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Phytochemical Screening and Antioxidant Activity of Ethanolic Extract of Cawat Hanoman Stem (*Bauhinia aculeata* L.) using DPPH Method

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Abstract

The free radical is an unstable molecule because contains one or two unpaired electrons. The antioxidant substance is a simple way to decrease the illness caused by free radicals. Cawat hanoman (*Bauhinia aculeata* L.) was known to contain tannin components one of the benefits as an antioxidant. This research aims to determine the antioxidant activity of the *B. aculeata* stem tested by qualitatively used thin-layer chromatography (TLC) and quantitatively using the DPPH method. *Bauhinia aculeata* stem was extracted using a maceration extract method with 96% ethanol. Antioxidant activity test was done qualitatively by eluent of ethyl acetate : methanol : purified water (6 : 2 : 1) using TLC and quantitatively using the DPPH method. The result of antioxidant activity from 96% ethanol extract of *B. aculeata* stem qualitatively showed the presence of yellow spots on a purple background at TLC after syringed DPPH 0.5 mM and quantitative test that resulted in an IC₅₀ of 21.862 µg/mL. These results indicate that 96% ethanol extract of *B. aculeata* has very strong antioxidant activity.

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INTRODUCTION

Free radicals are unstable molecule because contains one or more electrons unpaired (Phaniendra *et al.*, 2015). It tends to react with the molecules around it, if this reaction continues to occur in the body will cause negative effects that is the onset of degenerative diseases and cell damage (Liochev, 2013). Therefore, the body needs substance antioxidants that are able to ward off free radicals (Lobo *et al.*, 2010).

Natural antioxidants come from plants, like polyphenols compounds which has a hydroxyl group on molecular structure. Polyphenols compounds with hydroxyl groups has catch free radicals (Tungmunnithum *et al.*, 2018). Nowadays ample evidence from copious studies exists of polyphenols activity as antioxidative, anti-inflammatory and other various biological effects that

exert in the prevention of various pathologies including cardiovascular diseases and cancer (Mojzer *et al.*, 2016).

One of the endemic plants from Borneo Island especially in South Kalimantan is cawat hanoman (*Bauhinia aculeata* L.). Empirically this plant is used to increased male stamina. Previous research has been proven the 96% ethanolic extract of *B. aculeata* stem have polyphenols compounds and proven efficacious as an aphrodisiac (Wati *et al.*, 2018). *Bauhinia aculeata* contains phenolic compounds such as flavonoids and tannins (Margaretta *et al.*, 2011).

Previous research on *B. variegata* Linn which has the same genus with *B. aculeata* showed that antioxidant activity tests have been performed toward *B. variegata* Linn. The results showed that IC₅₀ value from *B. variegata* leaf, stem bark, and floral bud extract were 17.9, 19.5, and 17.2 µg/ml, respectively (Pandey *et al.*, 2012). Another

research report that methanolic extract of *B. variegata* barks showed IC_{50} value was 6.48 $\mu\text{g/ml}$, indicating that *B. variegata* extract has a very strong antioxidant activity (Sharma *et al.*, 2015).

Based on previous studies, *B. aculeata* stem is also expected to have antioxidant potential. This research aims to determine the antioxidant activity of the *B. aculeata* stem. Maceration method was chosen to withdraw the compound in *B. aculeata* stem used 96% ethanol as solvent (Wati *et al.*, 2018). The antioxidant assay in this research used 2,2-Dyphenyl-1-Picrylhyrazil (DPPH) method.

12 MATERIALS AND METHODS

Tools and materials

The tools used in this research was analytical balance (Scout Pro), desiccator, macerator, micropipette (Socorex), oven (Memmert UN55), refrigerator (Toshiba), vacuum rotary evaporator (IKA-RF10), UV-Vis spectrophotometer (T60), water bath (Memmert), and other glassware (Iwaki, Pyrex).

Material used in this research was *B. aculeata* stem obtained from Hantakan Village, Hulu Sungai Tengah District, South Kalimantan. Other materials consisting of amyl alcohol, anhydrous acetic acid (Brataco), DPPH (Sigma Aldrich), sulfuric acid, hydrochloric acid, iron(III) chloride, chloroform (Brataco), ethanol, quercetin (Sigma Aldrich), magnesium powder, methanol pro analytic, purified water, and thin-layer chromatography (TLC) plate silica gel 60 GF₂₅₄ (Merck).

Plant determination

Determination is carried out to identify and ascertain the identity of the plant species used. Determination is done using all parts of the plant carried out at the Department of Pharmacy Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta.

Extraction

A total of 300 g of *B. aculeata* stems simplicia were extracted by maceration method using ethanol 96% with a simplicia : solvent ratio of 1 : 5. The liquid extract obtained was then concentrated with a vacuum rotary evaporator. The thick extract is then evaporated again using water bath to obtain a fixed weight (Pandey *et al.*, 2011).

Phytochemical screening

Phytochemical screening including alkaloids, phenolic, flavonoids, saponins, steroids/triterpenoids, and tannins test. The test method used was a standard method with some modifications.

Alkaloids test

A total of 0.2 g extract was dissolved with 5 ml HCl 10% and filtered. The filtrate used as test solution.

1. Dragendorff test

As much as 1 ml filtrate were treated with 3-5 drops Dragendorff reagent (solution of potassium bismuth iodide). Formation of red precipitate indicates the presence of alkaloids (Tiwari *et al.*, 2011).

2. Mayer test

As much as 1 ml of filtrate were treated with 3-5 drops Mayer reagent (potassium mercuric iodide). Formation of a yellow precipitate indicates the presence of alkaloids.

3. Wagner test

As much as 1 ml of filtrate were treated with 3-5 drops Wagner reagent (potassium iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

Phenolic test

Ferric chloride test: As much as 0.3 g of extract was added with 3-4 drops FeCl_3 solution. Formation of bluish black color indicates the presence of phenols (Tiwari *et al.*, 2011).

Flavonoids test

As much as 0.5 g of extract added with 10 ml purified water, then added with 0.1 g magnesium powder, a few drops HCl 5 N, and 2 ml amyl alcohol solvent. The solution was shaken vigorously, then wait until it separated. The positive result showed the formation of orange or yellow in amyl alcohol layer (Muthia & Wati, 2018).

Saponins test

Foam test: As much as 0.5 g of extract was shaken for ± 1 minute with 5 ml of purified water. Foam produced that persists for at last 10 minutes indicates the presence of saponins (Muthia & Wati, 2018).

Steroids and triterpenoids test

Liebermann-Burchard test: As much as 0.5 g extracts were treated with 2 ml chloroform, shaken, and filtered. The filtrates were added with 1 ml of acetic anhydride, and 0.5 ml of concentrated sulphuric acid. The steroids positive results if there a formation of blue or green ring and the triterpenoids positive results give the formation of red or purple color (Muthia & Wati, 2018).

Tannins test

Gelatin test: As much as 0.2 g extract was added with 2-3 drops 1% gelatin solution containing sodium chloride. Formation of white precipitate indicates the presence of tannins (Tiwari *et al.*, 2011).

Antioxidant activity test

Qualitative test of antioxidant activity

Ethanollic extract of *B. aculeata* stem was spotted on the TLC plate silica gel CF₂₅₄. Extract was spotted on silica plate sized 1.5 cm x 10 cm by the upper limit and lower limit of 1 cm so that developer within 8 cm (Mustarichie *et al.*, 2017). The extract has been diluted was eluted in chamber with mobile phase of EtOAc : MeOH : H₂O (6 : 2 : 1). After the plate was eluted, then observed in visible light, ultraviolet (UV) with wavelength of 254 nm, 366

nm, and sprayed with DPPH 0.5 mM. The sample shows antioxidant activity characterized by the appearance of yellow spots against a purple background on the TLC plate (Muthia *et al.*, 2019).

Quantitative test of antioxidant activity

A total of 1.98 mg of DPPH was dissolved with methanol until 50 ml, then shaken until homogeneous and placed in dark bottle. As much as 2 ml ethanolic extract of *B. aculeata* stem with concentrations of 10, 20, 30, 40, and 50 µg/ml were reacted with 2 ml of 0.1 mM DPPH then incubated for 30 minutes in dark room at room temperature (25°C). Each absorbance sample was measured at a wavelength of 515 nm using a UV-Vis spectrophotometer. As a reference quercetin was used with the same treatment, with quercetin concentrations of 1, 2, 3, 4, and 5 µg/ml (Muthia *et al.*, 2019).

Data analysis

The antioxidant activity of the ethanol extract of *B. aculeata* stem is determined by the parameter of %inhibition of radical scavenging activity and IC₅₀ value. The value of %inhibition of radical scavenging activity can be calculated by the following formula (Mahdi-Pour *et al.*, 2012):

$$\% \text{ inhibition} = \left(\frac{A_0 - A}{A_0} \right) \times 100 \%$$

where A₀ is the absorbance of negative control (0.1 mM DPPH solution) and A is the absorbance in presence of extract. The result of the data is made a linear regression equation (y = bx + a) for the specified IC₅₀ value. The IC₅₀ value determination is done by entering the concentration sample as y and %inhibition value as x, where y = 50 and x is the IC₅₀ value.

RESULTS AND DISCUSSION

Determination was the first step carried out in this research, the aim was to find out and ascertain the taxonomic of a plant based on plant structure had been

seen from the shape of roots, stems, and leaves because some plants had a similarity. Determination result showed that plant samples used belongs to the Leguminosae family, with the species was *Bauhinia aculeata* L. The figure of *B. aculeata* is presented in **Figure 1**.



Figure 1. Stem of *B. aculeata* (a) and stem piece after dried (b)

Bauhinia aculeata stem was extracted with maceration method used 96% ethanol as solvent. The 96% ethanol is polar solvent which can dissolve antioxidant compounds such as flavonoids, phenols, and tannins (Ningsih *et al.*, 2017). The yield value of ethanolic extract of *B. aculeata* stem was 6.292% (b/v). Result of phytochemical screening indicated ethanolic extract of *B. aculeata* stem contained phenolic, flavonoids, saponins, and steroids compounds, as presented in **Table I**.

Table I. Phytochemical screening result of ethanolic extract of *B. aculeata* stem

Compounds	Reagent	Results	Figures	Conclusion
Alkaloids	Dragendorff	No red precipitate		-
	Mayer	No white-yellowish precipitate		-
	Wagner	No brown precipitate		-
Phenolic	FeCl ₃	Bluish green solution		+
Flavonoids	Mg powder + HCl + amyl alcohol	Orange color on amyl alcohol layer		+
Saponins	Aquadest, HCl	Stable foam		+
Steroids/ Triterpenoids	CHCl ₃ , Liebermann-Burchard	Blue-green color		+(Steroid)
Tanins	Gelatin 1% (b/v)	No white precipitate		-

The qualitative test of antioxidant activity was carried out using the TLC method. The mobile phase used was EtOAc : MeOH : H₂O (6 : 2 : 1) and sprayed with 0.5 mM DPPH. The results of TLC with several stain views are shown in **Figure 2**. There are three yellow spots on a purple background on the TLC plate with an R_f value of 0.88 (A); 0.75 (B); and 0.55 (C). These results indicate there are compounds that show antioxidant activity. Color changes occur because of the compounds in the sample that contribute hydrogen atoms to DPPH so that they are reduced to a more stable form (Pratiwi *et al.*, 2013).

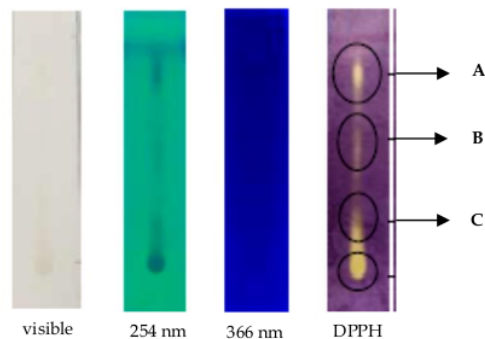


Figure 2. Chromatogram of *B. aculeata* stem extract with mobile phase EtOAc : MeOH : H₂O (6 : 2 : 1)

¹¹ Test of antioxidant activity from plant samples can be done by various methods. One of the easiest methods to do is the DPPH method (Atun *et al.*, 2019). Quantitative antioxidant activity tests were performed using a UV-Vis spectrophotometer with the DPPH method. DPPH molecules are free radical molecules that are stable in the presence of electron delocalization around the molecule. ¹ Before testing the antioxidant activity, the maximum wavelength of DPPH solution was determined and the operating time of DPPH solution in 96% ethanol was carried out. It aims to determine the best time and stable work from DPPH solution (Mustarichie *et al.*, 2017). The result showed maximum wavelength of DPPH solution was 515 nm, with incubation time of 30 minutes.

This study uses quercetin as a positive control because quercetin is an isolate from natural ingredients which is included in the flavonoid group and is proven as a powerful antioxidant compound (Ningsih *et al.*, 2017). Quercetin was reacted with ¹⁹ 0.1 mM DPPH and incubated for 30 minutes, until the color changed from purple to faded yellow. Quercetin in certain concentrations will cause a change in color from purple to fade to yellow due to the reduced conjugated double bond on DPPH. That happens because the capture of electrons by antioxidants that cause no chance of the electron resonating (Ichikawa *et al.*, 2019).

¹³ Absorbance data from each sample is then made into a curve and a linear regression equation is made. The equation is then used to calculate the IC₅₀ value. The standard curves of quercetin and ethanol extracts of hanoman loops are shown in Figures 3 and 4. The results showed that quercetin and *B. aculeata* stem had IC₅₀ values of 2.557 and 21.863 µg/ml, respectively. The %inhibition and calculation of antioxidant activity stated in IC₅₀ are presented in Table II.

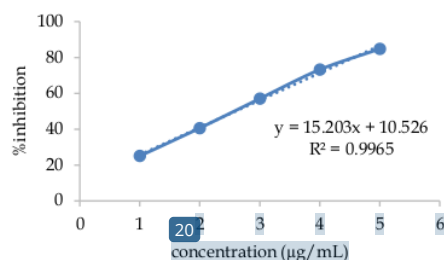


Figure 3. The antioxidant activity curve of quercetin

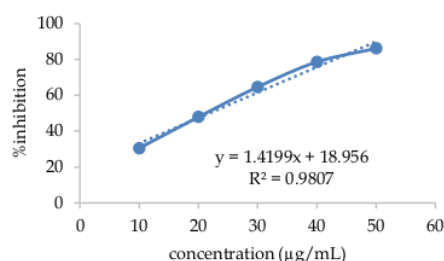


Figure 4. Antioxidant activity curve of ethanolic extract of *B. aculeata* stem

Table II. The IC₅₀ value of quercetin and ethanolic extract of *B. aculeata* stem

Sample	Concentration (µg/mL)	%inhibition ± SD*	IC ₅₀ (µg/mL)
Quercetin	1	25.046 ± 0.667	2.557
	2	40.605 ± 0.895	
	3	57.056 ± 0.814	
	4	73.211 ± 0.493	
	5	84.758 ± 0.629	
Ethanolic extract of <i>B. aculeata</i> stem	10	30.487 ± 0.871	21.863
	20	47.902 ± 0.524	
	30	64.634 ± 0.921	
	40	78.613 ± 0.988	
	50	86.125 ± 0.816	

*Mean ± Standard deviation is done in three replications

The smaller the IC₅₀ value, the higher the antioxidant activity (Mustarichie *et al.*, 2017). The IC₅₀ value of *B. aculeata* stem shows very strong antioxidant activity according to the antioxidant category, where if IC₅₀ < 50 µg/ml is said to have very strong antioxidant activity, if IC₅₀ between 51-100 µg/ml is said to have strong antioxidant activity, if IC₅₀ between 101-250 µg/ml is said to have moderate antioxidant activity, if IC₅₀ between 251-500 µg/ml is said to have weak antioxidant activity, and if IC₅₀ > 500 µg/ml is said to have no antioxidant

activity (Mustarichie *et al*, 2017). Although *B. aculeata* stem has an IC₅₀ greater than quercetin, *B. aculeata* stem is still very potential to be developed as an antioxidant. That is because quercetin is a pure compound that provides very strong antioxidant activity, while *B. aculeata* stem extract contains many compounds.

CONCLUSION

Based on research it can be concluded that 96% ethanol extract of *B. aculeata* stem contains phenolic, flavonoids, saponins, steroids, and tannins compounds. The extract can capture DPPH free radicals which are indicated by the presence of yellow spots on a purple background on the TLC plate sprayed with DPPH 0.5 mM using the mobile phase of ethyl acetate : methanol : purified water (6 : 2 : 1). The extract has a very strong antioxidant activity with an IC₅₀ value of 21.862 µg/ml.

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