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PHYTOCHEMICALS SCREENING AND ANTIOXIDANT ACTIVITY TEST OF ETHYL ACETATE FRACTION OF CHINESE BETLE LEAVES (Peperomia pellucida L.*)*

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ABSTRACT

Peperomia pellucida (Chinese betle) is considered as plant with antioxidant activity for inhibiting oxidation reaction. This study aims to determine the yield of secondary metabolites, total flavonoid levels and antioxidant activity. Dried leaves of Chinese betle were macerated using 70% ethanol as solvent. The extract was partitioned until the n-hexane and ethyl acetate fractions were obtained. Phytochemical screening test was followed by determining total flavonoid levels and antioxidant activity using UV-Vis spectrophotometry. Phytochemical screening results containing ethanolic extract of Chinese betle leaves (ECBL) showed positive results on the alkaloid, flavonoid, saponin and tannin. Meanwhile, the ethyl acetate (EAF) and n-hexane (HF) fractions showed positive results only for flavonoid presence. The total flavonoid contents of ECBL, EAF and HF were 3.30%, 2.54% and 2.03%, respectively. IC50 values of ECBL, EAF and HF were 7.51 ppm, 8.02 ppm and 8.04 ppm, respectively. The highest flavonoid content was found in ECBL. Antioxidant activity test of *Peperomia pellucida* ethanolic extract, ethyl acetate fraction and n-hexane fraction have potent antioxidant activity.

Keywords*: Antioxidant, Peperomia pellucida , DPPH*

INTRODUCTION

The metabolism that occurs within the body involves oxidation and reduction processes. The oxidation process can lead to the formation of harmful oxidants or free radicals for the body (Salamah & Lina, 2014). Free radicals can damage macromolecules such as lipids, cell membranes, DNA, and proteins, causing oxidative stress in cells. (Salamah & Lina, 2014). Antioxidants are compounds that can prevent oxidation reactions by providing their electrons to free radical molecules, thereby stopping the chain reactions caused by free radicals (Sitorus *et al*., 2013).

One type of plant that contains antioxidants is the Chinese betle leaves (Peperomia pellucida L.). The Chinese betle leaves has been traditionally used to treat several diseases, such as abscesses, boils, acne, skin inflammation, kidney disease, and stomach ache (Sitorus *et al*., 2013). According to research by Salamah and Lina (2014), ethanolic extract of Chinese betle leaves (ECBL) has antioxidant activity in inhibiting oxidation reactions. ECBL contain alkaloids, tannins, resins, flavonoids, steroids, phenols and carbohydrates (A. Pratiwi *et al*., 2021). The secondary metabolite compound flavonoids in Chinese betle leaves act as antioxidants that are used to inhibit and stop free radicals and accelerate the wound healing process by increasing or accelerating the proliferation of fibroblast cells and collagen fiber

production (Mulyani *et al*., 2018). Pratiwi *et al*., (2021) have also reported that ECBL has antioxidant activity.

This study aims to identify standard quality parameters of the extract based on specific parameters (organoleptic extract and phytochemical screening) and to determine the total flavonoid content and antioxidant activity of the extract and fraction of Chinese betle leaves (Peperomia pellucida L.) using DPPH (1,1-difenil-2-pikrilhidrazil). The mechanism of this method is the reaction between antioxidant functional group contained in the sample with DPPH. Antioxidants will donate their hydrogen atoms, the reby inhibiting the activity of free radical compounds (Sitorus *et al*., 2013).

RESEARCH METHOD

This study is a descriptive research to determine the content of secondary metabolites (alkaloids, flavonoids, triterpenoids, saponins, and tannins), total flavonoid levels, and antioxidant activity of the ECBL (*Peperomia pellucida* L.*)* using the DPPH method.

Tools

Analytical balance (OHAUS PX224 220 g/0,1 mg); *Rotary evaporator* (IKA®), *waterbath*; Measuring flask (Pyrex®); measuring cylinder (Pyrex®); *Beaker glass* (Pyrex®); kuvet; Spectrophotometer UV-Vis (Shimadzu UV Mini-1240).

Materials

Peperomia pellucida *L*. leaves; 70% ethanol (PT Brataco Indonesia); concentrated sulfuric acid; 2 M sulfuric acid; Dragendorff reagent; Mayer reagent; acetic anhydride; concentrated hydrochloric acid; chloroform (CV Sains Pratama) ; ferric chloride solution; aquadest (PT Brataco Indonesia); DPPH (Aldrich Chemistry); methanol (PT Brataco Indonesia); n-hexane (PT Brataco Indonesia); ethyl acetate (PT Brataco Indonesia); quercetin (Himedia laboratory); vitamin C powder (CV Merck).

Research Procedure

1. *Plant determination*

   Plant determination was carried out at Laboratory of Biology Study Program at IAIN Syekh Nur Jati Cirebon. This identification aims to examine and ensure the true identity and avoid errors in the plant samples used for study.

2. *Dry powder preparation*

   Chinese betle leaves (*Peperomia pellucida* L.) obtained from Kuningan Regency were collected and then sorted in fresh condition. Subsequently, the leaves were washed with flowing clean water. the clean sample were weighed and dried using an oven at 40^{o}C. In the end, the dry sorting process is carried out prior to powdering process (Depkes RI, 1979).

3. *Extraction procedure*

   Approximately 300 grams of crude herbs was soaked in 3 L of 70% ethanol and left for 5 days. The stirring was conducted everyday to obtain effective contact between powder and solvent (Sani *et al*., 2014). After 5 days, the extract was filtrated using flannel to obtain macerate for further process. The macerate was collected and subsequently concentrated on rotary evaporator (Wungkana, 2013). The solvent was let to evaporate at 70 ^{o}C on a water bath to get thick extract.

4. *Partition of ECBL*

   Two grams of extract are dissolved in 10 mL of 70% ethanol and placed in a separating funnel. Thirty mL of distilled water was added and shaken prior the addition of 30 mL of n-hexane for each process. The separating funnel is shaken and left to settle into two fractions. The *n*-hexane fraction is accordingly evaporated on a water bath until one-third remains, resulting in a non-polar fraction. The aqueous fraction was partitioned again using 2 x 30 mL of ethyl acetate solvent, shaken and left to obtain into two fractions. Then, the ethyl acetate fraction was evaporated on a water bath until one-third remains, resulting in a relatively polar fraction then the previous.

5. *Phytochemical Screening*
   a. Alkaloid identification (Harborne, 1987)

      Five hundred miligrams of ECBL was dissolved using 10 mL of chloroform and 3 drops of ammonia was added. This was then acidified using 2 drops of 2 M sulfuric acid. The acidic fraction was divided into 2 tubes and 3 drops of Dragendorff reagent was added to the first tube and 3 drops of Mayer reagent to the second tube. The presence of a white precipitate in Mayer's reagent indicates that the sample is positive for alkaloids, while a positive result for alkaloids in Dragendorff's reagent is indicated by the formation of a red precipitate.
   b. Flavonoid identification

      Five hundred miligrams of ECBL was placed in a spot plate, then a small amount of magnesium metal was added. To this, 1-2 drops of concentrated hydrochloric acid were appended with stirring it well. Appearance of pink color informs that the sample contains flavonoids.
   c. Triterpenoid

      Two grams of ECBL were dissolved using 25 mL of ethanol and heated. The sample then was filtered and then evaporated. The remaining residue is added to ether and transferred to a test tube, then added with Liebermann Burchard reagent (3 drops of anhydrous acetic acid and 1 drop of concentrated sulfuric acid). The appearance of red or purple color indicates the presence of triterpenoids.
   d. Saponin identification

      To 500 miligrams of ECBL, 5 mL of distilled water were added and this was heated for 5 minutes. The sample then was shaken for 5 minutes and let the foam to appear. A stable foam is indicator the presence of saponins.
   e. Tanin identification

      One gram of ECBL was boiled in distilled water for 5 minutes. Then, the solution was filtered and 5 drops of 1% (w/v) ferric(III) chloride were added. A positive test for tannins is indicated by the formation of a dark blue or black color.

6. *Evaluation of extract*
   a. Organoleptic analysis of extract

      The test was conducted to determine appearance, color, smell, and taste (Depkes RI, 2000).
   b. Moisture content test

      One gram of ECBL was placed in an evaporating dish. This was weighed to obtain constant mass. After that, the remains were put in an oven for drying process at 105 ^{o}C for 5 hours and weighed again. The difference between two consecutive weighings is no more than 0.25%. This method is used to determine the moisture content of the extract (Depkes RI, 2000).

7. *Total Flavonoid Test*
   a. Determination of quercetin maximum wavelength

      Determination of quercetin maximum wavelength was carried out by measuring the absorbance of quercetin solution in the range of 400-450 nm wavelength. The absorbance reading showed that the maximum wavelength of the quercetin standard is 431 nm. The maximum wavelength was used to measure the absorption of the extract
   b. Determination operating time

      A solution of 50 ppm quercetin was taken in an amount of 1 ml and added to 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M sodium acetate, and 2.8 ml of distilled water. The solution was measured for its absorbance at the maximum wavelength of 431 nm at 5-minute intervals until a stable absorbance was obtained. A curve was then created to show the relationship between absorbance, time, and the determined operating time.

c. Preparation of quercetin standard solution

Ten milligrams of quercetin were dissolved in 10 mL of ethanol p.a to prepare 1000 ppm quercetin standard solution. From the 1000 ppm quercetin standard solution, several concentrations were prepared, namely 20 ppm, 30 ppm, 40 ppm, 50 ppm, and 60 ppm. One milliliter of the quercetin standard solution was mixed with 0.1 ml of 10% aluminum chloride, 1 mL of 1 M sodium acetate and 2.8 mL of aquadest. The mixture was vortexed until homogeneous and allowed to stand for 30 minutes. Next, the absorbance of each concentration of the solution was measured using the UV-Vis spectrophotometry method at the maximum wavelength of 431 nm.

d. Measurement of blank solution absorbance

One millilitre of ethanol was added with 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M sodium acetate, and 2.8 mL of distilled water. The mixture was then allowed to stand for 30 minutes. Next, the absorbance of each concentration of the solution was measured using the UV-Vis spectroscopy method at a maximum wavelength of 431 nm.

e. Determination of total flavonoid content in ECBL

Twenty milligrams of the sample were weighed and dissolved in 10 mL of ethanol, resulting in a concentration of 2000 ppm. One mililitre of the test sample was added with 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M sodium acetate and 2.8 mL of distilled water. The mixture was vortexed until homogeneous and then allowed to stand for 60 minutes. Then, the absorbance of each concentration of the solution was measured using UV-Vis spectrophotometry method at the maximum wavelength of 431 nm.

f. Calculation of total flavonoid content

$$\% \text{ concentration} = \frac{C \times V \times df \times 10^{-3}}{\text{weight of sample}} \text{ x } 100\%$$

where:

C : concentration of sample (mg/L)
V : Volume of sample (L)
df : Dilution factor

8. Antioxidant activity test of ECBL

a. Preparation of DPPH solution

Two miligrams of DPPH were dissoved in 25 mL of methanol to obtain a concentration of 80 ppm the solution until homogeneous.

b. Preparation of blank solution

One mililitre of methanol was placed within a vial and added with 2 mL of DPPH solution (80 ppm), shaken until homogeneous and then incubated at 37˚C for 30 minutes.

c. Preparation of ECBL, EAF, HF and vitamin C stock solutions

Five milligrams of ECBL were mixed with 50 mL of methanol (100 ppm) and vortexed until homogeneous.

d. Preparation of concentration of ethanolic ECBL, ethyl acetate fraction, n-Hexane fraction, and vitamin C solutions

Sample solutions with concentrations of 2 ppm, 4 ppm, 6 ppm, 8 ppm and 10 ppm were prepared by diluting each stock solution of ECBL with volumes of 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL and 0.5 mL, respectively, then transferred to 5 mL volumetric flasks, added with 2 mL of DPPH solution and methanol up to the calibration mark.

e. Determination of maximum wavelength and operating time

The blank solution absorbance was determined using a UV-Vis spectrophotometer at a wavelength of 400-800 nm. The blank solution absorbance

was measured at 0 minutes (immediately after preparation) and at 10, 20, 30, 40, 50 and 60 minutes.

f. Antioxidant activity testing

Sample solutions with concentrations of 2 ppm, 4 ppm, 6 ppm, 8 ppm and 10 ppm were measured for their absorbance at a wavelength of 400-800 nm and their absorbance was recorded. In each test, the blank absorbance was also determined.

g. Calculation of antioxidant activity

Antioxidant activity was calculated using DPPH method, where the sample was reacted with a DPPH radical solution. The DPPH solution absorbance before and after the addition of the extract was calculated as the percentage inhibition (% inhibition) using the following formula:

$$\% \text{ Inhibition} = \frac{(blank\ absorbance - sample\ absorbance)}{blank\ absorbance}\ x\ 100\%$$

After obtaining the percentage inhibition from each concentration, the calculation was continued by linear regression using the equation y = bx+a where:
x = concentration (ppm)
y = Percentage inhibition (%)

The antioxidant activity is expressed as inhibition concentration 50% or IC50, which is the sample concentration that can scavenge 50% of the DPPH radical. The IC50 value is obtained from the X value by substituting Y with 50.

Data Analysis

The analysis of antioxidant activity data was calculated based on the percentage of inhibition (% inhibition) using formula below:

$$\% \text{ Inhibition} = \frac{(blank\ absorbance - sample\ absorbance)}{blank\ absorbance}\ x\ 100\%$$

After obtaining the percentage of inhibition from each extract concentration, further analysis was conducted using a regression equation (simple linear regression) represented as y = bx + a, where x represents the concentration (ppm) and y represents the percentage of inhibition (%). The antioxidant activity was evaluated by the inhibition concentration 50% (IC50), which refers to the concentration of the sample that can inhibit 50% of the DPPH radicals.

According to (Puspita *et al.*, 2021), the data analysis method used is descriptive and based on the determination of flavonoid content. The flavonoid content can be calculated using the following formula :

$$\% = \frac{\text{C (mg/L)} \times \text{V (L)} \times \text{Fp} \times 10^{-3}}{\text{sample (g)}}$$

RESULTS AND DISCUSSION

The obtained yield of Chinese betle leaves sample was 300 grams. The extract yield obtained was 17.75%. Based on these results, the extract yield meets the requirement as it is not less than 13.1% (Depkes RI, 2017). The concentrated ECBL was separated by partition using a separating funnel with n-hexane as a nonpolar solvent and ethyl acetate as a semipolar solvent. The partition separation of the ethanol extract yielded the n-hexane fraction and the ethyl acetate fraction.

Testing the characteristic parameters of the extract includes the identification of the extract, organoleptic properties, and moisture content. The results of testing the parameters of ECBL can be seen in Table I below:

Table I. Summary of Extract Parameters

Parameters	Results
Identity :	
extract name	Ethanolic extract of Chinese betle leaves
Latin name	*Peperomia pellucida* L.
Plant part(s)	Leaves
Organoleptic :	
Color	Dark brown, almost black
Odor	Distinctive odor
Form	Viscous extract
Moisture Content	24 %

Organoleptic evaluation is one specific parameter determined by using the senses and aims to provide initial simple recognition, but it is subjective in nature (Cahyani, 2017). Based on the organoleptic evaluation results conducted, ECBL has a dark brown to almost black color, with a distinctive odor, and a viscous consistency.

The moisture content test is conducted to determine the minimum limit or range of water content in an extract. Higher moisture content can promote the growth and proliferation of fungi and molds, thus reducing the biological activity and stability of the extract during storage. In the moisture content test, a result of 24% was obtained. Moisture content is considered to be at a high risk if it exceeds 10% (Cahyani, 2017). This indicates that ECBL was considered to be at a high risk due to the obtained result exceeding the 10% limit. Such a high moisture content can affect the formulation and stability of the extract. In addition, the extract was susceptible to be good medium for mold growth (Cahyani, 2017).

Partition of ECBL resulted in 2 fractions that should be observed to reveal informations regarding with their chemical constituents. For that, the phytochemical tests were conducted. The results of test were shown in Table II:

Table II. Phytochemical test results of extract of Chinese betle leaves (*Peperomia pellucida* L.)

Identification	Results for		
	Ethanolic extract	Ethyl acetate fraction	n- hexane fraction
Alkaloid	(+)	(-)	(-)
Flavonoid	(+)	(-)	(-)
Triterpenoid	(-)	(+)	(+)
Saponin	(+)	(-)	(-)
Tannin	(+)	(-)	(-)

(+): positive result
(-): negative result

The phytochemical screening results of ECBL showed positive results for alkaloids, flavonoids, tannins, and saponins. However, the n-hexane fraction (HF) and ethyl acetate fraction (EAF) only showed positive results for flavonoids. The variation in phytochemical screening results among the different samples is due to the presence of different secondary metabolite groups with distinct properties. These results differ from the phytochemical screening results of the extract and fractions conducted by Jannah *et al*., (2020). Their study showed positive results for alkaloid testing in both the EAF and the HF, as well as positive results for triterpenoid testing in the n-hexane sample and ethanolic extract. On the other hand, the study conducted by Arifah *et al*., (2018) reported the presence of false positive results for flavonoid compounds, indicated by the formation of an orange-brown color, and for triterpenoid compounds, indicated by the formation of a dark purple color.

False negative results for steroid compounds are indicated by the formation of a dark green color, false positive results for tannin compounds are indicated by the formation of a black precipitate, and false positive results for alkaloid compounds are indicated by the formation of a white precipitate. False positive results occur when a sample shows positive results for a compound that should actually show negative results. False negative results, on the other hand, occur when a sample shows negative results for a compound that should actually show positive results. False positive results are errors made during the research process where a sample shows positive results for a compound that should be negative. False negative results are errors made when a sample shows negative results for a compound that should be positive.

From the phytochemical screening, flavonoid was found in ECBL. Subsequently, it was important to determine the quantity of flavonoid existed in ECBL. Figure 1was the quercetin standard curve at maximum wavelength of 431 nm :

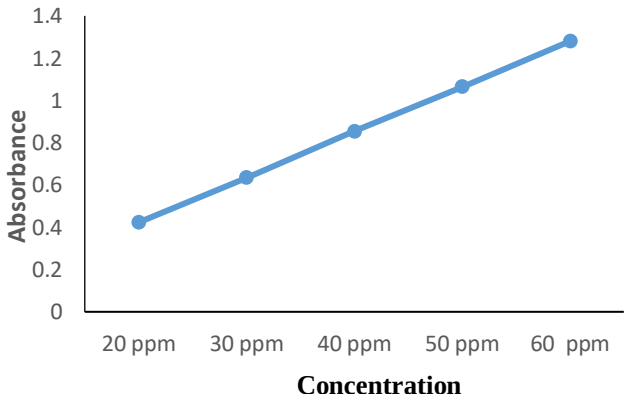

Figure 1. Quercetin Standard Curve

The coefficient of correlation (r) indicates the linearity relationship between two variables. The obtained value of r for the quercetin standard curve was 0.999. This result is slightly different from the r value obtained in the quercetin standard curve performed by Pratiwi (2020), which was 0.9979. However, both results indicate a good linear relationship between the variables as the obtained r value approaches one. Linearity refers to the ability of an analytical method to produce proportional values to the analyte concentration in the sample within a certain concentration range (Pratiwi, 2020).

The data was collected by measuring the absorbance values of a series of standard solutions. According to Pratiwi (2020), there is a linear relationship between absorbance and flavonoid concentration, meaning that higher measured absorbance values indicate higher

flavonoid concentrations. The absorbance data of the standard solutions will then be used to determine the flavonoid concentration using a standard curve, which is obtained from the relationship between quercetin concentration (mg/L) and sample absorbance. The results of the calculation of total flavonoid were provided in Table III.

Table III. Total Flavonoid Content

Sample	Absorbance	Total Flavonoid Content (%)
Ethanolic extract	1.472	3.30 %
Ethyl acetate fraction	1.090	2.54 %
n-hexane fraction	0.884	2.03 %

The results of antioxidant activity test were based on the comparison with the reference i.e. vitamin C. From the results given in Table IV, the best antioxidant could be identified based on the IC50 value.

Table IV. The results of antioxidant activity measurement

Sample	Concentration (ppm)	% inhibition	IC_{50}
vitamin c	0	0	1.97 ppm
	2	67.87	
	4	91.64	
	6	95.98	
	8	96.22	
	10	95.74	
ethanolic extract	0	0	7.51 ppm
	2	54.77	
	4	48.83	
	6	47.06	
	8	48.83	
	10	50.44	
ethyl acetate fraction	0	0	8.02 ppm
	2	44.97	
	4	44.73	
	6	49.87	
	8	48.99	
	10	46.58	
n-hexane fraction	0	0	8.04 ppm
	2	50.92	
	4	46.90	
	6	48.43	
	8	48.43	
	10	46.42	

After measuring the absorbance for the standard and sample solutions, the absorbance values of each tested sample can be obtained, allowing for the determination of the

percentage of free radical inhibition (% inhibition). The percentage of inhibition for each sample and the standard at the same concentrations of 2 ppm, 4 ppm, 6 ppm, 8 ppm and 10 ppm were as follows: ECBL sample: 54.77%, 48.83%, 47.06%, 48.83%, and 50.44%, EAF: 44.97%, 44.73%, 49.87%, 48.99%, and 46.58%, HF: 50.92%, 46.90%, 48.43%, 48.43%, and 46.42%, vitamin C (standard): 67.87%, 91.64%, 95.98%, 96.22%, and 95.74%. Vitamin C was used as a reference because it acts as an antioxidant by scavenging free radicals, preventing chain reactions. It exhibits high antioxidant activity, is readily available, and has higher polarity compared to other vitamins. Vitamin C contains a free hydroxyl group that acts as a free radical scavenger (Damanis *et al*., 2020). The obtained % inhibition values can be used to determine the values of a, b, and r to obtain the IC50 value. The linear regression coefficient (r) obtained is 0.6043 for the ethanolic ECBL sample, 0.692 for EAF, 0.612 for HF and 0.796 for vitamin C.

Based on the % inhibition values and IC50 values indicated in Table IV, it can be observed that ECBL had IC50 value of 7.51 ppm, EAF had IC50 value of 8.02 ppm and HF had IC50 value of 8.04 ppm. The sample showing the highest antioxidant activity was ethanolic ECBL, followed by the EAF and the HF, respectively. However, based on the obtained IC50 values, ECBL was not stronger than vitamin C as antioxidant.

The difference in antioxidant activity among the samples may be attributed to variations in the content of active compounds present in each sample, which affects their ability to scavenge free radicals (Mangela *et al*., 2016). Additionally, the use of different solvents with varying polarities can influence the types of compounds extracted (Mangela *et al*., 2016).

From the measurement of antioxidant activity of ECBL, EAF and HF of Chinese betle leaves, it can be concluded that they have the ability to scavenge free radicals and fall into the category of very strong antioxidants, as indicated by the IC50 values calculated being less than 50 ppm. According to Mangela *et al*., (2016), the smaller the IC50 value, the higher the antioxidant activity, and a compound is considered a very strong antioxidant if the IC50 value is less than 50 ppm, strong if it is in the range of 50-100 ppm, moderate if it is in the range of 100-150 ppm, weak if it is in the range of 150-200 ppm, and very weak if it is in the range of 200-1000 ppm.

CONCLUSION

Phytochemical screening of the ethanolic ECBL showed positive results for alkaloids, flavonoids, saponins, and tannins, while the ethyl acetate fraction and n-Hexane fraction only showed positive results for flavonoids..The total flavonoid content obtained from the ethanolic ECBL was 3.30%, while EAF and HF had total flavonoid contents of 2.54% and 2.03%, respectively. The highest total flavonoid content was found in the ethanol extract.

The ethanolic ECBL, ethyl acetate fraction and n-hexane fraction exhibited strong antioxidant activity. The IC50 values obtained were 7.51 ppm for the ethanol extract, 8.02 ppm for the ethyl acetate fraction, and 8.04 ppm for the n-hexane fraction. Vitamin C, used as the positive control, exhibited stronger antioxidant activity compared to the ethanolic extract, ethyl acetate fraction, and n-hexane fraction, with an IC50 value of 1.97 ppm.

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