Antioxidant Activity of Ethyl Acetate Extract of Sungkai Leaves (*Peronema canescens* Jack)

Rista Anggi Pramudia[1], Noviany Noviany[1], Sutopo Hadi[1], Jhon Fatriyadi Suwandi[2] and Nanik Siti Aminah[3]

[1]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, University of Lampung, Bandar Lampung, 35145, Indonesia*

[2]*Department of Pharmacology, Faculty of Medicine, University of Lampung, Bandar Lampung, 35145, Indonesia*

[3]*Faculty of Science and Technology, University of Airlangga, 60132, Indonesia*

noviany@fmipa.unila.ac.id

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Abstract: The mechanism of action of antioxidant is related to several diseases that cause health problems, such as: anti- inflammatory, anti-atherosclerotic, anti-thrombogenic and anti- tumor. This disease is caused by oxidative stress which occurs due to free radicals. Secondary metabolite compounds from medicines plants such as phenolic and flavonoids compounds are sources of antioxidant. The sungkai plant (*P. canescens* Jack) contains flavonoids, phenolic compounds, steroids, tannins, saponins and alkaloids. Therefore, this plant could be used as natural antioxidant source. The aim of the present study was to evaluated the antioxidant activity of the ethyl acetate extract of sungkai leaves (*P. canescens* Jack) using the DPPH method. The sungkai leaves powder was macerated with methanol for 3x24 hours and partitioned the concentrated extract with different solvents such as n-hexane, ethyl acetate, methanol and water. The ethyl acetate extract was selected to further fractionated using VLC method with n-hexane: ethyl acetate with gradient as eluent. The antioxidant activity was carried out using DPPH method with different concentration of 20, 40, 60, 80, and 100 ppm. The ability of antioxidant activity in the ethyl acetate extract of sungkai leaves was indicated by the change in DPPH colour from purple to yellow after incubation for 30 minutes. The ascorbic acid was used as positive control. The ethyl acetate extract was fractionated using VLC method to afford subfractions FEA, FEB, FEC, FED, and FEE. The antioxidant activity of FEA, FEB, FEC, FED, and FEE sub-fractions exhibited the strong antioxidant activity with the IC50 values of 0.848, 4.635, 3.277, 2.055, and 0.689 ppm, respectively. From the above findings, it is concluded that sungkai leaves have potential as natural antioxidant source particularly from ethyl acetate extract. However, the purification is still needed to identify the bioactive compounds which have potential as antioxidant activity.

1 INTRODUCTION

Diabetes mellitus, neurodegenerative disorders (Parkinson's disease, Alzheimer's disease and multiple sclerosis), cardiovascular diseases (atherosclerosis and hypertension), inflammation (Rani, 2017), are some types of diseases that triggered by excessive oxidation reactions in the body, causing various cell function disorders. Oxidative stress conditions that occur due to free radicals can cause oxidative damage at the cell, tissue and organ level which will accelerate the aging process and the emergence of diseases.

Antioxidants can decrease oxidative stress induced carcinogenesis by a direct scavenging of ROS and/or by inhibiting cell proliferation secondary to the protein phosphorylation. Antioxidants are molecules that are stable enough to provide electrons to free radicals and neutralize them, thereby reducing their damaging capacity (Lobo, 2010).

Based on the sourcess, antioxidants that can be utilized by humans are grouped into three, namely endogenous antioxidants (antioxidant enzymes) and synthetic antioxidants (Ayoka, 2022). The mechanism of action of antioxidants related to several diseases which can cause various health problems include: (1) anti- atherosclerotic effect, (2) anti-inflammatory, (3) anti- thrombogenic effect, (4) anti- osteoporotic effect, and (5) anti-tumor effect (Rahmi, 2023).

The concept of back to nature life that characterizes most Indonesians is in line with recommendations from the World Health Organization (WHO) to use

traditional medicines or herbal medicines for public health maintenance and disease prevention, especially in healing chronic diseases, degenerative diseases and cancer (Latief, 2021). Based on a review of ethnomedicinal literature, high antioxidant activity in plants is mainly due to the content of secondary metabolite compounds of the polyphenol type (Sharififr, 2009; Hasan, 2012; Noviany, 2012; Noviany, 2018; Noviany, 2020). These antioxidant compounds play an important role in the prevention and therapy of various diseases.

Plants that contain phenolic compounds or flavonoids are sungkai plants (*Peronema canescens* Jack) which is found in Indonesia. Based on the research that conducted by, it was reported that sungkai plants contain secondary metabolite compounds, such as flavonoids, alkaloids, saponins, tannins, phenolics and terpenoids (Maigoda, 2022). This plant is often found in Indonesia, including Sumatra and Kalimantan (Rahmi, 2023).

Recently, research on sungkai leaves has also been carried out on the results of testing that the ethanol extract of sungkai leaves has a total phenolic content of 5.64% (mgEAG/g) and a total flavonoid content of 142,247 mg EQ/g in a 1 mg sample and has antioxidant activity using the method DPPH (Maigoda, 2022).

Based on the information presented above, the leaves of the sungkai plant have the potential to act as antioxidants which contain flavonoids, thus encouraging the author to conduct further research. Therefore, in this research, antioxidant activity tests will be carried out, especially on the ethyl acetate fraction, which can attract polar compounds such as flavonoids.

2 METHODS

2.1 Tools and Materials

Sungkai leaves taken from Seputih Raman District, Central Lampung Regency. methanol (MeOH), n-hexane (n-C6H14), ethyl acetate (EtOAc), acetone (C3H6O), distilled water (H2O), ethanol (C2H5OH), TLC plate, DPPH (2,2-Diphenyl-2-Picrylhydrazyl) and ascorbic acid. Vacuum rotary evaporator, a set of vacuum liquid chromatography (VLC), UV lamp, a set of thin layer chromatography (TLC), capillary pipette, analytical balance, oven and agilent technology cary 100 UV-Vis spectrophotometer.

2.2 Sample Preparation

Sungkai plants were determined at the Bogoriense Herbarium in the Botany Division of the Biology Research Center-LIPI, Cibinong, Bogor. Sungkai leaves are cleaned with running tap water and then dried without being exposed to direct sunlight for one week until the grinding process can be carried out. The dried sungkai leaves are ground until fine to obtain 1000 grams of powder.

2.3 Extraction and Fractionation

The fine powder of the leaves is macerated with methanol for 3x24 hours. Next, the concentrated methanol extract was partitioned into liquid-liquid using n-hexane solvent, to obtain n-hexane fraction and methanol fraction. Then the n-hexane fraction is evaporated to produce n-hexane extract. The methanol fraction was added with water and partitioned using ethyl acetate solvent to obtain the methanol-water fraction and the ethyl acetate fraction. The partitioned fractions are then evaporated to produce a concentrated extract. The crude ethyl acetate extract was then fractionated using VLC method. The sample was dissolved in acetone and impregnated in silica gel, eluted using the eluent ethyl acetate: n-hexane 0% to ethyl acetate 100%. The elution results from each solvent were collected and monitored using TLC method. Fractions that show the same Rf value are combined in one container.

2.4 DPPH Method Antioxidant Activity Test

In this study, the antioxidant test used the DPPH method which has been carried out in previous research and has been modified [5].

2.4.1 Preparation of DPPH Solution

DPPH powder was weighed as much as 0.0078 grams, dissolved in methanol p.a in a 50 mL volumetric flask, lined with aluminum foil and mixed until homogeneous.

2.4.2 Preparation of Blank Solution

Pipetted 1 mL of 1 mM DPPH solution, added methanol p.a to the 10 mL mark, coated with aluminum foil, incubated at room temperature for 30 minutes, then measured the absorbance at a wavelength of 515 nm.

2.4.3 Preparation of ascorbic acid solution

Ascorbic Acid Solution 1000 ppm is made from 50 mg of ascorbic acid dissolved in methanol p.a, in a 50 mL volumetric flask. Then, the ascorbic acid stock solution was made to a concentration of 20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm in a 5 mL volumetric flask, plus 1 mL of DPPH solution, plus methanol p.a to the limit mark. Next, it was homogenized and incubated, then the absorbance was measured using UV-Vis spectroscopy at a wavelength of 515 nm.

2.4.4 Making a test solution for the ethyl fraction of sungkai leaves

A 1000 ppm stock solution was made by dissolving 5 mg of sungkai leaf ethyl fractions in methanol p.a in a 5 mL volumetric flask. Concentration variations of 20 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm were made, from the stock solution added 4 mL of methanol p.a and 1 mL of 1 mM DPPH solution, enough with methanol p.a in a 5 mL volumetric flask. Next, it was homogenized and incubated, then the absorbance was measured using UV-Vis spectroscopy at a wavelength of 515 nm.

2.4.5 Analysis of antioxidant data

Analysis of sample antioxidant data is determined by the amount of inhibition of DPPH radical absorption through calculating the percentage of absorption inhibition using the following calculation:

$$\%inhibition = \frac{(A\ beginning - A\ ater\ reaction)}{A\ beginning} x\ 100\%$$

The IC_{50} value is obtained by making a line equation that connects the % inhibition to the concentration of the test solution for each sample (20 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm) and the ascorbic acid reference (20 ppm, 40 ppm, 60 ppm , 80 ppm, and 100 ppm). IC_{50} is obtained by calculating the concentration of the test solution that can produce free radical inhibition (% inhibition) of 50 percent based on the linear regression line equation using the formula:

y = ax + b

Information:
Y= free radical resistance
X= concentration of test solution

Data obtained from the UV-Vis spectroscopy tool in the form of absorbance of controls and reagents from each test after reacting with sample and comparison test solutions at various concentrations, is used to calculate % inhibition (Maigoda, 2022).

3 RESULTS AND DISCUSSION

3.1 Sample Extraction

The samples were cleaned with running water, dried by drying in the sun without being exposed to direct sunlight. The drying process is carried out to reduce the water content in the sample so that the extraction process is easier. Then grind it and get 1 kg of sungkai leaf powder. The aim of grinding the sample is to expand the surface, help break down cell walls and membranes, thus making the extraction process easier.

Next, the sungkai leaf powder was macerated using methanol solvent for 3x24 hours to maximize the amount of secondary metabolites extracted by the solvent. The filtered maserate was combined and concentrated using a rotary evaporator with a temperature of 40°C and a rotation speed of 80 rpm to obtain a concentrated methanol extract of 38.5%. The concentrated methanol extract obtained was then dissolved with methanol solvent and partitioned using n- hexane solvent with a ratio of methanol extract: n-hexane (3:1) to separate non-polar compounds. The methanol extract was then suspended in water and partitioned with ethyl acetate solvent with a ratio of methanol extract: water: ethyl acetate (1:1:2).

The partitioned extract was concentrated using a rotary evaporator and weighed. The respective weights of n-hexane, ethyl acetate and methanol water extracts are 4.783 grams, 25 grams and 31 mL. Quantitative analysis of phytochemical screening of secondary metabolite compounds in n-hexane, ethyl acetate and MeOH-water extracts can be seen in Table 1.

Table 1: Phytochemical test of several sungkai leaf extracts

Phytochemical Test	N-hexane fraction	Ethyl acetate fraction	MeOH -water fraction
Saponins	++	+	++
Phenol	-	-	-
Terpenoids/ Steroids	Steroids	Steroids	-
Alkaloids	-	-	+
Flavonoids	-	+	+

The ethyl acetate extract of impregnated sungkai leaves was put into a KCV column and then pressed using a pressure glass under vacuum so that there were no voids in the sample. After the sample has been packaged, filter paper is added to the top to keep the sample surface stable when adding the eluent. The eluent used was 100% n-hexane, followed by a mixture of n-hexane: ethyl acetate with a gradient (0-100% ethyl acetate). In the KCV process, 13 fractions are produced. Next, each fraction was monitored using the TLC method with the eluent n- hexane: ethyl acetate (8:2). The resulting KCV fractions that have the same Rf value and separation pattern are then combined and concentrated using a rotary evaporator. Combining the fractions from the KCV results gives 5 fractions shown in Table 2 and Figure 1.

Table 2: Combined fractions of VLC results.

Codes of combined fractions	masses of sample (g)
FEA	0,1384
FEB	0,7317
FEC	1,2125
FED	0,3097
FEE	2,8690

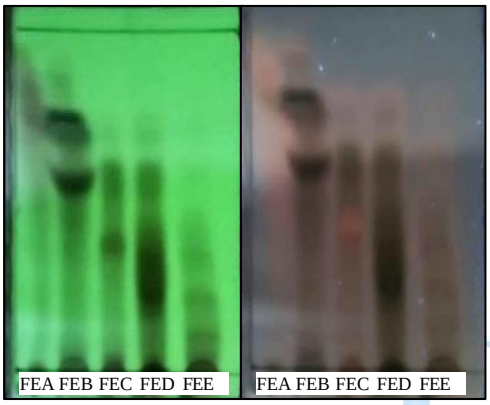

Figure 1. TLC results of 5 combined fractions at UV 254 nm (a) and at UV 366 nm (b).

3.2 DPPH Method Antioxidant Activity Test

Antioxidant activity of tested extracts was carried out with various concentrations of 20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm. The ability of antioxidant activity in the ethyl acetate fraction of sungkai leaves is shown by the change in the color of DPPH from dark purple, fading to yellow, after incubation for 30 minutes. After being reacted with DPPH, each fraction experienced a color change, the color change from purple to yellow indicated the presence of antioxidant activity. Fractions A, D, E, and the positive control are colored yellow, while fractions B and C are colored purple.

The absorbance of the five fractions and the positive control was measured using a UV- Vis spectrophotometer at a wavelength of 517 nm. From the absorbance data, the percentage of inhibition of each fraction and positive control can be calculated, to determine antioxidant activity by calculating the IC50 value. The IC50 value is calculated based on a linear regression equation which can be obtained by plotting the abscissa and ordinate axes. The abscissa axis showed the sample concentration while the ordinate axis showed the percent inhibition.

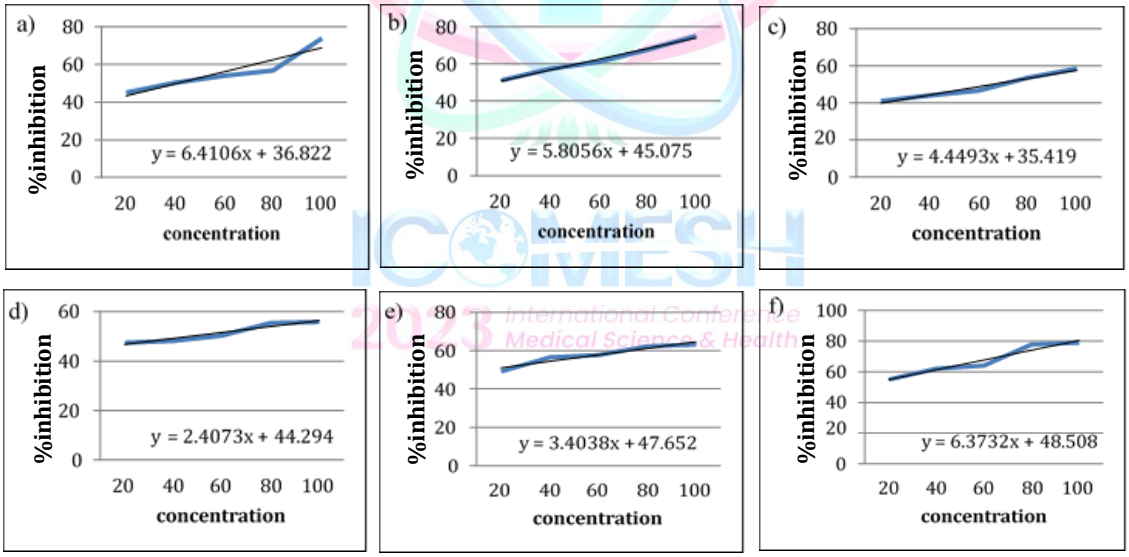

Figure 2. FEA linear regression curve (a); FEB (b); FEC (c); FED (d); FEE (e); positive control (f).

From the linear regression curve of the ethyl acetate fraction and positive control (Figure 2), the IC_{50} value can be calculated in the linear equation, so that the IC_{50} values obtained from FEA, FEB, FEC, FED, FEE, and positive control are 2.055 ppm, 0.848 ppm, 3.277 ppm, 2,370 ppm, 0.689 ppm, and 0.234 ppm. According to (Molyneux, 2004), antioxidant activity is divided into several categories, in table 2.

Table 2: Antioxidant activity category.

IC_{50} Value	**Category**
<50 ppm	strong
50 ppm – 100 ppm	medium
100 ppm -150 ppm	Weak
150 ppm – 200 ppm	very weak

Therefore, antioxidant activity from strong to weak is FEE>FEB>FEA>FED>FEC.

4 CONCLUSIONS

The results of the research can be concluded that each fraction reacted with DPPH experienced a color change from purple to yellow, which indicates the presence of antioxidant activity and the one that has relatively strong antioxidant activity is FEE with a value of 0.689 ppm.

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