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Antioxidant Activity Test of Red Belt Leaf Extract (*Piper crocatum*) and Black Belt Leaf (*Piper betle var nigra*) Using The DPPH Method

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Abstract: Antioxidant Activity Test Of Red Belt Leaf Extract (*Piper crocatum*) And Black Belt Leaf (*Piper betle var nigra*) Using The DPPH Method. Betel leaves (*Piper betle L.*) are among the most important agricultural products in the world, because the need for betel leaves as a raw material for traditional medicine has increased in the last decade. Betel leaves are known to contain phenolic compounds which have the potential to act as antioxidants. This study aims to determine the antioxidant activity of extracts of red betel leaves (*piper crocatum*) and black betel leaves (*piper betle var nigra*) using the dpph (*1,1-diphenyl-2-picrihydrazyl*) method. The research results showed that the antioxidant activity of the ethanol extract of red betel leaves, IC_{50}, was 45,92 µg/mL, the ethanol extract of black betel leaves obtained an IC_{50} value of 87,09 µg/mL, and the vitamin C comparator obtained an IC_{50} value of 4,11. Red betel leaf extract has higher antioxidant activity than black betel leaf extract.

Keywords: betel leaf (*piper betle L.*), antioxidant, dpph

Abstrak: Uji Aktivitas Antioksidan Ekstrak Daun Sirih Merah (Piper crocatum) dan Daun Sirih Hitam (Piper betle var nigra) dengan Metode DPPH. *Daun sirih (Piper betle L.) termasuk kedalam produk pertanian terpenting di dunia, karena kebutuhan daun sirih sebagai bahan baku obat tradisional dalam dasa warsa terakhir mengalami peningkatan. Daun sirih diketahui mengandung senyawa fenolik yang berpotensi sebagai antioksidan. Penelitian ini bertujuan untuk mengetahui aktivitas antioksidan ekstrak daun sirih merah (piper crocatum) dan daun sirih hitam (piper betle var nigra) dengan metode dpph (1,1-difenil-2-pikrihidrazil). Hasil penelitian menunjukkan bahwa antioksidan aktivitas ekstrak etanol daun sirih merah IC_{50} sebesar 45,92 µg/mL, ekstrak etanol daun sirih hitam diperoleh nilai IC_{50} sebesar 87,09 µg/mL, dan pembanding vitamin C diperoleh nilai IC_{50} 4,11. Ekstrak daun sirih merah memiliki aktivitas antioksidan lebih tinggi dibandingkan ekstrak daun sirih hitam.*

Kata kunci: *daun sirih (piper betle L.), antioksidan, dpph*

▪ INTRODUCTION

The betel plant (*Piper betle L.*) is one of the most important agricultural products in the world, because the need for betel leaves as a raw material for traditional medicine has increased in the last decade. Apart from the fruit which is the main product in processing, research also tested, there are leaves which are a by-product which shows similar contents, there are even more complex contents in the leaves which are very beneficial for human health. Good effects for the body are found in every part of the betel leaf, including red betel leaf and black betel leaf.

Red betel leaves were researched by Dewi in 2019 who stated that red betel leaves contain alkaloid metabolites, flavonoids, tannins and essential oils with antioxidant and antibacterial activity (Dewi *et al*., 2019). Meanwhile, black betel leaves contain the active substances alkaloids, flavonoids, tannins, saponins, phenolic compounds, carotenoids and steroids. This is proven by research conducted by Hastuty in 2020 which stated that black betel leaves were identified as containing alkaloids, flavonoids, tannins, saponins, phenolic compounds, carotenoids and steroids (Hastuty., 2020).

The flavonoid content in red betel leaf and black betel leaf extract is believed to inhibit free radicals in DPPH. Free radicals are molecules that lose one electron from their lone pair or in other words are the result of homolytic separation of a covalent bond. As a result of this homolytic breakdown, a molecule will split into free radicals which have unpaired electrons. Electrons need a partner to balance their spin value, so that radical molecules become unstable and will easily react with other molecules to form new radicals. Free radicals are the triggers of degenerative diseases such as cancer, diabetes mellitus and Alzheimer's (Fakriah *et al*., 2019).

To avoid the accumulation of free radicals which can cause cancer, it is necessary to consume antioxidant compounds. Antioxidants play a role in reducing, neutralizing and limiting the formation of free radicals in the body. The main function of antioxidants is to provide electrons to free radicals, so that free electrons in the body end up pairing. This process is able to inhibit or stop the damage that occurs in the body due to free radicals. (Arnanda *et al*., 2019).

Therefore, researchers were interested in testing the antioxidant activity of red betel leaf and black betel leaf extracts using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method. Where red betel leaves and black betel leaves will be used as samples. Because nowadays red betel leaves and black betel leaves are rarely used by people.

▪ METHOD

Tools And Materials

The tools used are analytical balance (Mettler Toledo – ME204), beaker glass (100 mL), measuring cup (50 mL), stir bar (20 cm), knife, measuring flask (100 mL), measuring flask (50 mL), funnel, Whatman filter paper No. 1, oven, blender, brown bottle (100 mL), measuring pipette (25 mL), micropipette, drop pipette (3 mL), vortex, vial (20 mL), sieve (25 mesh), stopwatch, UV-Vis spectrophotometry, Rotary Evaporator (Agustriani & Wijaya, 2022).

The ingredients used are red betel leaves, black betel leaves, DPPH powder (Merck), 96% ethanol (C_2H_5OH), ethanol p.a (C_2H_5OH), vitamin C ($C_6H_8O_6$), distilled water (H_2O).

Research Procedure

Simplicia Powder Preparation

1.5 kg of red betel leaves (*piper crocatum*) and black betel leaves (*piper betle var nigra*) each are cleaned of adhering dirt using running water. Then cut it into small pieces and air it at room temperature until dry. Next, mash it using a blender and sift it with a 25 mesh sieve.

Preparation of Betel Leaves Ethanol Extract

Extraction was carried out using the maceration method. As much as 300 grams of simplicia powder was macerated with 96% ethanol for 3 days. It is stirred every 2 hours and remaceration is carried out after that it is filtered with Whatman No. paper. 1 until the filtrate is obtained. The filtrate is then evaporated with a Rotary Evaporator until a thick extract is obtained.

Preparation of 0.1 mM DPPH Blank Solution

DPPH powder was weighed as much as 3.9 mg and dissolved in ethanol p.a to 100.0 mL.

Making Vitamin C Sample Solutions and Dressings

Weighed 5 mg each of red and black betel leaf extract, then dissolved it with ethanol p.a in a 50 mL volumetric flask to the mark, until a concentration of 10% was obtained. From a concentration of 10% it was diluted to concentrations of 10, 20, 40, and 80 µg/ml by adding ethanol p.a to each treatment.

1 mg of Vitamin C was dissolved in distilled water to 100 mL and obtained a concentration of 1%, from this concentration a series of concentrations of 1, 2, 4, and 8 µg/ml was made.

Determination of the Maximum Absorption Wavelength of DPPH

Measurement of wavelength (λ) by measuring 4.0 mL of 0.1 mM DPPH solution using UV-vis spectrophotometry with a wavelength of 400-600 nm to obtain an absorbance of ± 0,2-0,8.

Determining the operating time of 0.1 mM DPPH solution

Determining the operating time was determined by reacting 50 µl of the vitamin C comparison standard plus 4,0 mL of 0,1 mM DPPH solution, homogenizing with a vortex for 1 minute and then adsorbance measurements were carried out every five minutes for 1 hour at the maximum λ that had been obtained.

Blank Absorbance Measurement

Blank measurements using 4 mL of 0,1 mM DPPH solution were measured with a wavelength at maximum λ.

Antioxidant Activity Test with DPPH Method

A total of 4.0 mL of 0,1 mM DPPH was put into the vial, added 50 µL of ethanol extract of betel leaves with various concentrations then vortexed for 1 minute until homogeneous and incubate for 30 minutes at 37ºC, read the absorbance at the maximum λ obtained. Test activity standard vitamin C standard with the same treatment.

Data Analysis

Antioxidant activity in samples is determined from the amount of DPPH radical absorption inhibition by calculating the percentage of DPPH absorption inhibition.

$$\% \text{ inhibition} = \frac{control\ absorbance - sample\ absorbance}{control\ absorbance} \times 100\%$$

Information :
Control absorbance : Absorption of DPPH radical solution
Sample absorbance : Absorption of the sample solution in the DPPH solution

The calculation of the IC_{50} value uses a linear regression equation. IC_{50} is a value or number that indicates the concentration in the sample that is able to inhibit the activity of a radical by 50%. In determining the IC_{50}, the standard curve equation of percent inhibition is needed as the y axis and the concentration of antioxidant extract as the x axis. Where the smaller the IC_{50} value, the higher the antioxidant activity

▪ RESULT AND DISCUSSION

In this experiment, a thick extract of black betel leaves was obtained with a yield of 22% and thick extract of red betel leaves with a yield of 24%.

Maximum Absorption Wavelength

Determining the maximum wavelength (λ) aims to ensure that the absorbance of the sample is at the maximum wavelength so that maximum results are obtained. This is related to the sensitivity of the analysis, where the measurement of the change in absorbance that occurs for each concentration unit is the greatest at the maximum wavelength so that it will be obtained the best analysis sensitivity (Rantung *et al*., 2021).

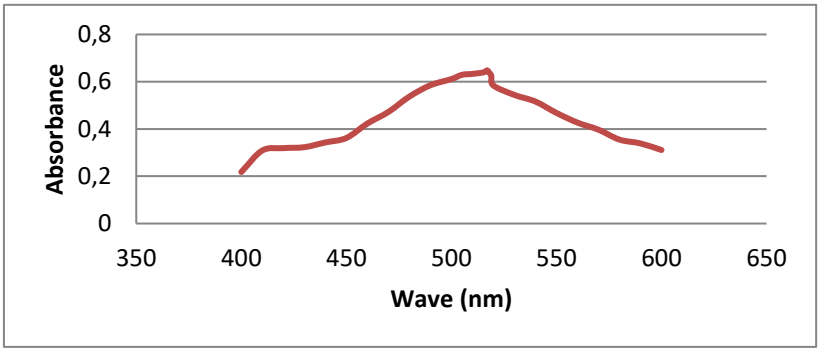

Figure 1. Maximum Wavelength Curve

From the scanning results, the maximum absorption wavelength of DPPH was obtained at 517 nm with an absorbance of 0,648. Measurements were carried out at a wavelength of 517 nm because DPPH provides strong absorption at this wavelength, so changes in absorbance due to reactions with antioxidants can be measured accurately (Damanis *et al*., 2020). From the results of the scanning carried out, it can be seen that the maximum absorption wavelength of DPPH radicals is in accordance with the theoretical maximum wavelength, namely at 517 nm.

Determination of Operating Time

The purpose of determining the operating time is to determine the stable measurement time of a compound, which can be seen by observing the absorbance. Determining the operating time is based on the time the absorbance value begins to stabilize, which is indicated by the difference in the absorbance value for each time

interval. The longer the measurement time, the greater the chance of damage and degradation of the compound being tested (Sadik, 2023).

Absorbance measurements for determining operating time were carried out by measuring the positive control standard solution of DPPH with vitamin C at 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 minutes using the wavelength The maximum obtained is at 517 nm. The operating time measurement results are shown in the graph below.

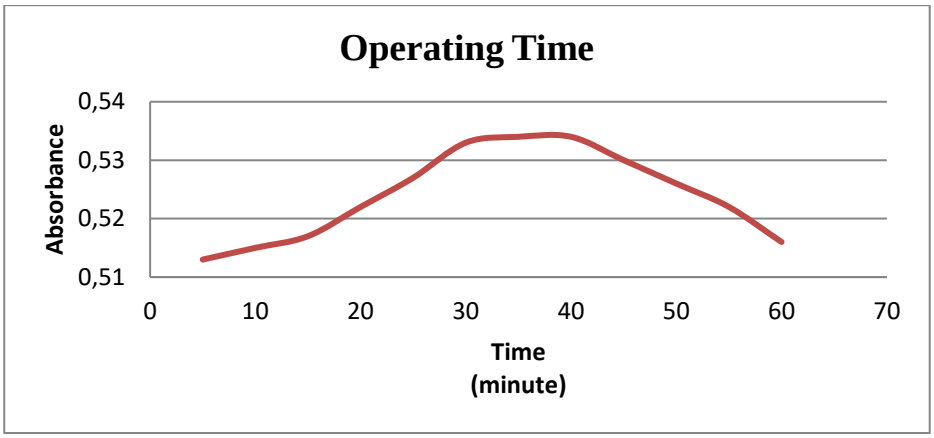

Figure 2. Operating time wavelength curve

From the results of absorbance measurements for determining operating time in the comparison solution which was added with 0.1 mM DPPH solution, the results were stable within 30 - 40 minutes with an absorbance of 0,534. Measurements at 30 minutes provide a consistent time point for evaluating the effectiveness of antioxidants in samples, because at this point the reaction has progressed well, the reaction speed between DPPH and antioxidants reaches a peak or balanced point (Kausar *et al*., 2023).

Antioxidant Activity with DPPH Method

The DPPH method was chosen for testing antioxidant activity because of the many advantages of this method. According to Al-Hmoud *et al* (2019), the advantages of the DPPH method are that it is simple, easy, fast, sensitive, requires a small amount of sample, and is easy to apply because the DPPH radical compound is relatively stable compared to other methods. This method has a linear relationship between antioxidant concentration and the resulting decrease in absorbance.

The weakness of the DPPH method is that the DPPH compound is very sensitive to light so that the DPPH compound is easily damaged when exposed to light. This is in accordance with Alam *et al* (2021) who stated that DPPH is sensitive to light and can reduce the accuracy of the reduction activity inspection process. Apart from that, the DPPH compound is also sensitive to temperature, where the right temperature for storage is at a low temperature, so storing it at the wrong temperature will damage the DPPH compound.

Preliminary tests were carried out as a first step with the aim of knowing and determining the presence of antioxidant activity in red betel leaf and black betel leaf extracts. This qualitative test was carried out by looking at the color comparison of a standard solution of betel leaf extract which was reacted with 0.1 mM DPPH, a negative

control (DPPH solution only), and a positive control (DPPH solution that had been mixed with a comparison standard solution of vitamin C).

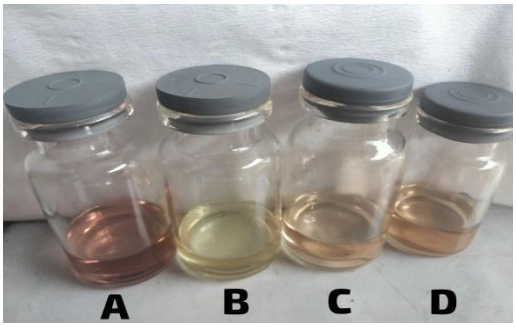

Figure 3. (A) DPPH Solution, (B) Vitamin C + DPPH Solution, (C) Black betel extract, (D) Red betel extract + DPPH Solution

From the test results, vitamin C and extracts of red betel leaves and black betel leaves showed positive antioxidant activity. The color change from purple to yellow in the vitamin C solution indicates that the compound is carrying out a hydrogen atom donating reaction to the DPPH free radical, as well as the color change in the betel leaf solution.

Antioxidant testing using the DPPH method in this study used ascorbic acid or vitamin C as a comparison (positive control). Axorbic acid functions as a secondary antioxidant by capturing free radicals and preventing chain reactions (Afriani *et al*., 2019).

Axorbic acid is a water-soluble vitamin. Axorbic acid functions as an effective antioxidant in inhibiting free radicals. Axorbic acid is chemically capable of reacting with most free radicals and oxidants in the body (Wibawa *et al*., 2020).

Various scientific evidence shows that the risk of chronic disease due to free radical compounds can be reduced by utilizing the role of antioxidant compounds such as vitamins C, E, A, carotene, phenolic acids, polyphenols and flavonoids. The main characteristic of antioxidant compounds is their ability to capture and stabilize free radicals (Syafriah *et al*., 2023).

The flavonoid compounds contained in betel leaves play a role in the mechanism of inhibiting lipid peroxidation. Flavonoids are able to donate one hydrogen atom from the phenolic hydroxyl (OH) group when reacting with free radicals. The flavonoids in betel leaves play a role in capturing and warding off free radicals. The mechanism of flavonoids as antioxidants occurs directly or indirectly (Hasan *et al*., 2023)

Table 1 Results of measuring the antioxidant activity of red betel leaf (*Piper crocatum*) and black betel leaf (*Piper betle var nigra*) extracts and comparing vitamin C using UV-Vis spectrophotometry

Table 1. Percent radical inhibition of betel leaf extract and vitamin C samples

Sample	Concentration (µg/ml)	Absorbance			Average Absorbance	Blank Absorbance (µg/ml)	% Inhibition
		1	2	3			
Black betel leaves	10	0.481	0.485	0.485	0.483	0.591	18.27
	20	0.442	0.440	0.443	0.441		25.38
	40	0.395	0.395	0.399	0.396		32.99
	80	0.317	0.314	0.318	0.316		46.53

Red betel leaves	10	0.422	0.420	0.423	0.421		28.76
	20	0.361	0.359	0.362	0.370		37.39
	40	0.292	0.289	0.291	0.290	0.591	50.93
	80	0.204	0.202	0.205	0.203		65.65
Vit C	1	0.394	0.391	0.395	0.393		33.50
	2	0.365	0.368	0.365	0.366		38.07
	4	0.281	0.281	0.284	0.282	0.591	52.28
	8	0.184	0.189	0.184	0.185		68.69

Data from Table 1 shows that as the concentration of the sample tested increases, the antioxidant activity in neutralizing DPPH free radicals also increases, this can be seen from the decrease in the absorbance value and the intensity of the purple color in the solution which is getting lower which reflects the reduction in the concentration of DPPH added to the sample and comparison.

The IC_{50} value was determined using the linear regression equation from the relationship curve of sample concentration to percent inhibition with the equation Y = ax + b, where the sample concentration (µg/ml) is the axis (X) and the inhibition percentage value is the axis (Y). The following is a graph of the IC_{50} values for red betel leaf and black betel leaf extracts (Badaring *et al*., 2020)

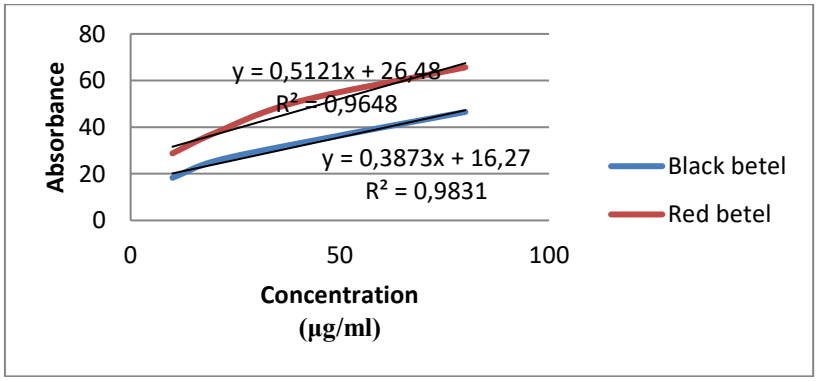

Figure 4. Graph of linear regression equation for antioxidant activity of red betel leaves and black betel leaves extracts

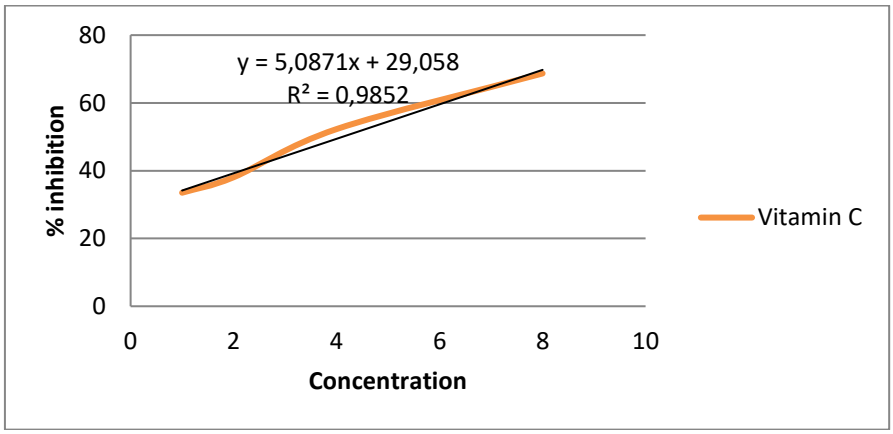

Figure 5. Linear regression equation graph of vitamin C antioxidant activity

From Figure 4 and Figure 5, it shows that there is a correlation between the concentration of vitamin C and red betel leaves and black betel leaves. It can be seen that the higher the concentration of vitamin C or betel leaf extract, the higher the % inhibition obtained, which is indicated by the R2 value. approaching 1. This indicates that vitamin C and red betel leaf and black betel leaf extracts have strong potential as antioxidants, with effectiveness increasing as the concentration increases. From the results above, the IC_{50} value of each sample can be calculated which can be seen in Table 2.

Table 2. Antioxidant power level

Sample	Linear Regression Equation	Value IC_{50} (μg/ml)
Black betel leaves	y = 0,3873x + 16,27 R^2 = 0,9831	87,09
Red betel leaves	y = 0,5121x + 26,48 R^2 = 0,9648	45,92
Vitamin C	y = 5,0871x + 29,058 R^2 = 0,9852	4,11

A compound is said to be a very strong antioxidant if it has an IC_{50} value of <50 μg/mL, strong if the IC_{50} value is 50-100 μg/ml, medium if the IC_{50} value is 100-150 μg/ml, and weak if the IC_{50} value is >150 μg/ml. The results of the research that has been carried out show that the antioxidant activity of samples of black betel leaves ethanol extract IC_{50} = 87,09 μg/mL (strong), and red betel leaves ethanol extract IC_{50} = 45,92 μg/mL (very strong). Meanwhile, the vitamin C solution obtained an IC_{50} value of 4,11 μg/mL (very strong).

Red betel leaves extract has stronger antioxidant activity than black betel leaves extract. This is proven by the fact that red betel leaves extract has an IC_{50} value of 45,92 (< 50 μg/mL), while black betel leaves extract has an IC_{50} value of 87,09 (50-100 μg/mL), the smaller the IC50 value, the higher the compound's ability to capture free radicals, which means its antioxidant activity is stronger. This can be caused by the flavonoid content as an antioxidant agent which is more abundant in red betel leaves extract than in black betel leaves. According to Mahmud *et al* (2021), the ethanol extract of red betel leaves contains flavonoid compounds of 23.16% and according to research conducted by Hartati (2020) found that the total flavonoid content in the ethanol extract of black betel leaves is around 7.56%.

▪ CONCLUSION

Betel leaves extract has the potential to be an excellent natural antioxidant. Where the level of antioxidant activity obtained quantitatively in the ethanol extract of red betel leaves with an IC_{50} value of 45,92 μg/mL (very strong), and the ethanol extract of black betel leaves obtained an IC_{50} value of 87,09 μg/mL (strong). Meanwhile, the vitamin C solution obtained an IC_{50} value of 4,11 μg/mL (very strong). The ethanol extract of red betel leaves has better antioxidant activity with an IC_{50} value < 50 μg/mL and is included

as a very strong antioxidant compared to the ethanol extract of black betel leaves which produces an IC_{50} value of 50-100 μg/mL and is included as a strong antioxidant.

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