

Optimization of Extraction Conditions for Phenolic Acids from the Leaves of *Melissa officinalis* L. Using Response Surface Methodology

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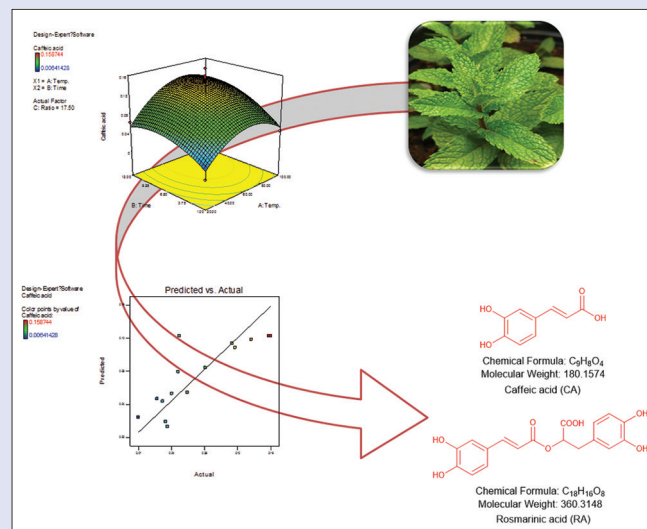
ABSTRACT

Background: *Melissa officinalis* L. is a well-known medicinal plant from the family *Lamiaceae*, which is distributed throughout Eastern Mediterranean region and Western Asia. **Objective:** In this study, response surface methodology (RSM) was utilized to optimize the extraction conditions for bioactive compounds from the leaves of *M. officinalis* L. **Materials and Methods:** A Box–Behnken design (BBD) was utilized to evaluate the effects of three independent variables, namely extraction temperature (°C), methanol concentration (%), and solvent-to-material ratio (mL/g) on the responses of the contents of caffeic acid and rosmarinic acid. **Results:** Regression analysis showed a good fit of the experimental data. The optimal condition was obtained at extraction temperature 80.53°C, methanol concentration 29.89%, and solvent-to-material ratio 30 mL/g. **Conclusion:** These results indicate the suitability of the model employed and the successful application of RSM in optimizing the extraction conditions. This study may be useful for standardizing production quality, including improving the efficiency of large-scale extraction systems.

Key words: Box–Behnken design, caffeic acid, *Melissa officinalis* L., response surface methodology, rosmarinic acid

SUMMARY

- The optimum conditions for the extraction of major phenolic acids from the leaves of *Melissa officinalis* L. were determined using response surface methodology
- Box–Behnken design was utilized to evaluate the effects of three independent variables
- Quadratic polynomial model provided a satisfactory description of the experimental data
- The optimized condition for simultaneous maximum contents of caffeic acid and rosmarinic acid was determined.



Abbreviations used: RSM: Response surface methodology, BBD: Box–Behnken design, CA: Caffeic acid, RA: Rosmarinic acid, HPLC: High-performance liquid chromatography.

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INTRODUCTION

Melissa officinalis L. (lemon balm) is a perennial herb that belongs to the family *Lamiaceae* and is found throughout the Eastern Mediterranean region and Western Asia.^[1,2] Therapeutic properties of *M. officinalis* L. are anti-inflammatory,^[3] hepatoprotective,^[4,5] antioxidant,^[6-9] sedative,^[10] anxiolytic,^[11] antifungal, anti-bacterial,^[12,13] antiviral,^[14] antilipidemic, antihistaminic,^[15] and antispasmodic^[16] activities, mainly due to the content of essential oils and phenolic acids.^[17] In particular, leaves of *M. officinalis* L. have been used as a medicinal plant for the treatment of headaches, rheumatism, and gastrointestinal disease.^[18] In the leaves of *M. officinalis* L., caffeic acid (CA) and rosmarinic acid (RA) are the representative phenolic acids.^[19] CA has antitumor^[20,21] and antifungal activities,^[22] while RA has antioxidant,^[23,24] anti-allergic, and immunosuppressive effects.^[25,26] Therefore, the biological activities of

phenolic acids from *M. officinalis* L. have recently been the subject of many studies.^[27-29]

Extraction is the first important step to recover bioactive compounds from plants for natural product research.^[30] Many factors such as extraction temperature,^[31] solvent-to-material ratio,^[32] extraction time, solvent

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composition, and extraction pressure,^[33] among others, may significantly influence the extraction efficacy. In general, optimization of a process can be determined by an empirical or statistical method. However, empirical methods have the limitations in perfect optimization.^[34] Response surface methodology (RSM) is a powerful tool for improving and optimizing extraction process variables.^[34-36]

MATERIALS AND METHODS

Plant materials

The dried leaves of *M. officinalis* L. were kindly supplied by Richwood Pharmaceutical Co. Inc. (Seoul, Korea) that also provided a certificate of identity and quality. A voucher specimen (YIPS-MO-160518) was deposited at the Herbarium of College of Pharmacy, Yonsei Institute of Pharmaceutical Sciences, Yonsei University, Incheon, Korea.

Reagents and apparatus

All organic solvents, such as hexane, chloroform, ethyl acetate, methanol, and n-butanol, used for extraction and chromatography were of analytical grade and were purchased from Daesan Chemical (Gyeonggi, Korea). Thin layer chromatography was performed on a precoated silica gel 60 F₂₅₄ (Merck, NJ, USA). high-performance liquid chromatography (HPLC) was carried out using an Agilent 1260 HPLC system.

High-performance liquid chromatography analysis

Chromatographic separation was carried out on an YMC hydrosphere C₁₈ column (250 mm × 4.6 mm, 5 μm) equipped with a C₁₈ guard column. The mobile phase consisted of acetonitrile (A) and 0.1% phosphoric acid (v/v) (B) at a flow rate of 0.1 mL/min. Analysis was performed using a linear gradient program at the following conditions: 0 min, 10% A and 90% B and 25 min, 90% A and 10% B. Then, the column was reconditioned with 90% B for 5 min. The column temperature was set at 40°C, and the analysis was monitored at 320 nm. Aliquots of 10 μL were injected into the HPLC. Standard working solutions were prepared by serial dilutions of 1.0, 0.5, 0.25, 0.125, and 0.0625 mg/mL and used for calibration curve. Good linearity of calibration curve for CA and RA was achieved with correlation coefficient of 0.999. The powdered leaves of *M. officinalis* L. (1.00 g) were precisely weighed and extracted with methanol solvent as indicated. Each sample solution was filtered through 0.2 mm membrane filter before being subject to HPLC analysis.

Experimental design

An optimization of extraction conditions for the extraction of CA and RA from the leaves of *M. officinalis* L. was conducted using RSM. Before conducting the RSM, the levels of RSM-independent variables for phenolic acid extraction were determined based on the preliminary experiments. In brief, 15 experimental runs were conducted with three independent variables, and three levels were developed according to the Box–Behnken design (BBD) as shown in Table 1. BBD has been widely used to experiment with the designing that evaluates nonlinear relationships between response values and factors. Compared with other designs, it has the advantage of reducing the number of experiments.^[37] The independent variables were extraction temperature (X_1 , °C), methanol concentration (X_2 , %, v/v, methanol/water), and solvent-to-material ratio (X_3 , mL/g), while the response variable was the amount of phenolic acids from the leaves of *M. officinalis* L.

Statistical analysis

Data were analyzed using the Design Expert 7.0 (Stat-Ease Inc., MN, USA) statistical software. The experimental data were applied to the quadratic polynomial model through which the regression coefficients were obtained. The generalized quadratic polynomial model used for the response surface analysis is shown by the following equation (1):

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

where X_i and X_j values are independent variables which influence the responses Y ; β_0 , β_i , β_{ii} , and β_{ij} values are the coefficients of regression model for intercept, linear, quadratic, and interaction terms, respectively; and k is the number of variables.

Verification of model

To verify the predictive value of the model, the optimum conditions with the maximum yield for phenolic acid were determined and used in the extraction test. The precision of the fitted model was verified by comparing the predicted value of the experimental value obtained from the three replicates.

RESULTS AND DISCUSSION

Model fitting

Optimization of the extraction condition was performed by BBD. According to the BBD design, 15 experiments were performed. Extraction

Table 1: Experimental design and responses of the dependent variables to extraction conditions

Standard order ^a	Run order ^b	Coded variables			Independent variables			Dependent variables (responses)	
		X_1	X_2	X_3	Extraction temperature (°C)	MeOH concentration (%)	Solvent ratio (mL/g)	CA (mg)	RA (mg)
12	1	1	-1	0	100	0	20	0.3447	3.5831
14	2	-1	0	1	20	50	30	0.1275	4.5935
6	3	-1	1	0	20	100	20	0.0168	0.6733
15	4	1	1	0	100	100	20	0.1719	3.8424
1	5	1	0	1	100	50	30	0.2580	5.3735
7	6	0	0	0	60	50	20	0.2225	4.938
2	7	-1	-1	0	20	0	20	0.1625	1.2892
3	8	0	1	-1	60	100	10	0.1343	2.8304
8	9	0	-1	-1	60	0	10	0.3775	3.386
13	10	0	-1	1	60	0	30	0.3826	3.385
9	11	0	1	1	60	100	30	0.2570	3.3593
10	12	-1	0	-1	20	50	10	0.1071	3.495
5	13	1	0	-1	100	50	10	0.1895	3.8823
4	14	0	0	0	60	50	20	0.1906	5.1888
11	15	0	0	0	60	50	20	0.2254	5.4789

^aNo randomized, ^bRandomized. CA: Caffeic acid; RA: Rosmarinic acid

temperature (°C), methanol concentration (%), and solvent-to-material ratio (mL/g) were selected as three variables that could potentially affect the contents of CA and RA. Quantitation of CA and RA was performed by HPLC analysis [Figure 1]. As shown in Table 1, the CA and RA contents were varied notably depending on extraction condition [Figure S1]. The analysis of variance (ANOVA) was statistically significant ($P < 0.05$) and suggested that the variables in the model can explain the experimental variation of CA and RA contents. Coefficient of determination (R^2), adjusted R^2 (adj R^2), and lack-of-fit values are shown in Table 2. The results suggested that the model was suitable for the experimental data.

Effect of extraction parameters on the yield of caffeic acid

The relationship between the CA contents and the extraction parameters is explained in the mathematical equation (2) as follows:

$$\text{CA contents} = 0.21 + 0.069X_1 - 0.086X_2 + 0.027X_3 - 0.007X_1X_2 + 0.012X_1X_3 + 0.029X_2X_3 - 0.078X_1^2 + 0.039X_2^2 + 0.036X_3^2 \quad (2)$$

In the models, the linear term of methanol concentration (X_2) had the most significant effect ($P < 0.01$) on CA Contents, followed by the

linear term of extraction temperature (X_1) [Table 3]. The linear term of solvent-to-material ratio (X_3) and quadratic term of X_1^2 and X_3^2 also showed a significant effect ($P < 0.05$) whereas interaction terms of X_1X_2 , X_1X_3 , and X_2X_3 were not significant. Table 2 shows the ANOVA of the fitted quadratic polynomial model for CA contents. The fitness of the predicted model for CA contents was verified by f -value of 24.8411 and $P = 0.0012$. In this response, the R^2 was 0.978 and the adj. R^2 was 0.939, indicating a high correlation between the observed and predicted values. In addition, P value for lack-of-fit was 0.3297, which is not significant relative to the pure errors. In general, lack-of-fit test for the model is used to verify the adequacy of the model.^[38] If the model does not fit well with the data, the lack-of-fit value will be significant, and the incorrect response surface values can be induced.^[39] In this study, statistical analysis demonstrated the suitability of predictive and experimental values and the usefulness of a quadratic polynomial model for optimization. To

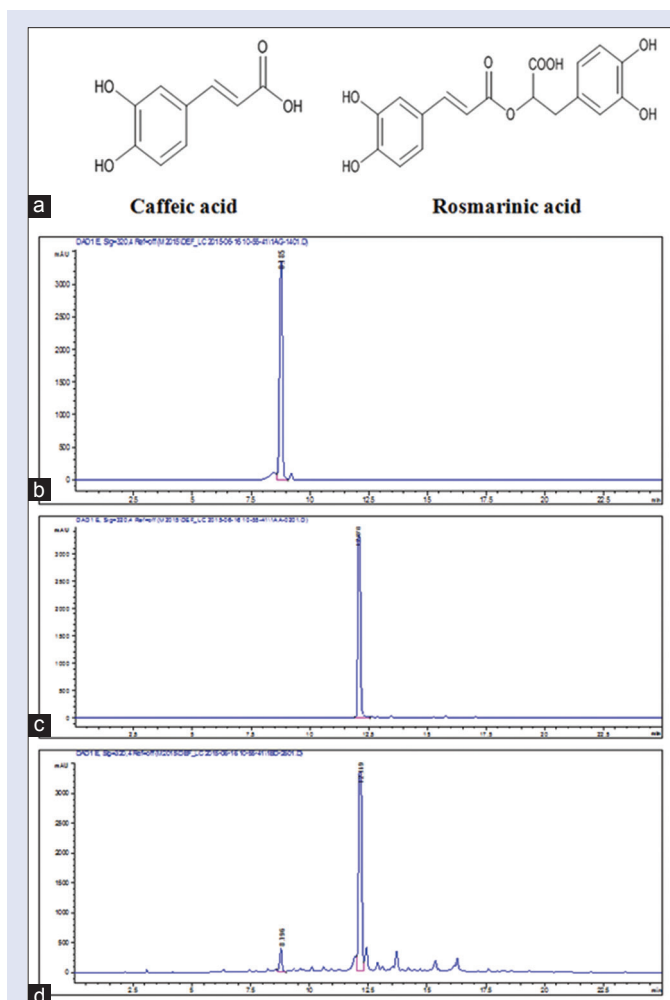


Figure 1: Chemical structures and high-performance liquid chromatography profiles. (a) Chemical structures of caffeic acid and rosmarinic acid, (b) high-performance liquid chromatogram of caffeic acid, (c) high-performance liquid chromatogram of rosmarinic acid, and (d) high-performance liquid chromatograms of *Melissa officinalis* L. extracts at the optimum conditions

Table 2: Analysis of variance for the response surface quadratic models

	Sum of square	Degree of freedom	Mean square	F	P
CA					
Model	0.1426	9	0.0158	24.8411	0.0012
Residual error	0.0032	5	0.0006		
Lack-of-fit	0.0024	3	0.0008	2.1808	0.3297
Pure error	0.0007	2	0.0004		
Total	0.1458	14			
R^2			0.978		
Adjusted R^2			0.939		
RA					
Model	23.6704	9	2.6300	4.3963	0.0588
Residual error	2.9912	5	0.5982		
Lack-of-fit	2.8446	3	0.9482	12.9396	0.0726
Pure error	0.1466	2	0.0733		
Total	26.6615	14			
R^2			0.973		
Adjusted R^2			0.954		

CA: Caffeic acid; RA: Rosmarinic acid

Table 3: Regression coefficients and their significances in the quadratic polynomial regression equations for caffeic acid and rosmarinic acid contents

	Coefficient	SE	t	P
CA				
Intercept	0.2128	0.0146	14.6050	0.0001
X_1	0.0688	0.0089	59.2926	0.0006
X_2	-0.0859	0.0089	92.5463	0.0002
X_3	0.0271	0.0089	9.1945	0.0290
X_1X_2	-0.0068	0.0126	0.2866	0.6154
X_1X_3	0.0120	0.0126	0.9073	0.3846
X_2X_3	0.0294	0.0126	5.4147	0.0675
X_1^2	-0.0781	0.0131	35.3043	0.0019
X_2^2	0.0392	0.0131	8.9128	0.0306
X_3^2	0.0358	0.0131	7.4127	0.0416
RA				
Intercept	5.2019	0.4466	11.6500	0.0001
X_1	0.8287	0.2735	9.1841	0.0291
X_2	-0.1173	0.2735	0.1841	0.6857
X_3	0.3897	0.2735	2.0305	0.2135
X_1X_2	0.2188	0.3867	0.3201	0.5960
X_1X_3	0.0984	0.3867	0.0647	0.8093
X_2X_3	0.1324	0.3867	0.1171	0.7461
X_1^2	-0.8795	0.4025	4.7743	0.0806
X_2^2	-1.9754	0.4025	24.0839	0.0044
X_3^2	0.0139	0.4025	0.0012	0.9739

SE: Standard error; CA: Caffeic acid; RA: Rosmarinic acid

visualize the relationship between the CA contents, which are dependent variables, and the extraction conditions, which are independent variables, we applied a quadratic polynomial model equation to construct three-dimensional (3D) surface plots [Figure 2]. As shown in Figure 2a, when solvent-to-material ratio was fixed at center point (20 mL/g), CA contents was increased slightly by decreasing methanol concentration from 75% to 0% and reached the maximum value at the fixed extraction temperature of 80°C. Figure 2b shows the correlation between the extraction temperature and the solvent-to-material ratio to the CA content at a fixed center point (50%) of the methanol concentration. Maximum CA contents were obtained at the highest solvent-to-material ratio (30 mL/g) and then increased slightly by increasing extraction temperature to 80°C. Figure 2c shows the correlation between methanol concentration and solvent-to-material ratio on the CA contents at a fixed extraction temperature of 60°C. Maximum CA contents were obtained at the lowest methanol concentration at a fixed solvent-to-material ratio of 10 mL/g. As a result, the response surface analysis and statistical analysis

showed that the CA contents were significantly increased when the methanol concentration decreased.

Effect of extraction parameters on the yield of rosmarinic acid

The relationship between the RA contents and the extraction parameters is explained in mathematical equation (3) as follows:

$$\text{RA contents} = 5.202 + 0.829X_1 - 0.117X_2 + 0.39X_3 + 0.219X_1X_2 + 0.098X_1X_3 + 0.132X_2X_3 - 0.88X_1^2 - 1.98X_2^2 + 0.014X_3^2 \quad (3)$$

In the models, the quadratic term of methanol concentration (X_2^2) had the most significant effect ($P < 0.05$) on RA content [Table 3]. The linear term of extraction temperature (X_1) also showed a significant effect ($P < 0.05$). However, the interaction terms X_1X_2 , X_1X_3 , and X_2X_3 with the other linear terms X_2 and X_3 and the quadratic terms X_1^2 and X_3^2 did not show a significant effect on RA contents. The

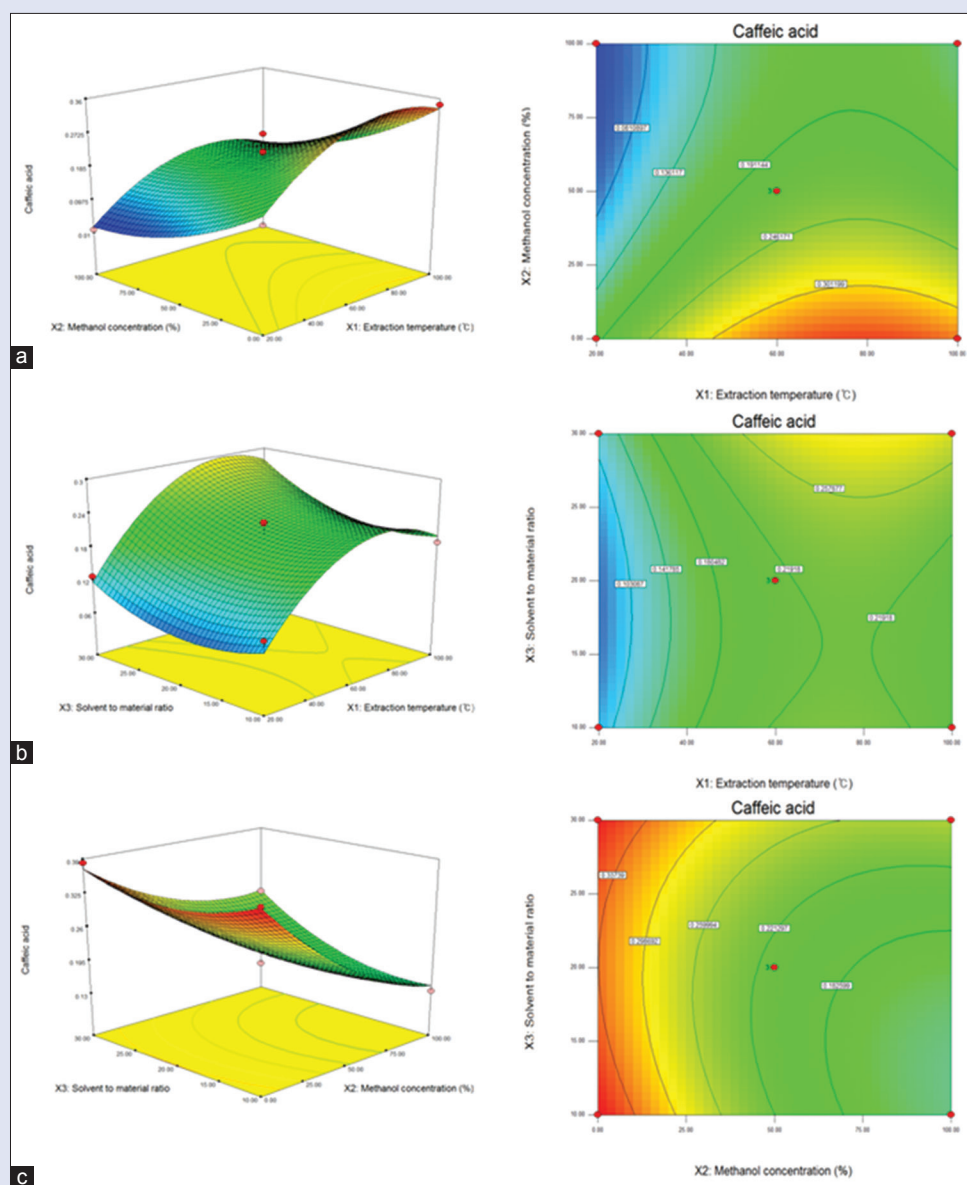


Figure 2: Response surface plot for the effects of extraction conditions for caffeic acid from the leaves of *Melissa officinalis* L., (a) extraction temperature and methanol concentration, (b) extraction temperature and solvent-to-material ratio, (c) methanol concentration and solvent-to-material ratio

fitness of the predicted model for RA contents was verified by f -value of 4.3963 and a P value of 0.0588. In this response, the R^2 and the adj. R^2 were 0.973 and 0.954, respectively, and P value for lack-of-fit was 0.0726 [Table 2]. These values represent a good match between the experimental and predicted values. To visualize the relationship between the RA contents, which are dependent variables, and the extraction conditions, which are independent variables, we applied a quadratic polynomial model equation to construct 3D surface plots [Figure 3]. As shown in Figure 3a, when solvent-to-material ratio was fixed at the center point (20 mL/g), RA contents were increased by increasing the extracted temperature from 20°C to 78°C and reached the maximum value by increasing methanol concentration from 0% to 49%. Figure 3b shows the correlation between extraction temperature and solvent-to-material ratio on the RA contents at a fixed methanol concentration of 50%. RA contents increased steadily when extraction temperature increased from 20°C to 87°C; however, the RA contents decreased when temperature exceeded 90°C. RA contents also increased when solvent-to-material

ratio increased from 10 to 30 mL/g. Figure 3c shows the correlation between methanol concentration and solvent-to-material ratio on the RA contents at a fixed extraction temperature of 60°C. The maximum RA content was obtained at a methanol concentration of 25%–75% and a solvent-to-material ratio of 29.52 mL/g. As a result, the response surface analysis and statistical analysis showed that the RA contents were significantly affected by the methanol concentration, extraction temperature, and solvent-to-material ratio.

Experimental validation of the optimum conditions

Verification experiments were performed using the recommended optimal conditions derived from RSM [Table 4]. The optimal conditions for both the maximum CA and RA contents were determined at a temperature of 80.53°C, methanol concentration of 29.89%, and solvent-to-material ratio of 30 mL/g. This predicted an extraction of 0.33 mg of CA content and 5.48 mg of RA content. Under the temperature of 80°C, methanol concentration of 30%, and solvent-to-material ratio

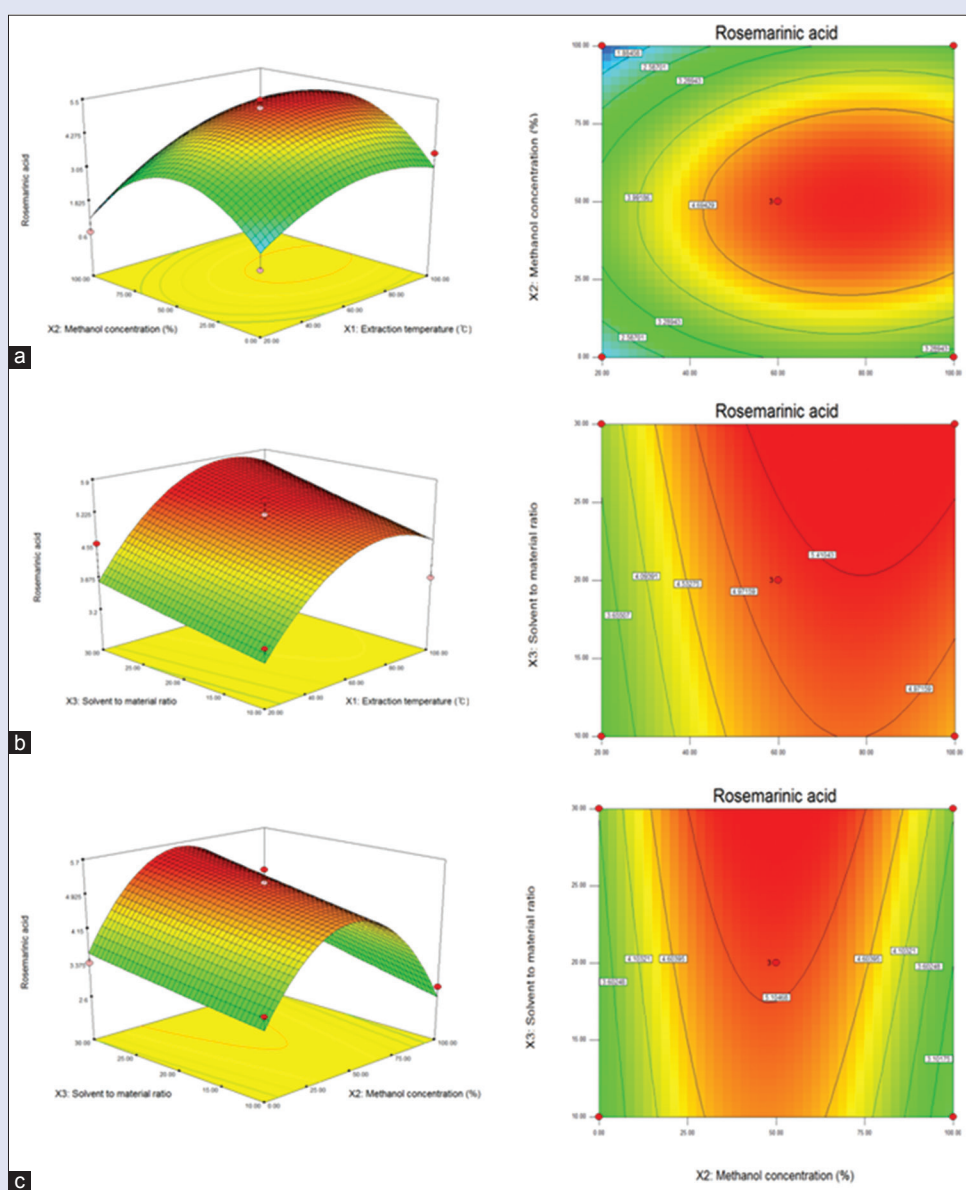


Figure 3: Response surface plot for the effects of extraction conditions for rosmarinic acid from the leaves of *Melissa officinalis* L., (a) extraction temperature and methanol concentration, (b) extraction temperature and solvent-to-material ratio, (c) methanol concentration and solvent-to-material ratio

Table 4: Predicted and experimental values of response variables under optimal conditions

Parameters	Optimum values	
	Predicted values ^a	Experimental values ^b
Extraction temperature (°C)	80.53	80
Methanol concentration (%)	29.89	30
Solvent to material ratio (mL/g)	30	30
CA (mg)	0.33	0.37±0.1
RA (mg)	5.48	5.62±0.4

^aPredicted using ridge analysis of response surface quadratic model, ^bMean±SD of triplicate determinations from different experiments. SD: Standard deviation; CA: Caffeic acid; RA: Rosmarinic acid

of 30 mL/g, the observed values were 0.37 ± 0.2 mg of CA content and 5.62 ± 0.4 mg of RA content. These results show that the actual experimental values are in good agreement with the predicted values in the regression model. Hence, the response surface modeling can be applied effectively to the prediction of extraction of major phenolic acids from *M. officinalis* L. leaves.

CONCLUSION

In this study, RSM was successfully employed to optimize the major phenolic acids extraction from *M. officinalis* L. leaves. The quadratic polynomial model provided a satisfactory description of the experimental data. These results showed that the optimized conditions with the simultaneous maximum content of CA and RA were determined, and methanol concentration was the most important factor for phenolic acid extraction from *M. officinalis* L. This study may be useful for standardizing production quality, including improving the efficiency of large-scale extraction systems.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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