Extraction Variables Optimization on Phenolics Content and Antioxidant Activities of *in-vitro* Propagated Leaves of *Curcuma caesia* Roxb. using Response Surface Methodology

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Curcuma caesia Roxb. (Zingiberaceae) is one of the endangered species in the genus *Curcuma* that possesses numerous beneficial bioactive compounds that are responsible for various pharmacological properties including anti-cancer, anti-asthmatic, anti-diabetic, and others. In the past few years, the number of *C. caesia* plants was reported to have drastically decreased due to over-harvesting activity. To conserve this species, plant tissue culture method was used as a propagation tool for the mass production of *C. caesia* plantlets. Hence, this study aimed to optimize the extraction of phenolics content and antioxidant activities using response surface methodology. In this study, central composite design (CCD) was used to investigate the effects of three independent variables, namely solvent-solid ratio (mL/g), methanol concentration (%), and extraction temperature (°C), on phenolics content and antioxidant activities. Based on the results, the optimal extraction condition was achieved using 54.02 mL/g solvent-solid ratio, 70% methanol concentration, and 70 °C of extraction temperature.

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INTRODUCTION

Curcuma caesia (*C. caesia*), which is commonly known as black turmeric, is a rhizomatous *Curcuma* species that belongs to the Zingiberaceae family. *Curcuma caesia* is native to India and grows well in temperate and subtropical regions (Mahanta *et al.* 2022). Morphologically, *C. caesia* has a close similarity to other *Curcuma* species except it has a deep ferruginous violet color on the lamina leaves and the rhizome's flesh is blue to black in color with an aromatic odor. Traditionally, the rhizome part of *C. caesia* has been extensively used as a medicine for the treatment of allergies, fever, asthma, toothaches, and leprosy (Fozia *et al.* 2012). In addition, the leaves also were reported to be used for the treatment of epilepsy, leukoderma, bronchitis, and hemorrhoids (Amalraj *et al.* 2017).

The phytochemicals analysis conducted on the rhizomes extract of *C. caesia* has found a wide range of phytochemical groups present, including phenolics, alkaloids, tannins, saponins, and others (Karmakar *et al.* 2011; Hait *et al.* 2019). The pharmacological studies conducted revealed that *C. caesia* extract possesses various biological activities, such as anti-acne, analgesic, anti-inflammatory, anti-asthmatic, anti-microbes, anti-diabetic, anticancer, antiulcer, hepatotoxicity, nephrotoxicity, neurotoxicity, and anti-proliferative effects (Karmakar *et al.* 2011; Paliwal *et al.* 2011; Das *et al.* 2012; Borah *et al.* 2019; Rai *et al.* 2020). The scientific evidence of pharmacological properties of *C. caesia* has led to over-harvesting activity, which significantly reduced the raw materials of *C. caesia* in their natural habitat (Borah *et al.* 2019). To produce a large number of raw materials of *C. caesia*, plant tissue culture technique was used as an alternative propagation method (Haida *et al.* 2020). In addition to the mass production of raw materials, plant tissue culture technique also can be used for plant secondary metabolites production (Haida *et al.* 2020).

The physiological profiles of *in vitro* propagated plants often diverge from those of conventionally grown plants due to differences in environmental conditions, plant growth and development and stress signaling pathways (Khanam *et al.* 2022; Pasternak and Steinmacher 2024). These physiological disparities can significantly influence the biosynthesis and accumulation of secondary metabolites in the plants (Qaderi *et al.* 2023). Plant tissue culture plants are grown in the laboratory under controlled environments, which allows for the precise regulation of physical and chemical factors (Reshi *et al.* 2023). This controlled environment enables the plants to have consistent physiological responses and leads to the stable production of secondary metabolites. Compared to the conventionally propagated plants which produce unstable secondary metabolites due to environmental factors, tissue culture technique offers a reproducible system for optimizing and maintaining secondary metabolites yields (Selwal *et al.* 2023; Prashant and Bhawana 2024).

Optimization of extraction variables is a way to maximize the extraction of phenolics content and antioxidant properties from the plant samples. To date, several designs of experiment have been employed to study the extraction of phenolics content from the plant sample such as one-factor-at a-time method, artificial neural network, and response surface methodology (Dalhat *et al.* 2021; Patra *et al.* 2021; Wilig *et al.* 2022; Weremfo *et al.* 2023). Among all the methods, response surface methodology is widely used for phenolics content extraction in various plant samples (Weremfo *et al.* 2023). The extraction process of phenolics content using the conventional method is time consuming and requires a high amount of sample. Compared to the conventional extraction method, response surface methodology can reduce the number of experimental units, which directly can reduce time, cost, and amount of sample used for the experiment (Wani *et al.* 2017). The advantage of response surface methodology is that the process can be modeled, and the statistical significance of independent variables can be simultaneously analyzed. As a result, the optimal conditions can be ascertained (Rittisak *et al.* 2022).

To date, there has been no study conducted on the extraction of phenolics content from *in vitro* propagated leaves of *C. caesia.* Hence, this is the first study that emphasizes the utilization of response surface methodology for phenolics extraction and antioxidant properties from *in vitro* propagated leaves of *C. caesia.* In this study, response surface methodology was employed to investigate the effect of solvent-solid ratio, methanol concentration, and extraction temperature, as well as the interaction on the model responses, including phenolics content and antioxidant activities, of *in vitro* propagated leaves of *C. caesia.* It was hypothesized that the extraction variables significantly influence the yield of phenolic compounds and antioxidant activities and the optimal conditions can be optimized through response surface methodology to maximize extraction efficiency from *in vitro* propagated *C. caesia* leaves.

EXPERIMENTAL

Extraction and Optimization using Response Surface Methodology

Plant materials and preparation

The raw materials of *C. caesia* were obtained from the plantlets that were propagated using plant tissue culture technique. The plantlets were grown on MSB5 medium (Murashige and Skoog 1962; Gamborg *et al.* 1968) supplemented with 15 BAP, 6 μM IBA, 50 g/L sucrose, and 3 g/L gelling agent (Fig. 1). The 12-week-old plantlets were harvested and cleaned using tap water to remove the attached media. The leaves were collected and placed in a paper bag and dried in an oven for 24 h at a temperature of 50 °C. The dried leaves were kept in an air-tight plastic bag. After that, the leaves were ground using pastel and mortar until it had become a fine powder.

Fig. 1. Example of a *C. caesia* plantlet that was harvested and used for the analysis

Extraction procedure

The powdered leaf samples of *C. caesia* were subjected to extraction using methanol with varying solvent-to-solid rations ranging from 33.18 and 66.82 mL/g and methanol concentrations between 16.36 and 83.64%. The extraction process was conducted in a water bath with water temperatures ranging from 43.2 and 76.8 °C for a duration of 105 min. A design matrix incorporating different combinations of these variables was generated using Design-Expert Software version 11.0 (Stat-Ease Inc., Minneapolis, MN, USA). Following extraction, the mixtures were filtered using Whatman No.1 filter paper and the resulting extracts were collected for further analysis.

Experimental design and statistical analysis

In this study, the optimization of three independent extraction variables namely as solvent-to-solid ratio (X_1), methanol concentration (X_2), and extraction temperature (X_3)

were performed using a central composite design (CCD). A total of 20 experimental runs were generated, with each variable coded at five levels: -α, -1, 0, 1, and α (Table 1). The experimental data were fitted to a second-order polynomial equation to model the relationship between the independent variables and the response parameters. The regression coefficients were determined, where x_i represents the independent variables influencing the dependent response *Y*. Additionally, β_0, β_i, β_{ii}, β_{ij}, and k correspond to the regression coefficients for the intercept, linear, quadratic, and interaction terms, as well as the number of parameters, respectively. The second-order polynomial equation is below:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_1 x_1 + \sum_{i=1}^{k=1} \sum_{j=2}^{k} \beta_{ij} x_i x_j + \sum_{i=2}^{k} \beta_{ii} x_i^2$$

The analysis of variance (ANOVA) was performed to assess the significance of the model and all experimental runs were conducted in triplicate. The optimized extraction conditions for all independent variables were determined and subsequently validated by comparing the predicted response values with the experimental values. The significant levels of the responses, interactions among variables, regression equations and three-dimensional (3D) surface plots were generated using Design-Expert software.

Analysis of Phenolics Content

Total phenolic content

The total phenolic content of the *in vitro* propagated leaves was determined following the method described by Haida *et al.* (2022), with minor modifications. Initially, 0.25 mL of the sample extract was mixed with 2.25 mL of distilled water in a vial. Subsequently, 0.25 mL of 2N Folin-Ciocalteu reagent was added and the mixture was incubated for 5 min. After incubation, 1 mL of distilled water and 2.5 mL of 7% sodium carbonate were added to the mixture, followed by an additional incubation for 90 min. The absorbance of the reaction mixture was measured at 750 nm using a UV-Vis spectrophotometer. The gallic acid was used as standard and the total phenolic content was expressed as mg gallic acid equivalent per gram dry weight of sample (mg GAE/g DW).

Total flavonoid content

The aluminum chloride colorimetric method as described by Marinova *et al.* (2005) was used for the quantification of total flavonoid content. Briefly, the extract (90.5 mL), 5% of sodium nitrite (0.15 mL), and distilled water (2 mL) were mixed and incubated for 5 min. After that, 10% of aluminum chloride (0.15 mL), 1 M of sodium hydrochloride (1mL), and distilled water (1.2 mL) were added in the reaction mixture. The absorbance was measured at 510 nm. The rutin was used as standard and the total flavonoid content was expressed as mg rutin equivalent per gram dry weight of sample (mg RE/g DW).

Total tannin content

Total tannin content was analyzed using Folin-Ciocalteu method, as described by Chandran and Indira (2016). A total of 0.1 mL of extract, 7.5 mL of distilled water, and 0.5 mL of 1 N of Folin-Ciocalteu reagent were mixed in the vial. The reaction mixture was incubated for 3 min and 1 mL of 35% of sodium carbonate and 0.9 mL of distilled water were added and incubated for 30 min. After the incubation, absorbance of the reaction mixture was measured at 700 nm. The tannic acid was used as standard and the total tannin content was expressed as mg tannic acid equivalent per gram dry weight of sample (mg TAE/g DW).

Analysis of Antioxidant Activity

DPPH free radical scavenging activity

The procedure for DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity was conducted according to a method described by Wong *et al.* (2006). Prior to experiment, 0.1 mM of methanolic DPPH solution was prepared and left at room temperature for 30 min for the solid DPPH to fully dissolved. After 30 min, initial absorbance of DPPH solution was measured at 515 nm using UV-vis spectrophotometer. An amount of 0.1 mL of extract was mixed with 1.5 mL of 0.1 mM of methanolic DPPH solution. The reaction mixture was incubated at room temperature for 30 min and absorbance was measured at 515 nm. Trolox was used as the standard and the DPPH free radical scavenging activity was expressed as mg trolox equivalent per gram dry weight of sample (mg GAE/g DW).

Ferrous ion chelating activity

The ability of the extract to chelate ferrous ion was measured using a protocol as described by Prieto *et al.* (1999). Briefly, an amount of 0.4 mL of extract and 0.05 mL of 1 mM of ferrous chloride were mixed in the vial. Then, 0.2 mL of 5 mM of ferrozine was added to initiate the reaction mixture and incubated for 10 min at room temperature. The absorbance of reaction mixture was measured at 562 nm.

Superoxide anion radical scavenging activity

The superoxide anion radical scavenging assay was conducted to a method described by Robak and Gryglewski (1988). The reaction mixture contained 0.5 mL of extract, 0.25 mL of 0.3 mM of nitro blue tetrazolium, 0.5 mL of 16 mM of Tris-HCl buffer (pH 8.0), and 0.25 mL of 0.936 mM of nicotinamide adenine dinucleotide. The initiation of reaction mixture was done by an addition of 0.25 mL of 0.12 mM of phenazine methosulfate. The reaction mixture was incubated for five min at a temperature of 25 °C and absorbance was measured at 560 nm against the blank sample.

RESULTS AND DISCUSSION

Optimization of Extraction Variables of Phenolics Content and Antioxidant Activities Using Response Surface Methodology

The effect of independent variables on phenolics content and antioxidant activities in *in vitro* propagated leaves of *C. caesia* were presented in Table 1. Based on the results, the lowest total phenolic (Y_1), total flavonoid (Y_2), and total tannin content (Y_3) exhibited from the experimental run number 1 (40 mL/g solvent to solid ratio, 30% methanol, 50 °C of extraction temperature) with 14.23 mg GAE/g DW, 4.34 mg RE/g DW, and 1.17 mg TAE/g DW, respectively. Meanwhile, experimental run number 4 (60 mL/g solvent-solid ratio, 70% methanol, 50 °C of extraction temperature) exhibited the highest total phenolic (23.84 mg GAE/g DW), total flavonoid (17.21 mg RE/g DW), and total tannin content (2.15 mg TAE/g DW).

Table 1. Central Composite Design with Responses of the Dependent Variables to Extraction Conditions of *in vitro* Propagated Leaves of *C. caesia*

Run	Independent Variables			Dependent Variables					
	X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6
1	40 (-1)	30 (-1)	50	14.23	4.34	1.17	11.82	43.81	54.75
2	50 (0)	50 (0)	43.18 (-α)	16.13	6.76	1.51	14.57	63.49	23.62
3	60 (1)	30 (-1)	50	20.87	10.71	1.96	19.11	42.56	56.77
4	60 (1)	70 (1)	50	23.84	17.21	2.15	13.39	89.19	18.87
5	60 (1)	70 (1)	70	21.17	14.71	1.68	10.05	86.21	19.98
6	50 (0)	50 (0)	60	17.22	10.43	1.63	15.89	74.06	26.86
7	50 (0)	50 (0)	60	17.11	8.93	1.64	15.8	74.08	21.63
8	50 (0)	16.36 (-α)	60	14.63	5.18	1.28	12.22	33.47	52.32
9	40 (-1)	70 (1)	50	15.72	10.41	1.53	13.44	86.42	17.77
10	66.82 (α)	50 (0)	60	21.18	10.26	1.82	12.95	78.74	40.73
11	33.18 (-α)	50 (0)	60	15.99	6.75	1.58	11.67	82.51	33.11
12	50 (0)	50 (0)	60	17.18	8.76	1.65	15.78	73.96	26.97
13	40 (-1)	30 (-1)	70	14.85	5.81	1.45	14.17	69.92	59.68
14	50 (0)	50 (0)	60	18.29	8.68	1.64	16.01	74.03	26.23
15	40 (-1)	70 (1)	70	17.33	11.54	1.71	14.96	81.92	13.91
16	50 (0)	50 (0)	60	17.1	8.76	1.65	15.91	74.18	26.71
17	50 (0)	50 (0)	76.82 (α)	19.13	11.34	1.57	16.42	84.68	28.04
18	50 (0)	83.64 (α)	60	17.86	15.43	1.76	15.43	83.73	17.59
19	60 (1)	30 (-1)	70	17.22	6.51	1.51	12.38	60.43	44.67
20	50 (0)	50 (0)	60	17.05	8.68	1.75	16.08	71.73	26.71

X_1 – Solvent-solid ratio (mL/g); X_2 – Methanol concentration (%); X_3 – Extraction temperature (°C); Y_1 – Total phenolic content (mg GAE/g DW); Y_2 – Total flavonoid content (mg RE/g DW); Y_3 – Total tannin content (mg TAE/g DW); Y_4 – DPPH scavenging activity (mg TE/g DW); Y_5 – Ferrous ion chelating activity (%); Y_6 – Superoxide anion scavenging activity (%)

As for antioxidant properties, the lowest value recorded was 10.05 mg TE/g DW (experimental run number 5; 60 mL/g solvent-solid ratio, 70% methanol, 70 °C of extraction temperature) for DPPH free radical scavenging activity, 33.47% of chelating activity (experimental run number 8; 50 mL/g solvent-solid ratio, 16.36% methanol, 60 °C of extraction temperature) for ferrous ion chelating activity and 13.91% of inhibition (experimental run number 15; 40 mL/g solvent-solid ratio, 70% methanol, 70 °C of extraction temperature) for superoxide anion radical scavenging activity (Table 1). Apart from that, the highest value recorded for each antioxidant activities were 19.11 mg TE/g DW (experimental run number 3; 60 mL/g solvent-solid ratio, 30% methanol, 50 °C of extraction temperature) for DPPH free radical scavenging activity, 89.19% of chelating activity (experimental run number 4; 60 mL/g solvent-solid ratio, 70% methanol, 50 °C of extraction temperature) for ferrous ion chelating activity, and 59.68% of inhibition (experimental run number 13, 40 mL/g solvent-solid ratio, 30% methanol, 70 °C of extraction temperature) for superoxide anion radical scavenging activity, respectively.

Fitting the model

Fitting the models represents the reliability of response surface methodology to predict optimal extraction variables and responses. The ANOVA analysis of total phenolic, total flavonoid and total tannin content were tabulated in Table 2. Based on the ANOVA analysis, the F-value for model response of total phenolic, total flavonoid, and total tannin content were significant at $p < 0.05$. In addition, the F-value of lack of fit recorded for total phenolic, total flavonoid, and total tannin content were not significant ($p > 0.05$), which indicated that the model proposed was well-fitted. Moreover, the value of adequate precision for all model responses were above four and the model proposed displayed a good coefficient of determination and adjusted coefficient of determination with the value above 0.85. By fitting the second-order polynomial, the equation for total phenolic (Y_1), total flavonoid (Y_2), and total tannin (Y_3) content were generated as Eq. 1, Eq. 2 and Eq. 3, respectively:

$$Y_1 = 151.85 - 6.63\, X_1 + 1.79\, X_2 - 3.24\, X_3 - 0.045\, X_1X_2 + 0.130\, X_1X_3 + 0.001\, X_2X_3 + 0.080\, X_1^2 - 0.014\, X_2^2 + 0.002\, X_3^2 \qquad (1)$$

$$Y_2 = 181.99 - 8.80\, X_1 + 1.93\, X_2 - 3.91\, X_3 - 0.046\, X_1X_2 + 0.169\, X_1X_3 - 0.006\, X_2X_3 + 0.106\, X_1^2 - 0.012\, X_2^2 + 0.002\, X_3^2 \qquad (2)$$

$$Y_3 = 2.86 - 0.33\, X_1 + 0.15\, X_2 - 0.05\, X_3 - 0.002\, X_1X_2 + 0.006\, X_1X_3 - 0.0003\, X_2X_3 + 0.005\, X_1^2 - 0.001\, X_2^2 - 0.0003\, X_3^2 \qquad (3)$$

The ANOVA analysis also revealed the linear, interaction, and quadratic effects between the extraction variables (Table 2). The linear effect of total phenolic and total flavonoid content showed that all extraction variables including solvent-solid ratio (X_1), methanol concentration (X_2), and extraction temperature (X_3) were significant at $p < 0.05$. For the linear effect of total tannin content, only solvent-solid ratio and methanol concentration were significant. Meanwhile, the extraction temperature was found to be not significant for total tannin content at $p > 0.05$. For the interaction effect, the significant interaction was observed between solvent-solid ratio and extraction temperature (X_1X_3). The combination of extraction variables between solvent-solid ratio with methanol concentration (X_1X_2) and methanol concentration with extraction temperature (X_2X_3) exhibited a non-significant interaction effect on total phenolic, total flavonoid, and total tannin content at $p > 0.05$. In terms of quadratic effect, only solvent-solid ratio (X_1^2) showed a significant effect on total phenolic content and methanol concentration (X_2^2) significantly affected the total flavonoid content. For the total tannin content, all extraction variables showed non-significant quadratic effect at $p > 0.05$.

Table 2. ANOVA Results for Coefficient of Quadratic Models for Total Phenolic, Total Flavonoid, and Total Tannin Content of *in vitro* Propagated Leaves of *C. caesia*

Source of Variation	Y_1		Y_2		Y_3	
	F-value	p-value	F-value	p-value	F-value	p-value
Model	20.68	0.0007	21.07	0.0006	17.27	0.0011
Linear						
X_1	32.12	0.0013	7.89	0.0308	7.65	0.0326
X_2	12.44	0.0124	67.26	0.0002	30.60	0.0015
X_3	10.73	0.0169	13.43	0.0105	0.4781	0.5151
Interaction						
X_1X_2	2.59	0.1583	1.35	0.2900	2.24	0.1847
X_1X_3	21.80	0.0034	13.84	0.0098	63.23	0.0002
X_2X_3	1.16	0.3234	0.2960	0.6060	0.4781	0.5151
Quadratic						
X_1^2	11.73	0.0141	0.0015	0.9702	2.87	0.1411
X_2^2	2.03	0.2039	7.26	0.0358	5.03	0.0661
X_3^2	2.09	0.1985	0.6221	0.4603	3.26	0.1211
Parameters used for the adequacy check of the model						
Lack of fit	6.07	0.0569	4.93	0.0772	6.29	0.0539
C.V. (%)	3.66		9.24		3.76	
R^2	0.9782		0.9886		0.9740	
$R^2_{Adjusted}$	0.9309		0.9321		0.9176	
Adequate precision	17.74		17.41		19.09	

X_1 – Solvent-solid ratio (mL/g); X_2 – Methanol concentration (%); X_3 – Extraction temperature (°C); Y_1 – Total phenolic content (mg GAE/g DW); Y_2 – Total flavonoid content (mg RE/g DW); Y_3 – Total tannin content (mg TAE/g DW)

The ANOVA analysis of antioxidant properties revealed that the F-value for model response of all antioxidant activities were highly significant at $p < 0.001$ (Table 3). Furthermore, the model proposed was well-fitted as the F-value recorded for lack of fit was not significant at $p > 0.05$ and adequate precision value was above four for all model responses. In addition, the model of antioxidant activities exhibited high coefficient of determination and adjusted coefficient of determination with R^2 and $R^2_{Adjusted}$ were above 0.95. The model proposed was fitted using a second-order polynomial equation and the Eq. 4, Eq. 5, and Eq. 6 were generated for DPPH free radical scavenging activity (Y_4), ferrous ion chelating activity (Y_5), and superoxide anion radical scavenging activity (Y_6), respectively:

$$Y_4 = 174.19 - 8.80\,X_1 - 1.21\,X_2 - 1.61\,X_3 + 0.068\,X_1X_2 + 0.102\,X_1X_3 - 0.012\,X_2X_3 + 0.108\,X_1^2 + 0.002\,X_2^2 - 0.002\,X_3^2 \quad (4)$$

$$Y_5 = 21.03 - 8.28\,X_1 + 7.32\,X_2 - 0.11\,X_3 - 0.089\,X_1X_2 + 0.135\,X_1X_3 - 0.063\,X_2X_3 + 0.104\,X_1^2 - 0.022\,X_2^2 - 0.0004\,X_3^2 \quad (5)$$

$$Y_6 = 945.20 - 36.68\, X_1 - 11.68\, X_2 - 2.91\, X_3 + 0.397\, X_1X_2 + 0.172\, X_1X_3 - 0.066\, X_2X_3 + 0.394\, X_1^2 + 0.047\, X_2^2 + 0.006\, X_3^2 \quad (6)$$

Table 3. ANOVA Results for Coefficient of Quadratic Models for DPPH Free Radical Scavenging Activity, Ferrous Ion Chelating Activity and Superoxide Anion Radical Scavenging Activity of *in vitro* Propagated Leaves of *C. caesia*

Source of Variation	Y_4		Y_5		Y_6	
	F-value	p-value	F-value	p-value	F-value	p-value
Model	432.67	< 0.0001	301.07	< 0.0001	38.05	0.0001
Linear						
X_1	52.77	0.0003	6.02	0.0496	3.69	0.1031
X_2	331.89	< 0.0001	1069.32	< 0.0001	76.70	0.0001
X_3	110.24	< 0.0001	190.08	< 0.0001	1.24	0.3077
Interaction						
X_1X_2	881.01	< 0.0001	33.53	0.0012	6.46	0.0440
X_1X_3	1564.74	< 0.0001	4.78	0.0715	2.31	0.1792
X_2X_3	52.77	0.0003	280.25	< 0.0001	0.3106	0.5975
Quadratic						
X_1^2	1549.66	< 0.0001	62.87	0.0002	37.26	0.0009
X_2^2	531.109	< 0.0001	371.37	< 0.0001	26.67	0.0021
X_3^2	25.56	0.0023	0.0218	0.8875	0.6343	0.4561
Parameters used for the adequacy check of the model						
Lack of fit	1.82	0.2352	2.77	0.1566	5.87	0.0599
C.V. (%)	0.8651		1.52		8.81	
R^2	0.9989		0.9985		0.9890	
$R^2_{Adjusted}$	0.9966		0.9952		0.9620	
Adequate precision	86.91		62.22		19.51	

X_1 – Solvent-solid ratio (mL/g); X_2 – Methanol concentration (%); X_3 – Extraction temperature (°C); Y_4 – DPPH scavenging activity (mg TE/g DW); Y_5 – Ferrous ion chelating activity (%); Y_6 – Superoxide anion scavenging activity (%)

In the linear effect analysis, all the extraction variables including solvent-solid ratio (X_1), methanol concentration (X_2), and extraction temperature (X_3) were significant for the DPPH free radical scavenging activity and ferrous ion chelating activity. As for superoxide anion radical scavenging activity, the extraction variables of solvent-solid ratio and extraction temperature showed a non-significant linear effect at $p > 0.05$. The interaction effect found that all extraction variables exhibited high significant interaction on DPPH free radical scavenging activity at $p < 0.001$. For the ferrous ion chelating activity, the interaction between the extraction variables of solvent-solid ratio with methanol concentration (X_1X_2) and methanol concentration with extraction temperature (X_2X_3) were significant. The interaction effect analysis of superoxide anion radical scavenging activity revealed that significant interaction only obtained between solvent-solid ratio with methanol concentration (X_1X_2). The quadratic effect analysis found that all extraction variables were significant on DPPH free radical scavenging activity. On the contrary, quadratic analysis of ferrous ion chelating activity and superoxide anion radical scavenging activity found that extraction temperature (X_3^2) exhibited non-significant quadratic effect at $p > 0.05$.

Response surface plots analysis

The relationship between the independent and dependent variables were demonstrated using the three-dimensional response surface plots (Figs. 1 to 3). Based on the response surface plots, solvent-solid ratio (X_1) at 60 mL/g recorded the highest accumulation of total phenolic, total flavonoid, total tannin content, and DPPH free radical scavenging activity. In contrast, the highest ferrous ion chelating activity was recorded from 40 mL/g solvent-solid ratio. Meanwhile, solvent-solid ratio did not significantly affect the superoxide anion radical scavenging activity as the linear p-value was above 0.05 (Table 3). In an earlier study conducted on *Polygonum aviculare*, the accumulation of total phenolic and total flavonoid content increased as the solvent-solid ratio increased from 20 to 100 mL/g (Wu *et al.* 2021). Generally, the increased phenolics compound yield as the ratio increased is due to high mass transfer gradient between the solvent and plant materials (Tabaraki *et al.* 2012; Lim *et al.* 2019). Similar findings were reported on *Olea europaea, Allium cepa*, and *Zingiber officinale* (Elboughdiri 2018; Pal and Jadeja 2019; Anuar *et al.* 2021).

The effect of methanol concentration (X_2) on all responses is also displayed in Figs. 1, 2, and 3. Based on the response surface plots, the increase of methanol concentration from 30 to 70% resulted in an increment of total phenolic, total flavonoid, total tannin content, DPPH free radical scavenging activity, and ferrous ion chelating activity. In contrast, superoxide anion radical scavenging activity showed a decrement trend as the methanol concentration increased from 30 to 70%. A study by Romero-Díez *et al.* (2019) stated that changes in solvent concentration will modify the physical properties of the solvents, such as viscosity, dynamic viscosity, polarity, dielectric constant, and the solubilities of compound, which directly influenced the yield of phenolics extraction and antioxidant properties. A similar effect was reported in the studies on *Trigonella foenum-graecum*, *Syzygium cumini*, and *Lavandula stoechas* (Akbari *et al.* 2019; de Sousa *et al.* 2021; Fadil *et al.* 2021).

As for extraction temperature (X_3), the response surface plots showed that increasing the extraction temperature from 50 to 70 °C led to an increase in all variables except total tannin content and superoxide anion radical scavenging activity. Based on Tables 2 and 3, the linear p-value of total tannin content and superoxide anion radical scavenging activity were above 0.05, which indicated a non-significant effect of extraction temperature on both responses. The extraction temperature is an important extraction variable that highly influences the yield of phenolics compound and antioxidant properties from the plant samples. During the extraction process, the plant tissue samples were softened and the solvent penetration rate into the plant samples were increased at high temperature (Al-Farsi and Lee 2008; Esmaeilzadeh and Dehghan 2020). Moreover, temperature is important to reduce the plant sample viscosity and increase the diffusion coefficient and mass transfer rate (Fidelis *et al.* 2020). Furthermore, higher temperature also helps to break certain chemical bonds and increase the solubility of chemical compounds, which resulted in high accumulation of phenolic compounds in the solvent (Kamarudin *et al.* 2020). However, heat sensitive compounds could degrade when exposed to high extraction temperature (Liew *et al.* 2019).

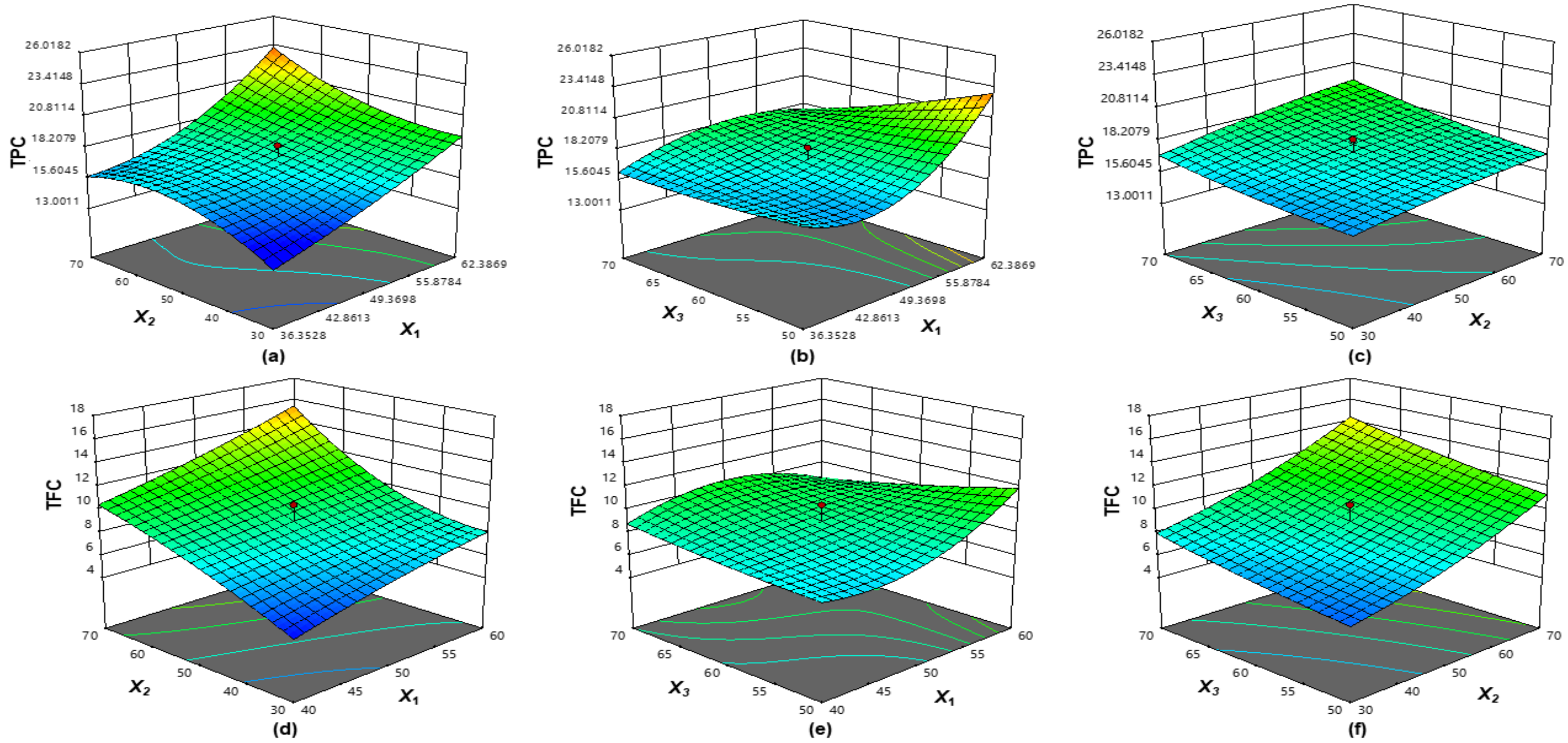

Fig. 1. The response surface plot of total phenolic and total flavonoid content: X_1 – Solvent-solid ratio (mL/g); X_2 – Methanol concentration (%); X_3 – Extraction temperature (°C); TPC – Total phenolic content (mg GAE/g DW); TFC – Total flavonoid content (mg RE/g DW)

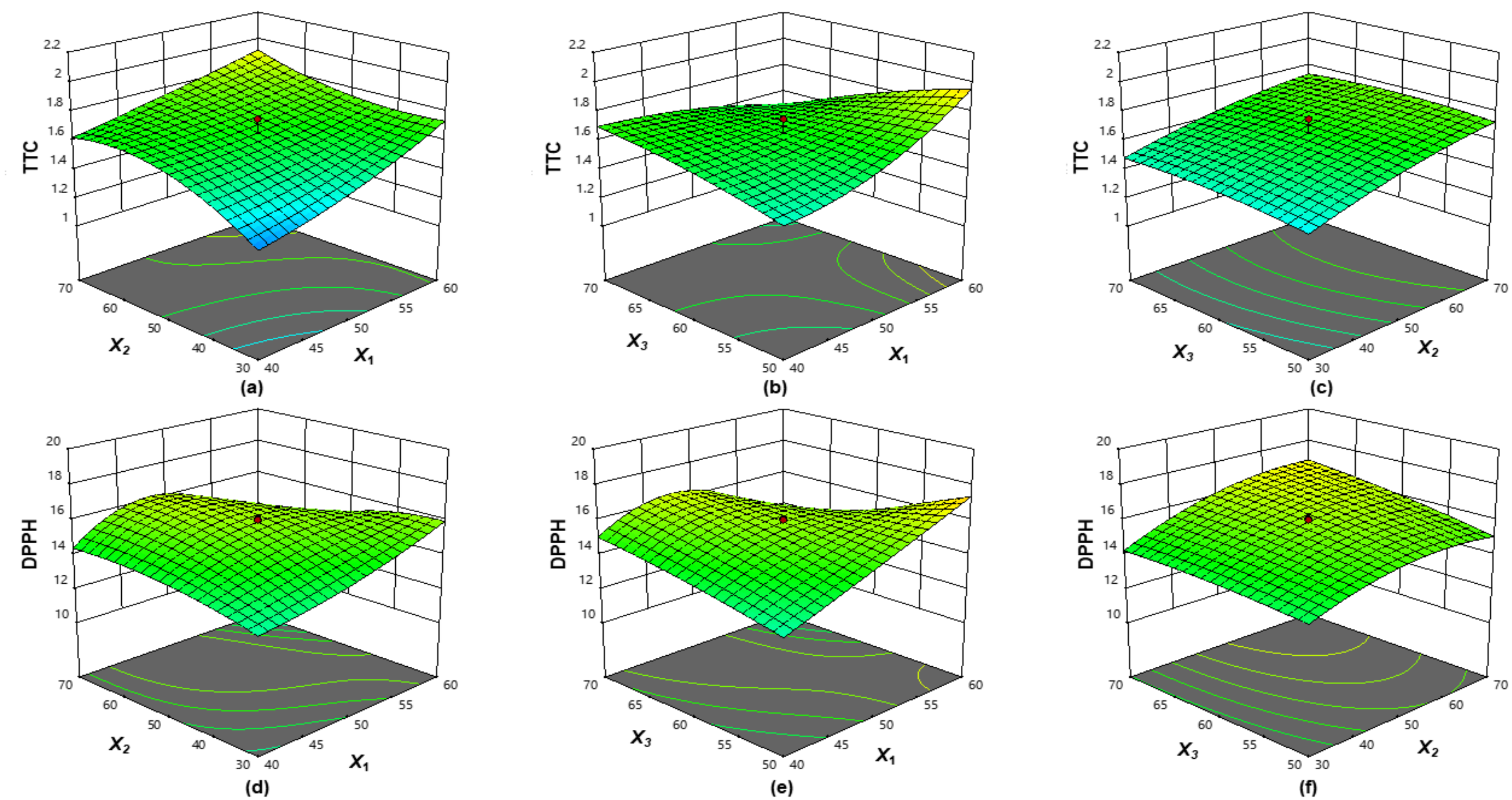

Fig. 2. The response surface plot of total tannin content and DPPH free radical scavenging activity: X_1 – Solvent-solid ratio (mL/g); X_2 – Methanol concentration (%); X_3 – Extraction temperature (°C); TTC – Total tannin content (mg TAE/g DW); DPPH – DPPH scavenging activity (mg TE/g DW)

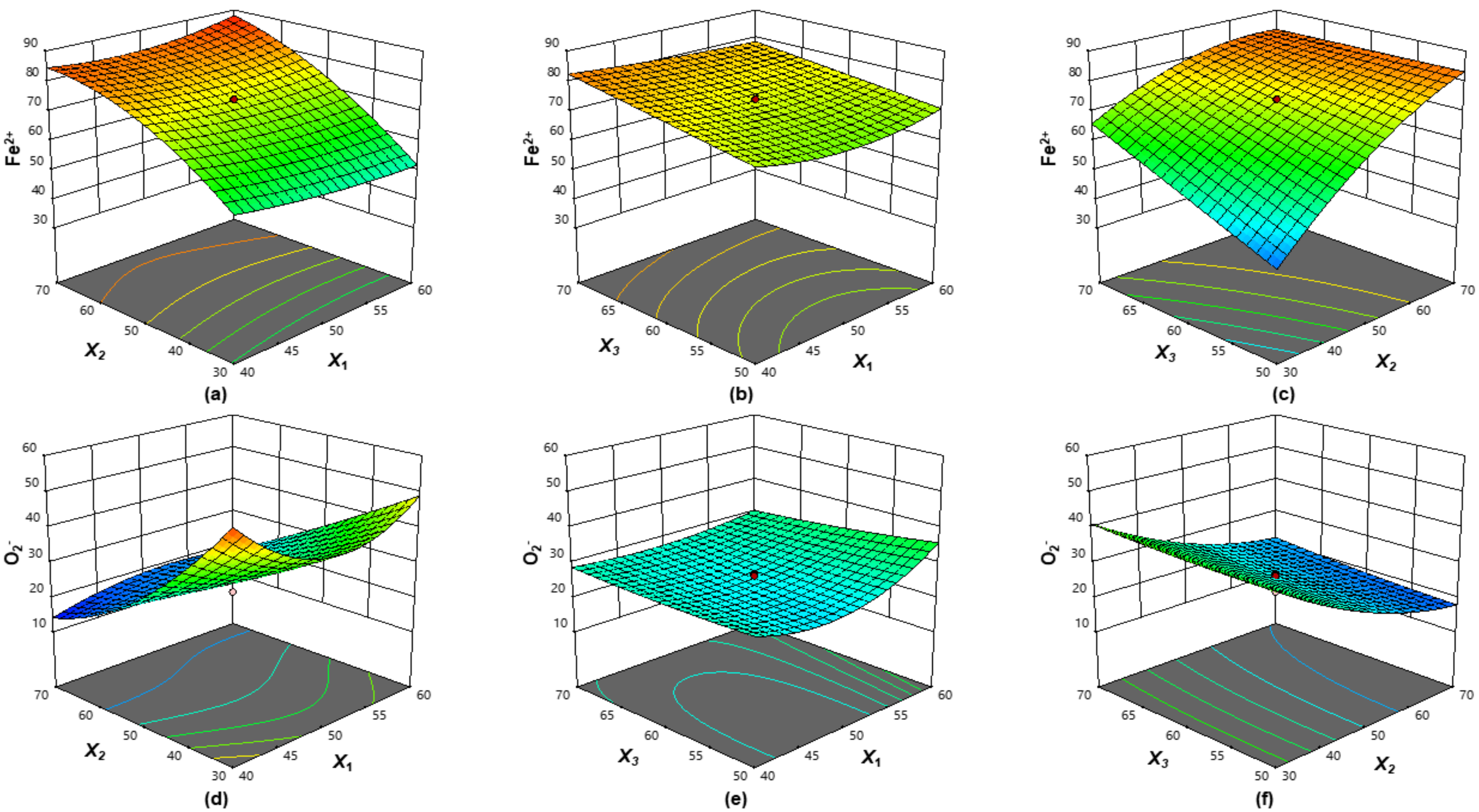

Fig. 3. The response surface plot of ferrous ion chelating activity and superoxide anion radical scavenging activity: X_1 – Solvent-solid ratio (mL/g); X_2 – Methanol concentration (%); X_3 – Extraction temperature (°C); Fe^{2+} - Ferrous ion chelating activity (%); O_2^- - Superoxide anion scavenging activity (%)

Optimization of process parameters and model validation

In this analysis, numerical analysis was performed to find the best possible combination that can maximize phenolics extraction and antioxidant properties from *in vitro* propagated leaves of *C. caesia*. Hence, the optimum extraction conditions generated for maximum phenolics extraction and antioxidant properties were obtained with 54.02 mL/g of solvent-solid ratio (X_1), 70% methanol concentration (X_2), and 70 °C of extraction temperature (X_3). Based on the optimum extraction conditions, the experimental value for each response recorded was 18.95 mg GAE/g DW for total phenolic content, 14.23 mg RE/g DW for total flavonoid content, 1.67 mg TAE/g DW for total tannin content, 15.07 mg TE/g DW for DPPH free radical scavenging activity, 85.38% for ferrous ion chelating activity, and 22.85% for superoxide anion radical scavenging activity (Table 4), respectively. All the experimental values were in 95% of the prediction confidence interval (CI) range that were obtained from the developed second-order models. In addition, the relative error was less than 10%, which indicated that the predicted and experimental values had achieved good correlation.

Table 4. Predicted and Experimental Values Under the Optimized Extraction Conditions for Maximum Phenolics Extraction and Antioxidant Properties from *in vitro* Propagated Leaves of *C. caesia*

Response	Predicted Value	95% CI Low	95% CI High	Experimental Value	Relative Error (%)
Y_1	20.11	18.94	21.27	18.95	5.77
Y_2	14.78	13.19	16.37	14.23	3.72
Y_3	1.72	1.61	1.83	1.67	2.91
Y_4	15.14	14.92	15.37	15.07	0.46
Y_5	84.05	82.10	86.00	85.38	-1.58
Y_6	21.97	16.93	27.01	22.85	-4.01

Y_1 – Total phenolic content (mg GAE/g DW); Y_2 – Total flavonoid content (mg RE/g DW); Y_3 – Total tannin content (mg TAE/g DW); Y_4 – DPPH scavenging activity (mg TE/g DW); Y_5 – Ferrous ion chelating activity (%); Y_6 – Superoxide anion scavenging activity (%)

CONCLUSIONS

This is the first study reported on application of response surface methodology on optimization of phenolics content and antioxidant activities from the *in vitro* propagated leaves of *Curcuma caesia.*

1. The results showed that the extraction conditions, including solvent-solid ratio, methanol concentration, and extraction temperature, were optimized for the maximum extraction of total phenolic content, total flavonoid content, total tannin content, DPPH scavenging activity, ferrous ion chelating activity, and superoxide anion scavenging activity, from the *in vitro* propagated leaves of *C. caesia.*
2. Based on the analysis, the optimum extraction conditions obtained for each variable were 54.02 mL/g of solvent-solid ratio (X_1), 70% methanol concentration (X_2), and 70 °C of extraction temperature (X_3).

3. Based on the optimal extraction conditions, the experimental value recorded for all responses were within the 95% of confidence interval which indicated that the extraction conditions were successfully optimized.
4. This study showed that the *C. caesia* that was propagated using plant tissue culture technique has a huge potential to be used as a source of plant secondary metabolites production. Hence, the *C. caesia* in their natural habitat can be protected and conserved from the over-harvesting activity.

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