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Recovery of Anthocyanins from *Hibiscus sabdariffa* L. Using a Combination of Supercritical Carbon Dioxide Extraction and Subcritical Water Extraction

Dwila Nur Rizkiyah [1], Nicky Rahmana Putra [1], Zuhaili Idham [1], Ahmad Hazim Abdul Aziz [2,*], Mohd Azizi Che Yunus [1,*], Ibham Veza [3], Irianto [4], Stanley Chinedu Mamah [5] and Lailatul Qomariyah [6,*]

1 Centre of Lipid Engineering and Applied Research (CLEAR), Ibnu Sina Institute for Scientific and Industrial Research, Universiti Teknologi Malaysia, UTM Johor Bahru Campus, Johor Bahru 81310, Malaysia
2 Faculty of Food Science and Nutrition, Universiti Malaysia Sabah, Kota Kinabalu 88400, Malaysia
3 Department of Mechanical Engineering, Universiti Teknologi PETRONAS, Seri Iskandar 32610, Malaysia
4 Department General Education, Faculty of Resillience, Rabdan Academy, Abu Dhabi P.O. Box 114646, United Arab Emirates
5 Chemical Engineering Department, Alex Ekwueme Federal University Ndufu-Alike, Abakaliki P.O. Box 1010, Ebonyi State, Nigeria
6 Department of Industrial Chemical Engineering, Institut Teknologi Sepuluh Nopember, Surabaya 60111, Indonesia
* Correspondence: hazim.aziz@ums.edu.my (A.H.A.A.); azizi@cheme.utm.my (M.A.C.Y.); laila.qomariyah@its.ac.id (L.Q.)

Abstract: Anthocyanins are one of the bioactive compounds in roselle that has many medicinal proposes. Anthocyanins are placed in the inner part of the roselle; therefore, combinations of two methods were applied to extract the anthocyanins. The first stage is employing supercritical carbon dioxide ($ScCO_2$) to break the particle surface or outer layer of the roselle based on the total phenolic compounds (TPC) recovery, and the second step was to apply subcritical water extraction (SWE) for the extraction of anthocyanins. The objective is to determine the best conditions to obtain high yields of total anthocyanins compounds (TAC) from the roselle (*Hibiscus sabdariffa*) by employing a combination of $ScCO_2$ and SWE. The optimal conditions of $ScCO_2$ (first stage) were 19.13 MPa, 60 °C, and 4.31 mL/min, yielding 18.20%, and 80.34 mg/100 g TPC, respectively. The optimum conditions of SWE (second stage) were 9.48 MPa, 137 °C, and 6.14 mL/min, yielding 86.11% and 1224.61 mg/100 g TAC, respectively. The application of integrated $ScCO_2$ and SWE proved successful in achieving high anthocyanins production and yield as compared to previous extraction methods. This approach may be used to extract the roselle with a greater anthocyanin's concentration than the prior method.

Keywords: roselle; anthocyanins; supercritical carbon dioxide; subcritical water; optimization

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1. Introduction

Roselle (*Hibiscus sabdariffa* L.) contains anthocyanins that are used to treat fever, liver, hypertension, and leukaemia [1]. Furthermore, a range of medicinal applications for roselle has been investigated worldwide [2]. Currently, fruit juice, drinks, and jam are popular products made from roselle. Roselle is often used in the production of jam and fruit juices in a few industries owing to their unusual flavour and colour, and their abundance of beneficial chemicals, particularly anthocyanins [3–5].

Anthocyanins are water-soluble pigments that can be found in most vascular plants, and a subgroup of large secondary plant substances called flavonoids. Anthocyanins have been revealed as one of the most promising ingredients in the food, beverage, cosmetics, and nutraceutical industries. This bioactive compound can improve sight acuteness, antioxidant capacity, control Type II diabetes, reduce the risk of coronary heart disease, and

prevent cancer development [6,7]. Anthocyanins as high polar compounds can be extracted from fruits, vegetables, flowers, leaves, stems, and roots. The compounds are found predominantly in inner cell layers, such as the epidermis and peripheral mesophyll cells [8,9].

$ScCO_2$ extraction is often used to extract anthocyanins compounds from roselle due to being an environmentally friendly solvent [10]. Abdul Aziz et al. Abdul Aziz, et al. [11] reported that $ScCO_2$ extraction provides high quality extraction of anthocyanins compounds that are safe for health and wellness products. Therefore, this method becomes an alternative extraction process instead of conventional extraction [12,13]. However, pure $ScCO_2$ cannot be applied to extract the anthocyanins in the inner part of the roselle due to different polarity between carbon dioxide and anthocyanins [14–20]. Commonly, ethanol is applied as an entrainer/modifier for $ScCO_2$ to extract the anthocyanins, where ethanol can break the particle surface roselle. According to the United Nations' sustainable development goals for good health and well-being, ethanol is classified as generally recognize as safe (GRAS) solvent. However, a higher amount of ethanol in the system will contribute to the solvent-solvent interactions, thus decreasing the recovery [21].

To overcome this problem, the combination of $ScCO_2$ and SWE can be applied to extract anthocyanins from roselle. First, the roselle was extracted by supercritical carbon dioxide in order to break and open the surface pore of roselle. Bound phenolic compounds contributing to cell wall formation are then conjugated with cell wall macromolecules, such as cellulose and protein via ester and glycosidic bonds [22]. Therefore, the TPC analysis is applied to determine the $ScCO_2$ extraction breaking particle cell wall of the roselle. After the roselle was extracted by $ScCO_2$ to open and break the surface pore, the raw material of the roselle is extracted by subcritical water.

The objective of this study is to establish the optimal conditions for recovering significant yields of total anthocyanins compounds (TAC) from roselle using $ScCO_2$ and SWE.

2. Materials and Methods

2.1. Roselle Preparation

Dried roselle (moisture content < 8%) was obtained from Ekomekar Resources, Terengganu. Furthermore, it was ground and sieved for a particle size of $355 < dp < 425$ µm.

2.2. Chemicals Used

Gallic acid, ethanol, KCL, Na_2SO_4, Na_2CO_3, and Folin–Ciocalteu were bought from Sigma-Aldrich, Schnelldorf, Germany. Distilled water is provided by CLEAR lab, UTM Malaysia.

2.3. Combination $ScCO_2$ and SWE to Extract the Anthocyanins from Roselle

In this work, a two-stage extraction including $ScCO_2$ (first stage), and SWE (second stage) was used. A Box–Behnken design (Version 13.0.4, Stat-Ease Corporation, Minneapolis, MN, USA) was developed to analyse the effects as shown in Table 1. The model, which includes linear and quadratic variables and interaction terms, was used to suit the polynomial equations of second order based on the experimental data as follows:

$$Y = B_0 + \sum_{i=1}^{k} B_i X_i + \sum_i \sum_j B_{ij} X_i X_j + \sum_{i=1}^{k} B_{ii} X_i^2 \quad (1)$$

where:

Y is an investigated response; B_0 is constant; B_i, B_{ii}, and B_{ij} are coefficients of linear, quadratic, and interaction terms, respectively; and X_i and X_j are independent.

The parameters of the $ScCO_2$ extraction were pressure (10 MPa to 20 MPa), flowrate (3 mL/min to 7 mL/min), and temperature (40 °C to 60 °C), and the responses were the total yield and TPC. The parameters of SWE were pressure (4 MPa to 12 MPa), temperature (100 °C to 140 °C), and water flow rate (4 mL/min to 8 mL/min) with the responses of global yield and TAC.

Table 1. The variables and responses of a combination of $ScCO_2$ extraction and SWE.

$ScCO_2$ Extraction						SWE					
Run	Variables			Responses		Run	Variables			Responses	
	Pressure, A (MPa)	Temp., B (°C)	Flow Rate, C (mL/min)	Yield, Y_1 (%)	TPC, Y_2 (mg/100 g)		Pressure, A (MPa)	Temp., B (°C)	Flow Rate, C (mL/min)	Yield, Y_1 (%)	TAC, Y_2 (mg/100 g)
1	15	60	5	13.86 ± 0.13	73.57 ± 1.25	18	4	100	6	68.3 ± 1.2	832.67 ± 3.21
2	10	50	3	4.62 ± 0.05	77.14 ± 1.23	19	8	140	8	85.24 ± 1.34	1224.76 ± 3.63
3	15	50	4	8.2 ± 0.12	85.85 ± 1.57	20	8	120	6	79.9 ± 1.42	984.43 ± 4.32
4	15	40	5	6.44 ± 0.11	85.91 ± 1.63	21	4	120	4	68.3 ± 1.3	939.41 ± 6.67
5	20	50	5	18.2 ± 0.12	81.91 ± 2.1	22	8	120	6	79.8 ± 1.2	1032.79 ± 2.43
6	15	50	4	7.47 ± 0.02	86.76 ± 2.34	23	12	100	6	58.88 ± 1.1	1108.27 ± 2.64
7	15	50	4	5.22 ± 0.1	88.19 ± 3.1	24	8	100	4	48.24 ± 0.93	906.55 ± 3.56
8	10	60	4	11.46 ± 0.12	96 ± 4.67	25	8	120	6	78.6 ± 0.84	995.65 ± 3.72
9	20	50	3	9.99 ± 0.03	76.91 ± 2.1	26	8	140	4	66.85 ± 0.93	912.29 ± 3.45
10	15	60	4	10.26 ± 0.21	82 ± 3.54	27	8	120	6	77.9 ± 1.3	991.78 ± 5.32
11	10	40	3	7.85 ± 0.13	75.71 ± 2.32	28	8	120	6	77.4 ± 1.21	992.98 ± 6.32
12	10	40	4	3.67 ± 0.02	82.1 ± 2.13	29	12	120	8	68.03 ± 0.87	1041.08 ± 2.98
13	20	40	4	10.06 ± 0.13	89.86 ± 3.02	30	4	120	8	35.91 ± 0.32	883.84 ± 2.56
14	10	50	5	4.37 ± 0.01	71.43 ± 1.54	31	12	120	4	64.85 ± 1.23	1040.67 ± 2.74
15	15	50	4	3.06 ± 0.01	82.95 ± 2.59	32	12	140	6	77.47 ± 1.87	1046.42 ± 2
16	15	50	4	7.41 ± 0.04	83.38 ± 2.56	33	4	140	6	59.47 ± 0.78	1015.96 ± 1.32
17	15	60	3	7.33 ± 0.03	80.29 ± 1.96	34	8	100	8	49.16 ± 0.56	789.92 ± 2.56

2.3.1. $ScCO_2$ Extraction (First Stage)

The apparatus includes a 5 mL extraction vessel, a CO_2 chiller, a CO_2 pump (Supercritical 24, Japan), an ethanol pump, a back pressure regulator with the heater (Jasco BP 2080, Japan), and an oven. The schematic design of $ScCO_2$ extraction utilizing ethanol as a co-solvent is shown in our previous study [3]. Roselle powder (3 ± 0.005 g) was inserted to the extraction vessel. The chiller's temperature was set to 6 °C, and the extraction time was set to 60 min. The back-pressure regulator's heater was set to 50 °C. After pump CO_2 worked, 0.24 mL/min of ethanol was pumped into the system (V_{Etoh}/V_{CO2}). The pressure was regulated based on the back pressure regulator, flowrate was controlled by a CO_2 pump and temperature was controlled by an oven.

2.3.2. SWE (Second Stage)

The system of SWE comprises of 5 mL extraction vessel, water pump, oven, and back pressure regulator (Solon, OH, USA). The schematic setup of SWE is published in our previous study [3,23]. The residue of roselle (200 ± 5 mg) from $ScCO_2$ was placed into an extraction vessel. the extraction time was set to 5 min. The pressure was regulated based on the back pressure regulator, the flowrate was controlled by a water pump, and the temperature was controlled by an oven.

2.4. TPC Analysis

The TPC analysis is followed by Rizkiyah, Jusoh, Idham, Putra, and Che Yunus Rizkiyah, Jusoh, Idham, Putra and Che Yunus [3]. The extract (1 mg) was added to 1 mL of ethanol and 5 mL of Folin–Ciocalteu solution (5 mL of Folin–Ciocalteu reagent + 50 mL of distilled water). After that, 2 mL of Na_2CO_3 solution (3 g of Na_2CO_3 + 100 mL of water) was added and rested for 30 min. The absorbance of the spectrophotometer UV-Vis (Jasco, Hachioji-shi, Japan) was 760 nm. The TPC was reported as mg gallic acid equivalents/100 g extract.

2.5. TAC Analysis

A pH differential technique was used to analyse the TAC and followed by Idham, Putra, Aziz, Zaini, Rasidek, Mili, and Yunus Idham, Putra, Aziz, Zaini, Rasidek, Mili and Yunus [14], and Rizkiyah, Putra, Idham, Che Yunus, Veza, Harny, Syahlani, and Abdul Aziz Rizkiyah, Putra, Idham, Che Yunus, Veza, Harny, Syahlani and Abdul Aziz [23]. Two dilutions of the same material were prepared using KCL (0.025 M) and Na_2SO_4 solutions (0.4 M), respectively. Both solutions were adjusted to pH 1.0 and 4.5, respectively, using hydrochloric acid. The absorbance used was 520 and 700 nm. The TAC was calculated as mg cyanidin-3-glucoside/100 g of dry roselle, as shown in Equation (3).

$$A = (A_{520} - A_{700})_{pH1{\cdot}0} - (A_{520} - A_{700})_{pH4{\cdot}5} \quad (2)$$

$$TAC\ (mg/L): A \times MW \times DF \times 1000/\varepsilon \times L \quad (3)$$

A is absorbance, MW is the molecular weight of cyanidin 3-glucoside (449.2 g/mol), DF is the dilution factor, ε is the cyanidin 3-glucoside extinction coefficient (26,900 L/(cm*mol)), and L is the cell path length (1 cm). TAC (mg/L) is transformed to mg of roselle extract per 100 g of dried roselle.

2.6. Characterization of Roselle Surface before and after $ScCO_2$ Extraction

Low vacuum scanning electron microscopy was used to examine the morphology of the roselle before and after $ScCO_2$ extraction (JEOL JSM-6390LV). Platinum (PT) was applied using an auto fine coater to the samples for this investigation (JEOL JFC-1600).

3. Results and Discussion

A dynamic extraction process combining integrated $ScCO_2$ and SWE was developed to recover anthocyanins from the roselle. The use of $ScCO_2$ extraction (first stage) as a pre-treatment technique was utilized to crack the surface pores of the roselle, followed

by the use of SWE (second stage) to extract the anthocyanins. RSM was used to optimize the pore-breaking procedure and anthocyanins recovery. Table 1 shows the design of the experiment for a combination of $ScCO_2$ extraction and SWE. Tables 2 and 3 provide the ANOVA for all response models for $ScCO_2$ and SWE, respectively. The results of optimizing conditions of $ScCO_2$ and SWE are shown in Table 4.

Table 2. ANOVA table and coefficient of models for the response of yield for $ScCO_2$ extraction.

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value	Sum of Squares	df	Mean Square	F-Value	*p*-Value
			Yield extract					TPC		
Model	211.42	9	23.49	5.38	0.02	517.89	9	57.54	3.78	0.04
A-Pressure	84.4	1	84.4	19.31	0.003	0.07	1	0.07	0.0046	0.94
B-Temperature	41.99	1	41.99	9.61	0.02	5.15	1	5.15	0.33	0.57
C-Flow rate	24.13	1	24.13	5.52	0.05	0	1	0.07	0.0047	0.94
AB	0.28	1	0.28	0.07	0.8	55.39	1	55.39	3.64	0.09
AC	24.56	1	24.56	5.62	0.05	41.3	1	41.3	2.71	0.14
BC	6.63	1	6.63	1.52	0.25	41.59	1	41.59	2.73	0.14
A^2	21.47	1	21.47	4.91	0.06	0.37	1	0.37	0.02	0.88
B^2	12.04	1	12.04	2.76	0.14	10.42	1	10.42	0.68	0.43
C^2	0.31	1	0.3126	0.07	0.79	180.31	1	180.31	11.85	0.01
Residual	30.59	7	4.37			106.5	7	15.21		
Lack of Fit	12.72	3	4.24	0.94	0.49	86.59	3	28.86	5.8	0.06
Pure Error	17.87	4	4.47			19.91	4	4.98		
Cor Total	242.01	16				624.39	16			
Std. Dev.			2.09					3.9		
Mean			8.2					82.35		
C.V. %			25.48					4.74		
R^2			0.87					0.83		

Table 3. ANOVA table for the response of yield for SWE.

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value	Sum of Squares	df	Mean Square	F-Value	*p*-Value
			Yield extract					TAC		
Model	2298.86	9	255.43	2.97	0.082	148,800	9	16,536	5.18	0.02
A-Pressure	173.61	1	173.61	2.02	0.19	39,840	1	39,840	12.48	0.01
B-Temperature	519.35	1	519.35	6.04	0.04	39,482	1	39,482	12.37	0.01
C-Flow rate	12.29	1	12.29	0.14	0.71	2473	1	2473	0.77	0.41
AB	187.96	1	187.96	2.19	0.18	15,023	1	15,023	4.71	0.06
AC	316.2	1	316.2	3.68	0.09	783	1	783	0.24	0.63
BC	76.23	1	76.23	0.89	0.37	46,029	1	46,029	14.42	0.01
A^2	262.36	1	262.36	3.05	0.12	386	1	386	0.12	0.73
B^2	96.74	1	96.74	1.12	0.32	288	1	288	0.02	0.77
C^2	561.91	1	561.91	6.53	0.04	4547	1	4547	1.42	0.27
Residual	602.09	7	86.01			22,342	7	3191		

Table 3. *Cont.*

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value	Sum of Squares	df	Mean Square	F-Value	*p*-Value
Lack of Fit	597.1	3	199.03	159.61	0.0001	20,890	3	6963	19.18	0.01
Pure Error	4.99	4	1.25			1452	4	363		
Cor Total	2900.94	16	255.43	2.97	0.082	171,200	16			
Std. Dev.			9.27					56.5		
Mean			67.31					984.68		
C.V. %			13.78					5.74		
R^2			0.8					0.87		

Table 4. The multiple responses optimization for combination $ScCO_2$ extraction and SWE.

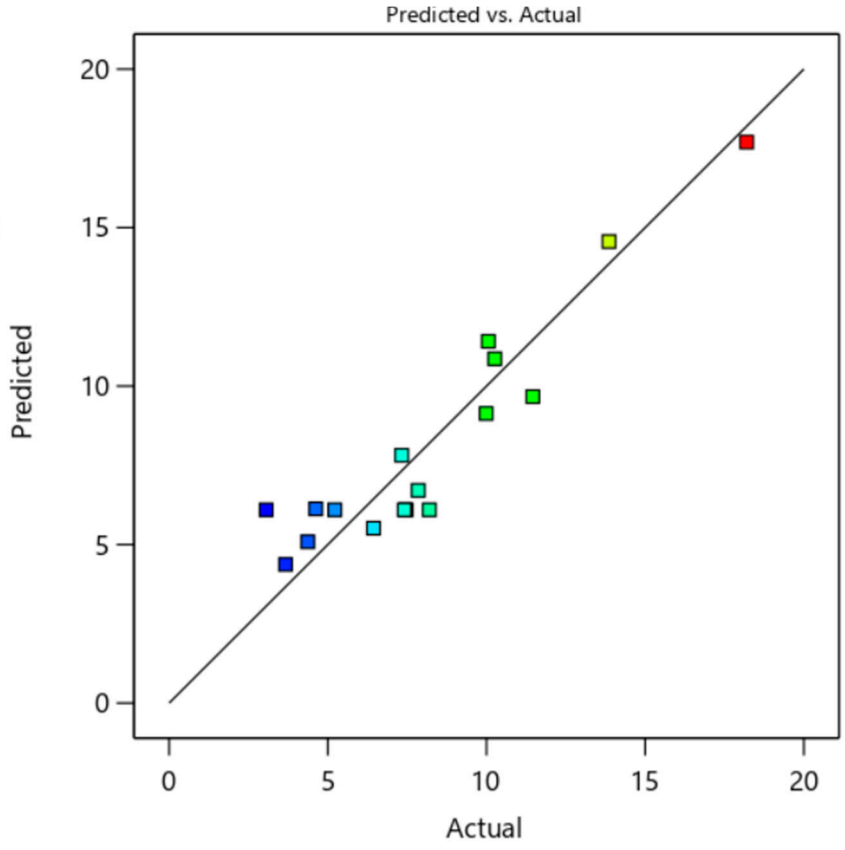
SC-CO_2 Extraction							
Parameters	**Set Goal**	**Values**	**Responses**	**Set Goal**	**Predicted**	**Observed**	**Error (%)**
Pressure, MPa	In range	19.13	Yield extract, %	Maximum	18.2	20.34	3.64
Temperature, °C		60	TPC, mg/100 g	Maximum	80.34	75.40	2.84
Flow rate, mL/min		4.31					
SWE							
Parameters	**Set Goal**	**Values**	**Responses**	**Set Goal**	**Predicted**	**Observed**	**Error (%)**
Pressure, MPa	In range	9.48	Yield extract, %	Maximum	86.11	83.23	3.34
Temperature, °C		137	TAC, mg/100 g	Maximum	1224.61	1144.21	6.55
Flow rate, mL/min		6.14					

3.1. Effect of Process Parameters on Yield for $ScCO_2$ Extraction

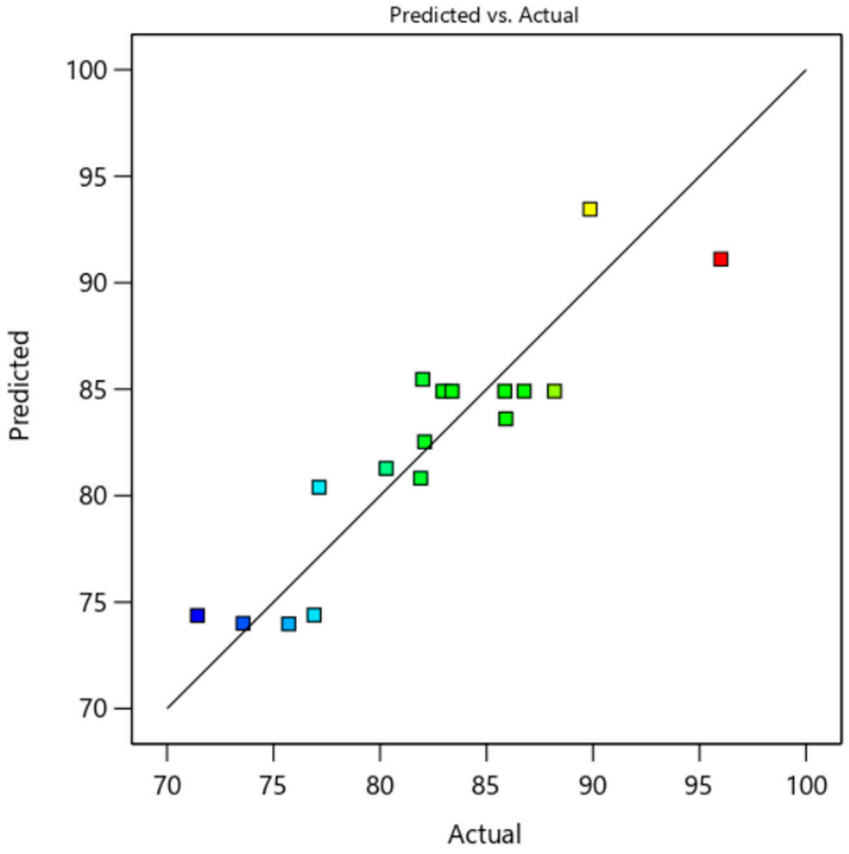
The extraction parameters were conducted based on preliminary data, a review of prior research, and the capabilities of the $ScCO_2$ system. Maximum pressure was set at 20 MPa based on the pressure limitation of the system. In addition, the highest temperature was set at 60 °C due to the prevention of the anthocyanin's degradations [15,24,25]. In addition, the flow rate was restricted to 5 mL/min to prevent the channelling process and the short residence time [26]. Table 2 shows an ANOVA table for the evaluation the quadratic effect, interactions, and coefficient factors. The quadratic model fits the experimental data satisfactorily (p-value < 0.05 and R^2 > 0.8). Hence, the quadratic model accurately analysed the correlation between the responses and parameters. As shown in Figure 1, the experimental data were correlated by the quadratic model data significantly (R^2 > 0.8). The equation model between parameters and total yield is shown in Equation (4).

$$Y_1 = 6.10 + 3.90A + 3.03B + 1.88C + 0.38AB + 2.40AC + 1.49BC + 3.09A^2 + 1.74B^2 + 0.32C^2 \quad (4)$$

As demonstrated in Figure 2a, increasing the pressure (10 to 20 MPa) at the constant flow rate (4 mL/min) increased the total yield. This is because the higher-pressure condition enhances the density of $ScCO_2$ [27]. The compound's solubility and solvent diffusivity were increased by enhancement of density [28,29]. According to Da Porto, Decorti, and Natolino Da Porto, Decorti and Natolino [21], higher pressure conditions (>20 MPa) significantly improved the total yield. On the other hand, this condition will increase the safety cost of the instrument during the extraction process.



(**a**)



(**b**)

Figure 1. Predicted vs. Actual responses: (**a**) Yield, and (**b**) TPC for $ScCO_2$ extraction.

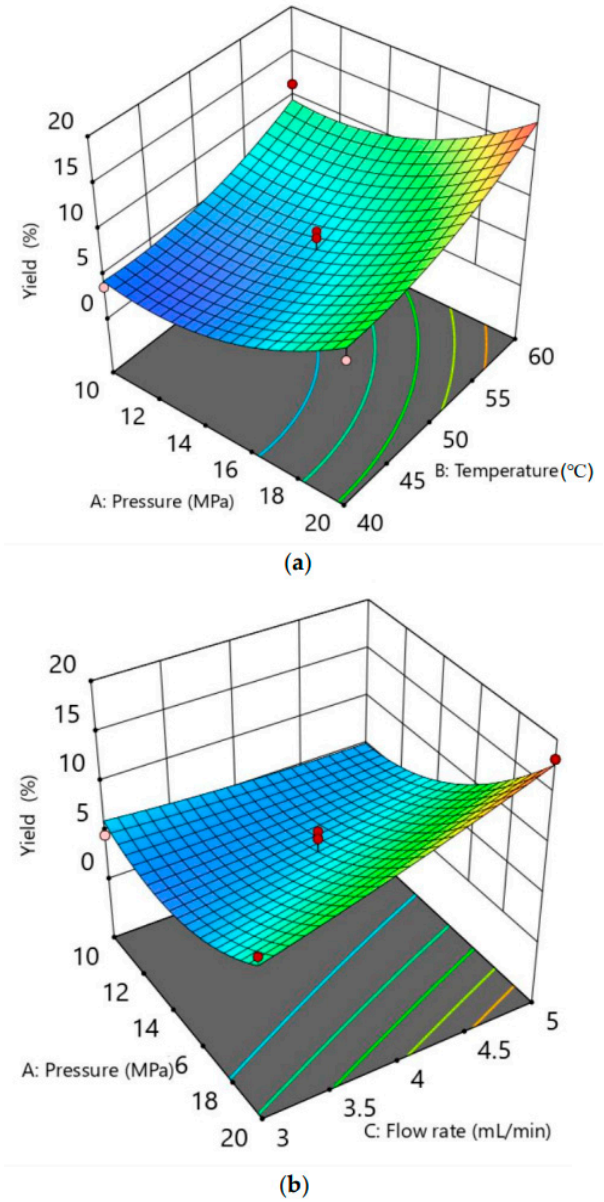


Figure 2. The effect of $ScCO_2$'s variables on total yield, % (**a**) effect of pressure and temperature at constant flow rate; (**b**) effect of pressure and flow rate at constant temperature.

Increasing the temperature from 40 °C to 60 °C at a constant flow rate (4 mL/min) and pressure (10 and 20 MPa) increases the total yield. The pattern is caused by the vapour solute condition as the temperature rise [30]. At a constant pressure of 20 MPa and a flow rate of 4 mL/min, raising the temperature from 40 °C to 60 °C reduces the total yield. As the density of solvents decreased with rising temperature, thus increased CO_2 and ethanol's solvation power. Temperature's influence on the total yield was more challenging to predict than pressures because of its two opposing effects on extract yield. At constant pressure, increasing the temperature reduces the density of the solvent and, as a result, its solvation power. In contrast, increasing the temperature raises the vapour pressure of the solutes, increasing their solubility in the supercritical solvent [31,32]. Furthermore, high temperatures may hasten mass transfer. Therefore, depending on whether the solvent density or the solute vapour pressure predominated, a temperature increase could have either a positive or negative effect [33].

The effect of flow rate is not significant, according to the *p*-value in Table 2 (*p*-value > 0.05). As a result, the flow rate does not affect the total yield extract. Figure 2b shows that

increasing the flow rate from 3 mL/min to 5 mL/min at a constant pressure of 10 MPa and 20 MPa slightly increases the yield extract. Flow rate is a mechanical factor in $ScCO_2$ extraction, but it does not affect the CO_2 density. Due to the low polarity of CO_2, most of the bioactive compounds extracted by Sc CO_2 were non-polar compounds [11]. The mass transfer resistance limited the amount of extract transported into the bulk of the solvent at higher flow rate conditions. In contrast, the CO_2 flow rate of 5 mL/min led to a reduced residence time of CO_2 in the extraction vessel. In both cases, the CO_2 left the extraction vessel unsaturated, decreasing the extract [26].

Most of the bioactive compound extracted by $ScCO_2$ was non-polar compounds due to low polarity of CO_2. Therefore, the competing effect of solute in the solvent can be reduced in subcritical water extraction (second step), thus the recovery of anthocyanins (polar compounds) can be enhanced. In this experiment, TPC recovery was more significant than total yield because TPC is associated with the rupture of surface pores in the roselle. Greater TPC recovery suggests that roselle pores are more damaged.

3.2. Effects of Parameters on the Responses of TPC for $ScCO_2$ Extraction

Table 2 shows that the quadratic model fits the experimental data ($R^2 > 0.8$). According to Table 2, the quadratic coefficients of flowrate had a substantial effect on the TPC to break and open the pores of roselle. The correlation of quadratic model between parameters and TPC is shown in Equation (5):

$$Y_2 = 84.91 + 0.11A - 1.06B + 0.1C - 5.35AB + 3.11AC - 3.74BC + 0.41A^2 + 1.62B^2 - 7.82C^2 \quad (5)$$

Figure 3a shows that increasing the pressure from 10 MPa to 30 MPa improves TPC recovery at a constant temperature of 40 °C and flow rate of 4 mL/min. Pressure increases the density of CO_2, whereas the high density of CO_2 contributes to increased solvent diffusivity [34]. Therefore, the solvation power of CO_2 can be increased to break the particle cell wall of the roselle. Increasing the extraction pressure causes an increase in fluid density, which increases the supercritical fluid's solvent power, improving its ability to break the cell wall and dissolve the phenolic compounds present in the matrix [35]. Higher phenolic compound recovery indicated that $ScCO_2$ extraction had already ruptured most of the particle cell wall.

At a fixed temperature of 60 °C and flow rate of 4 mL/min, increasing the pressure from 10 MPa to 30 MPa reduces TPC recovery. The roselle will be compacted within the extraction wall due to the high pressure. The limited mass contact area can reduce mass transfer between solvent and solute [36]. Therefore, based on the low concentration of TPC recovery, the penetration power of CO_2 is insufficient to breach the particle cell wall of the roselle.

Figure 3b shows that increasing flowrate 3 mL/min to 4 mL/min increase the TPC recovery, on the other hand the recovery of TPC was decrease at flow rate 4 mL/min to 5 mL/min. The mass transfer resistance limited the amount of phenolic transported into the bulk of the solvent whereas at CO_2 flowrate of 5 mL/min led to a reduced residence time of CO_2 in the extraction vessel. Therefore, the residence time of CO_2 was not enough to break and open the surface pore of roselle [11]. Bai et al. Bai, et al. [37] found that Increasing flow rate may improve the heat transfer performance of $ScCO_2$ Moreover, at the same injured area, the temperature of the fracture wall was always greater than that of $ScCO_2$; comparable to the behaviour of water, and the total heat transfer capacity of $ScCO_2$ increased as the injection flow rate increased. Therefore, the residence duration of CO_2 is more important than pressure and temperature in breaking and opening the pores of the roselle.

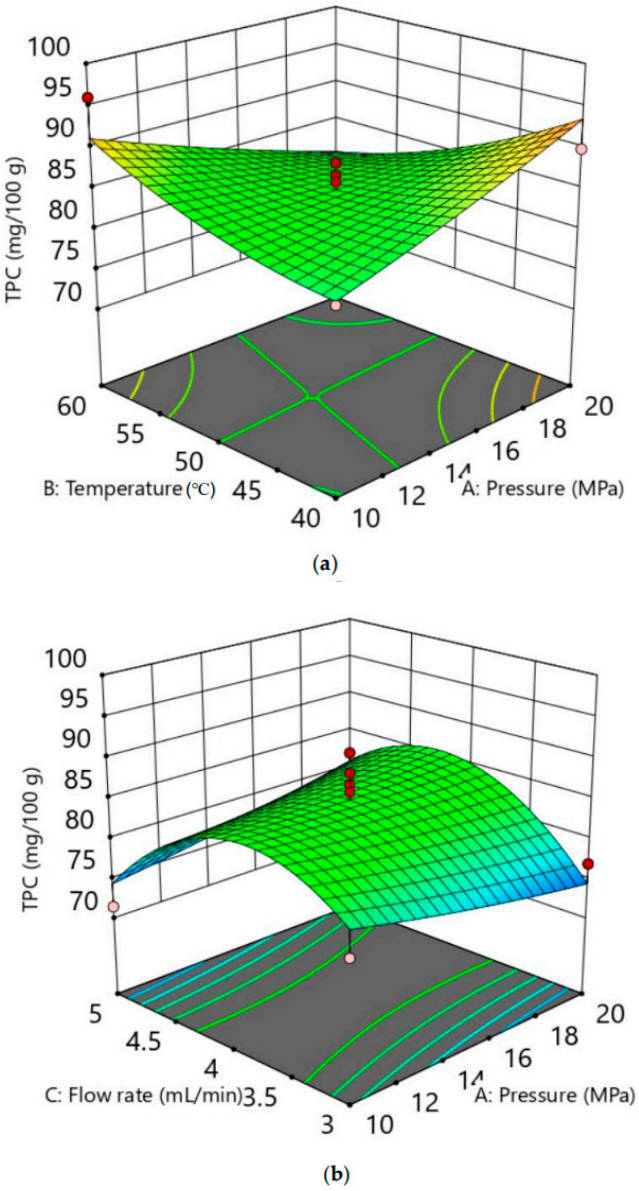


Figure 3. The effect of $ScCO_2$'s variables on TPC, mg/100 g, (**a**) effect of pressure and temperature at constant flow rate; (**b**) effect of pressure and flow rate at constant temperature.

3.3. Morphology of Roselle before and after $ScCO_2$ Extraction

The residue of the $ScCO_2$ extraction at optimum condition (20 MPa, 40 °C, and 4.875 mL/min) was analysed in order to validate the breaking surface pore of roselle as preliminary process; whereas the particle of roselle was extracted by $ScCO_2$ at optimum condition at. Figure 4a clearly shows that before the roselle was extracted there was no brokage of the cell wall or shrinkage of the cell. Meanwhile, there is obvious shrinkage and brokage of the cell walls after ScCO2 extraction in Figure 4b. Therefore, $ScCO_2$ is successfully broke and opened the roselle pore.

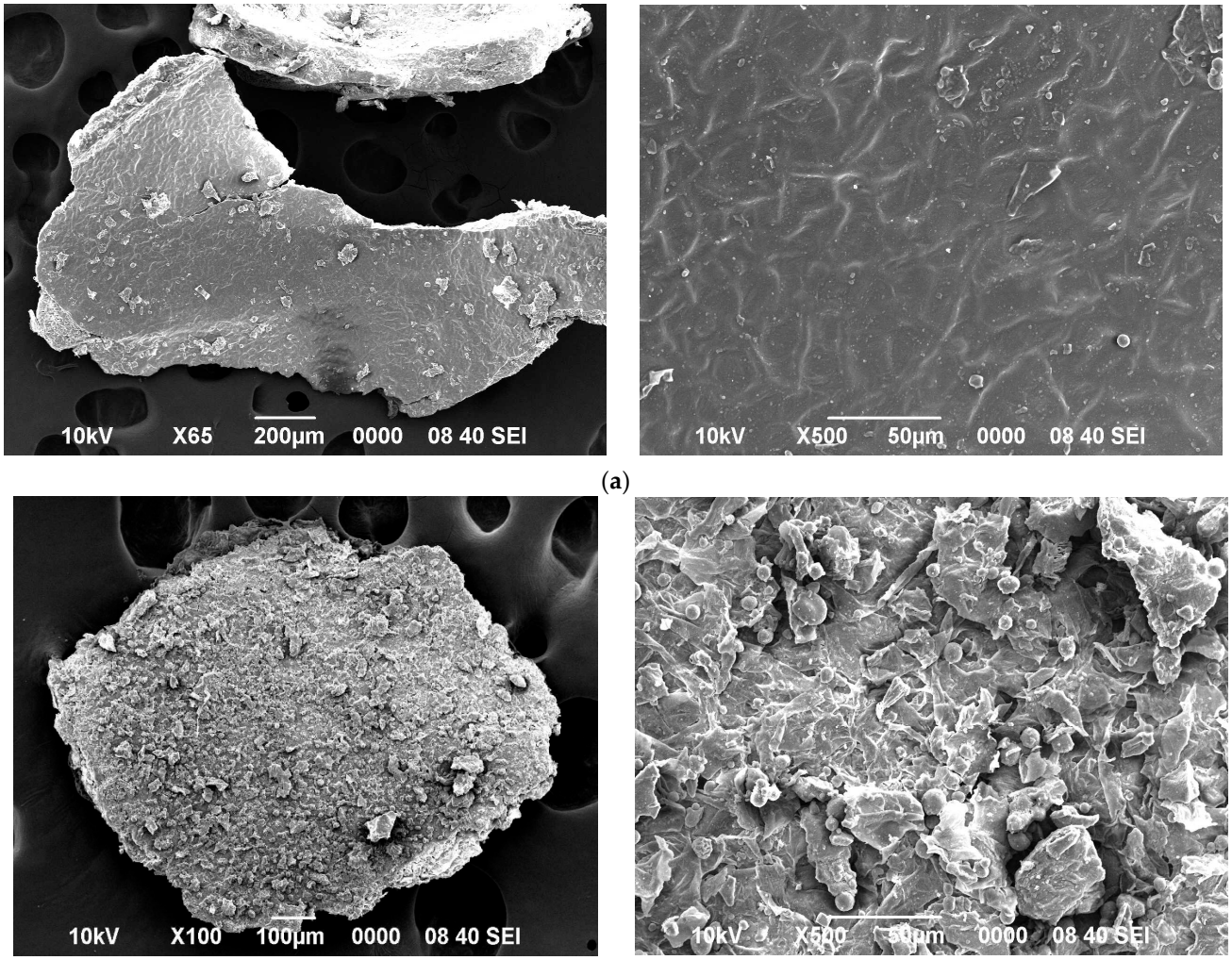


Figure 4. SEM Micrograph of roselle before (**a**) and after (**b**) $ScCO_2$ extraction.

3.4. Effect of Process Parameters on Total Yield for SWE

The parametric experiments were conducted based on preliminary data, a review of prior research, and the capabilities of the SWE system. A maximum operating pressure of 12 MPa has been stipulated per the specifications. In addition, the maximum temperature was adjusted at 140 °C to prevent the destruction of anthocyanins during the brief extraction period [3]. In addition, the flow rate of solvents was restricted to 8 mL/min to prevent the solvent channelling effect and decrease the residence time [38]. Figure 5 depicts the actual and predicted yield and TAC responses for SWE.

The ANOVA Table, as shown in Table 3, was done to analyse the quadratic effect, interactions, and coefficients of the treatment factors on the response variables. According to Table 3, the linear coefficients of temperature (B), quadratic flow rate (C^2), and interaction of pressure and flowrate (AC) had a significant ($p < 0.1$) impact on the total yield of SWE. The quadratic model fits the experimental data ($R^2 > 0.80$ and p-value < 0.1). The quadratic model between process variables and total yield is revealed in Equation (6).

$$Y_1 = 78.72 + 4.66A + 8.06B - 1.24C + 6.85AB + 8.89AC + 4.37BC - 7.89A^2 - 4.79B^2 - 11.55C^2 \quad (6)$$

Based on the p-value of the ANOVA table, the linear temperature significantly impacts yield recovery more than any other variable. As a result, the effect of pressure and flow rate on yield recovery is not dominant. Due to the high p-value, the interaction temperature

and flow rate variables are excluded. A high coefficient *p*-value improves the *p*-value of the quadratic model that fits the experimental data.

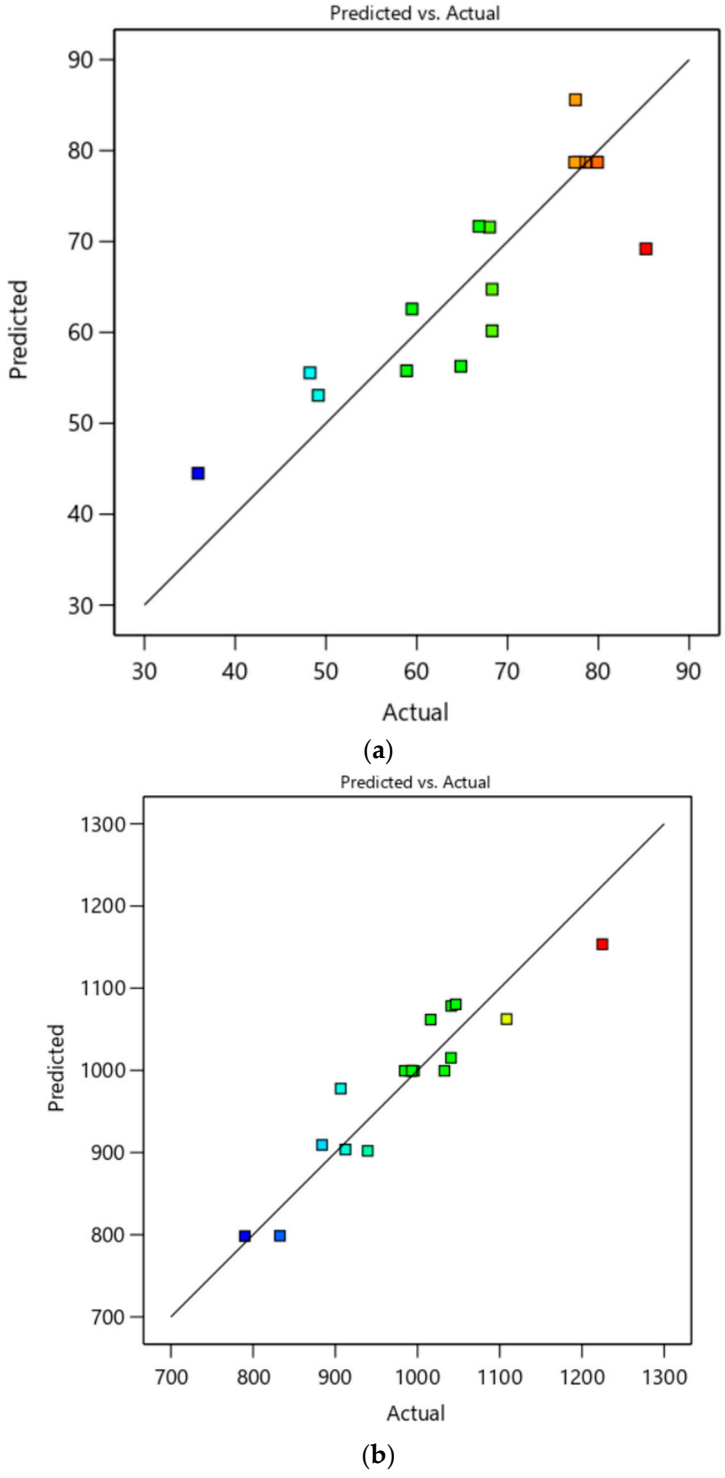


Figure 5. Predicted vs. Actual responses: (**a**) Yield and (**b**) TAC for SWE extraction.

Figure 6a shows that increasing pressure under a constant flow rate and temperature had no significant effect on recovery. When the temperature was raised from 100 °C to 140 °C at a constant flow rate and pressure (6 mL/min), the extract yield increased (4 MPa to 12 MPa). As the temperature rises, the predominance of the vapour solute state increases, resulting in the observed pattern [36]. As a result, the diffusivity of subcritical

water increases [39]. The capacity of the solvents to dissolve the target compounds is increased by increasing their diffusivity [27]. Cheng et al. Cheng, et al. [40] discovered that pressure has little influence on the dielectric constant of water. The temperature of the water is adjusted to control the dielectric constant to mimic various organic solvents. Using pressure during the extraction process has the primary benefit of keeping the water in a liquid state when its temperature exceeds the boiling point, allowing it to behave favourably throughout the extraction process [41,42].

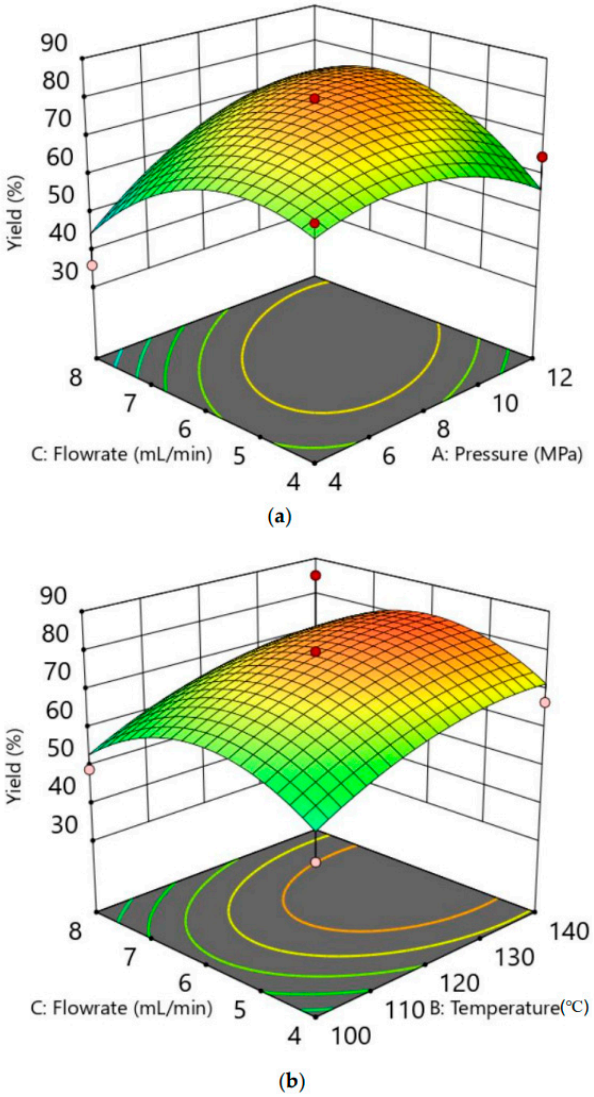


Figure 6. 3D response of the effect of SWE's variable on total yield, %: (**a**) effect of pressure and flow rate at constant temperature; (**b**) effect of temperature and flow rate at constant.

The pressure in the SWE aids in the solubilisation of analytes by forcing the solvent to enter the solid pores and rupture the matrix by enhancing the mass transfer of the solutes to the solvent [43]. Furthermore, when combined with high extraction temperatures, the pressure reduces the formation of air bubbles in the matrix, which is helpful in the procedure of preventing the solvent from reaching the analyte [44]. These conditions improve the analyte's solubility and the desorption kinetics of the sample matrix [45].

At a fixed flow rate (6 mL/min) and pressure (4 MPa and 12 MPa, respectively), the yield of extract increased when the temperature was raised from 100 °C to 140 °C as shown in Figure 6b. Furthermore, as the temperature rises, water viscosity decreases, increasing its diffusivity. As the viscosity of the solvent decreases, solute solubility increases [46]. Due to water's reduced viscosity and surface tension, these changes in its thermodynamic

properties increase the solubility of non-polar substances in water, increasing diffusivity and the mass transfer rate [42]. Mazzutti et al. Mazzutti, et al. [47] also stated that increasing the temperature above the boiling point of water reduces the subcritical water dielectric constant, making it similar to organic solvents and affecting its polarity and dissociation constant. However, Benito-Román et al. Benito-Román, et al. [48] discovered that one of the main disadvantages of SWE is the difficulty in controlling the heating/cooling times, which may result in excessively long exposure to high temperatures, causing thermal degradation of the bioactive compounds.

At constant pressure and temperature of 4 MPa and 120 °C, 4 mL/min to 8 mL/min flow rates reduce the yield extract. The amount of extract carried into the bulk of the solvent was limited by mass transfer resistance. At the same time, the residence time in the extraction vessel was reduced by a subcritical water flow rate of 8 mL/min. As a result, the subcritical water residence time was insufficient to carry out the solute removal [13,49]. Kim and Lim Kim and Lim [50] discovered that as temperature rises, water viscosity decreases, increasing penetration into solid particles, and thus facilitating compound dissociation from a complex matrix. High flow rates, on the other hand, may improve extraction capability by increasing total water volume and shortening residence times, inhibiting the formation of degradation products [51,52].

As the flow rate increased, the yields decreased because it accelerated mass transfer and increased surface velocity. The main disadvantage of using higher flow rates is that the amount of extract produced increases. As a result, the concentration of the final extract will be lower [46,53]. Extraction time and extract concentration are critical factors when determining the appropriate flow rate in practice, as the higher concentration extracts and shorter extraction times are preferred [54].

Lachos-Perez, Martinez-Jimenez, Rezende, Tompsett, Timko, and Forster-Carneiro Lachos-Perez, Martinez-Jimenez, Rezende, Tompsett, Timko and Forster-Carneiro [52] discovered that increasing flow rates could improve extraction yields of thermally labile components because exposure times of extracted components to thermally damaging conditions decrease with increasing continuous phase flow rates. Finally, the performance of mass transfer limited recovery will be independent of the continuous phase flow rate, and may even decrease for inaccessible components. In SWE, low flow rates (1–10 mL/min) are frequently used [43,55–57].

3.5. Effect of Process Parameters on TAC for Subcritical Water Extraction (SWE)

The quadratic model fits the experimental data ($R^2 > 0.80$ with a p-value < 0.05). The linear pressure and temperature had a significant effect ($p < 0.05$) on the TAC. This finding revealed that the regression models linking independent variables and responses could capture at least 80% of the variation in the response variables. The developed models accurately captured the actual relationships between the response parameters and characterised the data variation. The quadratic model fitted the experimental data of is shown in Equation (7):

$$Y_2 = 999.53 + 70.57A - 70.25B + 17.58C - 61.28AB + 13.99AC + 107.27BC + 9.59A^2 - 8.28B^2 - 32.86C^2 \quad (7)$$

Based on the p-value in the ANOVA table (p-value < 0.05) and the largest coefficient value in Equation (6), the pressure and temperature effects have a greater influence on TAC recovery than the flowrate effect (Figure 7a). Therefore, the influence of flowrate does not predominate in anthocyanins extraction. It is a finding that is inconsistent with TPC extraction using $ScCO_2$. It seems that the surface of the porous roselle has been compromised by $ScCO_2$. Consequently, subcritical water extraction is effective in extracting anthocyanins due to the absence of a competing solvent effect.

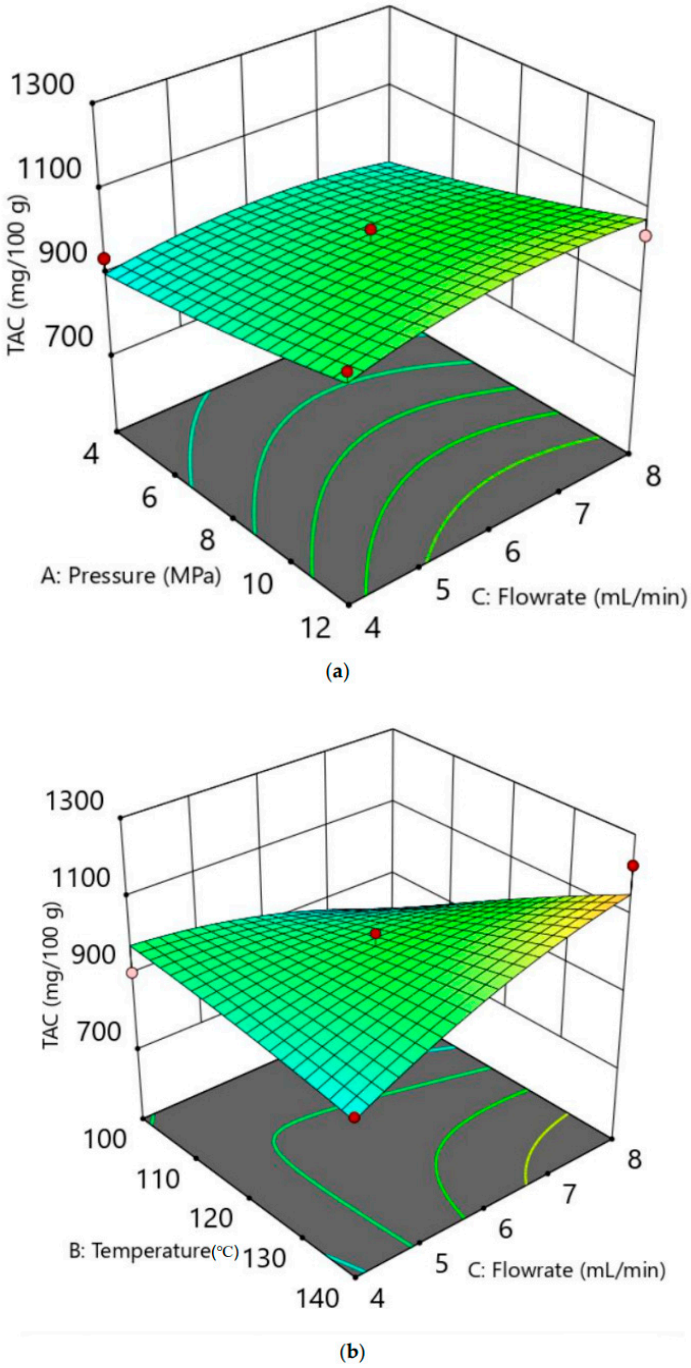


Figure 7. 3D response of the effect of SWE's variable on TAC, mg/100 g (**a**) effect of pressure and flow rate at constant temperature; (**b**) effect of temperature and flow rate at constant pressure.

Koyu et al. Koyu, et al. [58] also found that increased flow rates enabled larger volumes of solvent to come into contact with the plant material, which led to increased extraction of inactive metabolites and dilution of bioactive content, resulting in decreased anthocyanins recovery from *Morus nigra* L. fruits. A similar effect was seen in relation to lengthening the extraction duration, which resulted in reduced overall phenolic and flavonoid levels as a result of enhanced simultaneous extraction. Carr et al. Carr, et al. [59] also discovered that dynamic extraction is often quicker than static extraction due to the continual presence of new solvent (increasing the mass transfer driving force). It is likely that more water will be utilized to collect the material, and a more diluted result will be obtained. The selection of one technique over another will depend on whether lengthy exposure to water at a high

temperature may cause degradation (in the case of static extraction), or whether the product must be substantially concentrated in the extract mixture (in the case of dynamic extraction).

Figure 7b further illustrates that conditions of increasing pressure and temperature promote TAC recovery. Higher pressure may hasten the process by driving water into the matrix's pores, but normal pressure would not cause the pores to enlarge [60]. When temperature and pressure fluctuate, some water characteristics change; for example, when the temperature rises, the polarity of subcritical water decreases [48]. Polar and the polarity of anthocyanins may thus be differentiated. Due to the short residence time of subcritical water to transport solutes, the increasing flow rate had little effect on TAC recovery [23,43].

Moirangthem et al. Moirangthem, et al. [61] found the contradictive results in the anthocyanins recovery of subcritical water extracts of Manipur black rice bran and straw. Increasing of temperature of (160 °C) reduce the recovery of anthocyanins. This is due to the long extraction time, where length extraction enhances the residence time of solvent in the extraction vessel. The anthocyanins as flavonoids compounds were degraded rapidly at high temperatures during long-term heating over 30 min in pressurized hot water.

Sharifi et al. Sharifi, et al. [62] also found that the maximum concentration of anthocyanins was extracted from barberry (*Berberis vulgaris*) fruit at a temperature of 110 °C for 10 min. This decrease in anthocyanins' efficacy in response to an increase in temperature is likely due to a structural breakdown or alteration. Bridle and Timberlake Bridle and Timberlake [63] revealed that, when heated in an acidic medium, four anthocyanins' structures transform into the colourless carbinol base and chalcone forms. Destruction of anthocyanins may also be impacted by factors, such as pH, temperature, intermolecular pigmentation, ascorbic acid, and oxygen, present during various unit operating procedures. However, in the SWE process, where materials are often exposed to extremely high temperatures, the destruction or alteration of anthocyanins' structure and their consequent diminution were not anticipated. As high temperatures do not damage the anthocyanins recovery from roselle, the 5 min extraction time of this investigation is acceptable.

3.6. Multiple Responses Optimization of Integrated $ScCO_2$ Extraction and SWE

Multiple optimizations were carried out to determine the optimal parameters for the greatest number of multiple responses. Due to the two stages of the extraction process, known as integrated supercritical carbon dioxide and subcritical water extraction, there are two optimal conditions in this research. Thus, 19.13 MPa, 60 °C, and 4.31 mL/min were the optimal conditions for $ScCO_2$ (first stage), providing 18.20% yield and 80.34 mg/100 g TPC, respectively. Furthermore, 9.48 MPa, 137 °C, and 6.14 mL/min were the optimal conditions for the second stage of SWE, providing 86.11% and 1224.61 mg/100 g TAC, respectively. Table 4 shows the predicted and actual response for optimal condition, it indicates that the optimum condition is acceptable due to low percentage of error (%error < 10%).

3.7. Comparison the TAC Recovery with Previous Study

As seen in Figure 8, these findings are comparable to earlier research involving the extraction of anthocyanins compounds by different process. According to Idham, Putra, Aziz, Zaini, Rasidek, Mili, and Yunus Idham, Putra, Aziz, Zaini, Rasidek, Mili and Yunus [14], the best parameters for $ScCO_2$ extraction at a maximum TAC of 1197 mg/100 g were 27 MPa, 58 °C, and a cosolvent ratio of 8.86%. Although anthocyanins recovery is comparable between integrated $ScCO_2$ and SWE, the ratio of co-solvent is greater in the first stage of integrated $ScCO_2$ (8.86% > 4 %). Therefore, this procedure is preferred since it requires less ethanol and is safer than prior research. In addition, Redzuan et al. Redzuan, et al. [64] identified the ultrasonic-assisted extraction (UAE) for optimizing anthocyanins from roselle. Results reveal that a particle size of 0.125 mm, a solvent concentration of 10:1 mL/g solid, and an extraction duration of 15 min produced the maximum mass yield (64.72%), TAC concentration (70.97 mg/100 g), and AA concentration (90.05%). According to the findings, the combined $ScCO_2$ and SWE includes more anthoc65yanins than the UAE, although this method yields less anthocyanins than $SCCO_2$. Integrated $ScCO_2$ and SWE utilizes

a higher-pressure solvent, which improves the density and diffusivity of solvent for extracting anthocyanins. However, additional production and safety expenses arise from heightened pressure conditions. As a consequence, this technique may be substituted for the conventional method of anthocyanins extraction.

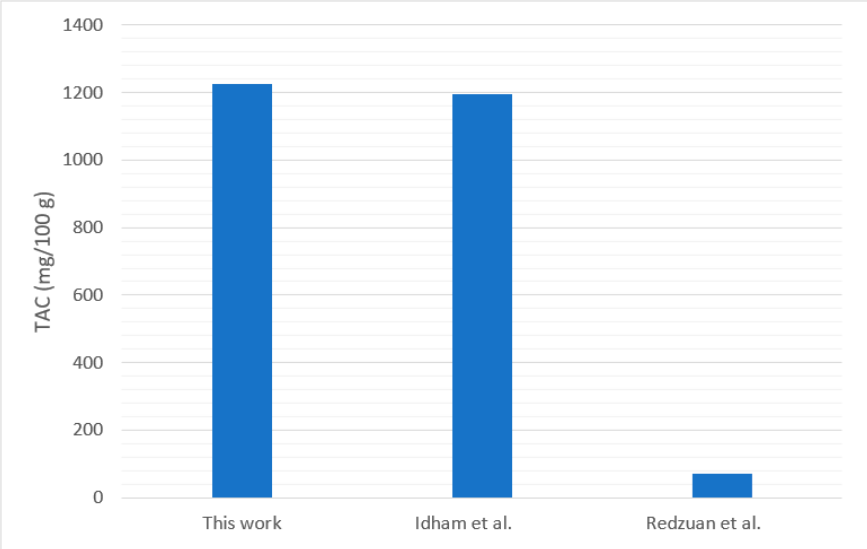


Figure 8. Comparison of the TAC recovery from roselle obtained in this study and in previous works [14,65].

4. Conclusions

Roselle has a long history of medicinal and geographical usage. It is used to treat fever, liver damage, hypertension, and leukaemia, among other illnesses. Anthocyanins are the beneficial bioactive molecules used to relieve this illness. Anthocyanins as high polar compounds can be extracted from fruits, vegetables, flowers, leaves, stems, and roots. The compounds are found predominantly in inner cell layers, such as the epidermis and peripheral mesophyll cells. The novel method, which integrates supercritical carbon dioxide and subcritical water to extract anthocyanins, has been developed. Determining the ideal conditions for recovering large yields of total anthocyanins compounds (TAC) from roselle using combined supercritical carbon dioxide ($ScCO_2$) and subcritical water extraction is the objective of this study (SWE). First, $ScCO_2$ is used to shatter the particle surface of roselle based on phenolic recovery, then SWE is used for the extraction of anthocyanins. Consequently, there are two ideal conditions, including $ScCO_2$ and SWE. 20 MPa, 40 °C, and 4.875 mL/min were the best conditions for $ScCO_2$ (first stage), producing a 21.11% yield and 93.55 mg/100 g TPC, respectively. Thus, 12 MPa, 140 °C, and 8 mL/min were the optimal conditions for the second stage of SWE, providing 86.11% and 1142.61 mg/100 g TAC, respectively. Application of combined $ScCO_2$ and SWE proved successful in achieving high anthocyanins production, concentration, and yield as compared to current and classic extraction methods. This approach may be used to generate roselle with a greater anthocyanin's concentration than the prior method.

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Data Availability Statement: Data available on request from the authors.

Conflicts of Interest: The authors declare no conflict of interest.

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