cup, test tube, petri dish, volume pipette, dropper pipette, analytical balance, spatula, stirring rod, test tube and rack, vortex, bottle, set of extraction tools, and UV-Vis Spectrophotometer. **Materials:** used were *Averrhoa bilimbi* leaves, acetic acid, chloroform, ammonia, $FeCl_3$, PbAcetate 5%, H_2SO_4 2 M, methanol, ethanol, n-hexane, gallic acid, sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl or DPPH, Tris-HCl buffer 1 M (pH 7.4), distilled water, quercetin standard, $AlCl_3$ 10%, and ascorbic acid.

Research Procedure

Sample Preparation

One kg of washed *Averrhoa bilimbi* leaves was dried and blended. The leaves were extracted by maceration with 70% ethanol in a 1:10 ratio. The sample was isolated for 3 days at room temperature, away from sunlight, and stirred every 24 hours for 5 minutes. Filtering was performed with filter paper and a vacuum to separate the pulp and filtrate. Concentration was carried out using a rotary evaporator until the solvent separated. A thick ethanol extract of *Averrhoa bilimbi* leaves was obtained. Fractionation was performed using 95% n-hexane. The thick ethanol extract of *Averrhoa bilimbi* leaves was partitioned using a separating funnel using hexane. The fraction was evaporated using a rotary evaporator to obtain the hexane extract.

Determination of Total Flavonoids

- Qualitative Flavonoid Test: *Averrhoa bilimbi* leaves extract was dissolved with a suitable solvent (Aquadest) in two test tubes. 3-4 drops of $FeCl_3$, and 5% Pb Acetate were added. [19]
- Quantitative Flavonoid Test:
    A. Preparation of a standard quercetin solution: Weigh 25 mg of the standard quercetin solution. Dissolve it in 25 ml of 96% ethanol to obtain 1000 ppm. Take 1 ml using a pipette to obtain 1000 ppm, and add 10 ml of 96% ethanol to obtain 100 ppm.
    B. Determination of Operating Time and Maximum Wavelength: Take 1 ml of the 100 ppm quercetin solution. Add 1 ml of 10% $AlCl_3$ and 8 ml of 5% acetic acid. The absorbance is measured at a wavelength of 200-600 nm.
    C. Preparation of a quercetin standard curve: A series of concentration solutions was prepared using quercetin as the standard. These concentrations were 40, 60, 80, 100, and 120 ppm. A 1 mL solution of each concentration was reacted with 0.2 mL of 10% AlCl3, 0.2 mL of 10% acetic acid, 3 mL of 96% ethanol, and 5.6 mL of distilled water. The absorbance of the series was read using UV-Vis spectrophotometry at the maximum wavelength.
    D. Determination of flavonoid content: *Averrhoa bilimbi* leaf extract was prepared at a concentration of 1000 ppm. A 1 mL sample of each 1000 ppm extract solution was taken. Variations in concentrations were made at 40 ppm, 60 ppm, 80 ppm, 100 ppm, and 120 ppm. 0.2 mL AlCl3, 0.2 mL 10% acetic acid, 3 mL 96% Ethanol, and 5.6 ml Aquades were added. The absorbance reading was carried out at the maximum wavelength.[19] The absorbance results obtained were then calculated using Microsoft Excel.

Antioxidant Activity Test using DPPH method

- Preparation of *Averrhoa bilimbi* leaves extract: Prepare 25 mg of *Averrhoa bilimbi* leaves extract. Then dissolve it using 50 ml of methanol p.a. in a beaker.
- Preparation of DPPH solution: Weigh 10 mg of DPPH. Dissolve it in 34 ml of methanol p.a. to the mark using a 100 ml volumetric flask (100 ppm) as a stock solution. Then, place it in a dark glass bottle.
- Preparation of standard ascorbic acid solution as a control: Weigh 10 mg of ascorbic acid. Dissolve it in 100 ml of methanol p.a. using a 100 ml volumetric flask to obtain a stock solution with a concentration of 1000 ppm. The test is performed by making six serial dilutions of the ascorbic acid reference solution: 2 ppm, 3 ppm, 4 ppm, 5 ppm, and 6 ppm.
- Determining the maximum DPPH wavelength: 1 ml of DPPH solution was placed in a test tube. Add methanol to a total volume of 5 ml, then homogenize and incubate at 37°C for 30 minutes. Measure the solution using a UV-Vis spectrophotometer at a wavelength of 515-518 nm and determine the maximum wavelength.
- Preparation of Blank: A blank 1 ml of DPPH solution was placed in a test tube, methanol was added to a total volume of 5 ml, and then covered with aluminum foil. The mixture was homogenized and incubated in the dark at 37°C for 30 minutes. Measure the blank solution using a UV-Vis spectrophotometer at a wavelength of 517 nm.
- Control measurements: 1 ml of each sample concentration was placed in a test tube. Add 1 ml of DPPH solution and 3 ml of p.a. methanol. Homogenize the solution. Incubate in a dark room at 37°C for 30 minutes. The absorbance of each sample was measured using a UV-Vis spectrophotometer at a wavelength of 517 nm.
- Antioxidant Activity Measurement: From each stock solution, 1 ml was pipetted to obtain concentrations of 25 ppm, 50 ppm, 75 ppm, 100 ppm, 125 ppm, and 150 ppm. Each concentration was made up to volume with methanol p.a. in a 5 ml volumetric flask. Then, 1 ml of each concentration was taken, and 2 ml of DPPH solution was added. The mixture was vortexed and incubated for 30 minutes at room temperature. The absorbance of each solution was measured at a wavelength of 515 nm, with four replicates. The absorbance results were then calculated using Microsoft Excel for IC_{50}. [10]

Data Analysis

Data analysis in the DPPH method antioxidant activity test was carried out by calculating the percentage of DPPH free radical inhibition, which was calculated using the formula:

$$\%\ Inhibisi = \frac{Abs\ Blank - Abs\ sample}{Abs\ Blank} x100$$

RESULT AND DISCUSSION

The extraction results from 1000 ml of *Averrhoa bilimbi* leaves extract, using a 70% ethanol solvent concentrated with a rotary evaporator, were in the form of a 200 ml thick extract. Then, it was fractionated using 96% n-hexane and reconcentrated with a rotary evaporator to obtain a 20 ml thick extract.

Total Flavonoid Content Results

The qualitative flavonoid test aims to determine the presence or absence of flavonoids in *Averrhoa bilimbi* leaves extract.

Table 1. Qualitative Test of Flavonoids

Test	Reagent	Observation	Result
Flavonoid	$FeCl_3$	Violet	+

The test showed a positive result for flavonoids. A positive result with $FeCl_3$ indicated a purple color change, formed due to a complex reaction between the Fe metal from $FeCl_3$ and the flavonoid hydroxyl group. The operating time was determined to determine the length of time required for the solution to reach constant absorbance. This was determined by measuring the absorbance of the quercetin standard solution at its maximum wavelength using a UV-Vis spectrophotometer. This was done by taking 1 mL of the quercetin solution and adding 1 mL of 10% $AlCl_3$ and 8 mL of 5% acetic acid. The study obtained a constant absorbance operating time. Analysis of the wavelength determination using a concentration of 100 ppm yielded a maximum wavelength of 229 nm. This maximum wavelength must be determined for use in sample content determination because it is at this wavelength that the maximum absorbance value is obtained, where the absorption change due to concentration is also maximum. [11]

Table 2. Results of determining operating time

Minutes to	Absorbance (229 λ)
0	0.229
1	0.228
2	0.227
3	0.229
4	0.229

Table 3. Absorbance value of quercetin standard solution

No.	Concentration (ppm)	Absorbance Average
1	40	0.237
2	60	0.238
3	80	0.511
4	100	0.546
5	120	0.774

The standard curve is intended to calculate the concentration of *Averrhoa bilimbi* leaves. The determination of the standard curve is obtained by reading the absorbance value of the quercetin standard solution with concentrations of 40 ppm, 60 ppm, 80 ppm, 100 ppm, and 120 ppm. The absorbance value of the quercetin standard solution can be seen in Table 3. Based on Table 3, the linear regression equation is obtained, namely Y = 0.0069x - 0.0916, and the correlation coefficient (r) is 0.92.

Determination of Flavonoid Content in *Averrhoa bilimbi* leaves

Flavonoids in *Averrhoa bilimbi* leaves extract were determined by entering absorbance values into a quercetin standard curve, resulting in an average total flavonoid content of 37.3 mgQE/g extract. The higher the flavonoid content, the greater the flavonoid's antioxidant benefits . [12]

Results of Antioxidant Activity Testing with a UV-Vis Spectrophotometer

Measurement of antioxidant activity using the DPPH method utilizes spectrophotometric principles. A compound with antioxidant activity will donate its hydrogen atom to bind with DPPH, forming reduced DPPH, indicated by a loss of purple color to pale yellow, accompanied by a decrease in absorbance. Absorbance

measurements in this study were performed at a maximum wavelength (λmax) of 515 nm for the DPPH solution, with absorbance being the maximum wavelength that can be used for measurements using the DPPH method as stated by.[5] The measurement data in Table 4 indicates DPPH radical scavenging activity, as evidenced by a decrease in DPPH radical absorbance values caused by the test samples at various concentrations and an increase in the percentage (%) of antioxidant activity. This is also visible with the naked eye, as the purple color fades and becomes slightly yellowish after a 30-minute incubation period. [7]

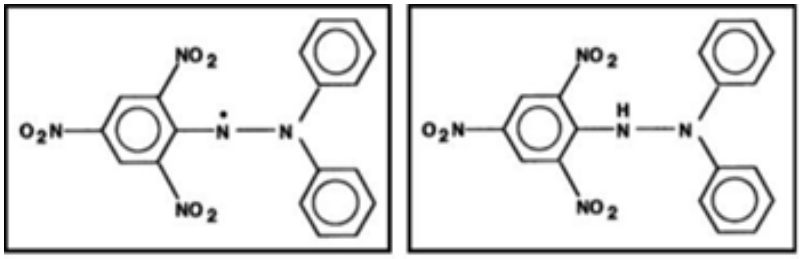

Figure 1. DPPH radical inhibition reaction (Molyneux, 2004)

This color change occurs due to the presence of compounds in the sample that donate hydrogen atoms to the DPPH radical so that it is reduced to a more stable form, namely DPPH-H (1,1-*diphenyl-2-picrylhydrazine*) (Figure 1) from the results of measuring the n-hexane fraction of *Averrhoa bilimbi* leaves extract shows that the higher the sample concentration, the lower the absorbance value of the DPPH solution in methanol. [1]

Table 4. Results of Antioxidant Activity Measurement of *Averrhoa bilimbi* leaves Extract Samples in UV-Vis Spectrophotometry

Concentration (ppm)	Abs Average (4 Replicate)	% Inhibition	Regression Equation	IC_{50} (µg/mL)
Blanko	0.012	0	Y= 0.3862x + 7.5293	6.58
25	0.223	20.63%	R= 0.956	
50	0.215	22.94%		
75	0.215	25.26%		
100	0.141	49.73%		
125	0.138	50.04%		
150	0.088	68.58%		

The regression equation between the concentration of *Averrhoa bilimbi* leaves extract sample (x-axis) and the percentage (%) of antioxidant activity (y-axis) is y = 0.3862x + 7.5293 with a correlation coefficient value (R^2) = 0.956. The positive correlation coefficient value illustrates that with increasing concentration of n-hexane fraction, the antioxidant activity is also greater. This relationship can be seen in the picture. [8]

The parameter used in testing antioxidant activity by capturing DPPH radicals is the Inhibitory Concentration (IC_{50}) value. The smaller the IC_{50} value, the more active the sample is as an antioxidant. Based on the linear regression equation obtained, the IC_{50} value or the concentration required to capture 50% of the DPPH radicals from the n-hexane fraction is 6.58 µg/mL. This value indicates that the n-hexane fraction of *Averrhoa bilimbi* leaves extract has very strong antioxidant activity, because it has an IC_{50} value ranging from 0-50 µg/mL. The positive control used in this study was Ascorbic acid. The use of a positive control was intended to compare how strong the antioxidant potential

contained in the n-hexane fraction of *Averrhoa bilimbi* leaves extract is with the antioxidants contained in the n-hexane fraction of *Averrhoa bilimbi* leaves extract.

Table 5. Results of Measurement of Antioxidant Activity of Ascorbic acid in UV-Vis Spectrophotometry

Concentration (ppm)	Abs Average (4 Replicate)	% Inhibition	Regression Equation	IC_{50} (µg/mL)
Blanko	0.012	0	Y= 0.0552x + 20.309	2.45
25	0.221	21.88%	R= 0.9883	
50	0.234	22.86%		
75	0.211	24.90%		
100	0.208	25.97%		
125	0.208	26.57%		
150	0.201	28.28%		

The linear regression equation obtained from the results of ascorbic acid antioxidant activity measurements in table 5 is y = 0.0552x + 20.309 with a correlation coefficient value of 0.9883. The IC_{50} value obtained from the results of ascorbate acid measurements is 2.45 µg/mL. This value indicates that ascorbic acid has very strong antioxidant activity, because it has an IC_{50} value of less than 50 µg/mL. The relationship between concentration and percentage inhibition of ascorbate acid can be seen in table 5.

The antioxidant activity test showed that the n-hexane fraction of *Averrhoa bilimbi* leaf ethanol extract exhibited very strong antioxidant activity, with an IC_{50} value of 6.58 µg/mL. In comparison, ascorbic acid used as the positive control showed an IC_{50} value of 2.45 µg/mL, indicating stronger antioxidant activity than the sample fraction. According to antioxidant activity classification, compounds with IC_{50} values below 50 µg/mL are categorized as very strong antioxidants. Therefore, both the n-hexane fraction and ascorbic acid were classified as very strong antioxidants, although ascorbic acid exhibited higher antioxidant potency due to its lower IC_{50} value.

The antioxidant activity observed in this study may be associated with the presence of flavonoid compounds and other phytochemical constituents in the extract. Flavonoids are known to act as free radical scavengers by donating hydrogen atoms or electrons to stabilize free radicals and inhibit oxidative chain reactions. The total flavonoid content obtained in this study was 37.3 mg QE/g extract, suggesting that flavonoid compounds may contribute to the antioxidant activity of the n-hexane fraction.

The antioxidant activity obtained in the present study was stronger than that reported by [18], who found that ethanol extract of *Averrhoa bilimbi* leaves had an IC_{50} value of 16.99 µg/mL. Similarly,[3] reported that 70% ethanol extract of *Averrhoa bilimbi* fruit exhibited an IC_{50} value of 74.63 ppm, which was categorized as a strong antioxidant. The lower IC_{50} value obtained in the present study indicates higher antioxidant potency compared to those previous studies. [9]

Differences in antioxidant activity among studies may be influenced by several factors, including plant part used, extraction solvent, fractionation process, phytochemical composition, environmental growing conditions, and extraction methods. In this study, fractionation using n-hexane may have concentrated certain bioactive compounds

responsible for antioxidant activity, resulting in stronger free radical scavenging activity. Fractionation separates compounds based on polarity, allowing specific metabolites to become enriched in particular fractions. [2]

Several phytochemical compounds reported in *Averrhoa bilimbi* leaves, including flavonoids, phenolics, tannins, and terpenoids, are known to possess antioxidant properties. These compounds can donate electrons to neutralize DPPH free radicals, thereby reducing oxidative stress. The strong antioxidant activity demonstrated by the n-hexane fraction suggests that semi-nonpolar compounds contained in the fraction may contribute significantly to its antioxidant potential.

The strong antioxidant activity observed in the n-hexane fraction may also be associated with the presence of less polar flavonoid compounds, such as flavonoid aglycones. Compared with glycosylated flavonoids, flavonoid aglycones generally possess higher lipophilicity, allowing better interaction with free radicals in lipid environments and improved membrane permeability. In addition, hydroxyl groups present in flavonoid structures play an important role in hydrogen donation and radical stabilization mechanisms. Therefore, the flavonoid content and other semi-nonpolar phytochemicals present in the n-hexane fraction may contribute synergistically to the antioxidant activity observed in the DPPH assay.

Overall, the findings of this study indicate that the n-hexane fraction of *Averrhoa bilimbi* leaf ethanol extract has promising potential as a natural antioxidant source and may be further developed for pharmaceutical, nutraceutical, or functional food applications. However, further studies are needed to identify specific active compounds responsible for the antioxidant activity and to evaluate their biological effects through in vivo studies.

LIMITATION

This study has several limitations. First, the antioxidant activity evaluation was conducted only using the DPPH method, which measures free radical scavenging activity in vitro and may not fully represent antioxidant activity under biological conditions. Second, the study focused only on the n-hexane fraction of *Averrhoa bilimbi* leaf ethanol extract without identification or characterization of specific active compounds responsible for the observed antioxidant activity. Third, the total flavonoid content was measured quantitatively, but individual flavonoid compounds were not analyzed using advanced analytical techniques such as HPLC or LC-MS. In addition, this study did not evaluate other phytochemical constituents that may also contribute to antioxidant activity. Finally, the biological activity of the extract was not assessed through in vivo studies; therefore, further research is needed to confirm its pharmacological potential, safety, and mechanism of action in biological systems.

CONCLUSION

Based on the research that has been done, it can be concluded that the total flavonoid content of the n-hexane extract of *Averrhoa bilimbi* leaves (*Averrhoa bilimbi* L.) quantitatively ranges from 37.5 mgQE/g extract or 3.75%. The n-hexane fraction of *Averrhoa bilimbi* leaves extract has antioxidant activity, which is indicated by an IC_{50} value of 6.58 µg/mL, while ascorbic acid, as a positive control, has an IC_{50} value of 2.45 µg/mL.

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AUTHOR CONTRIBUTION STATEMENT

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