The Potential of Rosella Floss (Hibiscus Sabdariffa l.) as a Dental Plaque Disclosing Agent

Vitri Nurilawaty[1], Tedi Purnama[1]*, Ai Emalia Sukmawati[2], Silvester Maximus Tulandi[2]

1. Department of Dental Health, Poltekkes Kemenkes Jakarta I, Indonesia.
2. Department of Pharmaceutical and Food Analysis, Poltekkes Kemenkes Jakarta II, Indonesia.

Abstract

Streptococcus mutans and other intracellular multipliers of bacteria make up plaque, a soft deposit that sticks tenaciously to the surface of teeth. These bacteria will adhere to the teeth and form colonies with food particles. If plaque is allowed to build up around the oral tissues, tartar will form, which can harm both the hard and soft tissues in the mouth. A contrast material, such as a dye (red/purple) in the form of a liquid or gel termed a dental plaque revealing, can be used to visualize plaque.

The rosella flower (Hibiscus sabdariffa L.) is one of the herbal plants that can be used as a complementary therapy for oral health issues; it has an antibacterial effect, remineralization properties, or does not dissolve calcium ions in tooth enamel in high concentrations, and it also contains anthocyanin in the form of a dense red pigment.

As a result, a dental plaque-disclosing preparation can be produced using the extract from this flower. The anthocyanin pigments found in these plants are unstable by a number of conditions, including changes in pH and temperature, so the aim of this study is to anticipate the optimal formula for dental plaque disclosing preparations utilizing rosella flower extract as the raw material.

The findings of this study show that anthocyanins will significantly deteriorate with the loss of their red color in the pH range of 4-5, whereas the most significant anthocyanin degradation caused by temperature influence takes place in the range of 50–60oC. The extract's ability to suppress the growth of Streptococcus mutans increases with extract concentration.

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Introduction

The quality and productivity of human resources are heavily influenced by human health. The oral cavity is an essential organ for the health of the body. Consequently, oral health is an integral component of overall human health. Poor oral health has negative effects not only on minors but also on elderly individuals.[1–3] The oral cavity is an optimal environment for the growth and development of microorganisms, particularly Streptococcus mutans, an acid-producing bacteria that can damage teeth due to the fermentation reaction of carbohydrates, including sucrose, fructose, and glucose. Therefore, if the oral cavity is not properly cleaned, food particles tucked into and affixed to the teeth, along with bacteria, will accumulate on the surface of the teeth and form a colony known as plaque.[4–6]

Plaque consists of microorganisms proliferating within an intracellular matrix and adhering tightly to the tooth surface. If plaque is permitted to build up around the oral tissues, tartar will form, damaging both hard and soft tissues in the oral cavity. Plaque cannot be removed by merely cleansing the mouth with water; it must be mechanically scrubbed away with a toothbrush.[7–9]

Caries is the most prevalent dental disease in modern society. Caries is a disease of the teeth's hard tissues, enamel, and dentine, caused by the activity of microorganisms against carbohydrates that produce acid.[10,11] A disclosing solution is a contrast agent used to observe plaque, such as a red/purple pigment in liquid or gel form. However, its use on humans has many drawbacks because it frequently contains toxic chemicals. Numerous problems are associated with the current use of chemical/synthetic drugs,

Corresponding author:
Tedi Purnama,
Department of Dental Health, Poltekkes Kemenkes Jakarta I, Indonesia.
E-mail: tedypurnama23@yahoo.com

so it is necessary to seek out more natural alternatives that are anticipated to have no adverse effects on host cells and are also cost-effective. According to the World Health Organization in Tulandi et al, 80-90 per cent of the world's population is estimated to consume natural remedies.[12,13]

Rosella (Hibiscus sabdariffa L.) is one of the herbal plants that can be used as an alternative treatment for oral health problems. The flower petals of this plant, in addition to having an antibacterial effect and remineralization properties or not dissolving high concentrations of calcium ions in tooth enamel, also contain a high concentration of anthocyanins in the form of deep red pigments. It is anticipated that the numerous advantages and benefits included in rosella flowers will meet the requirements for developing a dental plaque disclosing preparation.[14,15]

Rosella flowers (Hibiscus sabdariffa L.) contain critical chemical compounds, including glossy pepsin, anthocyanin, glucoside hibiscus, arginine, lignin, vitamin C, calcium, organic acids, polysaccharides, and flavonoids, according to scientific studies. This plant has been studied for its antiseptic, antibacterial, antihypertensive, diuretic, blood-circulation-improving, antispasmodic, anthelmintic, gum-healing, and disease-resistance-boosting properties, among others.[4,16] Another study on the concentration of rosella flower steeping on the solubility of calcium ions in tooth enamel revealed that the greater the concentration of rosella flower steeped water, the smaller the effect of solubility of enamel calcium ions on permanent teeth, while the normal pH range in the mouth is between 6.5 and 7.5.[14] Salivary acidity above the average pH value range of the oral cavity results in a remineralization process that restores minerals, whereas salivary acidity below the typical pH value range results in a demineralization process that removes minerals from the tooth enamel, a condition known as caries.[17,18]

An essential component of rosella flowers is the pigment anthocyanin, which gives many fruits and vegetables red, purple, and blue hues. Anthocyanins are of particular interest to researchers because they impart attractive shades to food systems and possess antioxidant properties and other health benefits. Moreover, anthocyanin pigments are relatively unstable and frequently degrade during processing and storage. Anthocyanin stability correlates with anthocyanin structure and is also affected by temperature, pH, radiation, presence of enzymes, phenolic acids, oxygen, sugar, Sulphur dioxide, and metal ions.[19,20] The antibacterial effect on Streptococcus mutans and the reddish-purple anthocyanin pigment present in rosella flowers and the high concentration of rosella flower extract reduces the demineralization of calcium in tooth enamel. However, rosella flower extract is relatively unstable and frequently endures degradation reactions. This is the basis of this research, as it is believed to have potential in product development for oral health from natural materials, including as a primary material for dental disclosing, but is relatively unstable in processing and storage.

Materials and methods

Equipment

Oven (memmert), grinder, sieve no. 40, Rotary evaporator (Eyela), Analytical balance (Sartorius), Ultrasonic (Branson), pH metre (Mettler), UV-Vis Spectrophotometer (Agilent Technologies), Water bath (memmert), Refrigerator were the primary pieces of equipment used in this research.

Materials

The following materials are utilized: Roselle flower petals, Aquadest, Ethanol 96% p.a. (Merck), Citrate Buffer (Merck), Phosphate Buffer (Merck), Dimethyl sulfoxide (DMSO) p.a. (Merck), Sodium hydroxide pellets p.a. (Merck), Hydrochloric acid 37% p.a. (Merck), Acetic anhydrous acid p.a. (Trypticase Soy Agar (TSA) Microbiology Medium (Merck), Trypticase Soy Broth (TSB) Microbiology Medium (Merck), and Amoxicillin BPFI.

Samples Preparation

Bogor's Spice and Medicinal Plants Research Institute (Balittro) provided the samples used in this investigation. The analysis was conducted at the Biology Research Centre of the Indonesian Institute of Sciences – Cibinong in Bogor, Indonesia. From extract preparation to testing, samples were prepared at the Natural Materials Laboratory, Department of Pharmaceutical and Food Analysis, Jakarta II Health Polytechnic, Ministry of Health.

Rosella Flower Extraction

One hundred grammes of pulverized, oven-dried at 40 degrees Celsius Rosella flowers

are weighed. Maceration employs ethanol: water (3:1) at a 1: 5 w/v ratio to the sample's mass in a securely sealed glass bottle. Thirty minutes of ultrasonic treatment, followed by a day and a night of rest. The ethanol layer is separated before being macerated until it is colorless. The ethanol layer collection is filtered. At 40 °C, a rotary vacuum concentrated ethanol filtrates to produce a viscous extract. Before analysis, the extract was kept at 4°C for storage.[21,22]

Phytochemical Test of Rosella Flower Viscous Extract

The secondary metabolites in rosella flowers were determined qualitatively through phytochemical analysis. Flavonoids, alkaloids, phenol hydroquinone, tannins, and anthocyanins are analyzed.

Steroid/triterpenoid Test

In a test tube, up to 1 mg of the extract was dissolved in 2 ml of DMSO. The mixture was combined with ten drops of anhydrous acetic acid and three drops of P sulfuric acid. The test result is positive if the solution turns red, blue, and green.

Flavonoid Test

One milligram of the extract was dissolved in 2 ml of DMSO and placed in a test tube, followed by adding 0.1 mg of magnesium powder, 0.4 mL of amyl alcohol, and 4 mL of alcohol, followed by vigorous shaking. The test result is positive if the amyl alcohol layer turns red, yellow, or orange.

Alkaloid Test

After dissolving 1 mg of the extract in 2 ml of DMSO and placing it in a test tube, adding a few droplets of 2 N sulfuric acid, and testing it with several alkaloid reagents, including Dragendorff, Meyer, and Wagner, the extract was found to contain alkaloids. The test result is positive if Wagner reagent precipitates are brown, Meyer reagent precipitates are yellowish white, and Dragendorff solvent precipitates are orange-red.

Saponin Test

Up to 1 mg of the sample is dissolved in hot water and spun to generate froth. The positive result is if the sample produces foam that persists for 30 minutes after adding one drop of 2 N HCl.

Hydroquinone Phenol Test

Along with 20 mL DMSO, 1 mg of sample extract was added. Add two 5% FeCl3 solution droplets to 1 milliliter of fraction solution. A positive result if the fraction extract solution is green or blue-green in color.

Tanin Test

3% FeCl3 reagent was added to a total of 1 g of sample. The formation of a dark blue color indicates a favorable outcome.

Anthocyanin Test

1 g of the extract was dissolved in distilled water and then divided between two test tubes. Test tube I was drizzled with 2 N NaOH drop by drop; a blue-green solution indicates the presence of anthocyanins. When HCl 2 N was added to test tube II and heated to 100 degrees Celsius, the red color did not dissipate.

Extract Analysis on the Effect of Changes in pH

100 mL of distilled water was diluted with 100 mg of rosella extract (stock solution). Then, 1 mL of rosella extract stock solution was placed in seven test tubes, and 10 mL of citrate-phosphate buffer solution was added according to the pH of each sample to be tested, including pH 3, pH 4, pH 5, pH 6, pH 7, and pH 8, along with a control tube containing diluted distilled water. The work was performed three times. Each solution was homogenized using a vortex mixer before being scanned using a UV-Vis spectrophotometer with a 200-700 nm wavelength range. Maximum measurements were conducted at a wavelength of 516 nm (16,21,22).

$$\% \text{ Degradation} = \frac{control\ absorbance - absorbance\ after\ treatment}{control\ absorbance} \text{ x } 100\% \quad (1)$$

Extract Analysis on the Effect of Temperature Changes

100 mL of distilled water was diluted with 100 mg of rosella extract (stock solution). Then, 1 mL of rosella extract stock solution was added to six test tubes, followed by adding 10 mL of distilled water to each, which was then homogenized using a vortex mixer. The absorbance was then measured at a maximal wavelength of 516 nm. This procedure was repeated three times, with one test tube serving as a control whose absorption was measured before treatment. In addition, the test tubes were stored for 1 hour at 20°C, 30°C, 40°C, 50°C, and 60°C while minimizing the influence of light. The percentage of deterioration is computed using the formula in Equation.[19,23,24]

Antibacterial Activity Analysis

Streptococcus mutans were cultured for 24 hours, and then one dose of the culture was homogenized with sterile TSB. Obtain 25%T (108 CFU/mL) by measuring %T with a UV Visible spectrophotometer with a wavelength of 580 nm. Prepare three Petri dishes, add 0.2 mL of the microbial suspension and 20-25 mL of TSA media to each, then homogenize and allow to solidify. A disc soaked in rosella flower extract diluted with DMSO at concentrations of 5, 10, 15, 20, and 30% w/v was affixed to a positive control soaked in amoxicillin, and a negative control drenched in DMSO. Observe the clear zone/inhibition zone after 18 to 24 hours of aerobic incubation at 30 to 35 degrees Celsius and 18 to 24 hours at room temperature. There are three repetitions.[25,26]

Data Analysis

The processing of data from each analysis (Effect of variations in pH, temperature, and antibacterial activity) was statistically analyzed using One-Way Anova with Minitab software version 16.

Results

Extraction of Rosella Flowers *(Hibiscus sabdariffa. L)*

Remaceration is used for extraction. Remaceration was accomplished by soaking 100 grammes of desiccated, previously ground simplicia in 5 times its weight in ethanol extractor liquid, sonicating the soaked powder for 30 minutes, leaving it for 24 hours, and then separating the ethanol layer. The ethanol layer was recrystallized until colorless, and the ethanol layer collection was filtered. At 40°C, the filtrate was evaporated in a rotary evaporator until it thickened. The obtained extract was then placed in the refrigerator. Because ethanol is a solvent from the alcohol group with polarity, it will be able to attract polar compounds in rosella flowers, such as the anthocyanin pigments that are the focus of this research; ethanol was used as the extraction solvent in this study. Because anthocyanin is a polar compound, water is added to the extraction solvent to increase the polarity of the solvent. Additionally, glycolysis can affect the solubility of anthocyanin pigments in ethanol. Remaceration is performed to maximize the extraction process of the secondary metabolites present in rosella flowers. In this investigation, the yield of the obtained extract was 25.48 per cent.

Phytochemical Test of Rosella Flower Ethanol Extract

Qualitative phytochemical screening of rosella flower ethanol extract was performed using the color and precipitate reaction test method. Alkaloids, flavonoids, phenol-hydroquinone, steroids/triterpenoids, tannins, saponins, and anthocyanins were tested. As shown in Table 1, the ethanol extract of rosella (Hibiscus sabdariffa L.) flowers contained flavonoids, phenol-hydroquinone, tannins, saponins, and anthocyanins.

Test Type	Reagent	Result
Alkaloid	Dragendorff	-
	Meyer	-
	Wagner	-
Flavonoid	Serbuk Mg, Aceton, Etanol	+
Phenol-hydroquinone	$FeCl_3$ 5%	+
Steroid/Triterpenoid	As.asetat anh + H_2SO_4 P	-
Tanin	$FeCl_3$ 3%	+
Saponin	HCl 2N	+
Anthocyanin	NaOH 2N+ HCl 2N	+

Note: (-) = undetected; (+) = detected

Table 1. The results of the analysis of secondary metabolites of rosella flowers.

Extract Analysis of Changes in pH

Because it is known that anthocyanin is a compound that is easily degraded under the influence of pH, temperature, and light exposure, an analysis of the color change of the ethanol extract of rosella flowers in response to pH changes was performed to obtain the correct color information so that in formulating dental disclosing preparations for plaque appearance on teeth will be maximized. In this investigation, the citrate-phosphate buffer was used as an extract diluent, and it was measured using a UV-Vis spectrophotometer with a 200 to 700 nm wavelength range. Figure 1 displays the outcomes of spectrophotometer measurements in the 200-700 nm wavelength range. The maximum obtained wavelength is 516 nm. The measurement yielded a spectrum with multiple peaks, indicating that anthocyanin also has various peaks. Because the aglycones in anthocyanin (flavylium cation) contain conjugated double bonds that allow them to be absorbed in the 500 nm wavelength region, measurements are performed in the visible light wavelength region.[23] This study measured the maximum

wavelength obtained in the visible light area. It appears like 516 nm.

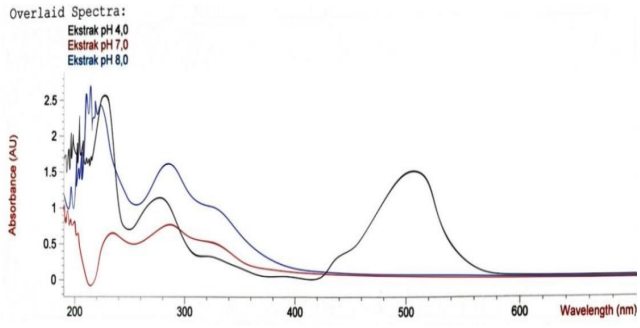

Figure 1. The spectrum of rosella extract in the range of 200-700 nm under conditions of acidic pH (pH 4, neutral (pH 7) and alkaline (pH 8).

In the UV-Vis spectrum of rosella flower ethanol extract, a typical spectrum of anthocyanin was obtained, exhibiting two primary groups of absorbance: one in the 220-280 nm wavelength region, followed by a small peak at 320-340 nm (UV region), and the other in the 430-520 nm (visible light area). At a pH of 4, there is a peak at the visible light wavelength of 516, which explains why anthocyanins retain their red color at this pH compared to neutral or alkaline pH, where there are no peaks at that wavelength. Table 2 and Figure 2 display the results of measurements made with a UV-Visible spectrophotometer to determine the effect of pH changes.

pH	Color	Absorbance Average	Degradation (%)
Control	Purple red	2.201	
3	Bright red	1.780	19.127
4	Light pink	1.502	31.758
5	Faded pink	0.508	76.919
6	Brownish green	0.108	95.093
7	Brown	0.086	96.092
8	Yellowish brown	0.073	96.683

Table 2. Results of UV-Visible Spectrophotometer analysis of changes in pH of the ethanol extract of rosella flowers (Hibiscus sabdariffa L.) at a wavelength of 516 nm.

This experiment demonstrates that the extract's color changes when diluted with a buffer solution appropriate for each pH. As shown in Figure 2, changing the solution's pH towards a more alkaline state causes the color to shift from brilliant red to bright pink, then fade, and finally become brownish green to yellowish brown. This is because, in an acidic environment, more anthocyanin pigments are produced in the form of colored flavylium cations or oxonium, and these flavylium cations change to a more stable hemiacetal form, which is colorless in chalcone form, as the pH increases towards alkaline.[20] Influenced by the number of flavylium cations with conjugated double bonds that can still be absorbed in the maximal wavelength in the visible light region, 516 nm, the absorbance rises. The one-way ANOVA analysis revealed significant differences between each treatment, with a p-value less than 0.05.

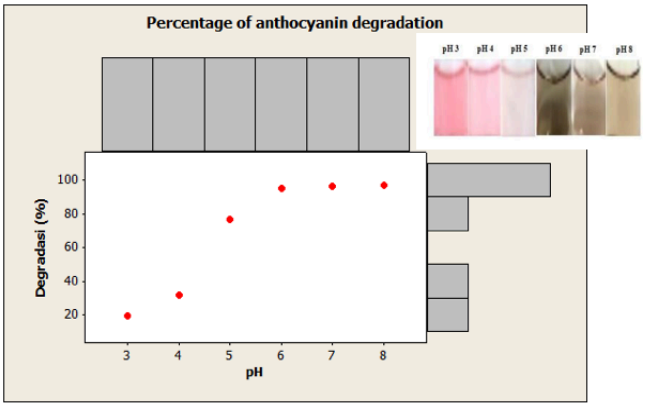

Figure 2. Percentage diagram of anthocyanin degradation to changes in pH. (Minitab version 16.)

Source	DF	SS	MS	F	P
pH	5	18353.09	3670.62	829 8.59	0.000
Error	12	5.31	0.44		
Total	17	18358.40			

S = 0.665 R-Sq = 99.97% *R-Sq(adj) = 99.96%*

Table 3. One-way ANOVA Statistical Test Results: Degradation (%) versus pH.

Extract Analysis of Temperature Changes

As it is known that anthocyanins are also sensitive to variations in temperature, some research indicates that anthocyanins will degrade as the temperature rises. In this test, we aim to determine the rate of degradation of anthocyanins in response to variations in temperature to determine the optimal formulation and storage temperatures. The results of temperature evaluation on anthocyanin degradation are shown in figure 3.

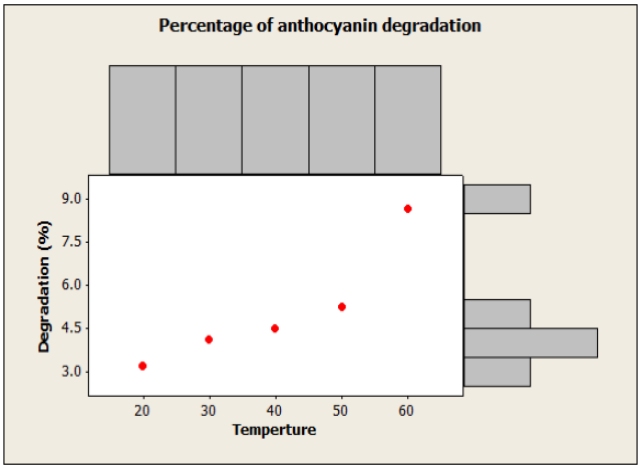

Figure 3. Percentage diagram of anthocyanin degradation with temperature changes (Minitab version 16).

Source	DF	SS	MS	F	P
Temperature	4	52.850	13.212	43.10	0.000
Error	10	3.065	0.307		
Total	14	55.915			
S = 0.5537		R-Sq = 94.52%		R-Sq(adj) = 92.32%	

Table 4. Results of One-way ANOVA Statistical Test: Degradation (%) versus Temperature (°C).

The lower the temperature, the lower the percentage of anthocyanin degradation, which is consistent that anthocyanin pigments are unstable pigments and will undergo a gradual degradation process during storage. As shown in Figure 3, the most significant degradation occurs when the temperature exceeds 50oC. Temperature rise causes the gallium ring in anthocyanins to open and form a colorless chalcone. In research conducted, it was determined that at high concentrations of solutions containing anthocyanin pigments, the process of degradation by light would be significantly reduced due to the difficulty of light penetrating the solution. According to this study, the rate of degradation caused by an increase in temperature is slower than that caused by a change in pH. Statistical analysis using a one-way ANOVA revealed a significant difference between treatments, with a p-value of 0.05; therefore, when formulating a dental plaque disclosing preparation using rosella extract as the raw material, a high concentration of rosella extract was required, and the preparation should be stored at cold temperatures to minimize the effect of light and temperature.[19,20,24]

Analysis of Antibacterial Activity against Streptococcus mutans

The ethanol extract of rosella flowers (Hibiscus sabdariffa L.) showed antibacterial activity against Streptococcus mutans, a bacterium involved in the formation of dental plaque. The 5; 10; 15; 20, and 30% extract concentration series were used in this test, with amoxicillin as the positive control. The results of the antibacterial activity test for the ethanol extract of rosella flowers are displayed in Table 5 and Figures 4 and 5. Observations after 24 hours of incubation at 37°C reveal that the diameter of the inhibition zone grew more prominent as the concentration of the extract increased. The One-Way Anova statistical analysis reveals a significant difference, with a p-value less than 0.05. This indicates that the extract's concentration significantly influences the antibacterial activity of the ethanol extract of rosella flowers (Hibiscus sabdariffa L.) against Streptococcus mutans bacteria. According to this research, a high concentration of rosella flower extract is highly recommended when formulating a dental plaque-disclosing preparation with rosella flower extract as the primary material.

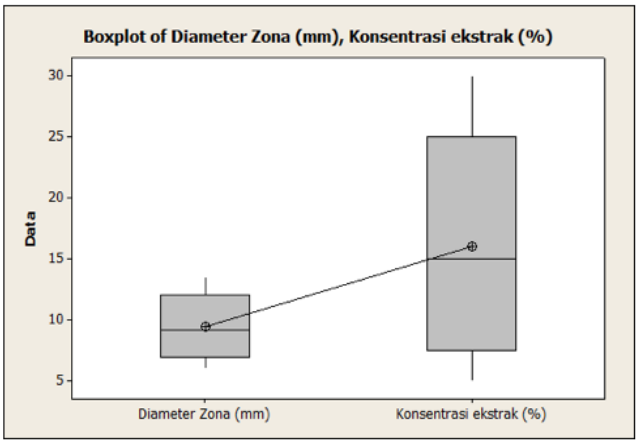

Figure 4. Boxplot diagram between zone diameter and extract concentration (Minitab version 16.)

Source	DF	SS	MS	F	P
Kons. ekstrak	4	97.023	24.256	219.18	0.000
Error	10	1.107	0.111		
Total	14	98.129			
S = 0.3327		R-Sq = 98.87%		R-Sq(adj) = 98.42%	

Table 5. One-way ANOVA Statistical Test Results: Zone (mm) versus Extract Concentration.

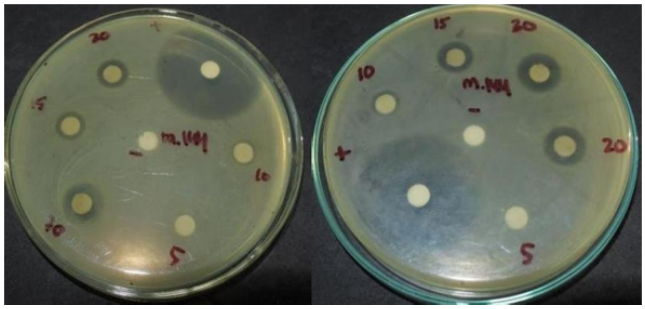

Figure 5. Activity test of rosella calyx (Hibiscus sabdariffa L.) extract using the agar diffusion method on Streptococcus mutans bacteria.

Discussion

This research acts to predict the formulation of disclosing dental plaque using rosella calyx extract (Hibiscus sabdariffa L.) as raw material so that the correct formulation can be obtained, as it is known that this plant contains anthocyanin pigment, a red pigment found in abundance in plants. To maintain the concentration and colour of anthocyanins, it is crucial to pay close attention to pH and temperature during the product development process's extraction and formulation phases, according to data derived from the research. Anthocyanins tend to degrade when the pH changes from acidic to alkaline. It is also essential to pay attention to the temperature from the extraction process to the formulation, as anthocyanin degradation can occur with an increase in temperature, albeit to a lesser extent than with changes in pH. In a separate study, Nurilawati et al. found that calcium ions' solubility decreases as the rosella petal extract concentration increases during the steeping procedure. At a pH of 4, the red colour of anthocyanin is still within the visible light spectrum, so this is a very suitable method if the formulation contains an acidic concentration. However, when oral conditions are acidic, a demineralization process of the tooth enamel occurs, so if a high concentration of extract is added to the formula, the demineralization process can be reduced.[14] This is supported by research which stated that at high concentrations of solutions containing anthocyanin pigments, the degradation process would This also supports the results of the antibacterial activity analysis performed; the higher the concentration of rosella calyx extract, the greater its antibacterial activity. The ethanol extract of rosella flowers (Hibiscus sabdariffa L.) can be used as a primary material in formulating dental plaque-disclosing preparations.[19,20,24]

In developing these preparations, a pH above the range of 4 to 5 must be avoided, as the likelihood of anthocyanin degradation to a colourless form is high in this pH range. Meanwhile, the most significant anthocyanin degradation occurred between 50 and 60 degrees Celsius due to temperature increase. The elevated temperature increases the proportion of anthocyanin degradation. Temperature rise causes the falvilium ring in anthocyanins to open and form a colourless chalcone. To obtain optimal conditions in the formulation of disclosing dental plaque preparations, it is advised to use the Response Surface Methodology design with multiple variables that influence anthocyanin stability so that the resulting preparations are more stable during processing and storage.

Conclusions

Rosella flower (Hibiscus sabdariffa L.) extract can be used as an essential material in the formulation of dental plaque-disclosing preparations, according to the findings of this study. The degradation of the anthocyanin compounds in rosella flower extract can be minimized by ensuring that the pH and temperature conditions do not exceed 4. The temperature of the process and the extract concentration in the concoction should not exceed 50^{o}C. To obtain optimal conditions in the formulation of dental plaque disclosing preparations, it is suggested to use the Response Surface Methodology design with multiple variables that affect anthocyanin stability so that the preparations obtained are maximized in processing and storage.

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Declaration of Interest

The authors report no conflict of interest.

Ethical Clearance

This research has received ethical approval from the Research Ethics Committee, Health Polytechnic of Jakarta I No.045/KEPK/VII/2022.

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