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Microwave-Assisted Extraction of *Hibiscus sabdariffa* Antioxidants: Method Development and Validation †

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Abstract: An increased understanding of antioxidant properties in edible flowers, especially *Hibiscus sabdariffa* (Roselle), shows the importance of a reliable determination of phenolic compounds in the flowers. This study reports the development and validation of the analytical microwave-assisted extraction (MAE) method for phenolic compounds. Prior to the optimization, a study for identifying phenolic compounds revealed that chlorogenic acid, protocatechuic acid, caffeic acid, and rutin were presented in Roselle. Three factors affecting MAE, viz. temperature, solvent composition, and sample to solvent ratio, were optimized employing a Box-Behnken Design (BBD) in conjunction with response surface methodology (RSM). The maximum extraction recovery was achieved using the extraction temperature of 68 °C, solvent composition of 59% MeOH in water, and 1:20 of ratio sample to solvent. The kinetics experiment confirmed full recoveries at 15 min. Subsequently, method validation showed a satisfactory result, including lower LOD and LOQ values ranging from 0.22 μg L^{-1} (rutin) to 0.62 μg L^{-1} (chlorogenic acid). Both precisions and accuracy met the acceptances by AOAC. Finally, the method was successfully applied to quantify phenolics in the two most common varieties of Roselle.

Keywords: edible flower; roselle; phenolic compounds; optimization; Box-Behnken design

1. Introduction

Edible flowers have gain popularity in culinary arts, where chefs all around the world have tried to incorporate their dish with one [1]. Actually, it was not only recently that people use the flower in daily consumption. In China dan Japan, the incorporation of flowers in their recipe has been made since thousands of years ago [2]. Greece and Rome, medieval France, Europe, Victorian England, or the Middle Eastern region had also used them as part of their diet [3]. In the different regions, various types and approaches in the cooking method are used. In Asia or other tropical areas, one of the most well-known edible flowers is *Hibiscus sabdariffa* or also known as Roselle [4].

Roselle is one to be the most consumed edible flower. Industries commonly use Roselle as a food coloring agent, while the cosmetic industry also uses its extract for lotion and shampoo mixture [5]. Traditionally, in some countries, the flower was made into juices, infused water, a blend in tea drink, and also served as herbal medicine [4,6]. Pleasant appearance and flavor are not the only consideration in consuming this flower; health benefits obtained from it are also taken into account. Exploration of Roselle extract has shown a great result in which several bioactive compounds are

found to be presented, such as phenolics, organic acids, and polysaccharides [7–9]. These compounds are responsible for antioxidant activity, antibacterial agent, anti-inflammatory, hepatoprotective and anti-cholesterol activities [5,8,10].

With respect to the importance of Roselle, the quality of the extract can be affected by extraction conditions and procedures [11]. Conventional methods, such as Soxhlet extraction and maceration, are commonly used. However, working with these methods requires a massive amount of solvent and time-consuming. Therefore, an alternative method, i.e., microwave-assisted extraction (MAE), is preferred. MAE has been proven to be helpful in extracting different types of analytes in a wide variety of edible flowers and plant parts [12–16]. The extraction rate is enhanced by a unique direct heating mechanism offered by MAE. The microwave energy reacts with the polar materials resulting in simultaneous and fast heating through ionic conduction and dipole rotation [17]. The short exposure time to high temperatures will provide high-quality extracts with better target compound recoveries [18].

The process efficiency of MAE strongly depends on the target compound and the matrix. Apart from temperature, solvent composition and ratio sample to solvent would also affect the extraction efficiency of MAE [19] because the solvent work as a medium for energy and mass transfer during the MAE process. Therefore, it is important to optimize the aforementioned MAE factors to achieve an effective and efficient process. Note that the interaction among the extraction variables is important; adoption of a chemometric approach is useful to develop a new extraction method. Integrating response surface methodology (RSM) and Box-Behnken Design (BBD) is promising for this purpose. Compared to the single-factor experimental design, a factorial design is more useful to evaluate the MAE factors simultaneously [20]. In regard to the number of extraction factors, BBD offers fewer runs over other factorial design [21].

The focus of the current study was to optimize a reliable analytical extraction for the determination of phenolic compounds in Rosella using BBD in conjunction with RSM. The optimized method is then validated and applied to determine phenolic compounds in different varieties of Roselle.

2. Material and Methods

2.1. Chemicals

Phenolic standards (analytical grade), including chlorogenic acid, protocatechuic acid, caffeic acid and rutin, HPLC-grade methanol, acetonitrile, and formic acid were purchased from Sigma (Madrid, Spain). Water was purified with a Mili-Q purification system (Millipore, Billerica, MA, USA).

2.2. Samples

Two samples of roselle were used in the study, namely red and pink roselle. Both flowers are obtained from local farmers in the dried form. Before the experiment, the dried roselle flower was ground into powder for 5 min, with 30 s break every 1-minute intervals. The powdered roselle was placed in a bottle sample and stored in a refrigerator at 4 °C.

2.3. Extraction of Phenolic Compounds

Microwave-assisted extraction (MAE) experiments were conducted in a MARS 6 240/50 (CEM, Matthew, NC, USA) equipped with the extraction vials, which are made of modified polytetrafluoroethylene (PTFE-TFM). Roselle powder was accurately weighed according to the experimental design to provide ratio sample to solvent 1:10, 1:15, and 1:20 and placed into an extraction vial. A precise volume of 15 mL with a different solvent composition (40, 70, and 100% MeOH in water) was used. The extraction was then conducted under the studied levels o temperature (30, 50, and 80 °C). Once the extraction was completed, the vessel was introduced to a 5 °C water bath for 30 s to allow it to cool down, reaching ambient temperature. Separation of the solid material and the solvent was conducted in centrifugation at 4000 rpm for 5 min. The extract was then adjusted to

its initial volume and filtered using a 0.22 μm nylon filter (Millipore) prior to injection in the UPLC–PDA system.

2.4. Identification and Quantification of Phenolic Compounds

The identification and quantification of individual phenolic compounds were conducted by chromatographic analysis using the ACQUITY UPLC H-Class system coupled to an ACQUITY UPLC Photodiode Array (PDA) detector. The system was controlled by EmpowerTM 3 Chromatography Data Software (Waters Corporation, Milford, MA, USA).

The PDA detector was set at the wavelength range 200–400 nm for the 3D scan, with a data collection rate of 40 pts s^{-1} to identify the compounds. A certain wavelength of 2D scans PDA detector (260, 280, and 320 nm), on the basis of the maximum absorbance wavelength with a data collection rate of 80 pts s^{-1} was used for compound quantification. For the purpose of optimization, 280 nm was chosen for peak integrations. A binary solvent system was used as the mobile phase. Solvent A was 0.01% acetic acid in water and solvent B was 2% acetic acid in acetonitrile. The analysis was run in 10 min using the following gradient program (%B): 0–0.3 min, 3.1–9.5%; 0.3–0.8 min, 9.5–15.6%; 0.8–5 min, 15.6–82.2%; 5–6 min, 82.2–100%; 6–10 min, 100-3.1%. The flow rate was set at 0.64 mL min^{-1} [22]. Phenolic compounds found in the Roselle extract were identified by comparing the retention time and spectra to those of standards. Additionally, a spiking procedure with corresponding standards was performed to confirm the identification.

2.5. Experimental Design and Statistical Analysis

Box-Behnken design (BBD) was used in this study to evaluate the effect of the independent variables on the extraction efficiency of microwave-assisted extraction (MAE). Response Surface Methodology (RSM) was then employed for optimization. The three-factor (x_1, temperature; x_2, solvent composition; and x_3, sample to solvent ratio) with the three-level design was performed (−1, 0, 1). Table 1 shows the range of the independent factors and their levels, while Table 2 presented the whole design, which consisted of 15 sets of experimental runs carried out in random orders. The response was the relative values with respect to the maximum response (%) of the total concentration of the studied compounds.

Table 1. Selected factors and their levels for Box–Behnken design.

Factors	−1	0	+1	Unit
x_1, temperature	30	55	80	°C
x_2, solvent composition	40	70	100	% methanol in water
x_3, solvent to sample ratio	1:10	1:15	1:20	g of sample:mL of solvent

Table 2. Box–Behnken design for three factors with their observed responses.

Run	x_1, Temperature	x_2, Solvent Composition	x_3, Solvent to Sample Ratio	Relative Values to the Maximum Response (%)
1	0	1	−1	45.08
2	0	−1	1	85.96
3	1	−1	0	88.98
4	−1	0	1	92.94
5	0	−1	−1	80.08
6	0	0	0	97.21
7	0	0	0	93.65
8	−1	−1	0	90.08
9	−1	0	−1	87.48
10	0	0	0	94.86
11	1	0	1	100.00
12	1	0	−1	85.15

13	−1	1	0	26.75
14	1	1	0	42.65
15	0	1	1	46.19

This approach was used to obtain the surface response by fitting the data to a polynomial model and also to evaluate the effects of each factor and the interaction effects between factors. If all factors are considered to be evaluated, the RSM can be expressed as follows:

$$y = f(x_1, x_2, x_3) \quad (1)$$

where y is the dependent factor while and x_1, x_2, and x_3 were independent factors (temperature, solvent composition, and solvent to sample ratio, respectively).

It is supposed that the x_i is continuous and controllable during the experiments. Since the objective was to optimize the response y, it was necessary to find the best estimation for the correlation between independent factors and the response surface. Generally, a second-order model is applied in RSM:

$$y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} {x_i}^2 + \sum_{i=1}^{k} \sum_{j=1, j \neq i}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

x_1, x_2, ..., x_k are the MAE factors that influence the extraction efficiency, y; β_o, β_{ii} (i = 1, 2, ..., k), β_{ij} (i = 1, 2, ..., k; j = 1, 2, ..., k) are unknown parameters, ε is a random error. The least-square method was used to estimate the β coefficients, while only second-order interactions were considered.

The construction and analysis of the experimental design and the response surface were performed using STATGRAPHICS Centurion XVI (Statpoint Technologies, Inc., USA) to reach the optimum conditions. This statistical tool utilized the quadratic model equation to build response surfaces. The Analysis ToolPak of an Excel of Microsoft Office Professional Plus 2013 was used to analyze the experimental data generated from single-factor experiments. The Analysis of Variance (ANOVA, p = 0.05) was used to determine the significance of the effect of studied variables. In the event that ANOVA suggested a significant difference, Least Significant Difference (LSD, p = 0.05) test was used to check the differences among the means.

2.6. Kinetic Study

The determination of the extraction rate of the studied phenolic compounds from Rosella was done by running the analysis using the optimum MAE condition over a period of time (5 to 30 min).

2.7. Performance of the Method

The validation of the analytical chromatographic method for the determination of phenolic compounds was performed based on the recommendations of ISO 17025 and ICH Guideline Q2 (R1) [23]. Linearity, the limit of detection and quantification of the method were assessed.

Linearity was evaluated to confirm that the method is appropriate over a specified interval of concentration. Five phenolic standards solutions were prepared by serial dilution for a concentration ranging from 0.5 to 10 μg L^{-1}. The limits of detection (LOD) and quantification (LOQ) of the chromatographic methods were 0.31 μg L^{-1} for protocatechuic acid, 0.62 μg L^{-1} for chlorogenic acid, 0.25 μg L^{-1} for caffeic acid, and 0.22 μg L^{-1} for rutin.

In regards to accuracy, as certified reference material (CRM) for phenolics in Rosella was not available, a specific statement cannot be made. Therefore, a spiking procedure was used to calculate extraction recovery (%R). Using the optimized MAE condition, extraction of Rosella samples with and without 1 mL of a mixture of the studied standard compounds were employed. The spiked mixture consists of chlorogenic acid, protocatechuic acid, caffeic acid, and rutin, in which the concentration of each compound was 100% of the concentration found in the extract. A comparison of the compounds level differences found in the spiked and non-spiked Roselle samples was made

with the level of corresponding compounds in the spiked solution to estimate the extraction recoveries.

Precisions were also calculated for the MAE method. The evaluation was conducted by repeating nine extractions on the same day for repeatability while three extractions in three consecutive days for intermediate precision. The precisions were expressed as the coefficient of variation (CV) of the responses.

3. Result and Discussion

3.1. Identification of Phenolic in Rosella

Prior to the method development, the type of phenolics presented in the Roselle sample was identified. For this purpose, a qualitative screening was performed by extracting 1 g Rosella in 10 mL of 50% MeOH. The extract was analyzed using UPLC-PDA system. The identification of phenolic compounds was conducted by comparing the retention time and the spectra found in the sample to the standard compounds. The result (Figure 1) revealed that Roselle contains chlorogenic acid and protocatechuic acid in which do not present in most edible flowers [24]. The other identified phenolic compounds in Roselle were caffeic acid and rutin.

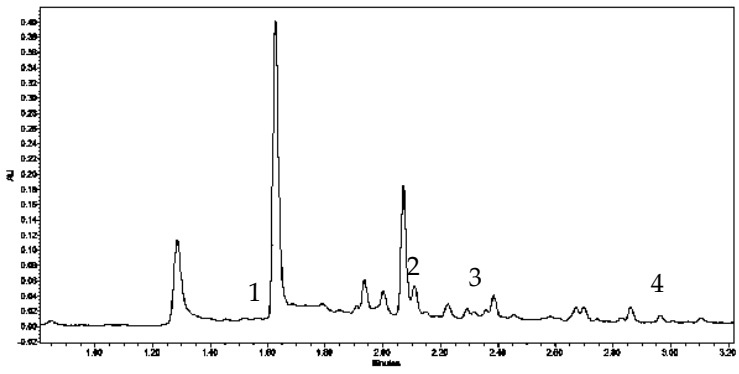

Figure 1. Chromatogram (280 nm) of the phenolic compounds presented in Roselle; 1: Protocatechuic acid, 2: Chlorogenic acid, 3: Cafffeic acid, 4: Rutin.

3.2. Effect of MAE Factors

The factors likely to affect the efficiency of microwave-assisted extraction (MAE), namely temperature (x_1, 30–80 °C), solvent composition (x_2, 50–100% MeOH in water), and ratio sample to solvent (x_3, 1:10–1:20 g mL^{-1}) were selected to be studied by BBD. The levels for the extraction variables were chosen based on previous information on literature working on the extraction of phenolic compounds from edible flowers [25]. In order to achieved an even response from the variables which have different range and unit, each of the variables were normalized and made into the range of −1 to +1. The responses showed in Table 2 were the relative values to the maximum concentration (%) of the studied compounds.

Based on the design, 15 experiments, including three center points, were performed to determine the effect contributed by each of the main variables and their combination to the extraction efficiency. The effect of the studied factors was evaluated using the analysis of variance (ANOVA). The mean square against an estimate of the experimental error was used to define the statistical significance of the effect of extraction factors. Pareto chart (Figure 2) was built, presenting the standardized effects of the main, interactions, and quadratic effects. Factors or combinations served as bars that cross the vertical line describe the ones to have a significant effect on the response ($p < 0.05$). Thus, four effects that consisted of two main effects (x_2 and x_3) and two combination effect (x_2x_2 and x_1x_2) with a p-value lower than 0.05 indicating that they were significantly influence the extraction recovery at the confidence level of 95%.

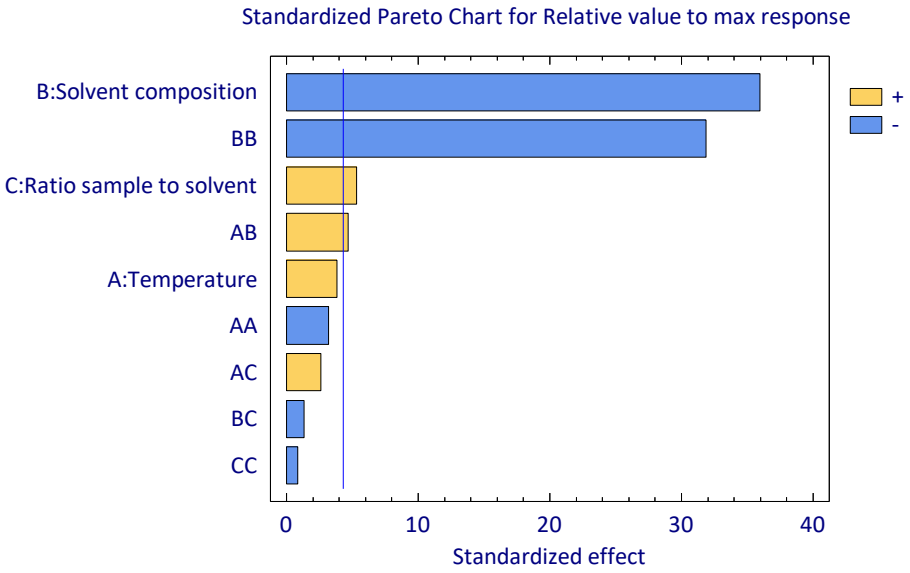

Figure 2. Standardized of main, interaction, and quadratic effects of MAE factors on the extraction yield.

As shown in Figure 1, solvent composition and its quadratic effect showed a negative effect in which increasing this factor will result in much lower extraction recovery. In contrast, a positive effect was found in the ratio sample to solvent, where an increase of recovery will be achieved by increasing the amount of the solvent in the mixture. The same trend is also found in the combination effect of temperature and solvent composition. Nonetheless, contribution by all factors was used to evaluate the fitting properties for the model. The second-order polynomial equation for the fitted model is:

$$y = 95.2378 + 2.43979x_1 - 23.0555x_2 + 3.4312x_3 - 3.02932x_1x_1 + 4.24956x_1x_2 + 2.34887x_1x_3 - 30.0947x_2x_2 - 1.19424x_2x_3 - 0.815874x_3x_3$$

where y is the extraction yield and x_i are the extraction factors (x_1, temperature; x_2, solvent composition; x_3, solvent to sample ratio).

The validity of the model was statistically evaluated by applying a lack-of-fit test. The test was carried out by calculating the variability of the current model residuals to the variability between observation at replicate settings for the factors. The p-value in ANOVA for the lack-of-fit test was 0.0506. Therefore the model was appeared to be adequate for the observed data at the confidence level of 95%. The statistical R^2 indicates that the model as fitted explains 97.60% of the variability in the response, with the standard deviation of the residuals to be 1.81. Whilst the mean absolute error of 3.03 is the average value of the residuals. Hence, the suggested model can be applied to estimate the optimum MAE factors to obtain the maximum extraction yield.

3.3. Optimization of MAE Condition

The objective of the optimization by RSM was to get the best combination between the three independent factors of the MAE, namely temperature (x_1), solvent composition (x_2), and ratio solvent to sample (x_3). Based on the predicted model, a three-dimensional mesh plot was constructed to predict the relationship between the independent factors and response. The highest response was obtained with the optimum MAE yield (103.54%) at the coordinate of 0.534496 for extraction temperature (x_1), −0.36511 for solvent composition (x_2), and 1 for ratio sample to solvent ($x3$). Based on the RSM (Figure 3), the optimum extraction recovery for the studied phenolic compound in Roselle can be achieved by applying 68 °C of extraction temperature using 59% methanol in water as the extraction solvent with a 1:20 ratio sample to solvent. Although the present study revealed similar

results as the previous one [26], the temperature used in the present study is much lower. This could guarantee better stability of the thermal labile phenolic compounds such as rutin [27]. It is therefore, the extraction yield reported here is superior to the previous studies. Additionally, the actual extraction technique could facilitate the higher ratio sample to solvent, allowing a lower amount of solute while increased the diffusion rate [28].

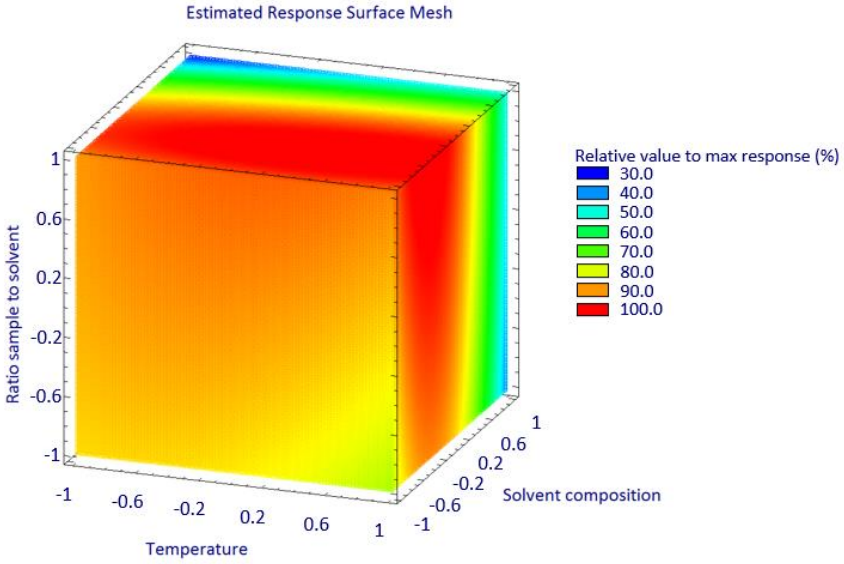

Figure 3. A 3D mesh of response plot for the studied MAE factors.

3.4. Kinetics Study

To achieved an efficient MAE for phenolic compounds in Roselle, extraction kinetics was performed by checking the extraction yield over a period of time. The extraction kinetics was evaluated by running extractions at the optimum MAE condition for 5 to 30 min. Afterward, the evaluation was conducted using the average of the relative value to the maximum yield of the extracted compounds (Figure 4). The ANOVA suggested that the extraction time significantly affected the extraction yield ($p < 0.05$). The proposed extraction time for phenolic compounds from Roselle was 15 min as within this time produced the highest yield (LSD, $p < 0.05$) compare to the longer extraction time. The decrease of the yield with longer extraction time might occur due to the degradation of the compounds or interaction between compounds as a result of microwave exposure [28,29].

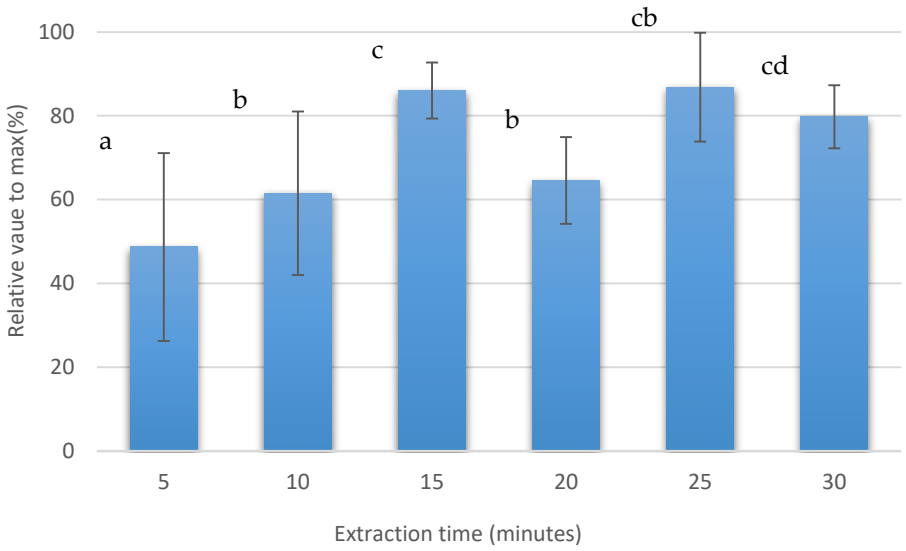

Figure 4. Extraction yield throughout the extraction time. The values followed by different letters are indicated as significantly different (p<0.05).

3.5. Method Validation

To check the reliability of the developed method, confirmation experiments were carried using the optimized MAE condition, viz, extraction temperature of 68 °C, 1:20 of ratio sample to solvent, 59% MeOH in water for the solvent, and extraction time of 15 min. Values for both precisions and accuracy were evaluated under the aforementioned optimized condition.

Two-level of precisions were studied, i.e., repeatability and intermediate precision. Although the values of coefficient of variation (*CV*) for the precisions were varied among the phenolic compounds, the data met the ICH requisite since the values were less than 10%. The recovery (%*R*) evaluation for indicating the accuracy was performed by comparing the concentration of both spiked samples to the concentration those of standards after the extraction procedure. The recoveries were between the acceptable values by the ICH, ranging from 92% (protocatechuic acid) to 119% (caffeic acid). The reported *CV* and *R* values indicate a high precision and accuracy of the developed method.

3.6. Real Sample Application

In order to assess the applicability, the developed and validated method was applied to extract real samples of two different Roselle varieties. The result showed that chlorogenic acid, the uncommon phenolic presented in flower, has the highest value in both samples, by 13.131 and 2.222 mg g^{-1} for red and pink Roselle, respectively. The total phenolic compounds in the darker colored Roselle was four times higher than the lighter one. This result was in accordance to the former study [30] which showed that darker colored Roselle contained much higher phenolic compounds compared to the less dark colored and white roselle in the early stage of maturity.

Table 3. The level of phenolic compounds in the studied Roselle samples.

Samples	Concentration (mg g^{-1})				
	Protocatechuic Acid	Chlorogenic Acid	Caffeic Acid	Rutin	Total
Red Roselle	0.130 ± 2.26	13.131 ± 1.83	0.152 ± 3.97	0.121 ± 0.51	13.534
Pink Roselle	0.034 ± 0.15	2.223 ± 1.71	0.533 ± 2.67	0.569 ± 3,30	3.361

4. Conclusions

A fast and reliable microwave-assisted extraction (MAE) for phenolic compound from Roselle was successfully optimized using BBD in conjunction with RSM. The optimum extraction yield can be achieved by applying the following MAE condition: temperature 68 °C, solvent composition 59% MeOH in water, ratio sample to solvent 1:20, and extraction time 15 min. The method validation reported a satisfactory result for precisions and accuracy to meet the ICH standard. Finally, the method was successfully identified and quantified four phenolic compounds in two varieties of Roselle with chlorogenic acid (13.131 mg g^{-1}) as the major phenolic in the sample.

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