Optimization of Roselle (*Hibiscus sabdariffa* Linn.) Anthocyanin Extraction Parameter by Response Surface Modeling and Potential of Roselle Agro-Waste as Alternative Sources of Anthocyanin

(Pengoptimuman Parameter Pengekstrakan Antosianin Rosel (*Hibiscus sabdariffa* Linn.) Mengikut Pemodelan Permukaan Tindak Balas dan Potensi Sisa Agro Rosel sebagai Punca Alternatif Antosianin)

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ABSTRACT

Roselle or *Hibiscus sabdariffa* Linn. is a perennial plant with a high concentration of anthocyanin in the calyx. This study optimized the extraction conditions for maximum anthocyanin yield in the calyx using response surface methodology (RSM) and face-centered central composite design (FCCCD), which showed that ultrasound-assisted extraction (UAE) method with 70% ethanol concentration (v/v) at 35 minutes had the highest total anthocyanin content (TAC) which at 5.277 mg Cya3G/g DW. Using this optimised extraction method, roselle agro-waste which are calyx residue, leaf and stem were extracted and tested for TAC. The highest TAC was found in the calyx residue (3.011 mg Cya3G/g DW), followed by stems (0.077 mg Cya3G/g DW), and leaves (0.073 mg Cya3G/g DW). This study also quantified the concentration of two major anthocyanins using high-performance liquid chromatography (HPLC) and compared the total phenolic content (TPC) and total flavonoid content (TFC) of calyx and calyx residue extracts. Our findings showed that the TAC of calyx residue extracts was significantly low compared to calyx extracts. However, there were no significant differences in the TPC and TFC values. These results suggest that calyx residue, which is typically discarded after processing of calyx products, is a good source of phenolics and flavonoids and compatible with fresh calyx. In addition, calyx residues are still a good source of anthocyanins, making it an added-value for the roselle industry.

Keyword: Anthocyanins; face-centered central composite design; HPLC; Response Surface Methodology; roselle

ABSTRAK

Rosel atau *Hibiscus sabdariffa* Linn. ialah sejenis tumbuhan berumur panjang yang mengandungi kepekatan tinggi antosianin di kaliksnya. Kajian ini mengoptimumkan keadaan pengekstrakan bagi mendapatkan hasil antosianin maksimum di kaliks menggunakan metodologi rangsangan permukaan (RSM) dan reka bentuk komposit berpusatkan muka (FCCCD) yang menunjukkan bahawa kaedah pengekstrakan dengan bantuan gelombang bunyi (UAE) menggunakan kepekatan etanol 70% (v/v) selama 35 minit mempunyai kandungan antosianin keseluruhan tertinggi (TAC) iaitu pada nilai 5.277 mg Cya3G/g BK. Dengan menggunakan kaedah pengekstrakan yang telah dioptimumkan ini, sisa pertanian roselle seperti sisa kaliks, daun dan batang diekstrak dan diuji untuk TAC. TAC tertinggi ditemui dalam sisa kaliks (3.011 mg Cya3G/g BK), diikuti oleh batang (0.077 mg Cya3G/g BK) dan daun (0.073 mg Cya3G/g BK). Kajian ini juga mengkuantifikasi kepekatan dua jenis antosianin utama menggunakan kromatografi cecair prestasi tinggi (HPLC) serta membandingkan kandungan fenol keseluruhan (TPC) dan kandungan flavonoid keseluruhan (TFC) antara ekstrak kaliks dan sisa kaliks. Hasil kajian kami menunjukkan bahawa TAC ekstrak sisa kaliks adalah jauh lebih rendah berbanding ekstrak kaliks. Namun begitu, tidak terdapat perbezaan yang signifikan dalam nilai TPC dan TFC. Hasil ini mencadangkan bahawa sisa kaliks yang biasanya dibuang selepas proses penghasilan produk kaliks adalah sumber yang baik untuk fenol dan flavonoid, sejajar dengan kaliks segar. Selain itu, sisa kaliks masih merupakan sumber yang baik untuk antosianin dan menjadikannya tambahan nilai bagi industri rosel.

Kata kunci: Antosianin; HPLC; Metodologi Rangsangan Permukaan; reka bentuk komposit berpusatkan muka; rosel

INTRODUCTION

Roselle, also known as *Hibiscus sabdariffa* L. is a plant that belongs to the Malvaceae family. The roselle plant is popular for its fleshy calyx, which are used to make drinks that have a blackcurrant-like flavour. It is widely used as a food colouring agent, sauces, jams, syrup, jellies, preservatives, and ice cream (Chumsri, Sirichote & Itharat 2008). Roselle is rich in phytochemicals such as anthocyanin, organic acids, pectin, polyphenols, and phytosterols which possess antioxidant properties (Bettiol et al. 2014; Kouakou et al. 2015; Wu, Yang & Chiang 2018).

Anthocyanin is a natural pigment from the flavonoid group which possesses anti-oxidative, anti-inflammatory, anti-diabetic, and anti-cancer effects (Mohd-Esa et al. 2010; Ojulari, Lee & Nam 2019; Sapian et al. 2022). Roselle calyx is rich in anthocyanin, mainly delphinidin-3-sambubioside (Dp-3-Sam) and cyanidin-3-sambubioside (Cy-3-Sam) (Pragalyaashree, Tiroutchelvame & Sashikumar 2018; Singh, Khan & Hailemariam 2017), which are potential antioxidants for delaying the aging process in the skin (Purwanto, Ariani & Pramitaningastuti 2019). Additionally, anthocyanin has the potential as a natural solar cell sensitizer because of its ability to absorb light (Shalini et al. 2020) with high photon conversion yield that creates strong currents to generate electricity (Norhisamudin et al. 2021).

In 2020-2022, Malaysia produced 167 metric tonnes of roselle with estimated 100-120 metric tonne of agro waste including stems, leaves, and calyx residue. The calyx residue still has a bright color, which might contain anthocyanins. Thus, stems, leaves, and calyx residue have the potential to be explored as a different source of anthocyanin to meet the high demand in the industry, which would enhance the added value of roselle plants.

However, anthocyanin is easily degraded due to the influence of temperature, light, pH, co-pigmentation, oxygen, sulphites, ascorbic acid, metal ions, and enzymes (Enaru et al. 2021; Wu, Yang & Chiang 2018). Therefore, in the development of an optimal extraction method, factors affecting extraction efficiency need to be considered. Among them are the selection of solvents, extraction temperature, extraction time, solute-to-solvent ratio, and extraction method (Chen et al. 2018). Ultrasonic-assisted extraction (UAE) is one of the most common advanced methods implemented for phenolic compound extraction, mainly roselle, due to its cost-effectiveness, quick extraction time, low solvent and time consumption, and eco-friendly processing technique compared to conventional methods (Hapsari & Setyaningsih 2021). Through cavitation forces, it encourages the rupture of plant tissue and improves solvent penetration into cells, resulting in the release of the intracellular component into the solvent, thus increasing the mass transfer phenomena of anthocyanin (Pinela et al. 2019). However, the efficiency of the extraction is also depending on the process variables which can be established using the response surface methodology (RSM).

RSM is a statistical and mathematical tool that has been employed to assess both the influence of independent variables and any potential interactions between them (Bezerra et al. 2008). It has several benefits, including a faster optimization process that saves time and cost by reducing the number of trials. For optimization purposes, the face-centered central composite design (FCCCD), is preferable as it is gives precise predictions of linear and quadratic interaction effects of parameters that influence the process (Witek-Krowiak et al. 2014). To maximize anthocyanin recovery while maintaining stability during the extraction process, roselle anthocyanin extraction should be optimized using RSM. Currently, there is limited data on the anthocyanin content of extracts obtained from roselle agro-waste, and no studies have optimized anthocyanin extraction parameters from roselle calyx residue, leaves, and stem using RSM. Therefore, there is a need to optimize the extraction process of anthocyanins from these sources to increase their value in the industry.

MATERIALS AND METHODS

CHEMICALS

Ethanol, methanol, acetonitrile, formic acid, potassium chloride powder, hydrochloric acid, sodium acetate, Folin-Ciocalteu reagent, sodium carbonate, gallic acid, aluminium chloride, potassium acetate, and quercetin were obtained from Sigma Aldrich meanwhile cyanidin-3-sambubioside, delphinidin-3-sambubioside were purchased from Extrasynthase.

SAMPLE PREPARATION

The roselle calyx, stems, leaves, and calyx residues were supplied from the roselle farm from Rhu Tapai, Kuala Terengganu. The samples were dried in a freeze dryer (Labconco 4.5 Plus Benchtop Freeze Dryer -80) and then ground into powder by using the grinder (Golden Bull Mulry Function Disintegrator SY-25). Prior to being used, these ground samples were kept in polyethylene vacuum bags and stored at room temperature.

EXTRACTION METHOD

The ultrasonic-assisted extraction (UAE) method was carried out according to Ali, Latip and Zain (2019) with modifications. A total of 3 g of roselle calyx powder was weighed and mixed with 60 mL of aqueous ethanol solutions (1:20). The extraction process was conducted in the sonicator (Elmasonic S 30 H, Elma Schmidbauer GmbH) at a fixed temperature of 60 °C for various extraction times and different percentages of aqueous ethanol solutions depending on the experimental design of RSM. Then, the roselle extract was filtered by using a vacuum filter (Heidolph ROTAVAC valve) lined with two pieces of filter paper (Whatman no. 1). The filtrate was then concentrated using a rotary evaporator (RotavaporR-210 Basic, Glass Assembley V ST 29/32,230V) to remove the ethanol until the viscous extract is obtained. The extract was dried using a freeze-dryer.

PRELIMINARY EXPERIMENT

To set the range of extraction parameters for the next optimization procedure, preliminary experiments were carried out. Three independent parameters were tested at their various screening levels which are ethanol concentration (0-100%), extraction time (10-240 min), and temperature (40-80 °C). During the preliminary experiment, one variable was fixed while the other variables varied. The appropriate levels for parameter optimization were set after the impact of those individual factors on the TAC yield was studied.

DESIGN OF EXPERIMENT

After the significant process variables (ethanol concentration, X_1, extraction time, X_2) had been identified through preliminary experiments, further optimization was to yield a higher concentration of anthocyanin using the response surface methodology of face-centered central composite design (FCCCD). The experimental matrix, model development, and data analysis were assessed by using Design Expert Software (version 13, Design-Expert Software, Stat-Ease Inc., Minneapolis, MN, USA). The experimental matrix consists of 13 experimental runs including 4 factorial points, 4 axial points, and 5 central points. All 13 runs were performed, and the responses are listed in Table 1. The optimal conditions that were obtained from FCCCD were verified by running an experiment on a sample and compared with predicted values. Then, the anthocyanin extraction from stems, leaves, and calyx residues were determined using the predicted optimal conditions.

DETERMINATION OF TOTAL ANTHOCYANIN CONTENT (TAC)

The TAC determination in calyx, stems, leaves, and calyx residue was measured by using the pH-differential method and referred to according to the method established by Giusti and Wrolstad (2001). Two buffer systems were prepared in this method including potassium chloride buffer (0.025 M) with pH 1.0 and sodium acetate buffer (0.4 M) with pH 4.5. About 10 mg of extract was diluted in 1 mL of distilled water to prepare the extract solution. Then, 300 µL of extract solution was mixed with buffer solution with pH 1.0 and 4.5 separately until 3 mL of test dilution was produced (1:10 dilution factor). Each of the solutions was incubated for 15 min before measuring the absorbance at 520 nm and 700 nm by using a UV-Vis spectrophotometer (Shimadzu UV Spectrophotometer UV-1800). All measurement was performed in a triplicate manner and TAC was calculated as described in Equation (1). All the data were expressed as mg cyanidin-3-glucoside equivalent per gram of dried weight (mg C3G/g DW).

$$TAC \ (mgC3G/gDW) = \frac{A \times MW \times DF \times V \times 1000}{\varepsilon \times l \times SW}. \quad (1)$$

where A is the $(A_{520nm} - A_{700nm})_{pH1.0} - (A_{520nm} - A_{700nm})_{pH4.5}$;

MW is the molecular weight for cyanidin-3-glucoside (449.2g/mol); DF is the dilution factor; V is the total volume of sample solution after extraction (L); ε is the (molar absorptivity) = 26,900 Lmol^{-1}cm^{-1} for C3G; l is the cell path length (cm); SW is the sample weight (g); and 10^3 is the factor conversion from g to mg.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS

The identification and quantification of major anthocyanin, Dp-3-Sam, and Cy-3-Sam were performed by using HPLC for the calyx and calyx residue extracts under the same extraction conditions. The samples were analyzed by using Waters 2535 quaternary gradient pump, Waters 2707 autosampler and Waters 2998 photodiode array (PDA), and HPLC column Kinetex Biphenyl (5 µm, 250 mm × 4.6 mm) with the gradient systems of 2 types of solvent; A (0.1% formic acid and water) and B (acetonitrile).

	% A 0.1% formic acid and water	% B Acetonitrile
0	100	0
13	80	20
20	70	30
25	20	80
30	100	0
35	100	0

1 mL of methanol was added to 20 mg of the sample in the vial with a volume of 7 mL. Samples were sonicated for 15 min and filtered through a 0.45 µM PTFE membrane syringe filter before being analyzed. The flow rate was fixed at 1 mL/min with an injection volume of 10 µL. The chromatograms were monitored at 520 nm. The quantification of the anthocyanins was done using standard calibration curve where R^2 value is 0.9996 for Dp-3-Sam and 0.9993 for Cy-3-Sam.

DETERMINATION OF TOTAL PHENOLIC CONTENTS (TPC)

The total phenolic content (TPC) was determined by the Folin-Ciocalteu colorimetric method according to Ali, Mohd and Latip (2019) with some modifications. Briefly, aliquots of 10 mg of samples were dissolved in 1 mL of distilled water. Then, 40 µL of the dissolved samples was mixed with 0.2 mL Folin Ciocalteu reagent, 0.6 mL of 7.5% sodium carbonate (Na_2CO_3), and 3.16 mL of distilled water. These mixtures were then incubated for 2 h in the dark at room temperature. The absorbance was recorded at 765 nm using a spectrophotometer (Shimadzu UV Spectrophotometer UV-1800). TPC was calculated based on the calibration curve (10-400 mg/L, $R^2 = 0.9914$) and the data were expressed as gallic acid equivalents (mg GAE/g dry weight). All measurements were performed in a triplicate manner and the average values were calculated.

DETERMINATION OF TOTAL FLAVONOID CONTENTS (TFC)

The total flavonoid content was evaluated according to the aluminium chloride colorimetric method described by Lin and Tang (2007) with modification. Briefly, aliquots of 10 mg of samples were dissolved in 1 mL of distilled water. This solution (0.5 mL) was mixed with 1.5 mL methanol, 0.1 mL of 10% aluminium chloride ($AlCl_3$),

0.1 mL of 1 M potassium acetate (CH_3COOK), and 2.8 mL of distilled water. The reaction mixture's absorbance was measured using a spectrophotometer (Shimadzu UV Spectrophotometer UV-1800) at 440 nm after being incubated at room temperature for 40 min. TFC was calculated based on the calibration curve (5-125 mg/L, $R^2 = 0.9853$) and the data were expressed as quercetin equivalents (mg QE/g dry weight). All measurements were performed in a triplicate manner and the average values were calculated.

DATA ANALYSIS

The experimental and analysis were performed in triplicate for each run and the results were reported as mean ± SD. The optimization process in experimental design (FCCCD) and TAC were analysed by one-way analysis of variance (ANOVA) to determine the significant differences between values, defined at the 5% level (p< 0.05) using the Design Expert Software (Version 13, Design-Expert Software, Stat-Ease Inc., Minneapolis, MN, USA). The interaction between the variables was illustrated in both two-contour and three-dimensional surface plots. The TAC compared between all parts of the roselle using One-Way ANOVA followed by Post-Hoc analysis meanwhile TFC and TPC were compared between calyx and calyx residue using Independent T Test and analysed by SPSS version 25.

RESULTS AND DISCUSSION

PRELIMINARY EXPERIMENT

Effect of Ethanol Concentration

Ethanol concentration affect phenolic compound extraction because it lowers the boiling point and alters the polarity of the mixed solvent (Rajha et al. 2014). The effects of ethanol concentration in TAC were experimented

using different concentrations of ethanol ranging from 0% to 100% vol/vol at constant extraction temperature (60 °C) and time (30 min) as shown in Figure 1(a). TAC rise with ethanol concentration, peaking at 75% ethanol, before drastically decreasing at 100% pure ethanol. This observation is supported by Bettiol et al. (2014) which reported that, the use of pure solvents did not give good extraction yields compared with an aqueous solvent solution. In addition, high solvent concentration can lead to altered solubility of anthocyanins resulting degradation (Khazaei et al. 2016). Aqueous ethanol enables extraction of more polar components in addition of ethanol aiding in the breakdown of cell membranes, which improved the solvent's permeability into the solid matrix (Meziant et al. 2014). Therefore, 75% ethanol concentration was chosen as the reference for the subsequent preliminary experiment.

EFFECT OF EXTRACTION TIME

One of the most important factors influencing TAC recovery from roselle is the extraction time. Hence, it is important to select optimal extraction time to avoid anthocyanin degradation due to prolonged exposure to high temperatures (Pham et al. 2019; Wu, Yang & Chiang 2018). The extraction time chosen in this study ranges from 10 to 240 minutes at a constant temperature of 60 °C and an ethanol concentration of 75%. According to Figure 1(b), the TAC increased significantly from 10 min to 30 min, and it was reduced sharply beyond 30 min and then increased again several times before decreasing significantly. Although prolonged extraction times might increase the contact between the plant sample and solvent, soften and weaken the cell wall, and therefore improve yield, however, exposure to high temperature in a longer period of extraction will cause structure alteration and the ability to decompose the wanted compound (Maciel et al. 2018; Ryu & Koh 2018; Sirichan et al. 2022). The extraction time of fewer than 30 min might lead to incomplete extraction (Peng et al. 2020) since it is not sufficient to break down the cell wall and allow the penetration of solvents (Pragalyaashree, Tiroutchelvame & Sashikumar 2018). Hence, anthocyanin cannot be released. Therefore, 30 min was selected to further study the impact of extraction parameters.

EFFECT OF TEMPERATURE

The other contributing factor in extracting anthocyanin is temperature. Figure 1(c) presents the impact of temperature on TAC that examined by using a varied temperature of 40 °C to 80 °C at a fixed ethanol concentration (75%) and extraction time (30 minutes). At 60 °C, the TAC peaked at 30 min, dropped sharply after that, and then gradually increased until 120 min. At 40 °C, the TAC starts to increase only after 60 min until it reaches 120 min while at 80 °C, the TAC value is the lowest compared to 40 °C and 60 °C at any time point. The result exhibited some stability at 40 °C and 60 °C and a mark in degradation at 80 °C (Maciel et al. 2018). An increase in extraction temperature will result in greater mass transfer as well as a higher extraction yield, but excessive temperature or exposure to ultrasound will degrade bioactive compounds (Sirichan et al. 2022). Therefore, temperatures of 40 °C to 60 °C could be applied to extract higher anthocyanin concentrations. However, 40 °C takes a longer time to yield a high TAC value as compared to 60 °C which yields the highest TAC value for only 30 min. When developing a novel extraction technique, faster extraction time is one of the required requirements. Hence, the current study has fixed the temperature at 60 °C but not chosen as an optimization process parameter.

## EXTRACTION PARAMETERS BASED ON RESPONSE SURFACE MODELLING

The experimental design trial was conducted to evaluate the effects of two extraction parameters on the total anthocyanin content of roselle calyx, including ethanol concentration (X_1) and extraction time (X_2). The experimental conditions and results of 13 runs are presented in Table 1. Total anthocyanin content in calyx roselle extracts ranged from 2.656 mg C3G/g DW to 5.322 mg C3G/g DW. Run 3 (ethanol concentration 75% and extraction time 35 min), has yielded the highest value of TAC which is 5.322 mg C3G/g DW. The current results were higher when compared to the value of the previous study by Maciel et al. (2018) that reported the highest total anthocyanin content as 2.70 mg CGE/g with an extraction temperature of 60 °C and extraction time of 20 min. However, total anthocyanins in roselle calyx are less than Zannou et al. (2020) finding, which are 6.80 mg D3S/g with almost similar extraction conditions. The differences in findings influenced by the conditions and growth methods (Kouakou et al. 2015), the stage of maturation (Maciel et al. 2018), environmental factors, genetic and the plant variety (Ali, Zainalabidin & Latip 2019).

FITTING THE RESPONSE SURFACE MODEL

Based on the sequential model sum of squares analysis, the regression model was chosen, at a 5% significance level, denoted by p-values <0.05. The p-value of each model is summarized by the sum of squares analysis shown in Table 2. The quadratic model is suggested as the ideal model since it provides the best fit to the experimental data with a p-value less than 0.0001 in explaining the linear effects, quadratic effects, and two-factor interaction effects of the parameters (ethanol concentration and extraction time) on the studied response of TAC yield at various extraction conditions. The empirical regression model that relates the two independent variables to the anthocyanin content is presented in Equation (2).

$$TAC = +5.19 - 0.9409X_1 - 0.1212X_2 - 1.21X_1^2 \cdot -0.2184X_2^2 - 0.0560X_1X_2. \quad (2)$$

The positive and negative signs show the direction of each term's effects while influencing the response requires either raising or lowering factor levels (Pinela et al. 2019). This study indicates a decreased of ethanol concentration (X_1) and extraction time (X_2) would increase the yield (TAC) for the linear effects. The quadratic effects of equations X_1^2 and X_2^2, were all negative indicating that the yield was declining as ethanol concentration and extraction time further increased. The negative sign on two factor interaction effects X_1X_2, showed the antagonism impacts among the extraction parameters on the yield (Halim et al. 2021).
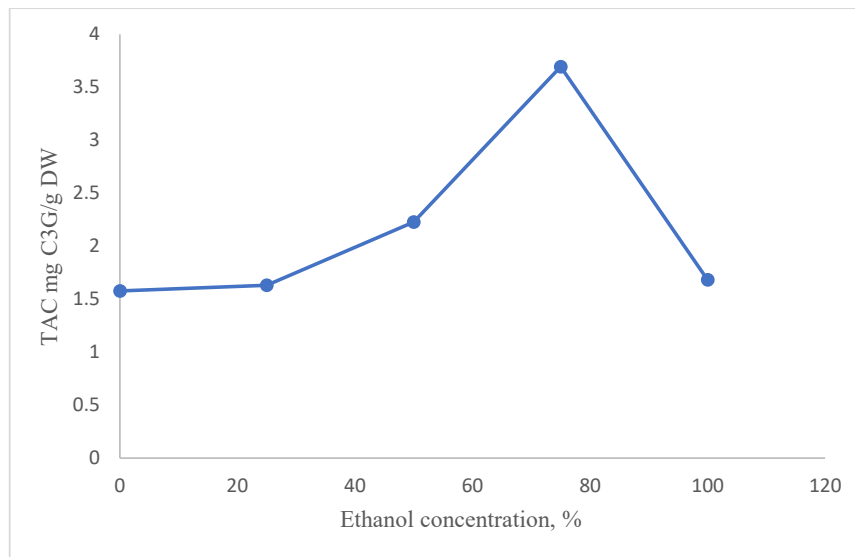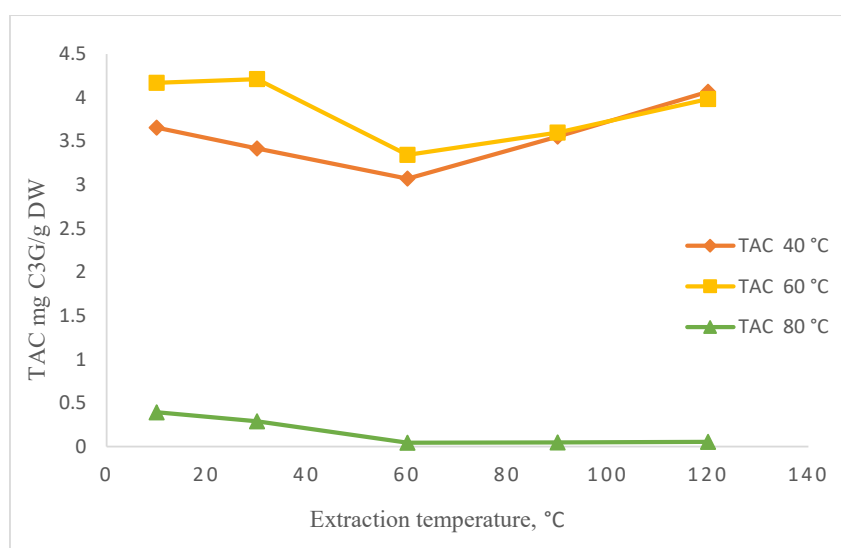

(a)
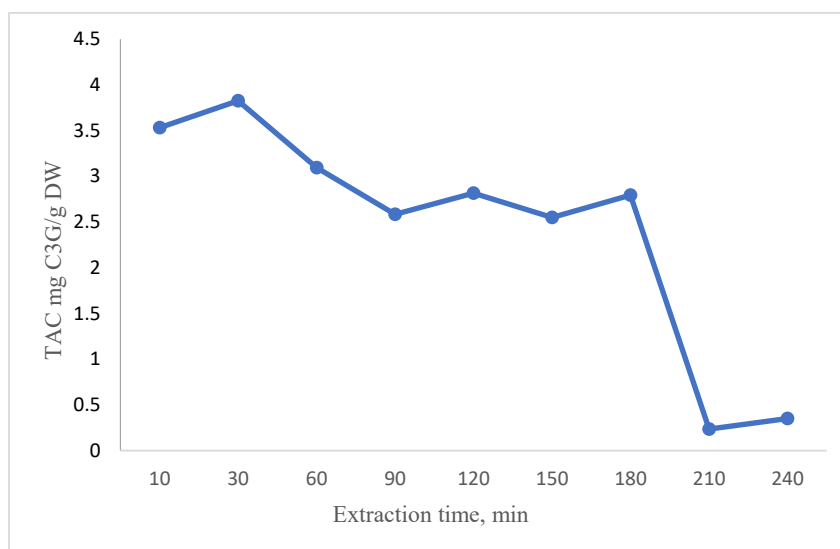
(b)

FIGURE 1. Preliminary experiment of (a) ethanol concentration, (b) extraction time, and (c) extraction temperature

TABLE 1. Experimental design trials in yielding anthocyanin content from roselle calyx using Central Composite Design

Run	Ethanol concentration (%)	Extraction time (min)	Total anthocyanin content (mgC3G/g DW)
1	75	60	4.860
2	100	35	3.045
3	75	35	5.322
4	75	35	5.170
5	75	35	5.200
6	50	60	4.623
7	75	10	5.149
8	50	35	4.980
9	50	10	4.730
10	75	35	5.098
11	100	10	2.987
12	100	60	2.656
13	75	35	5.112

TABLE 2. The sequential model sum of squares of polynomial models

Source	Sum of Squares	df	Mean Square	F-value	p-value (Prob > F)	
Linear vs Mean	5.40	2	2.70	4.83	0.0340	
2FI vs Linear	0.0126	1	0.0126	0.0203	0.8899	
Quadratic vs 2FI	5.53	2	2.77	470.56	< 0.0001	Suggested
Cubic vs Quadratic	0.0038	2	0.0019	0.2546	0.7847	

ANOVA ANALYSIS

A one-way analysis of variance (ANOVA) was used to assess the fitted response surface model's reliability, accuracy, and goodness of fit, as shown in Table 3 (Halim et al. 2021). The results showed that the model was highly significant, with an F-value of 372.33 and a p-value of <0.0001. The ethanol concentration was the most important factor with a p-value of <0.0001 and followed by extraction time with a p-value of <0.05. However, the interaction effects were not significant between the process factors for X_1X_2.

The presented results in Table 3 reflected a good fit because the value of R^2 (0.9963) is closer to 1. The predicted (0.9889) and adjusted (0.9936) R^2 values were more than 0.8 proving that the experimental and predicted data are well correlated; the difference is less than 0.2. The signal-to-noise ratio is estimated with adequate precision, and a ratio larger than 4 is appropriate. The obtained value for adequate precision was 48.8815 showing an adequate signal. The p-value of 0.7749 denotes an insignificant lack of fit which indicates that the model fits the experimental data well (Celli, Ghanem & Brooks 2015). There is a 77.49 % chance that a lack of fit F-value this large could occur due to noise. A low value of 1.69 (<10) for the CV was achieved which showed that the experimental values were correlated to a high degree of precision and a good deal of reproducibility (Liu et al. 2013). A lower PRESS value is preferable since it results in a high predicted R^2 value. The result obtained in this study is 0.1216, which implies that the model is adequate (Halim et al. 2021). These findings showed that the acceptable fitness of the generated model was significant to the process factor and fits within the chosen ranges.

The predicted responses *vs* actual responses displayed in Figure 2(a) are shown in a diagnostic plot, which is commonly used for data visualisation, to assess the regression's goodness-of-fit. The fitted plot, which displays the difference between the projected response values and the actual response values, which were randomly distributed along and close to the straight line, indicates that the created model is appropriate for use in the optimization of anthocyanin yield. The residuals were distributed linearly and adhered to the normal (linear) distribution, which is one of the essential requirements for the validity of ANOVA, as shown in Figure 2(b) (Halim et al. 2021).
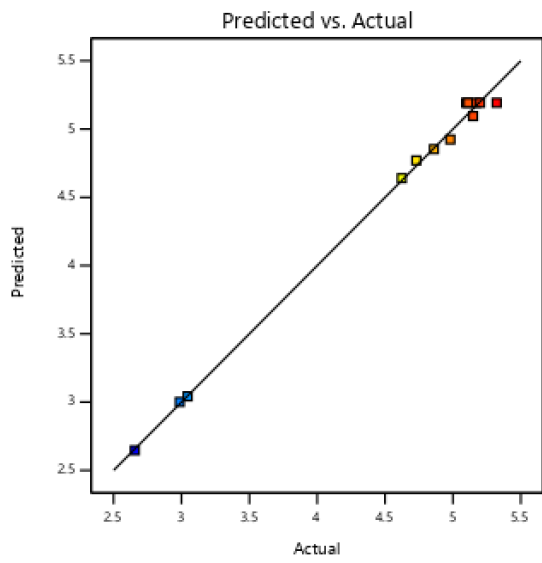
HPLC PROFILE OF ANTHOCYANIN CONTENT

The major anthocyanins of roselle were identified and quantified based on their retention times and compared with the certified reference materials. Figure 3 showed the HPLC chromatograms for the calyx and calyx residue extracts under the same extraction conditions (70% v/v ethanol concentration and 35 min extraction) which were all monitored at 520 nm. Generally, two major anthocyanins are detected in calyx and calyx residue extracts. The peaks were identified as Dp-3-Sam and Cy-3-Sam which were observed at retention times of 16.197 and 17.597 min (calyx) and 16.189 and 17.581 min (calyx residue), respectively. When compared to Cy-3-Sam, Dp-3-Sam had the highest content among the two anthocyanins in both runs (p<0.05). Similar results are following those obtained by Ali, Zainalabidin and Latip (2019) and Ifie et al. (2018). These two anthocyanins contribute to more than 85% of all anthocyanins in roselle extract (Amor & Allaf 2009).

Next, the contents of Dp-3-Sam and Cy-3-Sam in the two samples were quantified by using HPLC analysis. Quantitative analysis data for Dp-3-Sam and Cy-3-Sam in each run are given in Table 4. Among the extracted conditions, the content of Dp-3-Sam (873.18 ppm) and Cy-3-Sam (264.09 ppm) was found to be significantly higher in calyx extracts compared to calyx residue extracts; Dp-3-Sam (508.49) and Cy-3-Sam (172.46 ppm)

with ($p<0.05$). This finding corroborated the TAC values from the pH-differential method. However, Kouakou et al. (2015) reported the anthocyanin content of Dp-3-Sam and Cy-3-Sam as 21.28 mg/g DW and 17.11 mg/g DW respectively which were 5-12 times greater than the current result which is 4.37 w/w (Dp-3-Sam) dan 1.32 w/w (Cy-3-Sam). These variations can be related to various cultivars and extraction techniques (Ryu & Koh 2018).

TABLE 3. Analysis of variance (ANOVA)

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-Value, (Prob > F)
Model (quadratic)	10.94	5	2.19	372.33	<0.0001
X_1: Ethanol concentration	5.31	1	5.31	903.41	<0.0001
X_2: Extraction time	0.0881	1	0.0881	14.98	0.0061
X_1^2	4.04	1	4.04	687.55	<0.0001
X_2^2	0.1318	1	0.1318	22.41	0.0021
X_1X_2	0.0126	1	0.0126	2.14	0.1871
Residual	0.0412	7	0.0059	-	-
Lack of fit	0.0091	3	0.0030	0.3780	0.7749
Pure error	0.0321	4	0.0080	-	-
Correlation total	10.99	12	-	-	-

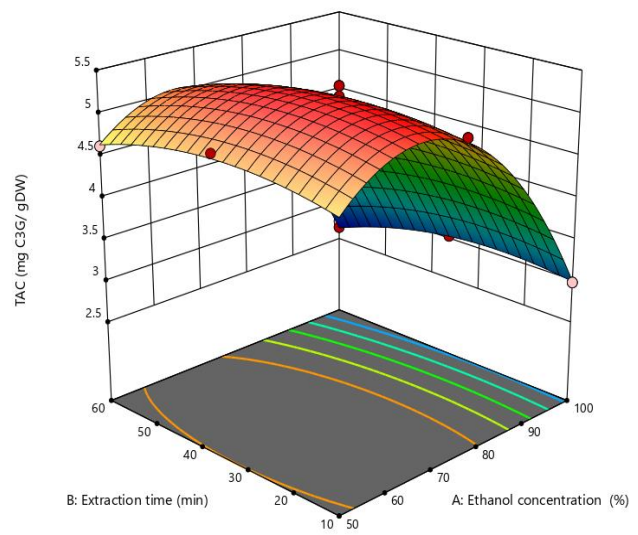| Credibility analysis of the regression equation					
Coefficient of variation (CV) %	1.69				
PRESS	0.1216				
R^2	0.9963				
Adjusted R^2	0.9936				
Predicted R^2	0.9889				
Adequate precision	48.8815				

(a) Predicted responses vs actual responsses
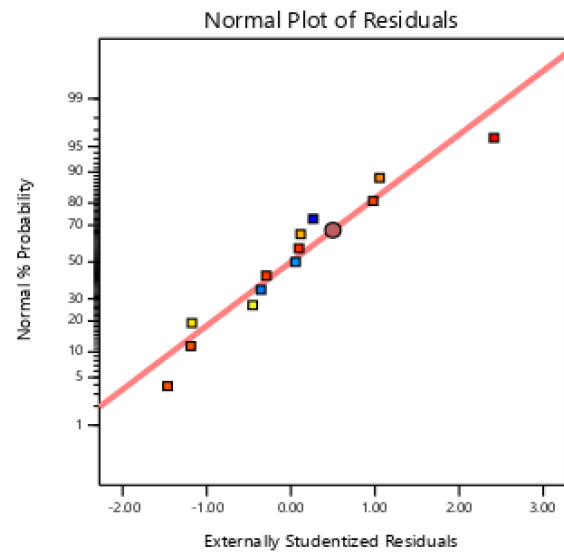
(b) The normal probability plot
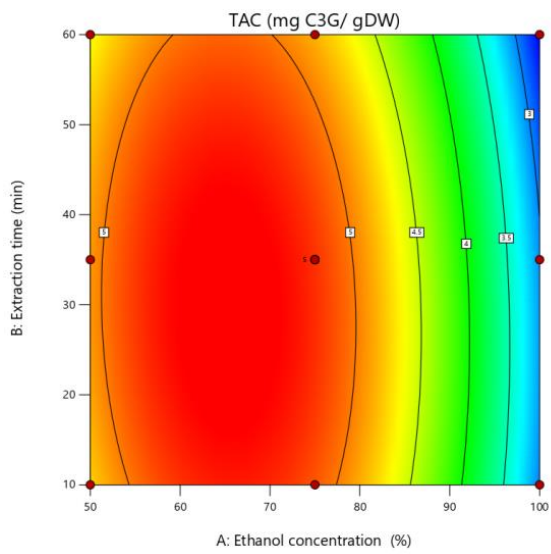
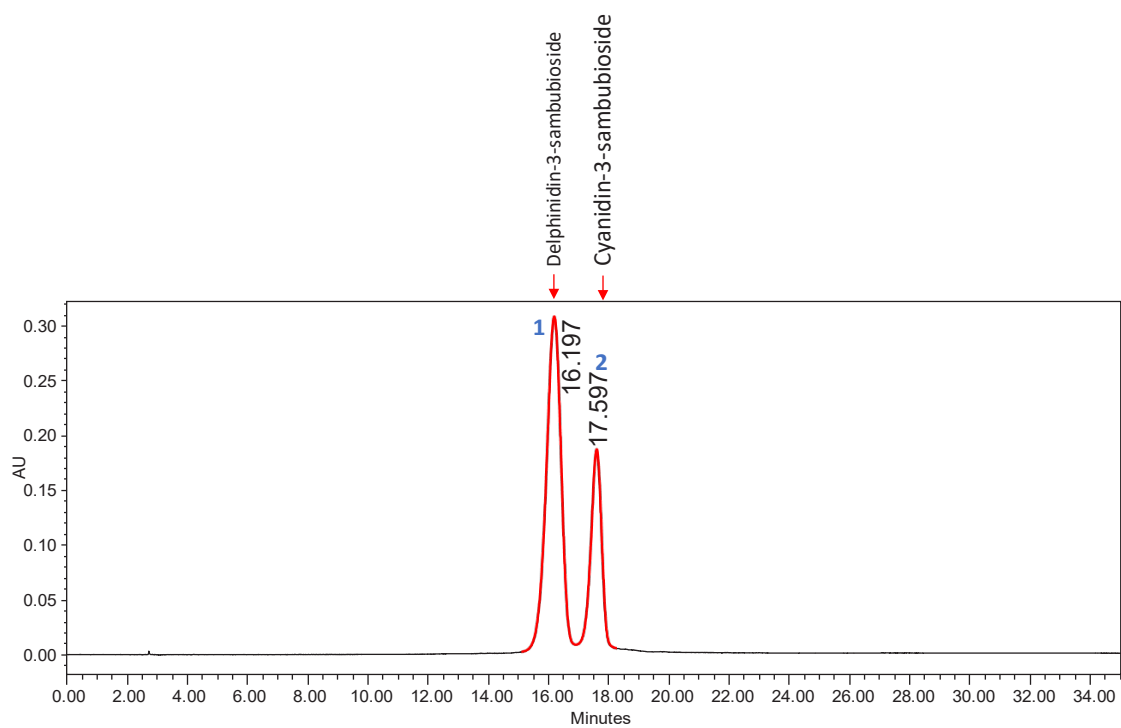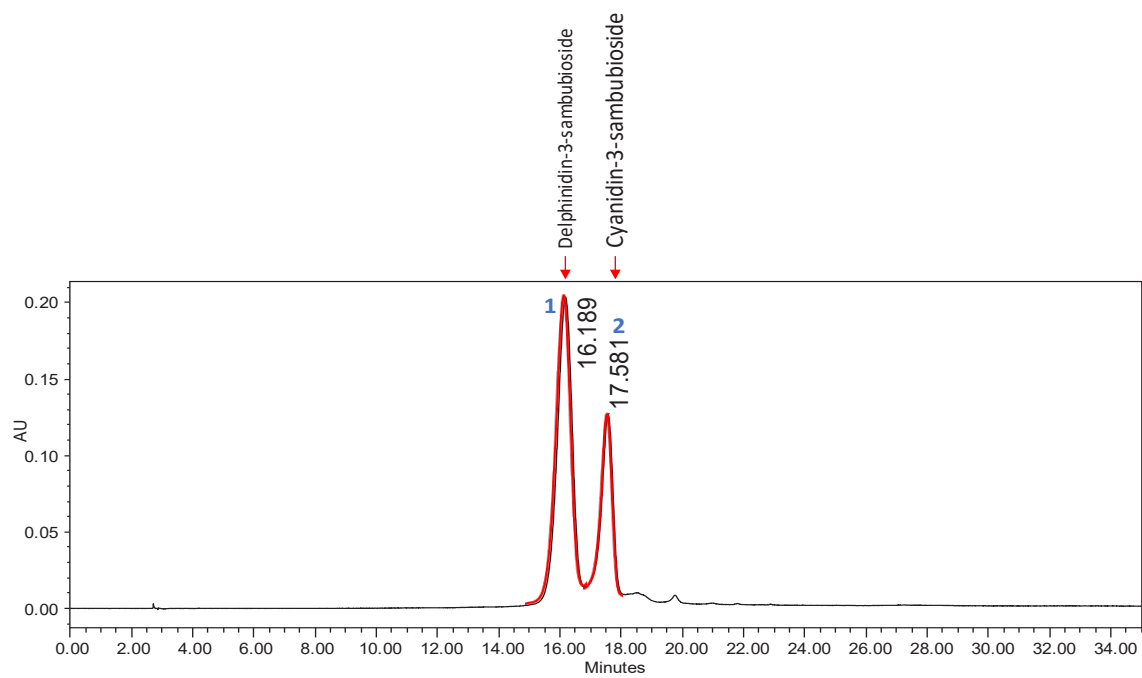(C) Contour plot

(d) Three-dimensinal surface plot

FIGURE 2. Diagnostic plots of the residual analysis and response surface plots
of the interaction effects of extraction parameters on TAC of roselle calyx

FIGURE 3. HPLC chromatogram of Dp-3-Sam and Cy-3-Sam in (a) calyx extracts,
(b) calyx residue extracts that were observed at 520 nm

TABLE 4. Quantitative analysis data of Dp-3-Sam and Cy-3-Sam in roselle extract

Sample	Concentration Dp-3-sam (ppm)	Percentage of Dp-3-sam in the sample (w/w)	Concentration Cy-3-sam (ppm)	Percentage of Cy-3-sam in the sample (w/w)
Calyx extract	873.18 ± 4.71[a]	4.37 ± 4.71[a]	264.09 ± 4.71[a]	1.32 ± 4.71[a]
Calyx residue extract	508.49 ± 1.88[b]	2.54 ± 1.88[b]	172.46 ± 1.57[b]	0.86 ± 1.57[b]

The data represented the mean of three replicates ± standard deviation. Different letters show significant effect ($p \leq 0.05$)

EFFECT OF EXTRACTION PARAMETERS ON TAC FROM ROSELLE CALYX

The major contributing factors that affect the extraction conditions in yielding higher TAC are ethanol concentrations and extraction time. Surface plots as shown in Figure 2(c) and 2(d) illustrates the interaction influence of two factors (ethanol concentration (X_1) and extraction time (X_2)) on the TAC value from roselle calyx at a fixed temperature of 60 °C. The maximum, average, and minimum TAC yield values are represented by the colored zones in red, green, and blue, respectively. A defined parabolic shape shows ethanol concentration is the main factor that contributed to the yield. The maximum yield of anthocyanin can be observed around the middle-level region (red zone) of ethanol concentration (60-75%), extraction time (20-40 min) at a constant extraction temperature of 60 °C, and TAC values declining beyond these ranges. According to Duy et al. (2019), the efficiency of 70% ethanol as a solvent was higher than that of 50% and 90% ethanol. Besides that, thermal stability tests discovered that very less significant loss was seen after 30 min of heating at 60 °C. Anthocyanins are generally stable when the heating period is around 30-45 min (Pragalyaashree, Tiroutchelvame & Sashikumar 2018). Therefore, the optimal conditions for anthocyanin extraction based on prediction from the design expert software were 70.193% v/v ethanol concentration and 34.233 minutes of extraction time, and significantly affect TAC recovery with the value of 5.332 mg C3G/g DW.

The obtained results were experimentally validated in triplicate using the approximate values of 70% v/v ethanol concentration and 35 min extraction duration at a constant extraction temperature of 60 °C. As a result, the verification's findings showed that the software-generated results and the results obtained through experiments were not significantly different where p>0.05. The highest TAC obtained from roselle calyx is 5.277 mg C3G/g DW, as stated in Table 5. Thus, optimal conditions for achieving this yield were 70% vol/vol of ethanol concentration and 35 min extraction time accordingly at a constant extraction temperature of 60 °C.

EFFECT OF PROCESS FACTORS ON TAC IN THE EXTRACT OF ROSELLE STEMS, LEAVES, AND CALYX RESIDUE

The optimal conditions obtained from FCCCD by using roselle calyx are 70% v/v ethanol concentration at 35 min with a constant temperature of 60 °C. The optimal conditions have been applied to roselle stems, leaves, and calyx residue and the TAC found has been demonstrated in Table 5. Roselle agro-waste contains anthocyanin whereas most TAC is found in calyx residue. The least TAC is found in stems and leaves. This study also showed that TAC in calyx is significantly higher than in calyx residue, stems, and leaves (p<0.05). The TAC in calyx residue is lower than calyx might be due to anthocyanin degradation. During the preparation of beverages, roselle calyx normally will be boiled at 100 °C. This high temperature can lead to anthocyanin degradation. Thermal degradation of anthocyanins can produce a variety of species depending on the degree and type of heating (Jiang et al. 2019). Anthocyanin can degrade in a variety of circumstances involving their A- or B-ring. It has been reported that the A-ring degradation results in the formation of formylphloroglucinal or phloroglucinol carboxylic acid while syringic, vanillic and protocatechuic acids were the main products of the anthocyanins degradation via B-ring cleavage (Yang et al. 2018). Besides that, anthocyanin would break down when heated into a chalcone structure, which would then change into a derivative of the coumarin glucoside with

the loss of the B-ring (Patras et al. 2008). This results in a lower TAC in the calyx residue than in the calyx.

Nevertheless, the TAC in calyx residue is considered high and can be an alternative source of anthocyanin other than calyx itself. Based on the data presented in Table 5, the TAC of calyx residue found in the current study is 3.011 mg C3G/g DW and it is higher compared to the TAC in calyx in the previous study (1.855 mg C3G/g DW) by Peredo Pozos et al. (2020) with nearly equivalent extraction conditions. This proved that agro-roselle waste, mainly calyx residue can be an alternative source of anthocyanin other than calyx itself. Meanwhile, TAC in stems and leaves is significantly lower than calyx residue, this low value proposes that extraction of anthocyanin is non-economical. Hence, it is crucial to fully utilize the calyx residue of roselle as a source of anthocyanin since it is often discarded after being processed as a food product.

TOTAL PHENOLIC (TPC) AND FLAVONOID CONTENT (TFC)

Results obtained in the present study showed that roselle calyx residue had the highest anthocyanin content of all the roselle agro-waste products (stems and leaves). Hence, calyx residue extract under optimal conditions was selected for the determination of TPC and TFC to compare with roselle calyx. According to Table 5, there are no significant differences between the TPC and TFC values of calyx residue and roselle calyx under the same optimal conditions ($p>0.05$). Zannou et al. (2020) reported the TPC value from roselle calyx that was extracted using ultrasound-assisted extraction (UAE) in 70% aqueous ethanol as 141.11 mg GAE/g which was four times greater than the present study. However, the TFC value reported was lower at 8.55 mg ECE /g. Apart from that, the TPC and TFC value of calyx residue, 30.09 mg GAE/g and 8.82 mg QUE/g, respectively, was considerable when compared to the value of TPC and TFC of roselle calyx that has been reported by Samadi and Fard (2020) which is 9.34 mg GAE/g and 4.76 QUE/g, respectively, when extracted using 80% ethanol.

Even though there is a significant difference in TAC between calyx and calyx residue, TPC and TFC values are not. This might be due to the boiling process of calyx to get the juice (before it becomes the calyx residue), the phenolic and flavonoid compounds were not extracted since the solvent used was water. Hence, there is still higher content of TPC and TFC in calyx residue. The highest TPC recovery was likely affected by the type of solvent used, polarity degree, polymerization degree, and the bonds that form with other ingredients in the sample which eventually form insoluble complexes. Since our study used a mixture of solvents which are ethanol and water, these mixtures were found to have better extraction of phenolic compounds. Water-only extraction is not as effective as hydroalcoholic solutions. This is due to the polyphenols' high solubility in organic solvents with lower polarity than water (Galgano et al. 2021). Thus, the calyx residue should be focused on as it still has a high content of bioactive compounds that can be utilized in various sectors.

TABLE 5. Total anthocyanin, phenolic, and flavonoid of the calyx, calyx residue, stems, and leaves under optimized conditions: 70% v/v ethanol concentration, 35 min extraction time, 60 °C extraction temperature

Sample	Calyx	Calyx residue	Stems	Leaves
TAC mg C3G/ g Dw	5.277 ± 0.130^a	3.011 ± 0.138^b	0.077 ± 0.005^c	0.073 ± 0.007^d
TPC mg GAE/ g DW	32.67 ± 1.746^a	30.09 ± 1.018^a	-	-
TFC mg QUE /g DW	11.34 ± 1.675^a	8.82 ± 0.893^a	-	-

The data represent the mean of three replicates ± standard deviation. Different letters show significant effect ($p \leq 0.05$)

Conclusion

In conclusion, this study has focused on the optimized extraction condition on TAC recovery from roselle. The obtained result indicates the optimal conditions for anthocyanin extraction are 70% ethanol concentration (v/v), 35 min extraction time at constant temperature 60 °C have yielded 5.277 mg C3G/g DW for calyx, 3.011 mg C3G/g DW calyx residue, 0.077 mg C3G/ g DW stems, 0.073 mg C3G/g DW leaves. This study also shows Dp-3-Sam present in both roselle calyx and calyx residue extracts from the same extraction conditions is higher compared to Cy-3-Sam. Although the amount of anthocyanin compounds in the leaves and stems is much lower than in the calyx, however, this study showed that calyx residue possesses the significant value of TPC and TFC which were 30.09 mg GAE/g and 8.82 mg QUE/g, respectively. This indirectly increases the added value of roselle plants. Hence, this study suggests that calyx residue which has been often discarded after being processed can be the alternative source for anthocyanin and can be fully exploited after the commercially significant part, i.e., the calyx, is harvested to meet the demands of various industries.

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