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Optimization of extraction technology of Astragalus polysaccharides by response surface methodology and its effect on CD40

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ABSTRACT

Astragali is a herbal medicine used as an adjuvant extensively in the treatment of various cancers of lung, digestive tract, urinary system, etc. Statistics-based experimental designs were applied to optimize the extraction conditions for Astragalus polysaccharides (AP). The optimum conditions were found to be: the values of extraction time 3 h, ratio of liquid to solid 4 and particle size 33.8 mesh. Under these optimized conditions, the maximum AP extraction rate was 16.32 (%), which well matches with the predicted value. Furthermore, the chemical composition was analyzed with HPLC, and the pharmacological effect of the AP was evaluated. Results showed that AP could significantly decreased CD40 expression rate and protein expression in rats with stomach cancer.

1. Introduction

In China, many plants used in traditional medicine have been considered for a long time to be potential sources for the development of alternative therapeutics for a long time (Xu, Chao, & Wan, 2009). Astragali is a herbal medicine used as an adjuvant extensively in the treatment of various cancers of lung, digestive tract, urinary system, etc. (Huang, Liu, Song, Liu, & Liu, 2009). As an important bioactive component of Astragalus, Astragalus polysaccharides (AP) has antioxidant, anti-hypertensive, and immunomodulatory activities (Grover, Yadav, & Vats, 2002; Mao, Xie, & Gu, 2002; Wu & Chen, 2004).

The variables for production of AP included extraction time, ratio of liquid to solid, and particle size. Optimization of extraction conditions has been used in enhancing the yield of many polysaccharides. However, there is no literature reported to optimize the extraction conditions for AP.

In the present work, a Box-Behnken design (BBD) of response surface methodology (RSM) has been used to optimize the extraction conditions for AP. The objective of the present study was to optimize the extraction conditions for AP and investigate its effect on CD40 expression rate and protein expression in rats with stomach cancer.

2. Material and method

2.1. Plant material

Fresh commercially obtained Astragalus roots from Sanxi province were purchased in a local herb market, Suzhou, China. The roots were ground in a rotary mill and then sieved (5–40 mesh).

2.2. Extraction of polysaccharides from astagalus

The dried plant material (300 g) was extracted twice with water (2 l) for 2.5 h at 100 °C. The combined extracts were concentrated to 250 ml using a rotary evaporator (BC-R203, Shanghai Biochemical Equipment Co., Shanghai, China) at 65 °C under vacuum. The proteins in the extract were removed by Sevag reagent (Wei, Zhou, Zang, & Jiang, 2007). After removal of the Sevag reagent, 100 ml of anhydrate ethanol was added before the mixture was maintained overnight at 4 °C to precipitate polysaccharides. The crude polysaccharides (25 g) was obtained by centrifugation at 3860*g* for 15 min.

2.3. Experimental design and optimization

RSM as a generic means for optimization was applied to optimize the extraction of ANP. The optimization was designed based on a three-factor Box-Behnken design with a total of 15 experimental runs that involved 3 factorial points and 3 replicates at the center points (Cai, Gu, & Tang, 2008). Based on the preliminary experiments and our previous studies, three extraction

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parameters, time ($x1$), ratio of liquid to solid ($x2$), and particle size ($x3$), were identified as key factors responsible for extraction yield. In view of the feasibility of ANP preparation, the ranges of the three factors were determined as follows: extraction time (2, 2.5, 3), Ratio of liquid to solid (3, 3.5, 4), and particle size (20, 30, 40) (Table 1). The experimental runs for Box-Behnken were shown in Table 2. Each experimental run was performed in duplicate except at the central point (13–15 runs) of the design. The response could be related to the selected variables by a second-order polynomial model. In this study, a second-order polynomial Eq. (1) was used to generate response surfaces.

$$Y = \beta_0 + \sum_i \beta_i x_i + \sum_i \beta_{ii} x_i^2 + \sum_{i \neq j} \beta_{ij} x_i x_j \quad (1)$$

where Y represents the predicted responses, x_i and x_j are the coded values of independent variables, β_0 is the intercept coefficient, β_i are the linear coefficients, β_{ii} are the squared coefficients, and β_{ij} are the interaction coefficients (Liang, 2008).

Prior to modeling, each independent variable was divided by its maximum value to treat the dimension uniformly. The least square technique was used to calculate the coefficients of variables in the models. The statistical significances were judged by Student's t-test at a probability of .01. The significance of the estimated effects was tested by analysis of variance. The accuracy of the statistical model used was described by the determination coefficient R^2. R^2 was the fraction of the data explained by the model, and value close to 1 indicated a good model. All calculation programs were coded in SAS 8.2 (SAS Institute Inc. Cary, NC, USA).

2.4. Verification of the results

Optimal conditions and the maximum predicted extraction yield were obtained using the second-order polynomial model of RSM. The practical acquired extraction yield was achieved under the optimal conditions. The experimental and predicted acquired results were compared in order to confirm the validity of the model.

2.5. HPLC analysis

AP were hydrolyzed with 2 M H_2SO_4 for 5–6 h at 121 °C in sealed glass test tube. After complete hydrolysis, content was neutralized with $BeCO_3$ and filtered. Monosaccharide composition of the hydrolysate was determined by HPLC (Waters Alliance, 2996-seperation module) using Supelco gel 610H column (30 cm × 7.8 mm) and RI (2414) detector with flow rate 0.4 ml/min at temperature 30 °C and mobile phase, 0.17% H_3PO_4 in water. The relative proportion of the peak area was calculated to estimate the monomer composition.

2.6. Animals

Male wistar rats 6–7 weeks old, weighing 200–250 g were purchased from the National Institute of Nutrition, Suzhou University, China, and maintained in the Central Animal House, Suzhou University. The animals were housed in groups of four or five in polypropylene cages and provided standard pellet diet and water ad libitum and maintained under controlled conditions of temperature and humidity, with a 12 h light/dark cycle. The animals were maintained as per the principles and guidelines of the ethical committee for animal care of Suzhou University in accordance with the China National Law on animal care and use.

Table 1
Factors and levels.

Factor	Low	Center	High
$X1$ (h)	−1 (2)	0 (2.5)	1 (3)
$X2$	−1 (3)	0 (3.5)	1 (4)
$X3$ (mesh)	−1 (20)	0 (30)	1 (40)

Table 2
Box-Behnken experimental design of the independent variables along with the observed values for the response (Y).

Run	$X1$	$X2$	$X3$	Y
1	−1	−1	0	3.9
2	−1	1	0	5
3	1	−1	0	10.5
4	1	1	0	16.9
5	0	−1	−1	8.8
6	0	−1	1	9.7
7	0	1	−1	14.1
8	0	1	1	12.4
9	−1	0	−1	7.7
10	1	0	−1	14.8
11	−1	0	1	9.8
12	1	0	1	15.5
13	0	0	0	13.9
14	0	0	0	13.9
15	0	0	0	13.9

2.7. Experimental design

The Institutional animal ethical committee, Suzhou University, China, approved the experimental design. A total of 40 male wistar rats were divided into five groups of eight each. Stomach carcinogenesis was developed in rats (group II–V) according to the method of Li and Xue (2006). Group III mice were orally administered AP (100 mg/kg b.w in 2 ml distilled water) once daily for 5 weeks. Group IV animals were orally administered AP (200 mg/kg b.w in 2 ml distilled water) once daily for 5 weeks. Group V animals were orally administered AP (300 mg/kg b.w in 2 ml distilled water) once daily for 5 weeks. Group I and II animals orally received an equal volume of saline. All animals were allow to free access to water and fed with standard commercial pelleted rat chaw. At the end of the experimental period all the animals were sacrificed by cervical dislocation.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from stomach cancer tissue samples frozen at −80 °C, and RNA concentration was determined by spectrophotometer. Five micrograms of RNA were reverse-transcribed into cDNA using oligo (dT) primers. The primer sequences used for RT-PCR were as follows: CD40 (30 bp), 5′-AGA-AGG-CTG-GCA-CTG-TAC-GA-3′ and 5′-CAG-TGT-TGG-AGC-CAG-GAA-GA-3′; β-actin (44 bp) internal control, 5′-CAA-CTC-CAT-CAT-GAA-GTG-TAA-3′ and 5′-CCA-CAC-GGA-GTA-CTT-GCG-CTG-3′. Relative levels of CD40 expression were expressed using the optical density ratio (CD40/β-actin), as determined by the Bio-Image Analysis system.

2.9. Western blotting

Tumors were cut into small pieces and lysed using chaps buffer (0.5% Chaps, 10 mM Tris–HCl, pH7.5, 1 mM $MgCl_2$, 1 mM EGTA, 5 mM mercaptoethanol, 10% glycerol, and 0.1 mM PMSF) for 30 min on an ice-bath. Protein concentrations were determined using the BCA assay with Varioskan flash (Thermo) at 595 nm. Fifty micrograms of protein from each treatment group was loaded on a 10% SDS–PAGE gels. Proteins were transferred to a nitrocellulose membrane (Millipore) and incubated overnight with antibodies for Western blots. Primary antibodies included rat CD40 antibody. Immunoreactive bands were visualized with the Odyssey infrared imaging system (Li-COR Biosciences).

2.10. Statistical analysis

All data are expressed as means ± standard deviations (SD). Differences between experimental groups were statistically analyzed using one-way ANOVA or Student's *t*-test for unpaired data when appropriate. $P < .05$ was considered statistically significant.

3. Result and discussion

3.1. Model fitting

The application of RSM yields the regression Eq. (2), which represents an empirical relationship between the response (DH) and the tested variables (in coded units):

$$Y = 13.9 + 3.9125 * X1 + 1.9375 * X2 + .25 * X3 - 2.0625 * X1 * X1 + 1.325 * X1 * X2 - 0.35 * X1 * X3 - 0.7625 * X2 * X2 - 0.65X2 * X3 + 0.1125 * X3 * X3 \quad (2)$$

The significance of each coefficient was determined using the Student *t*-test and *p* value in Table 3. The largest effect has the linear term of time ($X1$), ratio of liquid to solid ($X2$); followed by the quadratic term of time ($X1 * X1$) and the quadratic term of ratio of liquid to solid ($X2 * X2$). The interaction terms did not have significant influence ($P > .1$), which means that the interaction between the different factors did not influence the response. Considering our results, among the independent variables time played dominant role in the extent of extraction.

Table 3
ANOVA for *Y*.

Source	DF	SS	MS	*F*	Pr > *F*
*X*1	1	122.4613	122.4613	107.1871	0.000145
*X*2	1	30.03125	30.03125	26.28556	0.003686
*X*3	1	0.5	0.5	0.437637	0.537529
*X*1 * *X*1	1	15.70673	15.70673	13.74769	0.013887
*X*1 * *X*2	1	7.0225	7.0225	6.146608	0.055893
*X*1 * *X*3	1	0.49	0.49	0.428884	0.541463
*X*2 * *X*2	1	28.1775	28.1775	24.66302	0.004226
*X*2 * *X*3	1	1.69	1.69	1.479212	0.278177
*X*3 * *X*3	1	0.046731	0.046731	0.040902	0.847698
Model	9	203.8648	22.65165	9.794859	0.002133
Error	5	5.7125	1.1425		
Total	14	209.5773			

Table 4
Fit statistics for *Y*.

	Master model	Predictive model
Mean	11.38667	11.38667
R-square	97.27%	97.27%
Adj. *R*-square	92.37%	92.37%
RMSE	1.068878	1.068878
CV	9.387101	9.387101

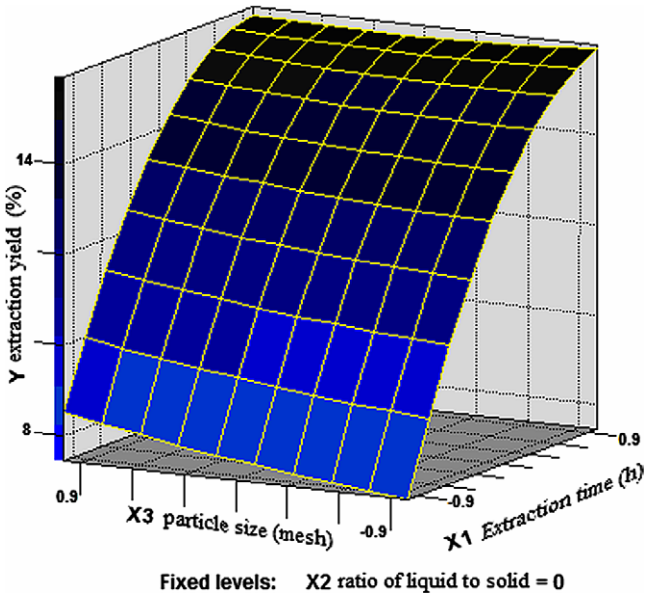

Fig. 2. Response surface curve for extraction yield of AP showing the interaction between extraction time and particle size at ratio of liquid to solid = 0.

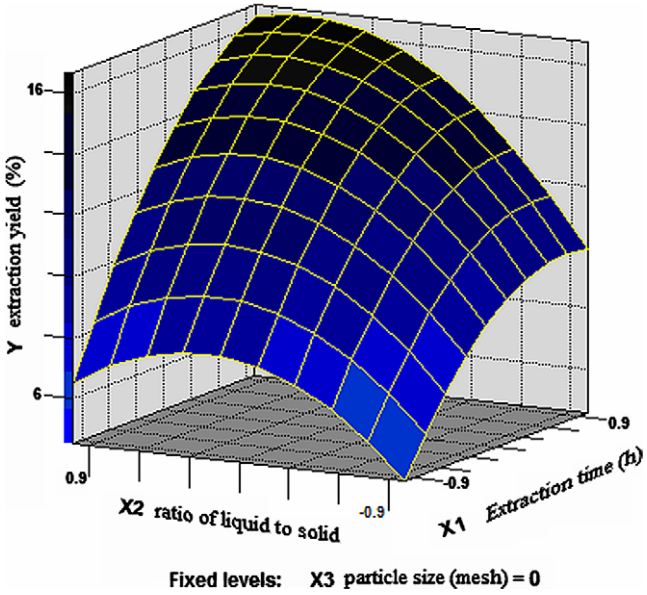

Fig. 1. Response surface curve for extraction yield of AP showing the interaction between extraction time and ratio of liquid to solid at particle size = 0.

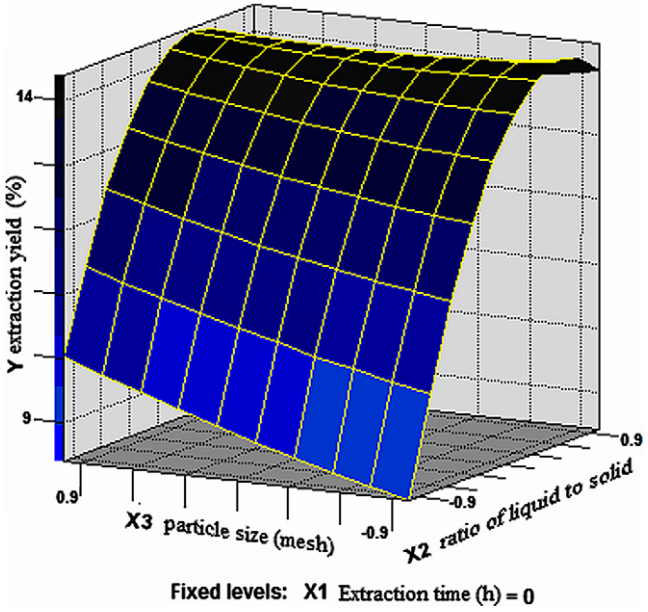

Fig. 3. Response surface curve for extraction yield of AP showing the interaction between ratio of liquid to solid and particle size at extraction time = 0.

The model has shown a good fit with the experimental data, since the coefficient of determination R^2 had a value of .973. This means that the fitted model could explain 97.27% of the total variability within the range of values studied. The analysis of variance (Table 4) showed high *F*-value, while *p*-value was less than .01, which implied that the model itself is significant. All these results imply a satisfactory mathematical description of the extraction process by the fitted model (Eq. (2)).

The response surface curves are plotted to explain the interaction of the variables and to determine the optimum level of each variable for maximum response. The response surface curves are shown in Figs. 1–3. Each demonstrates the effect of two factors while the other factors were fixed at zero level. The model predicted the optimal values (coded) of the three most significant variables were $X1 = 1$, $X2 = 1$ and $X3 = 0.44$. Correspondingly, the values of extraction time, ratio of liquid to solid and particle size were 3 h, 4 and 33.8 mesh, respectively. The maximum predicted yield of AP was 16.32 (%).

Table 5
Validation test for optimal extraction condition.

Optimum condition			Mean yield[a] (%)
*x*1 (h)	*x*2	*x*3 (mesh)	
3	4	33.8	16.14

[a] Mean ± standard deviation ($N = 3$).

3.2. Validation of the optimized condition

In order to confirm the optimized culture conditions, three additional experiments in shake flasks were performed using the predicted extraction conditions. The mean value of extraction yield was 16.14, which agreed well with the predicted value. This result demonstrates the validity of the response model (Table 5).

3.3. Analysis of chemical component of AP

The HPLC (Fig. 4a) analyses of the polysaccharides showed one main peak component, detected with an ELSD system. The preparative HPLC (Fig. 4b) separations of standard samples finally yielded five compounds corresponding to peaks 1, 2, 3, 4 and 5. From the comparison of retention time of the five standard compounds in HPLC analyses, we found that the polysaccharides were composed of glucose. That means that glucan appears as the major compound in the polysaccharides extract.

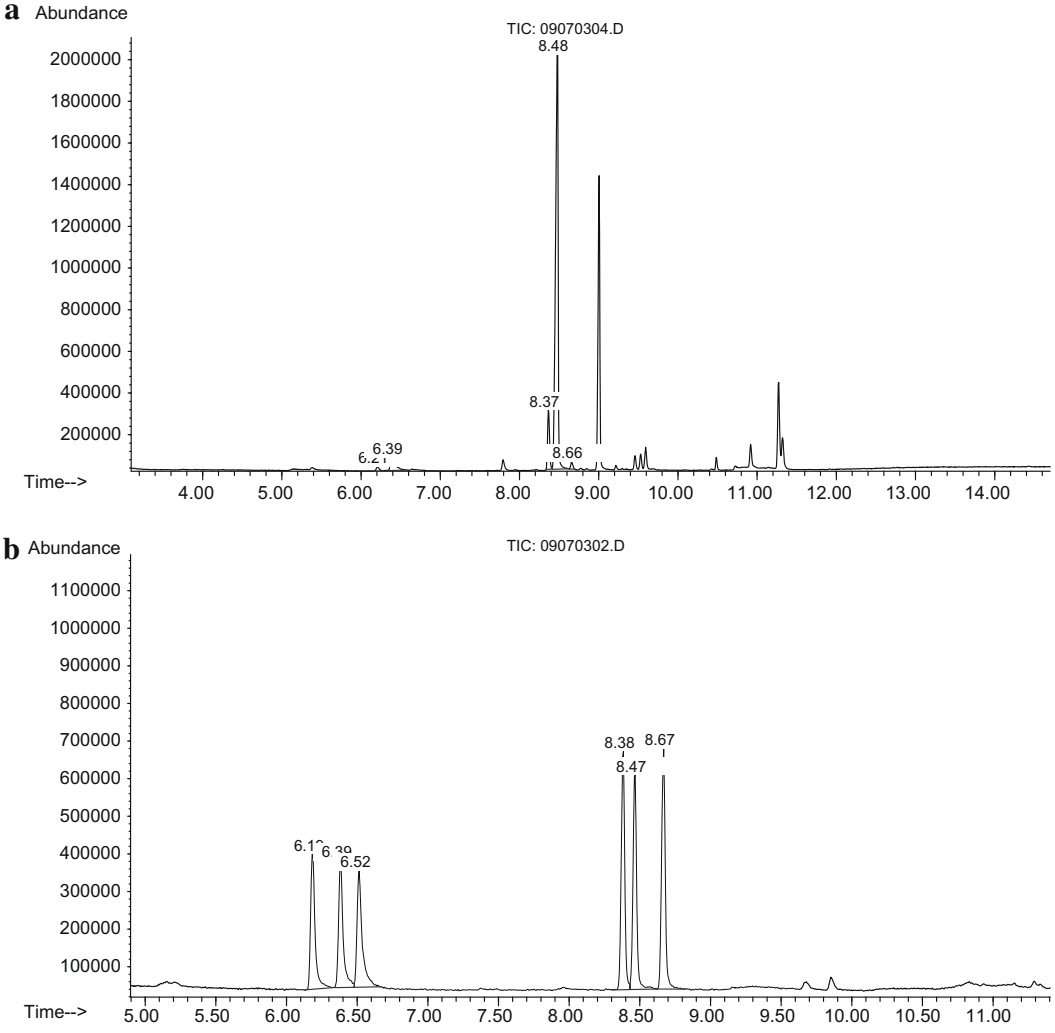

Fig. 4. (a) HPLC of AP and (b) HPLC of standard sample.

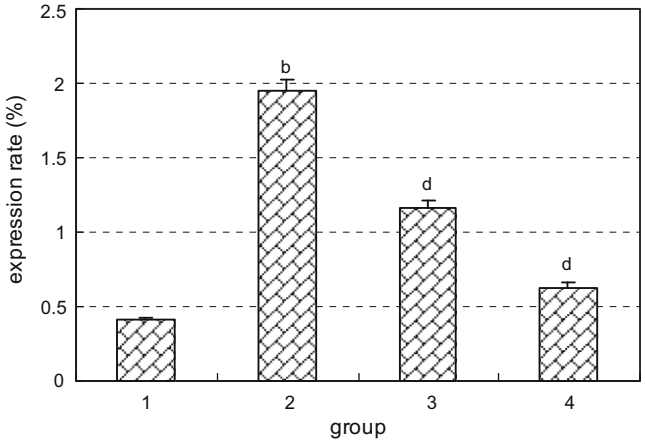

Fig. 5. Effect of AP on CD40 expression rate.

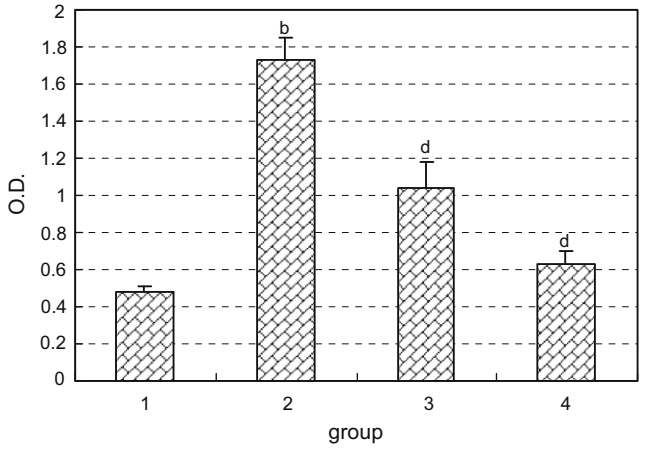

Fig. 6. Effect of AP on CD40 protein expression.

3.4. Effect of AP on CD40 expression rate

Data in Fig. 5 show significant increase in CD40 expression rate ($P < .01$) in rats with gastric cancer (group II) compared to normal control (group I). Treatment of rats (group III and IV) with ANP dose-dependently significantly ($P < .01$) decreased CD40 expression rate as compared to model control (group II).

3.5. Effect of AP on CD40 protein expression

Data in Fig. 6 show significant increase in CD40 protein expression ($P < .01$) in rats with gastric cancer (group II) compared to normal control (group I). Treatment of rats (group III and IV) with ANP dose-dependently significantly ($P < .01$) decreased CD40 protein expression as compared to model control (group II).

4. Conclusion

This study proved that statistical experimental designs offer an efficient and feasible approach for AP extraction optimization. A maximum AP production of 16.32 (%) was achieved with the following optimized factors: extraction time 3 h, ratio of liquid to solid 4 and particle size 33.8 mesh. Validation experiments were also carried out to verify the adequacy and the accuracy of the model. Results also showed that the AP can dose-dependently significantly ($P < .01$) decreased CD40 expression rate and CD40 protein expression in rats with stomach cancer.

Acknowledgement

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