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Determination of Total Phenolic Content in Muntingia Calabura L. Extract and Antioxidant Activity Test Using the Ferric Reducing Antioxidant Power Method

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Abstract

Natural antioxidants can be obtained from fruit, vegetables and plants, because they contain the largest compounds, namely phenolic compounds. Phenolic compounds are the largest group of compounds that function as natural antioxidants in plants. One plant that contains a lot of antioxidants is found in the cherry plant (Muntingia calabura L.). This study aims to determine the total phenolic content and antioxidant activity of the maceration and ultrasonic extraction methods of ethanol extract, ethyl acetate of cherry leaves (Muntingia calabura L.). Extraction of cherry leaves was carried out using maceration and ultrasonic methods using ethyl acetate solvent. The thick ethyl acetate and ethanol extracts of cherry leaves were measured for total phenolic content and antioxidant activity. The results of maceration extraction with ethanol solvent had a content of 58.498 mgGAE/g and ethyl acetate had a content of 58.820 mgGAE/g. The results of ultrasonic extraction with ethanol solvent had a content of 56.118 mgGAE/g and ethyl acetate had a content of 51.463 mgGAE/g. The total phenolic content of ethanol and ethyl acetate extracts of cherry leaves from the maceration and ultrasonic methods had significant differences. Ethanol and ethyl acetate extracts of cherry leaves using the maceration extraction method had antioxidant activity levels of 36,639 ppm and 39,361 ppm, while the ultrasonic method had antioxidant activity levels of 35,268 ppm and 39,179 ppm respectively. The antioxidant activity of ethanol and ethyl acetate extracts of cherry leaves from maceration and ultrasonic extraction methods has significant differences.

Introduction

Antioxidants are substances necessary in the body to neutralize free radicals and prevent damage caused by free radicals to normal cells in the body (Umboro & Yanti, 2020). Free radicals are molecules with one or more unpaired electrons, making them unstable, short-lived, and highly reactive, thus leading to an increase in free radicals within the body. Free radicals play a role in degenerative processes that gradually reduce tissue's ability to regenerate and repair itself, thereby maintaining its normal function (Sukatin et al., 2022).

Natural antioxidants are known to be beneficial as food ingredients due to their generally low toxicity levels. Natural antioxidant sources can be obtained from fruits, vegetables, and plants, as they contain significant amounts of phenolic compounds. Phenolic compounds have one or more phenol rings (polyphenols), which have hydroxyl groups attached to aromatic rings, making them easily oxidizable by donating hydrogen atoms to free radicals. Polyphenol derivatives function as antioxidants that can stabilize free radicals by complementing their

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electron deficiency and inhibiting the chain reaction of free radical formation (Dhurhania & Novianto, 2019).

Plants containing secondary metabolites such as flavonoids and phenols are known to function as free radical scavengers due to their antioxidant properties. One such plant rich in antioxidants is the kersen plant (Muntingia calabura L.). The kersen plant (Muntingia calabura L.) commonly grows in tropical regions. The benefits of the kersen plant for medicinal purposes are still relatively unknown. The parts of the kersen plant used for medicinal purposes are the fruit and leaves (Akuntansi, 2022). Kersen leaves contain secondary metabolites like flavonoids and phenolic compounds (Hakim & Saputri, 2022). Kersen leaves are beneficial for relieving coughs, and the ripe fruit can be used to treat jaundice (hepatitis). Kersen leaves contain flavonoids that are effective in lowering blood pressure, acting as antiseptics, antioxidants, hepatoprotectors, analgesics, anti-inflammatories, and antiplatelets (Akuntansi, 2022).

Methods

Equipment

The equipment used in this study includes an Ohaus Pioneer PA114 analytical balance, a 100 mL Buchner funnel, aluminum foil, a Shimadzu UV-Vis UV 1280 spectrophotometer, an ultrasonic bath, volumetric flasks (5 mL, 10 mL, 25 mL, 50 mL, 100 mL), a drying cabinet, a dropper pipette, a horn spoon, a rotary evaporator, porcelain dishes, a 250 mL Schott Duran Erlenmeyer flask, cuvettes, a stirring rod, a Socorex 825 micropipette (100-1000 µL), blue tips, a watch glass, a 100 mL beaker, and a maceration apparatus.

Materials

The materials used in this study are kersen leaves, 70% ethanol, 1% NaOH, Folin-Ciocalteu reagent, concentrated HCl, 1% FeCl3, TPTZ, ethyl acetate, sodium acetate, acetic acid, trichloroacetic acid, Whatman no.1 filter paper, methanol p.a, distilled water, gallic acid, and vitamin C (ascorbic acid).

Extraction

Kersen leaf extraction was carried out using maceration and ultrasonic methods with polar solvent (ethanol) and semi-polar solvent (ethyl acetate). Maceration was done by cleaning and drying the kersen leaves in a drying cabinet at 50°C for 3 days. The dried kersen leaves were then crushed into fragments. The fragments were soaked in 500 mL of 70% ethanol and 500 mL of ethyl acetate. The solution was stirred and left to stand for about 1 hour, a process repeated 3 times, and then left to stand for 3 days. The extract was filtered using Whatman no.1 paper, and the filtrate was evaporated using a rotary evaporator until a thick extract was obtained. The extract was further concentrated in a water bath to remove residual solvents. The yield was calculated using the following formula:

$$\text{Total Yield}(\%) = \frac{Total\ Extract\ Weight}{Total\ Dry\ Simplisia\ Weight}\ x\ 100\%$$

Fresh kersen leaves were cleaned and dried at 50°C for 3 days. The dried leaves were ground into a powder to maximize extraction efficiency. Ultrasonic Assisted Extraction involved placing 25 grams of kersen leaf powder into an Erlenmeyer flask, dissolving in 150 mL of 70% ethanol and ethyl acetate each (1:10), and extracting at temperatures of 30, 40, and 50°C for 10, 20, and 30 minutes using an ultrasonic bath at 47 kHz. The extract was filtered through Whatman no.1 paper and evaporated using a rotary vacuum evaporator at 100 mbar, 70°C, and 100 rpm to separate bioactive compounds from the solvent. The resulting thick extract was weighed and the yield calculated. Total phenol content and antioxidant activity were then determined (Isdiyanti et al., 2021).

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Determination of Total Phenolic Content

Total phenolic content was determined by weighing approximately 10 mg of gallic acid as a standard, dissolving it in methanol p.a. in a 25 mL volumetric flask, and preparing a series of gallic acid dilutions (20, 40, 60, 80, 100, and 120 ppm) in 10 mL volumetric flasks. Each concentration was mixed with 1 mL of Folin-Ciocalteu reagent (3.75% in water), incubated for 8 minutes, and then mixed with 1 mL of 1% NaOH (25 mL ad 50 mL distilled water) and incubated at room temperature for 1 hour. Absorbance was measured at 761.8 nm. A blank was prepared by mixing 0.5 mL of Folin-Ciocalteu reagent and 4.5 mL of 1% NaOH without the test solution. The absorbance data were used to create a calibration curve to determine the total phenolic content in the ethyl acetate and ethanol extracts of kersen leaves, expressed as Gallic Acid Equivalent (GAE). Test solutions were prepared by weighing approximately 200 mg of extract, dissolving it in methanol p.a., filtering through Whatman no.1 paper, and diluting to 25 mL in a volumetric flask. 2 mL of the extract solution was further diluted to 10 mL, and 1 mL of this solution was mixed with 3 mL of Folin-Ciocalteu reagent and 1 mL of 1% NaOH. Absorbance was measured at 761.8 nm (Silverman et al., 2023).

Antioxidant Activity Test Using the FRAP Method

The FRAP method was used to measure the antioxidant activity of kersen leaf extracts. The test began by preparing 250 mL of sodium acetate buffer (0.77 g sodium acetate dissolved in 4 mL of concentrated acetic acid and distilled water), 100 mL of 2N HCl (2 mL of concentrated HCl in distilled water), 2 mL of TPTZ solution (25 mg TPTZ in 2 mL of 2N HCl), 10 mL of FeCl3 solution (54 mg FeCl3 in distilled water), and 10 mL of trichloroacetic acid solution (1 g in distilled water). The FRAP reagent was prepared by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution, 2.5 mL of FeCl3 solution, and diluting to 100 mL with distilled water. Ascorbic acid solutions were prepared by weighing 10 mg of vitamin C, dissolving in ethanol p.a., and making serial dilutions (2, 6, 8, 10, 12 ppm) in 10 mL volumetric flasks. Sample solutions were prepared by weighing 1 g of each sample, dissolving in ethanol p.a., and making serial dilutions (10, 14, 18, 22, 26 ppm) in 10 mL volumetric flasks. Antioxidant testing involved mixing 1 mL of the sample with 3 mL of FRAP reagent, 1 mL of ethanol p.a., homogenizing, and incubating at 37°C for 30 minutes. The color change was observed, and absorbance was measured at 596 nm. A blank was prepared by mixing 3 mL of FRAP reagent, 1 mL of trichloroacetic acid, and 1 mL of ethanol p.a.

Data Analysis

Antioxidant Activity Test

Data analysis of antioxidant activity was based on the inhibition percentage of FRAP absorbance, calculated using the formula (Marjoni et al., 2015):

$$\% \text{ Inhibition} = \frac{Control\ Absorbance - Sample\ Absorbance}{Control\ Absorbance}\ x\ 100\%$$

Explanation:

Control Absorbance: Absorbance of 50 µM FRAP radical at 596 nm.

Sample Absorbance: Absorbance of 50 µM FRAP radical at 596 nm.

The IC50 value for each sample concentration was calculated using linear regression with extract concentration (µg/mL) on the x-axis and % inhibition on the y-axis. The IC50 value was determined when % inhibition reached 50%, calculated using the linear regression equation:

Y= A + Bx

Description:

Y: Absorbance

A: Y-intercept (when x = 0)

B: Slope

X: Concentration

Result and Discussion

Extraction

Table 1. Extraction Results of Ethyl Acetate Extract of Kersen Leaves (Muntingia calabura L.)

Extraction Method	Solvent	Wet Simplisia Weight	Dry Simplisia Weight	Solvent (mL)	Extract Weight	Yield (%)
Maceration	Ethanol	4000	500,07	500	26,4	5,279
	Ethyl Acetate	4000	500,13	500	14,4	2,879
Ultrasonic	Ethanol	4000	100,24	1500	25,6	25,539
	Ethyl Acetate	4000	100,27	1500	11,2	11,169

The data in Table 1 shows that extraction with 70% ethanol solvent resulted in the highest percentage yield compared to extraction using ethyl acetate solvent. The ethanol extract yield from ultrasonic extraction was higher than the ethanol extract yield from maceration extraction. The yield of maceration extraction using 70% ethanol solvent was 5.279%, while maceration extraction using ethyl acetate solvent yielded 2.879%. The yield from ultrasonic extraction using 70% ethanol solvent was 25.539%, whereas ultrasonic extraction using ethyl acetate solvent yielded 11.169%. The ability of 70% ethanol solvent to bind kersen leaf extract compounds is suspected to be the cause of the differences in yield. According to Sayuti (2017), factors that can affect the extract yield include the extraction method used, sample size, time and storage conditions, and the ratio of the sample to the solvent used. Additionally, the yield data is related to the amount of active compound content in a sample; thus, the higher the yield, the more active compounds it contains. The high bioactive compound content in a sample is indicated by the high yield value obtained.

Total Phenolic Content

Table 2. Total Phenolic Content Measurement Results of Kersen Leaves (Muntingia calabura L.)

Extraction Method	Solvent	Absorbance	Concentration (mg/L)	Total Phenolic Content (mgGAE/g extract)	Average Total Phenolic Content (mgGAE/g extract)
Maceration	Ethanol	0,491	11	55	58,498
		0,509	11,464	57,32	
		0,561	12,635	63,175	
	Ethyl Acetate	0,498	11,216	56,08	58,820
		0,512	11,531	57,655	
		0,557	12,545	62,725	
Ultrasonik	Ethanol	0,483	10,878	54,39	56,118
		0,447	10	50	
		0,568	12,793	63,965	
	Ethyl Acetate	0,395	8,896	44,48	51,463
		0,477	10,743	53,71	
		0,499	11,24	56,2	

The total phenolic content test was conducted using the UV-Vis Spectrophotometry method with 7.5% Folin-Ciocalteu reagent. Gallic acid was used as the standard because it is a natural and stable compound. A 1000 ppm gallic acid solution was added with 7.5% Folin-Ciocalteu reagent, resulting in a yellow color, and then 1% NaOH was added to create a basic environment for the reduction reaction of Folin-Ciocalteu by the hydroxyl groups of phenolic compounds in the kersen leaf extract. The hydroxyl groups of phenolic compounds react with the Folin-Ciocalteu reagent to form a blue molybdenum complex. The higher the phenolic content in an extract, the more intense the blue color formed (Pusparida et al., 2023). The total phenolic content test results for each sample showed that maceration ethanol extraction had a phenolic content of 58.498 mgGAE/g, maceration ethyl acetate extraction had 58.820 mgGAE/g, while ultrasonic ethanol extraction had 56.118 mgGAE/g and ethyl acetate extraction had 51.463 mgGAE/g. The total phenolic content of ethyl acetate extract from maceration extraction had the highest value at 58.820 mgGAE/g compared to ultrasonic extraction. The total phenolic content results from ultrasonic extraction with ethyl acetate solvent were also higher than the total phenolic content of 70% ethanol extract. The maximum wavelength used in this study was 761.8 nm, incubated for 1 hour after adding 1% NaOH.

Antioxidant Activity

Table 3. Antioxidant Activity Measurement Results of Vitamin C (Ascorbic Acid)

Sample	Concentration (ppm)	Absorbance	% Inhibition	IC_{50} (mgAAE/g)	Description
Vitamin C	2 ppm	0,793	1,654	38,686	Very Strong
	4 ppm	0,686	14,930		
	6 ppm	0,563	30,190		
	8 ppm	0,446	44,706		
	10 ppm	0,345	57,237		
	12 ppm	0,221	72,622		

The standard solution used in this study was ascorbic acid. Ascorbic acid was used as a comparator because it acts as a secondary antioxidant, capturing free radicals and preventing chain reactions. Vitamin C is a secondary antioxidant that can neutralize various extracellular radicals because it has free hydroxyl groups that act as free radical scavengers, and if it has polyhydroxy groups, it enhances antioxidant activity (Maryam et al., 2016). The antioxidant activity measurement of vitamin C in this study was 38.686 mgAAE/g, meaning that vitamin C can be considered a very strong antioxidant as it has an IC50 value < 50 ppm. According to Tristantini et al. (2016), a compound can be considered a very strong antioxidant if the IC50 value is < 50, strong (50-100), moderate (100-150), and weak (151-200). The smaller the IC50 value, the higher the antioxidant activity.

Table 4. Antioxidant Activity Measurement Results of Kersen Leaf Extract (Muntingia calabura L.)

Sample	Concentration (ppm)	Solvent	Absorbance	% Inhibition	IC50 (ppm)	Description
Maceration	10 ppm	Etanol	0,730	9,346567	36,639	Very Strong
	14 ppm		0,664	17,65922		
	18 ppm		0,548	31,96857		
	22 ppm		0,460	42,92804		
	26 ppm		0,411	49,00744	39,361	Very Strong
	10 ppm	Etil Asetat	0,754	6,410256		
	14 ppm		0,664	17,65922		
	18 ppm		0,559	30,6038		

	22 ppm		0,453	43,75517		
	26 ppm		0,345	57,15467		
UAE	10 ppm	Etanol	0,732	9,222498	35,268	Very Strong
	14 ppm		0,660	18,03143		
	18 ppm		0,563	30,10753		
	22 ppm		0,493	38,79239		
	26 ppm		0,415	48,46981		
	10 ppm	Etil Asetat	0,729	9,470637	39,179	Very Strong
	14 ppm		0,623	22,66336		
	18 ppm		0,531	34,07775		
	22 ppm		0,439	45,5335		
	26 ppm		0,374	53,63937		

In this study, the FRAP test was used with ascorbic acid solution as the standard. The addition of trichloroacetic acid aimed to precipitate the TPTZ complex. The addition of FeCl aimed to form a green to blue complex (Prussian blue). The reducing power is an indicator of the antioxidant potential of a compound. The reducing power in this reaction is measured by the ability of an antioxidant to convert Fe3+ to Fe2+. Compounds with reducing power in this reaction can act as antioxidants by stabilizing radicals by donating electrons or hydrogen atoms, thus converting radical compounds into more stable ones. The parameter used to indicate antioxidant activity is the Inhibition Concentration (IC50) value, which is the concentration of an antioxidant that provides 50% inhibition (Sami & Nur, 2017).

The regression results from the concentration (x) with the absorbance value (y) of the ascorbic acid solution obtained the equation y = 7.0897x – 12.738 with an R2 value of 0.999, and to calculate the antioxidant activity value, the average absorbance value of the sample was entered into this equation, resulting in an IC50 value for vitamin C of 38.686 mgAAE/g. The FRAP value is expressed in mg ascorbic acid equivalent/ g extract (AAE). From the antioxidant activity measurement results of kersen leaf extract using the maceration method with ethanol solvent, the equation y = 2.6148x – 16.884 with an R2 value of 0.984 was obtained, and the average absorbance value of the sample was entered into this equation, resulting in an IC50 value for ethanol extract of 36.639 ppm, while the ethyl acetate solvent obtained the equation y = 3.1896x – 26.297 with an R2 value of 0.999, resulting in an IC50 value of 39.361 ppm. The antioxidant activity measurement results of kersen leaf extract using the ultrasonic method with ethanol solvent obtained the equation y = 2.4814x – 15.74 with an R2 value of 0.997, resulting in an IC50 value of 35.268 ppm, while the ethyl acetate solvent obtained the equation y = 2.7802x – 16.966 with an R2 value of 0.993, resulting in an IC50 value of 39.179 ppm.

From this study, it can be concluded that the ethanol extract yield from ultrasonic extraction was higher compared to the ethanol extract yield from maceration extraction. The total phenolic content of ethyl acetate extract from maceration extraction had the highest value at 58.820 mgGAE/g compared to ultrasonic extraction. The antioxidant activity measurement results of vitamin C in this study were 38.686 mgAAE/g, meaning that vitamin C can be considered a very strong antioxidant as it has an IC50 value < 50 ppm. The antioxidant activity measurement results of kersen leaf extract showed that the antioxidant effect of ethanol extract was stronger compared to ethyl acetate extract. This is because the smaller the IC50 value of kersen leaf ethanol extract, the higher its antioxidant activity.

Conclusion

Based on the above discussion, it can be concluded that the ethyl acetate extract of kersen leaves using the maceration extraction method had a higher total phenolic content compared to the ultrasonic extraction method. The antioxidant activity of kersen leaf ethanol extract was

stronger compared to the ethyl acetate extract. This is because the smaller the IC50 value of kersen leaf ethanol extract, the higher its antioxidant activity.

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