Carbohydrate Polymers 111 (2014) 567–575

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Optimization of enzyme assisted extraction of polysaccharides from *Astragalus membranaceus*

Huaguo Chen[a,b], Xin Zhou[a,b,*], Junzeng Zhang[c]

[a] The Research Center for Quality Control of Natural Medicine, Guizhou Normal University, 116 Baoshan North Road, Guiyang, Guizhou 550001, PR China
[b] Key Laboratory for Information System of Mountainous Areas and Protection of Ecological Environment, Guizhou Normal University, 116 Baoshan North Road, Guiyang, Guizhou 550001, PR China
[c] Aquatic and Crop Resource Development, National Research Council Canada, 550 University Avenue, Charlottetown, PE C1A 4P3, Canada

ARTICLE INFO

Article history:
Received 12 March 2014
Received in revised form 10 May 2014
Accepted 16 May 2014
Available online 26 May 2014

Keywords:
Astragalus polysaccharides
Enzyme assisted extraction
Glucose oxidase
Response surface method
Antioxidant activity

ABSTRACT

Astragalus polysaccharide (*APS*) is known to have a variety of pharmacological activities. In the present study, enzyme assisted extraction of *APS* from *Astragalus mongholicus* using various enzymes were examined. Research found that glucose oxidase offered a better performance in enhancement of extraction yields of *APS* than other ones. Glucose oxidase assisted extraction process was further optimized by using response surface method (*RSM*) to obtain maximum yield of crude *APS*. The optimized extraction conditions were as follows: enzyme amount of 3.0%, enzyme treated time of 3.44 d, enzyme treated temperature of 56.9 °C and extraction solvent pH of 7.8. Under these conditions, the experimental yield was 29.96 ± 0.14%, which was well in close agreement with the value (30.19%) predicted by *RSM* model and increased more than 250% compared with none enzyme treated ones. Pharmacological test showed that enzyme assisted *APS* had a better antioxidant activity (about 2 times higher) than none enzyme treated ones.

1. Introduction

Astragalus membranaceus, also known as huáng qí (yellow leader), is a perennial member of the flowering legume (pea) family. It is indigenous to the northern and eastern parts of China and Mongolia where the *Astragalus* root has been used for several thousand years in a variety of herbal mixtures to treat diseases of the heart (Chen et al., 2003; Guo, Peng, & Yang, 1995), liver (Tan, Yin, & Yu, 2001) and kidneys (Meng et al., 2007; Sheng, Chen, Zhao, He, & Nan, 2005), to viral infections (Xiong, Yang, & Su, 1998), cancer (Cheng et al., 2004) and immune disorders (Ahmed, Hou, Battaglia, Picken, & Leehey, 2007). Chinese Medicine considers *Astragalus* herb to be one of the most important herbal tonics and as such it is valued for its ability to strengthen the primary energy of the body which we know as the immune system (Tan & Vanitha, 2004), as well as the metabolic (Mao et al., 2009), respiratory (Shen et al., 2008) and eliminative functions (Cai, Wang, & Chen, 2006). This fact is being increasingly substantiated by western research (Auyeung, Woo, Law, & Ko, 2012; Cui et al., 2003; Hong, Ko, Park, & Chang, 2013).

Astragalus polysaccharides (*APS*), one of biological active ingredients isolated from *A. membranaceus*, was proved to have the function of enhance immunity (Fan, Hu, et al. 2012; Guo et al., 2012), anti-inflammatory (Jiang, Qiu, Yang, He, Smith, & Li, 2010), anti-virus (Jiang, Wu, Gao, Song, & Li, 2010), anti-tumor (Li et al., 2008), antioxidant (Jia, Cao, Xu, Jeney, & Yin, 2012) and delaying senescence (Li et al., 2012). Besides, it is found that *APS* play an important role on the prevention and control of diabetes (Chen, Li, & Yu, 2008) and cardiovascular disease (Cheng et al., 2011; Li, Chen, Wang, Tian, & Zhang, 2009; Li, Chen, Wang, Tian, & Zhang, 2010; Li & Zhang, 2009), with no toxic record in clinic.

APS has attracted much interest because of its multiple important pharmacological actions. So optimizing extraction process to get maximum polysaccharides content is becoming a research focus in recent years (Mu, Zhu, Zhao, Zhou, & Jia, 2009). Enzyme assisted extraction is undoubtedly an emerging technology in the food industry since it offers many advantages such as high extraction yield, lower investment costs and energy requirements, high reproducibility at shorter times, simplified manipulation compared to conventional extraction method (Li et al., 2013; Nagendra Chari, Manasa, Srinivas, & Sowbhagya, 2013a). Thus, enzyme assisted

* Corresponding author at: Key laboratory for Information System of Mountainous Areas and Protection of Ecological Environment, Guizhou Normal University, 116 Baoshan North Road, Guiyang, Guizhou 550001, PR China. Tel.: +86 851 6700 414; fax: +86 851 6700 414.
E-mail address: alice9800@sina.com (X. Zhou).

http://dx.doi.org/10.1016/j.carbpol.2014.05.033
0144-8617/

Table 1
The coded experimental and predicted for *RSM* design using water as solvent.

Run	Enzyme amount (%) – X_1	Enzyme treated time (d) – X_2	Enzyme treated temperature (°C) – X_3	pH value – X_4	Extraction yield (%)	
					Experimental	Predicted
1	−1 (2.5)	0 (3)	−1 (50)	0 (7.5)	15.19	15.34
2	0 (3.0)	−1 (2)	−1 (50)	0 (7.5)	9.14	9.40
3	−1 (2.5)	0 (3)	0 (55)	−1 (7.0)	19.58	19.36
4	1 (3.5)	0 (3)	−1 (50)	0 (7.5)	17.43	17.12
5	0 (3.0)	−1 (2)	0 (55)	1 (8.0)	21.07	21.02
6	−1 (2.5)	0 (3)	1 (60)	0 (7.5)	23.66	24.08
7	0 (3.0)	0 (3)	1 (60)	1 (8.0)	26.95	26.93
8	1 (3.5)	1 (4)	0 (55)	0 (7.5)	26.90	27.12
9	1 (3.5)	0 (3)	0 (55)	−1 (7.0)	21.72	21.87
10	0 (3.0)	1 (4)	1 (60)	0 (7.5)	27.53	27.22
11	0 (3.0)	1 (4)	−1 (50)	0 (7.5)	18.81	18.63
12	−1 (2.5)	0 (3)	0 (55)	1 (8.0)	25.94	25.75
13	1 (3.5)	0 (3)	1 (60)	0 (7.5)	27.66	27.63
14	−1 (2.5)	1 (4)	0 (55)	0 (7.5)	24.09	24.23
15	0 (3.0)	0 (3)	−1 (50)	−1 (7.0)	10.82	10.77
16	0 (3.0)	0 (3)	0 (55)	0 (7.5)	28.28	28.21
17	−1 (2.5)	−1 (2)	0 (55)	0 (7.5)	16.55	16.26
18	0 (3.0)	1 (4)	0 (55)	1 (8.0)	28.89	28.86
19	0 (3.0)	−1 (2)	0 (55)	−1 (7.0)	13.97	14.12
20	1 (3.5)	−1 (2)	0 (55)	0 (7.5)	18.90	18.69
21	0 (3.0)	0 (3)	0 (55)	0 (7.5)	27.94	28.21
22	0 (3.0)	0 (3)	−1 (50)	1 (8.0)	21.76	21.89
23	1 (3.5)	0 (3)	0 (55)	1 (8.0)	28.38	28.56
24	0 (3.0)	1 (4)	0 (55)	−1 (7.0)	22.51	22.68
25	0 (3.0)	0 (3)	1 (60)	−1 (7.0)	25.17	24.97
26	0 (3.0)	0 (3)	0 (55)	0 (7.5)	28.27	28.21
27	0 (3.0)	0 (3)	0 (55)	0 (7.5)	28.24	28.21
28	0 (3.0)	0 (3)	0 (55)	0 (7.5)	28.30	28.21
29	0 (3.0)	−1 (2)	1 (60)	0 (7.5)	19.92	20.06

extraction may be an effective and advisable technique for the extraction of polysaccharides. To the best of our knowledge, there is little report that enzyme-assisted extraction method has been applied to extract polysaccharides from *A. membranaceus*.

Response surface methodology (*RSM*) (Fan, Cao, et al. 2012; Hollebeeck, Borlon, Schneider, Larondelle, & Rogez, 2013), as an effective statistical method, is widely used for optimizing complex processes since it can depict the complete effects of variables, and it is less laborious and time-consuming. In this paper, in order to obtain higher extraction yield and basic technological information for the *APS*, the approach of enzyme assisted extraction was used. *RSM* was employed to further optimize parameters of the influencing factors such as enzyme amount, enzyme treated time, treated temperature and pH value. Furthermore, *DPPH* assay was used to evaluate the antioxidant activity of enzyme-assisted crude *APS* and non-enzyme-assisted crude *APS*.

2. Materials and methods

2.1. Materials and reagents

A. membranaceus sample was collected from Charlottetown, Canada. After being ground by grinder machine, powder sample was passed though 20-mesh sieve, and then it was kept in sealed polyethylene bags at −20 °C until used. Amyloglucosidase, hemicellulase, glucose oxidase, bacterial amylase, fungal amylase were obtained from Bio-Cat Inc. Pectinase and cellulase were purchased from Enzyme Development Corporation. Vinozyme was bought from Sigma–Aldrich (USA). All solvents and chemicals used were of analytical grade.

2.2. Enzyme assisted extraction procedure

Enzyme assisted extraction of crude *APS* from *A. membranaceus* was carried out as detailed below. Calculated amounts of the above 8 enzymes were weighed, and distilled water was added to obtain stock solutions at the concentration of 0.4 mg mL^{-1}, respectively. The stock solutions were further diluted with distilled water and adjusted with acetate buffer to obtain serial solutions with desired concentration and pH value.

A batch of *A. membranaceus* powder (20 g) was homogenized for 2 min to increase the surface area for efficient enzyme treatment and mixed with the above 8 enzyme solutions (enzyme amount ranging from 0.5% to 5.5%), respectively. The mixtures were enzyme-treated in a designed temperature (ranging from 30 to 70 °C), time (ranging from 1 to 7 d), and pH values (ranging from 3.5 to 13.0). After enzyme-treated procedure, the mixtures were added with water to a final volume of 250 ml and extracted by an ultrasound method (Ultrasonic extraction apparatus, BILON-2000CT) for 30 min. The water extraction solutions were separated from insoluble residue through vacuum filter, and concentrated in a rotary evaporator under reduced pressure, and then precipitated by adding 95% ethanol to a final concentration of 80% (v/v) (for 36 h at 4 °C), and the precipitates were collected by centrifugation (6000 rpm, 15 min), washed with pure ethanol, acetone and finally lyophilized to obtain crude *APS*. The extract was dried under reduced pressure at 50 °C until its weight was constant and then was weighted with an electronic balance (AL204, Mettler Toledo Instrument Co. Ltd., Shanghai, China). The percentage polysaccharides extraction yield (%) is calculated as follows:

$$\text{Polysaccharides extraction yield (\%) (w/w)} = \frac{\text{Dried crude polysaccharides weight (g)}}{\text{Powder weight (g)}} \times 100\% \quad (1)$$

2.3. Experimental design

After determining the preliminary range of extraction variables through single-factor test, the response surface methodology

(*RSM*) with the Box–Behnken design (*BBD*) was employed to determine the optimal combination of extraction variables. As shown in Table 1, the ranges and center point values of four independent variables were based on the results of single factor experiments. The four independent variables chosen for this study were ratio of enzyme amount (X_1, %), enzyme treated time (X_2, d), enzyme treated temperature (X_3, °C), and pH value (X_4), while the dependent variable (response variable) was the extraction yield of *APS* (%). Each variable was prescribed into three levels, coded +1, 0 and −1 for high, intermediate and low value, respectively. Experimental data was fitted to the following second-order polynomial model:

$$Y = \sum A_0 + \sum_{i=1}^{k} A_i X_i + \sum_{i=1}^{k} A_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} A_{ij} X_i X_j \quad (2)$$

where Y is the response variable, X_i and X_j are the independent variables ($i \neq j$) affecting the response of Y, A_0, A_i, A_{ii} and A_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively.

2.4. DPPH assay

DPPH assay was used to evaluate the antioxidant capacity of crude polysaccharide from *A. membranaceus* in the present study. The *DPPH* free radical scavenging activity of each extract was determined according to the method described by Della (DellaGreca et al., 2009; Pedrini et al., 1993) with slight modification. In briefly, a 0.1 mM of ethanol *DPPH* solution was prepared. The initial absorbance of the *DPPH* in ethanol was measured at 520 nm and did not change throughout the period of assay. An aliquot (0.1 ml) of each extract (with appropriate dilution if necessary) was mixed with 2.5 ml of ethanol *DPPH* solution and incubated for 25 min at 30 °C in dark. Then the absorbance at 520 nm was measured. The *DPPH* free radical scavenging activity was expressed by IC_{50} value which was the concentration of extracts required to decrease the absorbance at 520 nm by 50%. Measurements were performed in triplicate.

2.5. Statistical analysis

Statistical analysis of the single-factor experimental data was performed with *SPSS* 13.0 software (*SPSS* Inc., Chicago, IL, USA). Stat-Ease Design-Expert 8.0.5 (Trial version, Stat-Ease Inc., Minneanopolis, MN, USA) was used for the experimental design and the regression analysis of experimental data.

The regression coefficients were determined by analysis of variance (*ANOVA*). The statistical significance of the regression coefficient was checked by Student's *t*-test, and the second-order model equation was determined by Fischer's *F*-test at a probability (*P*) of 0.001, 0.01 or 0.05. The adequacy of the model was evaluated by lack of fit, determination coefficient (R^2) and *F*-value obtained from *ANOVA*.

3. Results and discussion

3.1. Selection of enzyme type

It is well-known that plant cell wall consists of a rigid skeleton of cellulose embedded in a gel-like matrix composed of pectic compounds, hemi-cellulose and glycoprotein. Cellulase catalyzes the breakdown of cellulose into glucose, cellobiose and higher glucose polymers. Pectinase has the ability to disintegrate pectic compounds and pectin. Beta-glucosidase breaks the beta-1,4-glycosidic linkages in glycosides (Liang, Zhang, & Cong, 2012; Nagendra Chari et al., 2013a).

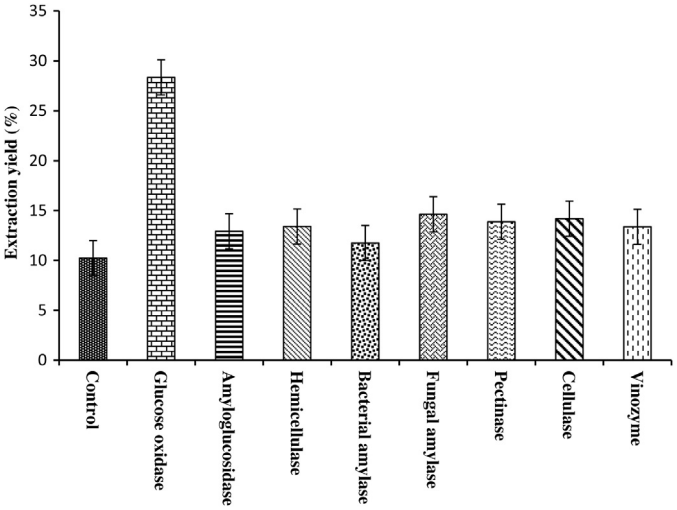

Fig. 1. Effect of different enzymes on extraction yield of *APS*.

In this study, 8 enzymes were tested on *A. membranaceus* root material. As shown in Fig. 1, the effect of different enzymes on *APS* yield varies. Some enzymes were having no effect, while others were found to have weak to strong activities. Glucose oxidase had the best performance among the enzymes tested. Zhu et al. (2014) reported an enzyme-assisted extraction method (cellulose:pectinase:trypsin the ratio of 2:2:1) for extracting polysaccharides from the fruits of *Hericium erinaceus*, and polysaccharides extraction yield increased by 67.62% when compared to hot water extraction. Zhang, Jia, Liu, Wu, and Ran (2011) used cellulase and papain to extract *Lycium barbarum* polysaccharide and obtained better result. In the present study, glucose oxidase was found to have the ability increasing extraction yield of *APS* at least 150% when compared to none enzyme treated ones. Thus, glucose oxidase was chosen as the optimal enzyme in the present work.

3.2. Effect of enzyme amount on yield of APS

Enzyme amount is an important factor to be considered. Too small, polysaccharides in raw material cannot be completely enzyme treated. Too high, it will cause high process cost (Narukawa, Hioki, & Chiba, 2012). In this study, the effect of different amount of enzyme used on *APS* content in the extracts was investigated. As shown in Fig. 2a, increase in enzyme (from 0.5% to 3.0%) used could improve *APS* extraction, and the increase leveled off at ratio of 3.0%. This result is in agreement with reports of other authors in extracting polysaccharides (Zhang et al., 2011; Zhu et al., 2014). Therefore an optimal ratio of 3.0% was chosen in this work.

3.3. Effect of enzyme treated time on yield of APS

Treatment time is also an important factor for *APS* extraction from plant materials. It will have an effect on the final concentration of *APS* in the extract, the efficiency of extraction and the energy cost (Jiao et al., 2012). In the present study, experimental results showed that *APS* content in extract increased with the increase of treatment time from 1 d to 3 d (Fig. 2b). Longer than 3 days, *APS* level was not found to further increase. It is reported that a long enzyme treatment time favors the production of polysaccharides (Hakala, Liitia & Suurnakki, 2013). Yin, You, and Jiang (2011) reported that the extraction yield of *Tricholoma matsutake* polysaccharides increased as the extraction time ascended from 1 to 3 h, the maximum yield of polysaccharides (6.82%) was observed when the extraction time was 3 h, after this point, the extraction yield

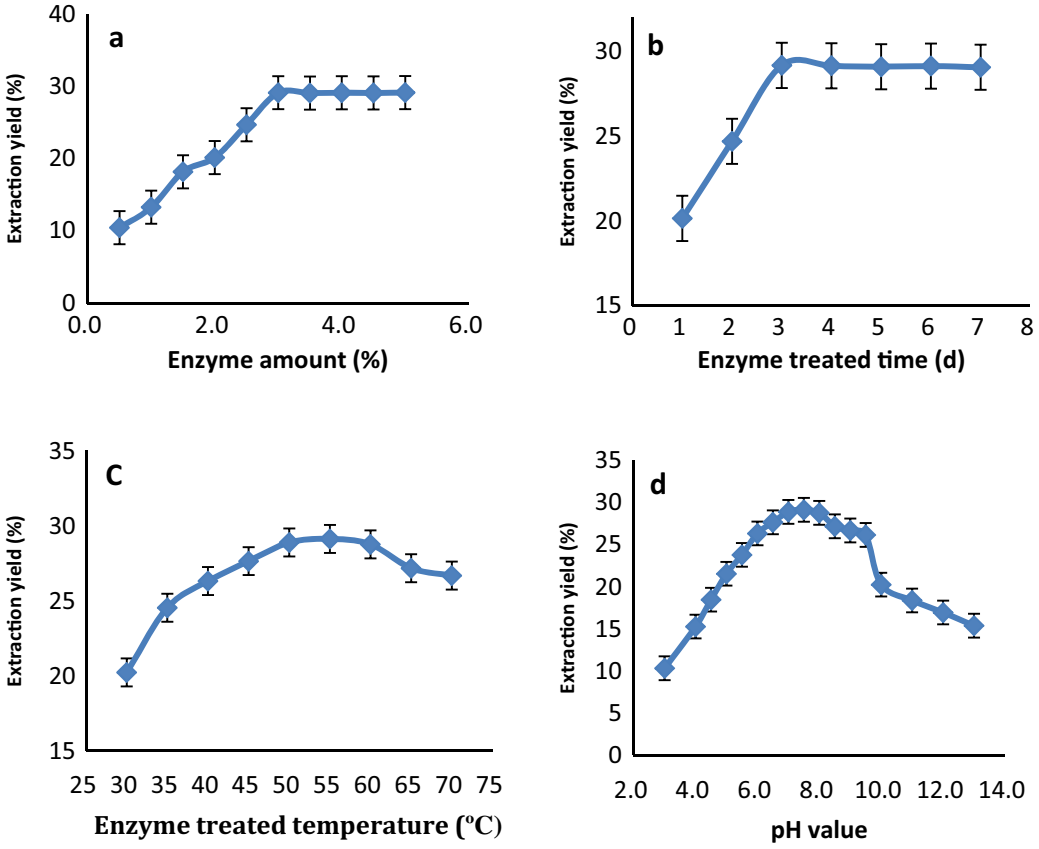

Fig. 2. Effects of different enzyme amount (a), enzyme treated time (b), enzyme-treated temperature (c) and pH value (d) on extraction yield of *APS*.

of polysaccharides started to maintain a dynamic equilibrium with the increasing of the extraction time, and no longer increased when the extraction time exceeded 3 h. This phenomenon maybe due to partial of the polysaccharide was hydrolyzed under some temperature and long extraction time. Thus, the treatment time chosen for *APS* extraction was 3 d in the present study.

3.4. Effect of enzyme treated temperature on yield of APS

Enzyme has an optimal working temperature (Nagendra Chari, Manasa, Srinivas, & Sowbhagya, 2013b), this also applies to the use of glucose oxidase for *APS* extraction. To investigate the effect of temperature on extraction of *APS*, various temperature points within 30–70 °C were tested in the present study, while keeping the treatment time and enzyme amount constant at 3 d and 3.0%, respectively. As shown in Fig. 2c, an increase of *APS* content in extract was observed with the elevation of temperature from 30 °C to 45 °C, and peaked at around 55 °C. Further increase of temperature, however, resulted in decreasing *APS* content. This result is in agreement with reports of other authors in extracting polysaccharides (Wang, Dong, & Tong, 2013; Yin et al., 2011). The increase of the polysaccharides diffusion coefficient and the enhanced solubility of the polysaccharides in the extracting solvent at higher temperatures caused the increase of the polysaccharides mass going out from the mushroom particles into the solution (Samavati, 2013). When temperature further increasing, the enzyme activity will gradually lose (Zhang et al., 2011). Thus, the optimal temperature was determined to be at 55 °C in this work.

3.5. Effect of pH value on yield of APS

To investigate the effect of pH value on extraction yield of *APS*, a pH range from 3.5 to 13.0 was tested while the temperature, treatment time and enzyme amount were kept at 55 °C, 3 d and 3%, respectively. An increase of *APS* content was observed with the increase of pH from 3.0 to 6.5, reaching the highest at pH 7.5, but further increase of pH resulted in decreasing *APS* content (Fig. 2d). Yin et al. (2011) reported that pH value could affect enzyme activity, different enzymes had their own optimal pH values. This might due to the changing of pH affected the spatial structure of enzyme, thus altered the enzyme conformation, enzymatic activity. Therefore, pH 7.5 was chosen as the optimal one based on the study.

3.6. Optimization of extraction parameters of APS

3.6.1. Statistical analysis and the model fitting

The *BBD* in the experimental design consisted of four factors, three levels and five replicates at the center point (Table 1), and the five center point runs were carried out to measure the process stability and inherent variability. The experimental conditions and the results of 29 runs with *BBD* design were presented in Table 1, and the results were means of triplicate tests. As shown in Table 1, the extraction yield of *APS* values (%) varied from 9.14% to 28.89%.

The results of extraction yield affected by enzyme amount, enzyme treated time, enzyme treated temperature and pH value were fitted with a second order polynomial equation, and the values of regression coefficients were calculated.

The effects of four variables were highly significant on extraction yield of *APS* (Table 2). The extraction yield value could be expressed by the following second order polynomial equations:

$$Y = 28.21 + 1.33X_1 + 4.10X_2 + 4.81X_3 + 3.27X_4 + 0.12X_1X_2 + 0.44X_1X_3 + 0.075X_1X_4 - 0.51X_2X_3 - 0.18X_2X_4 - 2.29X_3X_4 - 2.2X_1^2 - 4.42X_2^2 - 4.95X_3^2 - 2.11X_4^2 \quad (3)$$

Table 2
ANOVA for the effects of enzyme amount (X_1), enzyme treated time (X_2), enzyme treated temperature (X_3) and pH value (X_4) on the extraction yield of *APS* with water as solvent using predicted polynomial models.

Source	Sum of squares	*Df*	Mean square	*F*-value	*P*-value	Significant [a]
Model	898.29	14	64.16	854.44	<0.0001	Significant
X_1	21.28	1	21.28	283.37	<0.0001	***
X_2	201.55	1	201.55	2684.04	<0.0001	***
X_3	277.82	1	277.82	3699.69	<0.0001	***
X_4	128.18	1	128.18	1706.97	<0.0001	***
X_1X_2	0.05	1	0.05	0.70	0.4154	
X_1X_3	0.77	1	0.77	10.31	0.0063	**
X_1X_4	0.02	1	0.02	0.29	0.5927	
X_2X_3	1.06	1	1.06	14.12	0.0021	**
X_2X_4	0.12	1	0.12	1.72	0.2101	
X_3X_4	20.97	1	20.97	279.33	<0.0001	***
X_1^2	31.64	1	31.64	421.43	<0.0001	***
X_2^2	126.94	1	126.94	1690.44	<0.0001	***
X_3^2	159.18	1	159.18	2119.75	<0.0001	***
X_4^2	28.98	1	28.98	385.96	<0.0001	***
Residual	1.05	14	0.07			
Lack of fit	0.96	10	0.09	4.25	0.96	Not significant
Pure error	0.09	4	0.022			
Cor total	899.35	28				
R^2	0.9988					
Adj. R^2	0.9977					
Pred. R^2	0.9937					
Adequate precision	98.3672					

[a] **Significant at $P < 0.01$, ***Significant at $P < 0.001$.

The predicted values of extraction yield based on the above model were shown in Table 1.

The statistical significance of regression equation was checked by *F*-test, and *ANOVA* for response surface quadratic polynomial model were summarized in Table 2. The results of high model *F*-value (854.44) and low *P*-value ($P < 0.0001$) indicate that the models were highly significant. The determination coefficient (R^2) for model (0.9988) was close to 1.0, which represented the satisfactory correlation between actual and predicted values. The adjusted R^2 (Adj. R^2) value was 0.9977, which meant most variation (>99%) of the extraction yield could be predicted by the model, and less than 1% variations could not be explained by the models.

The lack-of-fit measured the failure of the model to represent the data in the experimental domain at points which are not included in the regression. The *F*-value of 4.25 and *P*-value of 0.96 for extraction yield implied that the lack of fit was insignificant relative to the pure error due to noise. Adequate precision compared the range of the predicted values at the design points to the average prediction error. Ratio greater than 4 indicated adequate model discrimination. In this research, the values were well above 4.

The *P*-values were used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interactions between the variables. The smaller was the value of *P*, the more significant was the corresponding coefficient. It can be seen from Table 2 that linear coefficients (X_1, X_2, X_3, X_4), quadratic term coefficient ($X_1^2, X_2^2, X_3^2, X_4^2$) and cross product coefficients (X_3X_4) were significant with very small *P* values ($P < 0.001$). Cross product coefficients of X_1X_3 and X_2X_3 were also significant ($P < 0.01$). The other term coefficients were not significant ($P > 0.05$).

3.6.2. Analysis of response surfaces

The *3D* response surface and *2D* contour plots are the graphical representations of regression equation. They provide a method to visualize the relationship between responses and experimental levels of each variable and the type of interactions between two test variables. The shapes of the contour plots, circular or elliptical, indicate whether the mutual interactions between the variables are significant or not. Circular contour plot indicates that the interactions between the corresponding variables are negligible, while elliptical contour plot indicates that the interactions between the corresponding variables are significant. In this study, the results of extraction yield of *APS* affected by enzyme amount, enzyme treated time, enzyme treated temperature and pH value is presented in Figs. 3 and 4.

Figs. 3a and 4a, which give the extraction yield of *APS* as a function of enzyme amount and enzyme treated time at fixed enzyme treated temperature (55 °C) and extraction pH (7.5), indicated that the extraction yield increased rapidly with increase of enzyme treated time from 2 d to 3 d, and increased with the increase of enzyme amount from 2.5 to 3.0. However, with the enzyme treated time increased from 3 d to 4 d and enzyme amount from 3.0 to 3.5, the extraction yield only has a slight increase. A similar trend has been reported for *Tremella fuciformis* polysaccharides (Chen, 2010), oligosaccharides and polymeric xylan (Hakala et al., 2013) and *L. barbarum* polysaccharides (Zhang et al., 2011).

The extraction yield of *APS* affected by different enzyme amount and enzyme treated temperature was seen in Figs. 3b and 4b, when the enzyme treated time and pH value were fixed at 3 d and 7.5, respectively. Enzyme amount and enzyme treated temperature had a positive impact on the extraction yield of *APS*. From two figures, we can conclude that the extraction yield of *APS* increased with the enzyme amount from 2.5 to 3.1, and but beyond 3.1, extraction yield of *APS* reached the plateau region where the yield was maximized and did not further increase the yield. The extraction yield of *APS* was found to increase rapidly with increase of enzyme treated temperature from 50 °C to 56 °C. It can be seen that the maximum extraction yield of *APS* can be achieved when enzyme amount and extraction temperature are around 3.1 and 56 °C, respectively. Samavati (2013), Wang et al. (2013) and Yin et al. (2011) reported the similar trend on extraction of plant polysaccharides.

Figs. 3c and 4c showed the *3D* response surface plot and the contour plot at varying enzyme amount and pH value at fixed enzyme treated time and enzyme treated temperature. It indicated that the maximum extraction yield of *APS* can be achieved when enzyme amount and pH value at the threshold level of around 3.1% and 7.6%, respectively.

Figs. 3d and 4d illustrated the *3D* response surface plot and the contour plot at varying enzyme treated time and enzyme treated temperature at fixed enzyme amount (3.0%) and pH value (7.5).

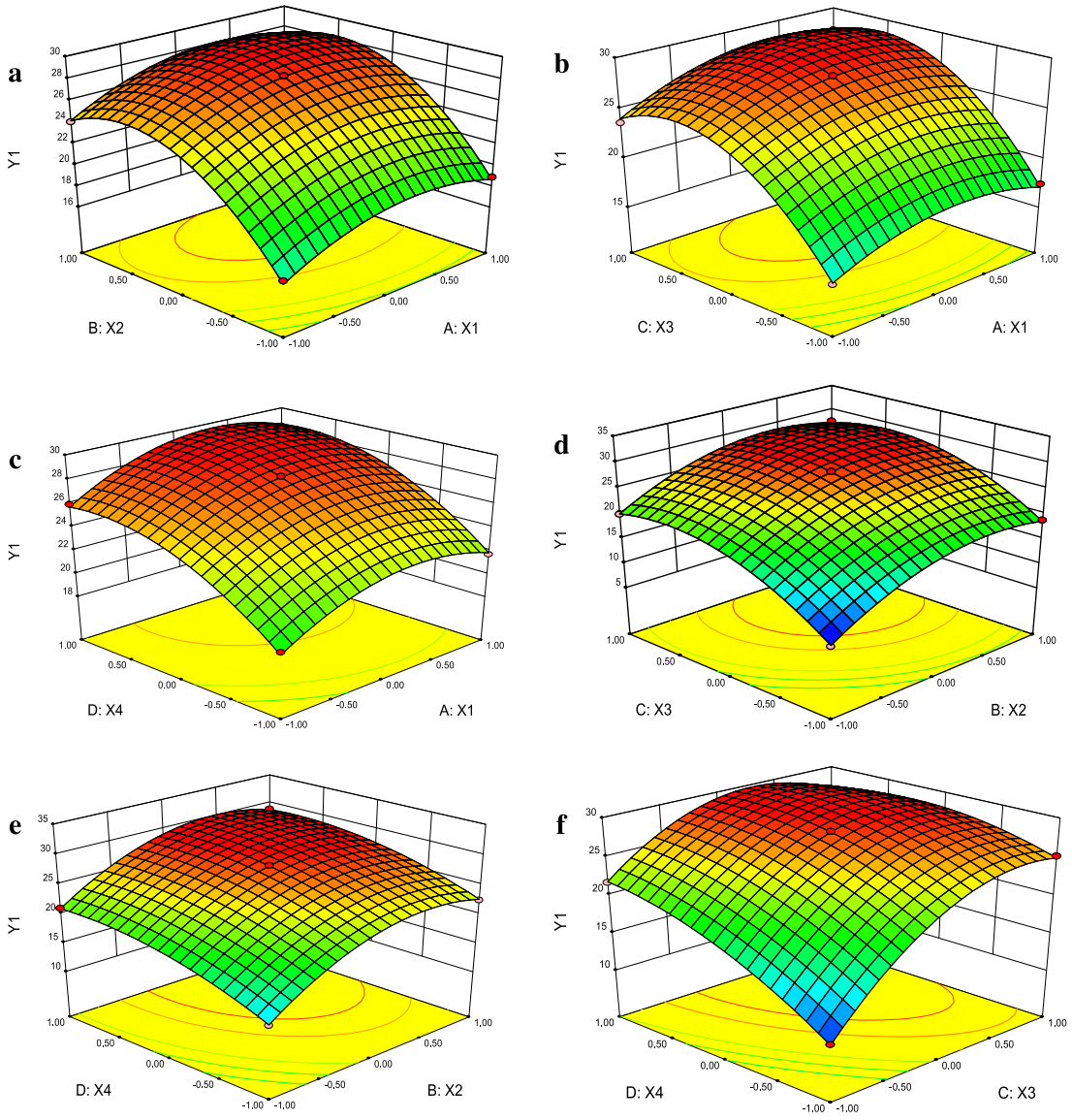

Fig. 3. Response surface (*3D*) showing the effect of enzyme amount (X_1), enzyme treated time (X_2), enzyme treated temperature (X_3) and pH value (X_4) on extraction yield of *APS*.

The extraction yield of *APS* increased with the increasing enzyme treated time from 2 to 3.2 d and reached the maximum value when the extraction temperature at the threshold level of 55 °C. So, extraction yield of *APS* increased with simultaneously increasing enzyme treated time and enzyme treated temperature.

In Figs. 3e and 4e, when the *3D* response surface plot and the contour plot were developed for the extraction yield of *APS* with varying enzyme treated time and pH value at fixed enzyme amount (3.0%) and enzyme treated temperature (55 °C). The yield increased rapidly with the enzyme treated time.

The extraction yield of APS increased with the increasing enzyme treated time from 2 to 3 d, and reached the maximum value rapidly at an extraction time around 3.1 d, but beyond this time, extraction yield of *APS* did not further increase.

The *3D* response surface plot and the contour plot based on the independent variable enzyme treated temperature and pH value were shown in Figs. 3f and 4f, while the other two independent variables, enzyme amount and enzyme treated time were kept at 3.0% and 3 d, respectively. An increase in the extraction yield of *APS* could be significantly achieved with the increasing of enzyme treated temperature. At higher temperatures, the viscosity of mucilage, decreases and makes the slurry less sticky. As a result, the mucilage can be easily released and the extraction yield increases (Zhang et al., 2011). In addition, different enzymes had their own optimal temperature range (Yin et al., 2011). It was observed that the extraction yield of *APS* increased with the enzyme treated temperature from 50 to 57 °C, meaning that further increase of enzyme treated temperature would not increase the extraction yield of *APS* any longer.

3.7. Verification of predictive model

Response surface optimization is more advantageous than the traditional single parameter optimization in that it saves time, space and raw material. In order to validate the adequacy of the model equations, verification experiment was carried out under the optimal conditions: enzyme amount 3.0%, enzyme treated time 3.44 d, enzyme treated temperature 56.9 and pH value 7.8. Good agreement must exist between the values predicted using model equations and the experimental values at the points of interest. To ensure the predicted result was not biased toward the practical value, experimental rechecking was performed using this deduced

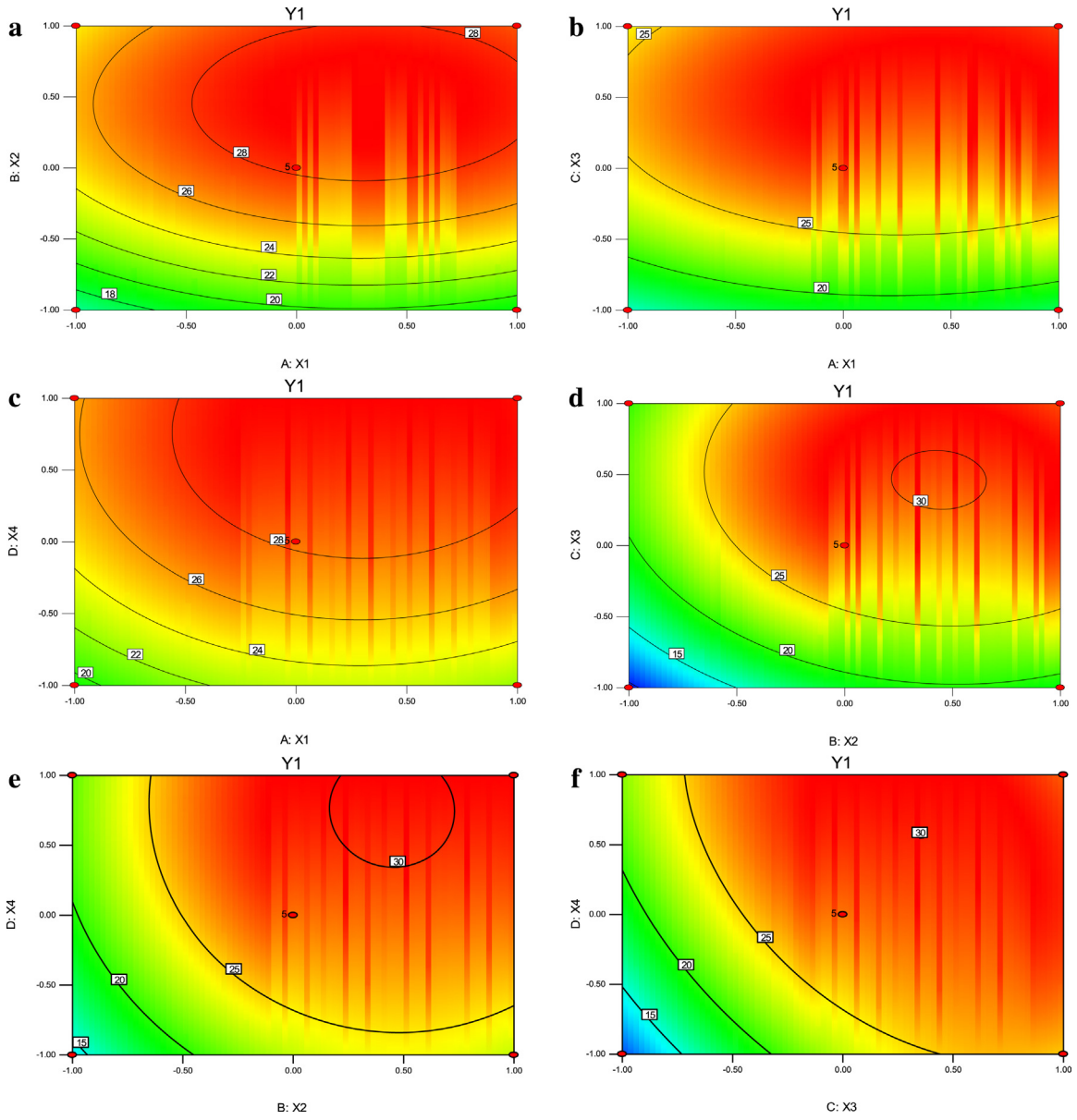

Fig. 4. Contour plots showing the effect of enzyme amount (X_1), enzyme treated time (X_2), enzyme treated temperature (X_3) and pH value (X_4) on extraction yield of *APS*.

optimal condition. This set of conditions was determined to be optimal by the *RSM* optimization approach and was also used to validate experimentally and predict the values of the response using the model equation. A mean value of 29.96 ± 0.14% (n = 5), obtained from real experiments, demonstrated the validation of *RSM* model. The validation result revealed that there was no significant difference between experimental and predicted values, suggesting that the response model was adequate for reflecting the expected

Table 3
Result of model validation experiments.

No.	Optimum conditions				Extraction yield of *APS* (%)	
	Enzyme amount (%)	Enzyme treated time (d)	Enzyme treated temperature (°C)	pH value	Experimental	Predicted
1	3.0	3.44	56.9	7.8	30.12	30.19
2	3.0	3.44	56.9	7.8	29.95	30.19
3	3.0	3.44	56.9	7.8	29.98	30.19
4	3.0	3.44	56.9	7.8	30.01	30.19
5	3.0	3.44	56.9	7.8	29.74	30.19
Average					29.96	30.19
6	0	3.44	56.9	7.8	11.21	
7	0	3.44	56.9	7.8	11.13	
8	0	3.44	56.9	7.8	11.11	
Average					11.15	

Table 4
Results of antioxidant ability for *APS* ($n = 3$).

No.	Blank samples (containing glucose without *APS*) (%)	IC_{50} ($\mu g\,ml^{-1}$)	*APS* extract purity (%) (Enzyme treated)	IC_{50} ($\mu g\,ml^{-1}$)	*APS* extract purity (%) (None enzyme treated)	IC_{50} ($\mu g\,ml^{-1}$)
1	0.5	–	30.03	1061.36 ± 36.29	30.25	2132.44 ± 40.31
2	1.0	–	35.67	843.54 ± 23.12	35.09	1729.26 ± 38.15
3	1.5	–	39.31	750.80 ± 20.26	38.73	1576.68 ± 36.53
4	2.0	–	42.59	678.36 ± 18.19	42.01	1492.39 ± 32.91
5	2.5	–	48.53	566.76 ± 20.14	47.95	1161.86 ± 29.17
6	3.0	–	61.08	421.82 ± 19.32	60.50	911.13 ± 25.38
7	3.5	–	72.43	290.05 ± 13.29	71.85	635.20 ± 19.35
8	4.0	–	75.11	224.35 ± 10.18	74.53	464.46 ± 18.76
9	4.5	–	85.52	152.69 ± 8.69	84.94	320.65 ± 14.52
10	5.0	–	92.72	91.59 ± 7.62	92.18	190.05 ± 21.15

optimization (Table 3). This result of analysis indicated that the experimental values were good agreement with the predicted ones, and also suggested that the model of Eq. (3) is satisfactory and accurate.

Furthermore, none enzyme treated process conditions were compared with the above optimized ones, as seen in Table 3, a mean value of extraction yield 11.15 ± 0.05% ($n = 3$) obtained from the none enzyme treated samples, extraction yield of enzyme treated condition increased 269% compared with the none enzyme treated ones.

3.8. Analysis of antioxidant capacity for APS

DPPH assay was used to evaluate the antioxidant capacity of different purity enzyme assisted extract of *APS* (purity from 30.03% to 92.72%) from *Astragalus mongholicus* in the present study. At the meanwhile, the antioxidant capacity of none enzyme treated extract of *APS* (purity from 30.25% to 92.18%) was also been compared. As shown in Table 4, the blank samples (containing glucose oxidase without *APS*) were without the ability of scavenging free radicals. However, for enzyme treated *APS* samples, with the purity of *APS* increasing, the IC_{50} for scavenging activity on DPPH free radical was decreasing, from 1061.36 ± 36.29 $\mu g\,ml^{-1}$ to 91.59 ± 7.62 $\mu g\,ml^{-1}$ for enzyme assisted extract. Compared with that of none enzyme treated extract (2132.44 ± 40.31 $\mu g\,ml^{-1}$ to 190.05 ± 21.15 $\mu g\,ml^{-1}$), which revealed that enzyme assisted extract of *APS* has a better antioxidant ability (almost 2 times higher) than that of none enzyme treated *APS* extract.

4. Conclusions

In the present study, 8 commercial enzymes were screened for the pre-extraction treatment of *A. mongholicus*, and glucose oxidase was found to be the most effective one for improving *Astragalus* polysaccharides yield. *RSM* was used to further optimize the optimum condition for extraction of crude *APS*. The coefficients of determination (0.9988) and the probability value ($P < 0.0001$) for the regression model demonstrate a very high significance for predicting the responses. In the case of water as solvent, optimal extraction conditions for crude *APS* are obtained as follows: enzyme amount 3.0%, enzyme treated time 3.44 d, enzyme treated temperature 56.9 and pH value 7.8. Under this condition, the mean experimental value of extraction yield (29.96 ± 0.14%) was achieved, which corresponds well with the predicted values and increased more than 250% compared with the none enzyme treated ones. Pharmacological test showed that enzyme assisted *APS* had a better antioxidant activity (about 2 times higher) than that of none enzyme treated ones.

Acknowledgement

The authors gratefully acknowledge the financial support of the present work by the Guizhou Province Science and Technology Plan projects (Nos. [2012]7024, [2012101]3-4, ZY[2011]3013 and [2011]4008).

References

Ahmed, M. S., Hou, S. H., Battaglia, M. C., Picken, M. M., & Leehey, D. J. (2007). Treatment of idiopathic membranous nephropathy with the herb *Astragalus membranaceus*. *American Journal of Kidney Diseases*, *50*(6), 1028–1032.

Auyeung, K. K., Woo, P. K., Law, P. C., & Ko, J. K. (2012). *Astragalus* saponins modulate cell invasiveness and angiogenesis in human gastric adenocarcinoma cells. *Journal of Ethnopharmacology*, *141*(2), 635–641.

Cai, X. F., Wang, Z. L., & Chen, L. F. (2006). Experimental treatment of chronic pelvic inflammatory disease in rats with acupoint injection of *Astragalus* parenteral solution. *Zhejiang Da Xue Xue Bao Yi Xue Ban*, *35*(4), 430–434, 439.

Chen, B. (2010). Optimization of extraction of *Tremella fuciformis* polysaccharides and its antioxidant and antitumour activities in vitro. *Carbohydrate Polymers*, *81*(2), 420–424.

Chen, J. X., Wang, S. R., Zhao, M. J., Lu, X. Y., Zhang, Z., & Qin, Y. (2003). Effect of different drug dosage to activate blood circulation and to nourish qi on cardiac function and structure of congestive heart failure rats after acute myocardial infarction. *Zhongguo Zhong Yao Za Zhi*, *28*(5), 446–449.

Chen, W., Li, Y., & Yu, M. (2008). *Astragalus* polysaccharides: An effective treatment for diabetes prevention in NOD mice. *Experimental and Clinical Endocrinology and Diabetes*, *116*(8), 468–474.

Cheng, X. D., Hou, C. H., Zhang, X. J., Xie, H. Y., Zhou, W. Y., Yang, L., et al. (2004). Effects of Huangqi (Hex) on inducing cell differentiation and cell death in K562 and HEL cells. *Acta Biochimica et Biophysica Sinica (Shanghai)*, *36*(3), 211–217.

Cheng, Y., Tang, K., Wu, S., Liu, L., Qiang, C., Lin, X., et al. (2011). *Astragalus* polysaccharides lowers plasma cholesterol through mechanisms distinct from statins. *PLoS ONE*, *6*(11), e27437.

Cui, R., He, J., Wang, B., Zhang, F., Chen, G., Yin, S., et al. (2003). Suppressive effect of *Astragalus membranaceus* Bunge on chemical hepatocarcinogenesis in rats. *Cancer Chemotherapy and Pharmacology*, *51*(1), 75–80.

DellaGreca, M., Cutillo, F., D'Abrosca, B., Fiorentino, A., Pacifico, S., & Zarrelli, A. (2009). Antioxidant and radical scavenging properties of *Malva sylvestris*. *Natural Product Communications*, *4*(7), 893–896.

Fan, J. P., Cao, J., Zhang, X. H., Huang, J. Z., Kong, T., Tong, S., et al. (2012). Optimization of ionic liquid based ultrasonic assisted extraction of puerarin from Radix Puerariae Lobatae by response surface methodology. *Food Chemistry*, *135*(4), 2299–2306.

Fan, Y., Hu, Y., Wang, D., Liu, J., Zhang, J., Zhao, X., et al. (2012). Effects of *Astragalus* polysaccharide liposome on lymphocyte proliferation in vitro and adjuvanticity in vivo. *Carbohydrate Polymers*, *88*(1), 68–74.

Guo, Q., Peng, T. Q., & Yang, Y. Z. (1995). Effect of *Astragalus membranaceus* on Ca2+ influx and coxsackie virus B3 RNA replication in cultured neonatal rat heart cells. *Zhongguo Zhong Xi Yi Jie He Za Zhi*, *15*(8), 483–485.

Guo, L., Liu, J., Hu, Y., Wang, D., Li, Z., Zhang, J., et al. (2012). *Astragalus* polysaccharide and sulfated epimedium polysaccharide synergistically resist the immunosuppression. *Carbohydrate Polymers*, *90*(2), 1055–1060.

Hakala, T. K., Liitia, T., & Suurnakki, A. (2013). Enzyme-aided alkaline extraction of oligosaccharides and polymeric xylan from hardwood kraft pulp. *Carbohydrate Polymers*, *93*(1), 102–108.

Hollebeeck, S., Borlon, F., Schneider, Y. J., Larondelle, Y., & Rogez, H. (2013). Development of a standardised human in vitro digestion protocol based on macronutrient digestion using response surface methodology. *Food Chemistry*, *138*(2-3), 1936–1944.

Hong, M. J., Ko, E. B., Park, S. K., & Chang, M. S. (2013). Inhibitory effect of *Astragalus membranaceus* root on matrix metalloproteinase-1 collagenase expression and procollagen destruction in ultraviolet B-irradiated human dermal

fibroblasts by suppressing nuclear factor kappa-B activity. *Journal of Pharmacy and Pharmacology*, *65*(1), 142–148.
Jia, R., Cao, L., Xu, P., Jeney, G., & Yin, G. (2012). In vitro and in vivo hepatoprotective and antioxidant effects of *Astragalus* polysaccharides against carbon tetrachloride-induced hepatocyte damage in common carp (*Cyprinus carpio*). *Fish Physiology and Biochemistry*, *38*(3), 871–881.
Jiang, J., Wu, C., Gao, H., Song, J., & Li, H. (2010). Effects of *Astragalus* polysaccharides on immunologic function of erythrocyte in chickens infected with infectious bursa disease virus. *Vaccine*, *28*(34), 5614–5616.
Jiang, J. B., Qiu, J. D., Yang, L. H., He, J. P., Smith, G. W., & Li, H. Q. (2010). Therapeutic effects of *Astragalus* polysaccharides on inflammation and synovial apoptosis in rats with adjuvant-induced arthritis. *International Journal of Rheumatic Diseases*, *13*(4), 396–405.
Jiao, J., Fu, Y.-J., Zu, Y.-G., Luo, M., Wang, W., Zhang, L., et al. (2012). Enzyme-assisted microwave hydro-distillation essential oil from *Fructus forsythia*, chemical constituents, and its antimicrobial and antioxidant activities. *Food Chemistry*, *134*(1), 235–243.
Li, S.-g., & Zhang, Y.-q. (2009). Characterization and renal protective effect of a polysaccharide from *Astragalus membranaceus*. *Carbohydrate Polymers*, *78*(2), 343–348.
Li, J., Bao, Y., Lam, W., Li, W., Lu, F., Zhu, X., et al. (2008). Immunoregulatory and antitumor effects of polysaccharopeptide and *Astragalus* polysaccharides on tumor-bearing mice. *Immunopharmacology and Immunotoxicology*, *30*(4), 771–782.
Li, R., Chen, W.-C., Wang, W.-P., Tian, W.-Y., & Zhang, X.-G. (2009). Extraction, characterization of *Astragalus* polysaccharides and its immune modulating activities in rats with gastric cancer. *Carbohydrate Polymers*, *78*(4), 738–742.
Li, R., Chen, W.-C., Wang, W.-P., Tian, W.-Y., & Zhang, X.-G. (2010). Antioxidant activity of *Astragalus* polysaccharides and antitumour activity of the polysaccharides and siRNA. *Carbohydrate Polymers*, *82*(2), 240–244.
Li, X. T., Zhang, Y. K., Kuang, H. X., Jin, F. X., Liu, D. W., Gao, M. B., et al. (2012). Mitochondrial Protection and anti-aging activity of *Astragalus* polysaccharides and their potential mechanism. *International Journal of Molecular Sciences*, *13*(2), 1747–1761.
Li, J., Zu, Y. G., Luo, M., Gu, C. B., Zhao, C. J., Efferth, T., et al. (2013). Aqueous enzymatic process assisted by microwave extraction of oil from yellow horn (*Xanthoceras sorbifolia* Bunge.) seed kernels and its quality evaluation. *Food Chemistry*, *138*(4), 2152–2158.
Liang, K., Zhang, Q., & Cong, W. (2012). Enzyme-assisted aqueous extraction of lipid from microalgae. *Journal of Agricultural and Food Chemistry*, *60*(47), 11771–11776.
Mao, X. Q., Yu, F., Wang, N., Wu, Y., Zou, F., Wu, K., et al. (2009). Hypoglycemic effect of polysaccharide enriched extract of *Astragalus membranaceus* in diet induced insulin resistant C57BL/6J mice and its potential mechanism. *Phytomedicine*, *16*(5), 416–425.
Meng, L., Qu, L., Tang, J., Cai, S. Q., Wang, H., & Li, X. (2007). A combination of Chinese herbs, *Astragalus membranaceus* var. *mongholicus* and *Angelica sinensis*, enhanced nitric oxide production in obstructed rat kidney. *Vascular Pharmacology*, *47*(2-3), 174–183.
Mu, L. X., Zhu, L., Zhao, A. H., Zhou, M. M., & Jia, W. (2009). Study on extraction and purification of polysaccharides from *Astragalus membranaceus*. *Zhong Yao Cai*, *32*(11), 1741–1745.
Nagendra chari, K. L., Manasa, D., Srinivas, P., & Sowbhagya, H. B. (2013). Enzyme-assisted extraction of bioactive compounds from ginger (*Zingiber officinale* Roscoe). *Food Chemistry*, *139*(1-4), 509–514.
Nagendra Chari, K. L., Manasa, D., Srinivas, P., & Sowbhagya, H. B. (2013). Enzyme-assisted extraction of bioactive compounds from ginger (*Zingiber officinale* Roscoe). *Food Chemistry*, *139*(1–4), 509–514.
Narukawa, T., Hioki, A., & Chiba, K. (2012). Aqueous extraction of water-soluble inorganic arsenic in marine algae for speciation analysis. *Analytical Sciences*, *28*(8), 773–779.
Pedrini, L., Guarnieri, C., Pisano, E., Masetti, L., Vaona, I., Muttini, A., et al. (1993). Prevention of reperfusion syndrome in acute muscular ischaemia with free radical scavengers and membrane-protecting compounds: An experimental study. *Cardiovascular Surgery*, *1*(4), 325–329.
Samavati, V. (2013). Polysaccharide extraction from *Abelmoschus esculentus*: Optimization by response surface methodology. *Carbohydrate Polymers*, *95*(1), 588–597.
Shen, H. H., Wang, K., Li, W., Ying, Y. H., Gao, G. X., Li, X. B., et al. (2008). *Astragalus membranaceus* prevents airway hyperreactivity in mice related to Th2 response inhibition. *Journal of Ethnopharmacology*, *116*(2), 363–369.
Sheng, B. W., Chen, X. F., Zhao, J., He, D. L., & Nan, X. Y. (2005). *Astragalus membranaceus* reduces free radical-mediated injury to renal tubules in rabbits receiving high-energy shock waves. *Chinese Medical Journal (English Edition)*, *118*(1), 43–49.
Tan, B. K., & Vanitha, J. (2004). Immunomodulatory and antimicrobial effects of some traditional Chinese medicinal herbs: A review. *Current Medicinal Chemistry*, *11*(11), 1423–1430.
Tan, Y. W., Yin, Y. M., & Yu, X. J. (2001). Influence of *Salvia miltiorrhizae* and *Astragalus membranaceus* on hemodynamics and liver fibrosis indexes in liver cirrhotic patients with portal hypertension. *Zhongguo Zhong Xi Yi Jie He Za Zhi*, *21*(5), 351–353.
Wang, S., Dong, X., & Tong, J. (2013). Optimization of enzyme-assisted extraction of polysaccharides from alfalfa and its antioxidant activity. *International Journal of Biological Macromolecules*, *62*(0), 387–396.
Xiong, D., Yang, Y., & Su, Y. (1998). Experimental study on treatment of viral myocarditis in mice by integrated traditional Chinese and Western medicine. *Zhongguo Zhong Xi Yi Jie He Za Zhi*, *18*(8), 480–482.
Yin, X., You, Q., & Jiang, Z. (2011). Optimization of enzyme assisted extraction of polysaccharides from *Tricholoma matsutake* by response surface methodology. *Carbohydrate Polymers*, *86*(3), 1358–1364.
Zhang, J., Jia, S., Liu, Y., Wu, S., & Ran, J. (2011). Optimization of enzyme-assisted extraction of the *Lycium barbarum* polysaccharides using response surface methodology. *Carbohydrate Polymers*, *86*(2), 1089–1092.
Zhu, Y., Li, Q., Mao, G., Zou, Y., Feng, W., Zheng, D., et al. (2014). Optimization of enzyme-assisted extraction and characterization of polysaccharides from *Hericium erinaceus*. *Carbohydrate Polymers*, *101*(0), 606–613.