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OPTIMIZATION OF SONICATION-ASSISTED EXTRACTION OF TOTAL FLAVONOID CONTENT AND ANTIOXIDANT ACTIVITY FROM *Celosia cristata* FLOWER BY RESPONSE SURFACE METHODOLOGY

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Abstract

In this work, flavonoid compounds were extracted from *Celosia cristata* flower using a sonication-assisted extraction (SAE) approach. Using response surface methodology (RSM), we optimized the extraction parameters (sonication time, particle size, liquid-solid ratio, and ethanol concentration) to increase the total flavonoid content (TFC) and ferric reducing antioxidant power (FRAP) activity of *C. cristata* flowers extracts (CFEs). The highest TFC (3.39 mg QE/g DW) was reported for 60 min extraction, 80 mesh particle size, 10 ml/g liquid-solid ratios, and 96% ethanol concentration. FRAP activity (175.63 µmol TE/g DW) was higher at 60 min extraction, 80 mesh particle size, 20 ml/g liquid-solid ratio, and 70% ethanol concentration. Overall, this study's findings showed that SAE was suitable and that RSM successfully improved the extraction conditions of flavonoid-antioxidant compounds from CFEs.

Keywords: *Celosia cristata flower, FRAP antioxidant, response surface methodology, sonication-assisted extraction, total flavonoid content*

Introduction

Chicken's comb flower (*Celosia cristata*) is a herbaceous plant of the Amaranthaceae family, which in one clump there are several main stems producing flowers, elongated heart-shaped leaves, and jagged edges[1]. The plant, in general, was found to contain phenolic compounds, tannins, flavonoids, and sterols. Various authors have published reports on identification and isolation of different compounds from the seeds and leaves of the plant[2]. In particular, its dry mature seeds are commonly used in traditional Chinese medicine to treat diseases as hypertension, palsy, cataract, diabetes, and caligo corneae[3].

Sonication or ultrasound-assisted extraction to break cell membranes has the advantage of reducing extraction time and increasing extract yield. However, the application of ultrasound damages the cell wall structure and accelerates the diffusion through the membrane[4]. Additionally, the extraction process is affected by the quality, technique, extraction time, temperature, type of solvent, solvent concentration, and material-solvent ratio[5]. The duration of the extraction process, the material-solvent ratio, the amount of extractant involved in the transfer determine the level of concentration differences, which are very important in the diffusion process, and can affect the content of compounds[6].

Response Surface Methodology (RSM) is a statistical and mathematical method used for modeling and analyzing problems, where several variables affect a response, the aim is to optimize the response[7]. The Central Composite Design (CCD), alongside the Box-Behnken, are the most widely used designs. This study, however, used the Box-Behnken technique because of its efficiency with fewer trial runs, especially for experiments with 3 or 4 factors[8]. According to Soraya[9] and Guntima[10], RSM succeeded in increasing the phenolic and antioxidant content of Lindau and Chromolaena odorata leaves. However, optimization data for *C. cristata* flowers is still lacking. *C. cristata* flowers extraction is influenced by four factors: extraction time, particle size, liquid-solid ratio, and ethanol concentration. Therefore this study aimed to develop a procedure to determine the optimal extraction of C. cristata flowers for a higher yield of flavonoid content and antioxidant activity.

Methods

Sample Preparation and Extraction

Samples of *C. cristata* flowers were separated from the dirt and washed thoroughly. The flower seeds are separated so that what is left is only the flower part. All samples were drained and dried in an oven at 40-50 °C for 4-5 days until the moisture content remained constant. The dry samples were then mashed with a blender and sieved with sizes of 60, 80, and 100 mesh. Simplicia weighed as much as 2 g and extracted using ethanol as much as 10 mL. Homogenization was carried out using a vortex for 15 minutes, then sonication was carried out with a sonicator. Then it was centrifuged at 10,000 g 4 °C for 15 minutes to obtain filtrate 1 and the precipitate. The extraction process was repeated on the resulting precipitate to obtain the second filtrate, the two filtrates were combined for the final sample[11]. The sample was dried by steam using a rotary evaporator until a final volume of 10 mL (0.2 g/mL) was obtained, then the sample was stored at -4 °C.

Experimental Design

The extraction optimization stage was to obtain the best extract rich in flavonoids and antioxidant activity. The research design was carried out using Response Surface Methodology (RSM). The Box-Behnken design (BBD) was chosen as a design with four factors and three levels. The independent variables or factors used in the extraction process are sonication time (A = 15, 45, 60 minutes), particle size (B = 60, 80, 100 mesh), liquid-solid ratio (C = 1:5, 1:10 , 1:20 g/ml) and ethanol concentrations (D = 50, 70, and 96%). Meanwhile, the dependent variables were total flavonoid levels and antioxidant activity. The 30 extracts from the Box-Behnken design were used to determine total flavonoids and test antioxidant activity using the FRAP method as a quality parameter of the extract[12].

Determination of Total Flavonoid Content (TFC)

C. cristata extract (10 µL) was reacted with 60 µL of methanol solution and 10 µL of 10% AlCl3

(methanol). Additionally, 10 µL of 10% CH3COOK (methanol) solution and 110 µL of distilled water was added to the final solution**,** respectively. The plates were incubated for 30 minutes at 37°C in the dark to allow the reaction.

The total flavonoid content was determined by reading the absorbance at a wavelength of 415 nm using an ELISA reader. Quercetin (dissolved in 80% ethanol) was used as standard at 25 ppm, 50 ppm, 75 ppm, 100 ppm, 125 ppm, 150 ppm, and 200 ppm. The results obtained are expressed in mg QE (Quercetin Equivalent)/g DW[13].

Determination of Antioxidant Activity by FRAP Method

Prepared FRAP reagent from TPTZ 10 mM (in HCl 40 mM), FeCl3 20 mM (in distilled water), and acetate buffer pH 3.6 in a ratio (1:1:10) were incubated for 30 minutes at 37 C in a dark room. A total of 10 L of sample and 300 L of FRAP reagent were put into the microplate. The solution was incubated for 30 minutes at 37 oC. Using the Trolox (µmol TE /g DW) standard[14], the results were subsequently measured by an ELISA reader at a wavelength of 593 nm.

Data Analysis

Extraction optimization results were analysed using Design-Expert software version 13 with the Box-Behnken model to describe the response of the combination of the four factors. The equation model was tested by analysis of variance (ANOVA) with = 0.05[7]. The test equation model is as follows:

$$Y = \beta_0 + \sum_{i=1}^{} \beta_0 Xi + \sum_{i=1}^{} \beta ii Xi^2 + \sum_{1=1,j=2}^{k-1,k} \beta i,jXiXj + \varepsilon$$

Information:

Y = Response, βo = Intercept, βi = Linear coefficient; βii = Quadratic coefficient; βij = Factor interaction coefficient, Xi = Treatment code for the i-th; Xj = Treatment code for the jth factor, k = Number of factors

Results and Discussion

Sample Extraction Optimazion

C. cristata dry powder was extracted using ethanol and influenced by three other variables: particle size, liquid-solid ratio, and sonication time using the combination resulting from the Box-Behnken design, as shown in Table 1. This table also presents the results of the response variables, namely the content of total flavonoids (TFC) and antioxidant activity consisting of FRAP (ferric-reducing antioxidant power).

Table 1: Variables of the Box-Behnken Design and response values as recorded

Runs	Factors				Responses	
	A	B	C	D	TFC	FRAP
1	0	-1	-1	0	1.02	59.017
2	0	-1	0	-1	1.34	100.878
3	-1	0	0	1	1.64	77.544
4	-1	0	1	0	2.31	121.461
5	1	0	1	0	1.69	175.628
6	1	-1	0	0	1.52	121.906
7	0	0	0	0	1.66	80.906
8	1	1	0	0	1.79	124.433
9	0	0	0	0	2.13	111.017
10	0	0	-1	1	0.79	46.878
11	0	0	0	0	1.25	95.822
12	0	1	0	-1	0.77	54.433
13	0	1	1	0	1.15	53.461
14	0	0	-1	-1	0.67	38.794
15	-1	0	-1	0	1.64	71.489
16	1	0	0	-1	0.96	55.767
17	0	0	1	1	1.49	104.794
18	0	1	0	1	1.05	88.156
19	0	0	0	0	1.63	167.128
20	0	0	1	-1	1.50	91.517
21	1	0	-1	0	1.20	92.683
22	-1	-1	0	0	2.56	110.294
23	1	0	0	1	3.39	88.767
24	0	0	0	0	1.25	95.6
25	0	-1	0	1	1.05	117.433
26	0	1	-1	0	0.93	65.461
27	0	0	0	0	1.60	102.794
28	0	-1	1	0	2.02	156.322
29	-1	0	0	-1	1.25	91.711
30	-1	1	0	0	1.20	104.183

A = sonication time (min), B = particle size (mesh), C = liquid-solid ratio (g/mL), D = ethanol concentration (%), TFC = total flavonoids content (QE/g DW), FRAP

= Ferric Reducing Antioxidant Power (µmol TE/g DW)

The results of the TFC values ranged from 0.673 to 3.391 mg QE/g DW, the minimum strength of the reducing antioxidant (FRAP) was 38.794, and the maximum value was 175.628 µmol TE/g DW. The lowest value of each response of TFC and FRAP had the same sonication of 45 minutes, particle size measuring 80 mesh, stomach to dissolved ratio 15g/75mL. The concentration of ethanol used for extraction was 50% for TFC and FRAP. Meanwhile, the highest value of TFC and FRAP responses was 60 minutes. The particle size of the second response is 80 mesh. Sample to solvent ratio 15g/150mL for TFC, 15g/300mL for FRAP. Ethanol concentration 75% for FRAP, 96% for TFC.

Based on the data obtained, the best sonication duration in the design was 60 minutes for flavonoid extraction and FRAP antioxidant assay. The best particle size is 80 mesh. The ratio of solute to solvent is 1:10 (TFC), 1:20 (FRAP), and the concentration of ethanol is 75% (FRAP) and 96% (TFC).

Model Selection on TFC and FRAP

Analysis of variance (ANOVA) for TFC and FRAP, is presented in Table 2. ANOVA was used to evaluate the fitted mathematical model to select the best model for TFC and FRAP at 95% confidence intervals. While the coefficient R^2 is used to evaluate the model's performance, where the model is considered correct if the coefficient value is close to one[15]. The results showed that the R^2 values were 0.707 and 0.715. The TFC and FRAP models are quadratic, explaining 70.7% and 71% of the data variability.

It shows that the four variables can influence the diversity of response values obtained; the remaining percent may be affected by other factors and less thorough during the research process. The adjusted and predicted R^2 values were used to compare the adequacy of the experimental and theoretical results. It was found that the TFC values had a difference in the value of less than 0.2, indicating that the two matched each other[16]. In contrast, the FRAP has a negative value. A negative R^2 prediction implies that the average of the overall scores may be a better predictor of response than the current design model.

Analysis of variance (ANOVA) was used to determine the response of total flavonoid (TFC) and antioxidant capacity (FRAP) produced to respond to the treatment given, such as using sonication time, particle size, liquid-solid ratio, and ethanol concentration in *C. cristata* extraction. The ANOVA results can be seen in Table 2.

F-statistics were used to test the regression model derived from the ANOVA study. The model is considered significant if the calculated F value is higher than the table at a low p-value < 0.05. From the results in Table 2, ANOVA showed significant regression for the quadratic model for TFC (F = 2.580, p = 0.039) and FRAP (F = 2.680, p = 0.034) at the 95% confidence level. In other words, the four independent variables have a significant effect on TFC and FRAP. The P-value of Lack of Fit TFC and FRAP is greater than 0.05, which means no deviations or inaccuracies to the model.

Table 2: ANOVA *Quadratic Model* for TFC and FRAP values

	P	F	R^2	AdjR^2	PreR^2	Adeq Prec	*Lack of Fit*
TFC	0.039	2.58	0.707	0.433	-0.452	6.317	0.204
FRAP	0.034	2.68	0.715	0.448	0.042	7.248	0.820

TFC = total flavonoids content (QE/g DW), FRAP = Ferric Reducing Antioxidant Power (µmol TE/g DW)

Effect of Extraction System on TFC & FRAP

The resulting model to determine the effect of the interaction between sonication time, particle size, liquid-solid ratio, and ethanol concentration on the total flavonoid content and the FRAP antioxidant activity test is quadratic, defined as the code value in Equations 1 and 2:

$$TFC = 1.590 - 0.003A - 0.219B + 0.326C + 0.242D + 0.410AB - 0.043AC + 0.510AD - 0.195BC + 0.143BD - 0.033CD + 0.395A^2 - 0.199B^2 - 0.196C^2 - 0.260D^2 \quad (1)$$

$$FRAP = 108.880 + 6.880A - 14.640B + 27.410C + 7.540D + 2.160AB + 8.240AC + 11.790AD - 27.330BC + 4.290BD + 1.300CD + 7.840A^2 - 2.150B^2 - 11.900C^2 - 27.060D^2 \quad (2)$$

Information: A = sonication time, B = particle size, C = liquid-solid ratio, and D = ethanol concentration

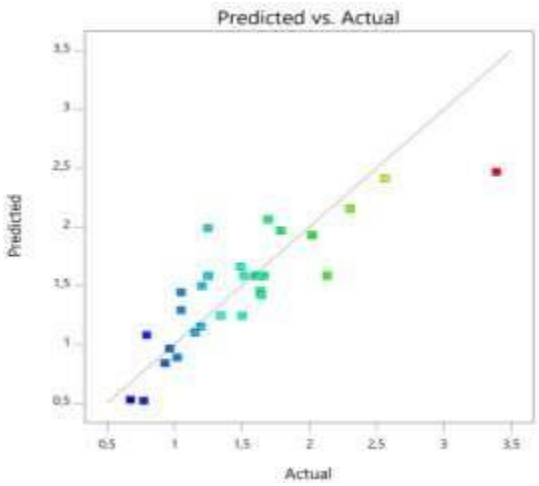
Equation 1 is the response equation given by TFC with a quadratic response model. Based on the p-value of the individual regression coefficient test on linear parameters, the most significant factor affecting the total TFC produced was the liquid-solid ratio with a value of 0.021 followed by ethanol concentration of 0.074, particle size 0.102, and sonication time of 0.981. Based on the interaction parameters between factors, the most significant combination was the interaction between sonication time and ethanol concentration 0.034, sonication time and particle size 0.079, particle size and liquid-solid ratio 0.386, particle size and ethanol concentration 0.521, sonication time and liquid-solid ratio. 0.848 solute and the least influential is the interaction between the liquid-solid ratio and the ethanol concentration of 0.882. The quadratic parameters gave the most significant effect by the quadratic sonication time of 0.032 followed by the quadratic of ethanol concentration 0.139, the size of the simplicia 0.250, and the lowest was the ratio of solvent/solvent 0.258.

Equation 2 is the response equation given by FRAP with a quadratic response model. The linear parameter with the most significant influence based on P-value on total FRAP activity was the liquid-solid ratio with a value of 0.002 followed by the particle size of 0.06, the ethanol concentration of 0.312, and sonication time 0.355.

The interaction parameters between the combination factors that have the most significant effect are the interaction of particle size and liquid-solid ratio 0.045, sonication time and ethanol concentration 0.359, sonication time and liquid-solid ratio 0.519, particle size, and ethanol concentration 0.736, sonication time and particle size 0.865. The lowest effect is the interaction between liquid-solid ratio and ethanol concentration of 0.919. Based on the quadratic parameter, the greatest effect was given by the quadratic of ethanol concentration 0.012, the quadratic ratio of solvent/solute 0.229, the quadratic sonication time 0.424, and the lowest effect was given by the quadratic size of the simplicia 0.825.

Based on the ANOVA results, the four variables can affect the high TFC and FRAP results because almost all the predicted values are close to the actual values. Figure 1 explains just about all the resulting points are adjacent to the diagonal line. It confirms that the regression model equation provides a fairly sure description of the experimental data. The image is used to find outliers, potential values resulting from the design. The color dots represent TFC, and FRAP results placed close to the zero axis indicate no constant error. The points are spread almost equally on the top and bottom x-axis, meaning that the proposed model is good, and there is no reason to suspect an independent violation or constant variance assumption[17].

a



b

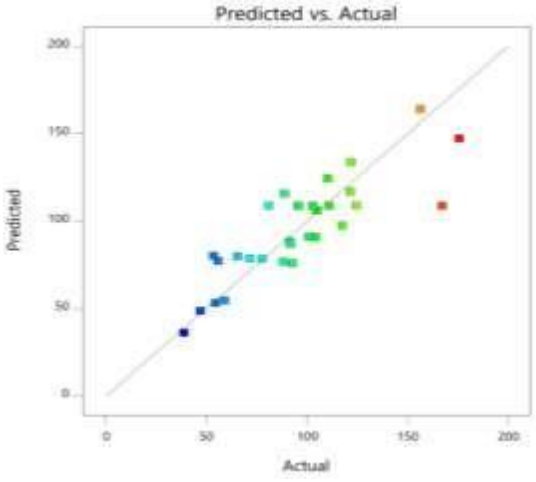
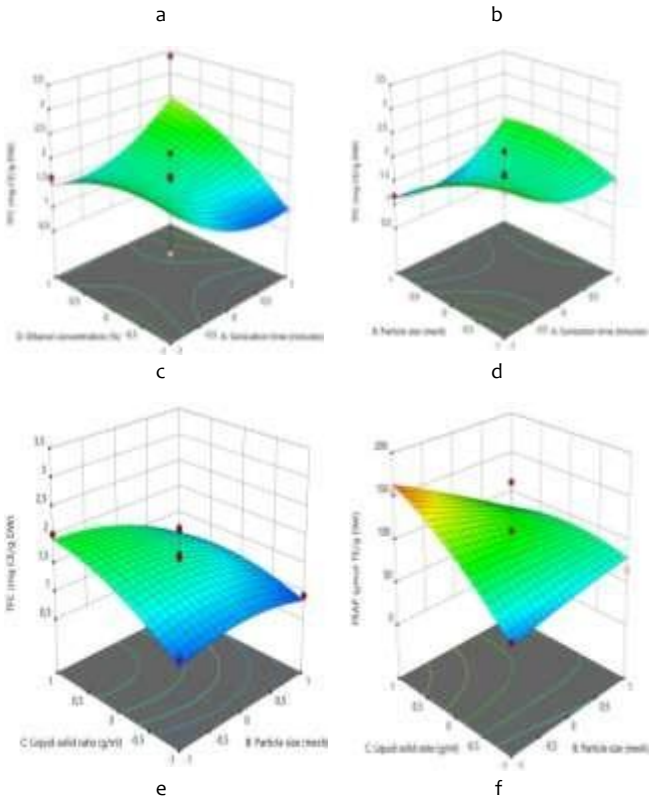


Figure 1: Predicted vs. actual for total flavonoid content (a) and FRAP (b) responses

RSM **Analysis**

Based on the polynomial model equations that have been generated, the experimental data were analyzed using Design-expert software. X and Y in Figure 2 of the three-dimensional response surface represent two factors: sonication time and particle size, sonication time and liquid-solid ratio, ethanol concentration, and sonication time. The Z-axis represents one of the four evaluation indices (TFC or FRAP). A three-dimensional response surface is formed, as depicted in Figure 2.

The response parameter is determined based on the slope of the response surface, and the greater slope, the faster the evaluation index increases. Furthermore, the contour plot reflects the interaction of the two factors. The rounded contour lines indicate a weak interaction of the two factors, and the distorted contour indicates a significant interaction of the two factors[18]. The color difference on the graph shows the total parameters generated. The parameter total will be higher in the red area and lower in the light blue area.



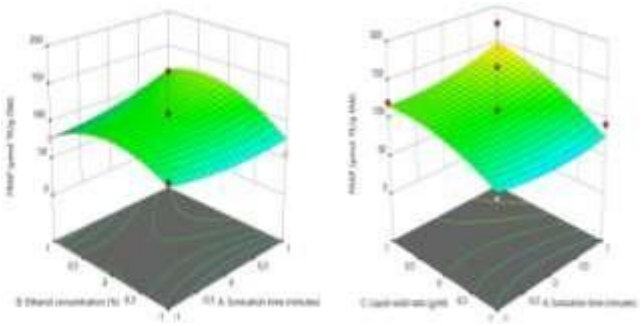


Figure 2: Plot response surface 3D for TFC (a-c) and FRAP (d-f)

Figure 2 (a-c) is the interaction surface between the factors on the TFC response. Figure 2 (a-c) shows that the decrease in particle size (B) and the increase in sonication time (A) of ethanol concentration (D) and liquid-solid ratio (C) led to an increase in the TFC value and vice versa. Figure 2 (d-f) illustrates that decreasing the size of the particle size (A) and increasing the value of sonication time (A), liquid-solid ratio (C), and ethanol concentration (D) increased the value of the FRAP response and vice versa.

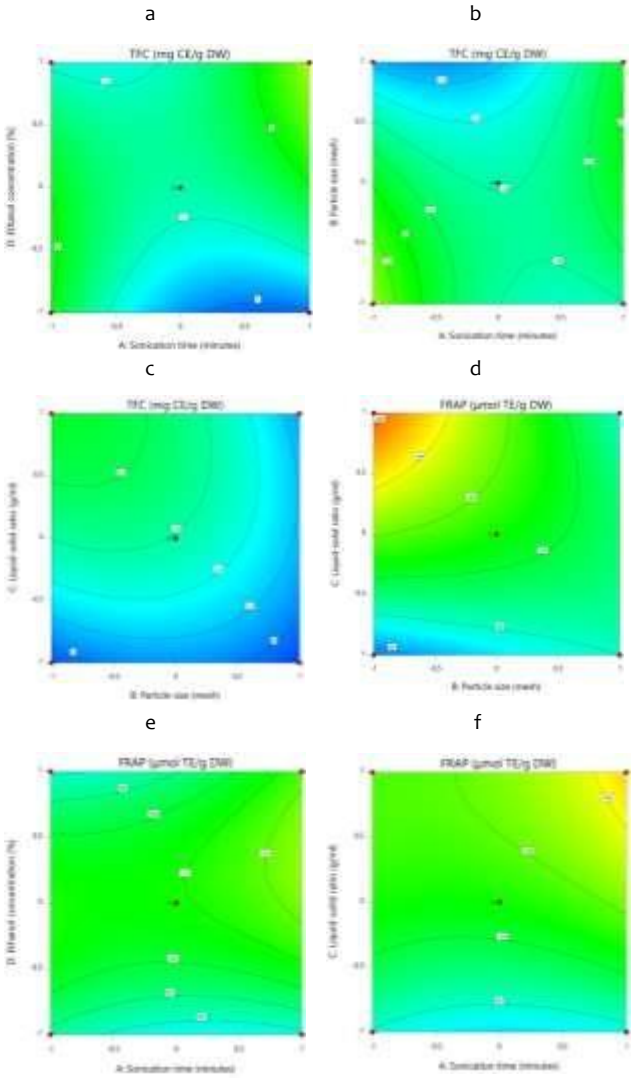


Figure 3: Contour plot for TFC (a-c) and FRAP (d-f)

The contour plot graph in Figure 3 shows a combination of parameters that influence the TFC and FRAP response values through different colors. The lines consisting of the points on the contour plot graph are a combination of four factors, and this combination is formed from the proportion of different factors and produces the same TFC and FRAP values. No rounded lines indicate that the interactions between the factors are not weak in the graph.

This study has the best sonication duration of 60 minutes for flavonoid extraction and FRAP antioxidant test based on the image data. The best particle size is 80 mesh. The ratio of solute to solvent is 1:10 and 1:20, and the ethanol concentration is 75 and 96%.

Optimization and Verification of TFC and Antioxidant Activity of *C. cristata*

The solution from the Design-Expert is to optimize the response of the TFC value and antioxidant activity by determining the criteria for the independent variables and the specified response. The set of experimental variables evaluated was generally selected based on the desirability value, with 1.00 as the highest score[19]. The criteria for determining the sonication time, particle size, liquid-solid ratio, and ethanol concentration were done by choosing the "in range" option. Because it is expected that the extraction process time is carried out at the particle size, temperature, and sonication time close to the midpoint or optimum point to get the TFC value and antioxidant activity at the optimum value. The following criterion is the maximum response because the four reactions are expected to give the highest value, close to each response's upper limit value.

The total response results from TFC range from 0.673 to 3,391, and the FRAP value ranges from 38,794 to 175,628 to maximize the importance of the results, which vary from 1-5. However, the result obtained in this analysis is 3.

Table 3: Optimum point solution and extraction verification of responses

	A	B	C	D	TFC	FRAP	*Desirability*
Predicted	-1	-1	1	-0.37	2.909	157.859	0.846
Actual	15	60	1:20	62.5	2.807	113.529	
P-*value*					0.234	1.776	

A = sonication time (minute), B = particle size (mesh), C = liquid-solid ratio (g/mL), D = ethanol concentration (%), TFC = total flavonoids content (QE/g DW), FRAP = Ferric Reducing Antioxidant Power (µmol TE/g DW)

Table 3 shows the desirability value of 0.846, 1.00 as the highest score[19]. P-value is higher than 0.05; there is no significant difference between the obtained and predicted values. The P-value obtained from TFC and FRAP > 0.05 indicates no significant

difference between the actual and predicted TFC and FRAP values, proving that the model is adequate.

Conclusion

Based on the results of the analysis that has been carried out, it can be concluded that the response surface method was successfully used to optimize the extraction parameters of TF compounds and the FRAP test of *C. cristata* using the Box-Behnken design. The solution to obtain the optimum conditions for the extraction process was obtained with a sonication time of 15 min, the particle size of 60 mesh. The Solid-liquid ratio and ethanol concentrations were 1:20 and 62.5%, respectively, with a desirability level of 0.846. Overall, this study's findings showed that SAE was suitable and that RSM successfully improved the extraction conditions of flavonoid-antioxidant compounds from CFEs.

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