Carbohydrate Polymers 95 (2013) 114–122

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Extraction, characterization and biological activities of polysaccharides from *Amomum villosum*

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ARTICLE INFO

Article history:
Received 11 October 2012
Received in revised form 27 February 2013
Accepted 2 March 2013
Available online 13 March 2013

Keywords:
A. villosum
Polysaccharide
Response surface methodology
Anti-cancer activity
Antioxidant activity

ABSTRACT

In the present study, the extraction, purification, characterization and biological activities of polysaccharides from the seeds of *Amomum villosum* (ASP) were investigated. Firstly, the optimal parameters for the extraction of ASP were obtained by using a Box–Behnken Design as follows: extraction temperature 78.8 °C, extraction time 71.3 min and ratio of water to raw material 39.3 ml/g. Then, the crude ASP was sequentially purified by chromatography of DEAE-52 and Sephadex G-100, resulting in three purified fractions of ASP-1, ASP-2 and ASP-3. The results showed that ASP-3 had a larger molecular weight and higher contents of protein, sulfuric radical and uronic acid than the other polysaccharide fractions. Furthermore, ASP-3 showed relative stronger inhibitory activity on the growth of HepG2 cells. Finally, ASP had strong free radical scavenging activities *in vitro* and significantly prevented the formation of malondialdehyde and enhanced the activities of antioxidant enzymes in CCl_4-induced liver injury mice.

1. Introduction

During the past decades, deep interest has been generated in the polysaccharides from plants, animals and microorganism due to their diverse and potentially significant pharmacological activities, such as anti-oxidation, hematopoietic activities, anti-mutagenic, immune regulation, anti-tumor, anti-inflammatory and hypoglycemic activities (Chemat, Lagha, AitAmar, Bartels, & Chemat, 2004; Gamal-Eldeen, Ahmed, & Abo-Zeid, 2009; Hromadkova, Ebringerova, & Valachovič, 1999; Li & Wang, 2005; Li, Zhou, & Han, 2006; Ookushi, Sakamoto, & Azuma, 2006; Tian et al., 2012; Ukai, Kiho, Hara, Kuruma, & Tanaka, 1983; Zheng et al., 2005). *A. villosum*, widely cultivated as an underplanted crop both in natural forests and tree plantations, is a major type of agroforestry farming in southern Yunnan and Guangdong, China. The seeds of *A. villosum* are medicinal herb of high economic value which have been used for the treatment of gastrointestinal diseases in China for hundreds of years (Zeng, Hu, Din, Chen, & Xu, 1999; Zhang, Liu, & Xu, 2005). It has been demonstrated that it is rich in volatile oil and polysaccharides that may contribute to the biological functions, such as anti-inflammation, antimicrobial and anti-nociceptive activities (Wu et al., 2004; Zhang, Wang, Wang, Li, & Lin, 2011). However, little attention has been devoted to the extraction and biological activities of polysaccharides from the seeds of *A. villosum*.

Recently, various novel techniques for the extraction of bioactive substances from plants have been developed, including supercritical fluid extraction, microwave-assisted extraction and ultrasonic-assisted extraction (Hromadkova et al., 1999; Ookushi et al., 2006). Compared with the first two methods, ultrasonic-assisted extraction has many advantages such as less extraction time, low extraction temperature and high extraction quotient. Therefore, in the present study, the ultrasonic-assisted method was performed for the extraction of ASP. And the extraction variables were optimized by employing Box–Behnken Design (BBD) for maximum polysaccharide yield. Furthermore, the crude ASP was purified by chromatography of DEAE-52 and Sephadex G-100. Then, the crude ASP and its purified fractions were characterized by chemical analysis, gas chromatography (GC), high-performance liquid chromatography (HPLC) and Fourier transform-infrared spectroscopy (FT-IR). Finally, the antioxidant and anticancer activities *in vitro* of crude ASP and its purified fractions were evaluated. In addition, the hepatoprotective activity *in vivo* of crude ASP was determined.

2. Materials and methods

2.1. Materials and reagents

The seeds of *A. villosum* were purchased from Yangchun Amomum plantation in Guangdong Province. Human hepatic cancer HepG2 cells were obtained from the Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China). The female Kunming

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http://dx.doi.org/10.1016/j.carbpol.2013.03.015

Table 1
Box–Behnken Design matrix and the response values for the yield of ASP.

No.	Extraction temperature (°C)		Extraction time (min)		Water to raw material ratio (ml/g)		Yield (%)
	X_1	Code x_1	X_2	Code x_2	X_3	Code x_3	
1	60	−1	60	−1	40	0	6.39
2	80	1	60	−1	40	0	11.15
3	60	−1	80	1	40	0	9.53
4	80	1	80	1	40	0	11.90
5	60	−1	70	0	30	−1	7.00
6	80	1	70	0	30	−1	11.37
7	60	−1	70	0	50	1	10.17
8	80	1	70	0	50	1	12.6
9	70	0	60	−1	30	−1	7.10
10	70	0	80	1	30	−1	9.02
11	70	0	60	−1	50	1	8.69
12	70	0	80	1	50	1	11.63
13	70	0	70	0	40	0	12.18
14	70	0	70	0	40	0	12.11
15	70	0	70	0	40	0	12.51
16	70	0	70	0	40	0	11.87
17	70	0	70	0	40	0	12.28

mice were purchased from the Experimental Animal Center of Guangzhou University of Traditional Chinese Medicine (Guangzhou, China). Nitroblue tetrazolium (NBT), reduced nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), DEAE-52 cellulose and Sephadex G-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Assay kits for protein, alanine aminotransferase (ALT), malondialdehyde (MDA), aspartate aminotransferase (AST), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were the products of Beijing Qison Biotechnology Co., Ltd. (Beijing, China). Other reagents were of analytical grade.

2.2. Preparation of crude ASP

The seeds of *A. villosum* were dried at 60 °C in an oven for 24 h and then ground up in a high speed disintegrator to obtain fine powder, all powder were passed through a 60-mesh sieve. Then the powder were defatted with petroleum ether (70–80 °C) and pretreated twice with 95% ethanol to remove oligosaccharides, some colored materials and small molecule materials. Finally, the organic solvent was volatilized to obtain pretreated dry powder. The dry powder (5.0 g) was extracted in an ultrasonic cleaner (KQ-400KDH, Kunshan Ultrasound Instrument Co., Jiangsu, China, 40 kHz) with distilled water, using designed ratio of water to raw material, extraction time and extraction temperature. The temperature of the bath was controlled with a constant temperature water-bath (501-A, Shanghai Instrument Co., Ltd.). After the treatment, the mixture was centrifuged at 5000 rpm for 20 min. The insoluble residue was re-extracted stated times to recover the residual water-soluble polysaccharides. The supernatants were collected, concentrated to a proper volume by using a vacuum rotary evaporator and mixed with four times volume of absolute ethanol. The mixture was stirred vigorously and then kept overnight at 4 °C. The precipitate was collected by centrifugation at 5000 rpm for 20 min and air-drying at 50 °C to a constant weight, affording the crude ASP.

The polysaccharide content of the crude ASP was measured by the phenol-sulfuric acid method using glucose as a standard substance (Masuko et al., 2005; Hou & Chen, 2008). Briefly, 1.0 ml polysaccharide sample solution was mixed with 1.0 ml 5% phenol and 5 ml 98% H_2SO_4. The absorbance at 490 nm was recorded after standing for 15 min. The polysaccharide yield (%) was then calculated using the following equation:

$$\text{extraction yield}(\%) = \frac{C \times N \times V_n}{W} \times 100 \quad (1)$$

where C is the concentration of polysaccharide calculated from the calibrated regression equation (mg/ml); N is the dilution factor; V_n is the total volume of extraction solution (ml); and W is the weight of defatted powder samples (g).

2.3. BBD for the extraction of polysaccharides

In the present study, BBD with three independent variables (X_1, extraction time; X_2, extraction temperature; X_3 ratio of water to raw material) at three levels was applied to determine the best combination of extraction variables for extraction of ASP. For statistical calculation, the variables were coded according to the following equation:

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

where x_i is the coded value of an independent variable, X_i is the actual value of an independent variable, X_0 is the actual value of an independent variable at center point, and ΔX_i is the step change value of an independent variable.

The range of independent variables and their levels are presented in Table 1. The whole design consisted of 17 experimental points carried out in random order, and the experimental data (Table 1) were fitted to the following second-order polynomial model:

$$Y = \alpha_0 + \sum_{i=1}^{3} \alpha_i X_i + \sum_{i=1}^{3} \alpha_{ij} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \alpha_{ij} X_i X_j \quad (3)$$

where Y is the predicted response (yield of ASP), α_0 is a constant, α_i, α_{ii} and α_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively. X_i and X_j represent the level of the independent variables ($i \neq j$).

2.4. Purification of crude ASP

The crude ASP was purified by DEAE-52 and Sephadex G-100 gel filtration chromatography according to the reported method with little modifications (Qiao et al., 2009). Briefly, the crude ASP solution (10 ml, 8 mg/ml) was applied to a column (2.6 cm × 30 cm) of DEAE-52 cellulose. Then, the column was stepwise eluted with 0, 0.1, 0.3 and 0.5 M NaCl solutions at a flow rate of 1 ml/min. The obtained elute (10 ml/tube) was collected automatically and the polysaccharides were detected by the phenol–sulfuric acid method. As a result, three fractions of ASP were obtained. Each fraction was

collected, concentrated, dialyzed and further purified through a column (2.6 cm × 60 cm) of Sephadex G-100, respectively, resulting in three purified fractions of ASP-1, ASP-2 and ASP-3. Finally, ASP-1, ASP-2 and ASP-3 were lyophilized for further research. The purified polysaccharide yield (%) was then calculated using the following equation:

$$\text{purified polysaccharide yield}(\%) = \frac{m_p}{m_c} \times 100 \quad (4)$$

where m_p is the weight of purified polysaccharide and m_c is the weight of crude polysaccharide.

2.5. Preliminary characterization of ASP

2.5.1. Determination of contents of carbohydrate, sulfuric radical, protein and uronic acid

The contents of carbohydrate in crude ASP and its purified fractions were determined by phenol–sulfuric acid method using glucose as the standard. The content of sulfate radical was determined according to the reported method (Dodgson & Price, 1962). The content of protein was determined by the method of Bradford using bovine serum albumin as the standard (Bradford, 1976). The content of uronic acid was determined according to the method of Blumenkrantz and Asboe-Hansen by using D-glucuronic acid as the standard (Blumenkrantz & Asboe-Hansen, 1973).

2.5.2. Analysis of monosaccharide composition of ASP

The monosaccharide compositions of crude ASP, ASP-1, ASP-2 and ASP-3 were analyzed by GC according to the reported method with slight modifications (Xu, Ye, Sun, Tu, & Zeng, 2011). Briefly, the polysaccharide sample was hydrolyzed with 2 ml trifluoroacetic acid (2 M) in an oven at 120 °C for 2 h. The hydrolyzate was repeatedly co-concentrated with methanol to dryness and acetylated by the addition of a mixture of methanol, pyridine and acetic anhydride. In a similar manner, the monosaccharide standards of rhamnose, xylose, arabinose, fucose, mannose, glucose and galactose were acetylated. Then, all the derivatives were analyzed by a 6890 N GC (Agilent Technologies, Santa Clara, CA, USA) equipped with flame ionization detector and an HP-5 fused silica capillary column (30 m × 0.32 mm × 0.25 mm). The operation conditions of GC were as follows: flow rates of N_2, H_2 and air were 25, 30 and 400 ml/min, respectively; the temperatures of oven, detector and inlet were 210, 280 and 250 °C, respectively. The injection volume was 1 μl aliquot for each run.

2.5.3. Molecular weight determination of ASP-1, ASP-2 and ASP-3

The molecular weight of ASP-1, ASP-2 and ASP-3 were characterized by high-performance liquid chromatography (HPLC) according to the reported method with slight modifications (Fu, Tian, Cai, Liu, & Li, 2007). Briefly, the purified polysaccharides (4.1 mg) were dissolved in distilled water (2 ml), passed through a 0.45 μm filter, and applied to a gel-filtration chromatographic column of TSK-GEL G3000SW$_{xl}$ column (7.5 mm × 300 mm, Tosoh Corp., Japan). The column was maintained at a temperature of 25 °C, eluted with 0.1 M Na_2SO_4 solution in PBS buffer (0.01 M, pH 6.8) at a flow rate of 0.8 ml/min. Preliminary calibration of the column was conducted using the dextrans with various molecular weight (5200, 11,600, 48,600, 148,000, 410,000, 668,000 and 1,482,000).

2.5.4. Infrared spectroscopy analysis of ASP

Crude ASP and its purified fraction powder was mixed with KBr powder, grinded and pressed for Fourier transform infrared (FT-IR) measurement. A FT-IR spectrum of the ASP was determined using the Nicolet 5700 spectrometer (Thermo Electron, Madison, WI, USA) at the frequency range of 4000–400 cm^{-1}.

2.6. Determination of antioxidant activities in vitro of ASP

2.6.1. Assay of superoxide radical scavenging activity

The superoxide radical scavenging activity of ASP was determined in accordance with the reported method (Li et al., 2006) with some modifications. Briefly, each 1.0 ml of NBT solution (156 μmol/L), PMS solution (60 μmol/L) and NADH solution (468 μmol/L) were mixed with 1 ml sample solution with different concentrations (0.1, 0.5, 1.0, 1.5, 3.0 and 5.0 mg/ml). The mixture was shaken well and incubated at 25 °C for 5 min. The absorbance at 560 nm was measured against the blank (water and 0.1 M phosphate buffer instead of ASP sample and NBT solution, respectively). The scavenging activity on superoxide radical was calculated according to the following formula:

$$\text{superoxide radical scavenging activity}(\%) = \frac{A_0 - A_1 + A_2}{A_0} \times 100 \quad (5)$$

where A_0 is the absorbance of control sample, A_1 is the absorbance of the tested sample, and A_2 is the absorbance of test sample solution without NBT solution. Ascorbic acid was used as positive control.

2.6.2. Assay of hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was determined in accordance with the reported method (Liu, Wang, Xu, & Wang, 2007). Briefly, 1 ml sample solution with different concentrations (0.1, 0.5, 1.0, 1.5, 3.0 and 5.0 mg/ml) was added to 2.4 ml of sodium phosphate buffer (0.1 M, pH 7.4) and 0.6 ml H_2O_2 solution (40 mmol/L). The mixture was incubated at 37 °C for 30 min and the absorbance of the solution was detected at 230 nm. The hydroxyl radical scavenging activity was calculated according to the formula below:

$$\text{hydroxyl radical scavenging activity}(\%) = \frac{A_0 - A_1 + A_2}{A_0} \times 100 \quad (6)$$

where A_0 is the absorbance of control sample, A_1 is the absorbance in the presence of tested samples, and A_2 is the absorbance of tested sample without H_2O_2 solution. The ascorbic acid was used as positive control.

2.6.3. Assay of DPPH radical scavenging activity

DPPH radical scavenging activity was determined in accordance with the reported method (Yuan, Bone, & Carrington, 2005). Briefly, 1 ml sample solution with different concentrations (0.1, 0.5, 1.0, 1.5, 3.0 and 5.0 mg/ml) was added to 0.2 ml DPPH solution (0.4 mmol/L) and 2.0 ml H_2O. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then, the absorbance of the mixture was measured at 517 nm. The DPPH radical scavenging activity was calculated according to the following formula:

$$\text{DPPH radical scavenging activity}(\%) = \frac{A_0 - A_1 + A_2}{A_0} \times 100 \quad (7)$$

where A_0 is the absorbance of control sample, A_1 is the absorbance in the presence of the tested sample, and A_2 is the absorbance of test sample solution without DPPH solution. The ascorbic acid was used as positive control.

2.7. Assay of inhibitory activity in vitro of ASP on HepG2 cell proliferation

The inhibition rates of crude ASP and its purified fractions on the growth of human hepatic cancer HepG2 cells were determined by MTT-based colorimetric method (Mosmann, 1983). Briefly, HepG2 cells were suspended in RPMI-1640 medium supplemented with 10% FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin

at a density of 1×10^5 cells/ml. The cell suspension was pipetted into a 96-well plate (50 μl/well) and inoculated at 37 °C in a humidified 5% CO_2 incubator (Sanyo, Japan) for 24 h. Then, 50 μl of test sample with different concentrations (0, 50, 100, 200 and 500 μg/ml in fresh growth medium) was added into each well separately. After 72 h incubation, MTT reagent (5.0 mg/ml) was added to each well (10 μl/well), and the plate was further incubated for 4 h. Then, 100 μl of 10% SDS in 0.01 M HCl was added into each well and kept overnight for the dissolution of formazan crystals. The absorbance of each well was read at 570 nm using an ELISA plate reader (TECAN Infinite F200, Switzerland). The inhibitory rate was calculated according to the formula below:

$$\text{inhibition rate}(\%) = \frac{1 - A_1}{A_0} \times 100 \quad (8)$$

where A_1 and A_0 are the absorbance of test sample and control, respectively.

2.8. Assay of hepatoprotective activity in vivo of crude ASP

2.8.1. Animal grouping and experimental design

In the present study, the female Kunming mice (8-week-old) of grade of specific pathogen free with body weight (BW) of 20.0 ± 2.0 g were used for the evaluation of hepatoprotective activity *in vivo* of crude ASP on carbon tetrachloride (CCl_4)-induced acute liver injury. All procedures were conducted in strict accordance with the Chinese legislation on the use and care of laboratory animals during the entire experimental period. Briefly, the mice were handled under standard laboratory conditions of a 12 h light/dark cycle in a room maintained at a temperature of 22.0 ± 0.5 °C and were free to access to food and water. After a 7-day acclimation period, these mice were randomly divided into six groups (eight for each). Mice in Group I (normal control) and group II (CCl_4 model control) were treated with 0.9% sodium chloride (25 ml/kg BW per day). Mice in Group III (positive control) were treated with silymarin (a hepatoprotective drug, 100 mg/kg BW per day). Mice in Groups IV (low dose of crude ASP), V (medium dose of crude ASP) and VI (high dose of crude ASP) were treated with 50, 100 and 200 mg/kg BW of crude ASP per day, respectively. All treatments were conducted by gastric gavage for 14 consecutive days. Then, CCl_4 administration consisting of an intraperitoneal injection of CCl_4 (150 μl/100 g BW of a 1:1 (v/v) mixture of CCl_4 and corn oil) was carried out (Simile et al., 2001). The mice in Groups II–VI received a single dose of CCl_4 on the 15th day, while Group I received an equal amount of corn oil instead of CCl_4. All mice were sacrificed at 24 h post-injection of CCl_4 or corn oil, and the blood samples were collected immediately and centrifuged at 3000 rpm for 10 min at 4 °C to afford the serums. The serums were stored at −18 °C until assay. The liver was excised and homogenized in 0.1 g/ml wet weight of ice-cold physiological saline. The homogenate was centrifuged at 4000 rpm for 10 min, and the supernatant was collected for further use.

2.8.2. Biochemical assay

The activities of ALT and AST in serum, protein content, level of MDA, and activities of SOD and GSH-Px in liver were determined by using commercial reagent kits according to the instruction manuals.

2.9. Statistical analysis

Analysis of the experimental design and data was carried out using Design Expert software of version 7.0 (Stat-Ease Inc., Minneapolis, USA). Analysis of variance (ANOVA) was carried out and the fitness of the polynomial model equation was expressed as the coefficient of determination R^2. The significances of the regression coefficients were tested by *F*-test. Multiple comparisons of means were done by the least significance difference (LSD) test. $P < 0.05$ was regarded as significant.

3. Results and discussion

3.1. Model fitting and optimization for the extraction of ASP

3.1.1. Model fitting

Using multiple regression analysis on experimental data by the software of Design Expert version 7.0, the correlation between response variables and test variables (the extraction yield of ASP) associated with the following second-order polynomial equation:

$$Y = -187.9 + 1.677125X_1 + 2.961375X_2 + 1.2835X_3 - 0.00597X_1X_2 - 0.00485X_1X_3 + 0.00255X_2X_3 - 0.00636X_1^2 - 0.01811X_2^2 - 0.01269X_3^2 \quad (9)$$

where Y represents the yield of ASP, X_1, X_2 and X_3 represent extraction temperature, time and ratio of water to raw material, respectively.

The variance of the quadratic regression model showed that the determination coefficient (R^2) was 0.9950, indicating that only 0.50% of the total variance was not explained by the model. The value of the adjusted determination coefficient (adjusted $R^2 = 0.9885$) also confirmed that the model was highly significant. Meanwhile, coefficient of the variation (CV) was 2.16, a very low value, clearly indicated that the degree of precision was very high and a large number of experimental data were very reliable. The model could be determined enough to predict within the range of experimental variables. The values of regression coefficient in Eq. (3) are listed in Table 2. Smaller the *P*-value is, more significant the corresponding coefficient is. Accordingly, *A*, *B*, *C*, *AB*, *AC*, A^2, B^2 and C^2 were significant ($P < 0.05$), while BC were not significant ($P > 0.05$) (Liang, 2008; Wu, Cui, Tang, & Gu, 2007).

3.1.2. Optimization for the extraction of ASP

Process variables and experimental data are shown in Table 1. In order to better understand the interactions of the variables, the response surface plots and contour plots for the model were produced by the Stat-Ease Design-Expert software. The shapes of the contour plots, circular or elliptical, indicate whether the mutual interactions between variables are significant or not. The response surface plots and contour plots as shown in Fig. 1 were generated using Design-Expert, which depicted the interactions between two variables by keeping the other variables at their zero levels for ASP yield. It was evident that these three-dimensional plots and their corresponding contour plots provided a visual interpretation of the interaction for two variables and facilitated the location of optimum experimental conditions. By using the software Design-Expert, the solved optimum values of the tested variables for the extraction of ASP were extraction temperature 78.8 °C, extraction time 71.3 min and ratio of water to raw material 39.3 ml/g, the maximum predicted extraction yield of ASP was 13.22%, which corresponded fairly well to that of real extraction (13.04 ± 0.36%, $n = 3$). The result suggested that the regression model was accurate and adequate for the prediction of ASP extraction.

3.2. Separation and purification of ASP

By using the optimal extraction conditions, crude ASP was obtained. Then, the crude ASP was firstly separated through an anion-exchange column of DEAE-52 cellulose. As the result, three independent elution peaks (F_1, F_2 and F_3) were obtained as shown in Fig. 2a. The three fractions were collected, concentrated, dialyzed

Table 2
ANOVA for response surface quadratic model.

Variables	Sum of squares	DF[a]	Mean square	*F*-value	*p*-Value Prob. > *F*
Model	70.13	9	7.79	153.38	<0.0001
A	24.26	1	24.261	477.44	<0.0001
B	9.57	1	9.57	188.38	<0.0001
C	9.25	1	9.245	181.98	<0.0001
AB	1.43	1	1.43	28.11	0.0011
AC	0.94	1	0.94	18.52	0.0036
BC	0.26	1	0.26	5.12	0.0581
A^2	1.70	1	1.70	33.55	0.0007
B^2	13.81	1	13.81	271.89	<0.0001
C^2	6.78	1	6.78	133.41	<0.0001
Residual	0.36	7	0.051		
Lack of fit	0.14	3	0.045	0.827864	0.5435
Pure error	0.22	4	0.055		
Correlation total	70.49	16			

[a] Degree of freedom.

and loaded into a column of Sephadex G-100, respectively. The column was eluted with deioned water, and the resulting elute was collected. As shown in Fig. 2b–d, each fraction generated one single elution peak, represent of ASP-1, ASP-2 and ASP-3, respectively. The three fractions were collected, concentrated, freeze-dried and purified products were obtained. The yields of ASP-1, ASP-2 and ASP-3 were 12.26%, 4.45% and 3.17%, respectively.

3.3. Preliminary characterization of ASP

3.3.1. Contents of carbohydrate, sulfate, protein and uronic acid in ASP

Table 3 shows the contents of carbohydrate, sulfate, protein and uronic acid in crude ASP and its purified fractions. The carbohydrate contents in crude ASP, ASP-1, ASP-2 and ASP-3 were 76.43%, 89.47%, 88.91% and 79.59%, respectively. Among all the polysaccharides tested, ASP-3 contained the highest contents of uronic acid and sulfate. The contents of protein in crude ASP, ASP-1, ASP-2 and ASP-3 were 1.94%, 0.37%, 0.78% and 2.85%, respectively.

3.3.2. Monosaccharide composition of ASP

The monosaccharide composition of crude ASP and its purified fractions (ASP-1, ASP-2 and ASP-3) were determined by GC, and the monosaccharides in ASP hydrolyte were identified by comparing the retention times with those of standards (Fig. 3a–e) and the results were presented in Table 3. As shown in Table 3, crude ASP was mainly composed of glucose (30.49%), arabinose (22.28%), galactose (23.31%) and mannose (24.92%). Other sugars, such as fucose, rhamnose and xylose, were not found in ASP. Furthermore, the monosaccharide composition of ASP-3 was different from that of ASP-1 or ASP-2. In addition, the contents of galactose and mannose in ASP-3 were relatively higher than those in ASP-1 and ASP-2.

3.3.3. Molecular weight of ASP

The linearity of the method was calibrated by using dextran standards of different molecular weight. The calibration curve of dextrans was plotted as the molecular weights on a log scale *versus* the retention time, then got a standard regression curve equation, $\log Mw = -0.42t + 6.872$, $r = 0.9989$ (t was the retention time, min).

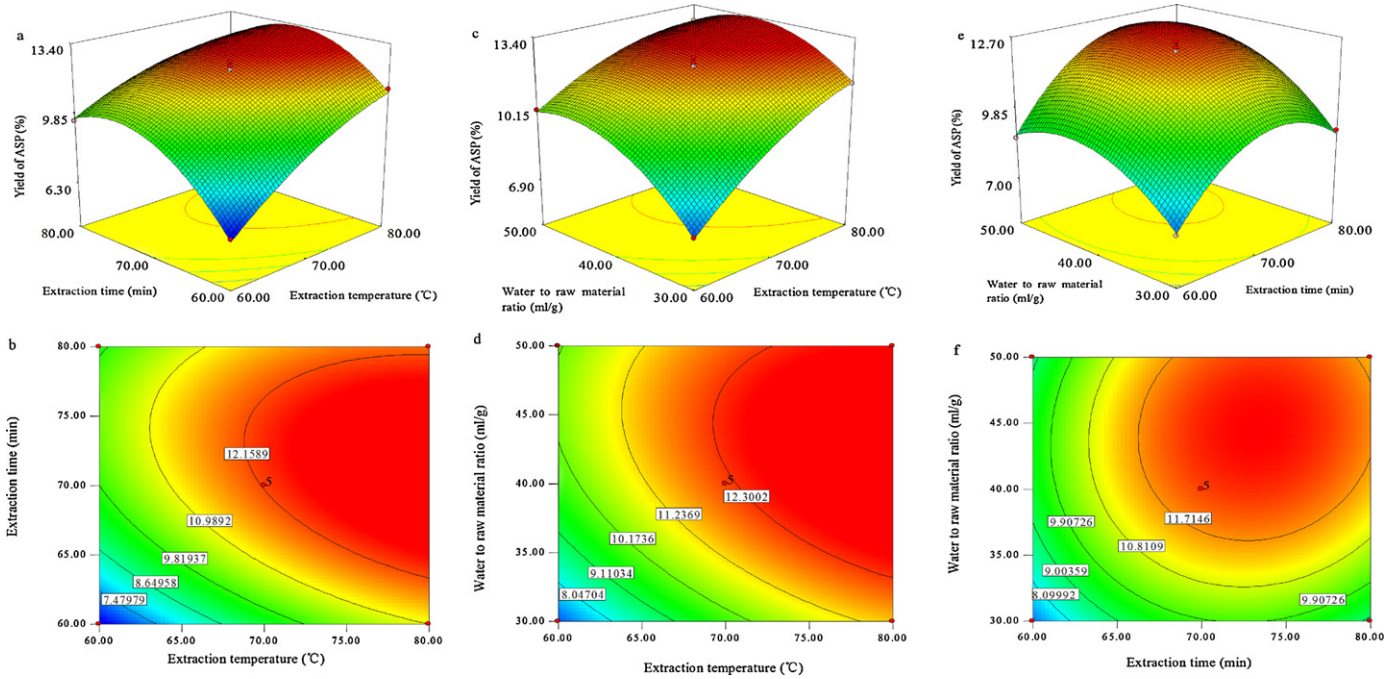

Fig. 1. Response surface plots (a, c and e) and contour plots (b, d and f) showing the effects of extraction temperature, extraction time and ratio of water to raw material their mutual effects on the extraction yield of ASP.

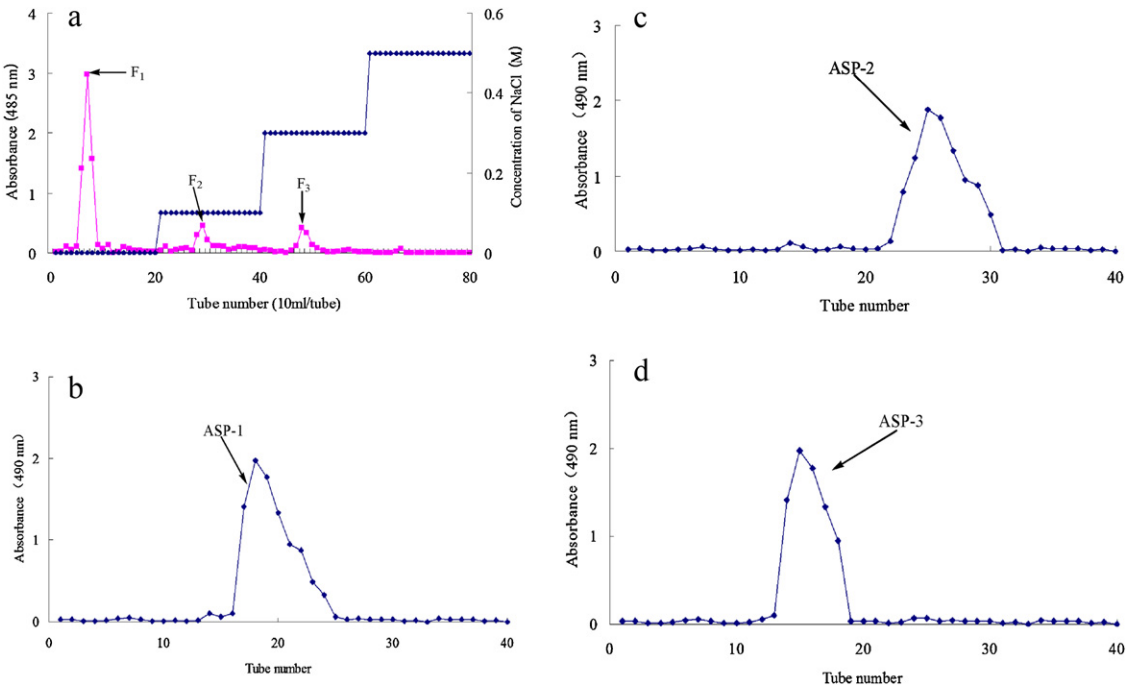

Fig. 2. Elution profiles of crude ASP on DEAE-52 cellulose anion-exchange chromatography column with 0–0.5 M NaCl elute (a) and elution curve of polysaccharide fractions (F_1, F_2 and F_3) from DEAE-52 cellulose on size-exclusion chromatography column of Sephadex G-100 (b–d).

Table 3
The chemical compositions and the contents of carbohydrate, protein, uronic acid and sulfate for crude ASP, ASP-1, ASP-2 and ASP-3.

Sample	Carbohydrate (%)	Sulfuric radical (%)	Protein (%)	Uronic acid (%)	Sugar component (%)			
					Glucose	Arabinose	Galactose	Mannose
Crude ASP	76.43	2.41	1.94	2.22	30.49	22.28	22.31	24.92
ASP-1	89.47	0.48	0.37	–[a]	33.91	49.53	7.42	9.14
ASP-2	88.91	1.77	0.78	1.62	50.89	21.22	5.19	22.70
ASP-3	79.59	2.58	2.85	3.55	20.17	16.53	24.51	38.79

[a] Not detectable.

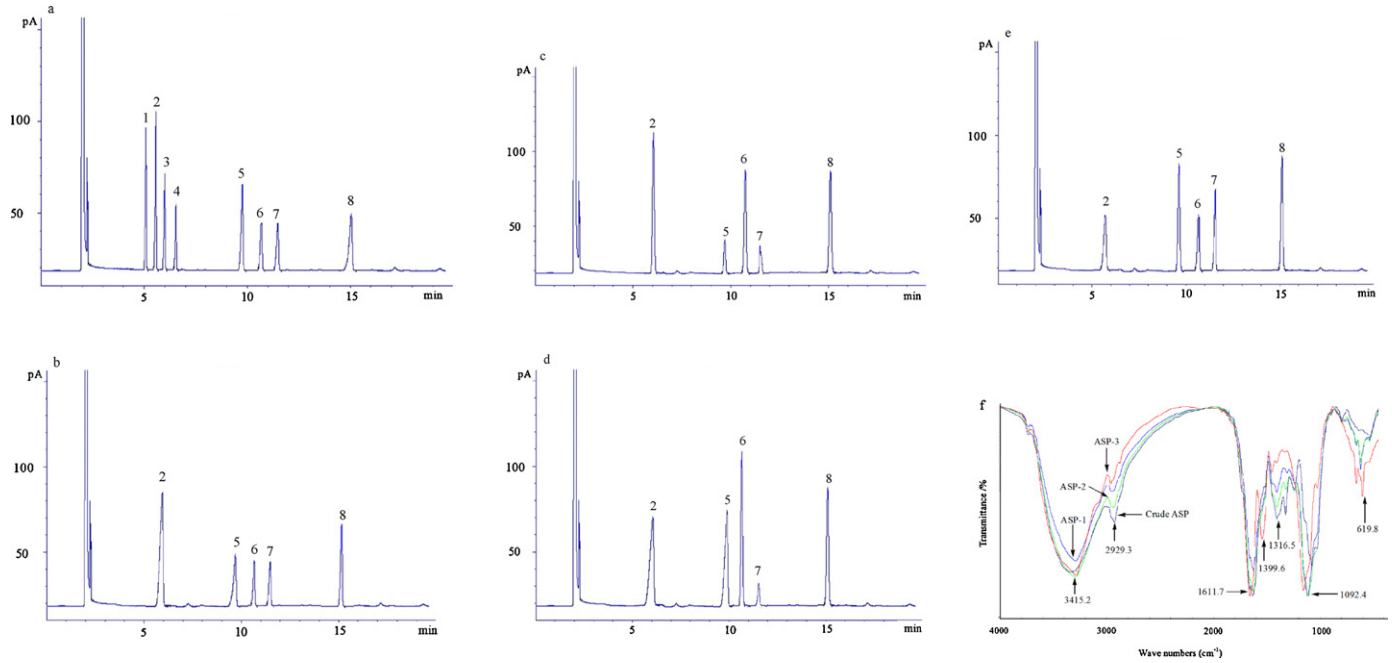

Fig. 3. GC spectra of sample references (a), crude ASP (b), ASP-1 (c), ASP-2 (d) and ASP-3 (e) (1, rhamnose; 2, arabinose; 3, fucose; 4, xylose; 5, mannose; 6, glucose; 7, galactose; 8, inositol) and FT-IR spectra of ASP (f).

The average molecular weights for ASP-1, ASP-2 and ASP-3 were determined to be 48.7, 53.5 and 61.4 kDa, respectively.

3.3.4. FT-IR spectrum of ASP

As shown in Fig. 3f, there was a strong and broad peak around the 3415 cm^{-1} of ASP infrared spectroscopy, which was belong to the hydroxyl groups stretching vibration (Wang, Chen, Jia, Tang, & Ma, 2012). The weak peaks around 2930 cm^{-1} and 1320 cm^{-1} were assigned to the C—H asymmetric stretching vibration (Lai, Wen, Li, Wu, & Li, 2010). The bands around 1612 cm^{-1} was the C=O asymmetric stretching vibration absorption peak (Cerna et al., 2003). A strong extensive absorption in the region of 1000–1200 cm^{-1} for stretching vibrations of C—OH side groups and the C—O—C glycosidic band vibrations were observed in the spectra (Barros et al., 2002). All these were characteristic absorptions of polysaccharides. Furthermore, absorption around 1071.9 cm^{-1} was the furan glycosides characteristic absorption peaks (Wang, 2011). The results of FT-IR characterization were in coincidence with the results of Table 3.

3.4. Antioxidant activity in vitro of ASP

3.4.1. Superoxide radical scavenging activity of ASP

Superoxide radical is a reduced form of molecular oxygen created by receiving one electron from mitochondrial electron transport systems. It can decompose to some reactive oxygen species (ROS), such as singlet oxygen and hydroxyl, which can initiate lipid peroxidation. As a result, the formation of superoxide radical can induce oxidative damage in lipids, proteins and DNA (Korycka-Dahl, Richardson, & Foote, 1978). Thus, the scavenging of superoxide radical is very important to antioxidant work.

The scavenging activities of crude ASP, ASP-1, ASP-2 and ASP-3 on superoxide radical are shown in Fig. 4A. The scavenging effects of crude ASP and its purified fractions were evident at all of the tested concentrations. And the superoxide radical scavenging activities of crude ASP, ASP-1, ASP-2, ASP-3 and ascorbic acid were positively correlated well with the increase of concentration from 0.1 to 5.0 mg/ml. ASP-3 and crude ASP showed stronger superoxide radical scavenging effect than ASP-1 and ASP-2. At a concentration of 5.0 mg/ml, the scavenging activities of crude ASP, ASP-1, ASP-2 and ASP-3 were 69.4, 52.5, 61.3 and 79.6%, respectively. The superoxide radical scavenging activity of ascorbic acid was 27.1% at a concentration of 5.0 mg/ml and was lower than that of ASP. The results indicated that ASP-3 has a strong scavenging activity on superoxide radical. The strong scavenging activity of ASP-3 might be attributed to its high contents of uronic acid and sulfuric radical.

3.4.2. Scavenging activity on hydroxyl radical of ASP

Hydroxyl radical is one of the most harmful reactive free radicals, it can react with all bio-macromolecules such as lipids, proteins, carbohydrate and DNA in cells, and then be toxic to cells (Huang, Ou, & Ronald, 2005; Xing et al., 2005). Therefore, the cleaning of hydroxyl radical is very important for the body from being oxidized injury.

The hydroxyl radical, generated by the Fenton reaction in the system, was scavenged by crude ASP, ASP-1, ASP-2, ASP-3 and ascorbic acid. The hydroxyl radical scavenging effects of crude ASP, ASP-1, ASP-2, ASP-3 and ascorbic acid were increased with the increase of concentration up to 5.0 mg/ml (Fig. 4B). The scavenging effects of crude ASP and ASP-3 were higher than that of ASP-1 and ASP-2. At a concentration of 5.0 mg/ml, the hydroxyl radical scavenging activities were 66.3, 48.4, 53.2 and 75.4% for crude ASP, ASP-1, ASP-2 and ASP-3, respectively. However, ascorbic acid showed higher hydroxyl radical scavenging activity than crude ASP. The results demonstrated that crude ASP and ASP-3 possessed strong scavenging activities on hydroxyl radical.

3.4.3. Scavenging activity on DPPH radical of ASP

DPPH radical is one of the relatively stable free radicals, it can accept an electron or hydrogen atom to formed a stable diamagnetic molecule and it is widely used in the evaluation of free radical scavenging activity (Yang, Zhao, Shi, Yang, & Jiang, 2008). The scavenging activities of crude ASP, ASP-1, ASP-2 and ASP-3 on DPPH free radical are shown in Fig. 4C. ASP had DPPH radical scavenging activity within the concentration of 0.1–5.0 mg/ml. The scavenging effect on DPPH radical of ASP-3 was higher than that of crude ASP, ASP-1 and ASP-2, and its scavenging activity was close to ascorbic acid at a concentration of 5 mg/ml. At a concentration of 5.0 mg/ml, the DPPH radical scavenging activity was 73.5, 33.2, 62.3 and 81.5% for crude ASP, ASP-1, ASP-2 and ASP-3, respectively. The results indicated that ASP had strong scavenging activity on DPPH radical.

3.5. Inhibitory effect of ASP on HepG2 cell proliferation

Human hepatic cancer line HepG2 is an ideal model for the study of cell proliferation (He et al., 2009). In the present study, the inhibitory effects of crude ASP, ASP-1, ASP-2 and ASP-3 on the growth of HepG2 cells were investigated by MTT assay. As shown in Fig. 4D, all polysaccharides exhibited a dose-dependent activity within the concentration range of 50–500 μg/ml, and the inhibitory effects of ASP-1, ASP-2 and ASP-3 increased significantly ($P < 0.05$) with the increase of sample concentration. At a concentration of 500 μg/ml, the inhibitory effects of crude ASP, ASP-1, ASP-2 and ASP-3 were 27.82, 53.64, 68.29 and 74.58%, respectively. Obviously, ASP-3 showed strong inhibitory effect on the growth of HepG2 cells.

The levels of anti-oxidation and reactive oxygen species are correlated well with the generation and malignant transformation of cancer cells. If compounds can enhance the level of anti-oxidation and clear the reactive oxygen species in cancer cells, they may inhibit the cells growth (Leng, Liu, & Chen, 2005). Therefore, the higher anticancer activity of ASP-3 might be attributed to its higher scavenging activity on free radicals, such as superoxide radical and hydroxyl radical.

3.6. Hepatoprotective activity in vivo of crude ASP

Table 4 shows the effects of crude ASP on the activities of ALT and AST in serums of CCl_4-induced hepatotoxicity mice. Apparently, a significant increase in ALT and AST activities ($P < 0.05$) were observed in serums between Group I (normal control group) and Group II (CCl_4 model control group), indicating that the CCl_4-induced hepatotoxicity model in mice was well-established. Furthermore, both crude ASP and silymarin treatments significantly decreased ($P < 0.05$) the activities of ALT and AST in serums in a dose-dependent manner (Groups IV–VI). However there was no significant difference between Group V and Group VI.

Effects of crude ASP on the activities of SOD, GSH-Px and levels of MDA in livers of CCl_4-induced hepatotoxicity mice are shown in Table 4. Apparently, a significant increase in MDA and marked decreases ($P < 0.05$) of antioxidant enzyme activities (SOD and GSH-Px) were observed in livers between Group I (normal control group) and Group II (CCl_4 model control group). The results confirmed again that the model was established successfully. Furthermore, both crude ASP and silymarin treatments significantly decreased ($P < 0.05$) the level of MDA and raised the activities of SOD and GSH-Px in mice livers. However, there was no significant difference between Group V and Group VI.

The hepatotoxicity induced by CCl_4 is the most commonly used model system for the screening of hepatoprotective

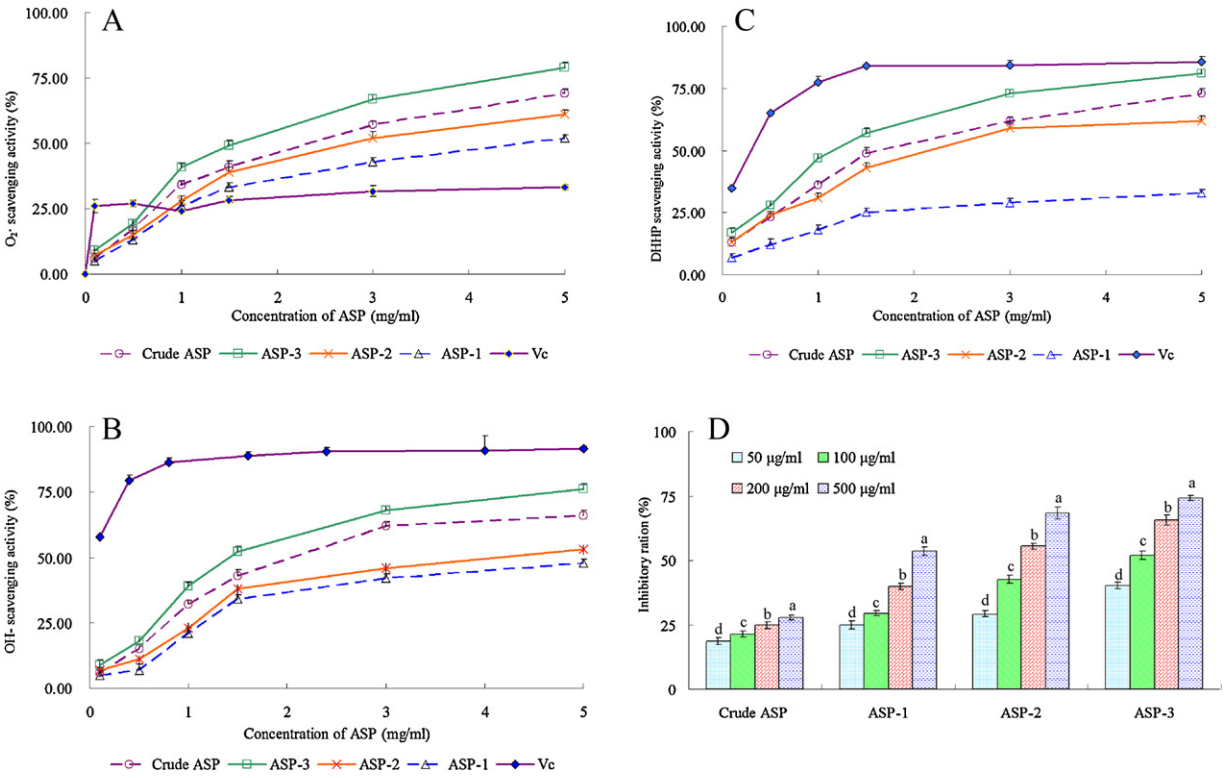

Fig. 4. Scavenging effects on (A) superoxide radical, (B) hydroxyl radical and (C) DPPH radical of crude ASP, ASP-1, ASP-2 and ASP-3. (D) Inhibitory effects *in vitro* of crude ASP, ASP-1, ASP-2 and ASP-3 at different concentrations against human hepatic cancer HepG2 cells. Values are presented as means ± SD ($n = 3$). Different alphabets (a–d) in superscript for each treatment (crude ASP, ASP-1, ASP-2 or ASP-3) denote significant difference ($P < 0.05$).

Table 4
Effect of crude ASP administration on the activities of ALT and AST in serums, and the activities of SOD, GSH-Px and level of MDA in livers of CCl_4-induced liver injury mice.

Group	ALT (U/L)	AST (U/L)	SOD (U/mg protein)	GSH-Px (μmol/L/mg protein)	MDA (nmol/mg protein)
I (normal control group)	16 ± 2^{d}	23 ± 2^{d}	359 ± 24^{b}	605 ± 82^{b}	0.61 ± 0.09^{b}
II (CCl_4 model control group)	132 ± 11^{a}	68 ± 3^{a}	289 ± 32^{c}	297 ± 35^{d}	1.18 ± 0.17^{a}
III (positive control group)	35 ± 5^{b}	22 ± 2^{d}	548 ± 39^{a}	839 ± 73^{a}	0.58 ± 0.08^{b}
IV (ASP treatment group, 50 mg/kg)	104 ± 10^{c}	53 ± 4^{c}	329 ± 34^{c}	478 ± 63^{c}	0.65 ± 0.05^{b}
V (ASP treatment group, 100 mg/kg)	39 ± 6^{b}	39 ± 4^{b}	402 ± 29^{b}	628 ± 73^{b}	0.62 ± 0.09^{b}
VI (ASP treatment group, 200 mg/kg)	36 ± 5^{b}	36 ± 4^{b}	381 ± 45^{b}	588 ± 47^{b}	0.56 ± 0.08^{b}

Data were presented as mean ± SD ($n = 8$) by one-way ANOVA followed by the Duncan's multiple-range tests, and values not sharing a common superscript letter donate significant difference ($P < 0.05$).

activity of plant extracts and drugs. It has been established that CCl_4 is accumulated in hepatic parenchyma cells and metabolically activated by cytochrome P450-dependent monooxygenases to form a trichloromethyl radical (CCl_3). The CCl_3 radical alkylates cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids, in the presence of oxygen, to produce lipid peroxides, leading to liver damage (Srilakshmi, Vijayan, Raj, Dhanaraj, & Chandrashekhar, 2010). Thus, antioxidant or free radical generation inhibition is important in protection against CCl_4-induced liver lesions. In the present study, experimental results showed that the activities of SOD and GSH-Px were significantly lower in mice of model control group as compared with those of normal group. It was likely that lower activities of SOD and GSH-Px resulted in the accumulation of free radicals which subsequently initiated lipid peroxidation and caused the elevation of MDA level, while pretreatment with crude ASP resulted in the decrease of MDA level and increase of SOD and GSH-Px activities in CCl_4-induced liver injury mice. The results suggested that polysaccharides from *A. villosum* had a significant protective effect against CCl_4-induced acute hepatotoxicity in mice.

4. Conclusion

BBD was employed to determine the optimal parameters for extraction of ASP in the present study. Through optimization, a maximum predicted yield of 13.22% was obtained with the following optimized conditions: extraction temperature 78.8 °C, extraction time 71.3 min and ratio of water to raw material 39.3 ml/g. Under these conditions, the experimental yield of polysaccharides was 13.04 ± 0.36%, which was close to the predicted yield value (13.22%). Then, three purified fractions of ASP-1, ASP-2 and ASP-3 were obtained from the crude ASP through sequential purification purified by chromatography of DEAE-52 and Sephadex G-100. Then, the crude ASP and its purified fractions were characterized by chemical analysis, GC, HPLC and FT-IR. Experimental results showed that crude ASP was mainly composed of glucose (30.49%), arabinose (22.28%), galactose (23.31%) and mannose (24.92%). In addition, the monosaccharide composition of ASP-3 was greatly different from that of ASP-1 or ASP-2. The contents of galactose and mannose in ASP-3 were relatively higher than those in ASP-1 and ASP-2. Furthermore, ASP-3 had the highest contents of protein, sulfuric radical and uronic acid among the

polysaccharides tested. The average molecular weights of ASP-1, ASP-2 and ASP-3 were determined to be 48.7, 53.5 and 61.4 kDa, respectively. Besides these, the anticancer and antioxidant activities of ASP were evaluated. The results demonstrated that all polysaccharides exhibited a dose-dependent anticancer activity within the concentration range of 50–500 μg/ml. And ASP-3 possessed strong scavenging activities on superoxide radical, hydroxyl radical and DPPH radical. For antioxidant activity *in vivo*, crude ASP significantly prevented the increase of serum ALT and AST levels, reduced the formation of MDA and enhanced the activities of SOD and GSH-Px in carbon tetrachloride-induced liver injury mice. ASP could be a new source of natural antioxidants with potential value in health food and medicine.

Acknowledgement

This research is supported by the China state "12th Five-year Plan" Scientific and Technological Support Scheme (2011BAI01B02).

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