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Antioxidant Activity of Ethanolic Extract and *N*-Hexane Fraction from Sikkam (*Bischofia Javanica* Blume) Stem Bark

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ABSTRACT

Objectives: The current investigation was conducted to examine the secondary metabolites content of sikkam (*Bischofia javanica* BLUME) stem bark. Antioxidant activity of the ethanolic extract and *n*-hexane fraction of sikkam stem bark were also evaluated.
Design: The design of this study was quantitative experimental, where quantitative antioxidant activity was tested by using the DPPH radical scavaging method and measured using a UV-Vis spectrophotometer.
Interventions: the variable that was intervened in this study was the concentration of extract used.
Main outcome measures: The main measurement carried out in this study is the absorbance value of DPPH which is converted into a 50 inhibitory concentration value (IC_{50}).
Results: Simplicia and ethanolic extract of sikkam stem bark exhibited secondary metabolites content of flavonoids, glycosides, tannins and triterpenoids. Ethanolic extract of sikkam stem bark (EESSB) and *n*-hexane fraction of sikkam stem bark (NHFSSB), exhibited very strong antioxidant activity with an IC_{50} of 12,248 µg/mL and 39,622 µg/mL.
Conclusion: The sikkam stem bark had contained flavonoids, glycosides, tannins, triterpenoid, and had a very strong antioxidant activity, where EESSB had a stronger activity than NHFSSB.

Keywords: *Bischofia javanica*, stem bark, antioxidant, secondary metabolites

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INTRODUCTION

Free radicals are defined as atoms or molecules that have unpaired electrons. Reactive oxygen species (ROS), such as superoxide radicals ($*O_2^-$), hydroxyl radicals (*OH), hydrogen peroxide (H_2O_2) can be produced during normal metabolic processes or due to exogenous agents and factors. Various diseases such as cancer, cardiovascular disease, osteoporosis, and degenerative diseases can occur due to the formation of ROS, which can cause oxidative damage to human cells [1].Antioxidants are chemical compounds that can donate one or more electrons (electron donors) to free radicals, so that free radical reactions can be inhibited. Antioxidants can protect the body against damage caused by reactive oxygen compounds, capable to inhibit the occurrence of degenerative diseases, cancer, inflammation, immune disorders, cardiac infarction, and aging [2]. Antioxidant compounds can be either synthetic or natural products. The use of synthetic antioxidants has led to development of carcinogenic adverse effects, present concern for the community. Therefore, it needs to find the antioxidants from alternative source including natural product [3,4]. The natural antioxidants are found for large quantity in plants and generally as phenolic compounds (polyphenolic) or in the form of flavonoids, tocopherols, organic acids polyphenols, coumarin, and lignin [5]. Generally, plants contain active compounds in the form of secondary metabolites such as alkaloids, flavonoids, tannins, glycosides, triterpenoids, steroids, and saponins. These metabolite compounds have been used as food additives and medicines, widely [6].

The stem bark of sikkam (*Bischofia javanica* BLUME) is empirically used by people of Simalungun North Sumatera to reduce concentration of plasma cholesterol, for treat of diarrhea and gastric ulcers. But less information is available about the secondary metabolites in sikkam stem bark. Hence, the present study purposed to probe secondary metabolites using phytochemical screening of simplicia and ethanol extract from sikkam stem bark. The antioxidant activity of this extract was investigated using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method.

MATERIALS AND METHODS

Plant and Chemicals Materials

The current study was conducted in the phytochemical laboratory and research laboratory, Faculty of Pharmacy, University of Sumatera Utara. Fresh old stem bark of sikkam was collected from Sudirman street, municipality of Medan, Sumatera Utara province, Indonesia. Sikkam was identified in Herbarium Medanense, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Sumatera Utara.

The aparatus used in this study were mortar and pestle, sieves, maceration containers, analytical scales, aluminum foil, whatmann filter paper, spatulas, measuring cups, measuring flasks, measuring flasks, beaker glass, test tubes, volume pipettes, rubber balls, water bath, and rotary evaporator.

Ethanol, *n*-hexane, ether, H_2SO_4, HCl, amyl alcohol, isopropanol, $CHCl_3$, methanol, were obtained from Merck. Mayer, Dragendorff, Bouchardat, Liebermann-Burchard reagent, Molisch reagent, Lead (II) acetate, Mg powder, $FeCl_3$ and DPPH (1,1-diphenyl-2-picrylhydrazyl).

Preparation of Simplicia

The fresh old stem bark of sikkam was cleaned from moss or other dirt and sliced with a width of 5–10 cm and long 10–20 cm approximately. After that, it was washed, then drained and dried for 45 days. Afterward, the dried stem bark was mashed into a fine powder (400 g).

Preparation of Ethanolic Extract and *n*-Hexane Fraction

The fine powder of sikkam stem bark (300 g) was extracted using 2250 mL of 80% ethanol (1:7.5) and putted in a closed container, then coated with aluminum foil for 5 days, protected from light and stirred frequently. The macerate was filtered with filter paper to obtain a filtrate 1. The residue was dried up and was extracted again with 750 mL of 80% ethanol (1:2.5) for 2 days, protected from light and stirred frequently. The macerate was filtered to obtain a filtrate 2. Afterward, filtrate 1 and filtrate 2 were collected and were evaporated using rotary evaporator at 40^{o}C and were dried using water bath to obtain thick extracts (ethanolic extract of sikkam stem bark; EESSB) [7].

The EESSB (50 g) was added with both 125 mL of 96% ethanol and 250 mL of hot water. After that, the fluid was homogenized and putted into a separating funnel, then extracted with 250 mL of *n*-hexane, until the water layer did not give a positive result for the Liebermann-Burchard reagent, to obtain *n*-hexane fraction of sikkam stem bark (NHFSSB).

Identification of Alkaloids

The simplicia powder of sikkam stem bark (500 mg) and EESSB (2 g) were added with 1 mL of HCl (2 N) and 9 mL of distilled water, respectively. Next, heated over a water bath for 2 minutes, then cooled and filtered. The filtrate (0.5 mL) was divided into a 3 of test tube:

1. First tube was added with 2 drops of Mayer reagent

2. Second tube was added with 2 drops of Bouchardat reagent

3. Third tube test tube was added with 2 drops of Dragendorff reagent

The sediment or turbidity was formed in at least two or three of the above experiments that indicating the presence of alkaloids [8].

Identification of Flavonoids

The simplicia powder of sikkam stem bark (10 g) and EESSB (2 g) were added with hot water, respectively. Next, boiled for 5 minutes and filtered immediately. Afterward, the filtrate (5 mL) was added with 100 mg of Mg powder and 2 mL of amyl alcohol solution, then shaked and allowed to separate. The color of red, yellow, orange in the amyl alcohol layer was formed that indicating the presence of flavonoids [9].

Identification of Glycosides

The simplicia powder of sikkam stem bark (3 g) and EESSB (2 g) were added with 30 mL of etanol 95% and 10 mL HCl (2 N), respectively. Next, refluxed for 2 hours, then cooled and filtered. The filtrate (20 mL) was added with 25 mL of distilled water and 25 mL of lead (II) acetate (0.4 M) solution, then shaked and immobilized for 5 minutes. Afterward, the fluid was filtered and the filtrate was extracted with 20 mL of admixture of isopropanol and $CHCl_3$ (2:3), repeated for 3 times. Next, the extract was collected and divided into 3 test tubes. After that, the tubes were evaporated at not exceed 50^{o}C. The residue was dissolved in 2 mL of water and 5 drops of Molisch reagent. Afterward, the tubes were added with 2 mL of concentrated H_2SO_4 (carefully) through the tube wall. The purple ring at the boundary of the two fluids was formed that indicating the presence of sugar bond [8].

Identification of Tannins

The simplicia powder of sikkam stem bark (1 g) and EESSB (2 g) were extracted with 10 mL distilled water, respectively. The filtrate was diluted with distilled water, until colorless. Afterward, the fluid (2 mL) was added with 1–2 drops of 1% $FeCl_3$. The color of blue or blackish blue or green or blackish green was formed that indicating the presence of tannins [10].

Identification of Saponins

The simplicia powder of sikkam stem bark (500 mg) and EESSB (2 g) were putted into a test tubes and were added with 10 mL of hot water, then cooled and shaked for 10 seconds. The stable froth as high as 1–10 cm for

less than 10 minutes (does not disappear with the addition of 1 drop of 2 N HCl) was formed that indicating the presence of saponins [8].

Identification of Steroids/Triterpenoids

The simplicia powder of sikkam stem bark (1 g) and EESSB (2 g) were submerged with 20 mL of ether, respectively, for 2 hours. Afterward, the macerate was evaporated using evaporator cup. Next, the residue was added with 10 drops of Liebermann-Burchard reagent. The color of greenish blue or purple red was formed that indicating the presence of steroids/triterpenoids [10].

Antioxidant Activity Analysis

Preparation of blank solution: DPPH 0.5 mM solution (concentration = 200 µg/mL) was pipetted (5 mL) and putted into a volumetric flask (25 mL), then dissolved and added to the mark line with methanol (concentration = 40 µg/mL).

Determination of maximum absorption wavelength: DPPH solution (concentration = 40 µg/mL) was homogenized and measured its absorption at a wavelength of 400–800 nm [11].

Preparation of raw standard sample solution: EESSB (10 mg) and NHFSSB (10 mg) were dissolved and putted into a volumetric flask (10 mL). Next, added to the mark line with methanol (concentration = 1000 µg/mL).

Preparation of test solutions: The solution of raw standard sample was pipetted (0.06 mL; 0.14 mL; 0.22 mL; and 0.30 mL) and transferred into a 10 mL of volumetric flask (concentration = 6 µg/mL, 14 µg/mL, 22 µg/mL, and 30 µg/mL). Next, the solutions were added with 2 mL of 0.5 mM DPPH (concentration = 40 µg/mL) and added to the mark line with methanol. Afterward, protected from light (placed in the dark) for 60 minutes and measured its absorption using UV-Visible spectrophotometers.

Determination of % inhibition and IC_{50} value:

The antioxidant ability was measured as a decrease in absorption of DPPH solution due to the addition of the test solutions. The absorption value of DPPH solution (before and after the addition of test solutions) was calculated as % inhibition and was performed using the following formula [12]:

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

The IC_{50} value was a number that shows the concentration of the test sample (µg/mL) which gives 50% of DPPH inhibiton. The calculation results were entered in the regression line equation, where the concentration of extract (µg/mL) as the X axis and the value of % (percent) inhibition (antioxidants) as the Y axis.

RESULTS AND DISCUSSION

Extraction and Fractionation

Extraction in this study using cold extraction method, namely maceration. Afterward, fractionation of ethanolic extract was carried out using *n*-hexane solvent. The EESSB from 300 g of simplicia powder was obtained 82.13 g with a rendemen percentage of 27.38% and extract color was blackish red. The NHFSSB was obtained 1.19 g with a rendemen percentage of 2.38% and fraction color was moss green. The sample size is affects for rendemen, where more finer the material used more higher the rendemen [6].

Phytochemical Screening

Characterization of simplicia powder and EESSB were carried out before phytochemical screening analysis. These characterization include determination of water content, water soluble extract content, soluble ethanol extract content, total ash content, and acid insoluble ash content. Characterization purpose for ensuring the uniformity of simplicia quality for qualify of standard requirements. Determination of water content describes the maximum limit of water content in simplicia, because the high amount of water can be a medium for the growth of bacteria and fungi that can damage the compounds contained in simplicia.

Based on the general requirements of Indonesian Materia Medika, the water content of simplicia not exceed 10%. Determination of water soluble extract and ethanol soluble extract content were carried out to provide an initial description of the amount of compounds that can be extracted with water and ethanol. Determination of total ash content was carried out for providing an overview of internal and external mineral content that originated from the initial process to the formation of simplicia associated with organic and inorganic compounds obtained internally and externally. Determination of acid insoluble ash content aims to examine the amount of ash obtained from external factors such as sand or silicate soil [13].

Phytochemical screening of simplicia and EESSB showed the presence of flavonoids, glycosides, tannins and triterpenoids compounds. The extraction principle of compounds for EESSB and simplicia powder is identical, because the ethanol solvent used for extraction is an alcoholic group that can attract all secondary metabolites polar, semipolar and nonpolar [14]. Flavonoids were screened with the addition of Mg powder and concentrated HCl, that produced discoloration of orange-red (flavones), red-dark red (flavonols), dark red-magenta (flavonones), and sometimes green or blue. Glycosides were easily dissolve in water by boiling them briefly in dilute acids or to hydrolyze parts of sugar and aglycone as indicated by the formation of a purple ring. The presence of tannins is characterized by discoloration of blue, blackish blue, green or greenish blue were formed with addition of $FeCl_3$ solution. The blackish blue discoloration on tannins analysis shows 3 hydroxyl groups. The Liebermann-Burchard reaction used widely for triterpenoid and steroid analysis, that exhibited blue-green discoloration to indicate the presence of steroids compound and red, pink or purple discoloration for triterpenoid compound [9]. Characterization of simplicia powder and EESSB shown in Table 1 and its phytochemical screening shown in Table 2.

Table 1: Characterization of simplicia powder and EESSB

No.	Characteristics	Results (%)	
		Simplicia Powder	EESSB
1.	Water content	5.99	6.65
2.	Water soluble extract content	25.56	38.56
3.	Ethanol soluble extract content	29.12	39.17
4.	Total ash content	4.94	0.49
5.	Acid insoluble ash content	5.78	0.05

Table 2: Phytochemical screening of simplicia powder and EESSB

Compounds	Simplicia Powder	EESSB	Results
Alkaloids	–	–	No precipitate formed
Flavonoids	+	+	Orange-red discoloration
Glycosides	+	+	Purple ring formed
Tannins	+	+	Blackish blue discoloration
Saponins	–	–	No stable foam formed
Triterpenoids	+	+	Red-violet discoloration

Antioxidant Activity Analysis

The analysis of antioxidant activity for EESSB was carried out using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical capture method. The principle of this method is the ability of the sample to inhibition the DPPH free radical oxidation process in methanol solution lead DPPH discoloration from purple to yellow, with IC_{50} values as one parameter to determine antioxidant activity in sample [15,16]. The discoloration becomes a benchmark for measurements on UV-visible spectrophotometer. Free radical capture gives a strong absorption at a wavelength of 516 nm. The EESSB and NHFSSB at concentrations of 6 µg/mL, 14 µg/mL, 22 µg/mL, and 30 µg/mL experienced discoloration from purple to yellow after 60 minutes incubation. The absorbance results was used to calculate the percent (%) inhibition of antioxidant compounds (samples) against DPPH. Percentage of free radical inhibition by EESSB and NHFSSB shown in Figure 1.

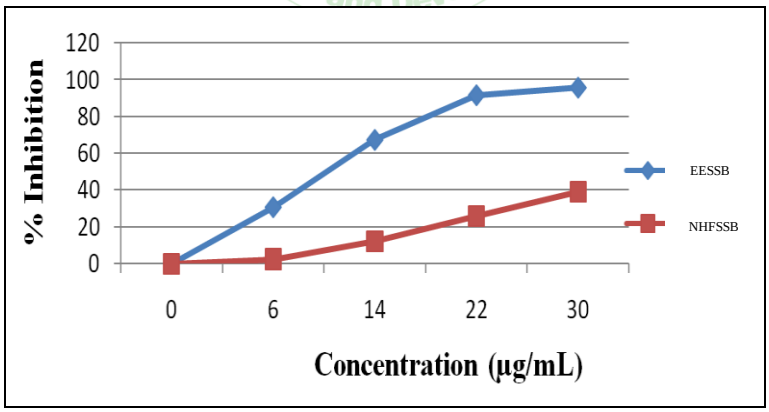

Figure 1: Graphic for antioxidant activity of EESSB and NHFSSB

The results of analysis free radicals inhibition by EESSB and NHFSSB showed that the increasing concentration of the test sample lead increasing DPPH inhibition activity, due to more hydrogen atoms from EESSB and NHFSSB were paired with electrons from DPPH free radicals, generate the decreases of absorption.

The EESSB had an IC_{50} value (12,248 µg/mL) with very strong category of antioxidant activity. The ethanol extract contains all secondary metabolites in the stem bark of sikkam, namely flavonoids, tannins, glycosides, and triterpenoids. Flavonoids and tannins contained in the plants stem bark has a function as scavengers of reactive oxygen species (ROS) [16,18]. Flavonoids have antioxidant activity for binding to harmful metal elements in the body. In plants, some phenolic compounds with potential antioxidants activity (flavonoids, tannins, and lignin) has a functioned as precursor compounds to capture of ROS. Flavonoid compounds have hydroxyl groups that can release protons in the form of hydrogen ions (only have one proton and do not have electrons), that lead radical

electrons of nitrogen atom in the DPPH compound bind to hydrogen ions and produce the reduced DPPH [16,19]. Tanin is generally defined as a polyphenolic compound that has biological activity as an antioxidant [20].

The class of compounds thought to have antioxidant activity in the *n*-hexane fraction are flavonoids and triterpenoids. However, the flavonoids contained in NHFSSB are nonpolar compounds and still bounded to the glycoside group, that generate inhibition for DPPH radicals scavenging and reducing antioxidant activity [21,22]. Side clusters that bind to flavonoids can inhibit antioxidant activity that rander flavonoids cannot donate its hydrogen and electrons for counteract free radicals as an effect of steric hidrance. The presence of other groups in the *n*-hexane fraction can also lead methylated flavonoids. The changes –H (as a source of protons for scavenging free radicals) into a methyl group ($–CH_3$) through a methylation reaction can reduce antioxidant activity [20,21]. The antioxidant potential of *n*-hexane fraction had a weak classified due to the presence of disturbances such as proteins, fats, and other compounds dissolved in non-polar solvents (*n*-hexane solvents). Proteins or fats in plants can donate its hydrogen atoms that lead scavenging to hydroxyl radicals of DPPH. The NHFSSB had an IC_{50} value of 39,622 µg/mL (as shown in Table 3) with very strong classified of antioxidant activity but weak compared to EESSB.

Table 3: The linear regression equation and IC_{50} value of EESSB dan NHFSSB

Sample	Linear Regression Equation	IC_{50} (µg/mL)
EESSB	Y = 3.287X + 9.740	12.248
NHFSSB	Y= 1.350X – 3.490	39.622

CONCLUSION

In conclusion, the simplicia and EESSB exhibited a secondary metabolites contained flavonoids, glycosides, tannins and triterpenoids. The sikkam stem bark had a very strong antioxidant activity, where EESSB had a stronger activity than NHFSSB. This finding might emphasize the potency of sikkam stem bark as antioxidant source from natural product.

CONFLICT OF INTERESTS

All author have no to declare

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