ANALYSIS OF TOTAL FLAVONOID CONTENT AND ANTIOXIDANT ACTIVITY ASSAY OF GUAVA VARIETY CRYSTAL (*Psidium guajava* L.) LEAVES EXTRACT

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Abstract

The study investigates the antioxidant activity and total flavonoid compounds in leaves crystal guava, a plant rich in vitamin C and high in antioxidant activity. The research involved maceration extraction of leaves cystal guava using methanol solvent, then fractionation with ethyl acetate and n-hexane solvents in stages to obtain extracts methanol, ethyl acetat and n-hexane; thereafter extracts were subjected to phytochemical screening, total flavonoid content determination, and antioxidant activity using the DPPH method. The study found secondary metabolite compounds which is found in leaves crystal guava such as flavonoids, alkaloids, steroids, terpenoids, saponins and tannins; that ethyl acetate extract had the highest flavonoid content of 171.91 mg/L and highest antioxidant activity with IC_{50} value of 9.59 ppm, followed by *n*-hexane and methanol extracts.

Keywords*: antioxidant, DPPH, guava variety crystal (Psidium guajava L.) leaves, Total Flavonoid Content (TFC)*

Introduction

Antioxidants are compounds that reduce free radicals in the body, which are reactive and less stable due to their lack of paired electrons. Phenolic and flavonoid compounds, known for their high antioxidant activity, effectively reduce free radicals by donating electrons to free radicals, preventing reactions with other compounds. (Priyanto *et al*., 2021; Ulmillah *et al*., 2023). Natural antioxidants significantly aid in combating various diseases like cancer, diabetes, and hypertension, which are caused by free radicals (Hartati *et al*., 2020).

Guava, rich in vitamin C and antioxidants, is a popular choice among the Indonesian people due to its crunchy, seedless guava crystal (*Psidium guajava* L.). Guava cultivation in Indonesia, particularly in Pancur Batu District, Deli Serdang Regency, North Sumatra Province, involves various methods beyond routine leaf pruning to increase flower and fruit quantity. Crystal guava leaves contain various secondary metabolite compounds including alkaloids, glycosides, flavonoids, isoflavonoids, polyphenols, tannins, terpenoids, steroids, and saponins (Priyanto *et al*., 2021; Ulmillah *et al*., 2023). Guava leaves contain flavonoid compounds that are dominant than other compounds, while the guava fruit skin contains vitamin C (Jamieson *et al*., 2022).

Flavonoids, the largest phenolic compound group, consist of 15 carbon atoms and are found in plants' leaves,

roots, wood, pollen, nectar, flowers, fruit, and seeds. Flavonoids are effective heart stimulants, hesperidin works on capillaries, and hydroxylated flavones function as diuretics and fat antioxidants (Sari and Hastuti, 2020). Flavonoid content in plants has various biological activities, including antibacterial, antifungal, anti-inflammatory, antidiabetic, and antioxidant properties, acting as an antidote to free radicals (Marpaung and Wahyuni, 2018). Flavonoids, with their hydroxyl groups, are antioxidants that help to protect against free radicals (Risna *et al*., 2023).

Flavonoid compounds contain chromophores, allowing their concentrations to be measured using UV-Vis spectrophotometry due to the high light absorption required in the analyte molecules (Kumalasari *et al*., 2023). UV-Vis spectrophotometry is commonly utilized for quantitative analysis rather than qualitative analysis (Sari and Hastuti, 2020). The DPPH technique is a simple, fast, and sensitive method for testing antioxidant activity, which prevents oxidative damage and disease caused by free radical reactions (Widyasanti *et al*., 2016).Ulmillah *et al*., (2023) conducted antioxidant activity testing on guava variety crystal using chloroform and methanol solvents. Results showed moderate IC_{50} values for chloroform extracts, strong IC_{50} values for methanol extracts, weak IC_{50} values for fruit flesh, and moderate IC_{50} values for peel and flesh. Plants' chemical compounds content and pharmacological activity are influenced by factors like climate, temperature, geography, and soil fertility in different growing areas (Meisarani and Ramadhina, 2018).

Extraction with the commonly used maceration method is ethanol solvent in determining antioxidant levels. In this study, extraction was carried out using methanol, ethyl acetate and n-hexane solvents. The procedure was carried out to see compounds with different levels of polarity. Methanol solvent will extract polar compounds, ethyl acetate will extract semi-polar compounds, and n-hexane will extract non-polar compounds. Based on this procedure, it will produce compounds with different levels of polarity (Ulmillah *et al*., 2023). Generally, flavonoid compounds dissolve in ethyl acetate solvent.

This study investigated the antioxidant activity and determination of the total flavonoid content of guava variety crystal in methanol, ethyl acetate and n-hexane extracts. The investigation began by extracting guava variety crystal leaves with methanol solvent, then dissolving them with ethyl acetate solvent to obtain total flavonoids, after that, to separate non-polar compounds, the investigation was carried out by fractionating the ethyl acetate residue by dissolving it with a little methanol and fractionating it using a solvent. n-hexane. The procedure after extraction which produces methanol, ethyl acetate and n-hexane extracts is carried out by phytochemical screening; antioxidant activity using the 1,1-diphenyl-2-picryl-hydrazyl or DPPH radical scavenging method; and determination of flavonoid content with the positive control quercetin.

Research Methods

Materials

This study used various materials to analyze of guava variety crystal (Ulmillah *et al*., 2023) leaves from Pancur Batu District, Deli Serdang Regency, North Sumatra Province. The materials used in this study were technical methanol, technical ethyl acetate, technical *n*-hekasan, ammonia 25% (Merck, German), HCl 37% (Merck, German), Mayer reagent, Wagner, Dragendorff, Mg metal, $FeCl_3$ 1% (Merck, German), H_2SO_4 (Merck, German), HCl 1M (Merck, German), ethanol 96% (Merck, German), *1,1-Diphenyl-2-Picrylhydrazyl* (DPPH)

powder (Sigma-Aldrich, America), aluminum foil, and vitamin C (Merck, German).

Instrumentation

This research utilized various tools including a vortex mixer Bransted International 50 Hz, waterbath, vacuum rotary evaporator Buchi R-300, and UV mini-1240 (UV-Vis Spectrophotometer Shimadzu).

Procedure

1) Extraction and fractionation

The extraction process involved weighing 2.5 kg of guava crystals powder, macerating them with methanol, and submerging them in distilled technical methanol solvent for 24 hours at room temperature. The extraction process involved weighing 2.5 kg of guava crystals powder, macerating them in distilled technical methanol solvent for 24 hours at room temperature. Maceration was carried out repeatedly to obtain a clear filtrate, then the filtrate was evaporated to produce methanol extract and dried. The methanol extract was dissolved using ethyl acetate solvent, and filtered to obtain filtrate and residue. The filtrate was evaporated to produce ethyl acetate extract, while the residue was dissolved with methanol solvent, then fractionated with n-hexane solvent using a separatory funnel. Fractionation was repeated until the n-hexan layer was clear, resulting in methanol and n-hexan layers. Each layer was evaporated using a rotary evaporator to produce methanol and n-hexan extracts.

2) Phytochemical screening test

The phytochemical screening of guava variety crystal leaves was conducted using methanol, ethyl acetate, and *n*-hexane extracts, including alkaloids, flavonoids, tannins, steroids, triterpenoids, and saponins (Lumbantoruan *et al*., 2023).

a. Alkaloids

The solid sample was dissolved in methanol and ammonia at a pH of 8-9, filtered, and then a 2M HCl solution was added and shaken. The results were tested in four test tubes, each with a different solution and reagent, with positive results indicated by a white, brown, or orange precipitate.

b. Flavonoids

A methanol extract was mixed with 10 drops of methanol, Mg tape, and concentrated HCl, resulting in a color change indicating a positive result.

c. Tannins

A dissolved solid sample in methanol was boiled and filtered, with $FeCl_3$ added to detect phenol groups. Dark blue and greenish black color indicates tannins, polyphenolic compounds. Positive results indicate phenol groups.

d. Steroids and Triterpenoids (Liebermann-Burchard Test)

A methanol solid sample was stirred with anhydrous acetic acid and concentrated H_2SO_4, indicating a purple to orange color for triterpenoid compounds and blue or green for steroid compounds.

e. Saponins

A test tube containing 1 mg of solid ethanol extract, distilled water, and HCl solution was shaken for 1 minute, heated for 3 minutes, and shaken vigorously for 10 minutes to detect saponin compounds.

3) Determination of *Total Flavonoid Content* (TFC)

Guava crystal extracts were dissolved in 96% ethanol, homogenized, mixed

with water, $NaNO_2$, $AlCl_3$, and NaOH, diluted, and allowed to stand for 15 minutes before re-dilution. The absorbance of the solution was measured using UV-Vis spectrophotometry at the maximum wavelength, and the TFC was calculated using the formula in Eq. (1) (Sukarti *et al*., 2020).

$$TFC = \frac{C \times V \times fp \times 10^{-6}}{m} \times 100\% \quad (1)$$

Description:
C = flavonoid concentration (mg/L)
V = sample volume (L)
fp = dilution factor
m = sample mass (g)

4) Antioxidant activity assay
   a. Preparation of DPPH mother solution
      The DPPH was weighed, dissolved in 96% ethanol, and homogenized in a dark glass bottle. The concentration was 400 ppm, and the mixture was incubated for 30 minutes.
   b. Determination of DPPH maximum lamda (λ)
      Vitamin C comparison solution was mixed with DPPH solution, homogenized, incubated, and measured for absorbance using UV-Vis spectrophotometry at the maximum wavelength.
   c. Preparation of blank solution
      The absorbance of DPPH solution was measured at a wavelength of 516 nm after being homogenized with 96% ethanol and left to stand for 30 minutes.
   d. Preparation of vitamin C comparison solution
      Vitamin C was dissolved in 100 ppm ethanol, then diluted with different concentrations of stock solution in a 5 mL flask. The concentrations were homogenized, allowed to stand for 30 minutes, and then measured using a UV-Vis spectrophotometer at 516 nm.
   e. Preparation of Test Solution
      A test solution of 10 mg guava crystal leaves was prepared, homogenized, and then divided into concentrations of 10–40 ppm, then added to a DPPH solution. The substance was incubated at 37 °C for 30 minutes, then measured by a UV-Vis spectrophotometer at 516 nm. The %inhibition can be calculated by Eq. (2). Meanwhile, the IC_{50} analysis value was calculated (Sembiring *et al*., 2016). From the equation Y = ax + b, the IC_{50} value can be determined through equation (3).

$$\% \text{ Inhibition} = \frac{(\text{Control absorbance} - \text{Sample absorbance})}{\text{Control absorbance}} \quad (2)$$

$$IC_{50} = \frac{50 - b}{a} \quad (3)$$

Results and Discussion

Extraction and fractionation

The study used maceration extraction on crystal guava leaves, producing 238.79 g of methanol extract with a blackish green color and a yield of 9.55%. This method is easy, does not require heating, and can be reused

solvent that has been rotary evaporator so as to prevent damage to active compounds (Sembiring *et al*., 2016). Methanol solvent is used because of its polar nature, damaging leaf cell walls and attracting various polar and non-polar compounds from guava variety crystals.

The process involved dissolving dry methanol extract in ethyl acetate solvent, filtering, evaporating, and drying to obtain a concentrated ethyl acetate extract, which was then dried to produce a concentrated green color. The insoluble residue was fractionated using *n*-hexane solvent, dissolving it in methanol, and added to the solvent. The resulting methanol filtrate and *n*-hexane phosphate phases were observed. The insoluble residue was fractionated, dissolved in methanol, and observed. The resulting methanol filtrate and n-hexane phosphate phases were evaporated, dried, and analyzed.

Figure 1. (A) Methanol extract; (B) Ethyl acetate extract; and (C) *n*-Hexane extract guava variety crystal (*Psidium guajava* L.) leaves

The solvent used in fractionation depends on the compound's polarity level, with non-polar compounds dissolved in non-polar solvents and compounds with different polarities dissolved similarly. Plants have varying affinity for solvent polarity, affecting secondary metabolite compounds, particularly phenolic compounds like flavonoids, using polar, semipolar, and non-polar solvents (Sembiring *et al*., 2016). The extraction and fractionation of guava variety crystal leaves yielded extracts from methanol, ethyl acetate, and *n*-hexane using different solvents (Table 1).

Table 1. Extraction results of guava variety crystal (*Psidium guajava* L.) leaves

Extract	Mass (g)	Content (%)
Methanol	107.33	4.29
Ethyl Acetate	90.21	3.61
n-Hexane	24.73	0.99

Phytochemical screening

The phytochemical screening of crystal variety guava (*Psidium guajava* L.) leaves produced extracts from methanol, ethyl acetate, and *n*-hexane in different solvents. (Table 2). The phytochemical screening revealed that methanol extract, ethyl acetate, and n-hexane extractions were all positive for flavonoids, alkaloids, saponins, tannins, steroids, terpenoids, and tannins.

Table 2. Phytochemical screening of guava variety crystal (*Psidium guajava* L.) leaves

Extract	Flavonoid	Alkaloid	Steroid	Terpenoid	Saponin	Tanin
Methanol	+	+	-	-	+	+
Ethyl Acetate	+	-	+	+	-	+
n-Hexane	+	+	+	-	-	+

Description:
(-) : Does not contain secondary metabolite compounds
(+) : Contains secondary metabolite compounds

Determination of Total Flavonoid Content (TFC)

The study determined Guava Variety Crystal's flavonoid content using the $AlCl_3$ colorimetric complex method, utilizing quercetin compound as a standard solution and reacting with $NaNO_2$ and NaOH (Sukarti *et al*., 2020) (Figure 2). Flavonoid content was determined by creating a standard solution curve, calculating flavonoid content percentage, and measuring absorbance at maximum wavelength, resulting in a maximum wavelength of 440 nm (Table 4).

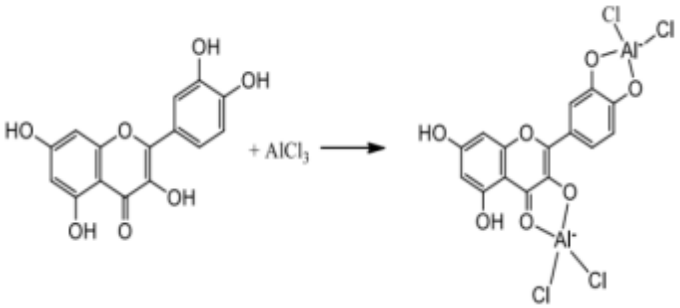

Figure 2. $AlCl_3$ complex formation reaction with flavonol compound

Table 3. *Total Flavonoid Content* (TFC) of guava variety crystal (*Psidium guajava* L.) leaves

Sample	Concentration (ppm)	Absorbance (440 nm)	Linear Equation	TFC (ppm)
Quercetin	10	1.214	y = 0.0988x + 0.05 R^2 = 0.9741	-
	8	0.888		
	6	0.763		
	4	0.509		
	2	0.263		
Methanol	10	0.364		57.99
Ethyl Acetate	10	0.855		171.91
n-Hexane	10	0.415		69.83

The standard curve in Figure 3 is obtained from the relationship between the concentration of quercetin (ppm) and the absorbance value at a wavelength of 440 nm resulting in a linear regression equation of y = 0.1141x + 0.0431 with a value of R_2 = 0.9869. The resulting linear equation can be used to determine the concentration of flavonoid compounds in methanol, ethyl acetate and n-hexane extracts whose results can be seen in (Table 3). Table 3 shows flavonoid content of methanol, ethyl acetate, and n-hexane extracts of guava variety crystal, with ethyl acetate extract having the highest compounds. Fenolic and

flavonoid compounds are antioxidants that can counteract free radical damage to healthy cells, preventing degenerative diseases through immune system destruction and lipid and protein oxidation (Sukarti *et al*., 2020).

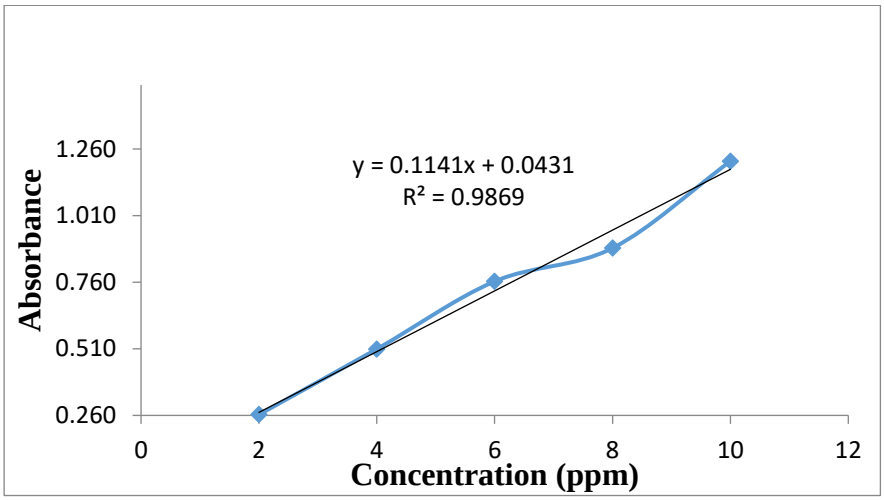

Figure 3. Quercetin standard solution curve

Antioxidant activity

The DPPH free radical scavenging method is utilized for antioxidant activity testing, measuring an extract's ability to reduce stable free radicals captured in an organic solvent at room temperature. The reaction between DPPH and flavonoid compounds involves three stages: electron delocalization, hydrogen reduction, and dimerization between phenoxyl radicals, transferring hydrogen radicals and re-reacting with DPPH radicals. The final stage involves the formation of complex compounds between aryl radicals and DPPH radicals, influenced by the stability and reaction potential of the molecular structure (Sembiring *et al*., 2016). The reaction between DPPH free radicals and antioxidant compounds results in the reduction of DPPH compounds, forming a stable yellow diphenylpycrilhydrazine complex compound (Dewi *et al*., 2018).

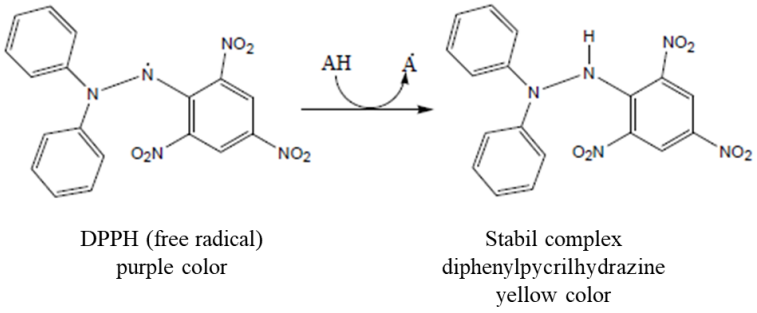

Figure 4. Reaction between antioxidants and DPPH free radicals

The DPPH method's antioxidant activity determination showed an inverse relationship between concentration and absorbance value, with higher concentrations resulting in smaller absorbance values. The regression equation reveals the relationship between concentration and % inhibition of methanol, ethyl acetate, *n*-hexane extracts and vitamin C as a positive control for antioxidant activity (Sembiring *et al*., 2016). The regression equation used to determine the IC_{50} value using equation (2) and (3).

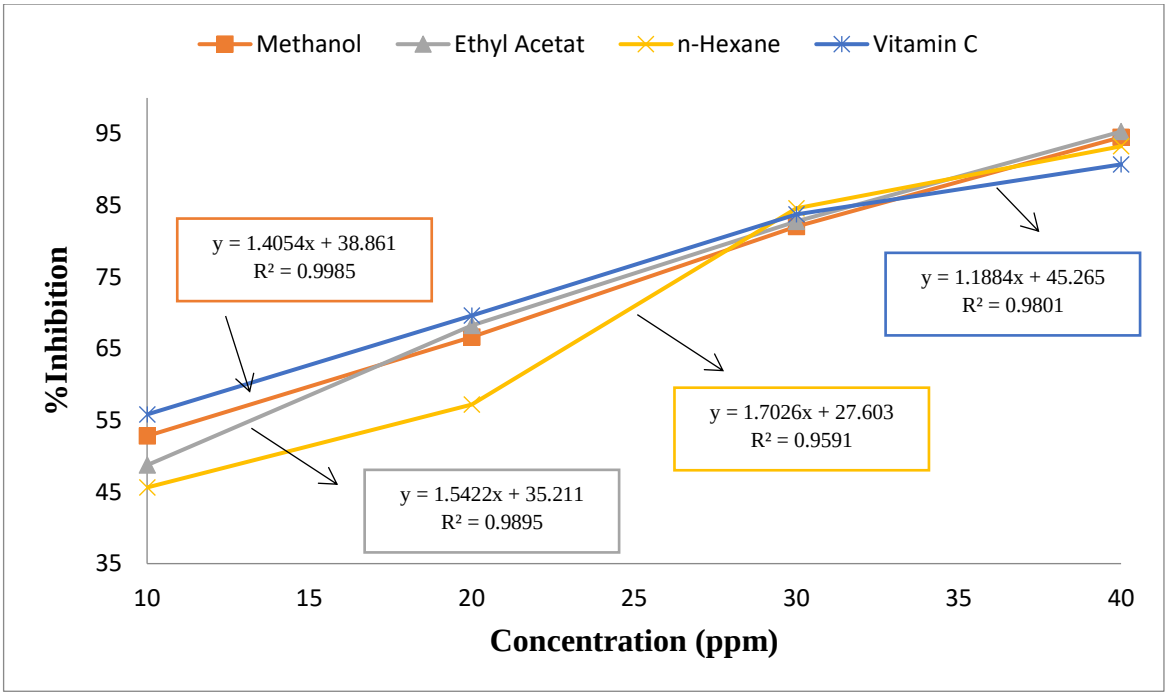

Figure 5. Antioxidant activity of guava variety crystal (*Psidium guajava* L.) leaves

Figure 5 shows DPPH free radical scavenging activity of methanol, ethyl acetate, *n*-hexane, and vitamin C extracts at different concentrations, with higher concentrations silencing against DPPH free radicals. Antioxidant capacity is determined by IC_{50} values, which indicate the amount of antioxidant in a sample needed to reduce 50% of free radicals (Dewi *et al*., 2017). The results of the IC_{50} value for Guava variety crystal (*Psidium guajava* L.) Leaf can be seen in (Table 3).

Table 4. Antioxidant activity of guava variety crystal (*Psidium guajava* L.) leaves

Sample	Concentration (ppm)	Absorbance (440 nm)	%Inhibisi	IC_{50} (ppm)
Vitamin C	10	0.984	55.820	3.98
	20	0.677	69.614	
	30	0.362	83.737	
	40	0.207	90.724	
Methanol	10	1.051	52.813	7.93
	20	0.744	66.622	
	30	0.400	82.032	
	40	0.122	94.524	
Ethyl Acetate	10	1.142	48.743	9.59
	20	0.708	68.238	
	30	0.384	82.780	
	40	0.105	95.302	
n-Hexane	10	1.211	45.631	13.15
	20	0.954	57.196	
	30	0.343	84.590	
	40	0.150	93.253	

Table 4 shows that methanol extract, ethyl acetate, and n-hexane crystal extract of guava varieties show high antioxidant activity. Table 4 shows that methanol extract, ethyl acetate, and n - hexane crystal extract of guava varieties showed high antioxidant activity. The IC_{50} value indicates antioxidant activity, the IC_{50} value is higher in the methanol extract because of the presence of phenolic compounds in the form of tannins based on phytochemical screening tests and flavonoids based on the total flavonoid content value, while the total flavonoid content is the least between ethyl acetate and n-hexane.

Research conducted by (Ulmillah *et al*., 2023) which tested the skin and flesh of crystal guava fruit from chloroform and methanol extract and the positive control ascorbic acid stated that the antioxidant content was low and had a high IC_{50} value. Meanwhile, based on the results of this research, all methanol, ethyl acetate and n-hexane extracts have high antioxidant activity where the IC_{50} value is low. Levels of flavonoid compounds are generally found in the leaves (Jamieson *et al*., 2022). Therefore, the possibility of high antioxidants is influenced by what part is taken and the soil nutrients that a plant contains. Each region has soil nutrients, such as samples taken at high altitudes.

Conclusions

The study reveals that the ethyl acetate extract from Guava variety crystal has the highest total flavonoid content at 171.91 mg/L, followed by *n*-hexane and methanol extracts. The highest antioxidant acitivity was the methanol extract from Guava variety crystal with an IC_{50} value of 7.93 ppm, followed by ethyl acetate and *n*-hexane extracts.

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