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Optimization of Extraction Condition and the Antioxidant Activity of *Momordica charantia* Leaves from Vietnam

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ABSTRACT

The growing interest in natural bioactive compounds has emphasized the need for efficient extraction techniques to maximize the yield of key phytochemicals. This study explores the ideal parameters, including extraction temperature, solvent concentration, and extraction duration, to optimize the extraction process and achieve maximum yields of total flavonoid content (TFC) and total phenolic content (TPC) from *Momordica charantia* leaves. Utilizing response surface methodology (RSM), the extraction process was optimized, revealing that the polynomial expressions for every model demonstrated significance in the obtained results. The result shows that the TPC (87.85 ± 0.24 mgGAE/g DW) and TFC (12.68 ± 0.17 mgQE/g DW) could be extracted higher in the optimum operating conditions at 72°C for 70 min and 70% ethanol concentration.

Keywords: *Momordica charantia*, Extraction, Total phenolic content, Total flavonoid content, Response surface methodology.

Introduction

Momordica charantia is commonly recognized by various names such as bitter melon, bitter gourd, pare, balsam pear, or karela.[1] It has been utilized both as a vegetable and a medicinal remedy for an extended period.[2] Cultivation of it is widespread in tropical and subtropical areas, including Vietnam, India, China, the Middle East, tropical Africa, Thailand, and the Americas. Cultivated through seeds, bitter gourd vines typically bloom within a month, with mature fruits appearing approximately two weeks later. In Vietnam, *Momordica charantia*'s size common is 15 – 25 cm long. It is harvested like squash, and it is ingested after being cooked while still in the green or early yellowing stage. Not only fruit, but all parts of the plant taste bitter.[3] In the traditional medicinal, *Momordica charantia* has been used in a variety of types such as fresh, juice, dry powders, dry slices, or fluid extracts. Besides, bitter gourd is commonly used for many different purposes such as making daily dishes, drinking water, bathing water, etc.

Previous reports have been published on the impact of bitter melon on the management of various illnesses such as anti-ulcerogenic,[4] antidiabetic,[5] anti-tumor,[6] anti-mutagenic,[7] immune-modulatory activities,[8] antioxidant,[9] anti-lipolytic, analgesic,[10] etc. Leaf extracts have antibacterial activities against *Staphylococcus*, *E. coli*, *Pseudomonas*, *Streptobacillus*, *Streptococcus*, and *Salmonella*.[11] The response surface methodology (RSM) is a potent mathematical system extensively employed across various industries for optimizing experimental conditions in technological operations.

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Because it evaluates the simultaneous influence of multiple factors and their interactions impacting one or more response variables, response surface methodology (RSM) is useful in optimizing or decreasing a variety of manipulated variables. Moreover, beyond serving as a visual aid for better clarity and understanding of the impacts of different factors on the extraction process, RSM also aids in identifying the optimal extraction region. Hence, in this study, the RSM technique was applied to pinpoint the optimal extraction conditions (time, temperature, and ethanol concentration) for achieving the highest concentration of flavonoid and phenolic compounds from *Momordica charantia* leaves.

Materials and Methods

Collection and Identification of Plant materials

Momordica charantia leaves were collected in September 2022 from the Con Cuong District of Nghe An Province, Vietnam (19.0554°N, 104.8784°E) and authenticated by the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology (VNMN202308). The dried leaves were milled to 2-3 mm, packed in a polyethylene (PE) pail (moisture content about 8-10%), and kept at a temperature range of 4-6°C until utilization.

Chemicals

The following chemicals - sodium carbonate, quercetin, aluminum chloride, ethanol, Folin-Ciocalteu's phenol reagent, and gallic acid were procured from Sigma-Aldrich (St. Louis, MO, USA). The remaining chemical reagents utilized in this study met the standards of analytical-grade quality

Plant extraction

Following drying with a heat pump drier at 40-50°C, the leaves were pulverized into a powder employing a mechanical mixer. Approximately 1g of the powdered dry leaves was precisely measured into a 60 mL spherical flask and combined with the solvent extract. Reflux systems with a temperature regulator and digital timer were part of the extraction process. To ascertain the extraction yield of TPC and TFC, the resultant extract was next filtered using conventional filtering using Whatman filter paper.

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Experimental design

Response surface methodology (RSM)'s central composite design (CCD) was applied in this study's experimental design. Stat-Ease Inc., located in Minneapolis, USA, produced the Design Expert program version 11.0.0, which was used to process the data. There are six repeats at the key points in each of the twenty experimental trials that make up the three-factor/five-level design. Temperature extraction (X_1, °C), and time extraction (X_2, min) and ethanol concentration (X_3, % v/v) are the three independent variables taken into account. Table 1 specifically presents the coded and non-coded forms of the five levels of values allocated to the independent variables. The polynomial regression equation for the TPC and TFC data obtained under various extraction conditions is formulated in the following equations (Eq. 1);

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

where Xi and Xj stand for the levels of the independent variables, where k is the number of variables, and Y is the response variables that need to be modeled. The coefficients consist of βo, which is the constant; βi, βii, and βij for linear and quadratic terms, respectively.

Table 1: Separate test variables, along with their coded and non-coded values, were employed for the Central Composite Design (CCD) matrix

Variables	Units	Coded and uncoded level of variables				
		-α	**-1**	**0**	**+1**	**+α**
Extraction Temperature	ºC	53.2	60	70	80	86.8
Extraction Time	min	49.8	60	75	90	100.2
Ethanol concentration	% (v/v)	43.2	50	60	70	76.8

TPC determination

The *Momordica charantia* leaf extracts' TPC was measured using a modified version of Singleton's methodology.[12] This method relies on evaluating the color change brought about by the reagent when sodium carbonate and phenolates are present. To make 5 ml of Folin-Ciocalteu's solution, mix 1 ml of the sample. After three minutes, add four milliliters of a 7.5% Na_2CO_3 solution to the mixture and dilute it with ten milliliters of deionized water. Let the mixture stand for 60 minutes at ambient temperature in the dark. The Agilent 8453 UV-Visible Spectrophotometer can be used to measure the color change by scanning the wavelength at 765 nm, which is the wavelength of maximum absorption. Using the established standard curve, the TPC of the *Momordica charantia* leaves extract was measured in milligrams of gallic acid. The results were given as mg GAE/g DW, or milligrams of gallic acid equivalent per gram of dry weight.

TFC determination

After making several adjustments, the total flavonoid content (TFC) of the *Momordica charantia* leaf extracts was determined using the methodology described by Chang.[13] 0.5 mL of 10% aluminum chloride, 1.5 mL of 75% ethanol, 2.8 mL of distilled water, and 0.1 mL of 0.1 M potassium acetate were mixed to create the *Momordica charantia* leaf extracts. The Agilent 8453 UV-Visible Spectrophotometer was used to measure the absorbance of the reaction mixtures at 415 nm after the mixture was left to incubate for 30 minutes at ambient temperature. The benchmark chemical used is quercetin, and the TFC is expressed as milligrams of quercetin equivalent per gram of dry material (mg QE/g DW).

Determination of DPPH radical scavenging activity

Using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, the antioxidant activity of *Momordica charantia* leaf extracts was evaluated. This method was modified from Xu and Chang's protocol.[14] 5.9 mg of DPPH should be dissolved in 100 ml of ethanol to create the DPPH solution. Specifically, 0.2 ml of the *Momordica charantia* leaf extract was mixed with 3.8 ml of the ethanolic DPPH solution. After giving the mixture a good shake for a minute, let it stand for half an hour at ambient temperature in the dark. Using the Agilent 8453 UV-Visible Spectrophotometer blank reagent as a reference, measure the absorbance at 517 nm. Three separate experiments were used to perform each measurement. The following equation (2) was used to compute the radical scavenging activity:

$$\text{Radical scavenging activity (\%)} = [1 - (Abs_{sample}/Abs_{control})] \times 100 \quad \ldots\ldots\ldots (2)$$

Inhibition activity against ABTS radical cation

Momordica charantia leaf extract's ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) radical cation inhibition activity was evaluated using methods somewhat modified from Biglari and Lo.[15,16] The ABTS reagent is made by combining 88 µl of 140 mM potassium persulfate ($K_2S_2O_8$) with 5 ml of 7 mM ABTS solution. After obtaining the mixture, it is put in a dark bottle and left at ambient temperature for sixteen hours. The Agilent 8453 UV-Visible Spectrophotometer was then used to standardize the absorbance of the ABTS reagent to 0.70 ± 0.05 at the wavelength of 734 nm using 95% ethanol. Ten microliters of the *Momordica charantia* leaf extract were mixed with about one milliliter of the ABTS reagent. After adding, the mixture was left to stand at ambient temperature for six minutes. At 734 nm, absorbance was measured using the Agilent 8453 UV-Visible Spectrophotometer in comparison to the blank reagent. Three separate experiments were used to perform each measurement. The radical inhibition activity was determined using the following equation (Eq. 3):

$$\text{Radical inhibition activity (\%)} = [1 - (Abs_{sample}/Abs_{control})] \times 100 \quad \ldots\ldots\ldots\ldots\ldots (3)$$

Results and Discussion

Fitting the response surface models

Using the Central Composite Design (CCD) to optimize the extraction process and establish relationships between response functions and process variables. The experimental design for the three associated response variables is shown in Table 2. In this study, the sequential sum of squares for the model was employed to select the models with polynomials of the maximum degree. Following the software recommendation, a second-degree polynomial model was selected and demonstrated the best fit for all three variables and independent responses.

Second-degree polynomial equations [Eqs. (3–4)] can be used to depict the experimental regression model, which shows the association between the responses and three variables assessed for phenolic and flavonoid content:

$$Y_1 = 86.45 + 5.36X_1 + 1.51X_2 + 3.71X_3 - 3.86X_1X_2 - 0.86X_1X_3 - 2.50X_2X_3 + 1.79X_1^2 - 1.22X_2^2 - 3.10X_3^2 \quad (3)$$

$$Y_2 = 12.96 - 0.015X_1 - 0.02X_2 + 0.09X_3 - 0.01X_1X_2 - 0.06X_1X_3 + 0.048X_2X_3 + 0.058X_1^2 + 0.019X_2^2 - 0.001X_3^2 \quad (4)$$

where X_1 is the temperature extraction, X_2 is the time extraction, X_3 is the ethanol concentration, Y_1 is total phenolic content, and Y_2 is total flavonoid content. The alternating negative and positive signs within each equation signify antagonistic and synergistic effects of the variables, respectively.

The parameters of the response surface methodology model were validated through analysis of variance (ANOVA) for the second-degree

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polynomial model, as summarized in Table 3. Upon conducting ANOVA, terms associated with the second-degree polynomial parameters model, exhibiting small p-values and large F-values, were suggestive of a more substantial impact on the corresponding response variables. The software was employed to create 3D surface plots depicting the fitted polynomial regression equations, offering a more vivid representation of how independent variables interact with responses. According to the results, TPC and TFC had coefficients of determination (R^2) of 0.9934 and 0.9879, respectively, indicating a well-fitted model. R^2 is defined as the percentage of explained variation to the total variation. The TPC and TFC exhibited coefficients of variation (C.V) at 0.9670 and 0.1324, respectively. A lower CV value suggests that the response model is more reliable. It was noted that the Lack of Fit did not demonstrate significance ($p < 0.05$) across all the examined models, affirming that the model fitness remained within the chosen range and was significant ($p < 0.05$) concerning the factors' effects.

Table 2: The experimental data obtained for the three responses based on the CCD matrix

Run no	Type	Extraction Temperature	Extraction Time	Ethanol concentration	Total phenolic content	Total flavonoid content
		X_1- ºC	X_2-min	X_3-%v/v	mgGAE/g	mgQE/g
1	Fact	80	60	70	98.21	12.91
2	Fact	80	60	50	86.10	12.94
3	Fact	80	90	50	87.17	12.75
4	Fact	60	90	70	86.56	13.11
5	Center	70	75	60	85.45	12.97
6	Fact	60	60	50	67.01	12.81
7	Center	70	75	60	86.80	12.96
8	Axial	70	49.8	60	79.57	13.04
9	Center	70	75	60	86.57	12.96
10	Center	70	75	60	86.49	12.94
11	Fact	80	90	70	86.78	12.95
12	Axial	70	100.2	60	85.82	12.99
13	Axial	86.8	75	60	100.02	12.78
14	Fact	60	90	50	81.01	12.71
15	Center	70	75	60	86.80	12.95
16	Center	70	75	60	86.67	12.96
17	Fact	60	60	70	80.08	13.06
18	Axial	53.2	75	60	82.39	12.82
19	Axial	70	75	43.2	71.35	12.79
20	Axial	70	75	76.8	83.42	13.08

Table 3: The projected second-order polynomial models' regression coefficients for the TPC and TFC

Source	Y_1 – TPC			Y_2 – TFC		
	Mean Square	F-value	p-value	Mean Square	F-value	p-value
Model	111.89	166.73	$< 0.0001^S$	0.0266	90.64	$< 0.0001^S$
X_1	392.88	585.46	$< 0.0001^S$	0.0031	10.74	0.0083^S
X_2	31.17	46.44	$< 0.0001^S$	0.0059	20.18	0.0012^S
X_3	187.77	279.81	$< 0.0001^S$	0.1252	427.50	$< 0.0001^S$
X_1X_2	118.89	177.16	$< 0.0001^S$	0.0013	4.27	0.0657^{NS}
X_1X_3	5.95	8.87	0.0139^S	0.0288	98.32	$< 0.0001^S$
X_2X_3	50.10	74.66	$< 0.0001^S$	0.0180	61.62	$< 0.0001^S$
X_1^2	45.95	68.47	$< 0.0001^S$	0.0474	161.76	$< 0.0001^S$
X_2^2	21.56	32.13	0.0002^S	0.0050	17.16	0.0020^S
X_3^2	138.54	206.45	$< 0.0001^S$	0.0013	4.54	0.0589^{NS}
Lack of Fit	1.08	4.13	0.0729^{NS}	0.0005	4.49	0.0624^{NS}
R^2	0.9934			0.9879		
Adjusted R^2	0.9874			0.9770		
C.V%	0.9670			0.1324		

S: significant ($p < 0.05$); NS: non-significant

Response surface analysis

The extraction conditions for achieving maximum TPC and TFC are influenced by three factors, namely extraction time, extraction temperature, and ethanol concentration. In the respective figures, three-dimensional model graphs display the surface response plots, demonstrating the fluctuation of two variables within the explored experimental range, while maintaining the other variable at its central level (0 levels).

Effects of process variables on the TPC

According to the data, the TPC measured in gallic acid equivalent (GAE) and extracted from *Momordica charantia* leaves ranged from 67.01 to 100.02. The results indicate that the TPC was highest in experimental run number 13 and lowest in experimental run number 6. The model's significance was indicated by the analysis of variance (ANOVA), which revealed a model F-value of 166.73 with a probability ($p < 0.0001$). This implies that the likelihood that a large F-

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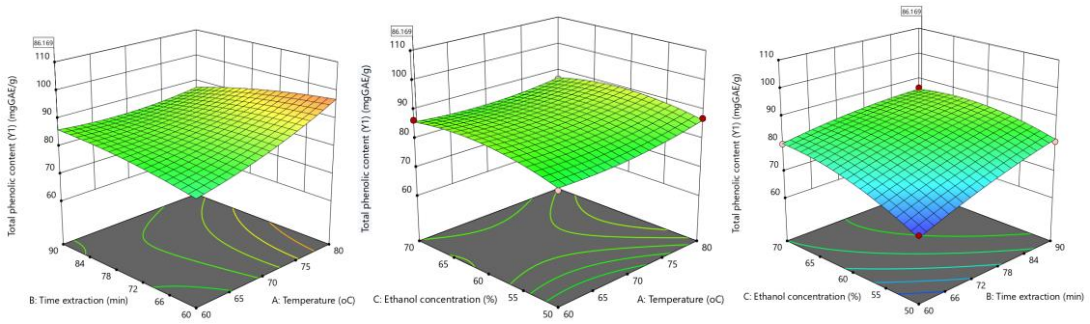
value might result from random variability alone is only 0.01%. Table 3 shows that all three linear parameters (X_1, X_2, X_3), interaction parameters (X_1X_3, X_2X_3), and quadratic parameters (X_1^2, X_2^2) had significant effects on the Total Phenolic Content (TPC) at ($p < 0.05$)

In Figure 1, the illustration depicts the effects of the variables and their interactions on the response. Figure 1a is a surface plot describing the effect of extraction temperature over time on TPC at a constant ethanol concentration of 60%. The phenolic content increases with the elevation of both temperature and extraction time. The temperature range of 75–80°C and the shortest extraction period of 60–75 minutes are conducive to achieving the maximum concentration of phenolics. However, there is no significant improvement in the total phenolic concentration when maintaining the temperature at the highest level of 80°C with an extended extraction time of up to 90 minutes, as the values decrease. Therefore, in this study, opting for a high temperature within a short duration was chosen to prevent the reduction of total phenolic content. In the short term, an increase in temperature will elevate the phenolic content. However, over an extended period, the effect is reversed due to the susceptibility of polyphenols to oxidation or degradation.[17] Moreover, as reported by Vajić and Chi, prolonged extraction time enhances the solubility of phenolics according to the second law of Fick's diffusion, suggesting that after a certain duration, an equilibrium state of the extraction process will be achieved.[18,19]

In Figure 1b, with a fixed extraction time of 75 minutes, the influence of temperature and ethanol concentration on the Total Phenolic Content (TPC) is depicted. The surface plot shows that in comparison to the lower ethanol concentration (50%) at a constant extraction temperature, the maximal phenolic content can be reached at the highest ethanol concentration (80%). Due to the polar nature of the solvents used, the phenolic content increases significantly.[20] Water and ethanol were used in this investigation since they are both safe and conducive to human health. This is akin to a study on phenolics conducted on spinach. Applying higher temperatures and sufficient solvent concentrations can potentially result in the softening of plant tissues, subsequently enhancing diffusion rates and increasing the production of phenolic compounds. However, exceeding a certain threshold, the phenolic content may begin to decrease, reaching a stable level as the extraction process completes and the system attains equilibrium.[21] Therefore, the optimal ethanol concentration is around 60–65% v/v, and the corresponding extraction temperature is 75–80°C for achieving the maximum Total Phenolic Content (TPC) in *Momordica charantia* leaves.

Figure 1c illustrates the surface response plot at a constant temperature of 70°C, depicting the relationship between extraction time and ethanol concentration. The surface plots show that raising the ethanol concentration at a set extraction period might result in a larger total phenolic content (TPC). Based on the results, at a constant extraction time of 90 minutes, 70% ethanol concentration yielded the highest total phenolic content (TPC) compared to 50% ethanol concentration. Nevertheless, an extended extraction time leads to the degradation of phenolic activity in *M. charantia* leaves. Therefore, the optimal TPC extraction process can be achieved when conducted at an ethanol concentration of 60–70% v/v and an extraction time of 75 minutes. Beyond this optimum, TPC has shown a decline.



a) b) c)

Figure 1: The response surface plot of TPC

Effects of process variables on the TFC

Within the overall range of 12.71 to 13.11 mg QE/g extract, the average total flavonoid content (TFC) of *Momordica charantia* leaves extracted under various circumstances is 12.91 mg QE/g extract. Experimental run number 14 had the lowest TFC efficiency, whereas experimental run number 4 had the highest TFC. An analysis of variance (ANOVA) yields a model F-value of 90.64 with a probability ($p < 0.0001$), meaning that the likelihood of noise producing such a high F-value is only 0.01%. Table 3 shows that TPC is strongly influenced at ($p < 0.05$) by all three quadratic parameters (X_1^2, X_2^2, X_3^2), interaction parameters (X_1X_2, X_1X_3, X_2X_3), and linear parameters (X_1, X_2, X_3). The impacts of the factors and their interactions are shown in Figures 2a–c.

A surface response curve at a fixed ethanol concentration of 60% is shown in Figure 2a, which also shows the relationship between extraction temperature and extraction time. The total flavonoid content (TFC) is influenced more by the extraction duration and less by the extraction temperature, according to the 3D plot. At any extraction temperature, the total flavonoid content (TFC) can be increased by prolonging the extraction period. Thus, in this investigation, the ideal concentration of flavonoids was obtained at an extraction temperature of 65–75°C and an extraction duration of 85–90 minutes.

The 3D surface plots in Figure 2b, with a fixed extraction time of 75 minutes, illustrate the interaction between extraction temperature and ethanol concentration. According to statistical research, the most significant factor ($p < 0.0001$) determining the total flavonoid content (TFC) is the concentration of ethanol. It is observed that at a fixed extraction temperature of 60°C, the TFC value increases as the ethanol concentration rises from 50 to 70% v/v. When increasing the extraction temperature at the lowest ethanol concentration, the TFC value increases, while raising the extraction temperature at the highest ethanol concentration results in a decrease in the TFC values can be explained by the increased mobility of particles, leading to the rupture of plant tissues, thereby allowing for higher solvent solubility until reaching a stable state and beginning to degrade towards lower values. TFC shows a diminishing trend above the optimal temperature and increases when the extraction temperature climbs below 70°C. This result aligns with previous reports by Thanh and Lu.[22,23] Thus, the optimal extraction temperature for maximum TFC is 60–70°C with an ethanol concentration of 65–70% v/v.

Figure 2c illustrates the surface response plot at a constant extraction temperature of 70°C, depicting the relationship between extraction time and ethanol concentration. The surface response plots illustrate that ethanol concentration is a predominant factor influencing the total flavonoid content (TFC) obtained from *Momordica charantia* leaves. The disruption of cell membranes, enhancing the solvent permeability into the solid matrix, increases with the rise in ethanol concentration. In this study, at the highest ethanol-water ratio (70%) compared to (50%), the highest total flavonoid content (TFC) is observed when increasing the extraction time from 80 to 120 minutes.

Optimization of extracting parameters and validation of the model

Finding the ideal circumstances to get the highest possible total flavonoid content (TFC) and total phenolic content (TPC) is the aim of this study. The final optimization results show that 72°C for 70 minutes

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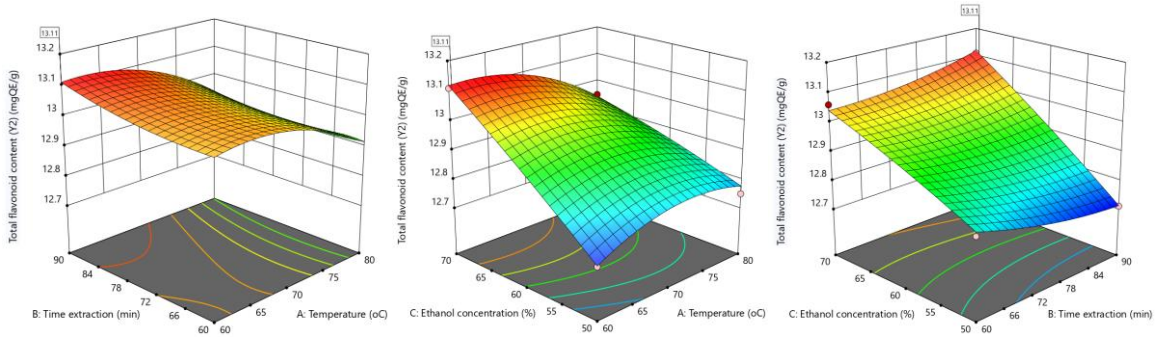
at a 70% ethanol concentration are the ideal ethanolic extraction conditions for *Momordica charantia* leaves. To maximize the total phenolic content (TPC) and total flavonoid content (TFC), the settings that were chosen were chosen. By comparing the surface response plots with the contour plots, which depict the relationship between the independent variables and the response of interest, optimal extraction conditions were determined. The ideal settings were validated by comparing the projected values from the response surface methodology model produced by the Design Expert 11.0.0 program with *M. charantia* leaves treated under the previously indicated optimized circumstances. At a 95% confidence level, the results, as shown in Table 4, show that the expected values of the responses nearly match the experimental values.



a) b) c)

Figure 2: The response surface plot of TFC

Table 4: Predicted and experimental values of responses under optimal conditions

Responses	Optimum extraction conditions			Maximum value		% difference
	X_1	X_2	X_3	Experimental[a]	Predicted	(CV)
TPC (mgGAE/g)	72 °C	70 min	70	87.85±0.24	88.448	0.68
TFC (mgQE/g)			%	12.68±0.17	13.020	2.61

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

DPPH and ABTS determination

Figure 3 illustrates the results of DPPH and ABTS inhibition by the extraction of *M. charantia* leaves under the optimal conditions of the extraction process. The concentration of the extract causing 50% inhibition of absorption, known as the IC_{50} value for each plant extract, was determined. In the current study, the extract from *M. charantia* leaves exhibited a DPPH activity inhibition of 82.15±0.27% and an ABTS activity inhibition of 93.26±0.34%.

Table 5: Experimental values of DPPH and ABTS under optimal conditions of the extraction

Responses[a]	**Extraction temperature**	**Extraction time** (min)	**Ethanol concentration** (%)	**Value**
DPPH (%)	72°C	70	70	82.15±0.27
ABTS (%)	72°C	70	70	93.26±0.34

[a]DPPH: 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability; ABTS: 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical cation inhibition.

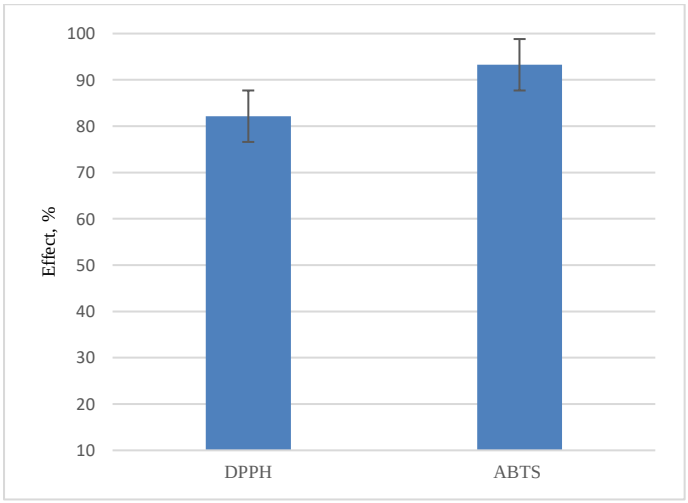


Figure 3: Antiradical activity of the *Momordica charantia* leaves extracts ($p<0.05$)

Conclusion

In conclusion, this study successfully optimized the extraction process for total flavonoid content (TFC) and total phenolic content (TPC) from *Momordica charantia* leaves using response surface methodology (RSM). The findings demonstrate that the optimal extraction conditions—72°C, 70 minutes, and 70% ethanol concentration—yielded maximum TPC (87.85 ± 0.24 mg GAE/g DW) and TFC (12.68 ± 0.17 mg QE/g DW). The significance of the polynomial models confirms the reliability of the optimization process, providing valuable insights for enhancing the efficiency of bioactive compound extraction from *Momordica charantia* leaves.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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