Antioxidant Activity Test and Determination of Total Phenolic Content of Combination Ethanol Extract of Guava Leaves and Green Betel Leaves

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Abstract. Guava and green betle leaves have been reported to provide numerous medicinal benefits, including hepatoprotective, anti-inflammatory, antidiabetic, antioxidant, antipyretic, and analgesic effects. The aim of this study was to ascertain the total phenolic content and the antioxidant activity of a mixture of ethanol extracts from green betel and guava leaves. The total phenolic content was determined using the folin ciocalteu method, and antioxidant activity was tested using the DPPH method. A UV-Vis spectrophotometer was used to assess both procedures. An 1:0, 2:1, 1:1, 1:2, and 0:1 ratio was utilized in the combination of guava leaf and green betel leaf ethanol extracts. The standard of reference for the antioxidant activity test was ascorbic acid. In comparison to the comparison concentrations of 1:0, 0:1, 2:1, 1:1, and 1:2 generated very strong antioxidant activity potentials in scavenging free radicals. The determination of total phenolic levels significantly showed differences in the amount of levels at all comparative concentrations. Based on results, it is concluded that the combination of guava leaf and green betel leaf ethanol extracts at a 2:1 ratio has the potential for very strong antioxidant activity and high total phenolic content when compared to other concentrations.

Keywords: Antioxidant, Combination of Extract, Green Betel Leaves, Guava Leaves, Total Phenolic Content

INTRODUCTION

An increase in unhealthy lifestyles, exposure to environmental pollution, and a less active lifestyle result in the risk of degenerative diseases (Karwiti et al., 2023). One of the main factors that contribute to the development of this degenerative disease is oxidative stress. Oxidative stress is a condition resulting from the ability of free radicals as oxidizing agents to obtain a stable state by attracting electrons from other molecules or cells in the body, so that they can trigger oxidative damage. (Sukweenadhi et al., 2020). This can be prevented by using antioxidants.

Antioxidants are chemical compounds that donate electrons to unpaired free radicals, thereby reducing the oxidizing effects of free radicals, so antioxidants are also

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known as compounds that can ward off free radicals. (Hidayati et al., 2017; Sukweenadhi et al., 2020). Natural compounds that have antioxidant activity with various working mechanisms can help prevent disease without causing negative effects/side effects. One of the natural compounds that has antioxidant potential is phenolic compounds (Chaudhary et al., 2023).

Phenolic compounds are the largest group of secondary metabolites in plants, where they are widely distributed in various plant organs at high levels such as in leaves, fruit, seeds or stems of the plant. (Zhang et al., 2022). Several plant sources that contain phenolic compounds and have been tested for their antioxidant activity are guava leaves and green betel leaves. Guava plants (Psidium guajava L.) were found to have high antioxidant activity (Angulo-López et al., 2021). Guava leaves contain the compound quercetin, which can be used to prevent premature skin aging and helps in curing cancer cells (Naseer et al., 2018). The green betel plant (Piper betle L.) has the main components, namely tannins, flavonoids (quercetin), eugenol, hydroxycavicol and chavibetol. The betel plant contains antioxidants from its extract which is believed to come from phenol and flavonoid compounds (Azahar et al., 2020). The antioxidant activity of guava leaves has been proven by (Darwis & Lubis, 2016) with an IC50 value of 22.39 ppm which shows very strong potential. Likewise, the antioxidant activity of green betel leaves has been carried out by (Gurning et al., 2021) with an IC50 value of 84,656 ppm which shows strong activity.

However, for the combination of guava leaves and green betel leaves, the potential for antioxidant activity proven by testing has not yet been obtained. Meanwhile, the results of research using a combination of two plant extracts that contain antioxidants can increase the potential for antioxidant activity to a higher level (Rudiana et al., 2023). This is proven by several studies regarding combinations of extracts, including a combination of ethanol extract of avocado leaves and guava leaves, which shows the results of strong antioxidant activity. (Douw & Wardani, 2023), a combination of basil and binahong leaf extracts with very strong antioxidant potential (Himawan et al., 2021). Therefore, research on the antioxidant activity of the combination of guava leaves and green betel leaves also needs to be carried out to obtain the highest activity, concentration and combination so that it can be used as an alternative natural medicinal preparation.

SUBJECT AND METHODS

Materials

The raw materials used are guava leaves and green betel leaves obtained in the area from Satyasakti Hamlet, Way Jepara District, East Lampung Regency, Lampung. Other ingredients used in this research were distilled water, ethanol, DPPH, folin-ciocalteu, gallic acid, Na_2CO_3, and vitamin C (ascorbic acid). The tools used in this research were a UV-Vis spectrophotometer, rotary evaporator, balance, measuring cup, jar, water bath, measuring flask, dropper pipette, volume pipette, filter paper, glass cuvette, oven, test tube and tube rack.

Sample Preparation

Guava leaves and green betel leaves that have been collected are then wet sorted, washed and dried using a combination of indirect sunlight and an oven at 50^{the}C. After the sample is dry, dry sorting is carried out and crushed to obtain simplicia powder. Guava leaf powder and green betel leaves were stored in different airtight plastic bags until the extraction process was carried out several days after drying the samples.

Extraction

Guava leaf simplicia powder and green betel leaf were each extracted using 70% ethanol using the maceration method. The sample was weighed as much as 500 g and then soaked in ethanol solution in a closed container protected from sunlight. This process is carried out 1 x 24 hours for 3 days with two repetitions and replacement of new solvent. The extraction results are filtered using Whatman paper to separate the dregs and filtrate. Next, the filtrate was concentrated at 40^{the}C using a rotary evaporator until the solvent evaporates so that a thick extract is obtained.

Phytochemical Screening

Phytochemical screening was carried out to see the chemical compounds contained in green betel leaf extract and guava leaf extract. Phytochemical screening was carried out based on the method described by (Harbone, 1998).

a. Phenolics: A total of 5 ml of extract is added with 3-4 drops of 1% FeCl3 solution. The formation of a blue or blue-black color indicates the presence of phenolics.
b. Flavonoids: A total of 2 ml of extract was added with 0.1 g Mg and 5 drops of concentrated HCL. The appearance of yellow, orange to color Red indicates the presence of flavonoids.
c. Alkaloids: As much as 1 ml extract Add 2 drops of chloroform and 2 drops of Mayer's reagent. The appearance of a white precipitate indicates the presence of alkaloids.
d. Saponins: Add 0.5 ml of extract to 5 ml of water, then shake vigorously for 10 seconds. Formation of foam during ±10 minutes and a height of 1-10 cm indicates positive saponin.
e. Tannins: A total of 2 ml of extract is added with 2-3 drops of 10% FeCl3 solution. The appearance of a dark blue or blackish green color indicates the presence of tannins.
f. Terpenoids and steroids: Add 1 ml of 2 ml of chloroform, 10 drops of acid to 1 ml acetate anhydrous (CH_3anhydrous COOH) and 3 drops of sulfuric acid through the wall of the test tube. The appearance of a blue or green color indicates the presence of steroids, while positivity for terpenoids indicates their presence color red or purple.

Table 1: Concentration of the combination of guava leaf and green betel leaf extracts.

Comparison	Guava leaves	Green betel leaves
1:0	100 mg	-
1:2	33 mg	67 mg
1:1	50 mg	50 mg
2:1	67 mg	33 mg
0:1	-	100 mg

Antioxidant Activity Test

Preparation of DPPH reagent (100 ppm): 5 mg DPPH was dissolved with ethanol in a 50 ml volumetric flask. The solution is stored at room temperature and protected from sunlight.

Preparation of standard solution of ascorbic acid (1000 ppm): Weigh 100 mg of ascorbic acid and dissolve it with ethanol in a 100 ml volumetric flask. after that, a concentration series of 2, 4, 6, 8 and 10 ppm was made.

Preparation of solution up to (1000 ppm): Extracts of guava leaves and green betel leaves are made in a concentration ratio of 1:0, 1:2, 1:1, 2:1, 0:1. Each sample concentration was made into a concentration series, namely 20, 40, 60, 80 and 100 ppm.

Antioxidant activity is carried out using DPPH as a free radical. A total of 2 ml was taken from each concentration of the test solution and standard ascorbic acid solution, then 2 ml of DPPH solution was added to each test tube, and incubated for 30 minutes. Through the highest wavelength (λ), namely 521 nm, the absorbance is measured and the percentage of DPPH radical inhibition is expressed as the IC value$_{50}$ which means that guava leaf and green betel leaf extracts can reduce 50% of DPPH free radicals.

The inhibition percentage is calculated using the formula:

$$\% \text{ inhibition} = \frac{Abs\ blanko - abs\ sample}{abs\ blanko} \times 100\%$$

Nilai IC_{50} calculated from the equation formed between the concentration of the test solution (x-axis) and the % inhibition (y-axis), with the equation y = a + bx, and the IC value$_{50}$ can be calculated using the formula:

$$IC_{50} = \frac{50 - a}{b}$$

Total Phenolic Content Test

Gallic acid standard solution (1000 ppm): Weigh 100 mg and dissolve with 60 ml of ethanol in a 100 mL volumetric flask, homogenize, then add enough ethanol to the mark.

Phenolic content was determined using a UV-Vis spectrophotometer. Samples of guava leaf and green betel leaf extracts were made in a ratio of 1:0, 1:2, 1:1, 2:1, 0:1 and made with 70% ethanol. Add 0.5 ml of reagent solution *folin-ciocalteu* and 7.5 ml of distilled water, wait for 2 minutes. Then, in a volumetric flask, 7% Na2CO3 is added up to the limit mark. After incubating for 40 minutes, transfer to a cuvette, and measure the absorbance of the solution at its maximum wavelength. A spectrophotometer was used to read absorbance at 765 nm. Analysis was carried out 3 times. Gallic acid was used to create a standard curve.

Data Analysis

Research data analysis was carried out using *software* IBM SPSS Statistics version 26. The research data was tested for normality using a test *Shapiro-Wilk*. The correlation between antioxidant activity and total phenolic content was assessed by calculating the Pearson correlation coefficient (r). Data analysis from the experimental results was carried out statistically at a confidence level of 95% (*p-value* < 0,05) (Milella et al., 2023).

RESULTS

Results of phytochemical screening on ethanol extract of guava leaves and green betel leaves.

Table 2: Phytochemical screening results.

Secondary Metabolytes Compounds	Guava Leaves	Green Betle Leaves	Observation
Phenolics	+++	++	Blackish blue coloration
Flavonoids	+++	+++	yellow or orange coloration
Alkaloids	+	+	presence of a white precipitate
Saponins	+++	+++	Stable persistent froth
Tannins	+++	+++	Blackish green coloration
Terpenoids	+++	+++	yellowish red coloration
Steroids	-	-	no color change

Notes: - = has no content (no color or foam appears); + = weak positive (light color or little foam); ++ = medium positive (quite dark color or quite a lot of foam); +++ = strong positive (very dark color or lots of foam).

Table 2 shows that guava leaves and green betel leaves contain secondary metabolite compounds, namely phenolics, flavonoids, alkaloids, saponins, tannins and terpenoids. The content of secondary metabolite compounds in guava leaves with very strong results is in phenolic compounds, flavonoids, tannin saponins and terpenoids, while in green betel leaves with very strong results it is shown in the content of flavonoids, saponins, tannins and terpenoids.

Table 3: Antioxidant activity test results.

Sample	IC_{50} Value (µg/mL)	Category
Ascorbic Acid	5,514	Very strong
Guava Leaves Extract Ethanol (1:0)	71,184	Strong
Green Betle Leaves Extract Ethanol (0:1)	77,737	Strong
Guava and Green Betle Leaves Extract Ethanol (1:1)	46,294	Very strong
Guava and Green Betle Leaves Extract Ethanol (1:2)	56,267	Strong
Guava and Green Betle Leaves Extract Ethanol (2:1)	16,890	Very strong

Notes: antioxidant activity category with IC50 value; <50 = very strong; 50-100 = strong; 101-150 = medium; 151-200 = weak; >200 = very weak

Table 3 shows based on IC values$_{50}$ The ascorbic acid standard had potentially very strong antioxidant activity (5.514 µg/mL) compared to the sample group. However, the results of antioxidant activity in the combination group of ethanol extract of guava leaves and green betel leaves with a ratio of 2:1 showed the results of obtaining IC values$_{50}$ which is potentially very strong (16,890 µg/mL) when compared with other combination groups.

Table 4: Results of determining total phenolic content.

Sample	Total Phenolics Compound (mg GAE/g)			Average (mg GAE/g)	SD
	I	II	III		
Guava Leaves Extract Ethanol (1:0)	42,286	43,873	44,508	43,556	± 1,145
Green Betle Leaves Extract Ethanol (0:1)	39,429	41,016	41,333	40,593	±1,020
Guava and Green Betle Leaves Extract Ethanol (1:1)	52,762	54,667	53,397	53,608	±0,970
Guava and Green Betle Leaves Extract Ethanol (1:2)	55,937	54,984	54,667	55,196	±0,661
Guava and Green Betle Leaves Extract Ethanol (2:1)	62,286	67,365	63,556	64,402	±2,643

Notes: GAE (Gallic Acid Equivallen), SD (Standar Deviation)

Based on table 4, it shows that the ethanol extract from plants combined with the total phenolic content contained is greater. The highest levels of results obtained from the combination of ethanol extract of guava leaves and green betel leaves were shown at a concentration ratio of 2:1 combination of ethanol extract (64.402 mg GAE/g).

Table 5: Correlation test results for total phenolic content and IC value$_{50}$.

	Up to total phenolics	Nilai IC$_{50}$
Up to total phenolics	1	-0,952**
Nilai IC$_{50}$	-0,952**	1

Notes: ** (significant correlation)

Table 5 shows that the total phenolic content to the IC50 value is related to the correlation coefficient value obtained, namely -0.952.

DISCUSSION

Phytochemical Screening

The results of phytochemical screening of ethanol extracts of guava leaves and green betel leaves contain phenolic compounds, flavonoids, alkaloids, saponins, tannins and terpenoids. Based on research results, guava leaf extract contains stronger phenolic compounds compared to green betel leaf extract. Phenolics have antioxidant,

chemopreventive, and various pharmacological properties. Phenol acts as an efficient free radical and reactive oxygen species (ROS) scavenger (Rudrapal et al., 2022).

Both plant extracts also did not show the presence of steroid compounds. This is different from research conducted by (Sudira et al., 2019) which states that guava leaf extract contains phenolic compounds, flavonoids, saponins, tannins, alkaloids, terpenoids and steroids. Meanwhile, research conducted by (Jumrah et al., 2023) shows that green betel leaf extract contains alkaloids, terpenoids, steroids, saponins, flavonoids and tannins. Differences in the results of phytochemical screening identification obtained from research that has been carried out can be caused by the influence of environmental factors, such as temperature, carbon dioxide, lighting, ground water, soil salinity and soil fertility. (Parbuntari et al., 2019).

DPPH radical Scavenging Activity

Testing the antioxidant activity of ethanol extract of guava leaves and green betel leaves using the DPPH method, this method is used to determine antioxidant activity by measuring the overall antioxidant capacity of the sample so that the hydrogen capture reaction by DPPH from the antioxidant substance is known. (Handayany et al., 2018; Theafelicia & Wulan, 2023). The magnitude of the antioxidant's ability to ward off free radicals can be expressed as the IC value$_{50}$ (*inhibitor concentration)*, namely the concentration of the test solution (antioxidant) which is able to reduce or inhibit 50% of the initial DPPH radicals within a certain time interval. The lower the IC value$_{50}$, the higher the antioxidant ability of DPPH in eliminating radicals. IC value$_{50}$ quantitatively describe the radical scavenging affinity (Gulcin & Alwasel, 2023).

Nilai IC_{50} in each ethanol extract combination of guava leaves and green betel leaves, the ratio (1:0) was obtained, namely 71,184 µg/mL, the ratio (0:1) was 77,737 µg/mL, the ratio (1:1) was of 46,294 µg/mL, ratio (1:2) of 56,267 µg/mL, ratio (2:1) of 16,890 µg/mL with positive control Vitamin C of 5,514 µg/mL. These results show that the combination (2:1) has the potential for very strong antioxidant activity as indicated by the IC value$_{50}$ the lowest. This happens because the 2:1 combination contains guava leaf extract in a greater concentration. Therefore, guava leaf extract is thought to provide the main contribution to antioxidant activity, which is supported by the results of antioxidant activity tests on single extracts of guava leaves which are higher than those of green betel leaves. The effect of increasing antioxidant activity was also caused by differences in the amount of secondary metabolite compounds in the ethanol extract of guava leaves and green betel leaves. Judging from the phenolic compounds contained in the ethanol extract of guava leaves, it is stronger than green betel leaves. This is in accordance with the statement (Maslukhah et al., 2016) that the higher the content of phenolic compounds in a material, the higher the antioxidant content.

Total Phenolics Content

The total phenolic content of the ethanol extract of the combination of guava leaves and green betel leaves respectively had a total phenolic content of 43,556 mg GAE/g (1:0), 40,593 mg GAE/g (0:1), 53,608 (1:1), (1:2), and (2:1). These results show that the 2:1 combination of extracts has a higher total phenolic content compared to

other comparison concentrations. This shows that when two extracts are combined and one of the extracts is increased, the combination ratio provides higher total phenolic levels than the combination of extracts with the same ratio. It is estimated that the ethanol extract of guava leaves plays a significant role in increasing total phenolic levels, considering that the results of measuring single guava leaf extracts have high total phenolic levels. The increase in total phenolic content in the 2:1 ratio is thought to be due to the use of a greater concentration of guava leaf ethanol extract. This is in accordance with the statement (Firdayani & Agustini, 2015) stated that the higher the concentration used, the higher the content of bioactive compounds in the extract.

Relationship between Total Phenolic Content and Antioxidant Activity

Relationship between total phenolic content and IC value$_{50}$ (antioxidant activity) was tested by statistical analysis, which showed a very strong correlation between total phenolic content and antioxidant activity (IC value$_{50}$), with a correlation coefficient of -0.952 (p-value < 0.01). The negative correlation coefficient indicates that an increase in total phenolic content is associated with a decrease in the IC value$_{50}$.

The relationship formed between phenolic compounds and antioxidant activity can be caused by phenolic compounds which are important antioxidants working through mechanisms *transfer* hydrogen atoms to free radicals (Kumar et al., 2014). The potential of phenolic compounds as antioxidants comes from the presence of hydroxyl groups in their phenol structure. This hydroxyl group donates a hydrogen atom when reacting with radicals through a mechanism *transfer* electrons, thereby inhibiting the oxidation process (Allo et al., 2022). The ability of the phenol group (-OH) to bind free radicals by giving its hydrogen atom through the process *transfer* These electrons cause phenol to change to phenoxyl radical. Phenoxyl radicals which are formed as a result of the reaction of phenol with free radicals will then stabilize themselves through a resonance effect. Therefore, phenol derivatives are good hydrogen donors that can inhibit reactions that occur with radical compounds. Phenolic compounds are also known as radical inhibitors (Hilma et al., 2020).

CONCLUSIONS

Both guava leaf extract and green betel leaf ethanol extract had moderate to high antioxidant activity at all extract concentration ratios. The antioxidant activity of guava leaves and green betel leaves was 2:1 higher than the antioxidant activity of all extract concentrations. This is because the total phenolics at the 2:1 concentration are higher than the total phenolics at all concentrations.

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