

Analysis of Total Anthocyanin Levels in Hibiscus (*Hibiscus rosa-sinensis* L.) Preparations by Differential pH Method Using UV-Vis Spectrophotometry

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Abstract. Red hibiscus is a plant that contains anthocyanins and flavonoids which act as natural dyes and preservatives. Anthocyanins are attractive coloring substances and are not harmful to health, therefore they are widely used in the food and pharmaceutical industries. The maximum allowable daily intake of anthocyanins is 0.25 mg/kg body weight. Excessive intake of anthocyanins can cause poisoning. This study aims to determine the total anthocyanin content in hibiscus extract. The method used to analyze anthocyanin levels is the differential pH method using UV-Vis spectrophotometry. The main advantage of this method is that it provides a simple way to measure very small amounts of substances. The results of the research explained that the qualitative analysis showed that the sample was positive for containing anthocyanins because the acid reagent HCl remained red, this was due to the formation of flavilium. The sample with the base reagent (NaOH) changes color to green due to the formation of a carbinol pseudobase structure. Quantitative analysis of anthocyanins showed that in 10.8876 grams of extract there was an anthocyanin content of 72.4731 mg/L at a dilution of 10 ml. The high level of total anthocyanin in the thick hibiscus extract preparation can be influenced by several things such as the temperature during the drying process, the length of maceration time, the concentration of the solvent used, and the high intensity of light.

Keywords: Anthocyanin; Hibiscus Extract; Differential pH.

Introduction

Hibiscus (*Hibiscus rosa-sinensis* L.) is a plant that generally grows in several countries with tropical and subtropical climates, including Indonesia, where it is usually used as an ornamental plant, hedge plant and cut flower and can be used as a herbal medicine (Purwaniati et al., 2020). The hibiscus plant has physical characteristics, namely beautiful flowers with attractive colors, varied shapes, containing honey, fragrant and slimy (Efendi et al., 2021). Red hibiscus is a plant that contains anthocyanins and flavonoids which act as natural dyes and preservatives (Elisa & Mulyanti., 2021). Anthocyanins are attractive coloring substances and are not harmful to health, therefore they are widely used in the food and pharmaceutical industries. Some plants that contain anthocyanins include roses, hibiscus, roselle, four o'clock and others. Meanwhile, in leaves, fruit and tubers, anthocyanins are found in caladium leaves, red spinach, purple sweet potatoes, red cabbage, strawberries, grapes, mulberries, dragons, and others (Priska et al., 2018). This substance is a polyphenol that has high antioxidant properties and has long been used as

a health food and food for various health benefits (Ooe et al., 2016). Anthocyanin, like flavonoids in general, have an aromatic ring containing polar substituent groups (hydroxyl, carboxyl, and methoxyl) and a glucosyl residue that together produce a polar molecule (Navas et al., 2012). Anthocyanin is a natural pigment from the flavonoid group which has three carbon atoms connected by an oxygen atom which connects two aromatic benzene rings (C_6H_6) in the main structure and produces blue flowers. It comes from the Greek word which means (Priska et al., 2018). According to Pratiwi & Priyani (2019) anthocyanins act as antioxidants thereby protecting against various cardiovascular diseases, cancer and diabetes. Apart from that, anthocyanins also have various biological activities, such as antioxidants, colon cancer prevention, and antihyperglycemic properties. Based on ADI (acceptable daily intake), the maximum allowable daily intake of anthocyanins is 0,25 mg/kg body weight. Excessive anthocyanin intake can cause poisoning (Sangadji et al., 2017). Anthocyanin color is greatly influenced by structure, pH, temperature, enzymes, UV radiation, co-pigmentation, and the presence of oxygen (Roy & Rhim, 2021).

Analysis of the total anthocyanin content in hibiscus preparations is important to determine the anthocyanin compound content in this plant. The method commonly used for anthocyanin analysis is the differential pH method using visible spectrophotometry (Purwaniati et al., 2020). The differential pH method is based on the effect of pH on the form of anthocyanins: at pH 3 and below, anthocyanins are in the flavyl form, while at higher pH, from 3 to approximately neutral, they are in the carbinol form (Pelletier et al., 2015). Spectrophotometers are used to measure relative energy if energy is transferred, reflected, or emitted as a function of wavelength. The main advantage of this method is that it provides a simple way to measure very small amounts of substances. In addition, the results obtained are very accurate because the values read are immediately recorded by the detector and output in the form of digital numbers or regression graphs. This measurement also uses simple tools and materials (Pratiwi & Priyani, 2019). Spectrophotometers are used to measure the concentration, maximum absorption wavelength, absorption value or light transmission of a solution sample. Measurements made with a spectrophotometer are a function of the absorption or transmission of light wavelengths (Afandi & Purwanto, 2018). The UV-Vis spectrophotometer is one of the instrument methods most commonly used in chemical analysis to detect compounds (solid/liquid) based on photon absorption (Irawan, 2019).

Research conducted by Purwaniati et al., (2020) showed that the anthocyanin content contained in fresh telang flower preparations using the differential pH method was greater than in the dosage form. This is because fresh telang flowers have not gone through a heating or preservation process. Apart from this, temperature differences can also affect the levels of anthocyanin produced. Research conducted by Cahyaningsih et al., (2017) after being tested using phytochemical screening and TLC analysis, showed that the Patikan Kebo herb used in this research contained several secondary metabolite compounds including flavonoids, tannins, steroids and anthraquinones. The TLC system used in this research can separate the phytochemical content and provide positive results that confirm the results of the phytochemical screening. The equation for this research is that it uses maceration extraction and phytochemical screening (qualitative analysis). According to research conducted by Gustriani et al., (2016) showed that purple cabbage extraction with 96% ethanol solvent had more optimal anthocyanins compared to extraction using lower concentrations. Meanwhile, research conducted by Zahroh & Agustini, (2021) showed that anthocyanin compounds were more effective in acidic solutions at pH 1.5, because the total anthocyanin content at pH 1.5 was higher than other pH solutions. Research conducted by Jamei & Babaloo., (2017) found that anthocyanin has high stability in acidic solvents with low pH. Increasing the pH value results in decreased anthocyanin stability due to damage.

Based on the problems above, research was carried out to determine the total anthocyanin content in hibiscus extract using the visible spectrophotometric differential pH method. The aim of this research is to analyze the anthocyanin content in hibiscus rosa-sinensis L. extract using acid and base reagents, as well as to analyze the total anthocyanin content of *hibiscus rosa-sinensis* L. extract using the differential pH method.

Methods

This study uses a qualitative method. Qualitative research is commonly known as a naturalistic research method because the research is carried out in natural conditions (natural settings). While the approach used in this study is a laboratory experiments in the laboratory to produce hibiscus extract which was then analyzed for anthocyanin levels using the differential pH method using visible spectrophotometry.

The tools used are an oven, maceration container, rotary evaporator, and visible spectrophotometry. The materials used are hibiscus flowers (*Hibiscus rosa sinensis* L), strong acid (HCl 2M), strong base (NaOH 2M), HCl 2N, distilled water, 96% ethanol, potassium chloride buffer (KCl), sodium acetate buffer (CH₃COONa).

Preparation of Materials and Solution

Prepare 1 kg of fresh red hibiscus flowers, then cut them into several pieces. The hibiscus flowers are then placed in the oven at 40°C to dry. 65 grams of hibiscus flowers that have been dried in the oven are ground using a blender. Then put it in a closed container (jar) and add 96% ethanol solvent in a ratio of 1:4, namely 65 grams: 260 mL. Then leave it for 2-3 days without being exposed to direct sunlight and stir every day for five minutes. The macerated mixture is filtered using filter paper to obtain the filtrate. The remaining organic material from maceration was then re-macerated for 2 days with the same treatment. The filtered filtrate is concentrated using a rotary evaporator until a thick extract is obtained.

Qualitative Analysis

Identification of anthocyanin compounds in hibiscus extract was carried out using phytochemical tests based on the addition of acids and bases. The 2M HCl and 2M NaOH solutions were prepared first. Identification with acid compounds was carried out by diluting 0.5 grams of thick extract with 1 mL of 96% ethanol, then adding 0.5 mL of 2M HCl and putting it in a test tube to be heated in Bunsen spirit for 5 minutes, the color was perfect red. Next, identification was carried out with basic compounds by taking 0.5 grams of thick hibiscus extract, then 1 mL of 96% ethanol was added, the mixture was dripped with 2M NaOH until the color changed to faded green.

Quantitative Analysis

Preparation of buffer solutions pH 1.0 and pH 4.5

To make a pH 1.0 buffer, 0.0405 grams of KCl is used, dissolved in distilled water in a 25 mL volumetric flask up to the mark. The pH was adjusted using 2N HCl to pH 1.0. Next, a pH 4.5 buffer solution was made by dissolving 0.82 grams of sodium acetate (CH₃COONa) with distilled water in a 25 mL volumetric flask until the limit mark. The pH was adjusted using 2N HCl until it reached pH 4.5.

Absorbance measurements at wavelengths of 510 nm and 700 nm

Absorbance was measured by preparing a thick extract (96% ethanol) at two types of pH, namely pH 1.0 and pH 4.5. A total of 1 gram of extract was dissolved in 25 ml of solvent, then two buffer solutions of pH 1 and pH 4.5 were prepared. The diluted extract

was then put into 2 beakers of 2 ml each. The first beaker was added with 10 ml of 96% ethanol solvent and 10 ml of 96% ethanol was added to the second beaker. Next, 4 mL of hibiscus extract solution was taken, then added with KCl buffer solution for pH 1.0 and for the second solution, 6 mL of sodium acetate buffer for pH 4.5 was added. Each was left for 15 minutes before being measured. Absorbance measurements were carried out for each solution at wavelengths of 510 and 700 nm and buffer pH 1 and buffer 4.5 as blanks.

Data analysis is done by means of the absorbance value obtained from measuring the pH 1 solution and pH 4.5 solution at wavelengths of 510 and 700 nm is then entered into the following absorbance value formula:

$$A = (A_{510} - A_{700}) \text{ pH } 1,0 - (A_{510} - A_{700}) \text{ pH } 4,5$$

Information:

A = Absorbance

Calculation of anthocyanin levels

The anthocyanin content in the sample was calculated using the equation:

$$\text{Total anthocyanins (mg/L)} = \frac{(A \times BM \times FP \times 1000)}{\epsilon \times b}$$

Information:

A = absorbance difference at pH 1 and pH 4.5 at wavelengths 510 and 700 nm

ϵ = molar absorptivity (26900 L/(mol.cm)),

b = cuvette thickness (1 cm)

BM = molecular weight (449.2 g/mol),

FP = dilution factor

Results and Discussion

The method used in this research is pH differential visible spectrophotometry to analyze total anthocyanin levels in hibiscus (*Hibiscus rosa-synthesis L.*) extract. This study used hibiscus as a sample because it contains anthocyanin which has the characteristic that it can change color under certain pH conditions (Meganingtyas & Alauhdin, 2021). Anthocyanin compounds were extracted using the maceration method with 96% ethanol solvent. The use of 96% ethanol solvent is because based on previous research, extraction with 96% ethanol solvent produces more optimal anthocyanins compared to extraction using lower concentrations (Gustriani et al., 2016). Apart from that, 96% ethanol has the advantage of being able to filter with wide polarity both polar and non-polar compounds (Puspitasari & Prayogo, 2017). The maceration process causes the solvent to penetrate the cell walls containing the active substance (Pratiwi & Priyani, 2019).

Yield Calculation

The results of extraction of hibiscus using 96% ethanol solvent resulted in a thick extract weighing 10.8876 grams with the weight of the simplicia used being 65 grams. Calculation of extract yield is:

$$\begin{aligned}
 \% \text{ Yield} &= \frac{\text{extract weight}}{\text{simple weight}} \times 100 \\
 &= \frac{10,8876 \text{ gram}}{65 \text{ gram}} \times 100 \\
 &= 16.75
 \end{aligned}$$

The yield obtained was 16.75%. This is different from research conducted by (Agustin & Ismiyati., 2015), namely the anthocyanin yield obtained in 96% ethanol solvent was 22.05%. This difference in results could be caused by differences in the number of simplicia used in the research. The higher the concentration of ethanol used, the more concentrated the resulting extract will be, because anthocyanin and ethanol have almost the same polarity level (Agustin & Ismiyati, 2015). The reaction between the sample and the solvent can affect the length of extraction time so that the solvent can penetrate better into the cell, resulting in many compounds being diffused out of the cell (Wijaya et al., 2018). Therefore, long extraction times can cause high yields. Other factors that influence the yield level are storage time, the ratio of the number of samples to the amount of solvent and type of solvent (Salamah & Widyasari, 2015).

Qualitative Analysis Results

Qualitative testing of anthocyanin levels in hibiscus extract using acid and base reagents aims to determine the presence of anthocyanins in the sample. The acid reagent used is 2M HCl which is marked by an unchanged red color and the basic reagent, namely 2M NaOH, is marked by a color change to green or fading blue indicating the presence of anthocyanin (H. Herlina et al., 2023). The following is a table of qualitative testing results for anthocyanins in **Table 1**.

Table 1. Hibiscus qualitative test results

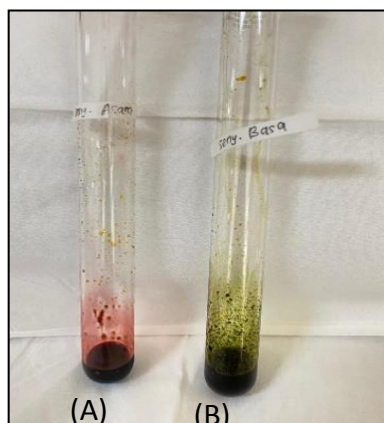
Sample	Reactor	Results	Information
Hibiscus extract	HCl 2M	The color changes to perfect red	(+) Antosianin
	NaOH 2M	The color changes to green	(+) Antosianin



Figure 1. Sample before adding HCl and heating



Figure 2. Sample before adding NaOH



Information:
 Figure 3 (A) = Acid sample
 Figure 3 (B) = Alkaline sample

Figure 3. Acid and base samples after treatment

Table 1 shows that in this study the results showed that all samples were positive for containing anthocyanins. The hibiscus extract in Figure 1 (A) was reacted with 2M HCl and heated using Bunsen spirit for 5 minutes so the color remained red but more perfect, this indicates the presence of anthocyanin compounds. The color change that appears in acid solutions is due to the formation of flavylium, namely the methoxy group in the anthocyanin structure is more abundant than the hydroxy group (Herlina et al., 2023). Meanwhile, the hibiscus extract that was reacted with 2M NaOH changed color to green as in Figure 1 (B), this indicates the presence of anthocyanins. The color change that occurs in basic solutions is due to the carbinol pseudobase structure (Herlina et al., 2023). Anthocyanins are red in acidic solutions because a low or acidic pH reaction occurs and the formation of protonated cyanidin molecules and forms positive ions or cations. Anthocyanin with an alkaline solution is green in color due to an increased pH reaction, the reaction will result in the molecule being deprotonated and forming a negative ion (Ifadah et al., 2022). Anthocyanins can also be influenced by the number of hydroxy and methoxy groups. The large number of hydroxy groups can cause the color to tend to be blue/green, generally unstable. Meanwhile, the phenomenon of the methoxy group dominating will result in a more stable red color (Pratiwi & Priyani, 2019).

Results of Quantitative Anthocyanin Analysis

Anthocyanin levels were analyzed using differential pH at pH 1 and pH 4.5. This is done because acidic conditions can denature plant cell membranes and cause flavonoids to dissolve so they leave the cells (Lestari & Hamzah, 2022). Measurement of the absorbance value of hibiscus extract with 96% ethanol solvent at pH 1 and pH 4.5 using UV-Vis spectrophotometry at wavelengths of 510 nm and 700 nm. The wavelength of 510 nm was used to measure the absorbance value because it is the maximum wavelength of anthocyanin. Meanwhile, the 700 nm wavelength aims to correct the sample (Lestari & Hamzah, 2022). Each sample was measured for absorbance 3 times, so that the results obtained were:



Figure 4. Absorbance pH 4,5

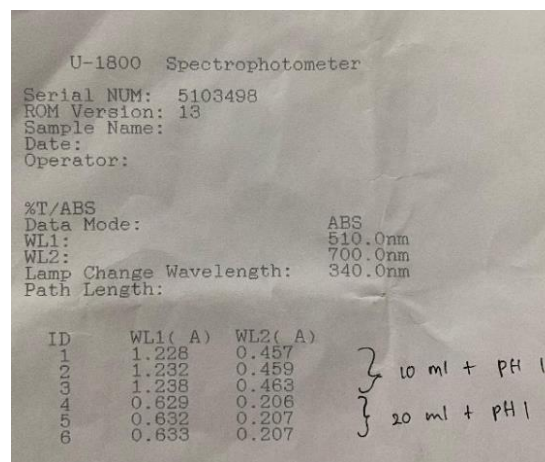


Figure 5. Absorbance pH 1

Information:

ID 1-3 dilution 10 mL

ID 4-6 dilution 20 mL

Table 2. Absorbance value of hibiscus extract at wavelengths of 510 nm and 700 nm

No	Sample	Absorbansi pH 1		Absorbansi pH 4.5	
		510 nm	700 nm	510 nm	700 nm
1.	Dilution 10 mL	1.228	0.457	0.618	0.283
		1.232	0.459	0.627	0.287
		1.238	0.463	0.630	0.290
		Average	1.232	0.625	0.286
2.	Dilution 20 mL	0.629	0.206	0.433	0.197
		0.632	0.207	0.424	0.195
		0.633	0.207	0.394	0.184
		Average	0.631	0.417	0.192

Based on the table above, the results show that redilution using 10 mL of 96% ethanol with a pH of 1 and a wavelength of 510 nm has an average absorbance value of 1,232, at a wavelength of 700 nm it is 0,459. Meanwhile, at pH 4.5 with a wavelength of 510 nm, the average absorbance was 0,625 and at a wave of 700 nm it was 0,286. The average absorbance value in redilution using 20 mL of 96% ethanol solvent with a pH of 1 and a wavelength of 510 nm was 0.631 and at a wavelength of 700 nm it was 0.206. Meanwhile, at pH 4.5 with a wavelength of 510 nm, it is 0.417 and a wavelength of 700 nm, namely 0.192. The absorbance results obtained at 10 ml and 20 ml dilutions were then successively calculated using the absorbance formula as follows.

$$\begin{aligned}
 A &= (A_{510}-A_{700}) \text{ pH } 1,0 - (A_{510}-A_{700}) \text{ pH } 4.5 \\
 (A) \text{ 10 ml} &= (1.232 - 0.459) \text{ pH } 1,0 - (0.625 - 0.286) \text{ pH } 4.5 \\
 &= 0.434 \\
 (A) \text{ 20 ml} &= (0.631 - 0.206) \text{ pH } 1.0 - (0.417 - 0.192) \text{ pH } 4.5 \\
 &= 0.2
 \end{aligned}$$

on re-dilution using 10 mL of 96% ethanol, the absorbance result was 0.434. Meanwhile, when re-diluting using 20 mL of 96% ethanol, an absorbance value of 0.2 was obtained. Anthocyanins can experience decomposition/change in composition at high temperatures, causing a decrease in absorbance (Sampebarra, 2018).

Based on the equation above, the total anthocyanin content contained in hibiscus extract can be calculated with solvent concentrations of 10 and 20 mL respectively using the following formula.

$$\begin{aligned}
 \text{Total anthocyanins (mg/L)} &= \frac{A \times BM \times FP \times 1000}{\varepsilon \times b} \\
 \text{Total anthocyanins (10 mL)} &= \frac{0,434 \times 449,2 \times 10 \times 1000}{26900 \times 1} \\
 &= 72.4731 \text{ mg/L} \\
 \text{Total anthocyanins (20 mL)} &= \frac{0,2 \times 449,2 \times 20 \times 1000}{26900 \times 1} \\
 &= 66.7955 \text{ mg/L}
 \end{aligned}$$

Dilution using 10 mL obtained anthocyanin levels of 72.4731 mg/L. Meanwhile, a 20 mL dilution resulted in anthocyanin levels of 66.7955 mg/L. The anthocyanin levels obtained in both treatments indicate that the anthocyanins contained in hibiscus plants are very high so they are good for use in studying acids and bases. The high level of total anthocyanin in the thick hibiscus extract preparation can be influenced by several things such as the temperature during the drying process, the length of maceration time, the concentration of the solvent used, and the high intensity of light (Armanzah & Hedrawati, 2016). This is supported by research Charmongkolpradit et al., (2021) namely that the lower the water content contained in purple corn, the higher the anthocyanin levels produced.

The anthocyanin levels in hibiscus extract were higher than the anthocyanin levels in butterfly pea flowers with the same treatment. This was proven in research conducted by Herlina et al. (2023) where the anthocyanin content in dried butterfly pea flowers was only 9.017 mg/L. Meanwhile, research conducted by Agustin & Ismiyati (2015) resulted in anthocyanin content in 25 grams of hibiscus with 96% ethanol solvent of 48.260 mg/L. This strengthens the statement that the high levels of anthocyanins in hibiscus are also influenced by solvent concentration.

Conclusion

The results showed that hibiscus extract contains anthocyanins with color changes that occur in acid and base reagents. The sample changes color to perfect red in the acidic reagent and turns green in the alkaline reagent. The anthocyanin content in the 10 mL dilution was 72.4731 mg/L, and in the 20 mL dilution it was 66.7955 mg/L. High levels of anthocyanin tend to be more stable, because the more acidic an anthocyanin solution is, the more colorful it will be.

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