Optimization of the extraction process for the seven bioactive compounds in Yukmijihwang-tang, an herbal formula, using response surface methodology

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ABSTRACT

Background: Yukmijihwang-tang (YJT) contains multiple bioactive compounds. Heat-reflux extraction was employed and optimized for the extraction of the bioactive compounds in YJT. **Objective:** The determination of optimal conditions with maximum yields of bioactive compounds, gallic acid, 5-hydroxymethylfurfural, morroniside, loganin, paeoniflorin, benzoic acid and paeonol, in YJT. **Materials and Methods:** The extraction ratio (ratio of water to herbal formula), extraction time and extraction number were set as individual values and the yields of the seven compounds were the response values that were optimized with a Box–Behnken design. **Results:** The optimal conditions obtained from response surface methodology (RSM) were 1:11.99 for the extraction ratio, 94.53 min for the extraction time and 2.21 for the extraction number. Under the optimal conditions, the response value of the experiment closely agreed with the predicted response value. **Conclusions:** The result suggests that RSM is successfully applied for optimizing the extraction of the marker compounds in YJT.

Key words: Bioactive compounds, heat-reflux extraction, optimal condition, response surface methodology, Yukmijihwang-tang

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INTRODUCTION

A herbal formula is prepared by boiling the herbal mixture with water before it is administered to the patients, and most *in vivo* and *in vitro* experimental models using a herbal formula as a treating agent have dealt with the water extract produced in the laboratory.[1,2] The therapeutic effect of a herbal formula is attributed to the synergistic property that results from the combination and interaction of bioactive constituents from herbal medicines.[3] Thus, the extraction method must be designed to produce efficiently the bioactive compounds from the herbal formula, so that those compounds can contribute to exert the curative effect.

Heat-reflux extraction (HRE) is a conventionally and widely used extraction method for the preparation of herbal medicine,[4-6] and it is close to the traditional extraction method of an herbal formula. There are many parameters determining the adequate conditions of an herbal extract, including extraction time, the number of extractions, and ratio of solvent to raw material, extraction temperature and pressure.[7,8] In the HRE process, water is boiled at 100°C and the evaporated vapor turns to water droplets in the attached condenser on the flask; hence, the temperature and pressure are not variables to be chosen as extraction parameters.

Yukmijihwang-tang (YJT, Liuweidihuang-tang in Chinese) is a widely used herbal formula in Korea and China. YJT consists of six herbal medicines including *Rehmannia glutinosa* Libosch. ex Steudel, *Dioscorea batatas* Decne., *Cornus officinalis* Sieb. et Zucc., *Paeonia suffruticosa* Andrews, *Poria cocos* F.A. Wolf, and *Alisma orientale* Juzep. Several pharmacological properties of YJT have been reported, such as renal protection,[9,10] regulation against autoimmune encephalomyelitis,[11] improving learning and memory,[12] protection against β-amyloid-induced paralysis and myelosuppression,[13,14] antiobesity[15] and antioxidant activity.[16] The main bioactive compounds of YJT are gallic acid, 5-hydroxymethylfurfural (5-HMF), morroniside, loganin, paeoniflorin, benzoic acid and

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paeonol which are analyzed using high performance liquid chromatography (HPLC)–ultraviolet–mass spectrometry, HPLC–diode array detector (DAD) or micellar electrokinetic chromatography.[17-20]

Response surface methodology (RSM) is a statistical technique to determine the optimum values of the independent variables to achieve the maximum response, and enables the user to investigate the interaction of the individual variables, which is considered more efficient than the traditional single parameter optimization because of the saving in time, space, and raw materials.[21] For those reasons, RSM has been employed in the extraction of chemical compounds from herbal medicines.[22-24]

The aim of this study was to optimize the extraction process for the seven bioactive compounds from YJT using RSM. The extraction factors, ratio of water to herbal formula, extraction time and extraction number, were chosen as the independent variables for the extraction and their influence on the yields of the compounds was studied through a Box–Behnken design (BBD). The content of the bioactive compounds was determined using HPLC–DAD analysis with a validated method. To the best of our knowledge, this is the first study on the optimization of chemical components from an herbal formula using RSM.

MATERIALS AND METHODS

Chemicals and reagents

High performance liquid chromatography-grade methanol, acetonitrile, and water were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). Gallic acid (1), 5-HMF (2) and benzoic acid (6) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Morroniside (3), loganin (4), paeoniflorin (5) and paeonol (7) were obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan). All of the compounds represented a purity of more than 98%. The chemical structures of the standard compounds are shown in Figure 1.

Compositional herbal medicines were purchased from the herbal medicine company, Kwangmyungdang Medicinal Herbs (Ulsan, Korea) [Table 1]. Herbal medicines were identified by Professor Je-Hyun Lee (Department of Herbology, Dongguk University, Korea) and Young Bae Seo (Department of Herbology, Daejeon University, Korea). A voucher specimen (2013-KE07-1-6) has been deposited in the Herbal Medicine Formulation Research Group of the Korea Institute of Oriental Medicine.

Extraction procedure of Yukmijihwang-tang

The herbal medicine mixture consisting of YJT was extracted with a 10-fold volume of distilled water (w/v) by boiling using reflux extractor. The extracted decoction was centrifuged at 3000 rpm for 10 min and the supernatant was lyophilized to create powder.

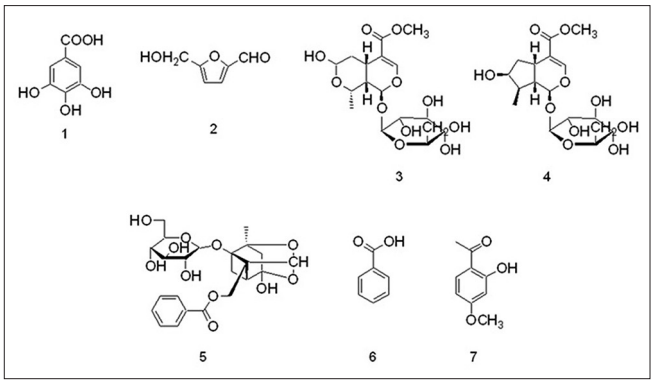

Figure 1: Chemical structures of standard compounds in Yukmijihwang-tang; gallic acid (1), 5-hydroxymethylfurfural (2), morroniside (3), loganin (4), paeoniflorin (5), benzoic acid (6) and paeonol (7)

Table 1: Composition of herbal medicine in YJT

Herbal medicine	Original region	Amount (g)
Rehmannia glutinosa Libosch. ex Steudel	Euiseong, Gyeongbuk, Korea	8.0
Dioscorea batatas Decne	Andong, Gyeongbuk, Korea	4.0
Cornus officinalis Sieb. et Zucc	Gurye, Jeonnam, Korea	4.0
Paeonia suffruticosa Andrews	Jecheon, Chungbuk, Korea	3.0
Poria cocos F.A. Wolf	Pyeongchang, Gangwon, Korea	3.0
Alisma orientale Juzep	Namyangju, Gyeonggi, Korea	3.0
Sum	-	25.0

YJT: Yukmijihwang-tang

Accurately weighed powders of YJT water extract (10 mg) were dissolved in 1 mL of HPLC grade-water and the solutions were filtered through a 0.2 μm syringe filter (SmartPor®, Woongki Science, Seoul, Korea) prior to HPLC analysis.

Chromatographic conditions

The analysis was carried out using a Hitachi HPLC–DAD system equipped with a solvent delivery unit, autosampler, column oven, and diode-array detector. The acquired data were processed using EZChrome Elite for Hitachi. Separation was performed on a Gemini C_{18} column (4.6 mm × 250 mm, 5 μm; Phenomenex, Torrance, CA, USA) at 35°C. The mobile phase, consisting of solvent A (1% aqueous acetic acid, *v/v*) and solvent B (acetonitrile with 1% acetic acid, *v/v*), was eluted using the gradient procedure, which was as follows: 5-40% (B) over 0-30 min, 40-100% (B) over 30-40 min, held for 5 min, and then re-equilibrated to 5% for 15 min. The flow

rate was 1.0 mL/min and the injection volume was set to 10 μL. The optimized detection wavelengths for standard compounds were set at 230, 272, and 280 nm.

Method validation

Accurately weighed standard compounds were dissolved in methanol at concentrations of 1000 μg/mL to produce a stock solution containing the seven standard compounds. The stock solution was diluted at five levels to make working solutions that were used to construct calibration curves in which the x-axis was the concentration of marker compound and the y-axis was the area of the marker compound. Linear regression and the coefficient of determination (r^2) of the compounds were calculated based on the calibration curves. The values of limits of determination (LOD) and limits of quantification (LOQ) were evaluated from the concentrations of each compound at signal-to-noise ratios of 3 and 10, respectively.

The precisions were measured by analyzing sample extracts at two concentrations of standard compounds of low and high levels on same day (intra-day) and three successive days (inter-day), which is represented by the values of the RSD. The recovery test that was used to evaluate the accuracy of the method was determined by assessing two different concentration levels of spiked compounds (low and high) for the samples. The recovery was calculated as follows:

$$\text{Recovery (\%)} = ([\text{Detected concentration} - \text{initial concentration}]/\text{Spiked concentration}) \times 100$$

Experimental design and statistical analysis

To determine the optimum condition for extraction of YJT, the preliminary range of the extraction variables, extraction ratio (ratio of water to the herbal formula), extraction time and the number of extractions, were investigated using a single-factor test. A three-level-three-factor BBD was employed to determine the optimal conditions for the extraction of the seven bioactive compounds in YJT.

Experimental data obtained from the BBD were fitted to a second-order polynomial model and the regression coefficients were obtained. The equation is as follows:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum \sum_{i<j} \beta_{ij} X_i Y_j$$

Where Y is the estimated response, β_0, β_j, β_{jj} and β_{ij} are the regression coefficients for intercept, linearity, square and interaction terms, respectively. X_i and X_j are the independent variables, which were coded.

The fitness of the second-order polynomial model was expressed by the lack of fit and coefficient of determination (r^2). *F*-test and *P* values resulting from the analysis of variance (ANOVA) were calculated to confirm the significance of the regression coefficients, which was determined at $P < 0.05$ or 0.01. The interaction and influence of the three variables on the yield of the bioactive compound was represented as three-dimensional response surface plots and contour plots, on which the optimal extraction condition was observed. The open-source software R (ver. 2.15.1; The R Foundation for Statistical Computing) was used to generate the experimental design, statistical analysis and regression model.

RESULTS AND DISCUSSION

Method validation

Using the developed HPLC methods, all of the bioactive compounds were well-detected and selective without any interference from endogenous constituents on chromatograms at their maximum absorption wavelengths [Figure 2]. On the basis of the calibration curves, the coefficient of determination (r^2) ranged from 0.9992 to 1.0000 for all analytes, which means good linearity. The ranges of LODs and LOQs were 0.01-0.09 μg/mL and 0.04-0.30 μg/mL, respectively [Table 2]. The precisions of the seven bioactive compounds represented as RSD values were 0.05-0.51% for intra-day precision and 0.01-1.10% for inter-day precision at two levels of concentrations [Table 3]. The recoveries of the seven marker compounds were in the range of 91.25-107.91%, with RSD values <4.1% over the concentration ranges [Table 4].

Model fitting

Preliminary experiments using single-factor tests determined the required range of ratio of water to

Table 2: Linear equations, coefficients of determination (r^2), LOD and LOQ for the bioactive compounds

Compound	Linear equation	r^2	Linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
Gallic acid	y=152447 x+41887	0.9999	1.56-50	0.02	0.08
5-HMF	y=319334 x+313197	0.9998	6.25-100	0.01	0.04
Morroniside	y=123459 x+125.68	0.9996	3.13-100	0.04	0.12
Loganin	y=56648 x+61532	0.9992	1.56-100	0.05	0.18
Paeoniflorin	y=42140 x+4838	1.0000	1.56-100	0.09	0.30
Benzoic acid	y=110322 x+27694	0.9992	0.78-25	0.04	0.12
Paeonol	y=197680 x+143817	0.9998	6.25-100	0.01	0.05

y: Peak area (mAU); x: Concentration of the compound (μg/mL). LOD: Limits of determination; LOQ: Limits of quantification; 5-HMF: 5-hydroxymethylfurfural

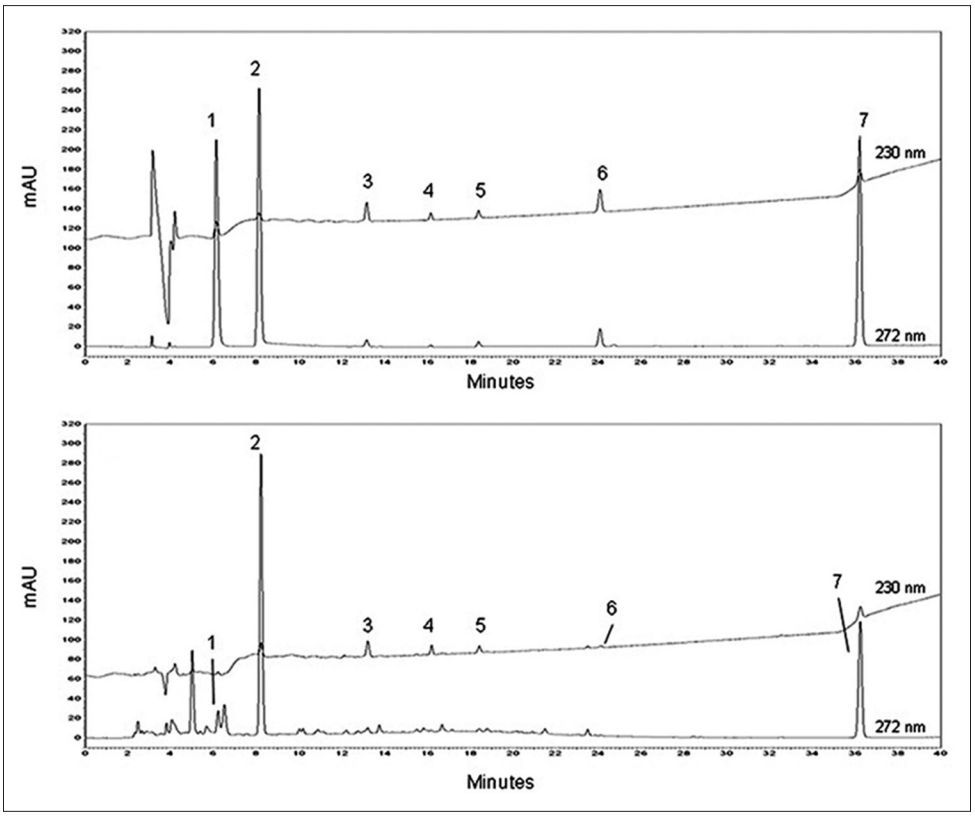

Figure 2: Chromatogram of standard compounds (a) and Yukmijihwang-tang water extract (b) at their optimum wavelength; gallic acid (1), 5-hydroxymethylfurfural (2), morroniside (3), loganin (4), paeoniflorin (5), benzoic acid (6), and paeonol (7)

Table 3: Intra- and inter-day precision of the bioactive compounds

Compound	Spiked concentration (μg/mL)	Intra-day (*n*=3)		Inter-day (*n*=3)	
		Detected concentration (μg/mL)	RSD (%)	Detected concentration (μg/mL)	RSD (%)
Gallic acid	4.00	3.89	0.09	3.71	0.07
	8.00	8.05	0.05	8.14	0.03
5-HMF	15.00	14.71	0.41	14.39	0.39
	30.00	30.15	0.21	30.30	0.18
Morroniside	10.00	10.23	0.07	10.25	0.06
	20.00	19.89	0.03	19.88	0.03
Loganin	10.00	9.62	0.14	9.47	0.21
	20.00	20.19	0.07	20.26	0.10
Paeoniflorin	15.00	15.26	0.51	13.47	1.10
	30.00	29.87	0.25	31.50	0.11
Benzoic acid	1.50	1.68	0.13	1.72	0.10
	3.00	2.91	0.06	2.89	0.06
Paeonol	15.00	14.88	0.10	14.95	0.03
	30.00	30.06	0.05	30.02	0.01

RSD (%) = (Standard deviation/mean) ×100. RSD: Relative standard deviation

herbal formula (X_1, 1:8-1:16), extraction time (X_2, 60-120 min) and the number of extractions (X_3, 1-3 repeats). A three-level-three-factor BBD comprising the 15 experiments listed in Table 5 was employed in this study, in which three replicates (runs 7, 9 and 11) were used to measure the pure error sum of squares. The sum of the yields of the seven marker compounds was treated as the response. The three factors used in this study were represented as three coded levels (–1, 0, 1) for each factor.

With the help of multiple regression analysis on the experimental data, the predicted response value was expressed by the following second-order polynomial equation using coded variables:

$$Y = 13.63 - 0.04X_1 + 0.26X_2 + 0.12X_3 + 0.02X_1X_2 + 0.19X_1X_3 - 0.28X_2X_3 - 0.51X_1X_1 - 0.71X_2X_2 - 0.24X_3X_3$$

Table 4: Recovery of the bioactive compounds

Compound	Concentration (μg/mL)			Recovery (%)	RSD (%)
	Initial	Spiked	Detected		
Gallic acid	6.21	4.00	10.12	99.67	3.04
		8.00	14.85	108.03	0.37
5-HMF	38.08	15.00	53.21	99.80	3.99
		30.00	69.61	104.95	0.41
Morroniside	23.66	10.00	33.87	99.61	1.19
		20.00	43.53	99.35	0.55
Loganin	25.00	10.00	35.11	99.98	1.68
		20.00	46.59	107.91	0.45
Paeoniflorin	32.09	15.00	46.29	97.59	4.07
		30.00	61.27	97.27	2.61
Benzoic acid	3.33	1.50	4.98	100.45	3.74
		3.00	6.21	96.15	3.15
Paeonol	29.87	15.00	43.67	92.00	2.45
		30.00	57.06	91.25	0.55

RSD (%) = (Standard deviation/mean) ×100. RSD: Relative standard deviation; 5-HMF: 5-hydroxymethylfurfural

Table 5: Box-Behnken design and the response values for yields of compounds

Run order	Coded and uncoded variables levels			Yield of compounds (mg/g)	
	X_1, ratio (ratio, mL)	X_2, time (min)	X_3, number (repeat)	Actual value	Predicted value
1	1 (1:16, 400)	0 (90)	1 (3)	13.26	13.16
2	−1 (1:8, 200)	1 (120)	0 (2)	12.55	12.69
3	−1 (1:8, 200)	−1 (60)	0 (2)	12.27	12.20
4	1 (1:16, 400)	0 (90)	−1 (1)	12.37	12.54
5	−1 (1:8, 200)	0 (90)	−1 (1)	12.88	12.99
6	0 (1:12, 300)	1 (120)	−1 (1)	13.34	13.10
7	0 (1:12, 300)	0 (90)	0 (2)	13.50	13.63
8	1 (1:16, 400)	−1 (60)	0 (2)	12.23	12.09
9	0 (1:12, 300)	0 (90)	0 (2)	13.58	13.63
10	0 (1:12, 300)	−1 (60)	1 (3)	12.57	12.82
11	0 (1:12, 300)	0 (90)	0 (2)	13.81	13.63
12	−1 (1:8, 200)	0 (90)	1 (3)	13.03	12.86
13	1 (1:16, 400)	1 (120)	0 (2)	12.58	12.65
14	0 (1:12, 300)	1 (120)	1 (3)	12.75	12.78
15	0 (1:12, 300)	−1 (60)	−1 (1)	12.05	12.02

Table 6: ANOVA for the fitted quadratic polynomial model for the extraction of compounds

	df	SS	MS	*F* value	*P* value
Model	9	3.8618	1.2873	7.1410	0.02169*
Residual	5	0.3004	0.0601		
Lack of fit	3	0.2514	0.0838	3.4167	0.23461
Pure error	2	0.0491	0.0245		

df: Degrees of freedom; SS: Sum of squares; MS: Mean square; ANOVA: Analysis of variance. **P* value shows the fitting to be significant at <0.05

Where Y is the yield of the seven compounds (mg/g), and the coded variables X_1, X_2 and X_3 represent the ratio of water to herbal formula, extraction time and extraction number, respectively.

An adequately fitted model can help the exploration and optimization of a fitted response surface, provide an adequate approximation to the true system and verify that none of the least squares regression assumptions are violated.[25] ANOVA was performed for the fitted quadratic polynomial model for extraction of the seven bioactive compounds [Table 6]. The coefficient of determination (r^2) was 0.9278 with no significant lack of fit at $P > 0.05$, indicating that the predicted model could explain 92.78% of the results and only 7.22% of the total variance was not explained by the model.

The significance of the model was evaluated using the *F*-value and *P* value, where the corresponding variables are more significant for larger *F*-values and smaller *P* values.[26] The *F*-value of 7.1410 and *P* value of 0.02169 imply that the model used to fit the response was significant and adequately represented the predicted results between the independent variables and the response.[27]

The regression coefficients of the predicted quadratic polynomial model were obtained for the coded variables and the significance of each coefficient was determined using Student's *t*-test and the *P* value, in which a larger *t*-value and smaller *P* value show the significance of the corresponding coefficient.[25] It was observed that the extraction time was significant in both linear ($P < 0.05$) and quadratic terms ($P < 0.01$), whereas the ratio of water to herbal formula was verified to be significant only for the quadratic term ($P < 0.05$). The other term coefficients (X_3, X_1:X_2, X_1:X_3, X_2:X_3, and X_3:X_3) were not significantly influential on the model ($P > 0.05$) [Table 7].

Analysis of response surface

The polynomial equation obtained from regression analysis was graphically visualized by a three-dimensional response plot and two-dimensional contour plots, where

Table 7: Regression coefficients of the predicted quadratic polynomial model

Variables	Estimate	Standard error	*t* value	*P* value
X_1	−0.03669	0.08666	−0.4234	0.68962
X_2	0.26177	0.08666	3.0205	0.02940*
X_3	0.12160	0.08666	1.4031	0.21952
X_1:X_2	0.01706	0.12256	0.1392	0.89474
X_1:X_3	0.18627	0.12256	1.5199	0.18900
X_2:X_3	−0.27835	0.12256	−2.2711	0.07234
X_1:X_1	−0.50958	0.12757	−3.9946	0.01038*
X_2:X_2	−0.71403	0.12757	−5.5973	0.00251**
X_3:X_3	−0.23776	0.12757	−1.8638	0.12137

*<0.05 or **<0.01

the interaction between variables and the effect of variables on the response can be observed. RSM plays a key role in determining the optimum values of the independent valuables that produce the maximum response.[21] The three-dimensional response plots and contour plots were obtained using two independent variables, while keeping the other variable set at the zero level. The interactions between the variables were determined through the shape of the contour plots. An elliptical contour plot indicates that the interaction between the variables is significant, while a circular contour plot means negligible interaction.[28]

As shown in Figure 3, the interaction between the ratio of water to herbal formula (X_1) and extraction time (X_2) is shown with the extraction number (X_3) set at the zero level in the response plot and contour plot. The yield of marker compounds increased with increasing ratio of water to herbal formula from 1:8 (200 mL) to 1:12 (300 mL) and increasing time of extraction from 60 min to 100 min. However, it was observed that the effect of the ratio had less influential on the yield than that of extraction time in the contour plot. The yield reached the maximum value of 13.6 mg/g when the ratio and extraction time were 1:11.9 and 94 min, respectively; however, there was a gradual decline in the response beyond those levels of the variables.

The response plot and contour in Figure 4 shows the interaction between ratio (X_1) and extraction number (X_3) with the extraction time (X_2) set at the zero level. It was found that increasing the ratio from 1:8 to 1:12 and increasing the extraction number from 1 to 2.5 increased the yield of the compounds, and the maximum value of the yield was observed within those levels.

Figure 5 describes the effect of extraction time (X_2) and extraction number (X_3) on the yield of compounds and the interaction between the two variables when the other variable (X_1) was kept at the zero level. The yield of compounds increased as the extraction time and extraction number increased, and the extraction time contributed to the increase in yield more than the extraction time in the contour plot. The highest level of yield was obtained at an extraction time of 95 min and extraction number of 2.5. The interactive effect of extraction time and extraction number on the yield of the compounds was not shown to be very weak ($P = 0.07234$) [Table 7].

As shown in Figures 3 and 5, and Table 7, the extraction time obviously affected the yield of compounds ($P = 0.02940$), but rather excessive extraction time could decrease the yield, which can be explained by the increasing extraction time accelerating chemical decomposition of marker compounds during the extraction process, resulting in reduced extraction yield.[29]

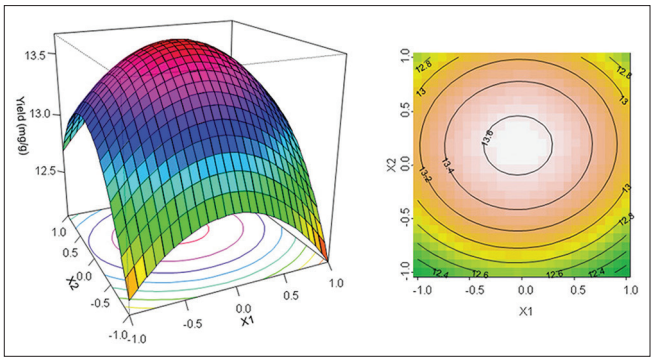

Figure 3: Response surface plot and contour plot of the ratio of water to herbal formula (mL, X1) and extraction time (min, X2)

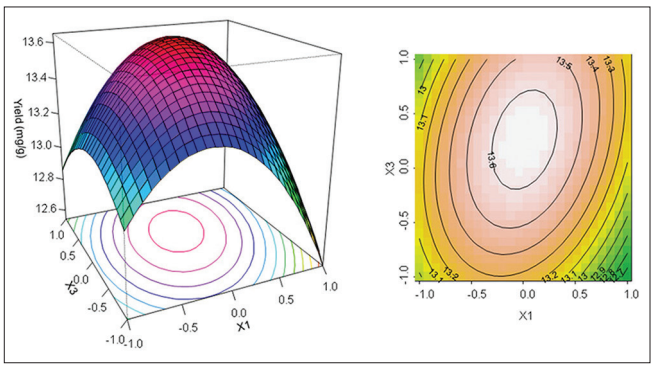

Figure 4: Response surface plot and contour plot of extraction volume (mL, X1) and extraction number (X3)

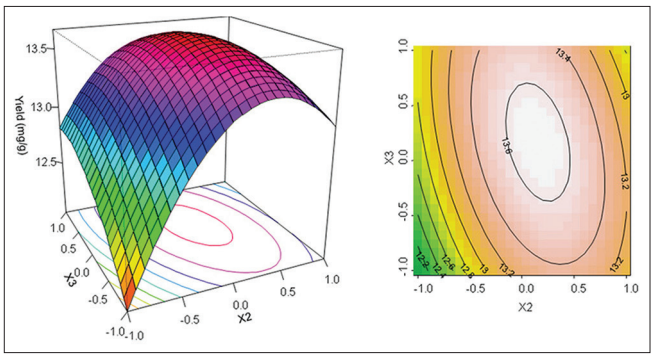

Figure 5: Response surface plot and contour plot of extraction time (time, X2) and extraction number (X3)

Optimization and verification of extraction by Response surface methodology

The aim of this study was to determine the optimal conditions producing the maximum extraction yield of chemical compounds from YJT. The conditions producing the maximum extraction of the compounds in YJT were determined based on a polynomial equation. The optimal condition of ratio of water to herbal formula, extraction time and extraction number was 1:11.99 (299.69 mL), 94.53 min and 2.21 repeats, respectively. The optimized extraction yield of total compounds was predicted to be 13.66 mg/g, which is very close to the actual

Table 8: Optimum conditions and the predicted and experimental values of the response at the optimum conditions

Condition	Ratio (mL)	Time (min)	Number (repeat)	Yield (mg/g)
Optimum	1:11.99 (299.69)	94.53	2.21	13.66 (predicted)
Modified	1:12 (300.00)	95.00	2	13.68 (actual)

value of 13.68 (mg/g) determined from the modified conditions [Table 8]. These results confirm that the model for the extraction of compounds from YJT was able to predict the experimental conditions.

CONCLUSIONS

In this study, RSM was employed to optimize the extraction conditions for the active compounds from YJT using the HRE method. Using the contour and surface plots from RSM, the optimum values for the ratio of water to herbal formula, extraction time and extraction number were determined. Under these conditions, the optimal extraction conditions for the seven bioactive compounds were 1:11.99 (299.69 mL), 94.53 min and 2.21 repeats for ratio of water to herbal medicine, extraction time and extraction number, respectively, and the obtained response was 13.66 mg/g, which closely agreed with the predicted value.

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