Optimization of Antioxidant Activity Extraction Conditions from the Stems of *Rourea oligophlebia* Merr. Using the Response Surface Methodology

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In the present study, the Box-Behnken design (BBD) of the response surface methodology (RSM) was used to investigate the effects of three factors; extraction temperature (°C), extraction time (min), and ethanol concentration (%), of *Rourea oligophlebia* Merr. stems on the responses of total phenolic content (TPC), total flavonoid content (TFC), and DPPH radical scavenging activity (DPPH). The optimal conditions from response RSM were observed to be 52% v/v of ethanol concentration, 61°C of extraction temperature, and 140 min of extraction time. The experimental values of TPC, TFC, and DPPH radical scavenging activity were 146.9±0.5 mgGAE/g DW, 18.3±0.2 mgQE/g DW, and 93.2±0.3%, respectively.

Keywords: *Rourea oligophlebia* Merr.; radical scavenging activity; total phenolic content; total flavonoid content; response surface methodology; extraction; Box-Behnken design

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Rourea is a genus of plants in the family Connaraceae with climbing shrubs and small trees, and has a widespread distribution in regions like the Amazon, the Pacific, Africa, and Asia [1]. With approximately 65 species and 129 varieties, it is frequently employed in ethnomedicine to treat various health issues such as rheumatism, diabetes, tumors, asthma, and diarrhea [2]. Local communities in Malaysia use various *Rourea* species for traditional medicine. For instance, *R. regusa* Planch, locally known as *akar semeling*, is used to treat respiratory diseases through the decoction of its roots [3]. Meanwhile, the Temuan indigenous people use the root decoction of *R. concolor* Blume, locally known as *akar semelit*, to treat kidney diseases, diabetes, lung tumor, and stomach tumor [4, 5]. In Chinese folk medicine, *R. minor* (Gaertn.) leaves are utilized as a styptic to treat minor injuries and wounds. However, caution should be exercised as the stems and roots are toxic, so they are commonly used as the tying material [6]. *Rourea* plant extracts and their active components have shown various significant biological activities, including antinociceptive, hypoglycemic, antibacterial, antiplasmodial, and antioxidant activities. The Thai ethnic group in Vietnam's indigenous knowledge includes the use of stems and roots from *R. oligophlebia* Merr. to treat conditions like back pain, injuries, bleeding, and to enhance sexual function [7]. Recently, studies indicate that *Rourea* species contain notable quantities of biologically active compounds, including flavonoids, lipids, phytosterols, triterpenes, phenolic acids, and coumarins [1, 8-10].

R. oligophlebia Merr. is a species discovered in the mountainous regions of central Vietnamese provinces such as Thanh Hoa, Nghe An (Que Phong, Quy Chau), Ha Tinh (Vu Quang), and Thua Thien Hue, Da Nang that is considered a sub-endemic species. An initial examination of the stems of *R. oligophlebia* Merr. revealed the presence of triterpenes, sterols, and phenolic compounds [11]. Recently, stems from *R. oligophlebia* Merr. were harvested from Ben En National Park, located in Thanh Hoa province, Vietnam. These stems are known to harbor compounds with potent antimicrobial properties against Gram-positive strains. Additionally, they have demonstrated cytotoxic effects against various cancer cell lines, including HepG-2, MCF-7, KB, and LU [7].

Response Surface Methodology (RSM) stands out as a potent statistical tool for fine-tuning experimental conditions and exploring pivotal processes while simultaneously reducing the number of required experimental trials. RSM enables the comprehensive assessment of the effects of independent variables, whether individually or in combination, within a process [12, 13]. An essential aspect of employing this method is the experimental validation of predicted model values. Consequently, RSM emerges as a valuable asset for optimizing technological processes compared to the conventional one-factor-at-a-time approach, which tends to be both costly and time-intensive.

In this investigation, we focused on optimizing the extraction parameters for TPC, TFC, and DPPH radical scavenging activity from the stems of *R. oligophlebia* Merr. collected in Que Phong district, Nghe An province, Vietnam. These bioactive constituents are renowned for their potent antioxidant properties, which hold promise in various applications such as antifungal and antibacterial treatments, combating malaria, alleviating bone pain, enhancing nerve function, and promoting overall health and vitality.

EXPERIMENTAL

Materials

Stems of *R. oligophlebia* Merr. (Que Phong, Nghe An) were collected in Que Phong district, Nghe An province, Vietnam in September 2021 and identified by the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. A voucher specimen was deposited at the herbarium of the School of Chemistry, Biology and Environment, Vinh University, Vietnam. The material was dried, crushed and stored at 4^0C for further experiments.

Methods

Total Phenolic Content (TPC)

The TPC of the *R. oligophlebia* Merr. stem extracts was measured according to the method reported in [14], with some modifications. This method measures color change caused by phenolate reagent in the presence of sodium carbonate. 1 mL of the sample was mixed with 5 mL of Folin-Ciocalteu's solution. After 3 minutes, 4 mL of 7.5% sodium carbonate solution was added to the mixture and adjusted to 10 mL with deionized water. The mixture was kept at room temperature in a dark environment for 60 min. The color change was determined by scanning the wavelength at 765 nm (Agilent 8453 UV-visible Spectrophotometer) since maximum absorbance was obtained. The TPC of the *R. oligophlebia* Merr. stem extract was determined as mg gallic acid equivalent using the standard curve prepared at different concentrations of gallic acid and reported as mgGAE/g dry weight (DW).

Total Flavonoid Content (TFC)

The TFC of the extracts from the *R. oligophlebia* Merr. stems was determined according to the method of Chang et al. (2022), with some modifications [15]. Specifically 1.0 mL of the *R. oligophlebia* Merr. stem extract was combined with 3 mL of 75% ethanol, 0.2 mL of 10% aluminium chloride, 0.2 mL of 0.1 M potassium acetate, and 5 mL of distilled water. This reaction mixture was allowed to stand for 30 minutes at room temperature, after which the absorbance was measured at 415 nm using an Agilent 8453 UV-visible spectrophotometer. Quercetin was utilized as the standard reference. The TFC was quantified in terms of milligrams of quercetin equivalents per gram of dry weight (mg QE/g DW).

DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of the R. oligophlebia Merr. stem extracts was evaluated using DPPH radicals based on the method in [16], with slight modification. The electron-donating effect for DPPH (a, a-diphenyl-picrylhydrazyl) of each extract was measured as follows: 0.5 mL of 0.5 mM DPPH was added into a test tube containing 1 ml of ethanol; after that 10 µL of sample and 990 µL of 100 mM sodium acetate buffer (pH = 5.5) were agitated at 200 rpm for 5 min in a dark room to induce responses. Finally, a UV spectrometer (Agilent 8453 UV-Visible Spectrophotometer) was used to measure the concentration of the remaining radical in 517 nm.

All determinations were determined by replicate experiments with triplicate analysis. The radical scavenging activity was calculated according to Eq. **1**.

Where, Abs_{sample} is the testing specimen absorbance and $Abs_{control}$ is the control reaction absorbance.

Experimental Design

RSM was used to determine the optimum levels of extraction time (min), extraction temperature (°C), and ethanol concentration (% v/v) as extraction medium on three responses TPC, TFC, and DPPH of the *R. oligophlebia* stem extracts. These three factors, namely extraction temperature (X_1), extraction time (X_2), and ethanol concentration (X_3) were coded into three levels (-1, 0, +1). Table **1** provides the information on the range and level of the experiment variables used in both coded and uncoded forms in this study.

$$\text{Radical scavenging activity (\%)} = \left(1 - \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right).100 \qquad (1)$$

Table 1. Coded levels of independent variables used in RSM design.

No.	Independent variables	Units	Coded symbols	Coded variable levels		
				-1	0	+1
1.	Extraction temperature	°C	X_1	50	60	70
2.	Extraction time	min	X_2	120	135	150
3.	Ethanol concentration	% v/v	X_3	40	50	60

The effects of the extraction conditions were evaluated using the program Design-Expert®, version 11.0.0. All analyses were performed in triplicate and all experimental results were expressed as mean ± SD. *P* values < 0.05 were significant. The response variable was fitted to be a second-order polynomial model as described in the following Eq. **2**.

Where, Y is the predicted response; β_o is the intercept coefficient; β_i is the linear coefficient; β_{ii} is the squared coefficient; β_{ij} is the interaction coefficient; X_i and X_j are the coded independent variables; and X_iX_j and X_i^2 are the interaction and quadratic terms.

RESULTS AND DISCUSSION

Fitting the Response Surface Models

The responses consisting of TPC, TFC, and DPPH radical scavenging activity for the *R. oligophlebia* Merr. stem extracts were optimized based on the Box-Behnken design (BBD). The experimental design and corresponding three response variables are presented in Table 2.

In this study, according to the sequential model sum of squares, the highest order polynomials were utilized to select the models where the additional coefficient estimates are significant and the models are not aliased. Hence, for all three independent variables and responses, a quadratic polynomial model was selected and fitted well as suggested by the software.

The final empirical regression model of their relationship between responses and the three tested variables for TPC, TFC, and DPPH radical scavenging activity could be expressed by the following quadratic polynomial equations (Eq. 3 - Eq. 5).

Where, Y_1 is the TPC, Y_2 is the TFC, Y_3 is the DPPH radical scavenging activity, X_1 is the extraction temperature, X_2 is the extraction time, and X_3 is the ethanol concentration (ethanol/water ratio).

The RSM model coefficients were validated by analysis of variance (ANOVA) of the response variables for the quadratic polynomial model summarized in Table **3**.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum\sum_{i<j} \beta_{ij} X_i X_j \quad (2)$$

$$Y_1 = 147.10 + 7.01X_1 + 7.32X_2 + 2.74X_3 + 3.39X_1X_2 - 3.56X_1X_3 - 2.59X_2X_3 - 16.98X_1^2 - 20.36X_2^2 - 12.61X_3^2 \quad (3)$$

$$Y_2 = 18.49 + 0.75X_1 + 0.96X_2 + 0.54X_3 - 0.45X_1X_2 - 0.48X_1X_3 - 0.23X_2X_3 + 2.74X_1^2 - 0.82X_2^2 - 0.84X_3^2 \quad (4)$$

$$Y_3 = 93.60 + 0.92X_1 + 1.46X_2 + 0.76X_3 - 0.60X_1X_2 - 0.58X_1X_3 - 0.78X_2X_3 - 2.76X_1^2 - 1.36X_2^2 - 0.90X_3^2 \quad (5)$$

Table 2. Experimental data obtained for three responses based on BBD design.

RUN	X_1 (°C)	X_2 (min)	X_3 (% v/v)	TPC, Y_1 (mgGAE/g)	TFC, Y_2 (mgQE/g)	DPPH, Y_3 (%)
1	50	135	60	117.07	15.28	90.35
2	50	135	40	104.23	13.05	87.85
3	60	135	50	149.74	18.63	93.75
4	60	150	60	122.06	17.97	92.77
5	50	120	50	99.26	12.72	86.32
6	60	135	50	146.03	17.85	92.71
7	70	135	40	125.09	15.48	90.68
8	70	150	50	127.05	16.23	91.43
9	60	120	40	101.03	15.24	88.34
10	60	150	40	121.98	17.52	92.63
11	50	150	50	105.98	15.63	90.63
12	60	120	60	111.47	16.59	91.61
13	60	135	50	145.01	19.05	94.21
14	70	135	60	123.68	15.81	90.88
15	60	135	50	145.29	18.39	93.79
16	60	135	50	149.45	18.51	93.55
17	70	120	50	106.76	15.12	89.52

Table 3. Analysis of variance (ANOVA) for the model.

Source	Y_1 – TPC		Y_2 – TFC		Y_3 – DPPH	
	F- value	**p- value**	**F- value**	**p-value**	**F- value**	**p- value**
Model	162.63	$< 0.0001^S$	51.97	$< 0.0001^S$	44.47	$< 0.0001^S$
X_1	114.02	$< 0.0001^S$	36.93	0.0005^S	33.53	0.0007^S
X_2	124.46	$< 0.0001^S$	61.31	0.0001^S	84.30	$< 0.0001^S$
X_3	17.49	0.0041^S	19.76	0.0030^S	23.11	0.0020^S
X_1X_2	13.37	0.0081^S	6.74	0.0357^S	7.13	0.0320^S
X_1X_3	14.74	0.0064^S	7.51	0.0289^S	6.55	0.0376^S
X_2X_3	7.79	0.0268^S	1.68	0.2355^{NS}	12.13	0.0102^S
X_1^2	352.57	$< 0.0001^S$	263.46	$< 0.0001^S$	159.09	$< 0.0001^S$
X_2^2	507.04	$< 0.0001^S$	23.43	0.0019^S	38.83	0.0004^S
X_3^2	194.37	$< 0.0001^S$	24.59	0.0016^S	16.88	0.0045^S
Lack of Fit	0.1767	0.9070^{NS}	0.1569	0.9200^{NS}	0.2049	0.8882^{NS}
R^2	0.9952		0.9853		0.9828	

The ANOVA analysis results for multiple regression and response surface quadratic model of Y_1, Y_2, and Y_3 were evaluated using the corresponding p and R^2 values. F values of Y_1, Y_2 and Y_3 were calculated to be 162.63, 51.97, and 44.47, respectively.

All leading to p value <0.05, suggesting the three models were statistically significant. The models' coefficient of determination (R^2) were 0.9952, 0.9853, and 0.9828, indicating that more than 99.52%; 98.53%, and 98.28% of the response variability were explained, and supporting a good accuracy and ability of the established model within the range limits used. The F values of Lack of Fit of Y_1, Y_2 and Y_3 were 0.907, 0.92, and 0.8882, respectively, implying that the Lack of Fit was not significant relative to the pure error. This indicated that the accuracy of the polynomial model was adequate.

Response Surface Analysis

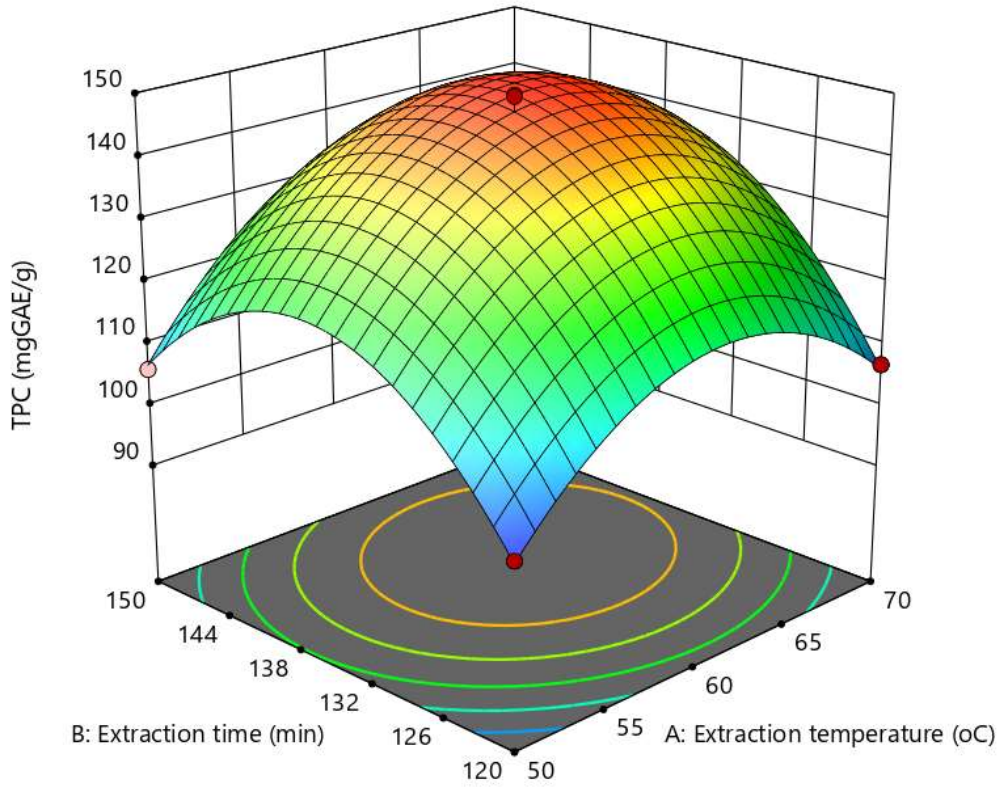
Three factors, temperature, time and ethanol concentration, affected the extraction condition of the maximum TPC, TFC, and DPPH radical scavenging activity. Three-dimensional model graphs were plotted, as shown in Figures 1 to 3. The response surface plots of the model were done by varying two variables, within the experimental range under investigation and holding the other variables at their central level [13, 23].

Response Surface Analysis of TPC

The response surface plots presented in Figure 1 delineate the extraction of the TPC from the *R. oligophlebia* Merr. stem extracts, showcasing the intricate interplay and impact of independent variables on TPC yields. Figure 1 and Table 3 reveal that all three factors - extraction temperature, extraction time, and ethanol concentration - exhibit negative quadratic effects ($p < 0.0001$).

In Figure 1(a), the surface plot elucidates the correlation between extraction temperature (X_1) and extraction time (X_2) concerning TPC yield while maintaining a fixed ethanol concentration of 50%. It becomes evident that the optimal phenolic content is attained within the temperature range of 57^0C to 67^0C across various extraction times. This observation can be attributed to the phenomenon wherein the diffusion coefficient and solubility of polyphenols escalate with rising temperature, consequently expediting the extraction process [17]. Nevertheless, it is imperative to adhere to an upper limit of temperature to prevent the degradation of thermosensitive phenolics during extraction, as highlighted in previous studies [13].

These findings parallel those reported in a previous study [18], wherein the total phenolic content derived from grape by-products exhibited an increase with rising temperature and a reduction in extraction time.


TPC (mgGAE/g)
150
140
130
120
110
100
90
150
144
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132
126
120
B: Extraction time (min)
50
55
60
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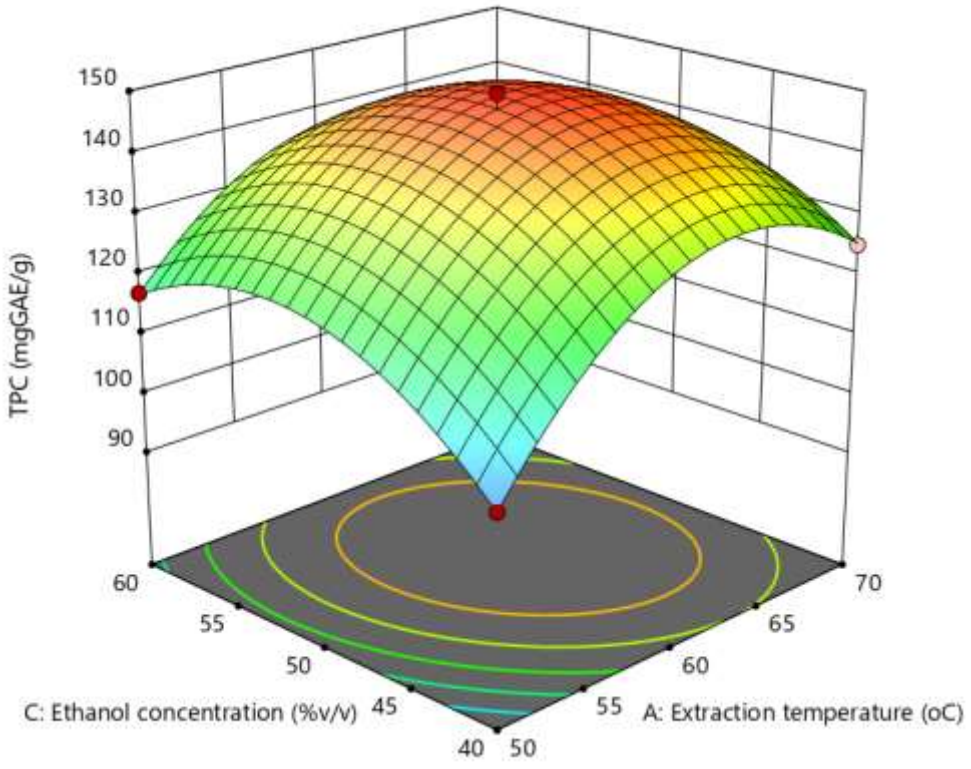
70
A: Extraction temperature (oC)

a)


TPC (mgGAE/g)
150
140
130
120
110
100
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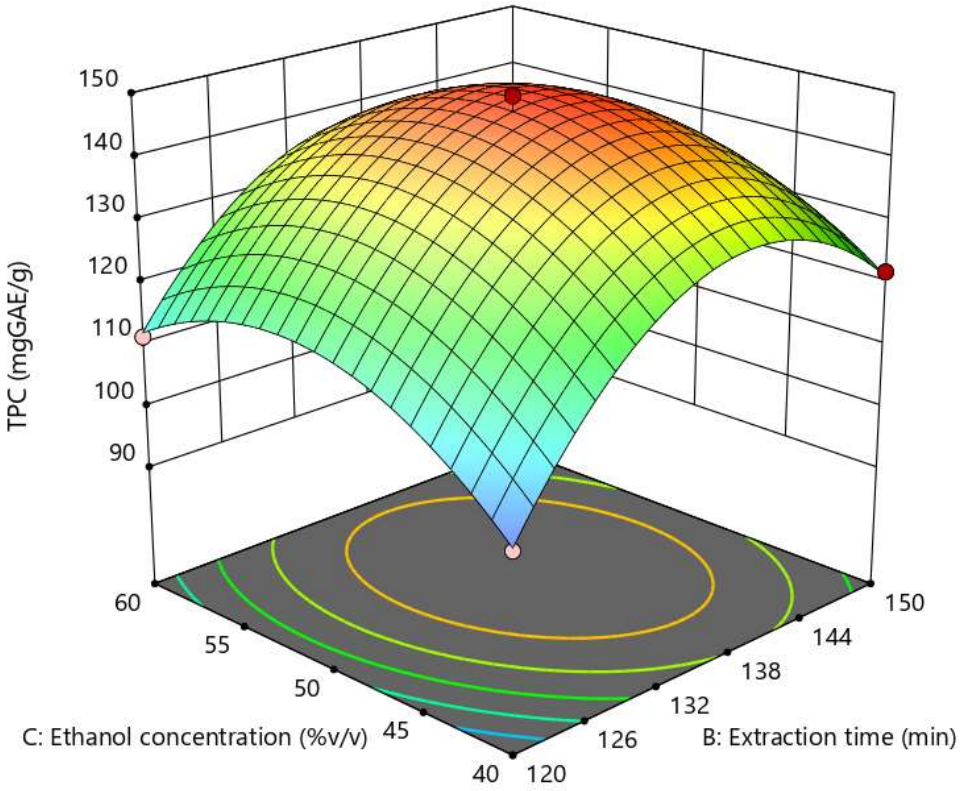
C: Ethanol concentration (%v/v)
50
55
60
65
70
A: Extraction temperature (oC)

b)



c)

Figure 1. Response surface plots of TPC.

The surface plot in Figure 1(b) illustrates the relationship between extraction temperature (X_1) and ethanol concentration (X_3) on the total phenolic content (TPC) at a fixed extraction time of 135 minutes. The highest TPC in the *R. oligophlebia* Merr. stem extracts was observed at the ethanol concentration ranging from 50% to 55%. This increase in phenolic content may be attributed to the inherent polarity of the solvents employed [19]. Ethanol and water were chosen as solvents for their favorable safety profiles compared to other organic solvents, as well as their suitability for human consumption [20].

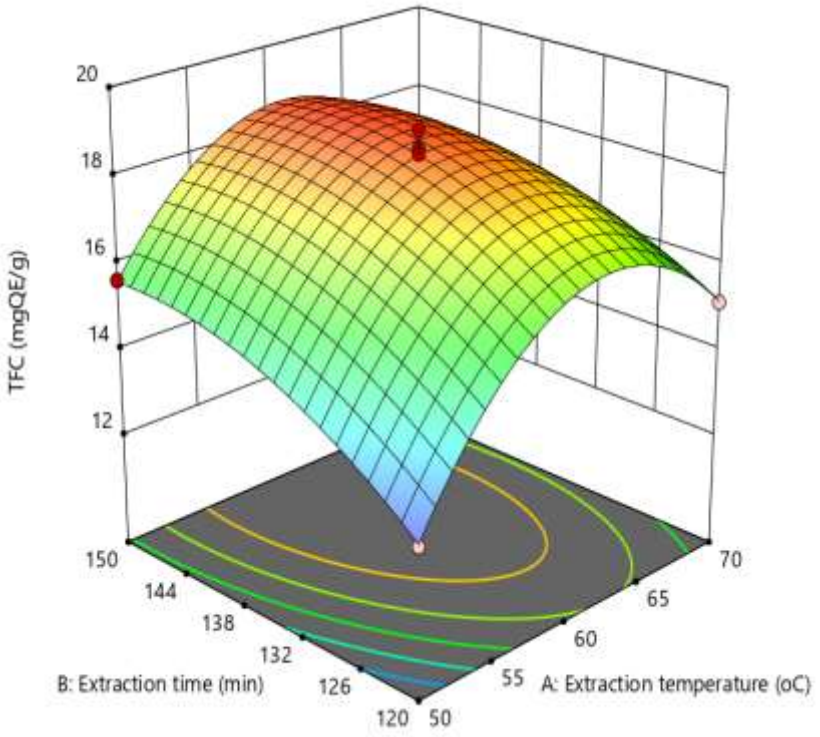
In Figure 1(c), the surface plot reveals that the maximum TPC in the *R. oligophlebia* Merr. stem extract could be achieved at the extraction time of 130 min to 140 min at a fixed ethanol concentration. The findings regarding the phenolic content exhibit a similar trend that was observed in a previous study [21], wherein an increase in ethanol concentration ratio and extraction time resulted in an elevation of the TPC until a peak level was attained.

Response Surface Analysis of TFC

The 3D plot in Figure 2(a) shows the response surface plot of extraction temperature (X_1) and extraction time (X_2) at a fixed ethanol concentration (50%). The 3D plot shows that extraction time exhibited a weaker effect, whereas extraction temperature represented a relatively significant effect on the flavonoid yield. An increase in the TFC could be significantly achieved with the increase of extraction time, at any level of extraction temperature. Therefore, the optimum TFC was achieved at 60-65°C of extraction temperature and 140-150 minutes of extraction time.

The 3D surface plot in Figure 2(b) shows the interaction between extraction temperature (X_1) and ethanol concentration (X_3) at a fixed extraction time. The flavonoids and their glycosides are thought to be efficiently extracted from plant materials by ethanol solvent. It was observed that the value of TFC in the *R. oligophlebia* Merr. stem extracts increased when ethanol concentration was increased from 40% to 60% at a fixed 50^0C extraction temperature. This result corresponds to the findings reported in [22].

Figure 2(c) shows the interaction between extraction time (X_2) and ethanol concentration (X_3) at the fixed extraction temperature of 60^0C. An increase in ethanol concentration promoted the breakdown of the cell membrane which enhanced the permeability of the solvent into a solid matrix. The highest TFC could be achieved when extraction was conducted at an ethanol to water ratio of 50 - 60% with increasing extraction time. A great increase in the yield also resulted when extraction time was increased in the range of 135 - 150 min.


TFC (mgQE/g)
20
18
16
14
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150
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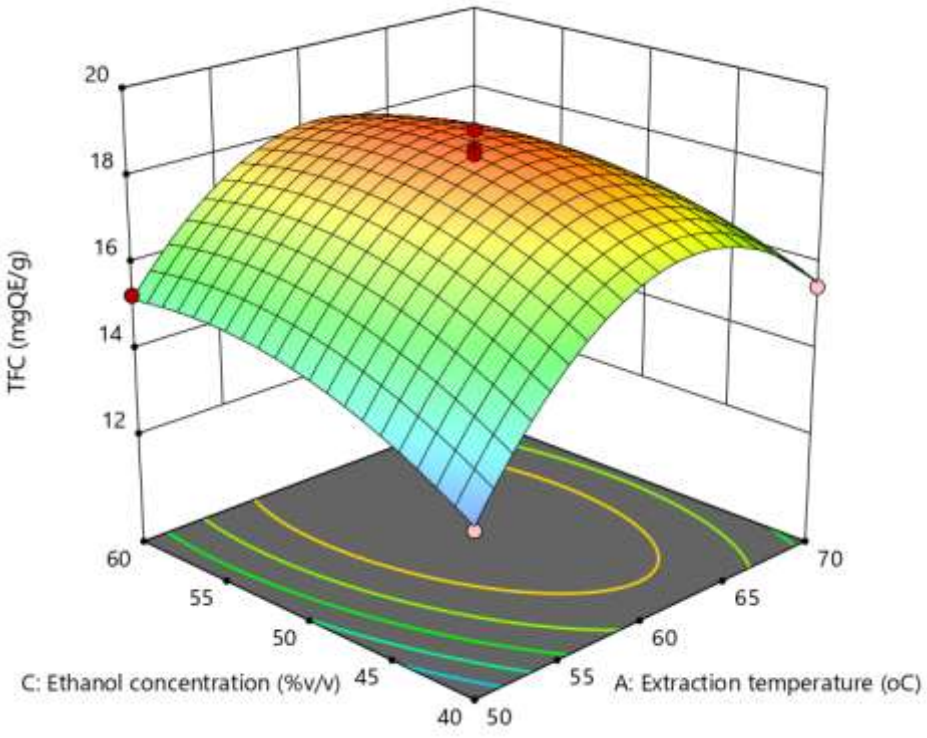
126
120
B: Extraction time (min)
50
55
60
65
70
A: Extraction temperature (oC)

a)


TFC (mgQE/g)
20
18
16
14
12
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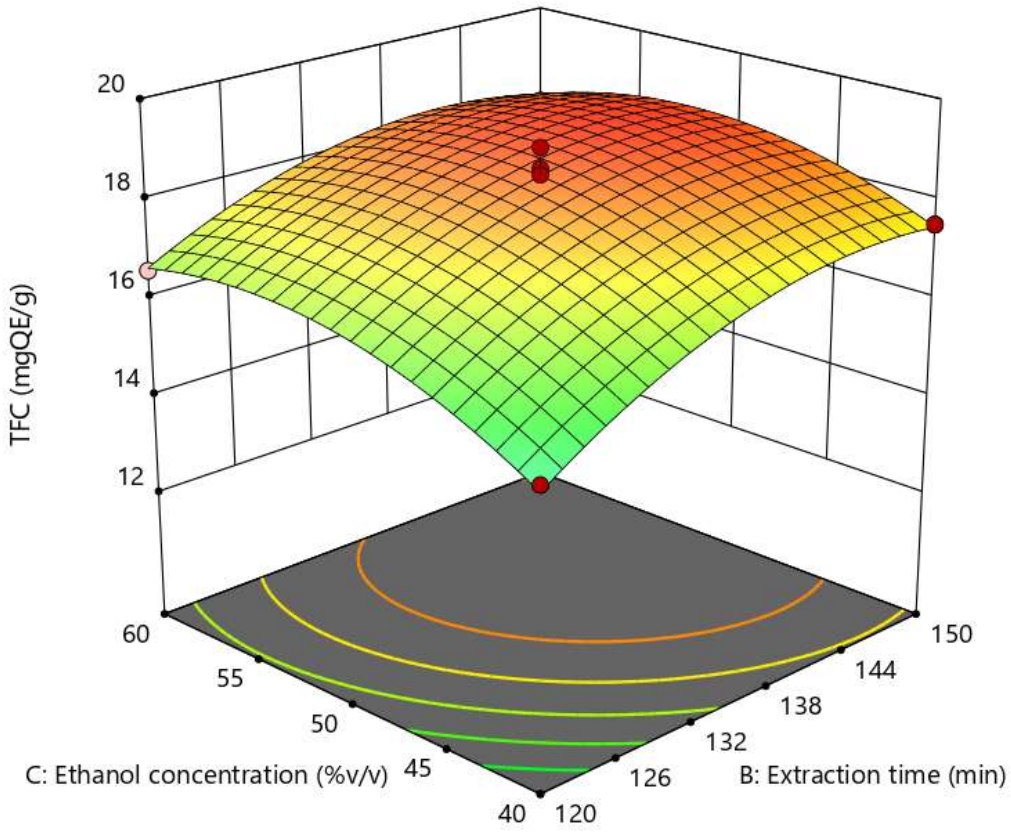
C: Ethanol concentration (%v/v)
50
55
60
65
70
A: Extraction temperature (oC)

b)



c)

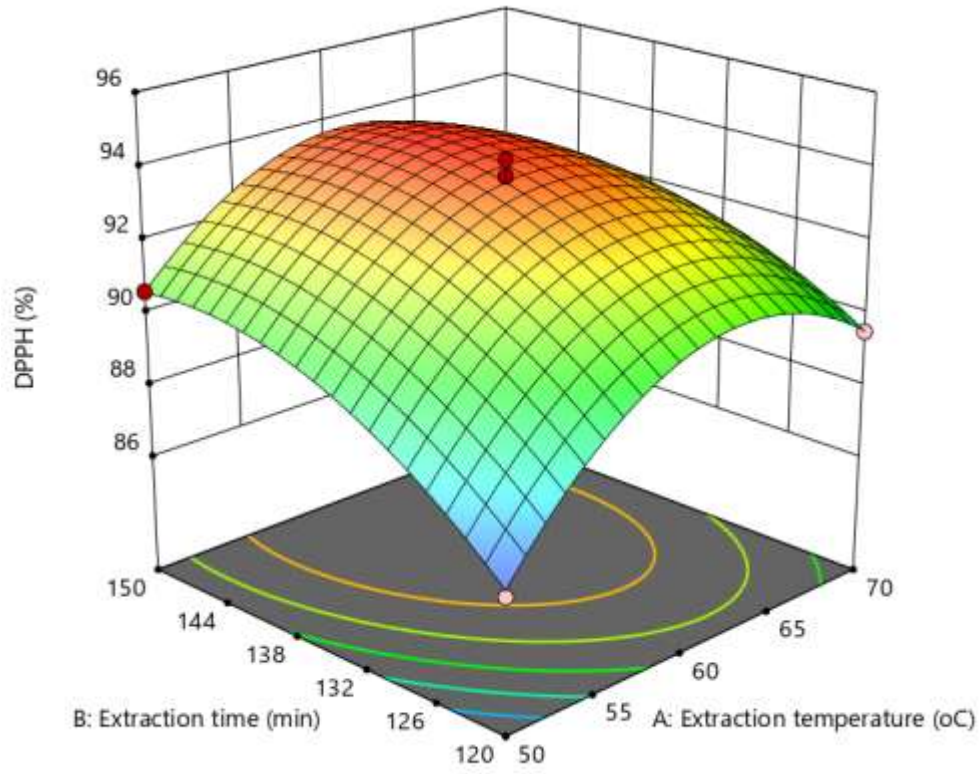
Figure 2. Response surface plots of TFC

Response Surface Analysis of DPPH

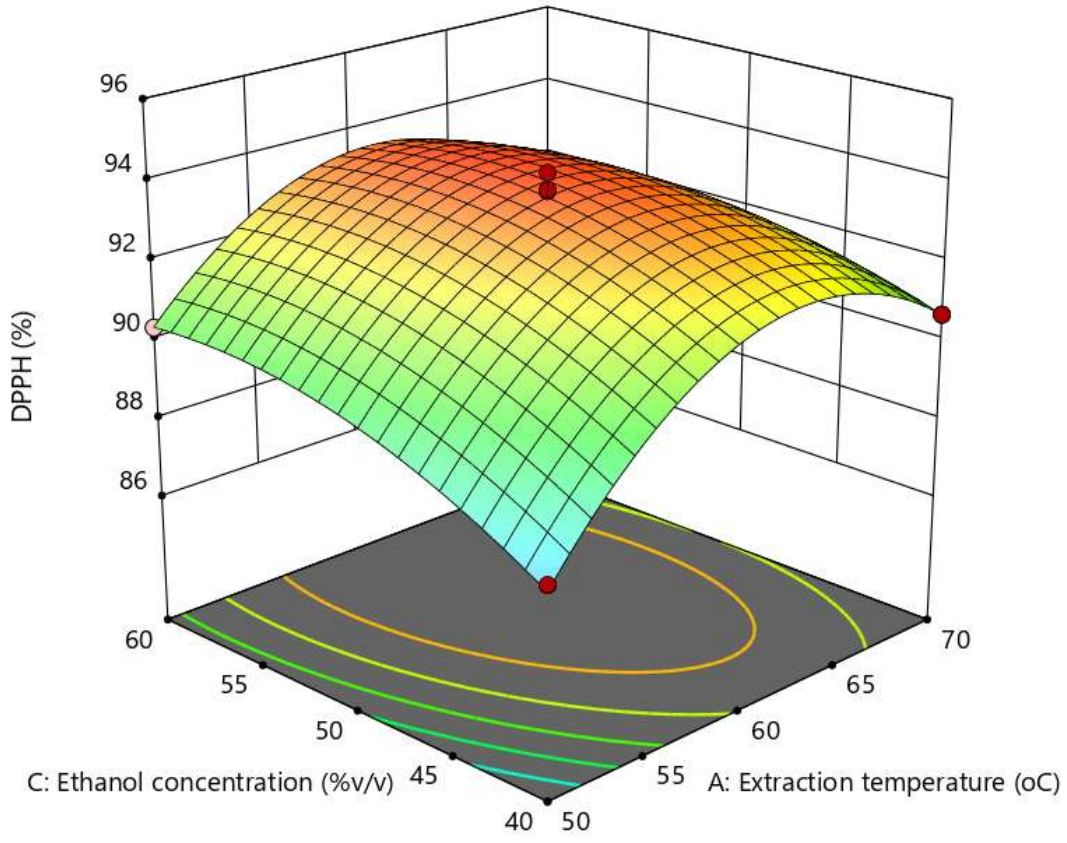
The 3D plots in Figure 3 illustrate the impact of extraction temperature, extraction time, and ethanol concentration on the DPPH radical scavenging activity of the *R. oligophlebia* Merr. stem extracts. Notably, the linear effects of all three factors on DPPH value were found to be significant.

Specifically, Figure 3(a) delineates the influence of extraction temperature and extraction time on DPPH activity while holding the remaining factor constant. It reveals a pattern where DPPH activity initially rises with increasing extraction temperature from 50°C to 63°C, followed by a decline as the temperature surpasses 63°C, echoing findings from a study by Chi et al. (2022) [23]. This can be explained by the fact that chemical reactions within the sample intensify as temperature rises, potentially altering the chemical structure of antioxidants, and leading to either an enhancement or reduction in DPPH scavenging ability [24]. Additionally, high temperatures may render some antioxidants unstable, causing them to degrade more easily [25]. Moreover, the plot demonstrates a direct correlation between extraction time and DPPH activity, as DPPH activity increased with prolonged extraction time, ranging from 120 to 150 minutes.

In Figure 3(b), which depicts the effect of ethanol concentration and extraction time on DPPH activity at a constant extraction temperature, a similar trend emerges. Here, DPPH activity escalates with increasing ethanol concentration, ranging from 40% to 60%, irrespective of the extraction temperature. The optimal DPPH activity for the *R. oligophlebia* Merr. stem extracts was achieved within a range of extraction temperature from 58 to 68°C, extraction time spanning 140 to 150 minutes, and ethanol concentration ranging between 55% and 60%.


DPPH (%)
96
94
92
90
88
86
150
144
138
132
126
120
B: Extraction time (min)
50
55
60
65
70
A: Extraction temperature (oC)

a)


DPPH (%)
96
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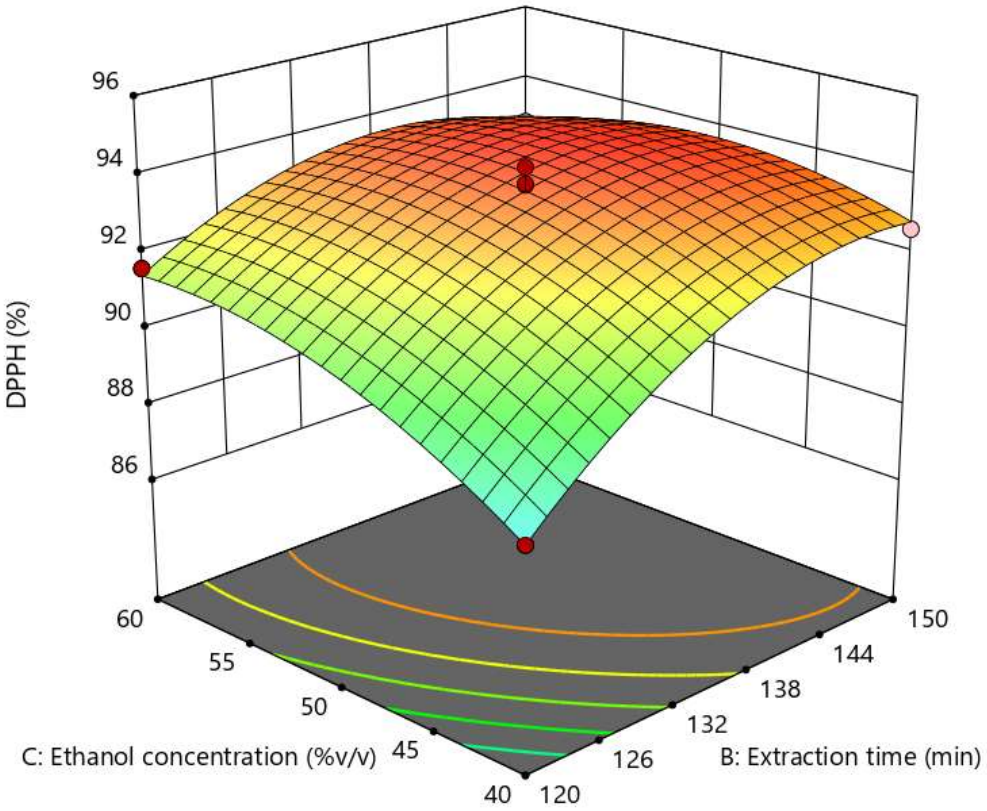
C: Ethanol concentration (%v/v)
50
55
60
65
70
A: Extraction temperature (oC)

b)



c)

Figure 3. Response surface plots of DPPH.

Optimization and Model Verification

The outcome of simultaneous optimization employing the desirability function approach delineated the most efficacious extraction parameters for the *R. oligophlebia* stem extracts, pinpointing 61°C for 140 minutes alongside 52% ethanol concentration as optimal for maximizing TPC, TFC, and DPPH radical scavenging activity. Table 4 presents both predicted and experimental values for the extraction of target compounds from the *R. oligophlebia* Merr. stem extracts. The experimental results yielded extraction yields for TPC, TFC, and DPPH radical scavenging activity of 146.9±0.5 mgGAE/g, 18.3±0.2 mgQE/g, and 93.2±0.3%, respectively. These experimental findings closely mirrored the predicted values (TPC = 148.128 mgGAE/g, TFC = 18.779 mgQE/g, and DPPH = 94.019%) derived from the corresponding regression models, exhibiting a coefficient of variation ranging from 0.83% to 2.55%.

Table 4. Comparison between predicted and experimental values for antioxidants from extracts of *R. oligophlebia* Merr. stems

S. No.	Responses	Optimum extraction conditions			Maximum value		% difference (CV)
		X_1	X_2	X_3	*Experimental*[a]	*Predicted*	
1.	TPC (mgGAE/g DW)	61°C	140min	52 %	146.9±0.5	148.128	0.83
2.	TFC (mgQE/g DW)				18.3±0.2	18.779	2.55
3.	DPPH radical scavenging activity (%)				93.2±0.3	94.019	0.87

X_1, extraction temperature (°C); X_2, extraction time (min); X_3, ethanol concentration (%); Y_1, TPC (mgGAE/g); Y_2, TFC (mgQE/g); Y_3, DPPH radical scavenging activity (%). [a]Responses are the means ± SD (n = 3).

CONCLUSION

In summary, we effectively utilized the BBD along with the RSM to pinpoint the best process parameters and create predictive models. Our experiments determined that the optimal combination of extraction temperature, duration, and ethanol concentration was 61°C for 140 minutes with 52% ethanol concentration. This resulted in a mean highest TPC of 146.9±0.5 mgGAE/g, TFC of 18.3±0.2 mgQE/g, and DPPH radical scavenging activity of 93.2±0.3%. Our findings highlight significant antioxidant activity in the stems of *R. oligophlebia* Merr., suggesting its potential as an antioxidant source.

REFERENCES

1. Osman, C. P., Zahari, Z., Adenan, M. I. and Zohdi, R. M (2019) A review on traditional uses, phytochemistry, and pharmacology of the genus *Rourea*. *Journal of Applied Pharmaceutical Science*, **9(09)**, 125–131.

2. Agbodjento, E., Klotoé, J. R., Sacramento, I. T., Dougnon, V. T., Hounkpatin, M., Dougnon J. T. and Atègbo, J. M. (2020) Current knowledge on traditional uses, phytochemistry, toxicity and biological activity of *Rourea Coccinea* (Schumach & Thonn.) Benth. *International Journal of Biosciences*, **16(4)**, 231–240.

3. Alsarhan, A., Sultana, N., Kadir, M. R. A. and Aburjai, T. (2012) Ethnopharmacological Survey of Medicinal Plants in Malaysia, the Kangkar Pulai Region. *International Journal of Pharmacology*, **8(8)**, 679–686.

4. Ong, H. C., Ahmad, N. and Milow, P. (2011) Traditional Medicinal Plants Used by the Temuan Villagers in Kampung Tering, Negeri Sembilan, Malaysia. *Studies on Ethno-Medicine*, **5(3)**, 169–173.

5. Ong, C. H., Chua, S. and Milow, P. (2011) Ethnomedicinal Plants Used by the Temuan Villagers in Kampung Jeram Kedah, Negeri Sembilan, Malaysia. *Studies on Ethno-Medicine*, **5(2)**, 95–100.

6. He, Z. -D., Ma, C. -Y., Tan, G. T., Sydara, K., Tamez, P., Southavong, B., Bouamanivong, S., Soejarto, D. D., Pezzuto, J. M., Fong, H. H. S. and Zhang, H. -J. (2006) Rourinoside and rouremin, antimalarial constituents from *Rourea minor*. *Phytochemistry*, **67(13)**, 1378–1384.

7. Dinh, T. N., Ha, M. V. T., Xuan, L. N., Thi, H. T., Thi, H. N., Ngoc, M. N. T., Huu, G. T., Kim, O. V. T. and Nguyen, T. L. (2021) A New Pyrrolidone Alkaloid and Other Constituents from *Rourea oligophlebia* Stems. *Journal of Chemistry*, **2021(6659106)**, 1–5.

8. Oliveira, P. V. D., Lemos, R. P. L. and Conserva, L. M. (2012) Chemical constituents of *Rourea doniana*. *Brazilian Journal of Pharmacognosy*, **22(2)**, 451–454.

9. Zhang, K. M., Jiang, J. Q. and Kong, L. (2008) Chemical constituents from *Rourea microphylla*. *Chinese Journal of Natural Medicines*, **6(5)**, 345–347.

10. Laikowski, M. M., dos Santos, P. R., Souza, D. M., Minetto, L., Girondi, N., Pires, C., Alano, G., Roesch-Ely, M., Tasso, L. and Moura, S. (2017) *Rourea cuspidata*: Chemical composition and hypoglycemic activity. *Asian Pacific Journal of Tropical Biomedicine*, **7(8)**, 712–718.

11. Thuc, D. N., Thuy, V. T., Mai, V. T. H., Thanh, L. N. and Quan, V. V. (2020) Chemical constituents from ethyl acetate extract of the stems of *Rourea oligophlebia* Merr. *Vietnam Journal of Chemistry*, **58(3)**, 298–301.

12. Baş, D. and Boyaci, I. H. (2007) Modeling and optimization I: Usability of response surface methodology. *Journal of Food Engineering*, **78(3)**, 836–845.

13. Silva, E. M., Rogez, H. and Larondelle, Y. (2007) Optimization of extraction of phenolics from *Inga edulis* leaves using response surface methodology. *Separation and Purification Technology*, **55(3)**, 381–387.

14. Singleton, V. L., Orthofer, R. and Lamuela-Raventós, R. M. (1999) [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology*, **299**, 152–178.

15. Chang, C. -C., Yang, M. -H., Wen, H. -M. and Chern, J. -C. (2022) Estimation of total flavonoid content in propolis by two complementary colometric methods. *Journal of Food and Drug Analysis*, **10(3)**, 178–182.

16. Baliyan, S., Mukherjee, R., Priyadarshini, A., Vibhuti, A., Gupta, A., Pandey, R. P. and Chang, C. M. (2022) Determination of Antioxidants by DPPH Radical Scavenging Activity and Quantitative Phytochemical Analysis of *Ficus religiosa*. *Molecules*, **27(4)**, 1–19.

17. Cacace, J. E. and Mazza, G. J. (2003) Mass transfer process during extraction of phenolic compounds from milled berries. *Journal of Food Engineering*, **59(4)**, 379–389.

18. Henrique, S. A., Gustavo, A. P. and Glaucia, M. P. (2016) Optimization of Extraction Parameters of Total Phenolics from *Annona crassiflora* Mart. (Araticum) Fruits Using Response Surface

Methodology. Food Analytical Methods, **10**, 100–110.

19. Dranca, F. and Oroian, M. (2016) Optimization of ultrasound-assisted extraction of total monomeric anthocyanin (TMA) and total phenolic content (TPC) from eggplant (*Solanum melongena* L.) peel. *Ultrasonics Sonochemistry*, **31**, 637–646.

20. Plaskova, A., Mlcek, J. (2023) New insights of the application of water or ethanol-water plant extract rich in active compounds in food. *Frontiers in Nutrition*, **2023(10)**, 1–23.

21. Zulkifli, S. A., Abd Gani, S. S., Zaidan, U. H. and Halmi, M. I. E. (2020) Optimization of Total Phenolic and Flavonoid Contents of Defatted Pitaya (Hylocereus polyrhizus) Seed Extract and Its Antioxidant Properties. *Molecules*, **25(4)**, 787, 1–17.

22. Gong, Y., Hou, Z., Gao, Y., Xue, Y., Liu, X. and Liu, G. (2012) Optimization of extraction parameters of bioactive components from defatted marigold (*Tagetes erecta* L.) residue using response surface methodology. *Food and Bioproducts Processing*, **90(1)**, 9–16.

23. Chi, T. P., Trang, B. T., Trinh, N. T. N., Tho, L. T. C., Thanh, N. T., Thang, T. D., Hang, H. T. L., Quang, L. D. and Tuan, N. N. (2022) Optimization of Ultrasonic-Assisted Extraction of Antioxidant Compounds in Black Shallots (*Allium ascalonicum*) from Vietnam using Response Surface Methodology. *Malaysian Journal of Chemistry*, **24(1)**, 26–35.

24. Ahmed, T., Rana, M. R., Maisha, M. R., Sayem, A. S. M., Rahman, M., Ara, R. (2022) Optimization of ultrasound-assisted extraction of phenolic content & antioxidant activity of hog plum (*Spondias pinnata* L. f. kurz) pulp by response surface methodology. *Heliyon*, **8(10)**, e11109, 1–17.

25. Uyen, L. P., Nhan, V. T. T., Hung, N. D., Sang, V. T. (2019) Impact of extract conditions on total phenolic compounds and antioxidant activities of Phu Quoc sim fruits (*rhodomyrtus tomentosa* (ait.) hassk.). *Journal of Science and Technology - Nguyen Tat Thanh University*, **2(1)**, 25–31.