

Optimization of Extraction of Phenolic and Antioxidant Activities from *Celosia cristata* Seeds Using Response Surface Methodology

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Abstract: This study aims to determine the optimal sonication extraction conditions to obtain the highest phenolic and antioxidant compounds from *Celosia cristata* L. (CCL) seeds. Effects of extraction variables including extraction time (15-60 min), particle size (60-100 mesh), solid-solvent ratio (1:5–1:20 g/mL), and ethanol concentration (50-96%) on the total content Phenolic (TPC) and total antioxidant activity (TAC) by DPPH, FRAP, and CUPRAC methods were determined and optimized using the response surface methodology (RSM). Particle size, solid-solvent ratio, and ethanol concentration significantly affected the yield of TPC and TAC, while extraction time had no significant effect on TPC and TAC in the tested ranges. Optimum CCL seed extraction conditions to achieve maximum TPC (1,687mg GAE/g fw) and TAC methods DPPH (4,352 $\mu\text{molTE/g fw}$), FRAP (8,773 $\mu\text{molTE/g fw}$) and CUPRAC (25,757 $\mu\text{molTE/g fw}$) were extraction time of 15 minutes, particle size of 100 mesh, solid-solvent ratio of 1:20 g/mL, and ethanol concentration of 88%. Under these conditions, the experimental values of TPC were 1.32 mg GAE/g fw, TAC-DPPH 3.23 $\mu\text{molTE/g fw}$, TAC-FRAP 6.54 $\mu\text{molTE/g fw}$, and TAC-CUPRAC 25.59 $\mu\text{molTE/g fw}$. Overall, the findings of this study demonstrate the success of RSM in optimizing the extraction conditions of phenolic and antioxidant compounds from CCL seeds.

Keywords: *Celosia cristata*; Response Surface Methodology; antioxidant; phenolic.

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1. Introduction

Plants with high nutritional value have received more attention in recent years due to their function as herbal medicines and dietary supplements to prevent chronic immune disorders. Researchers have claimed that chronic diseases can be treated or prevented using bioactive compounds extracted from plants because plants contain secondary metabolites and pharmacological components [1]. Secondary metabolites have been used as natural

antioxidants to control oxidative stress or cell damage [2]. One of the plants containing secondary metabolites is *Celosia cristata* L. (CCL).

CCL is an annual ornamental plant that belongs to the Amaranthaceae family and is widely distributed in Asia and Africa [3, 4]. In some countries such as Nigeria, China, and India, CCL leaves are widely consumed as nutritious green vegetables. The seeds, leaves, and flowers are also used as traditional medicine. In particular, CCL seeds have long been used for the treatment of hypertension, paralysis, cataracts, thrush, and hemorrhoids [5, 6] as an anti-inflammatory agent [7], and the decoction of the seeds has been reported to be helpful in diabetes mellitus [8].

Pharmacological investigations of CCL plant extracts have been shown to have antioxidant properties [9, 10]. In addition, cristatin and cementoside A, isolated from CCL seeds, have been reported to exhibit hepatoprotective activity in mice [6, 7]. Several studies have reported that CCL seed extract contains secondary metabolites such as flavonoids, glycosides, phenolics, saponins, tannins, triterpenoids, and squalene [6, 7, 11, 12]. A new phenolic compound, 3-geranyl-2,5-dihydroxy-benzaldehyde has been isolated from CCL seeds [13]. In addition, seven phenolic compounds, four flavonoids, three saponins, and one triterpenoid were isolated from CCL seeds [14].

All the gains mentioned above were carried out by the extraction method. Extraction aims to separate active components such as secondary metabolites from a plant using certain solvents, generally used to quantify and isolate compounds [15]. However, bioactive compounds in plant matrices are extracted mainly using conventional extraction methods such as soxhlet and maceration [16, 17]. Conventional extraction techniques require a lot of time and solvent, so they have a low-efficiency level. One of the extraction methods that can be used is sonication. Very high content of bioactive compounds is obtained using the sonication extraction method [18–22], and the extraction time in the sonication method is less than the conventional extraction method. Compared to other modern extraction methods, sonication instruments are cheaper and easier to work with.

Moreover, any solvent can be used to extract various pharmacologically and biologically bioactive compounds and thermosensitive compounds [23]. Therefore, the sonication method can be proposed as an efficient technique for extracting phenolic compounds and antioxidant compounds from chicken's comb seeds. However, the recovery of bioactive compounds from plants is potentially affected by the extraction conditions used, as well as various factors such as solvent type, extraction time and extraction temperature, sample-solvent ratio, sample size, pH known to affect the extraction yield, and the phytochemical content obtained [15, 24].

Although the bioactive properties of chicken's comb seed extract have been studied previously, no research has been conducted to optimize the extraction conditions for bioactive compounds from CCL seeds, so it is necessary to optimize the extraction. The method used in this extraction optimization process is Response Surface Methodology (RSM). RSM is an effective mathematical and statistical tool for performing, enhancing, and optimizing the independent factors that affect the response in a given set of experiments. Several studies have reported using RSM in determining the optimum extraction formulation that can produce optimum extracts in quantity and quality with RSM, such as *Rhododendron arboreum* [22], *Celastrus hindsii* leaf [25], *Hemerocallis citrina* [26], and Lemon Myrtle [27].

Therefore, this study aims to apply the RSM in optimizing extraction variables such as extraction time, ethanol concentration, particle size, and solid-solvent ratio for optimal acquisition of total phenolic content (TPC) and total antioxidant capacity (TAC) of CCL seeds.

2. Materials and Methods

2.1. Making of simplicia.

CCL seeds obtained from Sabisa Farm IPB were dried and oven-dried at 50°C, then blended and sieved to homogenize the simplicia size.

2.2. Optimization of CCL seed extraction.

A Box-Behnken Design (BBD) was used to determine the optimal extraction conditions for extracting metabolites consisting of 4 factors and 3 levels with extraction time, simplicia size, solid-solvent ratio, and ethanol concentration selected independent variables.

Table 1. Extraction variables and their levels used in BBD.

Extraction variable	Extraction variable symbol	Extraction variable level		
		-1	0	+1
Extraction time (minutes)	A	15	30	60
Particle size (mesh)	B	60	80	100
Solid-to-solvent ratio (g/mL)	C	1:5	1:10	1:20
Ethanol concentration (%)	D	50	70	96

2.2.1. CCL seed extraction.

2 g of homogeneous sample was extracted twice with ethanol using a sonicator (Decon, UK). The homogenate was centrifuged for 15 min at 10,000×G at 4°C (Kitman-T24, Japan). The supernatant was concentrated to a volume of 10 mL (final concentration = 0.2 g·mL⁻¹) using a rotary evaporator (HS-2005V, Korea) stored at -20 °C. The extract obtained from the extraction optimization process using RSM was then used for further analysis, namely quantitative analysis of phenolic compounds and analysis of antioxidant activity [28].

2.2.2. Measurement of total phenolic.

A total of 20 µL of sample in a microplate then added 100 µL of Folin-Ciocalteu reagent 50% (with distilled water) and allowed to stand for 5 minutes. A total of 80 µL Na₂CO₃ 7.5% (w/v in aqua dest) was reacted with the sample. Samples were incubated for 2 hours in the dark at room temperature. Then the absorbance was measured using a microplate reader at a wavelength of 750 nm. The total phenolic content is expressed as milligrams (mg) gallic acid equivalent per gram dry weight (mg GAE/g fw (fresh weight)) [29].

2.2.3. Antioxidant activity test.

Antioxidant activity analysis was carried out in vitro using three methods, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH), Cupric Ion Reducing Antioxidant Capacity (CUPRAC), and Ferric Reducing Antioxidant (FRAP).

Antioxidant Activity of DPPH. A total of 100 µL of the microplate sample solution was added with 100 µL of DPPH 125 µM (in ethanol). The solution was incubated for 30

minutes in the dark at room temperature. The solution was measured using a microplate reader at a wavelength of 517 nm. The standard used is Trolox [30].

Antioxidant Activity of CUPRAC. A total of 50 μL of the sample, 50 μL of 0.01 M CuCl_2 (in aquadest), 50 μL of neocuproin 0.0075 μM (in aqua dest), and 50 μL of buffered ammonium acetate pH 7 were placed on a microplate. The solution was incubated for 30 minutes in the dark at room temperature. The solution was measured using a microplate reader at a wavelength of 450 nm. The standard used is Trolox [31].

Antioxidant activity of FRAP. Frap reagent was prepared in advance by mixing 10 mM TPTZ (in 40 mM HCl), 20 mM FeCl_3 (in aqua dest), and acetate buffer pH 3.6 in a ratio (1:1:10). The reagents were incubated for 30 minutes at 37°C in a dark room. Then, 10 μL of the sample was put into a microplate, and 300 μL of FRAP reagent was added. The solution was incubated for 30 minutes at room temperature. The absorbance of the solution was measured using a microplate reader at a wavelength of 593 nm. The standard used is Trolox. Final units are expressed in $\mu\text{mol TE/g fw}$ [32].

2.3. Data analysis.

The optimal extraction conditions were determined using RSM, with three levels and four independent factors (Table 1). Four different responses, such as TPC and TAC of DPPH, FRAP, and CUPRAC as indicators of extraction optimization. Quadratic polynomial models are generated to relate the responses. The second-order polynomial equation resulting from the response is as follows (Equation 1):

$$Y(\text{respon}) = \beta_0 + \sum_{i=1}^4 \beta_i x_i + \sum_{i=1}^4 \beta_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^4 \beta_{ij} x_i x_j \quad (1)$$

Where, Y denotes the response; β_0 , β_i , β_{ii} , and β_{ij} denote the regression coefficients for intercept, linear, quadratic, and interaction, respectively; X_i and X_j represent independent factors; n denotes the number of factors involved [33].

3. Results and Discussion

3.1. Extraction optimization with RSM.

The experimental response values are listed in Table 2. The results showed that the extraction of CCL seeds obtained TPC 0.81-1.95 mgGAE/g fw, TAC-DPPH 0.83-4.65 $\mu\text{mol TE/g fw}$, TAC-FRAP 3.66 - 8.56 $\mu\text{mol TE/g fw}$, and TAC-CUPRAC 5.12 - 25.75 $\mu\text{mol TE/g fw}$. Variations of TPC, TAC-DPPH, TAC-FRAP, and TAC-CUPRAC depend on the extraction conditions used.

Table 2. Experimental design of CCL seed extraction variables for optimization of TPC, TAC-DPPH, TAC-FRAP and TAC-CUPRAC with BBD.

Run	A (minutes)	B (mesh)	C (g/mL)	D (%)	TPC (mgGAE/g)	TAC-DPPH ($\mu\text{mol TE/g}$)	TAC-FRAP ($\mu\text{mol TE/g}$)	TAC-CUPRAC ($\mu\text{mol TE/g}$)
1	30	60	1:5	70	0.87 $\pm$ 0.03	1.03 $\pm$ 0.18	4.66 $\pm$ 0.65	6.56 $\pm$ 0.22
2	30	60	1:10	50	1.19 $\pm$ 0.02	1.17 $\pm$ 0.12	5.72 $\pm$ 0.32	5.90 $\pm$ 0.44
3	15	80	1:10	96	1.00 $\pm$ 0.06	2.54 $\pm$ 0.16	8.48 $\pm$ 0.08	25.75 $\pm$ 0.98
4	15	80	1:20	70	1.47 $\pm$ 0.05	2.77 $\pm$ 0.23	6.60 $\pm$ 0.10	10.27 $\pm$ 0.92
5	60	80	1:20	70	0.84 $\pm$ 0.02	2.79 $\pm$ 0.41	8.49 $\pm$ 0.62	14.78 $\pm$ 1.11

Run	A (minutes)	B (mesh)	C (g/mL)	D (%)	TPC (mgGAE/g)	TAC-DPPH (μ mol TE/g)	TAC-FRAP (μ mol TE/g)	TAC-CUPRAC (μ mol TE/g)
6	60	60	1:10	70	1.29 $\pm$ 0.05	0.83 $\pm$ 0.51	3.66 $\pm$ 0.06	5.12 $\pm$ 0.14
7	30	80	1:10	70	1.29 $\pm$ 0.05	2.80 $\pm$ 0.07	6.15 $\pm$ 0.51	10.74 $\pm$ 1.42
8	60	100	1:10	70	1.41 $\pm$ 0.02	3.40 $\pm$ 0.07	7.60 $\pm$ 0.18	9.66 $\pm$ 0.45
9	30	80	1:10	70	1.12 $\pm$ 0.03	2.51 $\pm$ 0.13	5.42 $\pm$ 0.21	8.37 $\pm$ 0.56
10	30	80	1:5	96	0.91 $\pm$ 0.01	1.02 $\pm$ 0.94	4.83 $\pm$ 0.26	17.63 $\pm$ 1.01
11	30	80	1:10	70	1.08 $\pm$ 0.03	3.35 $\pm$ 0.10	4.03 $\pm$ 0.34	6.37 $\pm$ 0.09
12	30	100	1:10	50	1.56 $\pm$ 0.04	1.75 $\pm$ 1.63	7.04 $\pm$ 0.18	7.61 $\pm$ 0.28
13	30	100	1:20	70	1.95 $\pm$ 0.03	3.08 $\pm$ 1.06	8.47 $\pm$ 0.82	17.62 $\pm$ 0.40
14	30	80	1:5	50	1.08 $\pm$ 0.04	2.47 $\pm$ 0.59	5.60 $\pm$ 0.17	5.51 $\pm$ 0.24
15	15	80	1:5	70	1.23 $\pm$ 0.02	2.54 $\pm$ 1.43	6.34 $\pm$ 0.24	8.53 $\pm$ 1.04
16	60	80	1:10	50	1.12 $\pm$ 0.04	2.60 $\pm$ 0.18	5.34 $\pm$ 0.41	6.67 $\pm$ 0.13
17	30	80	1:20	96	1.11 $\pm$ 0.04	4.00 $\pm$ 0.16	8.56 $\pm$ 1.05	23.77 $\pm$ 0.52
18	30	100	1:10	96	1.15 $\pm$ 0.05	4.65 $\pm$ 0.14	5.24 $\pm$ 0.26	22.13 $\pm$ 0.40
19	30	80	1:10	70	1.34 $\pm$ 0.03	3.96 $\pm$ 0.02	6.74 $\pm$ 0.66	10.85 $\pm$ 0.28
20	30	80	1:20	50	1.41 $\pm$ 0.06	1.30 $\pm$ 0.29	7.30 $\pm$ 0.43	9.88 $\pm$ 0.40
21	60	80	1:5	70	1.20 $\pm$ 0.05	3.13 $\pm$ 0.14	4.98 $\pm$ 0.16	9.45 $\pm$ 0.25
22	15	60	1:10	70	1.14 $\pm$ 0.10	1.98 $\pm$ 0.09	3.74 $\pm$ 0.42	5.94 $\pm$ 0.65
23	60	80	1:10	96	0.81 $\pm$ 0.04	1.82 $\pm$ 0.20	5.21 $\pm$ 0.66	20.15 $\pm$ 1.92
24	30	80	1:10	70	1.26 $\pm$ 0.03	3.25 $\pm$ 0.32	5.22 $\pm$ 0.18	9.42 $\pm$ 0.09
25	30	60	1:10	96	1.03 $\pm$ 0.05	2.34 $\pm$ 0.47	5.68 $\pm$ 0.50	16.43 $\pm$ 0.60
26	30	100	1:5	70	1.14 $\pm$ 0.01	3.23 $\pm$ 0.21	4.98 $\pm$ 0.33	6.25 $\pm$ 0.41
27	30	80	1:10	70	1.24 $\pm$ 0.05	3.36 $\pm$ 0.06	5.30 $\pm$ 0.37	8.69 $\pm$ 0.46
28	30	60	1:20	70	1.30 $\pm$ 0.04	3.11 $\pm$ 0.05	4.98 $\pm$ 0.45	8.78 $\pm$ 0.63
29	15	80	1:10	50	1.30 $\pm$ 0.04	2.15 $\pm$ 0.18	5.81 $\pm$ 0.25	7.57 $\pm$ 0.36
30	15	100	1:10	70	1.37 $\pm$ 0.03	4.01 $\pm$ 0.24	7.11 $\pm$ 0.23	10.42 $\pm$ 0.17

3.2. Model installation and statistical analysis.

ANOVA and multiple regression analysis analyzed experimental data. After all the extraction factors (A, B, C, and D) are taken into account, the model for each response variable is obtained as follows:

$$\text{TPC} = +1.22 - 0.071A + 0.146B + 0.138C - 0.138D - 0.027AB - 0.153AC - 0.004AD + 0.095BC - 0.063BD - 0.034CD - 0.043A^2 + 0.109B^2 - 0.001C^2 - 0.105D^2 \quad (2)$$

$$\text{TAC-DPPH} = +3.21 - 0.117A + 0.805B + 0.303C + 0.409D + 0.133AB - 0.146AC - 0.292AD - 0.555BC + 0.432BD + 1.037CD - 0.269A^2 - 0.268B^2 - 0.281C^2 - 0.615D^2 \quad (3)$$

$$\text{TAC-FRAP} = +5.98 - 0.233A + 0.998B + 1.084C + 0.098D + 0.141AB + 0.814AC - 0.699AD + 0.792BC - 0.442BD + 0.506CD \quad (4)$$

$$\text{TAC-CUPRAC} = +9.074 - 0.221A + 2.079B + 2.599C + 6.893D + 0.015AB + 0.897AC - 1.174AD + 2.284BC + 0.998BD + 0.444CD + 0.485A^2 - 1B^2 + 1.078C^2 + 4.821D^2 \quad (5)$$

Table 3 shows the results of the analysis of variance derived from testing the factors that are significant to the response variable. Table 3 includes the sum of the squares and the probability of significance for the linear effects (A, B, C, and D), the quadratic effects (A^2 , B^2 , C^2 , and D^2), and the interaction effects of the variables (AB, AC, AD, BC, BD, and CD). Based on the table, factors with a probability value of less than 5% have a significant effect; the closer the value is to 0, the higher the influence of this factor.

Table 3. Analysis of the variance of the TPC, TAC-DPPH, TAC-FRAP, and TAC-CUPRAC prediction models for CCL seed extraction.

Source	TPC		TAC-DPPH		TAC-FRAP		TAC-CUPRAC	
	Sum of squares	p>F	Sum of squares	p>F	Sum of squares	p>F	Sum of squares	p>F
Model	1.12	0.029	21.02	0.0096	35.85	0.027	919.98	< 0.0001

Source	TPC		TAC-DPPH		TAC-FRAP		TAC-CUPRAC	
	Sum of squares	p>F	Sum of squares	p>F	Sum of squares	p>F	Sum of squares	p>F
A	0.060	0.169	0.166	0.538	0.650	0.441	0.589	0.687
B	0.255	0.009	7.78	0.0006	11.97	0.004	51.88	0.0016
C	0.229	0.013	1.10	0.126	14.11	0.0022	81.05	0.0002
D	0.228	0.013	2.02	0.044	0.1159	0.743	570.16	< 0.0001
AB	0.003	0.758	0.07	0.687	0.0796	0.786	0.0009	0.988
AC	0.094	0.091	0.086	0.657	2.65	0.131	3.22	0.352
AD	0.0001	0.959	0.34	0.379	1.95	0.190	5.51	0.228
BC	0.036	0.279	1.23	0.106	2.51	0.141	20.87	0.027
BD	0.016	0.473	0.75	0.201	0.7825	0.399	3.98	0.302
CD	0.005	0.693	4.30	0.006	1.03	0.336	0.787	0.642
A ²	0.013	0.521	0.499	0.292			1.61	0.507
B ²	0.082	0.112	0.493	0.294			6.86	0.181
C ²	5.926E-06	0.989	0.543	0.272			7.97	0.151
D ²	0.075	0.127	2.60	0.025			159.38	< 0.0001
Lack of Fit	0.381	0.079	5.00	0.233	16.71	0.373	38.32	0.383
R ²	0.722		0.770		0.631		0.946	
Adeq Precision	7.065		6.709		7.877		15.138	

ANOVA on the prediction model was performed to determine the effect of the extraction variable on the response and examine the effectiveness of the prediction model [34]. The model obtained is quite precise to predict the resulting TPC, TAC-DPPH, TAC-FRAP, and TAC-CUPRAC because it has a small p-value and an insignificant F-Lack of Fit value ($p>0.5$) [35], as well as any the model obtained, has a high adequate precision value (>4.0). R² is closer to 1. The closer the value is to 1, the better the model's performance in predicting the response [36]. This ANOVA result confirms that the obtained model can navigate the design space [37].

3.3. Interaction effect of extraction variables on response variables.

The interaction effect of extraction variables on the response can be identified by a 3-dimensional (3D) contour graph, which is indicated by a color spectrum. The highest projection value is limited by the smallest ellipse in the contour diagram. Elliptical contours are obtained when a perfect interaction exists between the extraction variables [38]. Based on Figures (1), (2), (3), and (4) show that the interactions that occur between the extraction variables in this study are not too striking. The 3D contour graph was formed according to the obtained equations to determine the optimal interaction of the extraction variables to maximize the yield of TPC, TAC-DPPH, TAC-FRAP, and TAC-CUPRAC extracts of chicken's comb seeds.

Figure 1 explains the effect of the interaction of extraction variables on TPC. A TPC of 1,223 mgGAE/g fw was obtained when all extraction variables were at point 0 (A = 30 minutes; B = 80 mesh; C = 1:10 g/mL; D = 70%). TPC increased by 23.82% when A was lowered (15 min) and B was increased (100 mesh) (Figure 1a). The interaction of B and C increased TPC by 32.43% when variables B and C were increased to 100 mesh and 1:20g/mL (Figure 1b). TPC increased by 14.33% during the interaction of C = 1:20g/mL and D = 60% (Figure 1c).

Based on Figure 2 explains the effect of the interaction of extraction variables on TAC-DPPH. A TAC-DPPH of 3.205 μ mol TE/g fw was obtained when all extraction variables were at point 0 (A = 30 min; B = 80 mesh; C = 1:10 g/mL; D = 70%). TAC-DPPH increased by 16.53% when B was increased to 100 mesh at constant A (30 min) (Figure 2a). The interaction of B and C increased TAC-DPPH by 16.48% when variable B was increased to 100 mesh at constant C (1:10g/mL) (Figure 2b). TAC-DPPH increased by 25.44% when increasing C = 1:20 g/mL and D = 96% (Figure 2c).

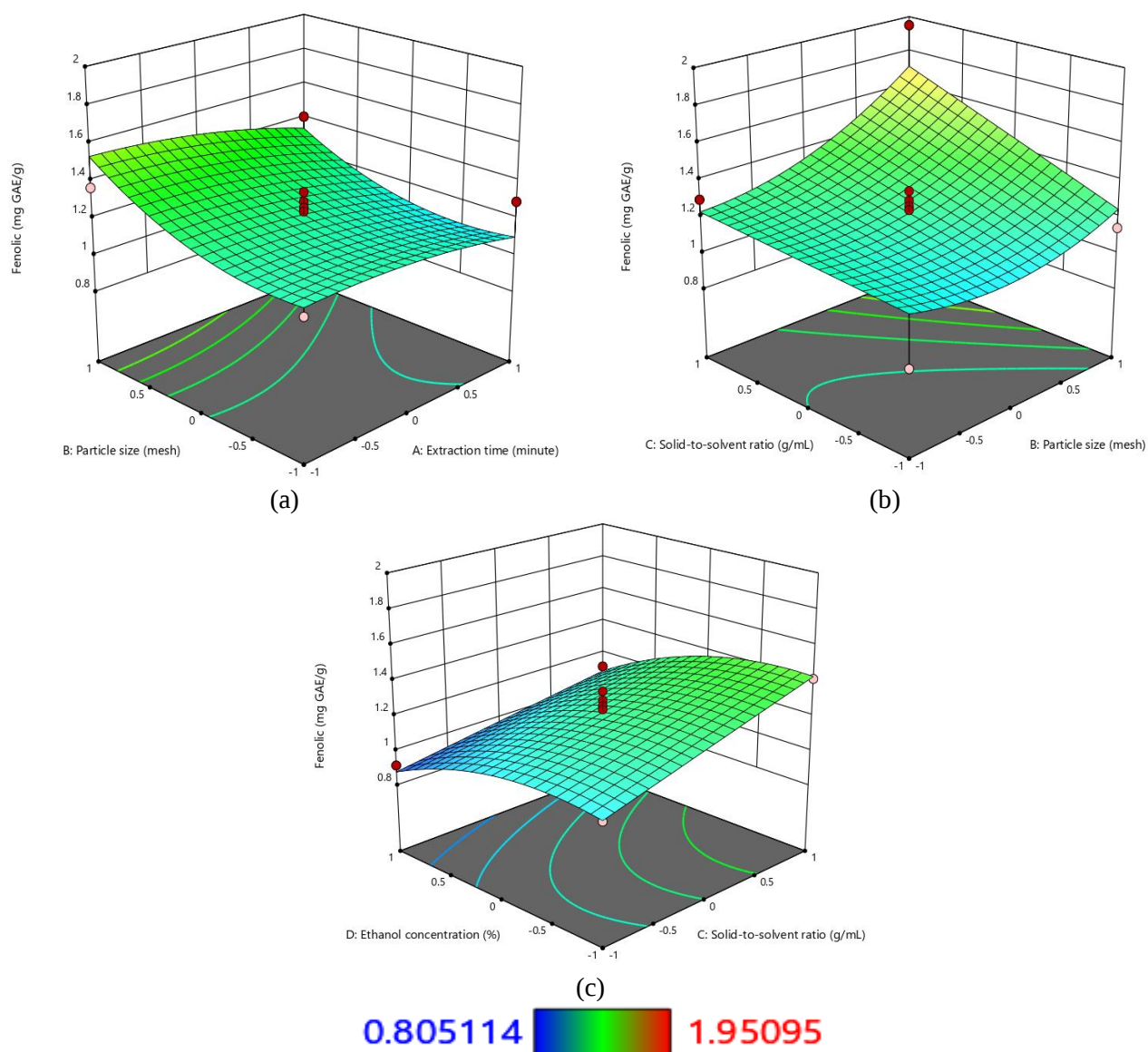
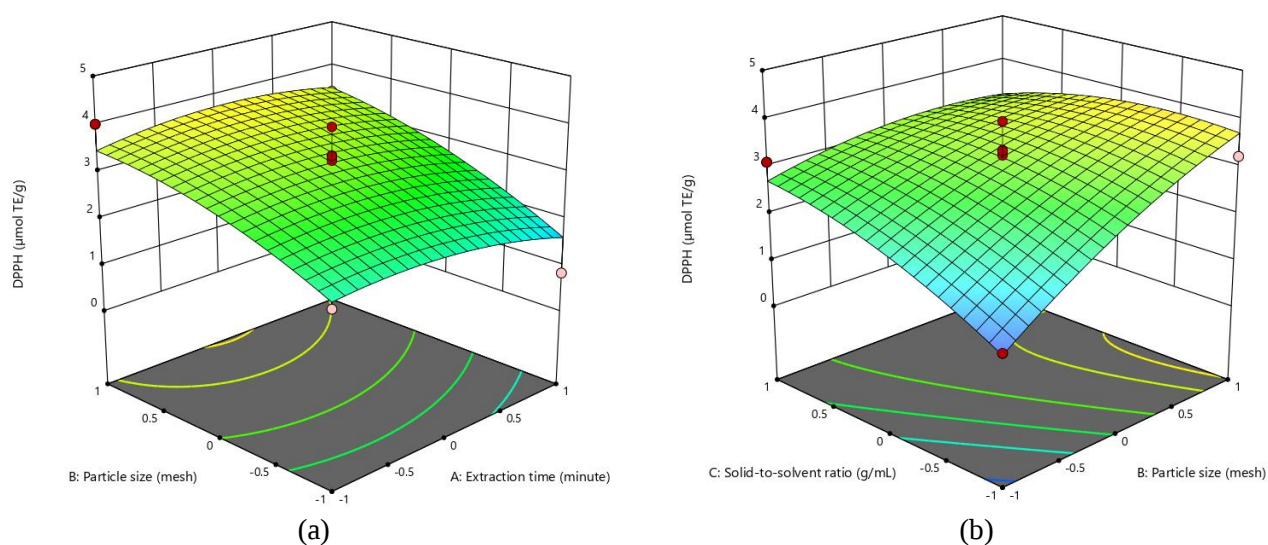


Figure 1. 3D contour graph of the effect of extraction time and particle size (a), particle size and solid-solvent ratio (b), as well as solid-solvent ratio and ethanol concentration (c) on the TPC of CCL seed extract.



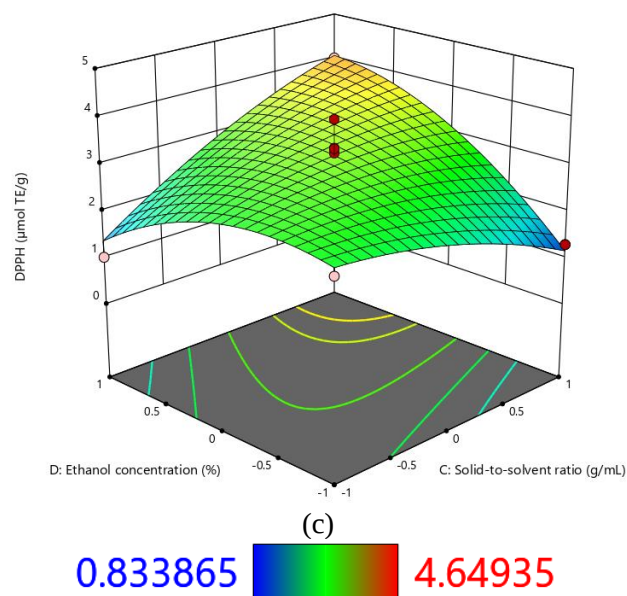


Figure 2. 3D contour graph of the effect of extraction time and particle size (a), particle size and solid-solvent ratio (b), as well as solid-solvent ratio and ethanol concentration (c) on TAC-DPPH of CCL seed extract

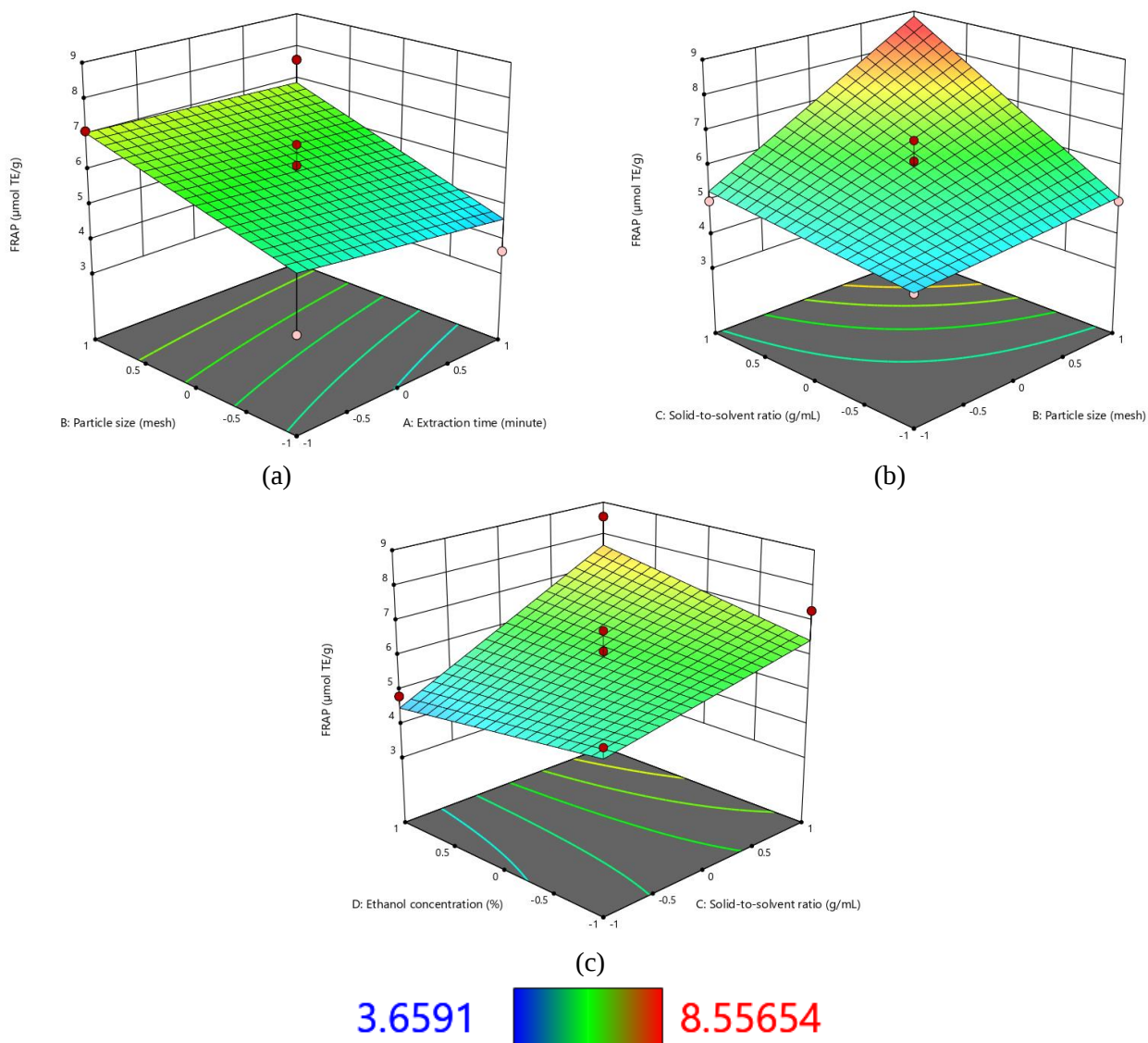


Figure 3. 3D contour graph of the effect of extraction time and particle size (a), particle size and solid-solvent ratio (b), as well as solid-solvent ratio and ethanol concentration (c) on TAC-FRAP CCL seed extract

Figure 3 explains the effect of the interaction of extraction variables on TAC-FRAP. A TAC-FRAP of 5,975 $\mu\text{mol TE/g fw}$ was obtained when all extraction variables were at point 0 ($A = 30 \text{ min}$; $B = 80 \text{ mesh}$; $C = 1:10 \text{ g/mL}$; $D = 70\%$). TAC-FRAP increased by 17.65% when A was lowered (15 min), and B was increased to 100 mesh (Figure 3a). TAC-FRAP increased by 34.68% when B and C were each increased to 100 mesh at 1:20g/mL (Figure 3b). TAC-FRAP increased by 25.52% when C and D were increased to 1:20 g/mL and 96% (Figure 3c).

Figure 4 provides an overview of the interaction effect of extraction variables on TAC-CUPRAC. A TAC-CUPRAC of 9,074 $\mu\text{mol TE/g fw}$ was obtained when all extraction variables were at point 0 ($A = 30 \text{ min}$; $B = 80 \text{ mesh}$; $C = 1:10 \text{ g/mL}$; $D = 70\%$). TAC-CUPRAC increased by 18.12% when A was lowered (15 min) and B was increased to 100 mesh (Figure 4a). TAC-CUPRAC increased by 65.02% when B and C were increased to 100 mesh at 1:20g/mL, respectively (Figure 4b). TAC-CUPRAC increased by 154.20% when C and D were increased to 1:20 g/mL and 96% (Figure 4c).

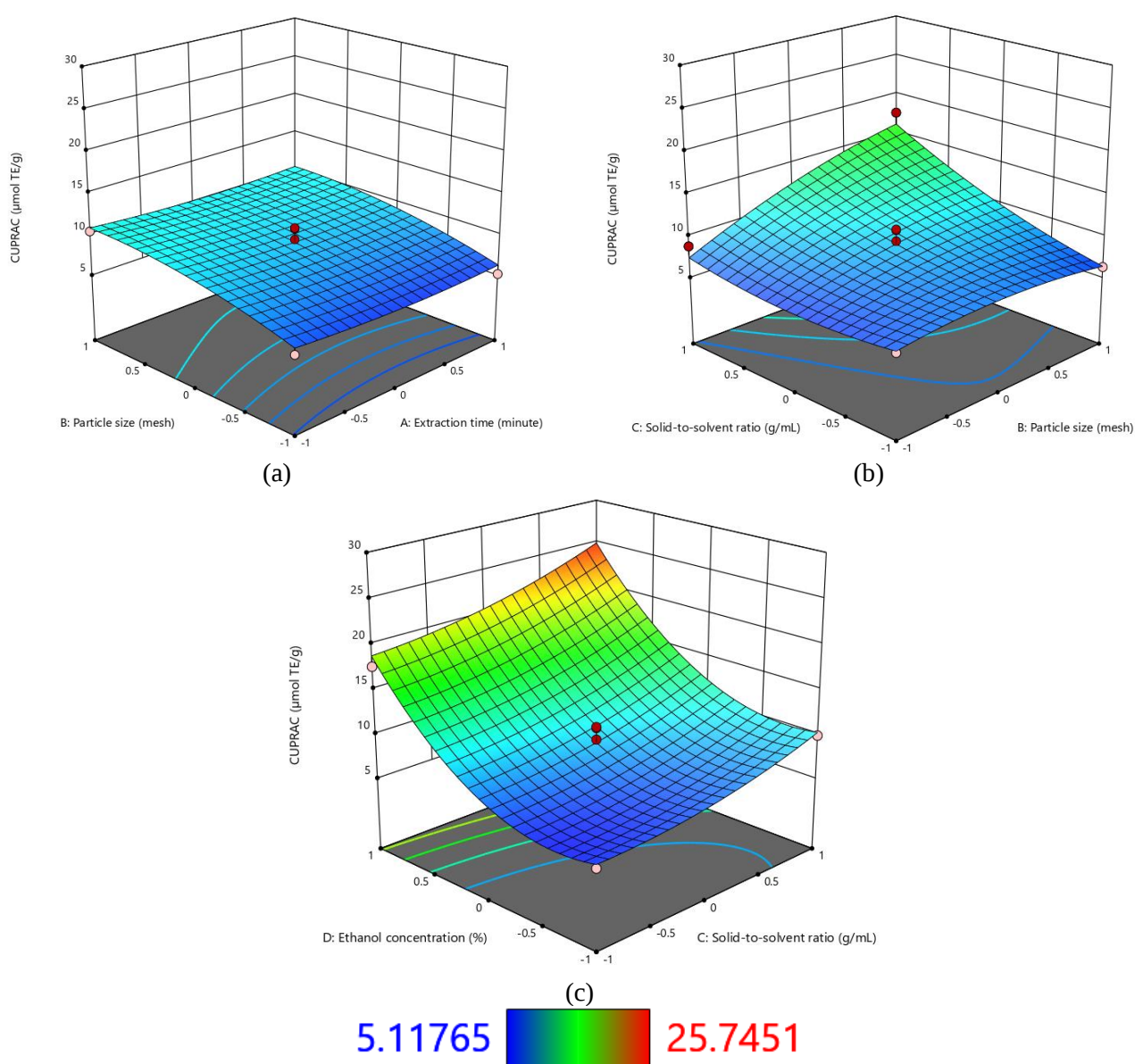


Figure 4. 3D contour graph of the effect of extraction time and particle size (a), particle size and solid-solvent ratio (b), as well as solid-solvent ratio and ethanol concentration (c) on TAC-CUPRAC CCL seed extract.

3.4. Effect of extraction variables on response variables.

Factors such as extraction time, particle size, solid-solvent ratio, and ethanol concentration were the most influential factors in the CCL seed extraction process. Therefore, this extraction factor plays an essential role in obtaining efficient extraction. Based on Figures (1), (2), (3), and (4) show the effect of the extraction variables used on the yield of TPC, TAC-DPPH, TAC-FRAP, and TAC-CUPRAC.

Based on the 3D contour graph, it is obtained a description of the effect of extraction time on the acquisition of each response variable in this study (TPC, TAC-DPPH, TAC-FRAP, and TAC-CUPRAC). Extraction time is a variable that does not show a significant difference in each response produced in this study ($p > 0.05$). This is in line with research [39]. In general, according to Fick's second law of diffusion, with the extension of the extraction time, the number of bioactive compounds extracted can increase, but the results in this study were that each time the extraction time was increased, the response rate decreased. The increase in extraction time from 15 to 30 minutes caused a decrease in each response variable obtained (TPC by 2.32%, TAC-DPPH 1.79%, TAC-FRAP 3.65%, and TAC-CUPRAC 7.20%). This is in line with research in extracting *Phoenix dactylifera*, which obtained the optimal extraction time of 15 minutes [40, 41]. This is probably due to the cavitation effect caused by ultrasound which can increase the contact area between the sample particles and the solvent, as well as increase the movement frequency and mass transfer rate of solvent molecules and sample particles in the system, and ultimately accelerate the melting of bioactive compounds and speed up the extraction process [42]. The decrease in antioxidant activity when the extraction time was increased was in line with the study of the extraction of *Myristica fragrans* [43]; this was due to the destruction of the natural antioxidants extracted and decreased the quality of the extract due to the formation of free radicals by ultrasonic waves with increasing sonication time [44]. In addition, long extraction times can lead to the degradation of sensitive phenolic compounds or increased solvent infiltration into the sample matrix, thus promoting the extraction of more contaminants than phenolic compounds [39, 45].

One of the extraction variables that significantly affect each response is the particle size used. The highest response was achieved with a particle size of 100 mesh. The increase in particle size from 80 to 100 mesh caused an increase in each response variable obtained (TPC by 20.56%, TAC-DPPH 12.29%, TAC-FRAP 16.48%, and TAC-CUPRAC 11.92%). This is because the smaller the particle size of the simplicia, the more active surface area and contact of the simplicia with the solvent so that more bioactive compounds can be extracted and cause a reduction in extraction time [46]. Previous studies on polyphenol extraction have shown that decreasing particle size in *Ginkgo biloba* leaves, *Punica granatum* bark, and *Vitis vinifera* seeds induce an increase in TPC [47–49].

The solid-solvent ratio is one of the extraction parameters that significantly affect the yield of TPA and TAC in this study. Based on Figures (1), (2), (3), and (4), the yield of TPC, TAC-DPPH, TAC-FRAP, and TAC-CUPRAC from CCL seeds increased with each increase in the solvent ratio. The highest increase in each response variable used the highest solid-solvent ratio of 1:20 (g/mL). This is in line with the ultrasonic perilla seed extraction study, and optimal results were obtained with a solid-solvent ratio of 1:20(g/mL) [50]. This may be because increasing the solid-solvent ratio increases the concentration gradient and consequently increases the rate of diffusion of the compound from the sample to the solvent, thereby increasing the extraction efficiency of the bioactive ingredients. In addition, increasing

the solid-solvent ratio can prevent saturation of the extraction medium and increase the extraction yield. Several studies have found that TPC increases rapidly with increasing ratio, at room temperature, as well as with the use of ultrasonic waves [51–53].

Generally, in binary solvent systems, one solvent can increase the solubility of polyphenols, while the other can increase desorption [54]. In a mixture of water and ethanol, water acts as a swelling agent, whereas ethanol breaks the bonds between the solute and the cell matrix [55]. Previous studies have reported that ethanol concentration is the main factor influencing the extraction ability of TPC and antioxidant activity of watermelon seeds and rinds [56], as well as argel leaves [36].

In this study, the ethanol concentration significantly impacted the yield of TPC, TAC-DPPH, TAC-FRAP, and TAC-CUPRAC. Increasing the ethanol concentration has a different effect on each response variable produced in the CCL seed extraction process. Increasing the ethanol concentration from 50% to 70% resulted in a decrease in TPC yield of 2.57%. The highest TPC was obtained when the ethanol concentration was 60%, and this is in line with research on the extraction of *Mentha piperita* [40] and the extraction process of *Solanum gilo* using RSM. The results showed that the best ethanol content for TPC was 61% [57]. This shows that the right combination of alcohol and water is more effective in extracting phenolic compounds. The proportion of ethanol in the extraction medium affects TPC recovery [51]. The extraction efficiency of phenolic compounds increases with a mixture of water and ethanol, while the use of pure solvents can reduce the extraction power [58].

In contrast, it was increasing the concentration of ethanol 50 to 70% on the antioxidant activity of CCL seed extract. There was an increase in the acquisition of TAC-DPPH 2.65% and TAC-FRAP 1.63%, and this increase was not too significant compared to the increase in TAC-CUPRAC, which was 30.51%. The increase in TAC-DPPH and TAC-FRAP was low because the measurement of DPPH and FRAP was more optimal for polar compounds. This is in line with the research on *Ilex guayusa* extraction optimization achieved by using 76% ethanol concentration to extract phenolic antioxidant compounds [59].

It differs in the increase in TAC-CUPRAC because CUPRAC is a sensitive method and is soluble in polar and non-polar compounds [60]. The solvent mixture can extract antioxidant compounds from both ends of polarity (compounds with the highest and lowest polarity), thereby increasing the yield of antioxidant compounds. These results indicate that the possible bioactive compounds from CCL seeds are semipolar [61].

3.5. Optimization and verification of CCL seed extraction process.

The optimal extraction conditions were formulated based on the 3D plot graph and model equations. This study carried out the extraction optimization process to achieve maximum TPC, TAC-DPPH, TAC-FRAP, and TAC-CUPRAC from CCL seeds. Extraction process conditions with an extraction time of 15 minutes, the particle size of simplicia 100 mesh, the solid-solvent ratio of 1:20 (g/mL), and ethanol concentration of 88% are recommended by the DX 13.0 program as the optimal formulation because this process condition has a high desirability value. The High is 0.918. The desirability value is used to determine the degree of accuracy of the recommended optimal formulation. The desirability value is close to one, the higher the optimization accuracy value obtained. So it can be concluded that the recommended extraction process conditions can produce a response that has characteristics that match the optimization target of 91.8% and is predicted to produce TPC 1.687 mg GAE/g fw, TAC -DPPH 4,352 $\mu\text{molTE/g fw}$, TAC-FRAP 8,773 $\mu\text{molTE/g fw}$, and TAC-CUPRAC 25,757 $\mu\text{molTE/g fw}$.

Table 4. Optimal conditions of extraction and validation of optimal conditions.

Optimum extraction conditions				Response variable	Maximum value	
A (minutes)	B (mesh)	C (g/mL)	D (%)		Prediction	Experiment
15	100	1:20	88	TPC (mg GAE/g)	1.69	1.32±0.09
				TAC-DPPH (µmolTE/g)	4.35	3.23±0.24
				TAC_FRAP (µmolTE/g)	8.77	6.54±0.71
				TAC-CUPRAC (µmolTE/g)	25.76	25.59±1.08

The model validation was carried out based on the formulation of the optimum extraction conditions suggested and carried out in triplicate, as shown in Table 4. The results obtained were TPC 1.32 mg GAE/g fw, TAC-DPPH 3.23 µmolTE/g fw, TAC-FRAP 6.54 µmolTE/g fw, and TAC- CUPRAC 25.59 µmolTE/g fw. Based on the T-test with a 95% confidence level to the experimental and predicted values, the p-value is 0.131 ($p>0.05$). This indicates that there is no significant difference between the experimental value and the observed predictive value. This analysis indicates that the experimental values are in line with those predicted and suggest that the model obtained is accurate.

4. Conclusions

The response surface methodology was successfully applied to optimize CCL seed extraction with sonication as the extraction method. Particle size, solid-solvent ratio, and ethanol concentration significantly affected the yield of TPC and TAC with $p\text{-value}<0.05$, whereas extraction time had no impact on TPC and TAC in the tested ranges. After the BBD technique's optimization process was obtained, the optimal processing parameters that met the requirements to produce the maximum CCL seed extract were obtained. Optimal extraction conditions were found at an extraction time of 15 minutes, the solid-solvent ratio of 1:20 (g/mL), the particle size of 100 mesh, and ethanol concentration of 88%.

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Conflicts of Interest

The authors declare no conflict of interest.

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