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The Phenolic, Flavonoid, and Anthocyanin Content From Methanol Extract of Senggani Fruit and Its Antioxidant Activity

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Abstract

Senggani fruit is an edible fruit that tastes sweet, has an attractive color, blue or reddish purple, and is thought to contain anthocyanin. The senggani fruit can be used as a source of natural dyes and is known to have active components as a source of antioxidants. This study aimed to measure the phenolic, flavonoid, and anthocyanin content and the antioxidant activity of the methanol extract of senggani fruit. Senggani fruits were extracted by maceration using methanol. The phytochemical screening test was performed. The phenolic, flavonoid, and anthocyanin content was measured using the Folin-Ciocalteu, $AlCl_3$, and pH differential, respectively. The antioxidant activity test was carried out using the DPPH method. The phytochemical screening test showed the methanol extract of senggani fruit contained phenols, flavonoids, alkaloids, saponins, and tannins. In this study, the total phenol content was 154.880 mg GAE/g, the total flavonoid content was 6.827 mg QE/g, and the anthocyanin level was 7.516 mg/100 g. The antioxidant activity using the DPPH method showed that the methanol extract of senggani fruit had moderate antioxidant activity, with an IC_{50} value of 99.79 ppm.

Keywords: Senggani, Melastoma malabathricum L, *Phenolic, Flavonoid, Anthocyanin, Antioxidant*

INTRODUCTION

Oxidative stress is a condition in which there is an imbalance between the formation of reactive oxygen species (ROS) and the capacity of enzymatic and non-enzymatic cellular antioxidants, resulting in membrane damage and damage to essential biomolecules in the body. This condition can cause cardiovascular disease, cancer, diabetes, and neurodegenerative diseases (Sies, 2020).

The body requires antioxidants to combat free radicals. Antioxidants can counteract ROS directly or indirectly inhibit ROS production (Gulcin, 2020). In the human body, endogenous antioxidant enzymes act against ROS, such as SOD, CAT, and GPx. However, when endogenous antioxidants cannot protect the body from ROS, exogenous antioxidants are required (Martemucci et al., 2022).

Phenolic, flavonoid, and anthocyanin compounds are phytochemical components widely found in plants. Studies have shown that these three compounds exhibit good antioxidant activity (Rafi et al., 2018). Several antioxidant mechanisms of these three compounds have been reported, including scavenging ROS, metal chelation, increasing endogenous antioxidant enzyme activity, and inhibiting ROS-producing pro-oxidant enzymes (Garcia & Blesso, 2021; Lv et al., 2021).

Senggani (*Melastoma malabathricum* L) belongs to the *Melastomataceae* family. The Senggani plant is commonly known as Cengkodok or Senduduk. Senggani plants are efficacious in treating digestive disorders (dyspepsia), bacillary dysentery, diarrhea, hepatitis, vaginal discharge (leukorrhea), thrush, excessive menstrual blood, and uterine bleeding (Ulung & Studi, 2014). Senggani fruit is an edible fruit that tastes sweet, has an attractive color, blue or reddish purple, and is thought to contain anthocyanin. The senggani fruit can be used as a source of natural dyes and is known to have active components as a source of antioxidants (Rifkowaty & Wardanu, 2016).

Phytochemical screening tests using thin-layer chromatography (TLC) showed the presence of phenolic and flavonoid compounds in the ethanol extracts of Senggani leaves, flowers, fruits, and stems (Nurmalasari et al., 2019). Research conducted by Rifkowaty et al. showed that wet extraction treatment using 70% ethanol and 3% citric acid with a ratio of materials and solvents of 1:4 resulted in antioxidant activity of 95.22% (Rifkowaty & Wardanu, 2016). Another study showed that the ethanol

extract of Senggani fruit had a strong antioxidant activity with an IC_{50} 52.03 ppm (D Kartikasari et al., 2018). The ethanol and chloroform fractions of Senggani fruit also had a strong antioxidant activity with IC_{50} 2.31 ppm and 1.12 ppm, respectively (Dian Kartikasari et al., 2018).

High antioxidant activities in fruits are mainly attributed to phenolic compounds, such as anthocyanins and other flavonoid compounds. Studies have shown a strong correlation between phenolic, flavonoid, and anthocyanin contents and antioxidant capacity. The greater the content of these compounds, the greater the antioxidant activity (Acero et al., 2019; Shibata et al., 2021). These findings suggest the importance of quantifying phenolic, flavonoids, and anthocyanins in Senggani fruits.

This study aimed to measure the phenolic, flavonoid, and anthocyanin content of the methanol extract of senggani fruit and its antioxidant activity. The existence of a strong relationship between phenolic, flavonoid, and anthocyanin content and antioxidant activity can provide a basis for further research focusing on the development of safer and cheaper natural antioxidants.

METHODOLOGY

Materials and Instrumentals

Materials

Senggani fruits (*Melastoma malabathricum* L), methanol, Folin-Ciocalteu reagent, Na_2CO_3, $AlCl_3$, DPPH (*2,2-diphenyl-1-picrylhydrazyl*), gallic acid, quercetin, KCl, and sodium acetate.

Instruments

Rotary vacuum evaporator (BUCHI), centrifuge (Minispin Eppendorf), spectrophotometer UV-Vis (Genesys 10S, Thermo Scientific), vortex, oven, microplate reader EZ read 400, micropipette, 96-well microplate.

Methods

Sample extraction

Senggani fruit was washed, drained, and then mashed using a blender. The samples were macerated using methanol for three days, after which the solvent was replaced every 24 h. The filtrate was concentrated using a rotary evaporator at 50 °C.

Phytochemical screening

Identifying secondary metabolites from the methanol extract of senggani fruit includes flavonoids, phenols, alkaloids, saponins, tannins, steroids/terpenoids, and glycosides (Farnsworth, 1966).

Determination of total phenolic contents

The Folin–Ciocalteu method was used to measure the total phenolic contents. Briefly, the supernatant (50 µL) was mixed with 2.5 mL of Folin–Ciocalteu reagent (10%) and 2.0 mL of Na_2CO_3 (7.5%), incubated at 45°C for 15 min. Afterward, the sample absorbance was measured using a UV-Vis Spectrophotometer at 765 nm. The absorbance of the sample was plotted against a standard curve (gallic acid was used as a standard). The phenol content of the samples was calculated. Measurements were performed in triplicate. Total phenolic content was expressed as gallic acid equivalents (GAE) in mg per gram of extract (mg GAE/g extract) (María et al., 2018).

Determination of total flavonoid contents

The total flavonoid contents were determined using the $AlCl_3$ method. The sample (1 g) was weighed into a 25 ml volumetric flask, calibrated with ethanol, and homogenized. An aliquot of 2 ml was mixed with 2 ml of 2% $AlCl_3$ solution in ethanol and incubated for 30 min at room temperature. Absorbance was measured at 415 nm using a UV-Vis spectrophotometer. The absorbance of the sample was plotted against a standard curve (quercetin was used as a standard). The levels of flavonoids in the samples were calculated. Measurements were carried out in triplicate. The total flavonoid content was expressed as quercetin equivalents (QE) in mg per gram of extract (mg QE/g extract) (Aryal et al., 2019).

Determination of total anthocyanin contents

Total anthocyanin content was measured using the pH differential method. The sample must first be dissolved in KCl buffer (pH 1) until an absorbance of less than 1.2 at 510 nm is achieved to establish the proper dilution factor. The absorbance of the aqueous solution was measured at 510 and 700 nm to determine the zero point. The maximum wavelength for cyanidin-3-glucoside was 510 nm, and sediments that were still present in the sample were corrected at 700 nm. The absorbance at 700 nm was zero when the sample was entirely transparent. Each sample was dissolved in a buffer solution based on a predetermined dilution factor (Df). After being diluted with KCl buffer (pH 1) and Na-acetate buffer (pH 4.5), the sample was incubated for 15 min before being measured. The absorbance of each solution at 510 and 700 nm was measured using distilled water as the blank. The absorbance of the dissolved sample (A) was calculated using the following formula (Le et al., 2019) Equation 1.

$$A = (A_{510}-A_{700})\ pH\ 1,0 - (A_{510}-A_{700})\ pH\ 4.5 \quad (1)$$

The following Equation 1 represents the overall anthocyanin concentration in cyanidin-3-glucoside equivalents (Equation 2).

$$\text{Anthocyanin (\%)} = \frac{A}{\varepsilon x L} \text{ x MW x Df x } \frac{V}{Wt} \text{ x 100\%} \quad (2)$$

where A is the absorbance of the dissolved sample, MW is the molecular weight of cyanidin-3-glucoside (449.2 g/mol), Df is the dilution factor, ε is the molar absorptivity of cyanidin-3-glucoside (26.900 L/mol), L is the cell path length (1 cm), and Wt is the sample weight (g).

Determination of antioxidant activity

The antioxidant activity measurement was performed using the DPPH (*2,2-diphenyl-1-picrylhidrazyl*) method. Briefly, 2 ml of sample solution (in methanol) was added to 2 ml of 0.1 mM DPPH solution. Covered with aluminum foil, then homogenized and incubated for 30 min. The absorbance was measured at the maximum wavelength of DPPH. Measurements were repeated three times. Prepare a blank solution containing DPPH solution in methanol. The data obtained were processed using linear regression analysis to determine the IC_{50} value. Data on the percentage of inhibition were required to calculate the IC_{50} value. Percent inhibition was estimated using the following formula Equation 3 (Sungthong & Srichaikul, 2018).

$$\text{\% Inhibition} = \frac{Abs\ blank - Abs\ sample}{Abs\ blank} \times 100\% \quad (3)$$

The percent inhibition values obtained are entered into the linear regression curve on the y-axis, and the concentrations are on the x-axis. The IC_{50} value was obtained using the linear regression equation: y = ax + b, where y is substituted for 50 and x is substituted for the IC_{50} value. The values of a and b in the equation y= ax + b appear on the curve.

RESULTS AND DISCUSSION

Sample extraction

Extraction resulted in a yield of 1.413%. Several factors affect the yield, including the type and concentration of the solvent, the sample particle size, and the length of the extraction time. The extraction process used in this study was the maceration method. Maceration is the most widely used traditional extraction method worldwide (Belwal et al., 2018). The principle of this method is based on solid-liquid separation using organic solvents or water as the liquid phase (Mohammed Golam Rasul, 2018). The solvent used during maceration will help the process of separating the active compound content from plant tissue (Kafelau et al., 2022). Methanol, ethanol, water, or a mixture of these solvents are generally used to extract phenolic compounds (Ćujić et al., 2016). The maceration method aims to minimize damage or loss of compounds during the extraction process. This method is particularly suitable for heat-sensitive compounds (Dali et al., 2022; Pandey & Tripathi, 2014). Particle size also greatly influences the extraction results in the maceration process. The smaller the particle size, the easier it is for the solvent to diffuse into the material; thus, withdrawing compounds from the material becomes more efficient (Budiastra et al., 2020).

Phytochemical screening

Phytochemical screening was used to determine the groups of secondary metabolites that exhibit biological activities in plants (Hasti et al., 2022). Phytochemical screening showed that the methanol extract of senggani fruit contained flavonoids, phenols, alkaloids, saponins, and tannins.

Table 1. The phytochemical screening

No	Secondary Metabolites	Findings
1	Alkaloid	+
2	Flavonoid	+
3	Steroid/Triterpenoid	-
4	Saponin	+
5	Tannin	+
6	Phenol	+
7	Glycosides	-

Total phenolic contents

The total phenolic content of senggani fruit extract was determined using the Folin-Ciocalteu method and gallic acid as the standard (María et al., 2018). The regression equation of the gallic acid standard curve was y = 0.0121x + 0.0292 with R^2 = 0.9998. The total phenolic content of the methanol extract of the senggani fruit was 154.880 mg GAE/g.

Table 2. Total phenolic contents

Replication	Abs	Total phenolic contents		Average
		%	mg GAE/g	
1	0.410	15.736	157.36	
2	0.395	15.116	151.16	154.880
3	0.407	15.612	156.12	

The Folin-Ciocalteu method is based on an oxidation-reduction reaction. The principle of this method is forming a blue complex that can be measured at 765 nm. Proton dissociation causes the phenolic compounds to transform into phenolic ions because phenolic compounds only interact with the Folin-Ciocalteu reagent in an alkaline environment. To create the alkaline conditions, Na_2CO_3 was used. A deeper blue shade is produced in line with the formation of additional phenolic ions. However, this reagent measures the total phenol and reacts with other reducing agents, such as sugars or ascorbic acid (Lamuela-Raventós, 2018). Gallic acid was used as a standard because it is a simple phenolic acid, a derivative of hydroxybenzoic acid with high antioxidant activity (Hudz et al., 2019).

Total flavonoid contents

The total flavonoid content of senggani fruit extract was determined using the $AlCl_3$ method and quercetin as the standard (Aryal et al., 2019). The regression equation of the quercetin standard curve was y = 0.0309x - 0.0324 with R^2 = 0.9995. The total flavonoid content of the methanol extract of the senggani fruit was 6.827 mg QE/g.

According to the Indonesian Herbal Pharmacopoeia, a total flavonoid content of not less than 0.90% can be calculated as quercetin (Kementerian Kesehatan RI, 2017). The results showed that the total flavonoids in the methanol extract of senggani fruit fulfilled the literature requirements. This indicates a quercetin compound in the methanol extract of the senggani fruit.

Table 3. Total flavonoid contents

.The principle of the $AlCl_3$ method is forming a stable complex between $AlCl_3$ and the ortho-hydroxyl group in ring A or ring B of the flavonoid compounds. The $AlCl_3$ addition caused a shift in the wavelength towards the visible region, marked by the formation of a yellow color. Because quercetin is stable in an acidic environment, sodium acetate was used to create an acidic environment and to maintain the wavelength in the visible range. Quercetin was used as the standard solution because of its ability to form complexes when reacting with $AlCl_3$ (Shraim et al., 2021).

Total anthocyanin contents

Total anthocyanins were determined using the pH differential method, namely pH 1.0 and pH 4.5 (Le et al., 2019). The anthocyanin content of the methanol extract of the senggani fruit was 7.516 mg/100 g.

Table 4. Total anthocyanin contents

Replication	pH	Absorbance		A	Average
		510 nm	700 nm		
1	1	1.073	0.547	7.485	7.516
	4.5	0.538	0.119		
2	1	1.070	0.547	7.547	
	4.5	0.544	0.124		

Note: A= Total anthocyanin contents (mg/100g)

At pH 1.0, anthocyanins exist in the form of oxonium compounds, and at pH 4.5, they are colorless carbinols. Anthocyanin pigments will form colored flavylium or oxonium cations as conditions become more acidic, especially closer to pH 1. The absorbance measurements will reveal greater amounts of anthocyanins. The flavylium cation transforms into a more stable, colorless hemiketal form and produces chalcones at pH 4.5, which is a weak acid. The monometric anthocyanin pigment was identified based on the difference in absorbance between the two buffer solutions. The sample measurement was performed at 510 and 700 nm because 510 nm is the maximum wavelength for cyanidin-3-glycosides, while 700 nm was used to correct precipitates in the sample. If the sample was clear, the absorbance at 700 nm was zero (Taghavi et al., 2022). This study used KCl (pH 1.0) and sodium acetate (pH 4.5).

DPPH Radical Scavenging Activity

The antioxidant activity was determined using the DPPH (*2,2-diphenyl-1-pikrilhidrazyl*) radical scavenging method. The principle of this method is that free radicals (DPPH solution) react with antioxidant compounds to produce the non-radical compound 1,1-diphenyl-2-picrylhydrazyl. As a result of electron donors from antioxidants, the DPPH radical becomes more stable, as indicated by a change from purple to yellow. To find out the remaining DPPH after adding the test solution was carried out by measuring the absorbance using a spectrophotometer at 517 nm (Munteanu & Apetrei, 2021).

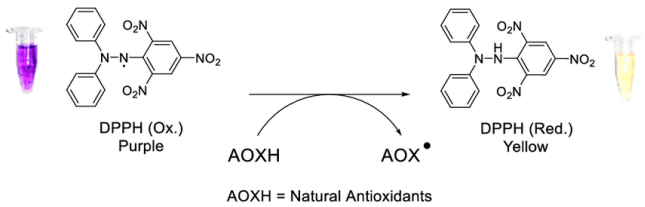

Figure 1. Mechanism of reducing DPPH radicals by antioxidants (Arce-Amezquita et al., 2019)

The correlation curve between the concentration and % inhibition as a percentage of free radical DPPH

inhibition from the methanol extract of senggani fruit is shown in figure 2.

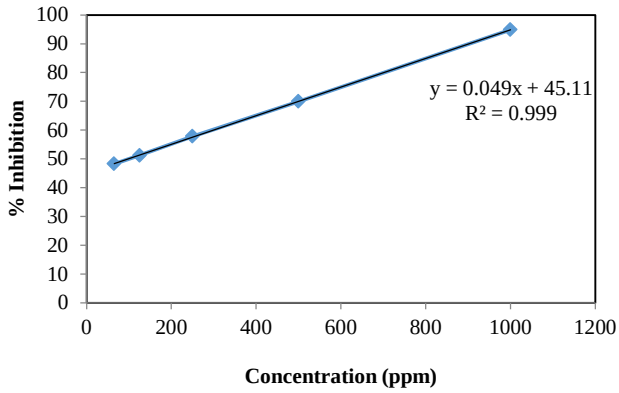

Figure 2. Correlation curve between concentration and % inhibition of methanol extract of senggani fruit

The calculation results of % inhibition and IC_{50} from the methanol extract of senggani fruits are presented in Table 5.

Table 5. DPPH Radical Scavenging Activity

Replicatio	Concentratio (ppm)	Absorbanc	% Inhibition	Average	IC_{50}
A1		0.066	94.50	94.86	
A2	1000	0.056	95.33		
A3		0.063	94.75		
B1		0.360	70.00	69.97	
B2	500	0.362	69.83		
B3		0.359	70.08		
C1		0.515	57.08	57.83	99.79
C2	250	0.503	58.08		
C3		0.500	58.33		
D1		0.588	51.00	51.22	
D2	125	0.587	51.08		
D3		0.581	51.58		
E1		0.629	47.58	48.27	
E2	65	0.623	48.08		
E3		0.610	49.16		

The methanol extract of the senggani fruit showed moderate antioxidant activity with an IC_{50} of 99.79 ppm. The potent antioxidant activity was related to the metabolite content, which had the potential to be an antioxidant in the methanol extract of senggani fruit. Flavonoids and phenols have the potential to act as antioxidants owing to the presence of –OH groups attached to the carbon of the aromatic ring. In polyphenolic compounds, antioxidant activity is closely related to the aromatic ring's side-chain structure and substitution (Vuolo et al., 2018).

The mechanism of phenols as antioxidants is that phenol can donate hydrogen atoms so that phenols can reduce DPPH radicals to a more stable form. The number and position of hydroxyl groups in the molecule influence phenolic compounds' free radical scavenging activity. The greater the number of hydroxyl groups, the stronger the antioxidant activity produced. Its ability to react with DPPH free radicals can affect antioxidant properties (Shahidi & Ambigaipalan, 2015).

Flavonoid compounds act as antioxidants because they contain hydroxyl groups that can cause hydrogen ions to release their protons. The hydrogen ion has only one proton and no electrons; thus, the radical electrons present in the nitrogen atom in the DPPH compound bind to hydrogen ions and produce reduced DPPH (Zheng et al., 2022). Flavonoids act as metal chelators, such as iron, copper, manganese, and cobalt. The hydroxyl (OH) and carbonyl groups of flavonoids can create stable metal complexes (Cherrak et al., 2016).

Flavonoids act as antioxidants by increasing the expression of antioxidant genes through nuclear factor erythroid 2-related factor 2 (Nrf2) activity. These genes play a role in synthesizing endogenous antioxidant enzymes such as SOD, CAT, and GPx (Suraweera et al., 2020). Flavonoids also regulate the oxidative state of cells by inhibiting pro-oxidant enzymes responsible for generating superoxides, such as NADPH oxidase (NO_X), cyclooxygenase (CO_X), xanthine oxidase (XO), and lipoxygenase (LO_X) (González-Paramás et al., 2018).

Anthocyanins belong to the class of flavonoids. Anthocyanins are natural pigments found in fruits, vegetables, and cereals that are red, blue, and purple to black. Several studies have shown that anthocyanins can prevent oxidation. The antioxidant activity of anthocyanins depends on their structures. For example, the orientation of the ring determines the ability to wire protons and transfer electrons. In addition, the number of free hydroxyl groups around the pyrone ring and its position also plays an important role in the antioxidant activity of anthocyanins (Tena et al., 2020). Several antioxidant mechanisms of anthocyanins have been reported, including 1). capture ROS, 2). stimulates the synthesis and activity of antioxidant enzymes, and 3). inhibits the action of enzymes that produce ROS (Bendokas et al., 2020).

CONCLUSION

The phytochemical screening test showed the methanol extract of senggani fruit contained phenols,

flavonoids, alkaloids, saponins, and tannins. In this study, the total phenol content was 154.880 mg GAE/g, the total flavonoid content was 6.827 mg QE/g, and the anthocyanin level was 7.516 mg/100 g. The antioxidant activity using the DPPH method showed that the methanol extract of senggani fruit had moderate antioxidant activity, with an IC_{50} value of 99.79 ppm.

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