Optimization of Anthocyanins Extracts from Roselle (*Hibiscus sabdarifa*) Petals Using Ultrasonic-Assisted Extraction Method

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Abstract Roselle petals contain high anthocyanins, a good antioxidant, and natural dark red colorants. Anthocyanins help in reducing risks of cardiovascular disease and colon cancer. But anthocyanins are easily affected by extraction process. Therefore, this study proposed a new method of extracting anthocyanins using ultrasonic-assisted extraction (UAE) from roselle petals with three extraction parameters. The extraction parameters are sample's particle size (0.125, 0.375 and 0.625 mm), solvent to solid ratio (10:1, 15:1 and 20:1 mL solvent/g solid) and extraction time (5, 10 and 15 min). The optimization of process parameters aims to achieve the highest extraction yield of extract, Total Anthocyanins Content (TAC), and Antioxidant Activity (AA). The results show that 0.125 mm of particle size, 10:1 mL solvent/g solid, 15 min of extraction obtained the best percent mass yield (64.72%), TAC (70.97 mg/L), and AA (90.05%). Method-wise, this study showed that the ultrasonic-assisted extraction gives better quality of roselle petals extracts than the maceration extraction.

Keywords Anthocyanins · *Hibiscus sabdarifa* · Roselle petals · Ultrasonic-assisted extraction · Antioxidants

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1 Introduction

Anthocyanins are generally accepted as the most critical group of water-soluble pigments, which occur naturally in plant foods [1]. More than 6,000 members of the polyphenol phytochemicals flavonoid family are found in various types of plant foods, fruits (eg: roselles, blackberries, raspberries), and vegetables [2]. These polyphenols are responsible for the purple, blue, orange, and red colors in many vegetables and fruits. Anthocyanin is very sensitive to temperature. Extraction temperature exceeding 40 °C can cause degradation of the active component and turning the extracts brown. Thus, there is a need to apply a more thermal sensitive extraction method to preserve the compound.

Anthocyanins is an excellent source of natural antioxidants that act as radical stabilizer and anti-inflammatory properties. Antioxidants also can prevent and reduce the cardiovascular and cancer diseases. Thus, these are the main reasons for their increasing popularity in the current population diet [3]. A clinical test on roselle extract has been done on a human prostate cancer cell and suggested that the extract could reduce the cancer cells' growth via intrinsic and extrinsic pathway [4]. Other than that, roselle petals also contain high anthocyanins, a good natural antioxidant source, and natural colorant [5]. However, they can quickly degrade due to the stability of anthocyanins is affected by the extraction parameters [6, 7].

Several extractions methods have been implemented to extract anthocyanins over the years [8]. However, most conventional extraction methods, such as hydrodistillation and maceration, have serious drawbacks, such as high solvent use and prolonged extraction time. A newly introduced extraction methods such as the Ultrasonic-Assisted Extraction (UAE) can solve the drawbacks aforementioned [8, 9].

UAE is a non-thermal technology that can serve as an alternative to traditional extracts [7]. Ultrasonic oscillators generate waves that propagate through the medium. The oscillation of rarefaction and compression causes bubble nuclei to form. As the bubbles' size increases, it will burst and generate a shear force that causes macro-turbulence in the medium. This phenomenon is due to cavitation, which can enhance the solvent's penetration deep into the sample matrix and increases the extraction efficiency for temperature sensitive materials. UAE method also contributes to environmental preservation by reducing the use of solvents, fossil energy, and the generation of hazardous substances. The utilization of ultrasonic radiation for extraction gives high reproducibility, simple operations, lower operating temperature, and lower loss of bioactive mixes contrast with the conventional method. Maceration and soxhlet have bound working conditions, especially temperature, limiting the extraction efficiency since temperature impacts the target solutes' kinetic and diffusivity.

Thus, this study aims to optimize the UAE's parameters for the anthocyanin's extraction from roselle petals. The optimization of process parameters aims to achieve the highest extraction yield of extract, Total Anthocyanins Content (TAC), and

Antioxidant Activity (AA). This study also compares the extraction quality between maceration extraction as the baseline of the extraction method with the UAE.

2 Methodology

2.1 Sample Preparation

A 2 kg of dried roselle petals were purchased from a local supplier, ground and sieved to achieve 0.125, 0.375, and 0.625 mm mean particle sizes. The process was conducted by utilizing blender and sieving instruments accessible in Block P Laboratory, Universiti Teknologi PETRONAS.

2.2 Ultrasonic-Assisted Extraction

A jacketed beaker was first set-up and loaded up with 50 mL of distilled water as the solvent used for the extraction. A 2.5 g of ground roselle petals were then inserted into the jacketed beaker. Tap water-line was fitted to the jacketed beaker to keep the extraction temperature below 40 °C. The ultrasonic generator and run clock were controlled at 20 kHz and 5 min, respectively. After the extraction was completed, the solvent was filtered using a Whatman-40 filter paper. The filtrate was transferred into a round bottom flask to remove the excess solvent using a rotary evaporator unit (BUCHI Rotavapor R-215). The concentrated extract was oven-dried (40 °C, 3–5 h) until no measurable weight loss is observed. The extract was then kept in a freezer (−20 °C) for further analysis. The design of experiment is shown in Table 1. A Box-Behnken design was chosen due to the limitation of samples and the simplicity of the design (3 factors and 3 levels, 5 replication of mid-point).

2.3 Analysis

2.3.1 Extraction Yield

Extraction yield was determined by weighing the samples after the drying process and comparing it to the sample's initial weight.

Table 1 Design of experiment for UAE extraction

Run	X_1: Solvent to solid ratio	X_2: Particle size	X_3: Time extraction
	mL/g	mm	min
1	10.00	0.125	10.00
2	20.00	0.125	10.00
3	10.00	0.625	10.00
4	20.00	0.625	10.00
5	10.00	0.375	5.00
6	20.00	0.375	5.00
7	10.00	0.375	15.00
8	20.00	0.375	15.00
9	15.00	0.125	5.00
10	15.00	0.625	5.00
11	15.00	0.125	15.00
12	15.00	0.625	15.00
13	15.00	0.375	10.00
14	15.00	0.375	10.00
15	15.00	0.375	10.00
16	15.00	0.375	10.00
17	15.00	0.375	10.00

2.3.2 Total Anthocyanins Content (TAC)

The determination of anthocyanins was done using the AOAC standard [10]. Anthocyanins were determined using a UV–visible spectrophotometer (UV-1800, Shimadzu, Japan) at 520 nm. A test portion should not exceed 10 mL due to the capacity of the cuvette used. A mixture of 1-part test portion and 4-part buffer was prepared for the analysis. The appropriate dilution factor was determined by diluting the test portion with pH 1.0 buffer until the absorbance at 520 nm is within the spectrophotometer's linear range. This dilution factor was used to prepared two dilutions of the test sample with one in h pH 1.0 buffer and the other with a pH 4.5 buffer. The absorbances were measured at a wavelength of 520 and 700 nm for pH 1.0 buffer and pH 4.5 buffer, respectively. The diluted test portions were recorded versus a blank cell filled with distilled water. Anthocyanin concentration was calculated and expressed as cyanidin-3-glucoside equivalents, as in Eq. 1.

$$Anthocyanin\ pigment\ (\text{mg CGE/L}) = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times l} \tag{1}$$

where A is absorbance value $(A_{520\text{nm}} - A_{700\text{nm}})$ of pH 1.0 – $(A_{520\text{nm}} - A_{700\text{nm}})$ pH 4.5, MW is molecular weight for cyanidin-3-glucoside (cyd-3-glu) (449.2 g/mol), DF is

dilution factor, l is path length (cm), ε is 26 900 molar extinction coefficient, for cyanidin-3-glucoside (L mol^{-1} cm^{-1}), 10^3 is factor for conversion from g to mg.

2.3.3 Antioxidant Activity (AA)

The determination of the Antioxidant Activity (AA) was done using 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The methodology was adopted from the referred literature [11]. A 5 mg/L methanolic DPPH stock solution was prepared and kept within the fridge until utilized. Next, 2.5 mL of extract was blended with a (1) mL of the methanolic DPPH solution and kept within the darkroom for 30 min. The absorbance was measured at 518 nm using a UV–Vis spectrophotometer (model Shimadzu UV-1800). The DPPH scavenging activity was determined using Eq. 2. A blank reading replaces the extracts with distilled water.

$$DPPH\ scavenging\ activity\ (\%) = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100\% \qquad (2)$$

2.4 Statistical Analysis

Experimental design and analysis via response surface methodology (RSM) was conducted using Design Expert V6.0.4 (Stat-Ease Inc., Minneapolis, USA) software. The software developed the model equation, plotting the response graphs, and predicting the optimum parameters over all three responses to obtain maximum yield, TAC, and the AA. The analysis was conducted on the regression fitness measured by the value of the coefficient of determination (R^2), analysis of variance (ANOVA), and p-value.

3 Results and Discussion

This work discussed the effect of each parameter (sample particle size, solid to solvent ration and extraction time) and their statistical fitness towards the responses (extraction yield, total anthocyanins content and antioxidant activity). The study also compares the quality of extracts obtained from the UAE to the maceration extraction method to measure the proposed UAE-method's efficiency.

3.1 *Effect of Sample Particle Size*

Particle size has been known to affect the composition and mass fraction of antioxidants in the extract [12]. Figure 1 illustrates that the maximum yield of 64.72% was obtained for the lowest particle size of 0.125 mm. The extraction yield decreases as the sample particle size increases from 0.125 to 0.625 mm. Lower particle size provides a higher total surface area for the extraction. This allows a higher mass transfer rate from the sample to the solvent. A study shows that larger particle size of wheat results in higher antioxidant properties compared with the smaller particle size [13]. This result is coherent with the surfaces plot in Fig. 2 that shows the TAC concentration.

In contrast, the highest TAC concentration of 65.32 mg CGE/L was observed at 0.375 mm. This high response can be explained by the total surface area available for mass transfer. Generally, by lowering the particle size, the total surface area accessible by the solvent is increased. This phenomenon promotes the extraction process and reduces the mass transfer resistance due to the distance traveled by the solvent. However, particle sizes that is too small can cause a negative impact on the results due to the loss of precious material in the grinding process. Mechanical shearing and tearing of the sample matrices cause the exposed solute loss to surrounding cause less amount being collected in the extraction process [13].

Figure 3 shows the highest percentages of DPPH radical scavenging activities were observed at a particle size of 0.125 mm. The result shows the highest antioxidant activity observed was 90.05%. This outcome corresponds with the research conducted by [13], whereby smaller particle sizes gave higher oil yield. Sample particle size can affect and regulate the mass transfer kinetics and solvent access into the soluble component. The reduction of sample particle size can increase the intrinsic capacity for the solvent's diffusion into the target component, which improves the

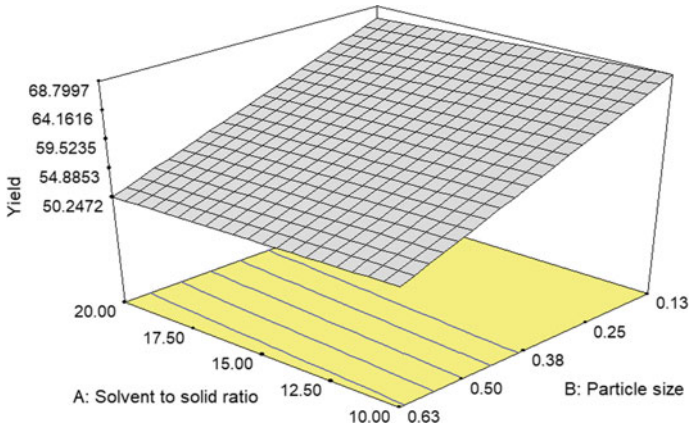

Fig. 1 Surface plots for the effect of mean particle size on extraction yield (0.125 mm, 15 min extraction time)

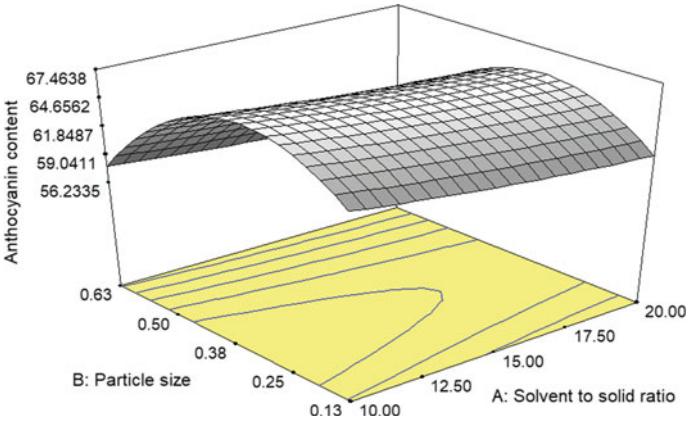

Fig. 2 Surface plots for the effect of mean particle size and solvent on solid ratio on total anthocyanins content at 10 min of extraction time

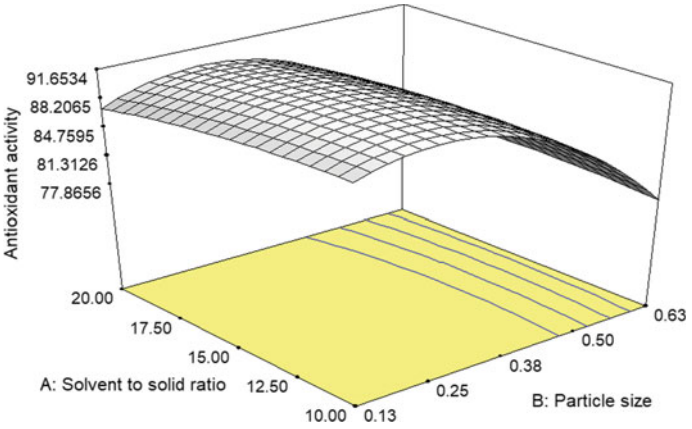

Fig. 3 Surface plots for the effect of mean particle size on antioxidant activity at 10 min of extraction time

extraction efficiency. Compared with the previous results, the AA corresponds to the yield extracted rather than the TAC amount obtained. This can be explained by the fact that TAC is the measure of anthocyanins extractable from the sample, whereby AA reflects on the extract activity. AA is a synergistic effect of the whole extract rather than the anthocyanin content obtained from the extract. Some other unknown component may contribute to the overall activity has yet to be determined.

Previous research showed that 52% of extract yield bioactive compounds from hazelnut shells with particle size 0.5–1.0 mm compared to 28.6% of yields for particle size between 1.00 and 2.0 mm [14]. Even so, as claimed by Makanjuola (2017), the smallest particle size may not always yield the highest antioxidant property.

Although particle size reduction corroborates an increase in extraction efficiency, a critical particle size might be reached, which will not significantly differ in the extraction efficiency [12]. Compared to anthocyanins extraction, results did prove smaller particle size yields higher oil yield.

3.2 Effect of Solvent to Solid Ratio

Solid to solvent ratio determines the amount of solvent need to be used for the extraction process. Figure 1 demonstrates that the ratio of 10:1 yielded the highest yield of 68.80%. Figure 1 also shows that the ratio did not significantly impact the curvature or shift of trend of the extraction across the particle size. The solvent to solid ratio for ultrasonic has a less effect on the percent mass yield of extraction since different ratios gave similar results. Each extraction method requires a different set of operating parameters to improve extracts and recovery of the bioactive compounds [9]. The saturation of the solvent causes this has not been achieved even at a ratio of 10:1. Saturation and solubility can be the factors affecting the yield of extracts. However, since the temperature and pressure of the process were kept constant, the solubility of the solute into the solvent should not change significantly. Like a study conducted by Giannoccaro et al. (2006), the lowest sugar amounts were obtained at 80 °C with solvent to solid ratio of 10:1 and 15:1 than with 1:5 [15].

The lowest solvent to solid ratio of 10:1 for ultrasonic extraction showed the highest TAC amount, which was 70.97 mg CGE/L. The solvent was kept fixed at 50 ml for ultrasonic extraction to suit the apparatus's available size capacity, whereas the mass of the sample was manipulated accordingly. The results indicated that the highest TAC was majorly produced by extracts with the lowest solvent to solid ratio (10:1). A research asserted that a lower solvent to solid ratio results in a greater concentration gradient, which acts as the driving force for the mass transfer between solute and solvent [7]. It is also reported that TAC was increased drastically with the increase of solvent to solid ratio. The increase of phenolic compounds may result from a higher amount of solvent that can penetrate plant cells [16].

For AA, an indistinct pattern was acquired in Fig. 3 contrasting and the yield rate pattern in Fig. 1. No critical contrast of AA observed was the solvent to solid ratio increases or decreases. The highest AA observed was 90.05% at a 10:1 ratio. It can be supported by the cumulative impact of present radical free searching exercises [17]. The antioxidant activity measured in this experiment considers the cumulative effect of all antioxidants present in the extracts that were also contributed from other extraction parameters.

A recent study by Mancini et al. (2018) confirmed that antioxidant capacity is an index derived from providing a single estimate of antioxidant activity from all bioactive compounds. The total bioactive compounds in the extracts from most natural product extracts were correlated with DPPH antioxidant assay. Hence, the high percentages of AA at the lowest solvent to solid can be deduced to be the effect of

DPPH scavenging activity other present bioactive compounds in the extracts other than anthocyanin content [18].

3.3 *Effect of Extraction Time*

Figure 4 shows the UAE produces the highest yield of 67.81% at the longest extraction time of 15 min and the smallest particle size of 0.125 mm. This indicates that longer extraction time gives a longer extraction process to take place, which allows the diffusion of solvent deep inside the sample matrix to extract the solvent from the core of the sample.

Figures 5 and 6 show the effect of extraction time on the TAC and AA, respectively. The highest TAC was produced at the extraction of 15 min, which yielded 70.97 mg CGE/L anthocyanins. There is no significant difference in the highest AA that was noticed across the extraction time tested. The highest percentage of AA was observed at 90.95% for ultrasonic at 15 min. The prolonged time of extraction potentially increases the loss of solvent with bioactive compounds by vaporization [19, 20]. Thus, 15 min at 40 °C as practices in the study is recommended to be performed for the anthocyanin's extraction.

Medina-Torres et al. (2017) mentioned that extraction efficiency is highly influenced by extraction time, specifically in UAE, where it is asserted that ultrasound increases as a function of time with two main stages; washing and diffusion extraction [21]. Washing occurs in the first 10 to 20 min of extraction, where approximately 90% of soluble components on the surfaces of the matrix dissipate, indicating rapid recovery of the phenolic compounds. Meanwhile, slow extraction is a stage where mass transfer from solute to solvent occurs only via diffusion, which could last up

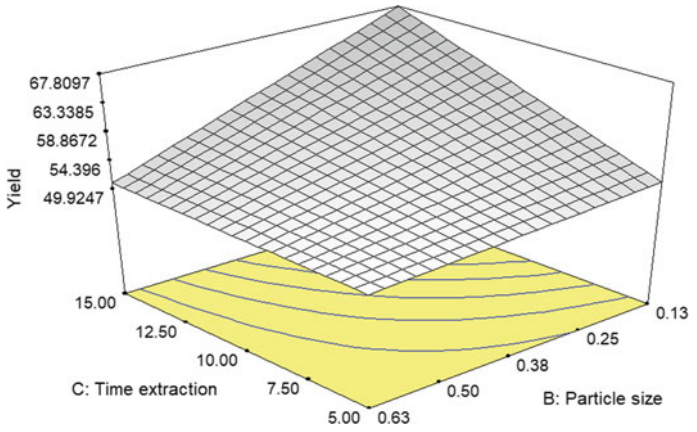

Fig. 4 Surface plots for the effect of time extraction ratio on yield at solvent to solid ratio on 15:1

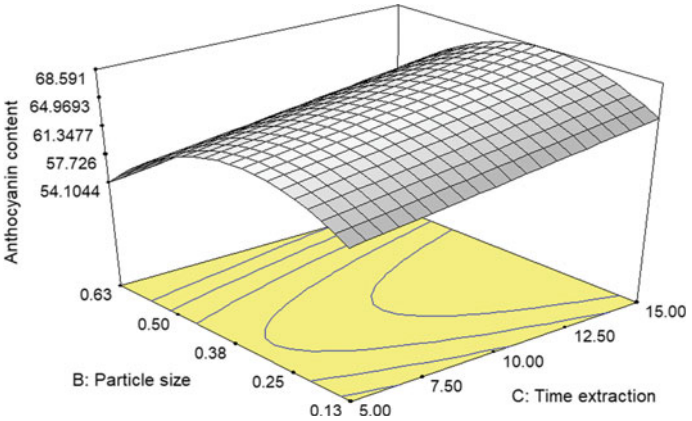

Fig. 5 Surface plots for effect of time extraction ratio on anthocyanin content at solvent to solid ratio on 15:1

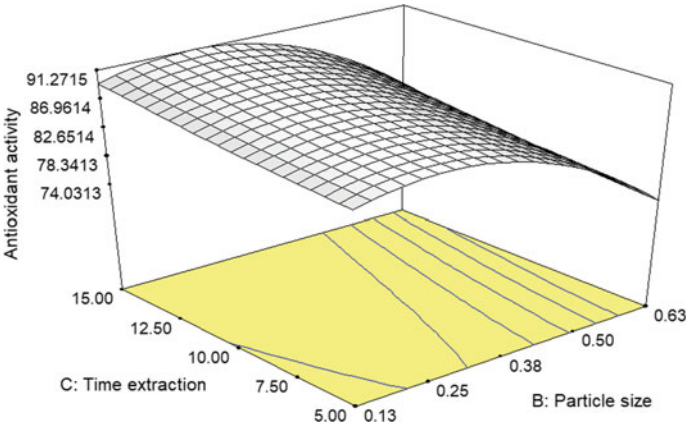

Fig. 6 Surface plots for the effect of time extraction ratio on antioxidant activity at solvent to solid ratio on 15:1

to 100 min. The ultrasonic extraction designed for this experiment was conducted in a relatively shorter period focusing on the critical washing stage.

3.4 Effect of Extraction Methods

This section of the study compares the two extractions method's performance, namely maceration and ultrasonic-assisted extraction. Maceration extraction was selected to be the control as it is the most basic method of extraction. The maceration extraction

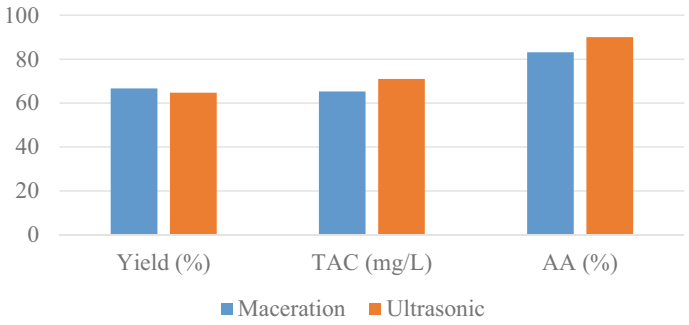

Fig. 7 Maximum yield, TAC, and AA for maceration and ultrasonic assisted extraction method

was conducted at room temperature while maintaining the UAE method's parameters except for extraction time that was increased from 1 to 3 h.

Figure 7 demonstrated that the maceration extraction method results in a slightly higher measure in the percentage of extraction yield (66.67%) compared to the UAE method (64.72%). This may be due to the substantial difference of sample size for both methods, which were 50.0 g for maceration extraction and 5.0 g of sample for UAE. The considerable distinction on this physical perspective was one of the contributing elements towards the higher percent mass yield created by maceration extraction [11].

However, for TAC, extraction using the UAE method produced 70.97 mg CGE/L compared to maceration extraction, which yielded 65.29 mg CGE/L. The UAE method utilizes acoustic cavitation assistance, making the development of bubbles rise due to the wave oscillation to the molecules. The collision between the molecules and bubbles results in a shear force, causing a decrease in particle size, thus encourages mass exchange [21].

The AA analysis of extracts shows that ultrasonic extraction obtained 90.05% DPPH scavenging activities, whereas the maceration extraction method showed 83.20% DPPH scavenging activity. These results demonstrated no significant difference, which are supported by previous studies [22–24]. The main difference between these two methods is the introduction of an ultrasonic probe to the system. Maceration extraction was maintained at room temperature (about 26 °C), while the introduction of the ultrasonic waves to the extraction calls for a need to keep the extraction at 40 °C. A slight disparity in operating temperature may cause the extraction content to be different. As temperature increases, the solubility of the solute in the solvent also increases. A higher amount of soluble solute extracted may lead to higher TAC and AA observed in the analysis.

Table 2 The proposed actual coefficient for the optimized equation for yield, TAC and AA for maceration and ultrasonic assisted extraction method

Coefficient	Maceration			UAE		
	Yield (%)	TAC (mg CGE/L)	AA (%)	Yield (%)	TAC (mg CGE/L)	AA (%)
Model	Quadratic	Quadratic	Quadratic	2FI	Quadratic	Quadratic
Mean	+80.50	+55.05	+96.72	+46.29	+53.24	+81.84
X_1	−3.13	−1.41	−1.037	−0.17	−0.39	+0.35
X_2	+54.90	+67.51	−0.380	+6.77	+63.02	+38.90
X_3	−13.34	+4.421	−1.21	+1.94	+0.91	−0.14
${X_1}^2$	+0.06	+0.04	+0.04	–	+0.01	−0.03
${X_2}^2$	−66.99	−98.59	−34.41	–	−103.40	−97.85
${X_3}^2$	+3.40	−0.53	+0.22	–	+0.002	+0.02
X_1X_2	+0.70	−0.07	−0.66	+0.16	+0.27	+0.67
X_1X_3	+0.45	+0.03	−0.02	−0.003	−0.03	+0.01
X_2X_3	−7.40	+0.84	+6.98	−2.87	+0.07	+0.23
R^2	0.9196	0.9662	0.9930	0.8880	0.9917	0.9996
Model *p*-value	0.0044	0.0002	<0.0001	0.0003	<0.0001	<0.0001
Standard deviation	2.07	1.14	0.57	2.19	0.59	0.17

3.5 Optimization of Extraction Condition

For optimization purposes, all three parameters; solvent to solid ratio (X_1), mean particle size (X_2), and extraction time (X_3) factors, and three responses were examined using Design-Expert Version 6.0.4. A second-order polynomial with interaction model was selected, and the proposed equation for the optimization purposes is shown in Table 2.

Table 2 showed that all the models are significant, with the coefficient of determination (R^2) at least 0.888. Tested with significant levels of 0.05, the predicted data shows a good agreement with the experimental runs with a 95% confidence level. It also can be seen that the most influential parameters in all responses are particle size (X_2 and ${X_2}^2$) as both are having the highest coefficient magnitude, as shown in Table 2. This can be supported as the particle size getting lower, the total accessible surface area increased, thus increasing the interaction between the solvent and solute. Meanwhile, the lowest influence on the extraction regime is dominated by the interaction between solvent to solid ratio and extraction time (X_1X_2). This shows that the extraction process still runs as lower solvent to solute saturation. Thus, increasing the solvent does not impact the result much.

The optimization of parameters and responses was conducted for both extraction methods. The parameters were set to 'in range' while the responses were set to

Table 3 Optimized condition for maceration and ultrasonic extraction method

Condition	Specification	Maceration	UAE
X_1	In range	10	10
X_2	In range	0.35 mm	0.24 mm
X_3	In range	3.00 h	15 min
Yield (%)	Maximum	66.38557	64.72
TAC (mg CGE/L)	Maximum	66.19	76.22
AA (%)	Maximum	83.38	91.60
Desirability		0.995	0.983

maximum. This is to ensure that the optimized condition could yield the maximum amount with the best quality of extracts. The results are tabulated in Table 3.

Maceration extraction acquired high desirability compared to the UAE method. However, the UAE method achieved the main objective of maximizing the anthocyanin content. Moreover, the UAE method only takes 15 min compared with maceration, which takes 3 h of extraction time. From an economic point of view, the UAE method is preferable because it is faster with better antioxidant quality. Even though the yield is 2% less than the maceration method, the quality of extract makes up for the lacking. The desirability function of both results is satisfactory as it is more than 95%. It can be concluded that the optimum condition for extraction of roselle petals with regard to maximizing extracts yield, TAC, and AA was 15 min of extraction time, the mean particle size of 0.24 mm, solvent to solid ratio of 10:1, and using the UAE method.

4 Conclusion

In this investigation, the examination of extraction techniques on roselle petals was conducted using the UAE method and proven to achieve a better result of extraction yield and total anthocyanin content when compared with the maceration extraction method. The results show that 0.125 mm of particle size, 10:1 mL solvent/g solid, 15 min of extraction obtained the best percent mass yield (64.72%), TAC (70.97 mg/L), and AA (90.05%). Method-wise, this study showed that the ultrasonic-assisted extraction gives better quality of roselle petals extracts than the maceration extraction. It is concluded that UAE method is a new sustainable, innovative green technology that can increase the extraction efficiency, reduce time and energy-consuming procedures. This study suggests the development of a kinetic and solubility model that takes into account the ultrasonics amplitude and frequency factors into consideration of the models.

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