Carbohydrate Polymers 84 (2011) 282–291

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Optimum extraction of acidic polysaccharides from the stems of *Ephedra sinica* Stapf by Box–Behnken statistical design and its anti-complement activity

Yonggang Xia, Haixue Kuang*, Bingyou Yang, Qiuhong Wang, Jun Liang, Yanping Sun, Yanhong Wang

Key Laboratory of Chinese Materia Medica (Heilongjiang University of Chinese Medicine), Ministry of Education, 24 Heping Road, Harbin 150040, China

ARTICLE INFO

Article history:
Received 25 September 2010
Received in revised form 1 November 2010
Accepted 16 November 2010
Available online 23 November 2010

Keywords:
Response surface methodology
Polysaccharides
Ephedra sinica Stapf
Extraction
Anti-complement activities

ABSTRACT

The response surface methodology was employed to study the extraction of acidic polysaccharides from the stem of *Ephedra sinica* Stapf. The quantitative effects of extraction temperature, time, number and ratio of water to raw material on yield of ephedra acidic polysaccharides were investigated with Box–Behnken design. The experimental data were fitted to three second-order polynomial equations using multiple regression analysis and also analyzed using the appropriate statistical methods. By solving the regression equation and analyzing 3-D plots, the optimum condition was at extraction temperature 100 °C, time 3.5 h, numbers 4 and ratio of water to raw material 13.6. Under these conditions, the experimental extraction yield, polysaccharide yield and uronic acid yield were 49.47 mg/g, 33.25 mg/g, and 22.08 mg/g, which were in good agreement with the predicted values. Ephedra acidic polysaccharide exhibited excellent anti-complement activity *in vitro*, indicating it could be a potential anti-complement therapeutic agent.

1. Introduction

Ephedra sinica Stapf (Mahuang), as a famous traditional Chinese medicine, has been used for thousands of years for its medicinal qualities as a diaphoretic, diuretic, anti-asthmatic to treat allergies, asthma, pneumonia, bronchitis, hay fever and colds (Comar & Kirby, 2005; Dunnick, Kissling, Gerken, Vallant, & Nyska, 2007; Kemper, Singla, & Gardiner, 2005; Nam et al., 2003; Roman et al., 2004; Schaneberg, Crockett, Bedir, & Khan, 2003; Trujillo & Sorenson, 2003; Young & Glennon, 1998). In the West, extracts of *E. sinica*, together with those of *E. intermedia* and *E. equisetina*, belonging to the genus Ephedra, were most commonly used in dietary supplements as a stimulant and to promote weight loss, and many people were using these products for the latter purpose (Boozer et al., 2001; Comar & Kirby, 2005; Gurley, Gardner, White, & Wang, 1998; Shekelle et al., 2003). However, it is unfortunate that using Mahuang-containing products for longer periods of time can cause serious adverse health outcomes (heart ailments, strokes); alkaloids are responsible for these side effects (Gurley et al., 1998; Haller & Benowitz, 2000; Samenuk et al., 2002; Shekelle et al., 2003). Thus the U.S. Food and Drug Administration (FDA) banned the sale of ephedra-containing supplements in 2004 due to evidence of adverse ephedra-related effects, which has therefore gained a lot of attention on the application of ephedra (Ganzera, Lanser, & Stuppner, 2005).

With the further studies, fortunately enough, it has been found that ephedrine alkaloids are not the only active ingredients accounting for all the effects mentioned above (Ding, Shi, Cui, Wang, & Wang, 2006; Zhong, Gong, Zhu, & Chen, 2010). Immunosuppressive effects of acidic polysaccharides from the stems of *E. sinica* have been demonstrated by carbon clearance test, delayed type hypersensitivity reaction and humoral immune response *in vivo* (Cheng, Zhu, & Xu, 2001; Kuang, Xia, Yang, Wang, & Wang, 2010; Meng, Yan, & Xu, 2007). Some purified polysaccharides have been isolated by ion exchange and gel-filtration chromatography from the stem of *E. sinica*. They were all typical acidic hetero-polysaccharides and consisted of xylose, arabinose, glucose, rhamnose, mannose, galactose, glucuronic acid and galacturonic acid by capillary electrophoresis (Kuang et al., 2010; Xia et al., 2010). Furthermore, ephedra acidic polysaccharide exhibited the higher immunosuppressive effects, which may be due to the higher uronic acid content by mice splenocyte proliferation activity *in vitro* (Kuang et al., 2010). These findings extended our understanding of the effects of Mahuang and its clinical application. Ephedra acidic polysaccharide may suggest therapeutic applications that related to Mahuang in future. Therefore, the finding of immunosuppressive effects of ephedra acidic polysaccharide may bring "New Spring" to Mahuang in the medical industry. However, so far there is no published information on the optimization of extraction conditions of ephedra acidic polysaccharide for further application due to its immunosuppressive activity.

Response surface methodology (RSM) defined as a collection of mathematical and statistical methods was initially developed and described by Box and Wilson (1951). Recently, it has been widely used to develop, to improve, or to optimize a product or

* Corresponding author. Tel.: +86 45182193001; fax: +86 45182110803.
E-mail address: hxkuang@hotmail.com (H. Kuang).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.11.035

process in many cases (Elavarasan, Kondamudi, & Upadhyayula, 2009; Gan, Abdul Manaf, & Latiff, 2010; Karacabey & Mazza, 2010; Myers, Montgomery, & Anderson-Cook, 2009; Paucar-Menacho, Berhow, Mandarino, de Mejia, & Chang, 2010; Sun, Liu, & Kennedy, 2010; Zhong & Wang, 2009). As a very popular class of designs for fitting a response surface, Box–Behnken design (BBD) provides efficient solutions compared with a three-level full-factorial design, reducing the number of required experiments by confounding higher-order interactions, which becomes more significant as the number of factors increases (Borkowski, 1995; White, Willis, Keshav, & Dutton, 2001). It provides information about the relative significance of main effects, as well as information about interaction effects that cannot be predicted by univariate techniques. The model results are easily interpreted and visualized in response surface plots (Lomasney, Guillo, Sidebottom, & Roper, 2009).

The objectives of this study were to explore the potential of Mahuang in producing ephedra acidic polysaccharide and to optimize the conditions for the extraction. RSM was applied to fit and to exploit a mathematical model representing the relationship between the responses (i.e. extraction yield, polysaccharide yield, and uronic acid yield) and variables (i.e. extraction temperature, time, number, and ratio of water to raw material). It may facilitate a deeper understanding of the process of polysaccharide extraction from the stems of *E. sinica* to provide theoretical references. In addition, as part of a continuing project on the mechanism of immunosuppressive action of polysaccharides from the stems of *E. sinica*, anti-complement effects of ephedra acidic polysaccharide *in vitro* have been investigated in the present study.

2. Materials and methods

2.1. Materials and reagents

The dry stems of *E. sinica* were collected in March 2007 from Datong of Shanxi Province, China and identified by Professor Zhenyue Wang of Heilongjiang University of Chinese Medicine. The voucher specimen (20070016) was deposited at Herbarium of Heilongjiang University of Chinese Medicine, Harbin, PR China.

Dimethyl sulfoxide (DMSO) was from Gibco, Grand Island, NY, USA. D-glucose, D-galacturonic acid, sulfuric acid, phenol, carbazole, hemolysin, gelatin, and sodium barbital were purchased from Sigma (St. Louis, USA). Normal human serum was collected from a healthy male volunteer. Rosmarinic acid was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). Water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). All other chemicals were of the highest grade available.

2.2. Extraction of polysaccharides from E. sinica

The dry stems of *E. sinica* (20 g) were ground to the particle size through standard mesh sieve No. 10 and were then extracted under reflux by distilled water in a designed extraction temperature, extraction time, number of extraction, and ratio of water to raw material. The water extraction solutions were filtered through a nylon cloth (Pore diameter: 38 μm) to remove the debris. The obtained supernatants were concentrated using a vacuum Rotavapor R-200 (Büchi, Switzerland) and then precipitated by 95% ethanol to a final concentration of 75% (v/v). After being left overnight at 4 °C, the precipitates were collected by centrifugation at 3000 rpm for 20 min, redissolved in water and dialyzed in a dialysis tube (MWCO 3500 Da, USA). Dry crude polysaccharides were then obtained by lyophilization. The extraction yield was measured and calculated as follows:

$$\text{extraction yield (mg/g)} = \frac{m_0}{m} \quad (1)$$

where m_0 (mg) is defined as the weight of dried crude polysaccharides; m (g) is defined as the dried raw material weight.

2.3. Determination of polysaccharide yield

Polysaccharide content was determined by the phenol–sulfuric acid method using glucose as a standard (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). 1.0 mL of dry crude polysaccharide sample solution was mixed with 1.0 mL of phenol (5%) and 5 mL of sulfuric acid (98%). After standing for 20 min, the absorbance at 490 nm was recorded. The polysaccharide yield (mg/g) was then calculated using the following equation:

$$\text{polysaccharide yield (mg/g)} = \frac{C \times D \times F}{W} \quad (2)$$

where C is defined as the concentration of polysaccharide calculated from the calibrated regression equation (mg/mL); D is defined as the dilution factor (mL); F is defined as the conversion factor; and W is defined as the weight of the dried raw material (g).

2.4. Determination of uronic acid yield

Uronic acid was determined by carbazole–sulfuric acid method (Bitter & Muir, 1962). In an ice bath, a dry crude polysaccharide sample solution (1.0 mL) was mixed with 6.0 mL of 0.9% (m/v) boric–sulfuric acid, which was obtained by combining 0.9 g of borax with 100 mL of sulfuric acid (98%). The mixtures were then heated in a boiling bath for 5 min and cooled to room temperature. After that, 0.2 mL of 0.1% (m/v) carbazole–ethanol complexes was added, which was obtained by combining 0.1 g of carbazoles with 100 mL of absolute alcohol. Subsequently the final mixtures were placed in a boiling water bath for 10 min and then were cooled to room temperature. After standing for 10 min, the absorbance at 530 nm was recorded. The uronic acid yield was calculated as follows:

$$\text{uronic acid yield (mg/g)} = \frac{C \times V}{W} \quad (3)$$

where C is defined as the concentration of uronic acid calculated from the calibrated regression equation (mg/mL); V is defined as the volume of extraction solution (mL); and W is defined as the weight of the dried raw material (g). Standard curve was obtained using galacturonic acid (0–100 μg/mL).

2.5. Experimental design

The effects of extraction condition on extraction yield, polysaccharide yield and uronic acid yield such as extraction temperature, extraction time, extraction number, ratio of water to raw material, ethanol concentration and particle size have been investigated by the single factor method. On the basis of the single factor experimental results, four major influence factors were confirmed, and then a response surface methodology was conducted to design experimental project. As shown in Table 1, the four factors chosen for this study were designated as X_1, X_2, X_3, and X_4 and prescribed into three levels, coded +1, 0, −1 for high, intermediate and low value, respectively. The four variables were coded according to the following equation:

$$X_i = \frac{x_i - x_0}{\Delta x}, \quad i = 1 - 4 \quad (4)$$

where X_i is a coded value of the variable; x_i is the actual value of the variable; x_0 is the actual value of the independent variable at the center point; and Δx is the step change of the variable.

Table 1
Factors and levels for BBD.

Factors	Symbol	Coded levels		
		−1	0	1
Extraction temperature (°C)	X_1	80	90	100
Extraction time (h)	X_2	2	3	4
Extraction number	X_3	2	3	4
Ratio of water to raw material (mL/g)	X_4	10	14	18

A second-order polynomial model corresponding to the BBD was fitted to correlate the relationship between the independent variables and the response (extraction yield, polysaccharide yield and uronic acid yield) to predict the optimized conditions. The computer-generated quadratic model is given as

$$Y = \beta_0 + \sum_{i=0}^{4}\beta_i X_i + \sum_{j=0}^{4}\beta_{ii}X_i^2 + \sum_{i=0}^{4}\sum_{j=0}^{4}\beta_{ij}X_iX_j \quad (5)$$

where Y is the predicted response; X_i and X_j are the coded independent variables; β_0 is the intercept coefficient; β_i is the linear coefficient; β_{ii} is the squared coefficient; and β_{ij} is the interaction coefficient. Analysis of the experimental design data and calculation of predicted responses were carried out using Design Expert software (version 8.0, Stat-Ease, Inc., Minneapolis, USA).

Design-Expert 8.0, trial version was used for the ANOVA analysis of the experimental data obtained. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination R^2 and the values of adjusted-R^2 of models were evaluated to check the model adequacies. The significance of each term in the equation is to estimate the goodness of fit in each case. The analysis of variance tables was generated, and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The p-values of less than 0.05 were considered to be statistically significant. The regression coefficients were then used to make statistical calculation to generate contour and dimensional maps from the regression models.

2.6. Anti-complement assay through the classical pathway

Anti-complement activity was carried out as described previously with proper modification (Cimanga et al., 1995; Lustep & Clark, 2001; Makridea, 1998; Min et al., 2003; Morgan & Harris, 2003). Briefly, a diluted solution of normal human serum (complement serum, 80 μL) was mixed with a gelatin veronal buffer (GVB^{2+}, 80 μL) with or without sample. Each sample was dissolved in DMSO, and it was used as negative control. The mixture was pre-incubated at 37 °C for 30 min, after which sensitized erythrocyte (sheep red blood cells, 40 μL) was added. After incubation under the same conditions, the mixture was centrifuged (4 °C, 1500 rpm) and the optical density of the supernatant (100 μL) was measured at 450 nm. Rosmarinic acid was used as the positive control. Anti-complement activity was determined as a mean of triplicate measurements and expressed as the 50% inhibitory concentrations (IC_{50}) values from complement-dependent hemolysis of the control. IC_{50} values were obtained from dose curves as described earlier (Master, Khan, & Poojari, 2005).

3. Results and discussion

3.1. Statistical analysis and the model fitting

The effects of four process variables (i.e. temperature (X_1), time (X_2), number (X_3) and ratio of water to raw material (X_4)) were studied during experimentation. Three responses of interest were the extraction yield, polysaccharide yield and uronic acid yield. These conditions seemed to be varied depending on the response required. Therefore, an optimum process should be investigated in order to obtain high extraction yield, polysaccharide yield and the uronic acid yield. The results of 29 runs using BBD design are presented in Table 2 that include the design, experimental values and the predicted values, where the predicted values of the responses were obtained from quadratic model fitting techniques by the software mentioned above. BBD with four factors and three levels, including five replicates at the center point, was used to fit a second-order response surface in order to optimize the extraction conditions. The five center point runs were carried out to measure the process stability and inherent variability.

The extraction yield was taken as the response Y_1, the polysaccharide yield was taken as the response Y_2, and the uronic acid yield was taken as the response Y_3. Predicted responses Y_1, Y_2, and Y_3 could be expressed by the following second order polynomial equations:

$$\begin{aligned} Y_1 = {} & 23.46 + 14.47 \times X_1 + 4.11 \times X_2 + 5.33 \times X_3 + 1.62 \times X_4 \\ & - 0.050 \times X_1 \times X_2 + 3.15 \times X_1 \times X_3 + 1.70 \times X_1 \times X_4 \\ & + 2.93 \times X_2 \times X_3 - 1.15 \times X_2 \times X_4 - 1.95 \times X_3 \times X_4 \\ & + 2.12 \times X_1^2 - 2.71 \times X_2^2 - 2.44 \times X_3^2 - 1.75 \times X_4^2 \end{aligned} \quad (6)$$

$$\begin{aligned} Y_2 = {} & 16.02 + 10.53 \times X_1 + 2.63 \times X_2 + 3.79 \times X_3 + 0.48 \times X_4 \\ & + 0.98 \times X_1 \times X_2 + 0.90 \times X_1 \times X_3 + 0.25 \times X_1 \times X_4 \\ & + 1.70 \times X_2 \times X_3 - 0.72 \times X_2 \times X_4 - 1.08 \times X_3 \times X_4 \\ & + 1.55 \times X_1^2 - 0.59 \times X_2^2 - 0.70 \times X_3^2 - 1.74 \times X_4^2 \end{aligned} \quad (7)$$

$$\begin{aligned} Y_3 = {} & 8.32 + 6.70 \times X_1 + 1.91 \times X_2 + 2.62 \times X_3 - 0.058 \times X_4 \\ & + 1.22 \times X_1 \times X_2 + 2.53 \times X_1 \times X_3 + 0.050 \times X_1 \times X_4 \\ & + 1.05 \times X_2 \times X_3 - 0.85 \times X_2 \times X_4 - 0.68 \times X_3 \times X_4 \\ & + 1.54 \times X_1^2 - 0.43 \times X_2^2 - 0.74 \times X_3^2 - 0.25 \times X_4^2 \end{aligned} \quad (8)$$

where X_1 denotes extraction temperature, °C; X_2 denotes extraction time, h; X_3 denotes number of extraction; X_4 denotes ratio of water to raw material, mL/g.

Analysis of variance (ANOVA) for the response surface quadratic models was presented in Table 3. The significance of each coefficient was determined using p-value, which is used as a tool to check the significance of each coefficient and the interaction strength between each independent variable. If p-value is the smaller, it is the bigger the significance of the corresponding coefficient (Khuri & Cornell, 1987). The significance of the F value depends on the number of degrees of freedom (DF) in the model, and is shown in the p-value column (95% confidence level). In general, the effects lower than 0.05 are significant.

Table 2
BBD with the experimental values and predicted values for extraction yield Y_1 (mg/g), polysaccharide yield Y_2 (mg/g) and uronic acid yield Y_3 (mg/g).

Run	X_1	X_2	X_3	X_4	Y_1 (mg/g)	Predicted Y_1 (mg/g)	Y_2 (mg/g)	Predicted Y_2 (mg/g)	Y_3 (mg/g)	Predicted Y_3 (mg/g)
1	80 (−1)	2 (−1)	3 (0)	14 (0)	6.4	4.25	5.3	4.80	2.9	2.05
2	100 (1)	2 (−1)	3 (0)	14 (0)	33.8	33.28	26.2	23.90	13.2	13.00
3	80 (−1)	4 (1)	3 (0)	14 (0)	12.2	12.56	6.5	8.11	3.2	3.41
4	100 (1)	4 (1)	3 (0)	14 (0)	39.4	41.40	31.3	31.11	18.4	19.26
5	90 (0)	3 (0)	2 (−1)	10 (−1)	10.3	10.38	8.5	8.23	4.1	4.10
6	90 (0)	3 (0)	4 (1)	10 (−1)	23.8	24.93	18.9	17.96	9.7	10.68
7	90 (0)	3 (0)	2 (−1)	18 (1)	18.8	17.51	11.1	11.35	6.3	5.33
8	90 (0)	3 (0)	4 (1)	18 (1)	24.5	24.26	17.2	16.78	9.2	9.21
9	80 (−1)	3 (0)	3 (0)	10 (−1)	7.9	9.45	5.5	5.07	2.8	3.01
10	100 (1)	3 (0)	3 (0)	10 (−1)	34.3	34.98	24.6	25.62	16.5	16.31
11	80 (−1)	3 (0)	3 (0)	18 (1)	7.8	9.28	5.8	5.54	2.0	2.80
12	100 (1)	3 (0)	3 (0)	18 (1)	41.0	41.62	25.9	27.09	15.9	16.30
13	90 (0)	2 (−1)	2 (−1)	14 (0)	11.3	11.80	8.1	10.00	4.2	3.68
14	90 (0)	4 (1)	2 (−1)	14 (0)	14.1	14.17	13.2	11.87	5.1	5.40
15	90 (0)	2 (−1)	4 (1)	14 (0)	14.5	16.60	12.1	14.19	6.5	6.81
16	90 (0)	4 (1)	4 (1)	14 (0)	29.0	30.67	24	22.85	11.6	12.73
17	80 (−1)	3 (0)	2 (−1)	14 (0)	5.8	6.50	3.9	3.45	1.6	2.33
18	100 (1)	3 (0)	2 (−1)	14 (0)	29.2	29.14	22.8	22.70	10.2	10.68
19	80 (−1)	3 (0)	4 (1)	14 (0)	12.8	10.85	9.2	9.23	3.6	2.51
20	100 (1)	3 (0)	4 (1)	14 (0)	48.8	46.09	31.7	32.08	22.3	20.96
21	90 (0)	2 (−1)	3 (0)	10 (−1)	12.8	12.12	10.1	9.85	4.5	4.94
22	90 (0)	4 (1)	3 (0)	10 (−1)	25.4	22.64	15.7	16.57	11.9	10.46
23	90 (0)	2 (−1)	3 (0)	18 (1)	16.9	17.65	13.2	12.27	5.7	6.53
24	90 (0)	4 (1)	3 (0)	18 (1)	24.9	23.57	15.9	16.08	9.7	8.64
25	90 (0)	3 (0)	3 (0)	14 (0)	27.7	23.46	15.1	16.02	10.3	8.32
26	90 (0)	3 (0)	3 (0)	14 (0)	21.4	23.46	16	16.02	7.5	8.32
27	90 (0)	3 (0)	3 (0)	14 (0)	21.3	23.46	15.6	16.02	7.4	8.32
28	90 (0)	3 (0)	3 (0)	14 (0)	21.3	23.46	15.8	16.02	7.6	8.32
29	90 (0)	3 (0)	3 (0)	14 (0)	25.6	23.46	17.6	16.02	8.8	8.32

3.1.1. Extraction yield

The analysis of variance (ANOVA) (Table 3) showed that this regression model was highly significant ($p < 0.01$) with F value of 40.14. The F value of 0.52 for lack of fit implies that it is not significant comparing to the pure error. The fitness of the model was further confirmed by a satisfactory value of determination coefficient, which was calculated to be 0.9757, indicating that 97.57% of the variability in the response could be predicted by the model (Table 4). The value of the adjusted determination coefficient (adjusted $R^2 = 0.9514$) also confirmed that the model was highly significant. Furthermore, the predicted extraction yield by the final quadratic model, along with the corresponding values observed, were given in Table 2, indicating that the agreement between the extraction yield predicted by the model and the experimental data is satisfactory, which suggests a good fit to the mathematical model (Eq. (6)). As shown in Table 3, the variable with the largest effect was the X_1, followed by the other linear terms of X_3 and X_2, which were extremely significant at $p < 0.0001$. It also can be seen from Table 3 that the linear coefficient (X_4), the quadratic term coefficients (X_1^2, X_2^2 and X_3^2) and the interaction term coefficients ($X_1 \times X_3$ and $X_2 \times X_3$) were significant, with small p-values ($p < 0.05$). The other term coefficients were not significant ($p > 0.05$) on the extraction yield.

3.1.2. Polysaccharide yield

Linear terms of X_1, X_2 and X_3 showed the largest effect ($p < 0.0001$) on polysaccharide yield (Table 3). It was followed by quadratic terms of ratio of water to raw material (X_4^2) ($p < 0.01$) and extraction temperature (X_1^2) ($p < 0.05$) and interaction term of extraction time and number of extraction ($X_2 \times X_3$) ($p < 0.05$), and the other interaction terms were however not significant ($p > 0.05$). The total determination coefficient (R^2) was 98.3% (Table 4), indicating a reasonable fit of the model to the experimental data. The value of the adjusted determination coefficient (adjusted $R^2 = 0.9659$) also confirmed that the model was highly significant. The coefficient of determination (R^2) of the predicted models in this response was 0.9107, suggesting a good fit to the mathematical model (Eq. (7)).

3.1.3. Uronic acid yield

The determination coefficient ($R^2 = 0.9733$) was shown by ANOVA of the quadratic regression model (Table 4), indicating that only 2.67% of the total variations were not explained by the model. The value of the adjusted determination coefficient (adjusted $R^2 = 0.9465$) also confirmed that the model was highly significant on the uronic acid yield. At the same time, the model was found to be adequate for prediction within the range of experimental variables, with the predicted determination coefficient (predicted $R^2 = 0.8806$) (Table 4). The "lack of fit F-value" of 0.90 implied the lack of fit was not significant relative to the pure error, and there is a 59.77% chance that this value could occur due to noise (Table 3). Thus, this model was found to be adequate to navigate the design space and further optimization on uronic acid yield, with a good fit to the mathematical model (Eq. (8)). The data in Table 3 indicated that the independent variables (X_1, X_2 and X_3), interaction between extraction temperature (X_1) and number of extraction (X_3) ($X_1 \times X_3$) and a quadratic term (X_1^2) significantly affected the yield of uronic acid ($p < 0.01$). Among them, the linear factor extraction temperature (X_1), extraction time (X_2) and number of extraction (X_3) were found to be extremely significant ($p < 0.0001$). Meanwhile, the temperature (X_1) was the major factor affecting the uronic acid yield.

3.2. Analysis of response surfaces

The relationship between the responses and the experimental variables can be illustrated graphically to investigate the interactions of the variables and to determine the optimal level of each variable for the maximum response by plotting three-dimensional response surface plots (Figs. 1–3) (Li, Liu, & Chi, 2008). Each plot shows a pair of factors by keeping the other factor constant at its middle level.

Table 3
Analysis of variance (ANOVA) for response surface quadratic model of ephedra acidic polysaccharide extraction determined from BBD.

	Sum of squares	DF	Mean square	*F*-value	*p*-Value	Significant
Extraction yield (mg/g)						
Model	3336.60	14	238.33	40.14	<0.0001	***
X_1	2511.41	1	2511.41	423.01	<0.0001	***
X_2	202.54	1	202.54	34.12	<0.0001	***
X_3	340.27	1	340.27	57.31	<0.0001	***
X_4	31.36	1	31.36	5.28	0.0375	*
$X_1 \times X_2$	0.010	1	0.010	1.684×10^{-3}	0.9678	
$X_1 \times X_3$	39.69	1	39.69	6.69	0.0216	*
$X_1 \times X_4$	11.56	1	11.56	1.95	0.1846	
$X_2 \times X_3$	34.22	1	34.22	5.76	0.0308	*
$X_2 \times X_4$	5.29	1	5.29	0.89	0.3612	
$X_3 \times X_4$	15.21	1	15.21	2.56	0.1318	
X_1^2	29.27	1	29.27	4.93	0.0434	*
X_2^2	47.75	1	47.75	8.04	0.0132	*
X_3^2	38.57	1	38.57	6.50	0.0232	*
X_4^2	19.88	1	19.88	3.35	0.0886	
Residual	83.12	14	5.94			
Lack of fit	46.99	10	4.70	0.52	0.8168	Not significant
Pure error	36.13	4	9.03			
Cor total	3419.72	28				
Polysaccharide yield (mg/g)						
Model	1661.39	14	118.67	57.68	<0.0001	***
X_1	1329.31	1	1329.31	646.11	<0.0001	***
X_2	83.21	1	83.21	40.45	<0.0001	***
X_3	172.52	1	172.52	83.85	<0.0001	***
X_4	2.80	1	2.80	1.36	0.2626	
$X_1 \times X_2$	3.80	1	3.80	1.85	0.1955	
$X_1 \times X_3$	3.24	1	3.24	1.57	0.2301	
$X_1 \times X_4$	0.25	1	0.25	0.12	0.7326	
$X_2 \times X_3$	11.56	1	11.56	5.62	0.0327	*
$X_2 \times X_4$	2.10	1	2.10	1.02	0.3292	
$X_3 \times X_4$	4.62	1	4.62	2.25	0.1561	
X_1^2	15.55	1	15.55	7.56	0.0157	*
X_2^2	2.25	1	2.25	1.09	0.3132	
X_3^2	3.19	1	3.19	1.55	0.2333	
X_4^2	19.62	1	19.62	9.54	0.0080	**
Residual	28.80	14	2.06			
Lack of fit	25.24	10	2.52	2.83	0.1640	Not significant
Pure error	3.57	4	0.89			
Cor total	1690.20	28				
Uronic acid yield (mg/g)						
Model	730.41	14	52.17	36.40	<0.0001	***
X_1	538.68	1	538.68	375.78	<0.0001	***
X_2	43.70	1	43.70	30.49	<0.0001	***
X_3	82.16	1	82.16	57.32	<0.0001	***
X_4	0.041	1	0.041	0.028	0.8684	
$X_1 \times X_2$	6.00	1	6.00	4.19	0.0600	
$X_1 \times X_3$	25.50	1	25.50	17.79	0.0009	**
$X_1 \times X_4$	1.000×10^{-2}	1	1.000×10^{-2}	6.976×10^{-3}	0.9346	
$X_2 \times X_3$	4.41	1	4.41	3.08	0.1013	
$X_2 \times X_4$	2.89	1	2.89	2.02	0.1775	
$X_3 \times X_4$	1.82	1	1.82	1.27	0.2785	
X_1^2	15.30	1	15.30	10.67	0.0056	**
X_2^2	1.18	1	1.18	0.82	0.3794	
X_3^2	3.54	1	3.54	2.47	0.1382	
X_4^2	0.41	1	0.41	0.29	0.6008	
Residual	20.07	14	1.43			
Lack of fit	13.88	10	1.39	0.90	0.5977	Not significant
Pure error	6.19	4	1.55			
Cor total	750.48	28				

* Significant at $p < 0.05$.
** Significant at $p < 0.01$.
*** Significant at $p < 0.001$.

3.2.1. *Extraction yield*

The 3D plot in Fig. 1a showed the effects of extraction temperature (X_1) and number (X_3) on extraction yield (Y_1). There was a rapid rise in extraction yield with increase in extraction temperature (X_1); however, extraction yield (Y_1) was found to rise slightly with number of extraction (X_3) varying from 2 to 4. In Fig. 1b, there was an upsurge in extraction yield (Y_1) with increase in extraction temperature (X_1), but extraction yield (Y_1) showed a slight escalating trend with increase in ratio of water to raw material (X_4). Similarly, Fig. 1c showed that extraction yield (Y_1) soared rapidly with increase in extraction temperature (X_1), but rose gently with increase in extraction time (X_2). Interestingly, Fig. 1a–c commonly demonstrated that extraction temperature was the major factor among the four factors causing significant effects on extraction yield. This finding was consistent with the previous literatures on polysaccharides (Hou, Lin, Lu, Fang, & Chen, 2006; Jiang, Qiao, & Zhang, 2005). Extraction yield (Y_1) affected by varying extraction time (X_2) and number of extraction (X_3) was shown in Fig. 1d.

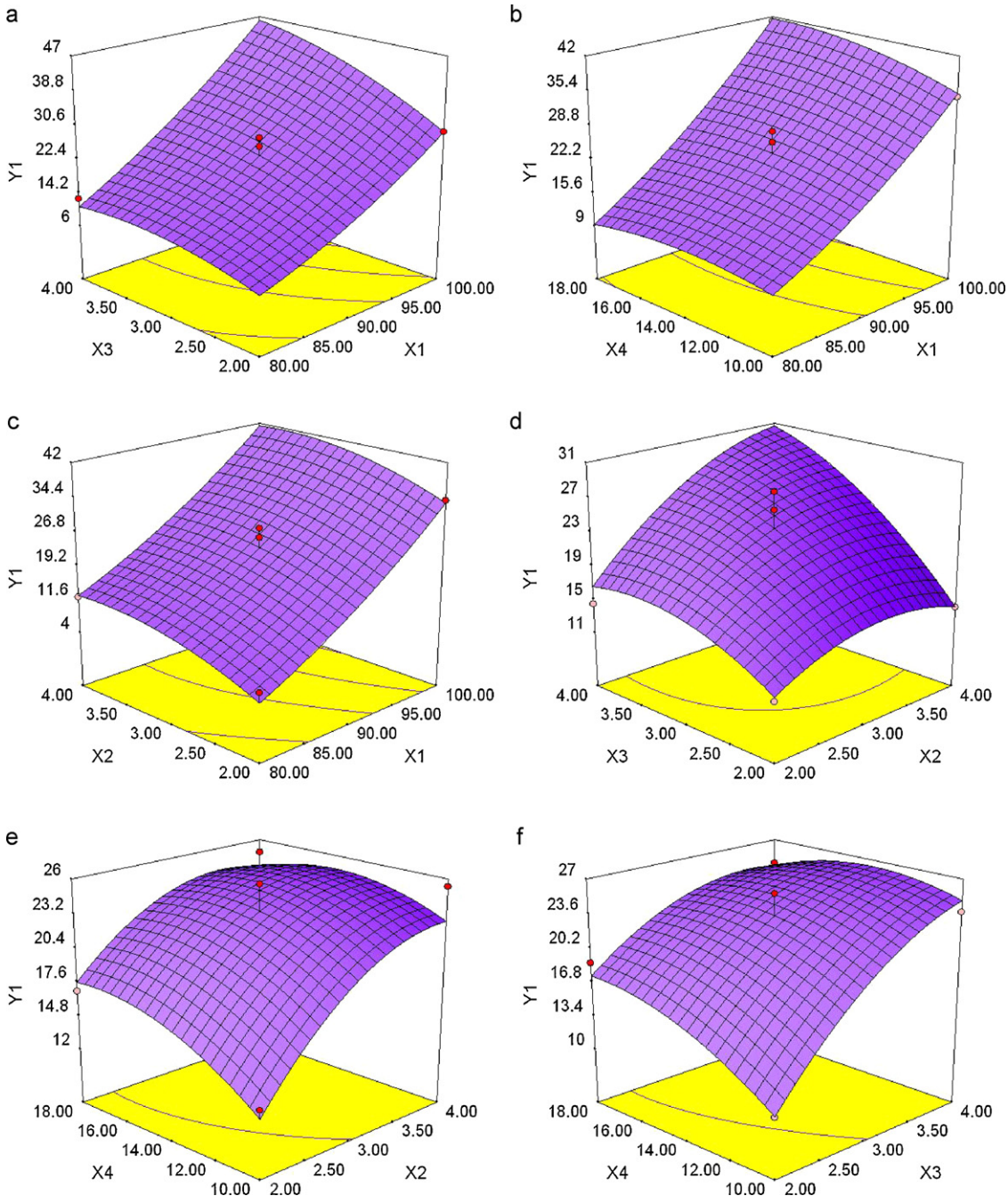

Fig. 1. Response surface plots showing the predicted values of extraction yield: the effects of two variables on the response extraction yield (Y_1, mg/g), with the other two fixed at 0 level (X_1: extraction temperature, °C; X_2: extraction time, h; X_3: number of extraction; X_4: ratio of water to raw material, mL/g).

It could be seen that contributions of extraction time (X_2) and number of extraction (X_3) to the effects on extraction yield (Y_1) were similar, demonstrating that extraction time (X_2) had a similar effect on extraction yield (Y_1) as number of extraction (X_3).

Table 4
Fit statistics for the response values.

Values	Y_1	Y_2	Y_3
Std. Dev.	2.44	1.43	1.20
Mean	21.48	15.41	8.37
PRESS	327.09	150.93	89.62
R^2	0.9757	0.9830	0.9733
Adj R^2	0.9514	0.9659	0.9465
Pred R^2	0.9044	0.9107	0.8806
Adeq precision	23.877	27.756	21.964

Fig. 1e indicated that extraction time (X_2) was the major factor affecting extraction yield (Y_1). The longer extraction time (X_2), the higher extraction yield (Y_1). Nevertheless, extraction yield (Y_1) first increased and then decreased with ratio of water to raw material (X_4) increased. In a trend similar to Fig. 1e and f showed that the increase in number of extraction (X_3) led to a rapid rise to extraction yield (Y_1), whereas ratio of water to raw material (X_4) showed a trend to first increase and then decrease extraction yield (Y_1).

3.2.2. Polysaccharide yield

With the similar trend to Fig. 1a, the response surface plot in Fig. 2a indicated that increasing extraction temperature (X_1) caused a significant positive effect on polysaccharide yield (Y_2),

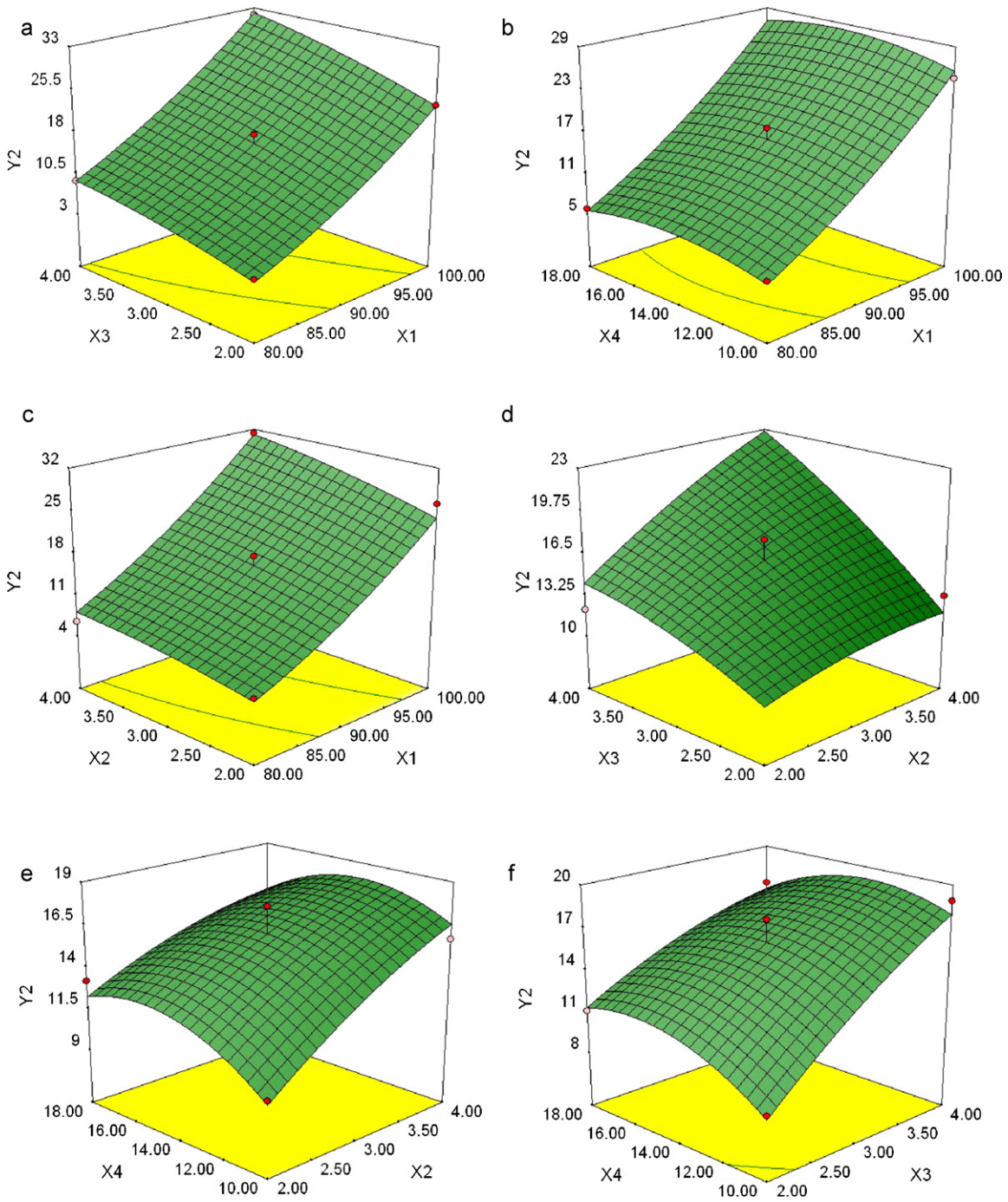

Fig. 2. Response surface plots showing the predicted values of polysaccharide yield: the effects of two variables on the response polysaccharide yield (Y_2, mg/g), with the other two fixed at 0 level (X_1, X_2, X_3 and X_4, see Fig. 1).

for polysaccharide yield (Y_2) rose more rapidly with an increase in extraction temperature (X_1) than in the case of number of extraction (X_3). As shown in Fig. 2b, there was a rapid increase in polysaccharide yield (Y_2) with increase in extraction temperature (X_1), but polysaccharide yield (Y_2) first increased and then declined with increase in ratio of water to raw material (X_4). Fig. 2c showed the trend very similar to Fig. 2a that polysaccharide yield (Y_2) soared quickly with increase in extraction temperature (X_1), but increased slightly with increase in extraction time (X_2). In this case, Fig. 2a–c also indicated that extraction temperature was the major factor which caused significant effects on polysaccharide yield. This phenomenon might be explained by the same principle (Zhong & Wang, 2009). As shown in Fig. 2d, with the same contribution to the increase in polysaccharide yield (Y_2), extraction time (X_2) and number of extraction (X_3) exhibited an equally positive effect on it. Fig. 2e indicated that the interactions between extraction time (X_2) and ratio of water to raw material (X_4) presented a weak effect on polysaccharide yield (Y_2). Extraction time (X_2) was the major cause affecting the increase in polysaccharide yield (Y_2). On the other hand, polysaccharide yield (Y_2) first increased and then decreased along with increasing ratio of water to raw material (X_4). Being similar to Fig. 2e, Fig. 2f exhibited that the increase in number of extraction (X_3) within the range caused a rapid linear rise to polysaccharide yield (Y_2), however as for ratio of water to raw material (X_4) it showed a parabolic curve (first increase and then decrease).

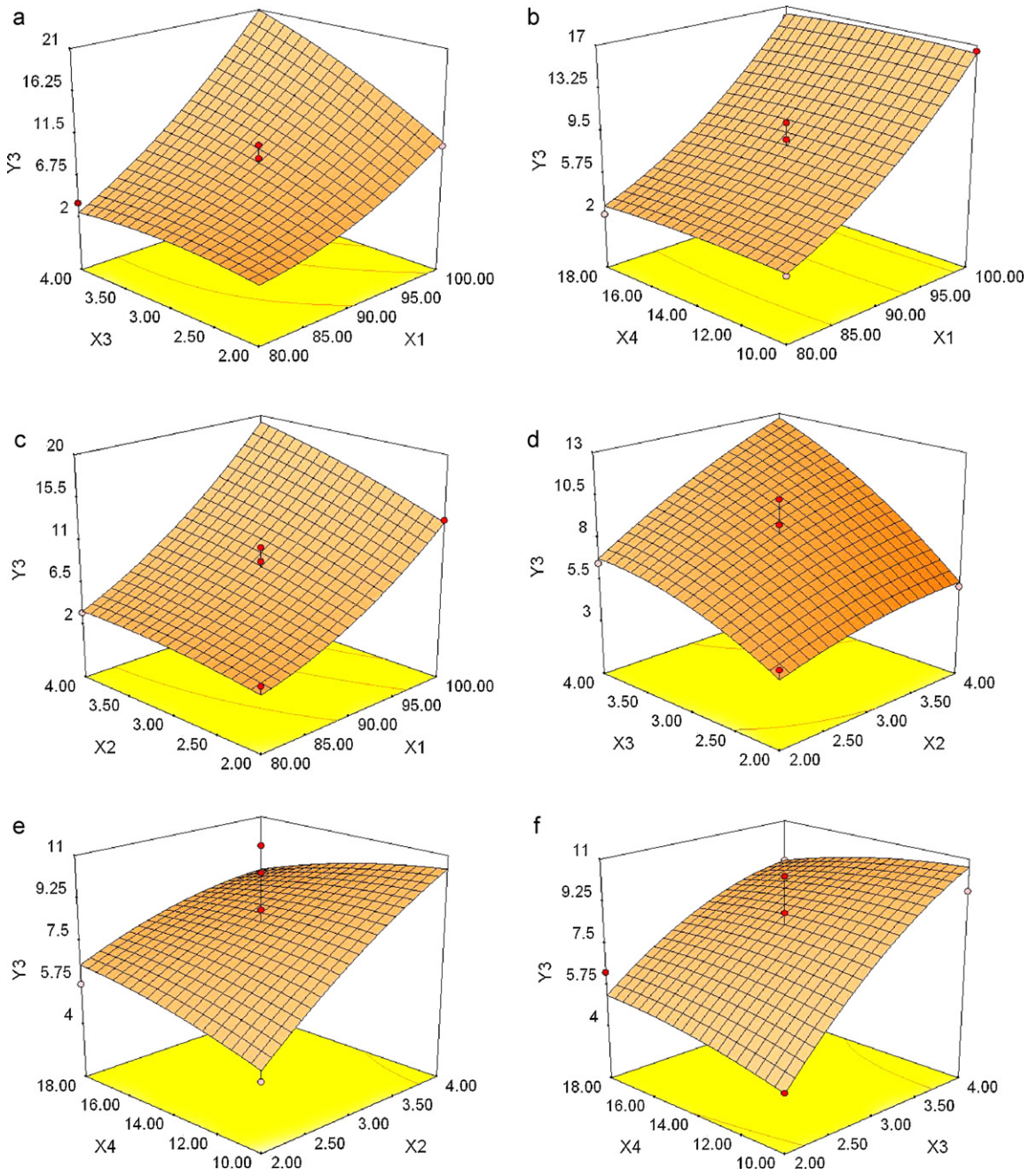

Fig. 3. Response surface plots showing the predicted values of uronic acid yield: the effects of two variables on the response uronic acid yield (Y_3, mg/g), with the other two fixed at 0 level (X_1, X_2, X_3 and X_4, see Fig. 1).

3.2.3. Uronic acid yield

Fig. 3a gave a similar figure to Fig. 2a; however, the only difference is that in the low level of extraction temperature (X_1) the increase in number of extraction (X_3) just caused a weak effect on uronic acid yield (Y_3). Similarly, in the low level of extraction temperature (X_1) as shown in Fig. 3a, there was a very small effect of ratio of water to raw material (X_4) on uronic acid yield (Y_3). And it came to the high level of extraction temperature (X_1) in the same case. The effect of the varying extraction time (X_2) on uronic acid yield (Y_3) was illustrated in Fig. 3c with the same trend as Fig. 3a. All the figures mentioned above in this section demonstrated that the effect of extraction temperature was the major effect causing dramatic variation on uronic acid yield. This phenomenon could be illustrated in this literature (Gan et al., 2010). Being very similar to Fig. 2d, Fig. 3d demonstrated that the effect of extraction time (X_2) and number of extraction (X_3) on uronic acid yield (Y_3) would be the same. Unlike Fig. 2e and f, Fig. 3e and f presented very different trends for the effect ratio of water to raw material (X_4) on uronic acid yield (Y_3) at different levels of extraction time (X_2) or number of extraction (X_3). As shown in Fig. 3e and f, an apparent increase in uronic acid yield (Y_3) was observed with increasing ratio of water to raw material (X_4) (extraction time (X_2) or number of extraction (X_3) was at low level), whereas uronic acid yield (Y_3) decreased with increase in ratio of water to raw material (X_4) (extraction time (X_2) or number of extraction (X_3) was at high level), which indicated that when extraction time or extraction number at high level ratio of water to raw material should be decreased to get a high uronic acid yield.

Table 5
Experimental and predicted values of the responses at optimum conditions.

Optimum condition				Y_1 (mg/g)		Y_2 (mg/g)		Y_3 (mg/g)	
X_1	X_2	X_3	X_4	Experimental[a]	Predicted	Experimental[a]	Predicted	Experimental[a]	Predicted
100 °C	3.5 h	4	13.6 mL/g	49.47	48.86	33.25	34.69	22.08	23.53

[a] Mean of triplicate determinations.

3.3. Optimization of extracting parameters and validation of the model

The optimum extraction conditions (extraction temperature 100 °C, X_2 extraction time 3.5 h, number of extraction 4, and ratio of water to raw material 13.6 mL/g) for the extraction yield, polysaccharide yield and uronic acid yield were estimated by solving the regression equation and analyzing the response surface plots. The predicted extraction yield, polysaccharide yield and uronic acid yield that were given by the Design Expert software under the above conditions was 48.86 mg/g, 34.69 mg/g and 23.53 mg/g, respectively (Table 5). The optimum extraction conditions were applied to three independent replicates to verify the prediction from the model. The mean experimental extraction yield, polysaccharide yield and uronic acid yield were 49.47 mg/g, 33.25 mg/g, and 22.08 mg/g, which were in good agreement with the predicted value of the model equation, confirming that the response model was adequate for the optimization.

3.4. Anti-complement activities of ephedra acidic polysaccharides

In our previous study, we reported a strategy for screening and comparison of immunosuppressive activities of polysaccharides from the stems of *E. sinica* by both specific and non-specific immune response (Kuang et al., 2010). The results in our study indicated that hot water-extractable acidic polysaccharides have shown obvious immunosuppressive effects by carbon clearance test, delayed-type hypersensitivity reaction and serum hemolysin analysis *in vivo*. As part of an ongoing program of research in our laboratory to investigate the mechanism of immunosuppressive action of polysaccharides from the stems of *E. sinica*, anti-complement activities of ephedra acidic polysaccharides were investigated *in vitro*.

The human complement system plays an important role in the host immune defense system against foreign invasive organisms, i.e. viruses, bacteria, and fungi, as well as an external wound. Its effects are normally beneficial to the host, but it can also cause adverse effects depending on the site, extent, and duration of complement activation (Cimanga et al., 1995; Min et al., 2006). Activation of the system may lead to pathologic reactions in a variety of inflammatory, degenerative and immunological mediated diseases such as multiple sclerosis, systemic lupus erythematosus, dermatological disease, rheumatoid arthritis, glomerulonephritis and asthma (Lustep & Clark, 2001; Makridea, 1998; Morgan & Harris, 2003; Xu, Zhang, Zhang, & Cheng, 2007). The ephedra acidic polysaccharides exhibited significant inhibitory effects on the classical pathway of the complement system, evidencing IC_{50} values of 50 μg/mL (Fig. 4), compared with a positive control, rosmarinic acid (IC_{50} 64 μg/mL). This showed that ephedra acidic polysaccharides exhibited comparable inhibitory effects on the complement system. Therefore, we have stronger reason to infer that immunosuppressive effects of ephedra acidic polysaccharides may be closely related to its inhibitory effects on the complement system, which reflects non-specific humoral immune response (Makridea, 1998; Xu et al., 2007), but the mechanism still needs to be studied.

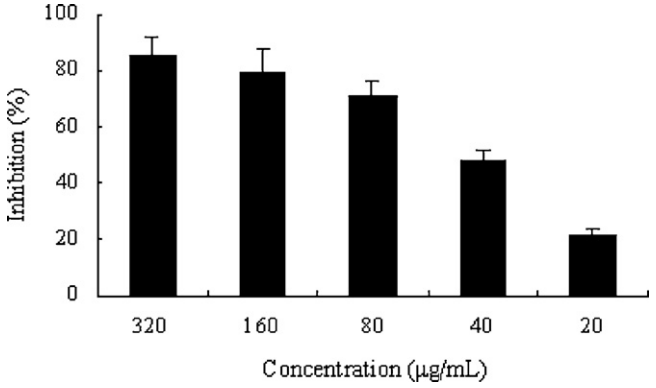

Fig. 4. Inhibitory effects of ephedra acidic polysaccharide on classical pathway of complement system (mean ± S.D., $n = 3$).

4. Conclusion

RSM was confirmed to be a useful tool for the optimization to estimate and optimize the experimental variables (extraction temperature, °C; extraction time, h; number of extraction; and ratio of water to raw material, mL/g). The coefficient of determination (R^2) for the three model equations was 0.9757, 0.9830 and 0.9733, respectively. And the probability value ($p < 0.0001$) demonstrated a very high significance for the regression models for predicting the responses. The optimum parameters were: extraction temperature of 100 °C, extraction time of 3.5 h, and number of extraction of 4 and ratio of water to raw material of 13.6 mL/g. This set of optimum parameters gives maximum predicted values of the three responses (extraction yield 48.86 mg/g, polysaccharide yield 34.69 mg/g and uronic acid yield 23.53 mg/g). Meanwhile, under these conditions, the mean experimental values (extraction yield 49.47 mg/g, polysaccharide yield 33.25 mg/g and uronic acid yield 22.08 mg/g) corresponded well with the predicted values. Furthermore, the present result clearly demonstrated the marked *in vitro* inhibitory activity of the ephedra acidic polysaccharide on the complement system, and the comparison with rosmarinic acid confirms that ephedra acidic polysaccharide is a potent complement inhibitor. To the best of our knowledge, the anti-complementary activities against the classical pathway of ephedra acidic polysaccharide are now being reported for the first time in this study.

Acknowledgements

Our work was supported by the Major State Basic Research Development Program of China (973 Program 2006CB504708), State Key Creative New Drug Project of 12th Five-year Plan of China (2011ZX11102), National Natural Science Foundation of China (No. 30973870) and Heilongjiang Research Based Innovation for Graduate Student (YJSCX2009-171HLJ).

References

Bitter, T., & Muir, H. M. (1962). A modified uronic acid carbazole reaction. *Analytical Biochemistry*, *4*, 330–334.

Boozer, C. N., Nasser, J. A., Heymsfield, S. B., Wang, V., Chen, G., & Solomon, J. L. (2001). An herbal supplement containing Ma Huang–Guarana for weight loss: A randomized, double-blind trial. *International Journal of Obesity*, *25*, 316–324.

Borkowski, J. J. (1995). Spherical prediction-variance properties of central composite and Box–Behnken designs. *Technometrics*, *37*, 399–410.
Box, G. E. P., & Wilson, K. B. (1951). On the experimental attainment of optimum conditions. *Journal of the Royal Statistical Society*, *13*, 1–45.
Cheng, R. M., Zhu, G. X., & Xu, Z. Y. (2001). Effect of different extracts from Ephedra on cell immunity. *Journal of Nanjing TCM University*, *17*, 234–236.
Cimanga, K., De, B. T., Lasure, A., Van, P. B., Pieters, L., Vanden, B. D., et al. (1995). In vitro anticomplementary activity of constituents from *Morinda morindoides*. *Journal of Natural Product*, *58*, 372–378.
Comar, K. M., & Kirby, D. F. (2005). Herbal remedies in gastroenterology. *Journal of Clinical Gastroenterology*, *39*, 457–468.
Ding, L. L., Shi, R. S., Cui, J., Wang, S. C., & Wang, Z. T. (2006). Advances in research of chemical constituents and pharmacological activities of Ephedra. *China Journal of Chinese Materia Medica*, *31*, 1661–1664.
DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.
Dunnick, J. K., Kissling, G., Gerken, D. K., Vallant, M. A., & Nyska, A. (2007). Cardiotoxicity of Ma Huang/caffeine or ephedrine/caffeine in a rodent model system. *Toxicologic Pathology*, *35*, 657–664.
Elavarasan, P., Kondamudi, K., & Upadhyayula, S. (2009). Statistical optimization of process variables in batch alkylation of *p*-cresol with *tert*-butyl alcohol using ionic liquid catalyst by response surface methodology. *Chemical Engineering Journal*, *155*, 355–360.
Gan, C. Y., Abdul Manaf, N. H., & Latiff, A. A. (2010). Optimization of alcohol insoluble polysaccharides (AIPS) extraction from the *Parkia speciosa* pod using response surface methodology (RSM). *Carbohydrate Polymers*, *79*, 825–831.
Ganzera, M., Lanser, C., & Stuppner, H. (2005). Simultaneous determination of *Ephedra sinica* and *Citrus aurantium* var. *amara* alkaloids by ion-pair chromatography. *Talanta*, *66*, 889–894.
Gurley, B. J., Gardner, S. F., White, L. M., & Wang, P. L. (1998). Ephedrine pharmacokinetics after the ingestion of nutritional supplements containing *Ephedra sinica* (Mahuang). *Therapeutic Drug Monitoring*, *20*, 439–445.
Haller, C. A., & Benowitz, N. L. (2000). Adverse cardiovascular and central nervous system events associated with dietary supplements containing *ephedra* alkaloids. *The New England Journal of Medicine*, *343*, 1833–1838.
Hou, S. J., Lin, C. Q., Lu, J., Fang, H. M., & Chen, C. G. (2006). Study on technology of extracting polysaccharides from polygonatum with water. *Anhui Chemical Industry*, *142*, 20–22.
Jiang, Q., Qiao, X., & Zhang, Z. (2005). Effect of extraction conditions on the yield of neutral polysaccharide from garlic (*Allillm Sativum* L.). *Journal of Chinese Institute of Food Science and Technology*, *5*, 101–105.
Karacabey, E., & Mazza, G. (2010). Optimisation of antioxidant activity of grape cane extracts using response surface methodology. *Food Chemistry*, *119*, 343–348.
Kemper, K. J., Singla, M., & Gardiner, P. (2005). Herbs and dietary supplements for asthma. *Clinical Pulmonary Medicine*, *12*, 67–75.
Khuri, A. I., & Cornell, J. A. (1987). *Response surfaces: Designs and analyses*. New York: Marcel Dekker Inc., pp. 105–205.
Kuang, H. X., Xia, Y. G., Yang, B. Y., Wang, Q. H., & Wang, Y. H. (2010). Screening and comparison of the immunosuppressive activities of polysaccharides from the stems of Ephedra sinica Stapf. *Carbohydrate Polymers*, doi:10.1016/j.carbpol.2010.08.056
Li, X. Y., Liu, Z. Q., & Chi, Z. M. (2008). Production of phytase by a marine yeast *Kodamaea ohmeri* BG3 in an oats medium: Optimization by response surface methodology. *Bioresource Technology*, *99*, 6386–6390.
Lomasney, A. R., Guillo, C., Sidebottom, A. M., & Roper, M. G. (2009). Optimization of capillary electrophoresis conditions for a glucagon competitive immunoassay using response surface methodology. *Analytical and Bioanalytical Chemistry*, *394*, 313–319.
Lustep, H. L., & Clark, W. M. (2001). Current status of neuroprotective agents in the treatment of acute ischemic stroke. *Current Neurology and Neuroscience Reports*, *1*, 13–18.
Makridea, S. C. (1998). Therapeutic inhibition of the complement system. *Pharmacological Reviews*, *50*, 59–87.
Master, H. E., Khan, S. I., & Poojari, K. A. (2005). Design and synthesis of low molecular weight compounds with complement inhibition activity. *Bioorganic Medicinal Chemistry*, *13*, 4891–4899.
Meng, D. L., Yan, S. H., & Xu, Z. Y. (2007). Effect of ephedran on erythrocytolysin formation of mouse caused by sheep erythrocyte. *Journal of Jiangsu University*, *17*, 379–381.
Min, B. S., Lee, S. Y., Kim, J. H., Lee, J. K., Kim, T. J., Kim, D. H., et al. (2003). Anticomplement activity of constituents from the stem-bark of *Juglans mandshurica*. *Biological Pharmaceutical Bulletin*, *26*, 1042–1044.
Min, B. S., Yun, B. S., Lee, H. K., Jung, H. J., Jung, H. A., & Choi, J. S. (2006). Two novel furan derivatives from *Phellinus linteus* with anti-complement activity. *Bioorganic Medicinal Chemistry Letters*, *16*, 3255–3257.
Morgan, B. P., & Harris, C. L. (2003). Complement therapeutics: History and current progress. *Molecular Immunology*, *40*, 159–170.
Myers, R. H., Montgomery, D. C., & Anderson-Cook, C. M. (2009). *Response surface methodology: Process and product optimization using designed experiments*. Hoboken: John Wiley & Sons, Inc., pp. 15–16.
Nam, N. H., Lee, C. W., Hong, D. H., Kim, H. M., Bae, K. H., & Ahn, B. Z. (2003). Antiinvasive, antiangiogenic and antitumour activity of *Ephedra sinica* extract. *Phytotherapy Research*, *17*, 70–76.
Paucar-Menacho, L. M., Berhow, M. A., Mandarino, J. M. G., de Mejia, E. G., & Chang, Y. K. (2010). Optimization of germination time and temperature on the concentration of bioactive compounds in Brazilian soybean cultivar BRS 133 using response surface methodology. *Food Chemistry*, *119*, 636–642.
Roman, M. C., Gray, D., Luo, G., McClanahan, R., Perez, R., Roper, C., et al. (2004). Determination of *ephedrine alkaloids* in botanicals and dietary supplements by HPLC–UV. *Journal of AOAC International*, *87*, 1–14.
Samenuk, D., Link, M. S., Homoud, M. K., Contreras, R., Theohardes, T. C., Wang, P. J., et al. (2002). Adverse cardiovascular events temporally associated with Ma Huang, an herbal source of ephedrine. *Mayo Clinic Proceedings*, *77*, 12–16.
Schaneberg, B. T., Crockett, S., Bedir, E., & Khan, I. A. (2003). The role of chemical fingerprinting: Application to *Ephedra*. *Phytochemistry*, *62*, 911–918.
Shekelle, P. G., Hardy, M. L., Morton, S. C., Maglione, M., Mojica, W. A., Suttorp, M. J., et al. (2003). Efficacy and safety of ephedra and ephedrine for weight loss and athletic performance. *The Journal of American Medical Association*, *289*, 1537–1545.
Sun, Y., Liu, J., & Kennedy, J. F. (2010). Application of response surface methodology for optimization of polysaccharides production parameters from the roots of *Codonopsis pilosula* by a central composite design. *Carbohydrate Polymers*, *80*, 949–953.
Trujillo, W. A., & Sorenson, W. R. (2003). Determination of ephedrine alkaloids in human urine and plasma by liquid chromatography/tandem mass spectrometry: Collaborative study. *Journal of AOAC International*, *86*, 643–656.
White, C., Willis, B., Keshav, N., & Dutton, S. (2001). Identifying and estimating significant geologic parameters with experimental design. *SPE Journal*, *6*, 311–324.
Xia, Y. G., Wang, Q. H., Liang, J., Yang, B. Y., Li, G. Y., & Kuang, H. X. (2010). Development and application of a rapid and efficient CZE method coupled with correction factors for determination of monosaccharide composition of acidic hetero-polysaccharides from *Ephedra sinica*. *Phytochemical Analysis*, 1235, doi:10.1002/pca.
Xu, H., Zhang, Y. Y., Zhang, J. W., & Cheng, D. F. (2007). Anti-complementary agents from natural products. *Chinese Journal of Natural Medicines*, *5*, 322–332.
Young, R., & Glennon, R. A. (1998). Discriminative stimulus properties of (−) ephedrine. *Pharmacology Biochemistry and Behavior*, *60*, 771–775.
Zhong, K., & Wang, Q. (2009). Optimization of ultrasonic extraction of polysaccharides from dried longan pulp using response surface methodology. *Carbohydrate Polymers*, *80*, 19–25.
Zhong, L. Y., Gong, Q. F., Zhu, J., & Chen, F. (2010). Comparative analysis of essential oil components in *Ephedra* and its processed products by GCMS. *Journal of Chinese Pharmaceutical Sciences*, *19*, 67–73.