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Full Length Research Paper

Optimization of forsythoside extraction from *Forsythia suspensa* by Box-Behnken design

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The optimum conditions for the extraction of forsythoside from *Forsythia suspensa* were determined using response surface methodology. A Box-Behnken design was performed to evaluate the effects of ratio of water to raw material, extraction temperature and time on yield of forsythoside. Statistical analysis of results showed that, the linear and quadric terms of these three variables had significant effects, and evident interaction existing between extraction temperature and ratio of water to raw material was found to contribute to the response at a significant level. Furthermore, Box-Behnken design used for the analysis of treatment combinations gave a second-order polynomial regression model, which was in good agreement with experimental results, with R^2=0.9809 (P<0.05). The optimal conditions for the yield of forsythoside obtained using response surface methodology and canonical analysis were ratio of water to raw material of 21.38, extraction temperature of 72.06°C and extraction time of 2.56 h. Under the optimum conditions, the experimental value of 6.62±0.35% was well in close agreement with value predicted by the model, thus indicating suitability of the model employed and the success of response surface methodology in optimizing the extraction conditions.

Key word: *Forsythia suspense*, Forsythoside, optimum conditions, response surface methodology, Box-Behnken design.

INTRODUCTION

Forsythiaside, a phenylethanoid glycoside, is abundant in a well-known Chinese herbal medicine Lian-Qiao, which is the fruit of *Forsythia suspensa* (Thunb.) vahl (Figure 1). The herb has been widely used as an important source of medicine for gonorrhea, inflammation, pyrexia and ulcer (Cuellar et al., 1998; Lee et al., 2010; Li and Chen, 2005; Ozaki et al., 2000; Piao et al., 2008; Rouf et al., 2001), especially acute upper respiratory tract complaints caused by viruses and/or bacteria infection (Han et al., 2007; Zhou et al., 2011). Forsythiaside together with phillyrin is commonly used as chemical markers for quality control of Lian-Qiao raw material (Qu et al., 2008) and the derivable preparations (Cao et al., 2006). Pharmacological studies demonstrated that forsythiaside possesses strong antioxidant (Qu et al., 2008; Wang et al., 2008), antibacterial (Qu et al., 2008) and antiviral (Xia et al., 2011; Li et al., 2011) activities, and also exhibits a slow relaxation effect against norepinephrine induced contraction of rat aorta (Lizuka and Nagai, 2005). Moreover, it is reported that forsythiaside could significantly protect DNA damage caused by hydroxyl radicals (Pan et al., 2003) and inhibit protein kinase C (PKCα) with an IC_{50} value of 1.9 μM (Zhou et al., 1998). Therefore, nowadays more and more attention was casted on forsythiaside in the field of pharmaceutical, biochemical, food and cosmetic industry, and so on.

To gain the optimum extracting process of forsythiaside from *F. suspensa*, orthogonal design has been used to study the factors that affect extracting conditions of forsythiaside (Zhao and Xuan, 2010). However, the experimental data gained by orthogonal design methodology was not precise enough and the mathematical model developed was not good enough to fit with the experimental data of forsythiaside extraction (Liu and Xiang, 2007). Therefore, it is very essential to develop a simple, reliable and accurate method for the extraction of the active constituent.

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Abbreviations: BBD, Box-Behnken design; **RSM,** response surface methodology.

The yield of fosythside can be calculated by the Equation 1. From the equation, it can be seen that the yield (D) increase along with the increase of the content (C) or the total volume (V) of prepared sample, and it reduces following the increase of quality of raw material (M). In generally, the quality of raw material is constant, whereas the content and the total volume of prepared sample are affected by numerous parameters. For example, a higher content can be obtained by increasing extraction temperature or time. But the content may reduce when the extraction temperature or the time is further increased. In additional, the content will be decreased and the total volume of prepared sample will be increased along with extraction number and ratio of water to raw material increasing. Therefore, the four parameters exhibit an important effect on the yield of fosythside.

When many factors and interactions affect desired response, response surface methodology (RSM) is an effective tool for optimizing the process (Sun et al., 2010; Cao et al., 2008; Varun et al., 2010). RSM is a collection of statistical and mathematical techniques that are based on the fit of empirical model to the experimental data obtained in relation to experimental design. The optimization process of this methodology involves studying the response of the statistically designed combinations, estimating the coefficients by fitting it in a mathematical model that fits best the experimental conditions, predicting the response of the fitted model and checking the adequacy of the mode. The common design, that is, central composite design (CCD) and Box-Behnken design (BBD), of the principal response surface methodology have been successfully applied for optimizing conditions in food and pharmaceutical research (Babar et al., 2007; Bazhdanzadeh et al., 2011; Chaudhary et al., 2010; Yang et al., 2009, 2010). The main advantage of RSM is to reduce number of experimental trials needed to evaluate multiple variables and their interactions. So it is less laborious and time consuming than other approaches required optimizing a process. Meantime, the experimental data obtained by RSM was fitted with a second-order polynomial equation and the model gained was fitted with the experimental data.

Up to now, no detailed investigation has been conducted on optimization of forsythoside extraction from the fruiting bodies of *F. suspensa* by response surface methodology. Therefore, the purpose of this study was to employ a BBD (3 factors and 3 levels) to optimize the process parameters of forsythoside extraction from the fruiting bodies of *F. suspensa* so as to facilitate the further and reasonable exploration of this treasured herb.

MATERIALS AND METHODS

F. suspensa was collected from Shanxi Province (China) and verified by Professor Yanbing Li (Heilongjiang University of traditional Chinese Medicine, Harbin, China). Forsythoside was purchased from the Chinese Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile was of high performance liquid chromatography (HPLC) grade from Tedia (Fairfield, USA). Acetic acid (A.R. grade) for analysis was purchased from Hangzhou Reagent Company (Hangzhou, PR China). Water was purified by a Milli-Q water system (Millipore, Bedford, MA, USA).

Forsythoside extraction

F. suspensa (2000 g) were ground in a blender to obtain a fine powder. Each dried pretreated sample (20 g) was extracted by water in a designed temperature, extraction time, ratio of water to raw material and extraction number. The water extraction solutions were separated form insoluble residue by centrifugation (3000 rpm for 5 min), and then freeze-dried to obtain crude extraction.

Extraction rate of forsythoside

The content of the forsythoside was measured by HPLC method (Xia et al., 2010). On the basis of the forsythoside content of prepared samples measured by the standard curve, the extraction rate of forsythoside was calculated by the equation:

$$D(\%) = \frac{C \times V}{M} \times 100\% \quad (1)$$

Where D represents the extraction rate of forsythoside (%), V is the total volume of prepared sample (ml), C is the forsythoside content of the prepared sample (g/ml), and *M* is the quality of raw material (g).

Experimental design

Extraction processes were affected by numerous parameters. Because it was impossible to identify the effects of all parameters, it was necessary to select the parameters that had major effects (Bas and Boyaci, 2007). It was reported that extraction temperature, extraction time, ratio of water to raw material and extraction number had significant effects on forsythoside production from *F. suspensa* (Zhao and Xuan, 2010). So the yield of forsythoside would be improved by optimizing the four parameters. Figure 2 represents the brief procedure of the experiment design. Four parameters (extraction temperature, extraction time, ratio of water to raw material and extraction number) were screened using single-factor experiment, which had great influence on the yield of forsythoside. After the identification of the important parameters, the direction in which improvements lie was determined and the levels of the parameters were identified.

On the basis of single-factor experiment, a BBD with three independent variables (X_1, extraction temperature; X_2, extraction time; X_3, ratio of water to raw material) at three levels was performed. For statistical calculations, the relation between the coded values and actual values were described as the following equation:

$$x_i = \frac{(X_i - X_0)}{\Delta X} \quad (2)$$

Where x_i is a coded value of the variable; X_i is the actual value of variable; X_0 is the actual value of the X_i on the center point; and ΔX

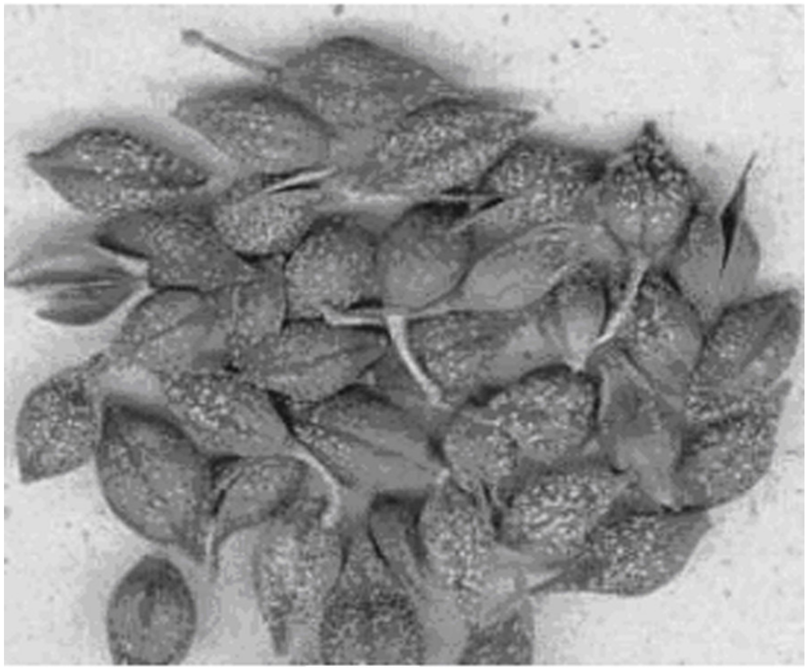

Figure 1. The real picture of *Forsythia suspensa*.

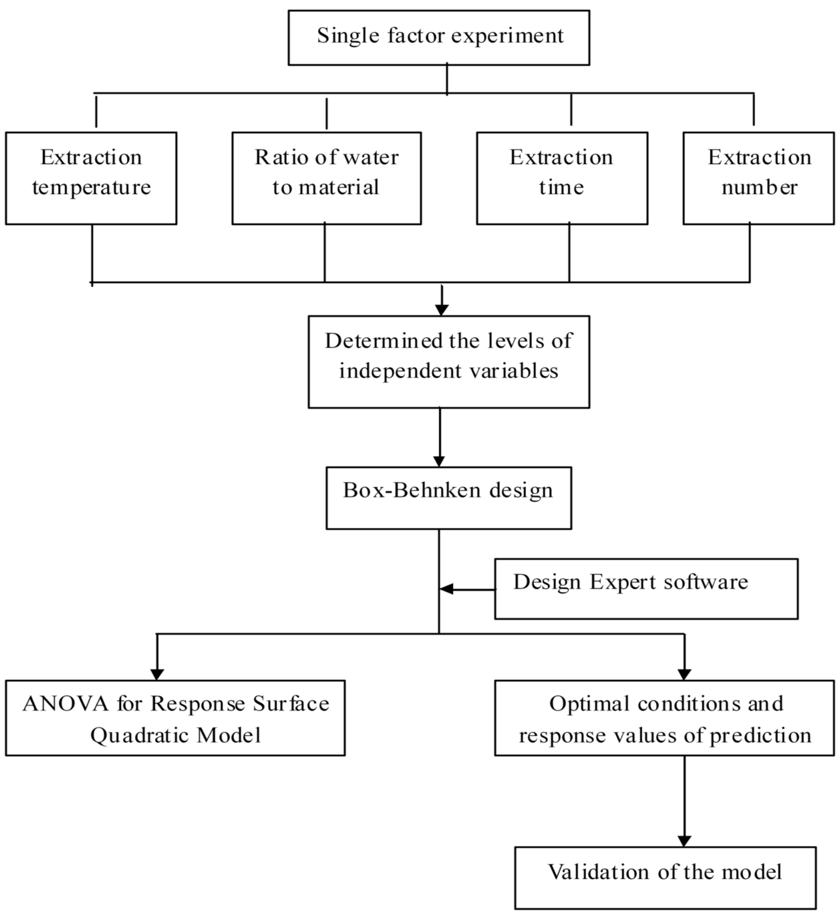

Figure 2. The technological process reflecting the brief procedure of experiment design.

Table1. Variables and experimental design levels for response surface.

Independent variable	Coded symbol	Level		
		-1	0	1
Extraction temperature	X_1	60	70	80
Ratio of water to raw material	X_2	15	20	25
Extraction time	X_3	2	2.5	3

Table 2. The Box-Behnken experimental design with three independent variables.

S/N	X_1 (Extraction temperature, ℃)	X_2 (Ratio of water to raw material)	X_3 (Extraction time, h)	Extraction yield (%)
1	60.00	15.00	2.50	4.388
2	80.00	15.00	2.50	5.48
3	60.00	25.00	2.50	5.23
4	80.00	25.00	2.50	5.51
5	60.00	20.00	2.00	5.282
6	80.00	20.00	2.00	5.798
7	60.00	20.00	3.00	5.744
8	80.00	20.00	3.00	6.038
9	70.00	15.00	2.00	4.898
10	70.00	25.00	2.00	5.654
11	70.00	15.00	3.00	5.348
12	70.00	25.00	3.00	5.864
13	70.00	20.00	2.50	6.428
14	70.00	20.00	2.50	6.512
15	70.00	20.00	2.50	6.71

is the step change value. The range of independent variables and their levels are presented in Table 1, which was based on the results of preliminary experiments. As seen from Table 2, the whole design consisted of 15 experimental points carried out in random order. Three replicates (treatment 13-15) at the center of the design were used to allow for estimation of a pure error sum of squares. The response value in each trial was the average of three repetitions. Data from the BBD was analyzed by multiple regressions to fit the following quadratic polynomial model.

$$Y = A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=1+1}^{3} A_{ij} X_i X_j \qquad (3)$$

Where, Y represents the response function; A_0 is the constant; A_i, A_{ii} and A_{ij} are the coefficients of the linear, quadratic and interactive terms, respectively. The terms X_iX_j and X_i^2 represent the interaction and quadratic terms, respectively. And accordingly X_i and X_j represent the coded independent variables. According to the analysis of variance, the regression coefficients of individual linear, quadratic, and interaction terms were determined. The regression coefficients were then used to make statistical calculation to generate dimensional and contour maps from the regression model. Design-expert software (version 7.0) was used to analyze the experimental data. The P-values of less than 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Single factor analysis method

Effect of different extraction temperature on yield of forsythoside

Extraction temperature is one of the important factors affecting the yield of forsythoside. At higher temperature, a higher extraction rate extracted from fruit can be obtained. High temperature solvent will promote forsythoside on the cell wall to be distributed to the solvent, and to weaken or undermine the integrity of the cell wall so that more solvent can be contacted with forsythoside (Sun et al., 2002). To study the effect of different temperatures on the extraction rate of forsythoside, the extraction process was carried out under the condition of different extraction temperature of water bath from 30 to 80℃, while other extraction condition were as follows: ratio of water to raw material of 20, extraction time of 2.5 h, and extraction number of 3. Figure 3a shows the effect of different temperatures on yield of forsythoside from *F. suspensa*.

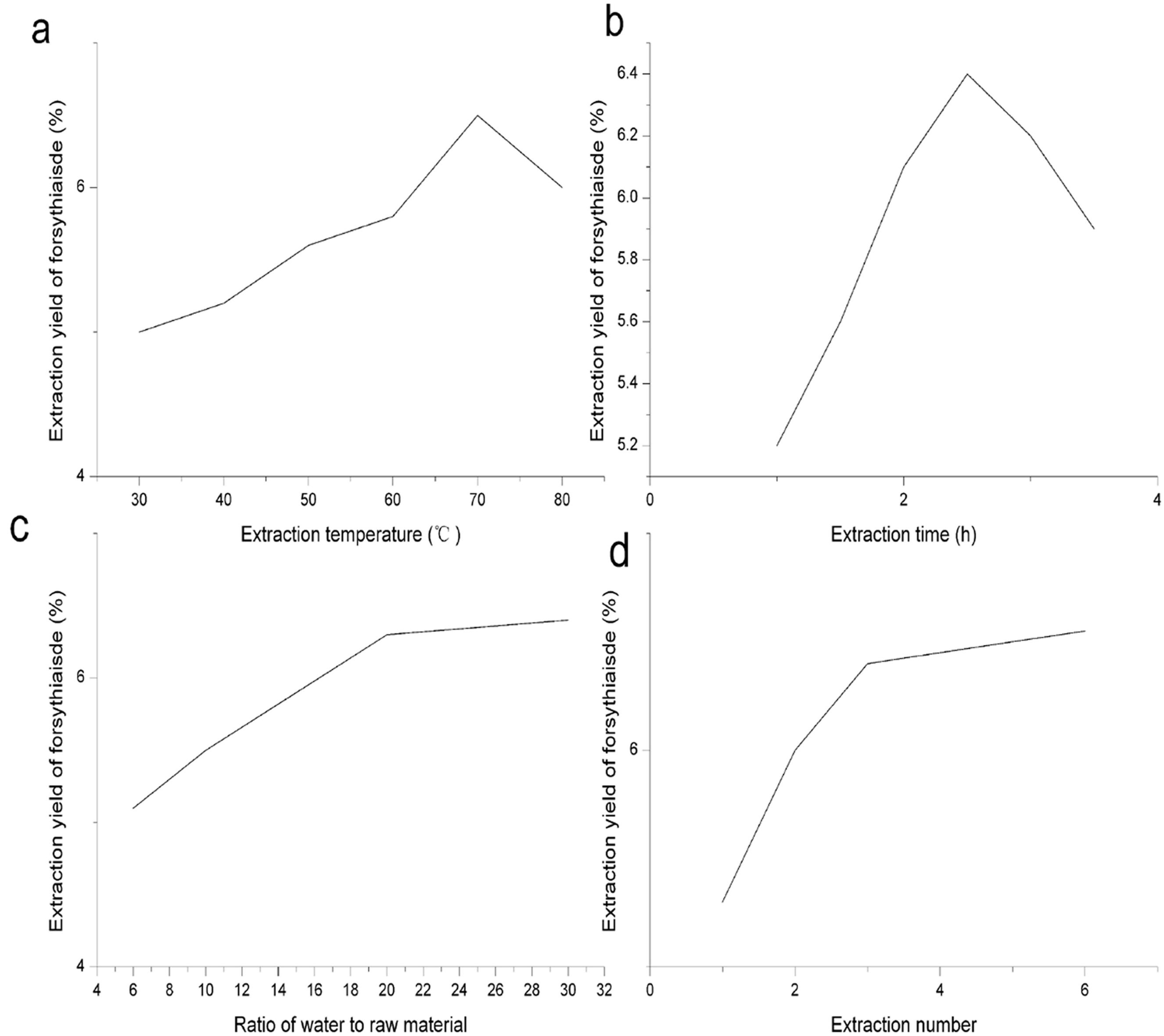

Figure 3. The curve showing the effect of different temperature (a), time (b), ratio of water to raw material (c) and extraction number (d) on yield of forsythoside.

Figure 3a indicates that when the temperature increased from 30 to 70℃, the total yield of forsythoside rose gradually, and then reached the peak at 70℃, and finally dropped from 70 to 80℃. This phenomenon could be explained that, with the increase in temperature, solvent viscidity declined and the movement of molecular accelerated, which led to the dissolution of forsythoside for the enlargement of diffusion coefficient and the increase of solubility. However, much higher temperature promoted the oxidation of forsythoside. In addition, high temperature could cause the loss of solvent, resulting in a lower yield. Therefore, the center point of extraction temperature was considered to be 70℃ in this experiment.

Effect of different extraction time on yield of forsythoside

In a general way, extraction rate will be increased along with extraction time increase. However, longer extraction time increases the chance of oxidation of forsythoside. Therefore, extraction time is an important parameter of forsythoside extraction. It was, respectively, set at 1, 1.5, 2, 2.5, 3 and 3.5 h to examine the effect of extraction time on the yield of forsythoside when the other reaction conditions were as follows: extraction temperature of 70℃, ratio of water to raw material of 20 and extraction number of 3.

As shown in Figure 3b, the forsythoside yield increased when the extraction time was extended from 1 to 2.5 h, whereas it declined when the extraction time was further extended from 2.5 to 3.5 h. The reason was that it may induce more chemical reaction on the longer extraction time and then cause oxidative conversion of forsythoside. Therefore, the extraction time of 2 to 3 h was adopted in the work.

Effect of ratio of water to raw material on yield of forsythoside

Different ratio of water to raw material will significantly affect extract yield (Govender et al., 2005). If ratio of water to raw material is too small, forsythoside in raw material cannot be completely extracted up. When ratio of water to raw material is increased, the extraction rate of forsythoside also increases (Zhao and Xuan, 2010). However, this will cause high process cost when ratio of water to raw material is too big. Therefore, suitable ratio of water to raw material should be selected for extraction of forsythoside. In this studies, ratio of water to raw material as an important extraction parameter was set at 6, 8, 10, 15, 20, 25 and 30 to investigate the influence of different ratio of water to raw material on the yield of the forsythoside, while other extraction conditions were as follows: extraction temperature of 70℃, extraction time of 2.5 h and extraction number of 3. As is shown in Figure 3c, the forsythoside yield increased with increasing ratio of water to raw material, and reached highest value when the ratio was 20. A possible explanation is that increase in ratio of water to raw material may increase diffusivity of the solvent into cells and enhance desorption of the polysaccharides from the cells (Ray, 2004; Volpi, 2005). However, the results from this experiments indicate that when the ratio was higher than 20, the forsythoside yield maintained a mild slope. This might be due to the reason that most of forsythoside had been excessively dissolved when the ratio was 20 and only a small portion of forsythoside was retained in the fruiting bodies of *F. suspensa*. Therefore, the ratio of water to raw material of 15 to 25 was adopted in the work.

Effect of number of extraction on yield of forsythoside

Multiple-step extraction was an important method to improve the yield of forsythoside from the *F. suspensa*. To study the extraction number on the extraction rate of forsythoside, the samples were extracted with water for different number of extraction (1, 2, 3, 4, 5, and 6) when the other three factors were as follows: extraction temperature of 70℃, ratio of water to raw material of 20 and extraction time of 2.5 h.

It can be seen from Figure 3d that the yield of forsythoside had obviously increased within the number of extraction (1 to 3), whereas it did not significantly vary when the number was further increased. This result indicates that extraction number of 3 was sufficient for the extraction of forsythoside.

Optimization of the extraction progress

Mathematical model and optimization of extraction conditions

Response surface optimization is more advantageous than the traditional single parameter optimization in that it saves time, space and raw material. According to the method of BBD experiment and the levels of independent variables chosen based on the values obtained in the single factor experiment, extraction temperature (X_1, ℃) , extraction time (X_2, h), and ratio of water to raw material (X_3, v/g), which had a great effect on the extraction rate of forsythoside, were selected as design variables in the RSM, the extraction rate of forsythoside (Y,%) was employed as a response value, and the three factors and three levels RSM test were designed (Tables 1 and 2). Zero experiment was carried out for three times.

There were a total of 15 runs for optimizing the three individual parameters in the current BBD. The experimental data were statistically analyzed by multiple regression analysis using the Design-Expert software 7.0; the response variable and the test variables were related by the following second-order polynomial equation:

$$Y = -53.83350 + 0.92268*X_1 + 1.73580*X_2 + 7.05250*X_3 - 4.06000E\text{-}003*X_1*X_2 - 0.011100*X_1*X_3 - 0.024000*X_2*X_3 - 5.61750E\text{-}003*X_1^2 - 0.033450*X_2^2 - 1.09100*X_3^2 \quad (4)$$

Performing the analysis of ANOVA for the quadratic model was required to test the significance and adequacy of the model. Table 3 shows that the model, the linear coefficients (X_1, X_2, X_3), quadratic term coefficients (X_1^2, X_2^2, X_3^2) and cross product coefficients (X_1*X_2) were significant, with very small P-values ($P < 0.05$). The other term coefficients were not significant ($P > 0.05$). The analysis also showed that the extraction temperature was the most significant single parameter which influenced forsythiaside yield followed by ratio of water to raw material and extraction time. From Table 4 it can be seen that the determination coefficient (R^2=0.9809) was showed by ANOVA of the quadratic regression model, indicating that only 1.91% of the total variations were not explained by the model. The value of the adjusted determination coefficient (Adj R^2 =0.9464), also confirmed that the model was highly significant. At the same time, a relatively lower value of the coefficient of variation (CV = 2.52%) indicated a better precision and reliability of the experiments carried out.

In this study, the aim of optimization was to find the conditions which gave the maximum yield of forsythiaside.

Table 3. Regression coefficients of the predicted quadratic forsythiaside model.

Source	Sum of squares	DF	Mean square	F-value	p-Value	Significant
Model	5.21	9	0.58	28.46	0.0009	***
X_1	0.60	1	0.60	29.28	0.0029	**
X_2	0.57	1	0.57	28.27	0.0031	**
X_3	0.23	1	0.23	11.41	0.0197	*
X_1X_2	0.16	1	0.16	8.11	0.0359	*
X_1X_3	0.012	1	0.012	0.61	0.4715	
X_2X_3	0.014	1	0.014	0.71	0.4383	
X_1^2	1.17	1	1.17	57.32	0.0006	***
X_2^2	2.58	1	2.58	127.03	< 0.0001	***
X_3^2	0.27	1	0.27	13.51	0.0144	*
Residual	0.10	5	0.020			
Lack of Fit	0.060	3	0.020	0.95	0.5498	Not significant
Pure Error	0.042	2	0.021			
Cor Total	5.31	14				

*Significant at P<0.05, **Significant at P<0.01, *** Significant at P<0.001.

Table 4. Fit statistics for the response values.

Statistics	Y
Mean	5.66
R^2	98.09%
Adj.R^2	94.64%
CV	2.52%

By solving the regression equation and analyzing the response surface plots, the optimum extraction conditions (extraction temperature f 72.06℃, extraction time of 2.56 h, and ratio of water to raw material of 21.38) were estimated. The predicted extraction yield given by the Design Expert software under the above conditions was 6.62%.

The interaction between the variables

Response surfaces were plotted using Design-Expert version 7.0 software to study the effects of parameters and their interactions on forsythiaside yield. Three-dimensional response surface plots, as presented in Figures 4 to 6, were very useful to see the interaction effects of the factors on the responses (Li et al., 2008). These types of plots showed effects of two factors on the response at a time. In all the presented figures, the other factor was kept at level zero.

Figure 4 represents the effects of extraction temperature, extraction time and their reciprocal interactions on extraction yield of forsythiaside. An increase in extraction rate was observed with the increase of extraction temperature and extraction time at first but the trend was reversed when the extraction temperature and extraction time reached a certain value. These data were consistent with the conclusion of the single factor test. Therefore, both extraction temperature and extraction time would have independent optimum condition parameters.

Figure 5 shows the three-dimensional plot of the response surface for the forsythiaside yield as related to extraction time and ratio of water to raw material with temperature and number set at 70℃ and 3, respectively. Extraction time exhibited an important effect on the forsythiaside yield, which did not continue to increase significantly until the extraction time was over 2.5 h. And further increases in extraction time, resulted in little decrease in the yield of forsythiaside. With respect to ratio of water to raw material, the influence of this parameter was not as significant as that of extraction time. As shown in this figure, the amount of forsythiaside extracted increased at first when the ratio was increased, but prolonging the contact time led to a slight increase in the forsythiaside yield.

It was observed from the Figure 6 that the yield of forsythiaside significantly increased with increasing temperature at a given ratio of water to raw material, especially at low temperature and ratio of water to raw material. If the given ratio of water to raw material was higher than a certain value (about 20 V/g), while temperature was rising, the forsythiaside yield increased at low ratio of water to raw material levels, but once the temperature reached the high levels, the forsythiaside yield slightly decreased.

Validation of the model

In order to validate the adequacy of the model equations, a verification experiment was carried out under the optimal conditions (within the experimental range):

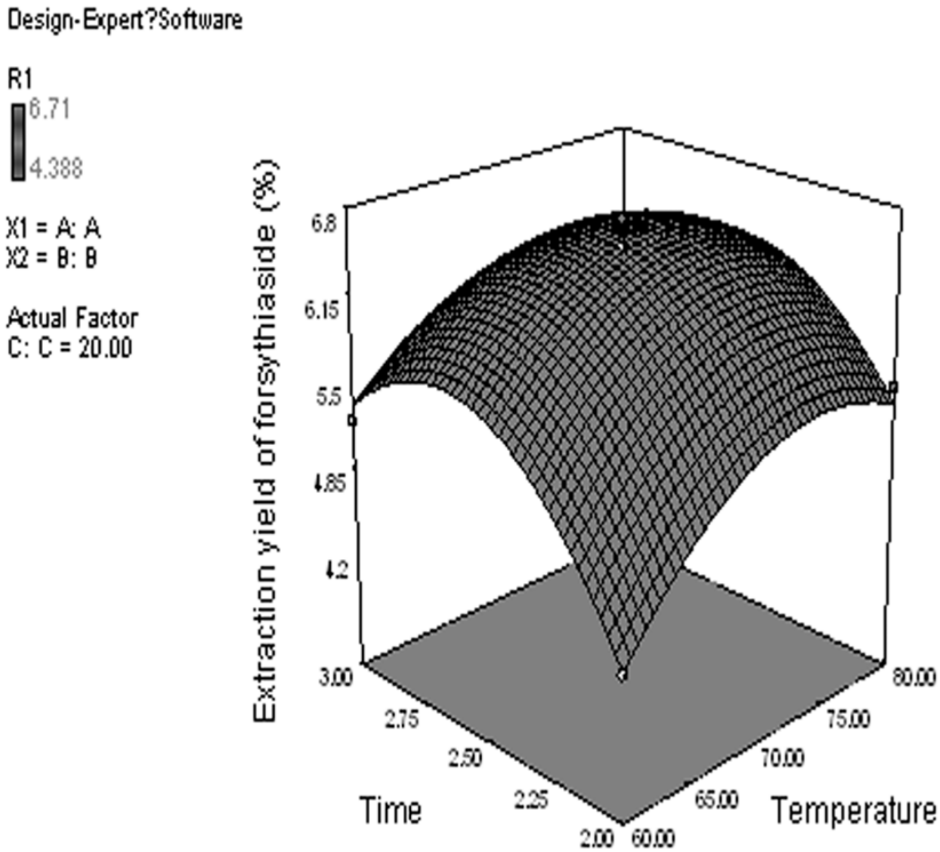

Figure 4. The response surface (3D) showing the correlative effects of temperature and time on yield of forsythoside.

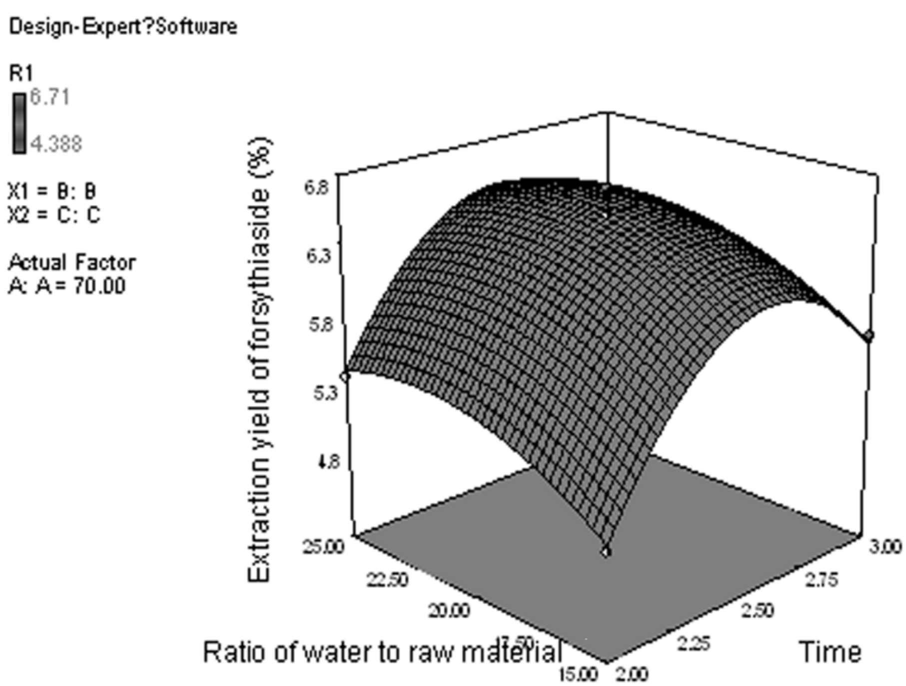

Figure 5. The response surface (3D) showing the correlative effects of time and ratio of water to raw material on yield of forsythoside.

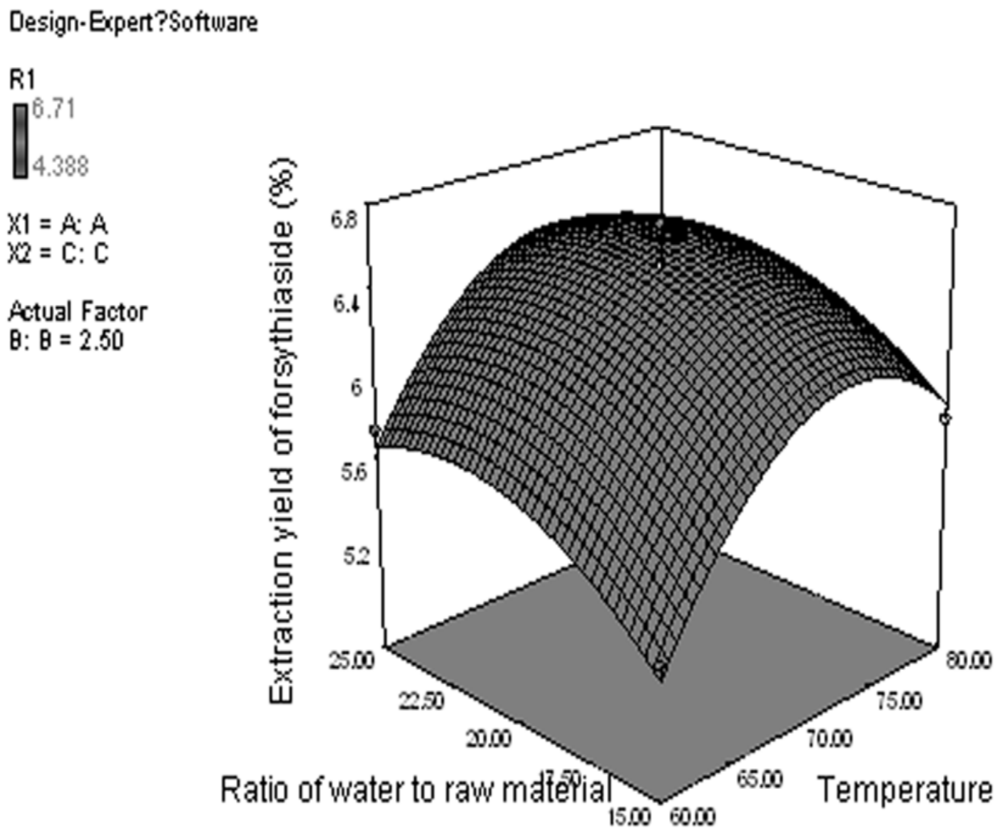

Figure 6. The response surface (3D) showing the correlative effects of temperature and ratio of water to raw material on yield of forsythoside.

extraction temperature of 72.06℃, extraction time of 2.56 a verification experiment was carried out under the optimal conditions (within the experimental range): extraction temperature of 72.06℃, extraction time of 2.56 h, ratio of water to raw of 21.38. This set of conditions was determined to be optimum by the RSM optimization approach and was also used to validate experimentally and predict the values of the responses using the model equation. A mean value of 6.62±0.35 (N= 3), obtained from real experiments, demonstrated the validation of the RSM model, indicating that the model was adequate for the extraction process (Table 5).

Conclusion

In the studies, the effect of experimental parameters on the yield of forsythiaside was established using RSM. The four main findings could be drawn from the information presented in this paper.

1. The importance of the independent variables on the yield could be ranked in the following order: extraction temperature (X_1)> ratio of water to material (X_2) > extraction time (X_3).
2. The dependent variable and independent variable were related by the following second-order polynomial equation: $Y = -53.83350 + 0.92268*X_1 + 1.73580*X_2 + 7.05250*X_3 - 4.06000E\text{-}003*X_1*X_2 - 0.011100*X_1*X_3 - 0.024000*X_2*X_3 - 5.61750E\text{-}003*X_1^2 - 0.033450*X_2^2 - 1.09100*X_3^2$
3. Analysis of variance represented a high coefficient of determination value (R^2=0.9809), thus ensuring a satisfactory adjustment of the second order regression model with the experimental data.
4. The optimal experimental yield of 6.62% was obtained when the optimum conditions were extraction temperature of 72.06℃, extraction time of 2.56 h and ratio of water to raw material of 21.38. Under these optimized conditions, the experimental yield of forsythiaside agreed closely with the predicted yield.

In summary, this study clearly shows that response surface methodology was one of the suitable methods to optimize the operating conditions and maximize the yield of forsythoside.

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Table 5. Predicted and experimental values of the responses at optimum conditions.

Optimum condition			Extraction yield (%)	
Extraction temperature	**Ratio of water to raw material**	**Extraction time**	**Experimental**	**Predicted**
72.06	21.38	2.56	6.62±0.35	6.62

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