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Optimisation of extraction conditions for polysaccharides from the roots of *Isatis tinctoria* L. by response surface methodology and their *in vitro* free radicals scavenging activities and effects on IL-4 and IFN-γ mRNA expression in chicken lymphocytes

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ABSTRACT

Response surface methodology along with Box-Behnken design was firstly applied to optimize the extraction conditions for the polysaccharides from the roots of *Isatis tinctoria* L. The experimental data obtained were fitted to a second-order polynomial equation using multiple regression analysis. The 3-D response surface and the contour plots derived from the mathematical models were applied to determine the optimal conditions. The optimal conditions were extraction temperature (X_1) 99.5 °C, extraction time (X_2) 3.75 h and ratio of water to raw material (X_3) 11.84 (v/w). Under these conditions, the maximal observed value extraction yield of Isatis root polysaccharides (IRPSs) was (11.19 ± 0.04)%, which was agreed with predicted value 11.17%. Pharmacological experiments indicated that IRPS have an appreciable ABTS radical scavenging ability *in vitro* and could increase IL-4 and IFN-γ mRNA expression in chicken lymphocytes obtaining maximum promoted effects of 70% and 115%, respectively. This study may facilitate a deeper understanding of IRPS to provide theoretical references.

1. Introduction

Isatis tinctoria L. (woad, Brassicaceae) has a long and well documented history as an indigo dye plant in temperate climates (Oberthür, Graf, & Hamburger, 2004). In China, *I. tinctoria* L. is a known traditional Chinese herb that comes from the roots of woad. It is also known as Radix isatidis (Condurso et al., 2006; Malcolm & Bruce, 1971). The herb also named as Ban Lan Gen in Chinese has been used for its medicinal properties in traditional Chinese medicine (TCM) for over several thousand years (Hamburger, 2002; Liau, Jong, Lee, & Chen, 2007). It is described in detail in the Chinese Materia Medica classics, *Shen Nong Ben Cao Jing* (Kong, Zhao, Shan, Xiao, & Guo, 2008; Peng, Fan, & Wu, 2005). This herb is cultivated in various regions of northern China, namely Hebei, Beijing, Heilongjiang, Henan, Jiangsu, and Gansu (Malcolm & Bruce, 1971; Qin et al., 2001). The root harvested during the autumn and dried is processed into granules, which are most commonly consumed dissolved in hot water or tea (Sun, Tong, & Bi, 2002). The product, called Banlangen Keli, is very popular throughout China, and used as an herbal antibiotic, antiseptic and anti-viral (Cen, Zhou, & Li, 2008; Chang, Luan, & Li, 2008; Qin et al., 2001; Sun et al., 2002; Wu & Xie, 2006). It is also used for pharyngitis, laryngitis, erysipelas, and carbuncle, and to prevent hepatitis A, epidemic meningitis, cancer and inflammation (Chen, 2009; Cui, Xue, Yang, & Hao, 2001). In some cases, it is an effective alternative to Western prescription antibiotics (Chen, 2009; Cui et al., 2001; Liu, Ding, & Lin, 2001; Liu, Luo, Li, & Xiong, 2001; Yang, Yang, & Xia, 2008). Also, Ban Lan Gen Instant Beverage (Isatis Tea Beverage) is the suitable tea for people in fever that is most popular beverage in China (Liu et al., 2001a,b).

Studies have shown that polysaccharides from roots are the main bioactivital components of *I. tinctoria* L. (Qiu et al., 2007; Xu & Lu, 1991). Isatis root polysaccharides (IRPSs) significantly increased the weight of spleen and number of white blood cells in peripheral blood in normal ICR mice, and antagonized the immunosuppressive actions of hydrocortisone. Also, the result indicates that IRPS is capable of increasing humoral and cellular immune functions and enhancing the functions of reticuloendothelial system, and might be a good immunopotentiator (Xu & Lu, 1991). Besides, IRPS have

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the effects on T subpopulations of piglets in the immune response to porcine reproductive and respiratory syndrome attenuated virus vaccine. IPRS could improve the proportion of CD3+ and CD8+ in the piglets peripheral blood and boost the production of special antibody (Zhang et al., 2007).

However, to the author's knowledge, there are almost no previous reports regarding the extraction process of IRPS. Hot water technology is the main and most conventional extraction method for polysaccharides mentioned in recent studies (Yan et al., 2011). Therefore, efficient extraction conditions for IRPS are needed to be optimum. However, so far there is no published information on the optimization of extraction conditions of IRPS for further application. Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes (Gan & Latiff, 2011a,b; Gan, Abdul Manaf, & Latiff, 2010; Myers, Montgomery, & Anderson-Cook, 2009; Paucar-Menacho, Berhow, Mandarino, de Mejia, & Chang, 2010; Sun, Liu, & Kennedy, 2010; Zhong & Wang, 2009). The main idea of RSM is to use a sequence of designed experiments to obtain an optimal response, and the experiments will be more easily arranged and interpreted using this efficient (Gan & Latiff, 2011a,b; Sun et al., 2010).

The objectives of this study were to optimize the conditions for the extraction of IRPS. It may facilitate a deeper understanding of the process of polysaccharide extraction from the roots of *I. tinctoria* L. to provide theoretical references. Besides, enhancing effect of IRPS on chicken peripheral blood lymphocytes and their *in vitro* free radicals scavenging activities were investigated.

2. Materials and methods

2.1. Materials

The samples of *I. tinctoria* L. were collected in Tianjin (2010), in China. All the collected *I. tinctoria* L. were immediately dried at 50 °C and stored in a dry and dark place. Samples were ground and sieved using a grinder and were passed through a 40-mesh sieve to obtain pretreated samples. All used solvents and chemicals were of analytical grade.

2.2. Extraction of IRPS

Each pretreated sample was extracted by hot deionized water, which was filtered with a vacuum pump (Division of Millipore, Waters, USA) and a filter (HA-0.45, Division of Milli-pore, Waters, USA) before use, in designed extraction temperatures, extraction times, and ratio of water to raw material. The extract was left to cool at room temperature, filtered, and then precipitated using 150 ml of 95% ethanol, 100% ethanol and acetone, respectively. After being left overnight at 4 °C, the precipitates were collected by centrifugation at 3000 rpm for 20 min, redissolved in deionized water and dialyzed in a dialysis tube (MWCO 3500 Da, USA) and then freeze-dried to obtain IRPS. The polysaccharide extraction yield (%) is calculated as follows:

$$\text{Extraction yield}\,(\%) = \frac{\text{weight of dried crude polysaccharide extraction}\,(\text{g})}{\text{weight of powders}\,(\text{g})} \times 100 \quad (1)$$

2.3. Experimental design

Response surface methodology (RSM) is an empirical statistical technique employed for multiple regression analysis by using quantitative data obtained from properly designed experiments to solve multivariate equations simultaneously (Ahmed, Rico, Martin-Diana, & Barry-Ryan, 2011; Fernández-Silva, Sanmartín, Silva, Moldes, & Prieto, 2011; You, Regenstein, & Liu, 2010). Box-Behnken, a spherical and revolving design, has been applied in optimisation of chemical and physical processes (Li et al., 2011; Maiti, Rathore, Srivastava, Shekhawat, & Srivastava, 2011) because of its reasoning design and excellent outcomes. The purpose of the center points is to estimate the pure error and curvature.

Table 1
Factors and levels.

Factors	Symbol	Coded levels		
		−1	0	1
Temperature (°C)	X_1	80	90	100
Time (h)	X_2	1	2.5	4
Ratio (mL/g)	X_3	5	10	15

On the basis of the single factor experimental results, three major influence factors and the ranges of each factors were confirmed as extraction temperature 80–100 °C, extraction time 1–4 h and ratio of water to raw material 5–15 mL/g, and then Box-Behnken Design (BBD) was conducted to design experimental project. The experiments with different extraction temperature (X_1), extraction time (X_2) and ratio of water to raw material (X_3), were employed simultaneously covering the spectrum of variables for the percentage extraction of IRPS in the BBD. In order to describe the effects of extraction temperature (X_1), extraction time (X_2) and ratio of water to raw material (X_3) on percentage of IRPS extraction, batch experiments were conducted.

As shown in Table 1, the three factors chosen for this study were designated as X_1, X_2, and X_3 prescribed into three levels, coded +1, 0, −1 for high, intermediate and low value, respectively. The coded values of the extraction parameters were determined by the following equation:

$$X_i = \frac{x_i - x_0}{\Delta x}, \quad i = 1, 2, 3 \quad (2)$$

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

The complete quadratic equation used is as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=0}^{3} \beta_{ii} X_i^2 + \sum_{i=0}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad (3)$$

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). And a statistical program in Design Expert software 8.0 was used for regression analysis of the data obtained and to estimate the coefficient of the regression equation. The equations were validated by the statistical tests called the ANOVA analysis. The significance of each term in the equation is to estimate the goodness of fit in each case. Response surfaces were drawn to determine the individual and interactive effects of test variable on the response. Additional confirmation experiments were subsequently conducted to verify the validity of the statistical experimental design.

2.4. ABTS radicals scavenging assay

The radical scavenging activity of IRPS against ABTS was measured using the method with some modifications (Luo, Fan, & Luo,

2011; Zhou et al., 2011). ABTS was dissolved in 0.01 M PBS (pH 7.4) to a 7 mM concentration. $ABTS^+$ was produced by reacting the ABTS stock solution with 2.45 mmol/L potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The ABTS radical cation solution was diluted to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30 °C for 30 min. Each sample (0.2 ml) with various concentrations (0.0125, 0.0500, 0.1000, 0.2000, and 0.3000 mg/mL) was mixed with 2.0 ml of diluted ABTS radical cation solution. After reaction at room temperature for 20 min, the absorbance at 734 nm was measured immediately and recorded. Vitamin C was used as standard. Each sample was measured in triplicate and averaged. This activity is given as percentage $ABTS^+$ scavenging that is calculated by the following formula:

$$\text{ABTS scavenging effect}(\%) = \frac{A_0 - (A_s - A_b)}{A_0} \times 100 \quad (4)$$

where A_0: A_{734} of ABTS without sample, A_s: A_{734} of sample and ABTS and A_b: A_{734} of sample without ABTS.

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

Total RNA was extracted from chicken lymphocytes frozen at 80 °C, and RNA concentration was determined by spectrophotometer. Five micrograms of RNA were reverse-transcribed into cDNA using oligo (dT) primers. PCR products were obtained by amplification cDNA from normal chicken lymphocytes using specific primers as follows: IL-4 (305 bp), 5′-TACTTACAGCTCTCA GTG-3′ and 5′-TTGGTGGAAGAAGGTACGT-3′; IFN-γ (317 bp), 5′-GCTGACGGTGGACCTAT T-3′ and 5′-TCCTCTGAGACTGGCTCCTT-3′; β-actin (280 bp) internal control, 5′-ACCTCGCA CTGGATTTCG-3′ and 5′-TGTCAGCAAT GCCAGGGT-3′. PCR was carried out under the following conditions: 5 min at 95 °C, 1 min denaturation at 95 °C, 1 min annealing at 60 °C, 1 min extension at 72 °C for 40 cycles, with an additional 10 min extension for the last cycle. Relative levels of IL-4 and IFN-γ expression were expressed using the optical density ratio (IL-4/β-actin and IFN-γ/β-actin), as determined by the Bio-Image Analysis system (Bio-Profil Celbio, Milan, Italy).

2.6. Statistical analyses

Statistical analysis was performed using Design-Expert (Version 8.0, Stat-Ease) and SPSS (Version 17.0) statistical software. Significance of difference between two groups was evaluated using Student's *t*-test. For multiple comparisons, one-way analysis of variance (ANOVA) was used.

3. Result and discussion

3.1. Statistical analysis and the model fitting

The design matrix and the corresponding results of RSM experiments to determine the effects of the three independent variables including extraction temperature (X_1), extraction time (X_2), and ratio of water to raw material (X_3) are shown in Table 2. By applying multiple regression analysis on the experimental data, the predicted model was obtained by the following second-order polynomial function:

$$\text{Extraction yield} = 8.61 + 1.22X_1 + 1.20X_2 + 0.57X_3 + 0.19X_1X_2 + 0.14X_1X_3 + 0.052X_2X_3 + 0.36X_1^2 - 0.47X_2^2 - 0.17X_3^2 \quad (5)$$

The above model can be used to predict the extraction yield within the limits of the experimental factors. Fig. 1 shows that the actual response values agree well with the predicted response values.

Table 2
Box-Behnken design matrix (in coded level of three variables) and response values for the extraction yield of IRPS.

Run	Coded variable levels			Extraction yield (%)
	X_1 (temperature)	X_2 (time)	X_3 (ratio)	Experimental values
1	−1	−1	0	6.22
2	1	−1	0	8.30
3	−1	1	0	8.31
4	1	1	0	11.16
5	−1	0	−1	7.20
6	1	0	−1	9.35
7	−1	0	1	7.96
8	1	0	1	10.65
9	0	−1	−1	6.23
10	0	1	−1	8.44
11	0	−1	1	7.39
12	0	1	1	9.81
13	0	0	0	8.59
14	0	0	0	8.62
15	0	0	0	8.65
16	0	0	0	8.65
17	0	0	0	8.51

The significance of each coefficient was determined using the *F*-test and *p*-value in Table 3. The ANOVA of the quadratic regression model demonstrated that the model was highly significant, as was evident from the *F*-test with a very low probability value ($p < 0.0001$). The corresponding variables would be more significant if the absolute *F*-value becomes greater and the *p*-value becomes smaller (Gan & Latiff, 2011a,b; Qiu et al., 2010). The data in Table 3 indicated that the variables with the largest effect were the linear terms of extraction temperature (X_1), extraction time (X_2), and ratio of water to raw material (X_3) and the quadratic term of extraction temperature ($X_1 \times X_1$), and extraction time ($X_2 \times X_2$) ($p < 0.001$). Besides, the interaction effects of extraction temperature and extraction time ($X_1 \times X_2$) and the quadratic term of ratio of water to raw material ($X_3 \times X_3$) were also found significant ($p < 0.01$). Meanwhile, the extraction temperature (X_1) was the major factor affecting the yield. The lack of fit test measures the failure of the model to represent data in experimental domain at points which are not included in the regression. The "lack of fit *F*-value" of 4.164 implied the lack of fit was not significant relative to the pure error.

The total determination coefficient (R^2) was 0.9981, indicating a reasonable fit of the model to the experimental data. In addition

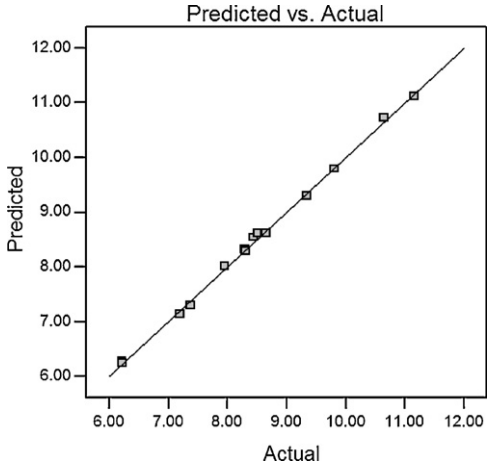

Fig. 1. Predicted response versus actual value.

Table 3
ANOVA for response surface quadratic model.

Variables	Sum of squares	DF	Mean square	*F* value	*p*-Value Prob. > *F*
Model	27.801	9	3.089	412.690	<0.0001[a]
X_1-temperature	11.923	1	11.923	1592.944	<0.0001[a]
X_2-time	11.488	1	11.488	1534.801	<0.0001[a]
X_3-ratio	2.631	1	2.631	351.513	<0.0001[a]
$X_1 \times X_2$	0.150	1	0.150	20.066	0.0029[b]
$X_1 \times X_3$	0.075	1	0.075	10.026	0.0158
$X_2 \times X_3$	0.011	1	0.011	1.434	0.2701
$X_1 \times X_1$	0.539	1	0.539	71.984	<0.0001[a]
$X_2 \times X_2$	0.918	1	0.918	122.706	<0.0001[a]
$X_3 \times X_3$	0.126	1	0.126	16.807	0.0046[b]
Residual	0.052	7	0.007		
Lack of fit	0.040	3	0.013	4.164	0.1009[c]
Pure error	0.013	4	0.003		
Cor total	27.854	16			

[a] Means significance ($p < 0.001$).
[b] Means significance ($p < 0.01$).
[c] Not significant.

the adjusted coefficient of determination ($R^2_{Adj} = 0.9957$) and the coefficient of variation (C.V.% = 1.02%) are shown in Table 4. These values indicated that the accuracy and the general availability of the polynomial model were adequate, and the R^2_{Pred} of 0.9765 was in reasonable agreement with the R^2_{Adj}. The "Adeq. precision" of 73.2957 indicated that this model could be used to navigate the design space.

3.2. Optimization of the process

In this study, the aim of optimization was to find the conditions which give the maximum extraction yield of polysaccharides. 3D response surface and 2D contour plots were the graphical representations of regression function. The optimal values of the selected variables were obtained by solving the regression equation using the Design-Expert software. They showed the type of interactions between two tested variables and the relationship between responses and experiment levels of each variable. Two variables within the experimental range are depicted in 3D surface plots when the third variable is kept constant at zero level and different shapes of the contour plots indicated different interactions between the variables. Fig. 2a and a′ shows the 3D surface plot and the contour plot of the effect of extraction temperature (X_1) and extraction time (X_2) on extraction yield. It can be seen that the extraction temperature (X_1) demonstrated an exponential increase at a range of 80–100 °C on extraction yield. This occurrence might due to the induced cell wall disruption and solubilisation of cell wall materials at higher temperature (Guo, Zou, & Sun, 2010; Luo & Chen, 2010). The effect of extraction time (X_2) also displayed an increase on the response at a range of 1–4 h. It is generally believed that the high extraction yield for polysaccharides is due mainly to the high extraction temperature and the long extraction time (Basedow & Ebert, 1977; Cui, Xu, Shu, Xu, & Tao, 2006; Lorimer, Mason, Cuthbert, & Brookfield, 1995; Ouyang, Zhang, Wang, & Deng, 2004; Shen, Zhu, & Zhang, 2004; Sun, Gu, & Ding, 2006). Fig. 2b and b′ depicts the effect of extraction temperature (X_1) and ratio of water to raw material (X_3) on the extraction yield. It was observed that extraction yield increased with the increase in ratio of water to raw material (X_3). This steady increase in the extraction yield with increasing ratio of water to raw material could be due to the addition of increasing amounts of solvent molecules to the blend which may affect the extent of polysaccharides gelatinization and thus the rheological properties of the raw material (Liang, 2008). Fig. 3c and c′ shows the effect of extraction time (X_2) and ratio of water to raw material (X_3) on the extraction yield. It was observed that extraction yield increased with the increase in ratio of water to raw material (X_3). Also, extraction yield increased with the increase in ratio of water to raw material (X_3). By analyzing the plots, the predicted maximum value (11.17%) of the tested variables for extraction yield, lied in the following condition: extracting temperature (X_1) 99.5 °C, extracting time (X_2) 3.75 h, and ratio of water to raw material (X_3) 11.84. In the optimal conditions, the experimental yield was (11.19 ± 0.04)%, which agreed with the predicted value. Therefore, it confirmed that these conditions were optimal for extraction yield.

3.3. Effect of scavenging ABTS radicals

The ABTS radical cation decoloration assay, which employs a specific absorbance (734 nm) at a wavelength well separated from the visible-light range and requires only a short reaction time, has been widely applied to evaluating the total antioxidative activity in both lipophilic and hydrophilic samples (Wu et al., 2006). As shown in Fig. 3, the ABTS radical was scavenged by IRPS in a concentration dependant manner with the maximum percentage of inhibition 64.3%, closed to that of vitamin C, observed at 0.3 mg/ml. This method, used for the screening of antioxidant activity, is applicable to both lipophilic and hydrophilic antioxidants (Long, Kwee, & Halliwell, 2000). Compared with the results of Shirwaikar, Shirwaikar, Rajendran, and Punitha (2006), the results in Fig. 3 imply that the activity of IRPS may have an appreciable scavenging power on hydroxyl ABTS radicals.

3.4. Effect of IRPS with various concentration on IL-4 and IFN-γ mRNA expression in chicken lymphocytes

IL-4 is a cytokine that induces differentiation of naive helper T cells (Th0 cells) to Th2 cells. Upon activation by IL-4, Th2 cells subsequently produce additional IL-4 (Sokol, Barton, Farr, & Medzhitov,

Table 4
Analysis of variance for the fitted quadratic polynomial model of extraction yield.

Item	Std. dev.	Mean	C.V.%	Press	R^2	R^2_{Adj}	R^2_{Pred}	Adeq. precision
Value	0.0865	8.47	1.02	0.65	0.9981	0.9957	0.9765	73.2957

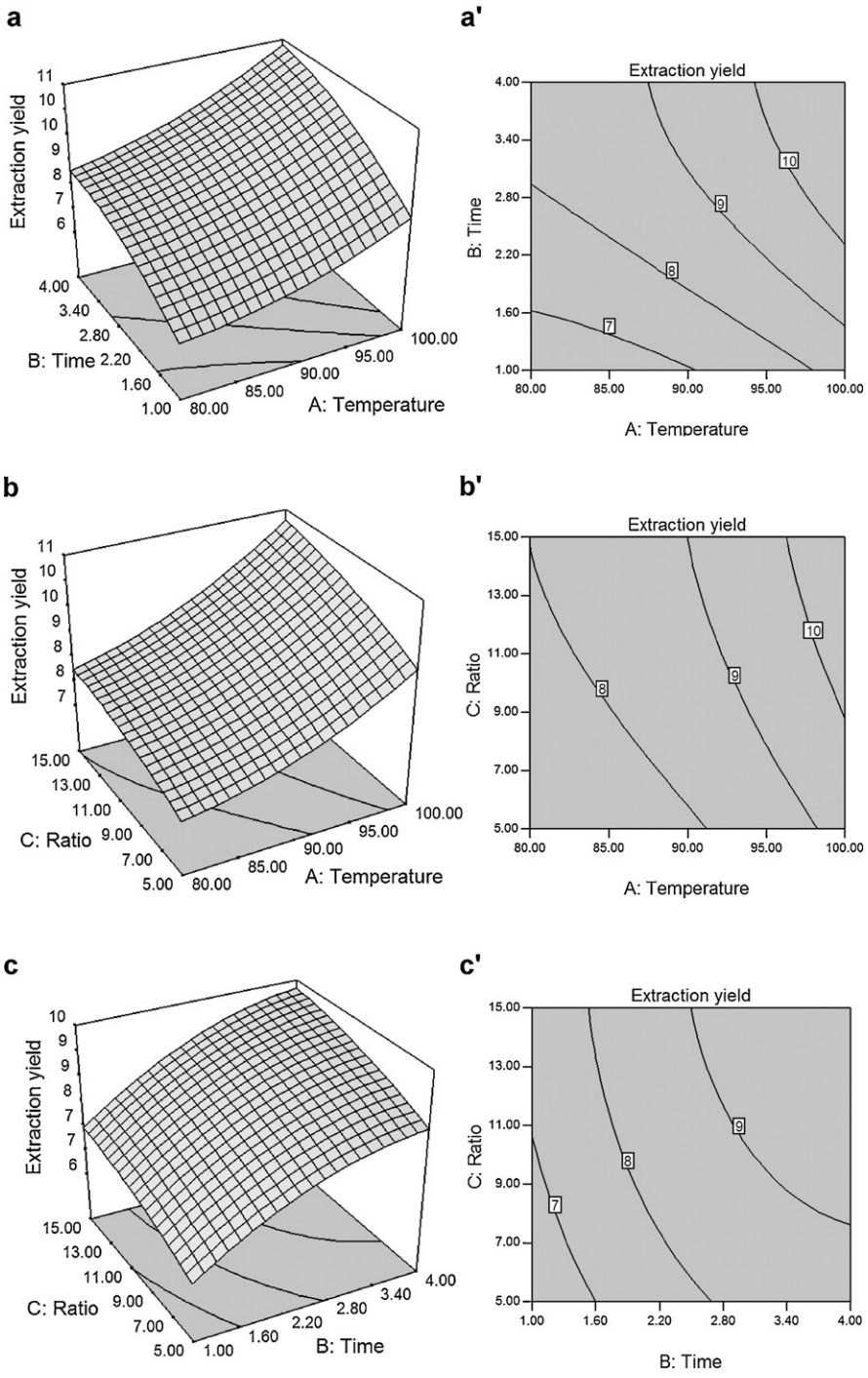

Fig. 2. Response surface plots (a–c) and contour plots (a′–c′) showing the effect of extraction temperature (X_1), extraction time (X_2) and ratio of water to raw material (X_3) on the extraction yield of IRPS.

Table 5
Effect of IRPS with various concentration on IL-4 and IFN-γ mRNA expression in chicken lymphocytes.

Content (μg/mL)	Relative level of IL-4 mRNA expression (IL-4/β-actin)	Promoted effect (%)	Relative level of IFN-γ mRNA expression (IFN-γ/β-actin)	Promoted effect (%)
0	0.110 ± 0.017	–	0.092 ± 0.010	–
50	0.157 ± 0.024[a]	43	0.149 ± 0.022[a]	62
100	0.187 ± 0.028[a]	70	0.198 ± 0.030[a]	115
200	0.171 ± 0.026[a]	55	0.196 ± 0.029[a]	113
300	0.143 ± 0.021[a]	30	0.165 ± 0.025[a]	79
400	0.113 ± 0.017[b]	3	0.134 ± 0.020[a]	46

[a] Means significance ($p < 0.01$) compared with the control group.
[b] Not significant ($p > 0.05$) compared with the control group.

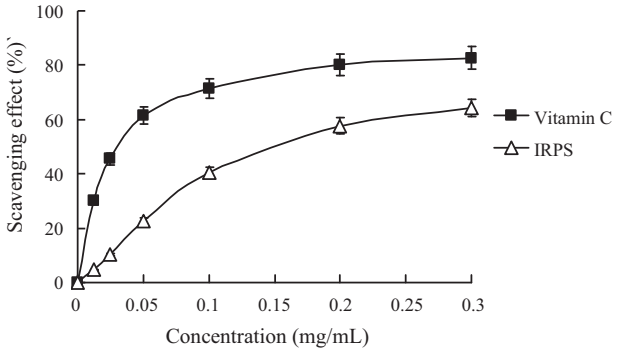

Fig. 3. The scavenging effect of IRPS on ABTS radicals. Results are presented as means ± standard deviations ($n = 3$). Differences are considered to be statistically significant if $p < 0.05$ when compared to control.

2008). And it has many biological roles, including the stimulation of activated B-cell and T-cell proliferation, and the differentiation of CD4+ T-cells into Th2 cells. It is a key regulator in humoral and adaptive immunity (Hershey, Friedrich, Esswein, Thomas, & Chatila, 1997). IFN-γ is a dimerized soluble cytokine that is the only member of the type II class of interferons, which is secreted by Th1 cells, Tc cells and NK cells (Gray & Goeddel, 1982). The importance of IFN-γ in the immune system stems in part from its ability to inhibit viral replication directly, and most importantly from its immunostimulatory and immunomodulatory effects (Schoenborn & Wilson, 2007). In the experiments, chicken lymphocytes were incubated with different concentrations of IRPS (50, 100, 200, 300 and 400 μg/mL) to find out the optimal concentration and the optimal promoted effect. As seen in Table 5, IRPS concentration of 100 μg/mL induced both IL-4 and IFN-γ expression peaks in a similar manner, where there were 0.187 ± 0.028 of IL-4/β-actin with 70% of promoted effect of IL-4 mRNA expression, and 0.198 ± 0.030 of IFN-γ/β-actin with 115% of promoted effect of IFN-γ mRNA expression. Besides, Table 5 shows that IRPS could increase IL-4 and IFN-γ mRNA levels in response to concentrations of 50, 100, 200 and 300 μg/mL as determined with RT-PCR ($p < 0.01$).

4. Conclusion

RSM was used to determine the optimum process parameters that gave a high extraction yield. The coefficient of determination (R^2) for the model equation was 0.9981. And the probability value ($p < 0.0001$) demonstrated a very high significance for the regression models for predicting the responses. By analyzing the second-order polynomial model, a maximum extraction yield 11.17% was obtained under the following condition: extraction temperature of 99.5 °C, extraction time of 3.75 h, and ratio of water to raw material of 11.84. Under this condition, the mean experimental value extraction yield (11.19 ± 0.04)% corresponded well with the predicted value. Free radicals scavenging activities *in vitro* indicated that IRPS have appreciable radical scavenging effects on ABTS radical. On the other hand, IRPS could increase IL-4 and IFN-γ mRNA expression in chicken lymphocytes *in vitro*.

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References

Ahmed, L., Rico, D., Martin-Diana, A. B., & Barry-Ryan, C. (2011). Optimization of application of delactosed whey permeate treatment to extend the shelf life of fresh-cut tomato using response surface methodology. *Journal of Agricultural and Food Chemistry*, *59*, 2377–2385.

Basedow, A. M., & Ebert, K. H. (1977). Ultrasonic degradation of polymers in solution. *Advances in Polymer Science*, *22*, 83–84.

Cen, Q., Zhou, L. L., & Li, T. (2008). Membrane separation technique and its application in the field of traditional Chinese medicine. *Journal of Shenyang Pharmaceutical University*, *25*, 77–80.

Chang, Y. D., Luan, G. H., & Li, L. D. (2008). Determination of contents of indirubin in Xiaoke capsules made from 36 medicinal herbs. *China Tropical Medicine*, *8*, 659–661.

Chen, Q. (2009). Pharmacological action and clinical application of Radix Isatidis. *Chinese Pharmaceutical Affairs*, *23*, 607–608.

Condurso, C., Verzera, A., Romeo, V., Ziino, M., Trozzi, A., & Ragusa, S. (2006). The leaf volatile constituents of *Isatis tinctoria* by solid-phase microextraction and gas chromatography/mass spectrometry. *Planta Medicine*, *72*, 924–928.

Cui, F., Xu, H., Shu, C., Xu, Z., & Tao, W. (2006). Optimization of processing parameters for extraction of soluble *Grifola frondosa* polysaccharides by response surface methodology. *Food Science*, *27*, 142–147.

Cui, S. Y., Xue, Y., Yang, J. L., & Hao, J. (2001). Advantages of Ban Lan Gen. *Chinese Traditional and Herbal Drugs*, *32*, 670–671.

Fernández-Silva, I., Sanmartín, P., Silva, B., Moldes, A., & Prieto, B. (2011). Quantification of phototrophic biomass on rocks: optimization of chlorophyll – A extraction by response surface methodology. *Journal of Industrial Microbiology and Biotechnology*, *38*, 179–188.

Gan, C. Y., Abdul Manaf, N., & 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.

Gan, C. Y., & Latiff, A. A. (2011a). Extraction of antioxidant pectic-polysaccharide from mangosteen (*Garcinia mangostana*) rind: Optimization using response surface methodology. *Carbohydrate Polymers*, *83*, 600–607.

Gan, C. Y., & Latiff, A. A. (2011b). Optimisation of the solvent extraction of bioactive compounds from Parkia speciosa pod using response surface methodology. *Food Chemistry*, *124*, 1277–1283.

Gray, P. W., & Goeddel, D. V. (1982). Structure of the human immune interferon gene. *Nature*, *298*, 859–863.

Guo, X., Zou, X., & Sun, M. (2010). Optimization of extraction process by response surface methodology and preliminary characterization of polysaccharides from *Phellinus igniarius*. *Carbohydrate Polymers*, *80*, 345–350.

Hamburger, M. (2002). *Isatis tinctoria* – From the rediscovery of an ancient medicinal plant towards a novel anti-inflammatory phytopharmaceutical. *Biomedical and Life Sciences*, *1*, 333–344.

Hershey, G. K., Friedrich, M. F., Esswein, L. A., Thomas, M. L., & Chatila, T. A. (1997). The association of atopy with a gain-of-function mutation in the alpha subunit of the interleukin-4 receptor. *New England Journal of Medicine*, *337*, 1720–1725.

Kong, W. J., Zhao, Y. L., Shan, L. M., Xiao, X. H., & Guo, W. Y. (2008). Investigation on the spectrum–effect relationships of EtOAc extract from Radix Isatidis based on HPLC fingerprints and microcalorimetry. *Journal of Chromatography B*, *871*, 109–114.

Li, W., Wang, Z., Sun, Y. S., Chen, L., Han, L. K., & Zheng, Y. N. (2011). Application of response surface methodology to optimise ultrasonic-assisted extraction of four chromones in Radix Saposhnikoviae. *Phytochemical Analysis*, doi:10.1002/pca.1282

Liang, R. J. (2008). Optimization of extraction process of *Glycyrrhiza glabra* polysaccharides by response surface methodology. *Carbohydrate Polymers*, *74*, 858–861.

Liau, B. C., Jong, T. T., Lee, M. R., & Chen, S. S. (2007). LC–APCI–MS method for detection and analysis of tryptanthrin, indigo, and indirubin in Daqingye and Banlangen. *Journal of Pharmaceutical and Biomedical Analysis*, *43*, 346–351.

Liu, Y. H., Ding, S. P., & Lin, A. H. (2001). Study on antiendotoxin of chloroform extract form Radix Isatids. *Chinese Journal of Hospital Pharmacy*, *21*, 326–328.

Liu, X. Q., Luo, X. T., Li, X., & Xiong, Y. X. (2001). Pharmacy advantages of Ban Lan Gen. *Primary Journal of Chinese Materia Medica*, *15*, 43–45.

Long, L. H., Kwee, D. C., & Halliwell, B. (2000). The antioxidant activities of seasonings used in Asian cooking. Powerful antioxidant activity of dark soy sauce revealed using the ABTS assay. *Free Radical Research*, *32*, 181–186.

Lorimer, J. P., Mason, T. J., Cuthbert, T. C., & Brookfield, E. A. (1995). Effect of ultrasound on the degradation of aqueous native dextran. *Ultrasonics Sonochemistry*, *2*, 55–57.

Luo, C., & Chen, Y. S. (2010). Optimization of extraction technology of Se-enriched *Hericium erinaceum* polysaccharides by Box-Behnken statistical design and its inhibition against metal elements loss in skull. *Carbohydrate Polymers*, *82*, 854–860.

Luo, A., Fan, Y., & Luo, A. (2011). In vitro free radicals scavenging activities of polysaccharide from Polygonum Multiflorum Thunb. *Journal of Medicinal Plants Research*, *5*, 966–972.

Maiti, B., Rathore, A., Srivastava, S., Shekhawat, M., & Srivastava, P. (2011). Optimization of process parameters for ethanol production from sugar cane molasses by *Zymomonas mobilis* using response surface methodology and genetic algorithm. *Applied Microbiology and Biotechnology*, *90*, 385–395.

Malcolm, C. E., & Bruce, B. S. (1971). Distribution and variation of indole glucosinolates in Woad (*Isatis tinctoria* L.). *Plant Physiology*, *48*, 498–503.

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.

Oberthür, C., Graf, H., & Hamburger, M. (2004). The content of indigo precursors in *Isatis tinctoria* leaves – A comparative study of selected accessions and post-harvest treatments. *Phytochemistry*, *65*, 3261–3268.

Ouyang, X., Zhang, X., Wang, H., & Deng, Z. (2004). Research on extraction methods of polysaccharide from Agrocybe chaxingu Huang Mycelia. *Edible Fungi of China*, *23*, 35–37.

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.

Peng, J., Fan, G., & Wu, Y. (2005). Isolation and purification of clemastanin B and indigoticoside A from Radix Isatidis by high-speed counter-current chromatography. *Journal of Chromatography A*, *1091*, 89–93.

Qin, Q., Hou, H., Qiu, L., Wu, H., Wei, M., & Pan, Z. (2001). An anti-oxidative activity of a fraction and its subfraction from 'Ban lan gen', root of *Isatis tinctoria* L. *Chinese Journal of Clinical Pharmacy*, *10*, 373–375.

Qiu, Y., Hu, Y. L., Cui, B. A., Zhang, H. Y., Kong, X. F., Wang, D. Y., et al. (2007). Immunopotentiating effects of four Chinese herbal polysaccharides administered at vaccination in chickens. *Immunology, Health, and Disease*, *86*, 2530–2535.

Qiu, L., Zhao, G., Wu, H., Jiang, L., Li, X., & Liu, J. (2010). Investigation of combined effects of independent variables on extraction of pectin from banana peel using response surface methodology. *Carbohydrate Polymers*, *80*, 326–331.

Schoenborn, J. R., & Wilson, C. B. (2007). Regulation of interferon-gamma during innate and adaptive immune responses. *Advances in Immunology*, *96*, 41–101.

Shen, A., Zhu, Z., & Zhang, W. (2004). Studies on the extraction technology of water-soluble polysaccharides from mulberry leaf. *Canye Kexue*, *30*, 277–279.

Shirwaikar, A., Shirwaikar, A., Rajendran, K., & Punitha, I. S. (2006). In vitro antioxidant studies on the benzyl tetra isoquinoline alkaloid berberine. *Biological & Pharmaceutical Bulletin*, *29*, 1906–1910.

Sokol, C. L., Barton, G. M., Farr, A. G., & Medzhitov, R. (2008). A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nature Immunology*, *9*, 310–318.

Sun, F., Gu, W., & Ding, X. (2006). Study on the extraction technology of polysaccharide from Dioscorea opposite Thunb. *Journal of Food Science and Biotechnology*, *25*, 79–83.

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.

Sun, L., Tong, L., & Bi, K. (2002). A study on the quantitative method of Indigo and lndirubin in Fufang Banlangen Keli. *Chinese Journal of Modern Applied Pharmacy*, *19*, 138–140.

Wu, L. C., Hsu, H. W., Chen, Y. C., Chiu, C. C., Lin, Y. I., & Ho, J. A. (2006). Antioxidant and antiproliferative activities of red pitaya. *Food Chemistry*, *95*, 319–327.

Wu, J., & Xie, Y. X. (2006). The studies of chemical ingredients and quality control methods on the roots of *Isatis indigotica*. *LiShiZhen Medicine and Materia Medica Research*, *17*, 2067–2068.

Xu, Y. M., & Lu, P. C. (1991). Experimental studies on immunostimulatory effects of the *Isatis indigotica* polysaccharide. *Zhong Xi Yi Jie He Za Zhi*, *11*, 357–359, 325–326.

Yan, Y., Yu, C., Chen, J., Li, X., Wang, W., & Li, S. (2011). Ultrasonic-assisted extraction optimized by response surface methodology, chemical composition and antioxidant activity of polysaccharides from *Tremella mesenterica*. *Carbohydrate Polymers*, *83*, 217–224.

Yang, Y. J., Yang, Y. M., & Xia, Z. N. (2008). Study on the antibacterial activity of Radix Isatidis injection. *Guide of China Medicine*, *6*, 19–21.

You, L., Regenstein, J. M., & Liu, R. H. (2010). Optimization of hydrolysis conditions for the production of antioxidant peptides from fish gelatin using response surface methodology. *Journal of Food Science*, *75*, C582–587.

Zhang, H. Y., Zhao, X. M., Cui, B. A., Xia, P. A., Qiu, Y., Hu, M., et al. (2007). Effects of IRPS on T subpopulations in the immune response to porcine reproductive and respiratory syndrome attenuated virus vaccine. *Chinese Journal of Immunology*, *23*, 134–137.

Zhong, K., & Wang, Q. (2009). Optimization of ultrasonic extraction of polysaccharides from dried longan pulp using response surface methodology. *Carbohydrate Polymers*, *80*, 19–25.

Zhou, H. C., Lin, Y. M., Li, Y. Y., Li, M., Wei, S. D., Chai, W. M., et al. (2011). Antioxidant properties of polymeric proanthocyanidins from fruit stones and pericarps of *Litchi chinensis* Sonn. *Food Research International*, *44*, 613–620.