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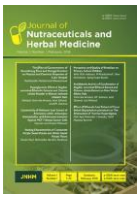

Determination Of Total Phenolic, Flavonoid Contents, And Antioxidant Activity Evaluation Of Ethanolic Extract From *Plumeria Alba*

Monicia Attasih[1], Muhtadi[2]*, Dwi Bagus Pambudi[3], Muhammad Sa'ad[4]

[1]Pharmacy Faculty, Universitas Muhammadiyah Surakarta, Jl. A. Yani Pabelan Kartasura Surakarta, Jawa Tengah Indonesia 57102
[2]Department of Pharmacy, Faculty of Health Sciences, Universitas Muhammadiyah Pekajangan Pekalongan, Jl. Raya Pekajangan No. 1A Kedungwuni. Pekalongan, Jawa Tengah Indonesia 51172
[3]Department of Pharmacy, Sekolah Tinggi Ilmu Kesehatan Nasional Surakarta, Jl. Raya Solo - Baki, Kwarasan, Kec. Grogol, Kabupaten Sukoharjo, Jawa Tengah 57552

*Corresponding Author. email : muhtadi@ums.ac.id

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Abstract

A polluted environment and an unhealthy lifestyle can result in an increase in the number of free radicals in the body. This condition can be reduced by consuming antioxidant-rich plants. This study aims to determine the antioxidant activity of the ethanol extract of the Cambodian plant (*Plumeria alba* L.) and its correlation with phenolic and flavonoid levels. Sample extraction was carried out using the maceration method, antioxidant activity was measured using the DPPH and CUPRAC methods, and total flavonoid and phenolic levels were measured using the spectrophotometer method. The results showed that the ethanol extract of *P. alba* L stems had inactive antioxidant activity, while the ethanol extract of *P. alba* L flowers and leaves had weak antioxidant activity with IC_{50} values of 266.72 ppm and 312.96 ppm. The ethanol extract of *P. alba* L flowers and leaves has an antioxidant capacity of 208.82 and 207.81 mg trolox equivalent/g extract. The total phenolic content measured from the ethanol extract of *P alba* L flowers and leaves was 2.28 and 48.43 mg gallic acid equivalent/g extract, while the flavonoid content was 29.73 and 31.26 mg quercetin equivalent/g extract. The antioxidant activity of the extract was positively correlated with phenolic and flavonoid levels.

Keywords: Plumeria alba L, DPPH, CUPRAC, Phenolic Content, Flavonoid Content;

INTRODUCTION

Polluted air due to vehicle or industrial pollution, exposure to ultraviolet rays from the sun, the habit of consuming foods high in fat are causes of an increase in the rate of free radical production in the body (Khaira, 2010). Free radicals can cause chronic health problems such as cardiovascular disease, cataracts, inflammation and cancer. Antioxidants are able to prevent the effects caused by free radicals by preventing the formation of free radicals or encouraging the decomposition process. There are two antioxidant systems, namely: endogenous and exogenous

which come from foods rich in antioxidants. Food and its main components are the main sources of antioxidants, besides that synthetic antioxidants have been reported to be dangerous for health. This results in an increase in the search for compounds that have high antioxidant activity and are safe for the body (Lobo et al., 2010). Previous research that was carried out in vitro showed that the methanol extract of *Plumeria alba* L. leaves has antioxidant activity and contains nine types of flavonoid compounds. and phenolics (Dawood et al., 2016). Other research also states that *P. alba* leaf extract is a promising source of antioxidants, this is due to the presence of phytoconstituents such as polyphenols, terpenoids and glycosides (Chaudhuri et al., 2015). It was also reported that the ethyl acetate and n-butanol fraction extracts of *Plumeria alba* L leaves showed antiarthritic activity and thus had the potential to treat rheumatoid arthritis (Choudhary, Kumar, Gupta, et al., 2014). The water-ethanol fraction of *P. alba* leaf extract has gastroprotective abilities, protecting the gastric mucosa from damage (Choudhary, Kumar and Singh, 2014).

Plumeria alba flower extract also has antioxidant activity, this is shown by an inhibition value of 81% higher than *Plumeria rubra* flowers and cytotoxic activity against colon cancer cells with an IC50 of 259.90 µg ml-1 (Rahman, 2014). The methanol extract of *P. alba* flowers contains saponin compounds, while the n-hexane and ethyl acetate extracts contain terpenoids. In all solvent phases, *P. alba* L flower extract was positive for alkaloid, flavonoid and tannin compounds (Fathoni et al., 2019). In another study, the ethanol extract of *P. alba* flowers had antibacterial activity against Streptococcus pyogenes (Jiwantono et al., 2017) and the common uro-gastro pathogen *Escherichia coli* (Syakira and Brenda, 2010). *P. alba* stem extract has curative antimalarial activity because it contains terpenoid compounds, glycosides, coumarin and anthracene (Boampong et al., 2013).

This paper reports the results of research that focuses on testing 3 parts of the *P. alba* plant, namely: flowers, leaves and stem bark for antioxidant activity using the DPPH and CUPRAC methods and their correlation with total phenolic and flavonoid contents.

METHODS

Tool

The tools needed for this research are: drying cabinet, collision, analytical balance, maceration vessel, bekker glass (Pyrex), volume pipette, measuring pipette, weighing bottle, Buchner funnel, vacuum rotary evaporator (Heidolph), water bath (Memmert), mortar and stamper, porcelain cup, micropipette (Socorex), macropipette (Socorex), eppendorf, 1 mL cuvette, measuring flask (Iwaki), test tube, spatula, blue tip, and UV-Vis spectrophotometer (Shimadzu-Mini UV-Vis).

Material

The materials used consist of: flowers, leaves and stems of white frangipani (Plumeria alba L.), technical ethanol 96%, DPPH (Sigma), ethanol p.a 96%, distilled water, Folin-Ciocalteu's reagent 10%, Na_2CO_3 7.5% , gallic acid (Sigma), AlCl3 10%, sodium acetate (Merck), CuCl2, ethanolic neocuproine, ammonium acetate buffer, and trolox (Aldrich).

Sample Collection and Extraction

The flowers, leaves and stems were collected from the Pracimaloyo Public Cemetery Area, Sukoharjo, dried using a drying cabinet for 4 days, crushed using a crusher to become simplicia

powder. The next stage was maceration with 96% ethanol (1:7.5), the maceration was filtered using a Buchner funnel. The results of the filtering are evaporated using a vacuum rotary evaporator, then placed in a water bath to obtain a thick extract (Putra et al., 2017).

Preliminary Test Using the DPPH Method

This preliminary test uses a method that has been carried out (Antasionas et al., 2017). The initial step was to add 500 µL of sample solution in Eppendorf, then 500 µL of DPPH 0.4 mM in 96% ethanol and finally 500 µL of 96% ethanol. The solution mixture was incubated for 45 minutes in the dark. The next step is to read the absorbance at a wavelength of 516 nm using a UV-Vis spectrophotometer, the desired output is % FRC.

Testing antioxidant activity using the DPPH method

Each sample that has the potential to have antioxidant activity was made into a solution with a concentration of 1200 ppm, then diluted to obtain 6 concentration series. For each concentration series, 50 µL was taken, 50 µL of 0.4 mM DPPH solution was taken, and 50 µL of ethanol p.a was placed in an Eppendorf. The next step was incubation for 45 minutes in a dark place, the absorbance was measured using a UV-Vis spectrophotometer with a max lambda of 516 nm.

Testing antioxidant activity using the CUPRAC method

Samples that have antioxidant potential are weighed at 10 mg, dissolved in 10 ml of ethanol to obtain a solution with a concentration of 1000 ppm. Then dilution is carried out to obtain a sample solution of 500 ppm. 1 ml of the sample solution was taken, 1 ml of CuCl2 was added. H2O 0.01 M, 1 ml Neocuproine ethanolic 0.0075 M, 1 ml Buffer ammonium acetate 1 M and finally 0.1 ml distilled water. Incubation was carried out for 30 minutes, then the absorbance was read at lambda max 454 nm. Antioxidant capacity is expressed in trolox equivalents/g extract (Haeria, Nurshalati Tahar, 2013).

Determination of total phenolic contents

A sample of 5 mg was taken, dissolved in 5 ml of ethanol. 500 µL of each sample solution was taken, mixed with 2.5 ml of 10% Folin-Ciocalteu's reagent, incubated for 3 minutes. Next, 2.5 ml of 7.5% NaHCO3 was added, incubation was carried out for 45 minutes in a dark place. The absorbance of the sample was measured using a UV-Vis spectrophotometer at λ 782 nm, the phenolic content obtained was expressed in mg gallic acid equivalent/g extract (Stanković, 2011).

Determination of flavonoid contents

The extract was dissolved in ethanol p.a until a concentration of 1000 ppm was obtained, then 500 ml was taken. The next stage is mixed with 1.5 ml of ethanol p.a., 0.1 ml of 10% AlCl3, 0.1 ml of 1 M sodium acetate and finally 2.8 ml of distilled water. Then keep the solution mixture for 30 minutes. Absorbance was measured at a wavelength of 426 nm, flavonoid levels were expressed as mg quercetin equivalent/g extract (Pourmorad et al., 2006).

Data analysis

The research data were processed using the Microsoft Excel 2007 program to calculate the linear regression equation, average and standard deviation. Data is displayed in the form of tables and graphs. This research used 2 replications for each test.

RESULTS AND DISCUSSION

Maceration of *Plumeria alba* L.

The samples that have been collected are cleaned, then dried to prevent mold growth and prevent enzymatic reactions caused by microbial activity so that the chemical content does not change (Handayani et al., 2014). The extraction stage uses the maceration method where the powdered sample and 96% ethanol are placed in a tightly closed inert container at room temperature. This method has the advantages, namely, it is easy to carry out, the yield obtained is high, the equipment and procedures used are simple and it prevents damage to thermolabile compounds. However, the disadvantages of this method are that it uses more solvent, takes a long time, it is possible that some compounds are lost and is difficult to extract at room temperature (Mukhriani, 2014). Ethanol 96% was chosen as a solvent because it has broad extraction power and can be used to extract compounds whose structures are unknown (Saifudin, 2014). The yield of extraction results for each part can be seen in Table 1.

Table 1. Yield from the extract of flower, leave and stem bark of *Plumeria alba*

Sample	Extract Weight (gram)	Simplicia Weight (gram)	Yield (%)
Flower	25.67	300.01	8.55
Leaf	20.95	300.05	6.98
Stem bark	32.06	200.04	16.03

After maceration, the weight of each extract was obtained, calculated using the calculations in Appendix 1. The final result of these calculations was the yield in the form of percent. The ethanol extract of *P. alba* stems had the largest yield, namely 16.03%, then the *P. alba* flower extract was 8.55% and the lowest was the ethanol extract of *P. alba* leaves at 6.98%. The highest yield of ethanol extract from *P. alba* stems shows that this extraction process is efficient in extracting *P. alba* stems (Dewatisari et al., 2018). Meanwhile, this extraction process is less efficient in extracting *P. alba* leaves.

Preliminary Test of the DPPH

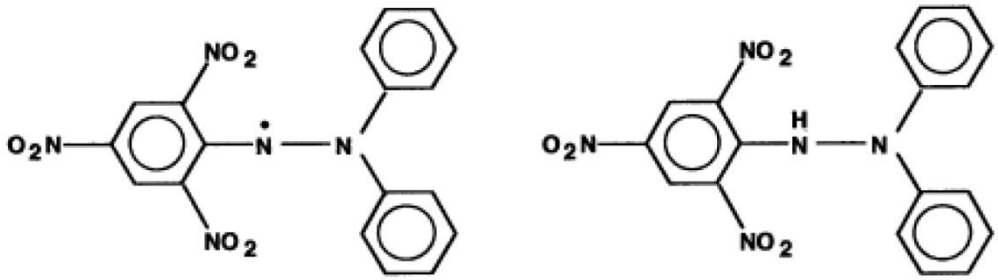
Method Preliminary tests in this research used the DPPH method to determine which extracts have the potential to have antioxidant activity. This method was chosen because it is fast, easy, simple, requires little sample and is sensitive (Hanani et al., 2005). The antioxidant activity of each extract is expressed in the percentage of free radical capture, which shows the number of free radicals that can be captured or reduced by the sample (Nurani, 2013). The

results of this test are shown in Table 2, this shows that those with potential antioxidant activity are *Plumeria alba* flower and leaf extracts.

Table 2. Preliminary test results of extract of flower, leave and stem of *Plumeria alba*

Sample	Absorbance of DPPH	Absorbance of Sample	Free Radical Scavenger (%)
Flower	1.00	0.240	71.73
Leave	1.00	0.365	63.50
Stem bark	1.00	0.533	46.70

Measurement of antioxidant activity using the DPPH method is based on the sample's ability to capture free radicals. The active compound in the extract releases hydrogen atoms which will be captured by free radicals from DPPH to produce the compound 1,1-diphenyl-2-picrylhydrazyl, the compound reaction is in Figure 1. Its presence can be seen by changing the color from purple to yellow (Nurani, 2013). Based on the data above, the highest percentage of free radical capture in the ethanol extract of *P. alba* flowers is 71.73%, the ethanol extract of *P. alba* leaves is 63.5% and the lowest is the extract *P. alba* stems amounted to 46.7%. The percentage of free radical capture indicates whether the extract has antioxidant potential or not. If we look at the percentage of free radical capture that exceeds 50% in the ethanol extract of *P. alba* flowers and leaves, then the antioxidant activity of the two ethanol extracts is seen and the phenolic and flavonoid levels are measured.



(a) (b)

Figure 1. Diphenylpicrylhydrazyl form of free radical (a), Diphenylpicrylhydrazyl reaction result with extract (b)

Measurement of Antioxidant Activity by the DPPH Method

In this method, the parameter used to interpret the results is IC50, which means the concentration required to inhibit 50% of free radicals, obtained by linear regression between concentration (x) and FRC (y)(Molyneux P, 2004). In this study, vitamin E was used as positive control, which is known to have very strong antioxidant activity with an IC50 value of 21.76 (Lung and Destiani, 2018). The results of measuring the antioxidant activity of 3 ethanol extracts and vitamin E are in Table 3.

Table 3. Results of Measurement of Antioxidant Activity by the DPPH Method

Sample	Linear Regression Equations	IC_{50} (ppm)
Flower of *P. alba*	Y = 0.181x + 1.724	266.72
Leave of *P. alba*	Y = 0.158x + 0.552	312.96
Vitamin E	Y = 1.875x + 16.785	27.97

Based on the table above, the lowest IC50 value is vitamin E with 27.97 ppm, then the ethanol extract of *P. alba* flowers with an IC50 of 266.72 ppm and the highest is the ethanol extract of *P. alba* leaves with an IC50 of 312.96 ppm. The level of strength of antioxidant activity can be divided into 4 groups, namely, very strong if IC50 <50 ppm, strong if IC50 50-100 ppm, moderate if IC50 100-150 ppm, weak if IC50 150-200 ppm (Molyneux P, 2004). Based on this category, vitamin E as a positive control has very strong antioxidant activity as indicated by an IC50 value of less than 50 ppm. Meanwhile, for extracts of P. alba flowers and leaves, the strength of antioxidant activity is very weak, as indicated by an IC50 value of more than 200 ppm. The high IC50 values for both extracts are probably due to the use of the less polar ethanol solvent. This is supported by other research which states that the IC50 value for *P. alba* flowers is 2.96 ppm in ethyl acetate solvent, 72.44 ppm in methanol and 116.63 ppm n-hexan (Fathoni et al., 2019).

Measurement of Antioxidant Activity by the CUPRAC Method

Apart from using the DPPH method to see antioxidant activity, we also use the CUPRAC (Cupric Reducing Antioxidant Capacity) method. This method was chosen because the CUPRAC reagent is selective, the reagent is more stable, relatively fast and efficient because the standard redox potential of the Cu (I/II)-Neukoprin complex is 0 .6 V is higher than a single Cu2+ or Cu+ pair of 0.17 V and is easy to implement even though laboratory equipment is limited (Özyürek et al., 2011). In the CUPRAC method, a solution of CuCl2, neocuproine, and ammonium acetate is mixed at pH 7 to produce a redox reagent, namely bis (neocuproine) copper (II) chelate. This reagent can work at pH 7, so ammonium acetate is needed which functions to maintain it at pH 7 (Apak et al., 2007). This method measures the antioxidant capacity to reduce bis (neocuproine) copper (II) chelate, the structure of the reagent can be seen in Figure 2 and the redox reaction equation can be seen below (Özyürek et al., 2011):

$$\mathbf{n\ Cu(Nc)_2^{2+} + electron\ reducing\ agent\text{-}n \leftrightarrow n\ Cu(Nc)_2^{+} + electron\ oxidized\ products\text{-}n + n\ H^+}$$

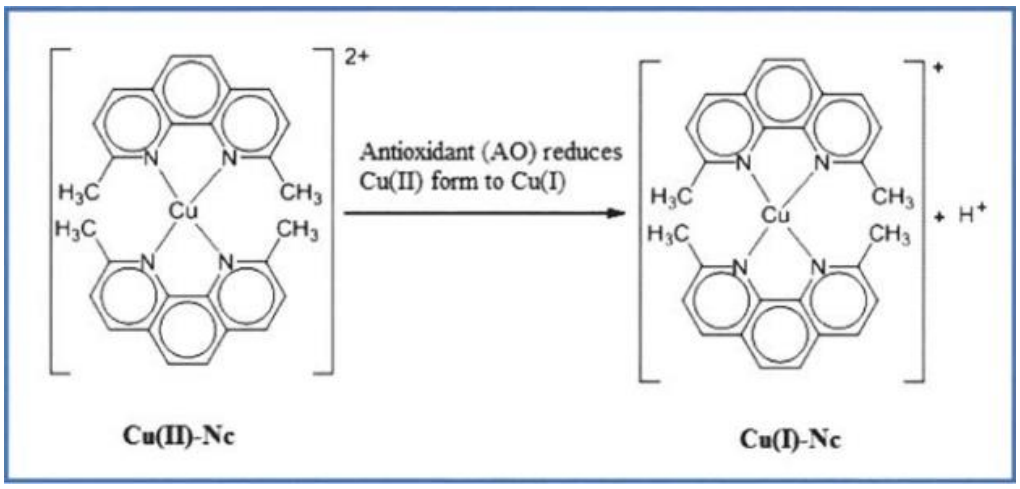


Figure 2. Reaction of bis (neocuproine) copper (II) chelate with antioxidants

In this study, Trolox was used to create a standard curve. Trolox was made in various concentrations and the absorbance was measured, then graphs and standard curve equations were obtained which were attached in Figure 3. The wavelengths of the two extracts were measured and calculated to produce the antioxidant capacity as in Table 4.

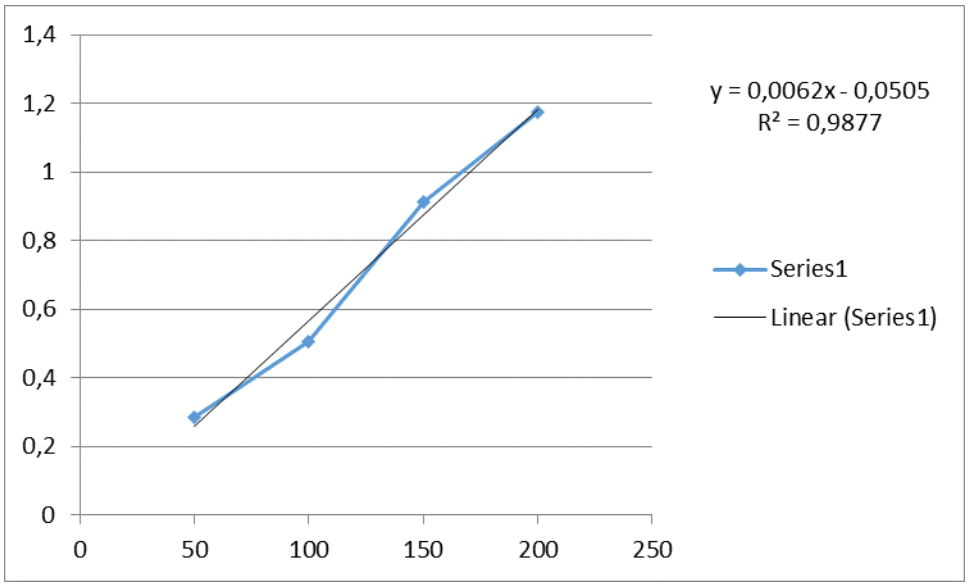


Figure 3. Trolox standard curve

Table 4. Measurement of Antioxidant Activity by CUPRAC Method

Sample	Antioxidant Capacity (mg Trolox equivalent/g extract)

Flowers of *P. alba*	208.82 ± 4.51
Leaves of *P. alba*	207.81 ± 5.73

Based on Table 4, the antioxidant capacity of the ethanol extract of *P. alba* flowers is 208.82 mg trolox equivalent/g extract, the antioxidant capacity of 1 gram of ethanol extract of *P. alba* flowers is equivalent to 208.82 mg trolox. Meanwhile, the antioxidant capacity of the ethanol extract of *P. alba* leaves is 207.81 mg trolox equivalent/g extract, which means that the antioxidant capacity of 1 gram of ethanol extract of *P. alba* leaves is equivalent to 207.81 mg trolox. Both extracts have almost the same antioxidant capacity.

Determination of total phenolic contents

In this study, phenolic content was measured using the Folin-Ciocalteu method. The principle of this method is that a reaction occurs between the extract and the Folin-Ciocalteu reagent in an alkaline medium to produce a phospho molybdic-phosphotungstic-phenol complex which will be read spectrophotometrically (Hatami et al., 2014). Apart from gallic acid samples, the absorbance was also measured to obtain a standard curve. The results of the sample absorbance measurements are then entered into a linear regression equation, where concentration is (X) and absorbance is (Y). The equation was obtained (Y=0.007x+0.107) with an R value of 0.988, which means that the greater the concentration of gallic acid, the greater the absorbance. The standard curve equation graph is in Figure 4. Then the results of the sample absorbance readings are entered into the equation. The results of measuring phenolic levels are in Table 5, which are expressed in mg gallic acid equivalent/g extract, which means that 1 gram of extract is equivalent to several mg gallic acid.

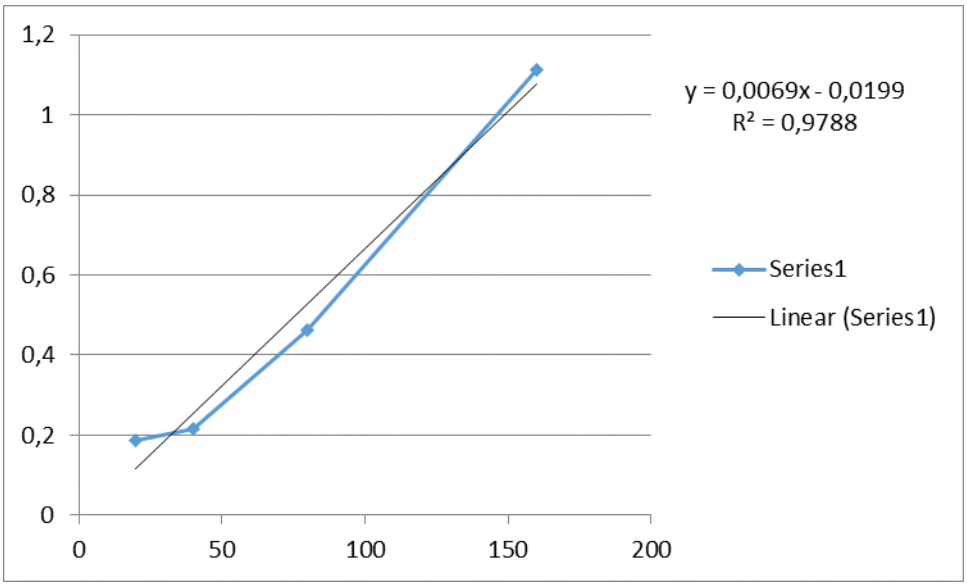


Figure 4. Gallic acid standard curve

Table 5. Measurement Results of Phenolic Contents

Sample	Phenolic Content (mg gallic acid equivalent/g extract)
Flowers of *P. alba*	62,28 ± 2,64
Leaves of *P. alba*	48,43 ± 5,21

Based on table 5 above, it shows the phenolic content of each sample. *P. alba* flowers have a phenolic content of 62.28 mg gallic acid equivalent/g extract, higher than *P. alba* leaves which is 48.43 mg gallic acid equivalent/g extract. The low phenolic content in *P. alba* leaves was due to sampling during the day, when the sun intensity was high. In research (Ibrahim and Jaafar, 2012) it is stated that there is a relationship between the level of solar radiation and the phenolic and flavonoid content, where higher levels of solar radiation can reduce the production of phenolics and flavonoids.

Determination of flavonoid contents

The measurement of flavonoid levels in this study used the aluminum chloride colorimetric method. The principle of this method is the formation of a stable acid complex between aluminum and the C-4 keto group and the C-3 or C-5 hydroxy groups of flavonoids and phenolics (Kamtekar et al., 2014), the complex formed will produce a yellow color which is then read in spectrophotometry. If the color formed becomes more intense, it indicates that there are more flavonoid compounds (Rizki et al., 2015). The standard curve was created using quercetin because, in previous research, quercetin had the greatest absorbance among the 14 other standard flavonoids (Chang et al., 2002). The quercetin that has been measured is then made into a standard curve, the curve and equation are attached in Figure 5. For samples whose absorbance has been measured, the flavonoid content is calculated using the formula attached in Appendix 6. The flavonoid content in each sample is expressed in mg quercetin equivalent/g extract. The results of measuring the flavonoid levels of *P. alba* flower and leaf extracts are shown in Table 6.

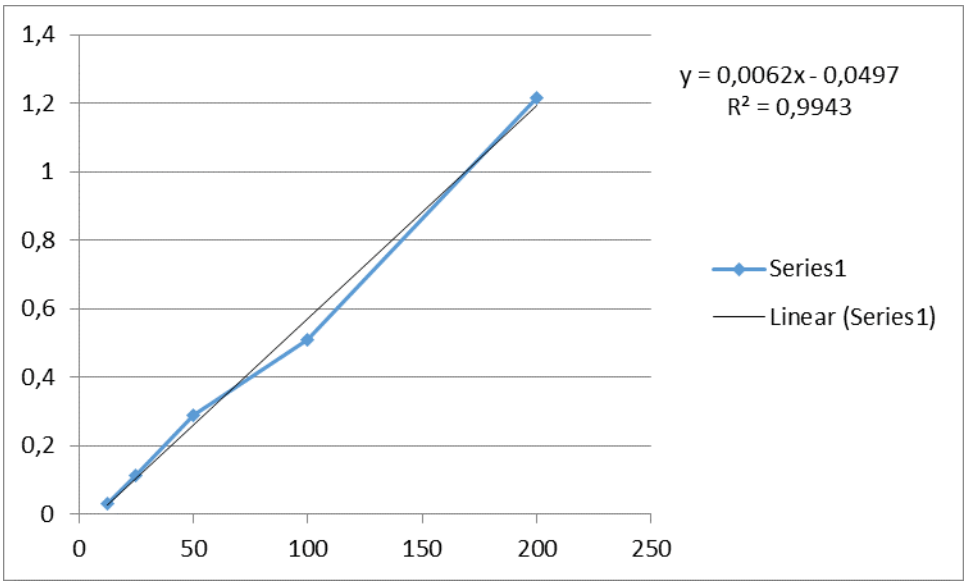


Figure 5. Quercetin standard curve

Table 6. Results of Measurement of Flavonoid Contents

Sample	Flavonoid Contents (mg quercetin equivalent/g extract)
Flowers of *P. alba*	29.73 ± 6.74
Leaves of *P. alba*	31.26 ± 4.82

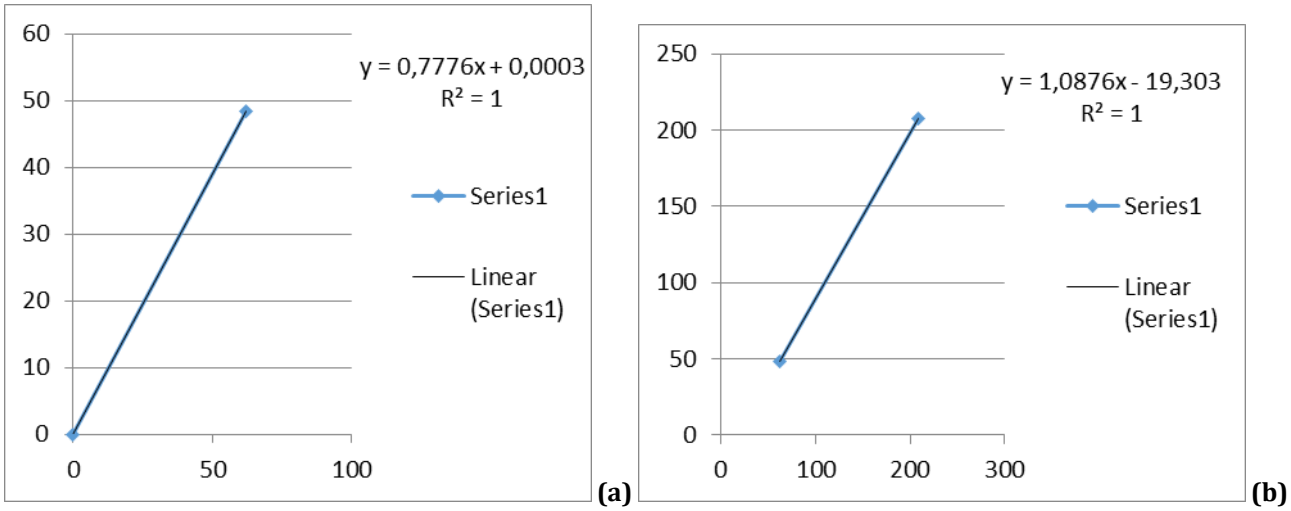
Based on table 6 above, the highest flavonoid content in *P. alba* leaves is 31.26 mg equivalent of quercetin/g extract, which means that 1 gram of ethanol extract of *P. alba* leaves is equivalent to 31.26 mg quercetin. *P. alba* flowers have a flavonoid content of 29.73 mg quercetin equivalent/g extract, in 1 gram of ethanol extract of *P. alba* flowers the equivalent of 29.73 mg quercetin, less than *P. alba* leaves. This is because photosynthesis occurs in the leaves so that the flavonoid biosynthetic pathway produces more flavonoids than other plant parts (Saboonchian et al., 2014).

The correlation of antioxidant activity with phenolics and flavonoids

Phenolic compounds are known to have activity as antioxidants through electron transfer (Pertiwi, 2018; Kusumowati et al., 2011), because phenolic compounds are able to prevent oxidation so it is thought that the higher the phenolic content, the higher the antioxidant activity (Djapiala et al., 2013; Mellanisa et al., 2011). Based on data analysis, a correlation between the levels was carried out. Phenolics as the axis (X) and antioxidant activity as the axis (Y). The correlation results between phenolic content and antioxidant activity (DPPH and CUPRAC) were obtained as shown in Figure 6. From these results, the correlation value of phenolic content with the DPPH and CUPRAC methods each had a positive R2 = 1 value. This is significant if there is a

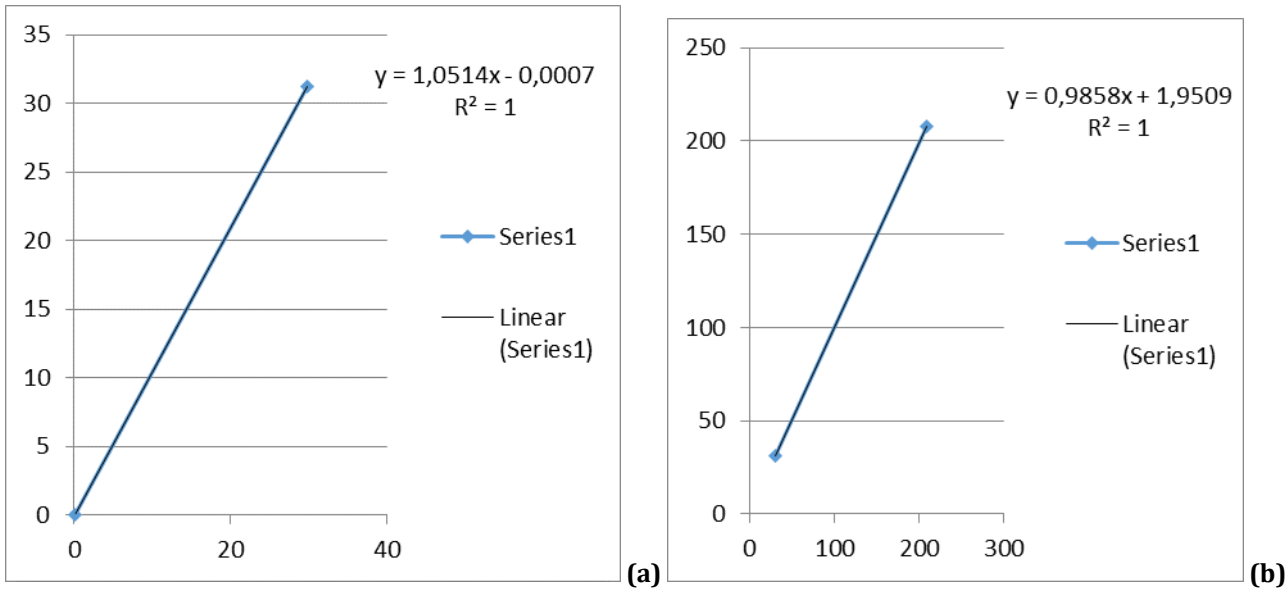
relationship between phenolic content and antioxidant activity in the DPPH and CUPRAC methods.

Flavonoid compounds are also thought to have a relationship with the antioxidant activity of an extract. The correlation value was obtained from a linear regression equation where flavonoid content was the (X) axis and antioxidant activity was the (Y) axis. The correlation results are shown in Figure 7. It was found that the correlation value of flavonoid levels with antioxidant activity (DPPH and CUPRAC) was each positive with a value of R2 = 1. This shows that flavonoid levels influence the antioxidant activity of P. alba flower and leaf extracts.



(a) (b)

Figure 6. Relationship between Phenolic Content and Antioxidant Activity using (a) DPPH and (b) CUPRAC methods



(a) (b)

Figure 7. Relationship between Flavonoid Levels and Antioxidant Activity using (a) DPPH and (b) CUPRAC methods

CONCLUSION

Based on the results of research that has been carried out, *P. alba* L stem extract has the weakest antioxidant activity. In the DPPH method, *P. alba* L. flower and leaf extracts did not have potent antioxidant activity with IC50 values of 266.72 ppm and 312.96 ppm. *P. alba* L. flower and leaf extracts had an antioxidant capacity of 208.82±4.51 and 207.81±5.73 mg trolox equivalent/g extract. The phenolic content of P. alba L. flower and leaf extracts was 62.28±2.6 and 48.4±5.21 mg gallic acid equivalent/g extract. Meanwhile, the flavonoid content of *P. alba* L flower and leaf extract was 29.73±6.7 and 31.26±4.82 mg quercetin equivalent/g extract. Antioxidant activity was positively correlated with the levels of total phenolic compounds and flavonoids.

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