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Determination of total phenol and total flavonoid, and antioxidant activities of chocolate leaves (*Zephyranthes candida* (Lindl.) Herb.)

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ABSTRACT

Introduction: *Zephyranthes candida* plants or known as chocolate flowers are usually only used as ornamental plants, many people in Indonesia do not know that this plant has medicinal properties. The purpose of this study was to determine total phenol levels, and total flavonoids and determine the antioxidant activity of chocolate flower plants.

Methods: This study was an experimental study using cacao flower leaves, ethanol, aqua dest, ABTS, gallic acid, quercetin, AlCl3, Folin-Ciocalteu reagent, Na2CO3, FeCl3, sodium acetate, and Vitamin C. Samples of cacao flower leaves (Zephyranthes candida) were collected from Leles, Garut Regency, West Java. The material taken is collected and then sorted wet to separate the plant parts that are not needed, then washed with running water until clean. Then dried in a drying cabinet at a temperature of 40-50°C to dry. Furthermore, dry sorting is carried out to separate foreign objects that enter during drying. The dried samples were mashed using a blender. Then the simplicia powder is stored in a tightly closed container at room temperature and is ready to be extracted. Then the extract will be analyzed to determine the total phenol content, flavonoid content, antioxidant activity, and thin layer chromatography.

Results: The results of the determination of total phenol and total flavonoid levels and the antioxidant activity of brown flower leaves showed that the total phenol content of the brown flower ethanol extract was 20.5803 ± 0.15 mgGAE/gram. And the total flavonoid content of the brown flower ethanol extract was 1.8411 ± 0.0641 mgQE / g. The test results of the antioxidant activity of brown flower leaves showed that these plants had a very strong antioxidant activity with IC50 of 18.2106.

Conclusions: The results of the research to determine the total phenol and total flavonoid content and antioxidant activity of cacao flower leaves showed that the total phenol content of the ethanol extract of cacao flower was 20.5803 ± 0.15 mgGAE/gram. Moreover, the total flavonoid content of the cacao flower ethanol extract showed a very strong antioxidant activity.

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INTRODUCTION

Free radicals are atoms or molecules (groups of atoms) that have unpaired electrons, besides that free radicals are very reactive, radicals can be stabilized by taking electrons from other molecules and starting chain reactions or extending chain bonds that can damage tissues. In the metabolic process, free radicals are formed when food is converted into energy so that free radicals such as superoxide anions, hydroxyl, and others are easily formed because, in the metabolic process, electron leakage often occurs. In addition, compounds such as hydrogen peroxide (H2O2), ozone, and others, are not free radical compounds but are easily transformed into free radicals. As a result, these free radicals can cause degenerative diseases such as cancer, diabetes, and others. Chemically, antioxidant compounds are electron donating compounds (electron donors). We need compounds that can reduce free radicals, namely antioxidants.[1,2]

Antioxidants are compounds that can reduce and ward off the negative effects of free radicals. Antioxidants donate one electron for free radicals, antioxidants bind to free radicals so that free radicals become stable. Antioxidants are needed by the body to protect the body from free radical attack. Antioxidants are compounds or chemical components which in certain levels or amounts can inhibit or slow down the damage caused by the oxidation process.[3] Compounds that have antioxidant activity can be found in plants, including polyphenols, vitamin C, vitamin E, -carotene, and flavonoids. These antioxidants will stimulate the body's ionic response so that it can bind free radicals so that they are stable and can prevent degenerative diseases such as cancer.[2,3]

The genus Zephyranthes has been used as traditional medicine in many countries. Plant parts such as tubers and leaves have been used to treat various diseases. Zephyranthes candida leaves have been used by indigenous people in Africa for the treatment of diabetes mellitus. Zephyranthes candida plants or

known as cacao flowers are usually only used as ornamental plants, many people in Indonesia do not know that this plant has medicinal properties. This plant has previously been studied for its antibacterial, antimicrobial, and antidiabetic activity. The research on antidiabetic activity of methanolic leaf extract and different fractions of Zephyranthes candida in streptozotocin-induced diabetic rats showed that Zephyranthes candida leaves contain flavonoids, glycosides, terpenoids, saponins, alkaloids, and tannins.[4]

The purpose of this study was to determine the levels of total phenols, and total flavonoids and to determine the antioxidant activity of cacao flower plants. This research is expected to be useful in providing information and knowledge regarding the levels of total phenol, total flavonoids, and antioxidant activity in cacao flower leaves as an alternative to natural antioxidants.

METHODS

Study Design

The type of research conducted is an experimental laboratory study. This study used cacao flower leaves, ethanol, aqua dest, ABTS, gallic acid, quercetin, AlCl3, Folin-Ciocalteu reagent, Na2CO3, FeCl3, sodium acetate, and Vitamin C. Samples of cacao flower leaves (Zephyranthes candida) were collected from Leles, Garut Regency, West Java.

Data Collection

The tools used in this research are stainless knife, analytical balance, measuring cup, glass beaker, glass funnel, maceration vessel, distillation apparatus, aluminum foil, desiccator, vacuum rotary evaporator, filter paper, silicate crucible, furnace, test tube, cuvette, volumetric flask, UV-VIS spectrophotometry, microscope, micropipette, water bath, dropper, filter paper, stir bar, electric stove, oven, and Erlenmeyer. The collected material was determined in the Bandungense herbarium of the School of Biological Sciences and Technology (SITH) of the Bandung Institute of Technology (ITB), to ensure the identity of the test plants.

Zephyranthes candida (Lindl.) Herb Extract Preparation

The material taken is collected and then sorted wet to separate the plant parts that are not needed, then washed with running water until clean. Then dried in a drying cabinet at a temperature of 40-50°C to dry. Furthermore, dry sorting is carried out to separate foreign objects that enter during drying. The dried samples were mashed using a blender. Then the simplicia powder is stored in a tightly closed container at room temperature and is ready to be extracted.

The extraction method used in this research is maceration. A total of 500 grams of cacao flower leaf simplicia powder obtained from drying was extracted using the maceration method with 5000 mL ethanol 96% (1:10) in a tightly closed container. Soaked for the first 6 hours, stirring occasionally, then allowed to stand for 18 hours. The procedure was repeated 2 times with the same solvent and the total volume was half the volume of the solvent in the first extraction. The filtrate obtained is then combined and concentrated or evaporated using a vacuum rotary evaporator at a temperature of 40°C until a thick extract is obtained.

Characteristics of Simplicia

Drying loss

Put 1 to 2 grams of simplicia in a crucible that had previously been heated to the determination temperature and had been thawed. Simplicia flattened in a crucible. Put in the oven, heated at a temperature of 105°C then weighed and repeated heating until a constant weight is obtained. In the drying stage, allow the crucible and lid to cool in a desiccator to room temperature.

Water content

5 grams of simplicia put into a dry flask. Put 200 mL of water-saturated toluene into the flask, connect the device, and pour the saturated toluene into the receiving tube through the cooler to the neck of the reservoir. Heat the pumpkin for 15 minutes. After that, wait for it to cool, then continue the distillation for 5 minutes. Cool the receiving tube to room temperature, and calculate the volume of water and toluene after separating completely.

Water soluble content

5 grams, put into an Erlenmeyer, added 100 mL of water that had been saturated with chloroform, shaken many times with a shaker for the first 6 hours, and then left for 18 hours. Then filtered, 20 ml of the filtrate was evaporated to dryness in an evaporating dish that had been tared, then the remainder was heated at 105°C until the weight remained constant.

Ethanol soluble content

Put 5 grams of powder. Placed in a corked flask, added 100 ml of ethanol while being shaken repeatedly with a shaker for the first 6 hours and then left for 18 hours. Then filtered quickly by avoiding the evaporation of ethanol. Then 20 ml of the filtrate was evaporated to dryness in an evaporating dish that had been weighed then the rest was heated at 105°C to a constant weight.

Total ash content

Put 2 to 3 grams of the substance that has been weighed carefully, put into a crucible that has been heated and tara. Incandescent slowly until it becomes charcoal then put into a kiln and incandescent until constant weight. The total ash content is calculated on the material that has been air-dried.

Water soluble ash content

The ash obtained from the total ash content was boiled with 25 mL of water for 5 minutes. Then the insoluble part was filtered with ash-free filter paper and then washed with hot water. The residue and ash-free filter paper were then ignited to become ash, then weighed to a constant weight. The difference in weight corresponds to the amount of ash dissolved in water. The water-soluble ash content is calculated on the material that has been dried in the air.

Water soluble acid content

The ash obtained from the total ash content was heated with 25 mL of dilute hydrochloric acid for 5 minutes, then the acid-insoluble part was collected, filtered with ash-free filter paper, washed with

hot water, and ignited until it became ash, cooled, and then weighed to weight. constant. The acid insoluble ash content is calculated on the material that has been dried in the air.

Determination of Total Phenol Content

A standard solution of gallic acid 1000 g/mL was prepared by dissolving 0.1 grams of gallic acid with ethanol p.a to reach a volume of 100 mL. 200 L, 400 L, 600 L, 800 L, and 1000 L of the stock solution were pipetted and each was added with ethanol pa up to 10 mL to obtain various concentrations of 20 g/mL, 40 g/mL, 60 g/mL. , 80 g/ml, and 100 g/mL. Determination of the maximum wavelength was carried out by taking 100 L of gallic acid at 1000 g/mL, then adding 5 mL of Folin-Ciocalteu reagent (which had been dissolved in 1:10 aqua dest) and 4 mL of 7.5% sodium carbonate solution. The solution was allowed to stand for 15 minutes. Furthermore, the absorbance measurement was measured in the wavelength range of 400-800 nm. The maximum absorbance measurement results are shown at a wavelength of 761 nm.[5]

Put 0.5 mL of the reference gallic acid solution, added 5 mL of Folin-Ciocalteu reagent (which had been diluted with 1:10 aqua dest), and 4 mL of 7.5% sodium carbonate solution. The mixture was allowed to stand for 15 minutes, then absorbance was measured at a wavelength of 761 nm. After obtaining the absorbance of each comparison solution, a calibration curve was made and a linear regression equation was obtained.

The mother liquor of cacao flower extract with a concentration of 1000 g/mL was diluted again with distilled water to obtain a concentration of 400 g/mL. A total of 0.5 ml of the test sample was added with 5 mL of Folin-Ciocalteu reagent (which had been dissolved in 1:10 aqua dest) and 4 mL of 7.5% sodium carbonate solution. The solution was allowed to stand for 15 minutes, then absorbance was measured at a wavelength of 761 nm. Total phenol was calculated using the linear regression equation of the gallic acid calibration curve.

Determination of Total Flavonoid Content

Weighed 0.1 grams of quercetin and dissolved in 100 mL of ethanol p.a to 1000 g/mL. From a standard solution of quercetin 1000 g/mL, then 1 mL was taken and dissolved in 10 mL of ethanol pa, so that a concentration of 100 g/mL was obtained, then made several concentrations of 2 g/mL, 4 g/mL, 6 g/mL, 8 g /ml, and 10 g/ml.

Determination of the maximum wavelength was carried out by taking 100 L of quercetin 100 g/mL, added with 1.5 mL of ethanol pa, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M sodium acetate, and 2.8 mL of distilled water. and allowed to stand for 30 minutes at room temperature. Furthermore, the absorbance measurement was measured in the wavelength range of 400-800 nm. The maximum absorbance measurement results show a wavelength of 433 nm.

A total of 0.5 mL of the comparison solution was added with 1.5 mL of ethanol p.a, 0.1 mL of 10% aluminum (III) chloride, 0.1 mL of 1 M sodium acetate, and 2.8 mL of distilled water. After being allowed to stand for 30 minutes, the absorbance was measured at a maximum wavelength of 433 nm. After obtaining the absorbance of each comparison solution, a calibration curve was made and a linear regression equation was obtained.

The cacao flower extract solution with a concentration of 1,000 g/ml was diluted again with distilled water to obtain a concentration of 500 g/mL. A total of 0.5 ml of the test sample was added with 1.5 mL of ethanol p.a, then 0.1 mL of 10% aluminum (III) chloride, 0.1 mL of 1 M sodium acetate, and 2.8 mL of aqua p.i were added. After being incubated at room temperature for 30 minutes, the absorbance of the mixture at a wavelength of 433 nm was measured using a UV-VIS spectrophotometer. Total flavonoids were calculated using the linear regression equation of the quercetin calibration curve.[6]

Antioxidant Activity

A 7.4 mM ABTS was dissolved in distilled water. Potassium persulfate 2.6 mM dissolved in distilled water. Then each solution was allowed to stand for 12 hours. The free radical cation ABTS (ABTS') was prepared by reacting ABTS solution and 1:1 potassium persulfate solution and then stored in the refrigerator before use. 1 mL of ABTS solution was put into a 5 mL volumetric flask filled with ethanol p.a after which it was scanned with a UV-VIS spectrophotometer at a wavelength of 400-800 nm.[5]

In the standard solution of vitamin C, 1000 ppm of vitamin C was made as a mother liquor by diluting 10 mg of vitamin C with ethanol p.a after which it was made up to 100 mL. From the mother liquor, a concentration series of 3, 4, 5, 6, 7, and 8 ppm were made. From each concentration, 1 mL was taken, then 1 mL of ABTS was added, then 5 mL was added to the limit with ethanol p.a, measured at the wavelength of the scanning results, and measurements were carried out 3 times. Absorbance results made a curve of the relationship between absorbance and % inhibition.[5]

Thin Layer Chromatography (TLC)

Testing of thin layer chromatogram of ethanolic extract of cacao leaves with silica gel GF254 as stationary phase and ethyl – n-hexane as mobile phase (8:2). The chamber was first saturated with the mobile phase for 30 minutes with filter paper as a barrier that the solution was saturated. A total of 10 mg of ethanol extract of cacao leaves was dissolved in 1 mL of ethanol. The extract solution was spotted on a GF254 silica gel TLC plate. The TLC plate is inserted into the chamber which has been saturated with the mobile phase and eluted to the mark. After eluting, the TLC plate was removed from the chamber and dried. The dried plates were then observed with UV light with wavelengths of 254 nm and 366 nm. Then sprayed with a spot viewer and seen in visible light.[7]

RESULTS

Table 1. Characteristic result of *Zephyranthes candida* (Lindl.) Herb.

Characteristic	Result (%)	Standard (%)
Dry loss	8,27	-
Ethanol soluble content	7,11	-
Water soluble content	3,37	-
Water content	6	10
Total ash content	8,83	10
Acid soluble ash content	1,53	-
Water soluble ash content	6,70	-

Table 2. Metabolite seconder compound of *Zephyranthes candida* (Lindl.) Herb.

No.	Compound	Result	
		Simplicia	Extract
1	Fenol	+	+
2	Flavonoid	+	+
3	Tanin	+	+
4	Saponin	+	+
5	Alkaloid	+	+
6	Steroid/Triterpenoid	+	+

DISCUSSION

This study used cacao flower leaves taken from Leles, West Java Regency. Before being used or tested the cacao flower sample was determined with the aim of ensuring that the sample used was the correct cacao flower plant with the Latin name Zephyranthes candida. Then the samples obtained were carried out characteristics including macroscopic, microscopic, drying shrinkage, ethanol soluble extract content, water-soluble extract content, water content, total ash content, water-soluble ash content, and acid insoluble ash content.

The drying shrinkage test aims to see how much compound is lost during the heating or drying process. In determining the drying shrinkage content, the drying shrinkage result was 8.27%, this indicates that the compound that evaporates or is lost during the drying process is 8.27%. Determination of ethanol soluble extract content and water-soluble extract content was carried out to see compounds that

Table 3. Phenol total result of *Zephyranthes candida* (Lindl.) Herb.

Sample	Concentration (ppm)	Absorbance	Asam Galat Galat Acid content (ppm)	phenol total content (gGAE/ 100g sample)	Average content (gGAE/ 100g sample) ± SD
extract	400	0,751	82,9677	20,7419	20,5803 ± 0,15
		0,744	81,8387	20,4587	
		0,746	82,1613	20,5403	

Table 4. Flavonoids total result of *Zephyranthes candida* (Lindl.) Herb.

Sample	Concentration (ppm)	Absorbance	Quersetin content (ppm)	Flavonoid total content (gGAE/ 100g sample)	Average content (gGAE/ 100g sample) ± SD
extract	500	0,779	9,5129	1,9038	1,8411 ± 0,0641
		0,765	9,2201	1,8440	
		0,749	8,8782	1,7756	

Table 5. Antioxidant result vitamin C of *Zephyranthes candida* (Lindl.) Herb.

Control Absorbance	Cocentration (ppm)	Absorbance			Average Absorbance	% Inhibitioni	IC50
		A1	A2	A3			
0.688	3	0,544	0,543	0,545	0,544	20.9302	5.5455
	4	0,459	0,460	0,461	0,460	33.1395	
	5	0,385	0,383	0,384	0,384	44.186	
	6	0,313	0,314	0,312	0,313	54.5058	
	7	0,215	0,214	0,216	0,215	68.75	
	8	0,169	0,168	0,170	0,169	75.436	

Table 6. **The antioxidant measurement of Chocolate Flowers' Leaves Ethanol Extract.**

Control Absorbance	Concentration (ppm)	Absorbance			Average Absorbance	% Inhibitioni	IC50
		A1	A2	A3			
0,688	15	0,600	0,625	0,642	0,622	9,5930	18,2246
	16	0,577	0,516	0,573	0,555	19,3314	
	17	0,428	0,464	0,448	0,446	35,1744	
	18	0,366	0,379	0,390	0,378	45,0581	

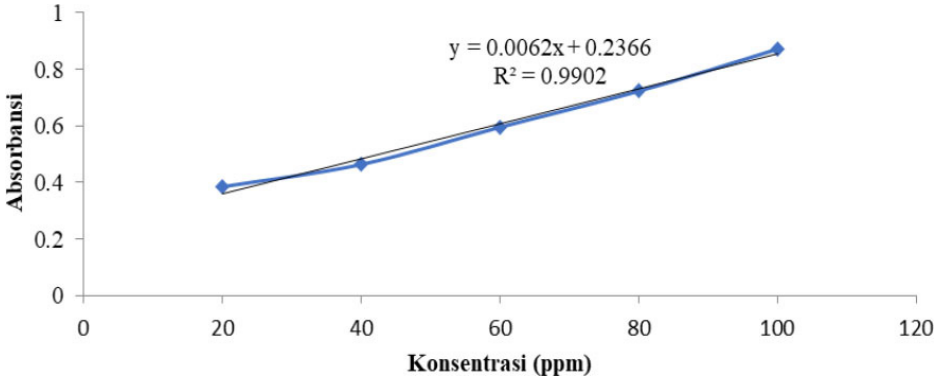

Figure 1. Callibration curve of Galat Acid.

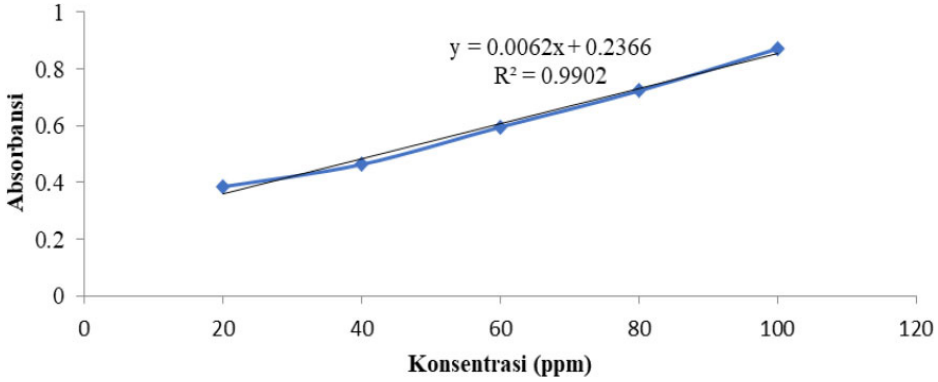

Figure 2. Callibration curve of Quercetin.

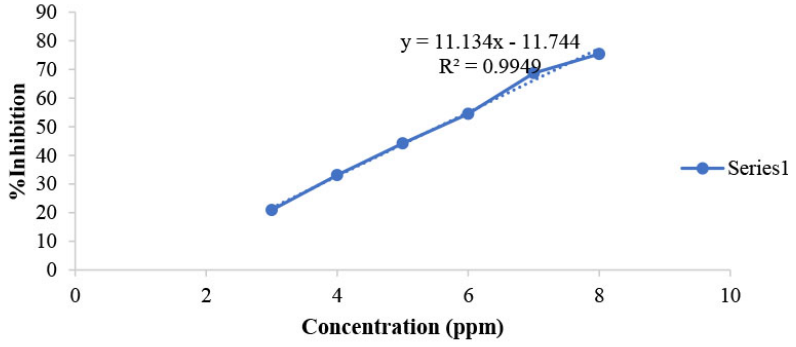

Figure 3. Callibration Curve of Vitamin C.

could be extracted with ethanol and water solvents in simplicia. From the test results, it was found that the ethanol soluble extract content was greater than the water-soluble extract content, which was 7.11% and the water-soluble extract content was 3.37%. This means that more compounds are dissolved in ethanol than those extracted in water.

In the determination of the water content obtained levels of 6% which still meets the requirements, namely not more than 10%. A lot of water content can be a place for microbial growth because water is a medium for the proliferation of microorganisms so that the shelf life is not long and water is also a place for enzymatic processes to occur so that they can decompose the active substance compounds. Determination of the total ash content was carried out to see the level of mineral content in the simplicia. The result of the total ash content is 8.83%, where the provisions of the total ash content are not more than 10%, so the results of the total ash content obtained are included in the standard. The acid insoluble ash content was carried out to see the levels of acid insoluble compounds such as heavy metals. The result of acid insoluble ash content is 1.53%. Water soluble ash content was carried out to see metals that were not soluble in water such as alkali metals and alkaline earth metals, the results obtained were 6.70%. Furthermore, phytochemical screening was carried out on simplicia and extracts which aimed to determine the class of compounds contained in the plant and to see when the extraction process and concentration of extracts did not damage the compounds contained in simplicia. The results of phytochemical screening showed that the content of cacao flowers was phenol, flavonoid, alkaloid, steroid/triterpenoid, tannin, and saponin.

Simplicia extracted by maceration method with the aim that the content that is not resistant to heating is not damaged. Simplicia was macerated with

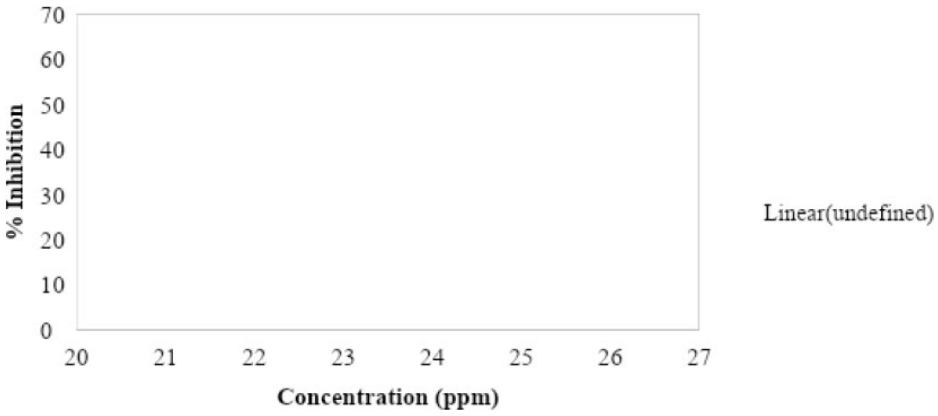

Figure 4. Callibration Curve of Sample.

96% ethanol because ethanol is a universal solvent that can attract polar and nonpolar compounds, besides the volatile nature of 96% ethanol and its ability to inhibit microbial growth are also considerations for choosing a solvent.[8] The concentrated ethanol extract obtained was 59.91 gram with a yield of 11.98%. Determination of total phenol content used Folin Ciocalteau method. This method is a very simple method and Folin Ciocalteau reagent is used because phenolic compounds can react with Folin to form a solution whose absorbance can be measured. The standard solution used is gallic acid which is a natural and stable phenolic. In the way phenol works, Na2CO3 is added to create an alkaline atmosphere because phenolic compounds bind to Folin's reagent in an alkaline environment so that proton dissociation occurs in phenolic compounds into phenolic ions.[9]

Measurement of the maximum wavelength was carried out to determine the wavelength required for the standard solution to achieve maximum absorption. The results of measuring the wavelength of the standard solution of gallic acid obtained that the maximum wavelength is 761 nm. Next, measure the absorbance of the standard solution of gallic acid from various concentrations with the maximum wavelength obtained. Then the absorbance results are made into a calibration curve. The linear regression equation obtained is y= 0.0062x + 0.2366 with an R2 value of 0.9902. The result of the total phenol content of cacao flower extract was 20.5803 ± 0.15 mgGAE/gram extract, meaning that in every gram of ethanolic extract of cacao flower there was phenolic equivalent to 20.5803 ± 0.15 gallic acid.

The determination of the total flavonoid content of quercetin was chosen as a comparison because it belongs to the flavonoid group that is widely found in plants and is known to have many biological activities, especially antioxidants. visible (visible). The principle of flavonoid assay is that there is a reaction between flavonoids with yellow complex AlCl3 and with the addition of sodium acetate to form a pink complex compound whose absorbance is measured.

In determining the maximum wavelength of quercetin, the maximum wavelength is 433 nm. The linear regression equation obtained is y= 0.0468x+0.3335 with an R2 value of 0.9947. Based on the results of the study, the total flavonoid content of cacao flower extract was 1.8411 ± 0.0641 mgQE/g extract, meaning that in every gram of ethanol extract of cacao flower leaves there is a phenolic equivalent to 1.8411 ± 0.0641 quercetin.

The antioxidant activity test used the ABTS method (2,2-Azinobis 3-ethyl benzothiazoline 6-sulfonic acid). ABTS has a higher sensitivity and the reaction mechanism is the ability of antioxidant compounds to stabilize free radical compounds by donating proton radicals. water so that it can detect compounds that are lipophilic and hydrophilic. This method uses IC50 as a parameter to determine the concentration of antioxidant compounds capable of inhibiting 50% oxidation. The curve of the relationship between concentration and percent inhibition to determine the IC50 value. The IC50 value of vitamin C is 5.5455 ppm which belongs to the very strong antioxidant group and the results from the cacao flower leaf extract obtained an IC50 value of 18.2106 ppm which is also included in the very strong group, namely <50 ppm.

TLC analysis of the extract was carried out by spotting on a TLC plate eluted with ethyl: n-hexane as mobile phase in a ratio of 8:2 with GF254 stationary phase with a size of 1x7 cm. On spraying with H2SO4, purple, blue, red, and yellow stains are seen with an Rf of 0.85 cm where the blue and yellow colors mean flavonoids, purple steroids and red is probably chlorophyll. In the view of the FeCl3, black color was obtained with an Rf of 0.31 cm which indicated a positive phenol. In AlCl3 obtained a yellow color with an Rf of 0.73 cm. In addition, citroborate obtained a yellow color with an Rf of 0.73 which indicates a positive flavonoid.

CONCLUSION

The results of the research to determine the total phenol and total flavonoid content and antioxidant activity of cacao flower leaves showed that the total phenol content of the ethanol extract of cacao flower was 20.5803 ± 0.15 mgGAE/gram. And the total flavonoid content of the cacao flower ethanol extract was 1.8411 ± 0.0641 mgQE/g. The results of the antioxidant activity test of cacao flower leaves showed that cacao flower leaves had a very strong antioxidant activity with an IC50 of 18.2106.

DISCLOSURE

Author Contribution

All authors have contributed to this research process, including conception and design, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article, collection and assembly of data.

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Conflict of Interest

There is no conflict of interest for this manuscript.

Ethical Consideration

This research was approved by the Health Research *Ethics* Committee of Mathematics and Sciences Faculty of Garut University. Letter of exemption Ref. No. 11.7899/UG.22/LL/2022

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