Carbohydrate Polymers 112 (2014) 63–72

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Purification, characterization and immunomodulatory effects of *Plantago depressa* polysaccharides

Hong Zhao[a,b], Qiuhong Wang[a,*], Yanping Sun[a], Bingyou Yang[a], Zhibin Wang[a], Guifang Chai[a], Yongzhou Guan[a], Weiguo Zhu[c], Zunpeng Shu[a], Xia Lei[a], Haixue Kuang[a,*]

[a] *Key Laboratory of Chinese Materia Medica, Heilongjiang University of Chinese Medicine, Ministry of Education, Harbin 150040, China*
[b] *Key Laboratory of Biological Medicine Preparations, Institute of Pharmacy, Jiamusi University, Jiamusi 154007, China*
[c] *Chemistry Department, Henan Key Laboratory of Chemical Biology and Organic Chemistry, Key Laboratory of Applied Chemistry of Henan Universities, Zhengzhou University, Zhengzhou 450052, China*

ARTICLE INFO

Article history:
Received 1 April 2014
Received in revised form 21 May 2014
Accepted 22 May 2014
Available online 2 June 2014

Keywords:
Plantago depressa
Acidic polysaccharide
Purification
Partial structure
Immunomodulatory effects

ABSTRACT

Four purified polysaccharide fractions from seeds of *Plantago depressa* (PDSP-1, PDSP-2, PDSP-3 and PDSP-4) were obtained by isolation and purification using DEAE-52 cellulose and Sephacryl S-400 HR chromatography. Basic physicochemical properties including molecular weight, chemical composition, FT-IR and glycosidic linkage of these fractions were investigated. They seemed to be homogeneous acidic protein-bound heteropolysaccharides with high molecular weight of over 1000 kDa and contained a lot more β-type glycosidic linkages than α-type. PDSP-3 mainly contained mannose, arabinose and fucose, and the others were rich in arabinose, fucose and galacturonic acid. The immunomodulatory effects of them were assessed by splenocyte proliferation index and production of NO and TNF-α from macrophages. They all showed significant immunomodulatory activities, and PDSP-3 presented the strongest effect. Their observed differences in biological activities were probably due to their structure differences. And monosaccharide compositions, linkage types and molecular weight may affect their immunomodulatory activities.

1. Introduction

In recent decades, numerous polysaccharides isolated from natural resources are relatively common with tremendous structural diversities and biological properties (Lee et al., 2013; Li et al., 2013; Schepetkin & Quinn, 2006; Shukla, Joshi, & Rawat, 2010; Skjåk-Bræk & Martinsen, 1991; Wasser, 2002). Owing to non-cytotoxic properties, these polysaccharides among naturally occurring substances have attracted extensive attention to be immunomodulators, exhibiting a variety of beneficial pharmacological effects, such as anti-tumor, anti-inflammatory and anti-atherosclerotic activities (Chen et al., 2004; Hirazumi & Furusawa, 1999; Takata, Yamamoto, Yanai, Konno, & Okubo, 2005; Thakur, Connellan, Deseo, Morris, & Dixit, 2011; Wong, Lai, & Cheung, 2011). A possible mechanism is that these polysaccharides bind to different receptors on innate immune cells, such as macrophages or other monocytes, to activate them to release pro-inflammatory factors, cytokines and chemokines, which help the host to constitute an intensive immune response (Thomas, Harding, & Moore, 2000; Wakshull et al., 1999).

Plantago depressa Willd. (also called "Pingcheqian" in China), an edible and medicinal herb, belongs to the genus *Plantago* of the family *Plantaginaceae*, widely distributed in China, Korea, Russia, Pakistan and India (Yan et al., 2009), according to Scientific Database of China Plant Species (2011). In particular, its dry mature seeds are very popular known in traditional Chinese medicine (TCM) to treat a wide variety of diseases especially edema, being recorded for the first time in *Shen Nong Ben Cao Jing* thousands of years ago (Hou J.P. and Jin Y., 2005). Currently in China, the genus *Plantago* contains various species for officinal use, of which *Plantago asiatical* and *P. depressa* are both the original plants of Semen Plantaginis recorded in National Pharmacopoeia Committee (2010) with the same activities, such as fever-reducing, diuretic, stranguria-treating, eyesight-improving and expectorant activities. So far, most literatures mainly concentrated on various chemical components, such as phenylethanoid glycosides, phenolics, iridoids and various polysaccharides, from *P. asiatical* more than *P. depressa* (Huang et al., 2009a; Hu, Nie, Min, & Xie, 2012; Kim, Lee, & Chang, 2009; Miyase et al., 1991; Ravn, Nishibe, Sasahara, & Xuebo, 1990; Ye, Hu, & Dai, 2011; Ye & Jiang, 2011; Yin, Nie, Zhou, Wan, & Xie,

* Corresponding authors. Tel.: +86 451 82193001; fax: +86 451 82110803.
E-mail addresses: qhwang668@sina.com (Q. Wang), hxkuang@hotmail.com (H. Kuang).

http://dx.doi.org/10.1016/j.carbpol.2014.05.069
0144-8617/

2010; Yin et al., 2012). In particular, polysaccharides from *P. asiatical* were reported to be very important effective components for their anti-oxidant, murine dendrite cell maturation-inducing, defecation-promoting, and short-chain fatty acid-producing activities (Huang et al., 2009a,b; Yin et al., 2010). However, frequency of using *P. depressa* decreases gradually especially in polysaccharide applications, which causes the waste of *P. depressa* resources. Up to now, little information of polysaccharides from seeds of *P. depressa* (PDSPs) was available especially regarding their purification, characterization and immunological activities.

Therefore, we report here the immunomodulatory activities of four polysaccharide fractions prepared from *P. depressa* seeds. Briefly, the purified fractions were characterized by chemical analysis, high performance liquid chromatography (HPLC), Fourier transform infrared spectroscopy (FT-IR), and gas chromatography–mass spectrometer (GC–MS). The immunomodulatory effects of PDSP fractions were evaluated by *in vitro* splenocyte proliferation assay, and determination of NO and TNF-α production in RAW 264.7 macrophage cells.

2. Materials and methods

2.1. Materials

The dry seeds of *P. depressa* were collected from Wuchang in Heilongjiang Province of China in March 2012 and identified in accordance with *Chinese Pharmacopeia* (Commision, 2010) by Prof. Zhenyue Wang of Heilongjiang University of Chinese Medicine. RPMI medium 1640 was obtained from Gibco-BRL (Gaithersburg, MD, USA). Penicillin and streptomycin were obtained from Sigma-Aldrich (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (Con A), 1-phenyl-3-methyl-5-pyrazolone(PMP), obtained from Beijing Reagent Plant (Beijing, China). DEAE-52 was purchased from Whatman International Ltd. (Maidstone, Kent, UK). DEAE-Sepharose Fast Flow and Sephacryl S-400 HR were from the Pharmacia Co. (Sweden). All other reagents were of the highest grade available.

2.2. Preparation of crude PDSPs

In order to remove lipids, the seeds of *P. depressa* (1.0 kg) were pre-extracted with 95% ethanol (10 L × 3) at 75 °C for 2 h. Then the dried residue was extracted with 10 times of distilled water (100 °C, 2 h, 3×) and filtered. After removal of the residue by filtration, the filtrate was deproteinated by the method of Sevag, Lackman, and Smolens (1938). The resulting aqueous solution was extensively dialyzed (cut-off Mw 3500 Da) against tap water for 48 h and distilled water for 48 h. The solution in the dialysis tube was then concentrated and precipitated with 95% ethanol to a final concentration of 85% (v/v) and then incubated at 4 °C overnight. After centrifugation (3000 rpm, 15 min, 20 °C), the precipitate was washed with anhydrous ethanol and acetone in turn, and then lyophilized to obtain the crude polysaccharides (PDSPs, 124.6 g).

2.3. Isolation and purification of crude PDSPs

The crude PDSPs were sequentially purified by chromatography of DEAE-52 and Sephacryl S-400 according to the previously reported method (Kuang, Xia, Yang, Wang, & Wang, 2011; Xu, Zhang, Zhang, & Chen, 2007) with slight modifications. PDSPs (8 g) was dissolved in distilled H_2O and passed through two series connected resin columns (Amberlite FPA90-Cl (Cl^- form), 8.5 × 80 cm, i.d.) and Amberlite IRC-84 (H^+ form), 8.5 × 80 cm, i.d.) eluting with distilled H_2O and 0.5 M NaCl solution to yield fractions Fr. A (3.5 g) and Fr. B (2.3 g). Fr. A (3 g) was applied to DEAE-52 cellulose column (5 × 70 cm, i.d.) eluted with water, 0.1, 0.2, 0.3 and 0.4 M NaCl solution to yield subfractions Fr. A-1 (1.19 g), Fr. A-2 (1.03 g), Fr. A-3 (0.51 g)and Fr. A-4 (0.22 g), of which Fr. A-1 and Fr. A-2 was further purified by Sephacryl S-400 eluting with water to afford PDSP-1 (960 mg) and PDSP-2 (836 mg) (Fig. 1A–C). Fr. B (2 g) was also chromatographed over DEAE-52 cellulose column (5 × 70 cm, i.d.) eluted with water, 0.1 M, 0.2 M and 0.3 M NaCl solution to afford Fr. B-1 (1.09 g) and Fr. B-2 (0.81 g) (Fig. 1D), which were selected to be further purified by Sephacryl S-400 eluted with water to afford PDSP-3 (912 mg) and PDSP-4 (668 mg) (Fig. 1E and F). Each fraction was collected at a flow rate of 2 mL/min and monitored by the phenol–sulfuric acid method at 490 nm and spectrophotometer method at 280 nm.

2.4. Homogeneity and molecular weight determination

The homogeneity and the molecular weight of these four PDSP fractions were determined using high performance gel permeation chromatography (HPGPC) with ELSD detection on a Shodex sugar KS-805+ guard column KS-G (Shodex, Japan) (300 × 7.8 mm). The molecular weights of the PDSP fractions were estimated by referencing a calibration curve, and Dextran T-series (T-10, T-40, T50, T-70, T-130, T-500 and T-2000) were used as calibration standards.

2.5. Determination of contents of carbohydrate, protein and uronic acid

The total carbohydrate content of each PDSP fraction was determined according to the phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The protein content was determined according to Bradford method (Bradford, 1976). The uronic acid content was determined according to the carbazole–sulfuric acid method (Bitter & Muir, 1962).

2.6. Monosaccharide analysis

Monosaccharide compositions of purified PDSP fractions were measured using Reverse-phase HPLC as described previously (Dai et al., 2010; Lv et al., 2009). Ten milligram of each polysaccharide sample was dissolved in 2 mL of 2 M trifluoroacetic acid (TFA). The monosaccharides were conventionally converted into their PMP derivatives and analyzed.

2.7. Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectra of purified PDSP fractions were recorded with a FTIR spectrophotometer (FTIR-8400S, Shimadzu Co., Japan) using KBr disks method. The dried sample was ground with spectroscopic grade potassium bromide (KBr) powder and then pressed into 1 mm pellets and the spectra were recorded in a transmittance mode from 4500 to 400 cm^{-1}.

2.8. Determination of glycosidic linkages

Glycosidic linkage information was elucidated by methylation analysis, carried out by three steps, including hydrolysis, reduction and acetylation, according to Kim and Carpita (1992). The products obtained from the methylation process were analyzed by GC–MS on an Agilent 7890A instrument equipped with a HP-5 fused-silica capillary column (30 m × 0.32 mm × 0.25 μm) and an Agilent 5975C MS detector.

2.9. Splenocyte proliferation assay

The splenocyte proliferation assay was carried out using MTT-based colorimetric assay according to the reported method with slight modifications (Luo et al., 2009). The cell suspension was

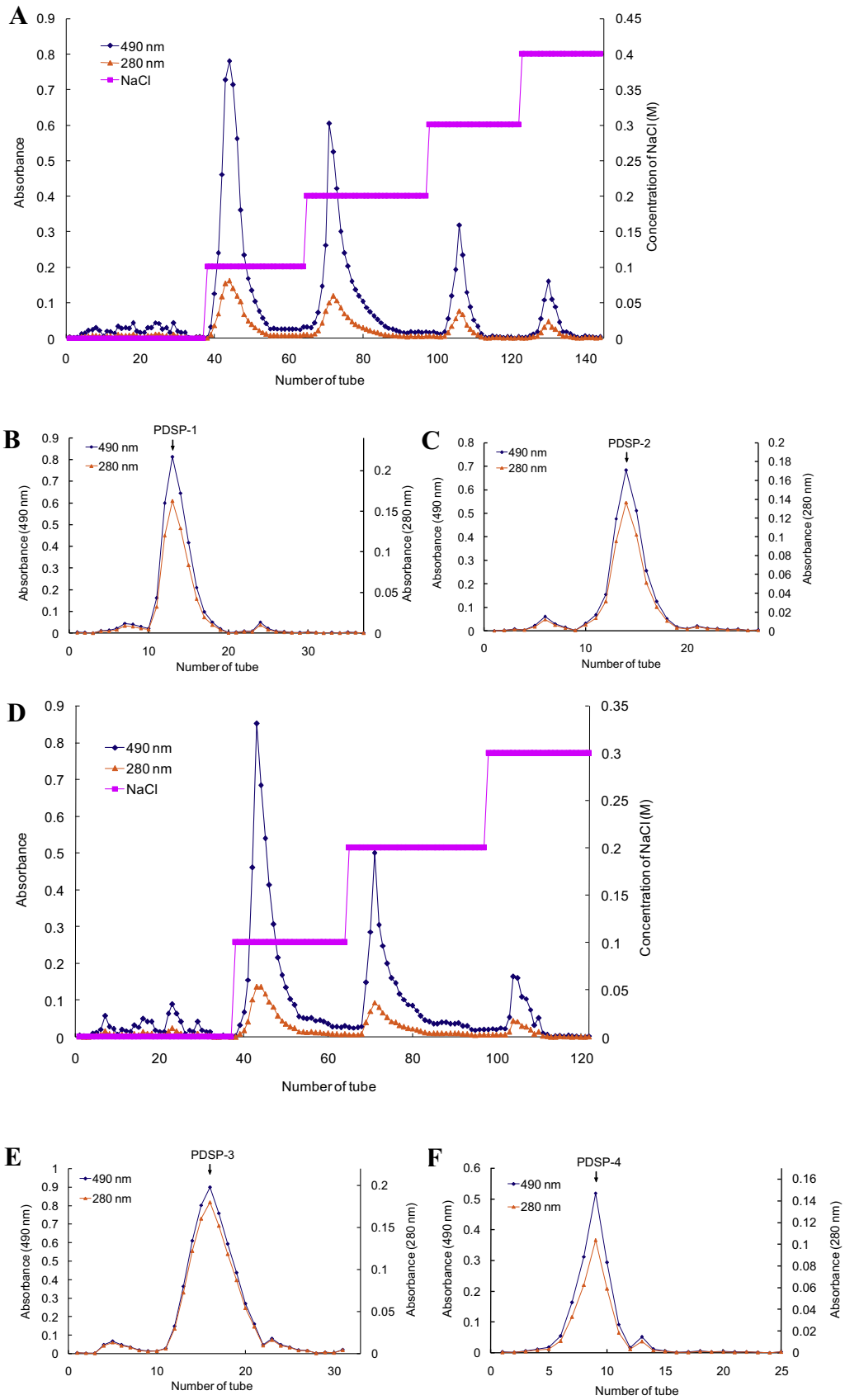

Fig. 1. Ion exchange chromatography (DEAE-52 cellulose) of Fr. A, eluted with distilled water and NaCl (0–0.4 M) (A); elution profile of PDSP-1 and PDSP-2 on Sephacryl S-400 column, respectively, eluted with distilled water (B and C); ion exchange chromatography (DEAE-52 cellulose) of Fr. B, eluted with distilled water and NaCl (0–0.3 M) (D); elution profile of PDSP-3 and PDSP-4 on Sephacryl S-400 column, respectively, eluted with distilled water (E and F).

planted in the 96-well culture plate with or without ConA (5.0 μg/mL) or LPS (10.0 μg/mL). Samples (at 5, 25 and 125 μg/mL concentrations) were added to each cell. The plates were set up for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. After 44 h, 20 μL of MTT solution (5 mg/mL) were added to each well and incubated for another 4 h. After aspirating the supernatant from the wells, 100 μL of dimethylsulfoxide (DMSO) was added to dissolve the formazan crystals, and the absorbance was evaluated in an ELISA reader (Bio-Rad, USA) at 570 nm after 15 min. The splenocyte proliferation index (SPI) was calculated as the absorbance value for mitogen-cultures divided by the absorbance value for non-stimulated cultures.

2.10. Measurement of NO and TNF-α production

Nitric oxide (NO) production was assayed by measuring the nitrite concentration in the supernatant of cultured macrophages using a colorimetric assay with Griess reagents as earlier described (Jung et al., 2008; Wang et al., 2009). Briefly, macrophage cells RAW 264.7 (5×10^5 cells/mL) were seeded in 96-well plate. Cells were left overnight for attachment and the purified PDSP sample was added to the wells in different concentrations (12.5, 25, 50, 100 or 200 g/mL). DMEM medium and LPS (10 μg/mL) were used as a blank and positive control, respectively. After incubation for 48 h, the culture supernatant was added to Griess Reagent in the ratio of 1:1 in a 96-well plate. After incubation for 15 min at room temperature, the absorbance was measured at 540 nm in an ELISA reader (Bio-Rad, USA). A serial dilution of nitrite ($NaNO_2$) was used as a standard reference curve. In addition, the levels of tumor necrosis factor-α (TNF-α) was also measured at 450 nm according to the instructions of commercial ELISA kits (BD Biosciences Pharmingen, San Diego, USA). Cytokine quantities in the samples were calculated from standard curves of recombinant cytokines using a regression linear method.

2.11. Statistical analysis

The data were expressed as mean ± standard deviation (SD) of three replications and evaluated by one-way analysis of variance (ANOVA) followed by the Duncan's multiple-range tests. *p*-Values of less than 0.05 were regarded as significant. All statistical analyses were performed using statistical software (SPSS, Version 20.0).

3. Results and discussion

3.1. Homogeneity and molecular weight of PDSP fractions

The crude polysaccharides of *P. depressa* were obtained with the yield of 12.46% and two fractions (Fr. A and Fr. B) isolated using resin columns were further fractionated on DEAE-52 cellulose column with gradient elution to give four and three elution peaks, respectively: Fr. A-1, Fr. A-2, Fr. A-3, Fr. A-4, Fr. B-1 and Fr. B-2 (Fig. 1A and 1D). From the results, these fractions were all considered to be protein-bound polysaccharides due to the simultaneous maximum absorbance peaks at 490 nm (phenol–sulfuric acid assay) and 280 nm (spectrophotometer), according to He et al. (2013). Finally, four of these fractions were selected to be further purified on Sephacryl S400 column to obtain four sub-fractions namely PDSP-1, PDSP-2, PDSP-3 and PDSP-4, respectively. As detected by an ultraviolet (UV) spectrum at 490 nm and 280 nm, a single symmetrical absorbance peak was observed (Fig. 1B, C, E and F), which indicated that PDSP-1, PDSP-2, PDSP-3 and PDSP-4 were all thought to be homogeneous.

For further verify the homogeneity and determination of the molecular weight of PDSP-1 PDSP-2, PDSP-3 and PDSP-4, HPGPC with ELSD detection on a Shodex sugar KS-805+ guard column using dextran standards was employed. From HPGPC profile in Fig. 2, each purified polysaccharide appeared as a symmetrical sharp peak to prove its homogeneity, according to Chen et al. (2011) and Liu et al. (2007). Based on the calibration with standard dextrans, the average molecular weights of PDSP-1 PDSP-2, PDSP-3 and PDSP-4 were estimated to be 1698.1, 1240.4, 1495.5 and over 2000 kDa, respectively (Table 1).

3.2. Chemical compositions of PDSP fractions

The carbohydrate content, protein content, and monosaccharide compositions of four PDSP fractions were summarized in Table 1. From the results, they all showed high carbohydrate contents and the carbohydrate content of PDSP-3 was relatively higher than the other fractions. The total carbohydrate contents of PDSP-1, PDSP-2, PDSP-3 and PDSP-4 were determined to be 90.7%, 89.1%, 93.1% and 91.9%, respectively, with a purity of >99.8%, using the phenol–sulfuric acid method. Their protein contents were measured to be around 5%, and these PDSP fractions could be proved to be protein-bound polysaccharides because the Sevag method has been repeated many times to remove free proteins (Peng, Zhang, Zeng, & Xu, 2003; Peng, Zhang, Zeng, & Kennedy, 2005; Wang, Luo, & Liang, 2004). The content of uronic acid for PDSP-2 and PDSP-3 was relatively lower than that of PDSP-1 and PDSP-4. As seen from Table 1 and Supplementary Fig. S1, PDSP-1 was composed of mannose, galacturonic acid, arabinose and fucose, and arabinose was the main monosaccharide. PDSP-2 was composed of mannose, galacturonic acid, xylose, arabinose and fucose, and arabinose was the main monosaccharide. Being different monosaccharide composition from PDSP-1 and PDSP-2, PDSP-3 was composed of nine monosaccharides, which were mannose, ribose, glucuronic acid, galacturonic acid, glucose, galactose, xylose, arabinose and fucose, exhibiting an arabinomannan-like structure. From the results, galacturonic acid mainly existed in PDSP-1 (22.2%), whereas PDSP-3 consisted of small content (8.1%) of galacturonic acid. With the different amount of monosaccharides in PDSP-3, PDSP-4 was composed of mannose, glucuronic acid, galacturonic acid, glucose, galactose, xylose, arabinose and fucose.

3.3. FT-IR spectroscopy of PDSP fractions

FT-IR spectroscopy is typically used for the qualitative measurement of organic functional groups (Zhang et al., 2013). The FT-IR spectra were shown in Supplementary Fig. S2 with some differences in the intensity of bands. The absorption bands within the range of 3600–3000 cm^{-1}, 3000–2800 cm^{-1}, 1400–1200 cm^{-1} and 1200–700 cm^{-1} were the characteristic absorption peaks of polysaccharides. All the infrared spectra of these four fractions displayed a strong and broad absorption peak at 3404 cm^{-1} for characteristic of O—H and a weak C—H band at 2925 cm^{-1} (Wang, Chen, Jia, Tang, & Ma, 2012a). The weak absorption at 1544 cm^{-1}, corresponding to the bending vibration of the N—H (—CONH—), proved the presence of protein (Chen, Xie, Nie, Li, & Wang, 2008; Liu et al., 2012). The absorption peak at 1635 cm^{-1} and 1423 cm^{-1} could be assigned to the antisymmetric and symmetric COO— stretches, and the band at 1251 cm^{-1} was ascribed to O—H (—COOH) variable angle vibration, indicating that they were all acidic polysaccharides (Liu et al., 2012; Wu et al., 2013). PDSP-3 and PDSP-4 exhibited a stronger absorption peak at 1043 cm^{-1}, which indicated the pyranose ring (He et al., 2007; Zhao, Kan, Li, & Chen, 2005). There are two types of end carbon–glucoside bonds: α- and β-type glycosidic linkages. A characteristic absorption peak at 896 cm^{-1} was obviously observed, but the shoulder absorption peak at 771 cm^{-1} was almost imperceptible, which indicated that the four fractions contained many more β-type glycosidic linkages than α-type glycosidic linkages (Barker, Bourne, Stacey, & Whiffen,

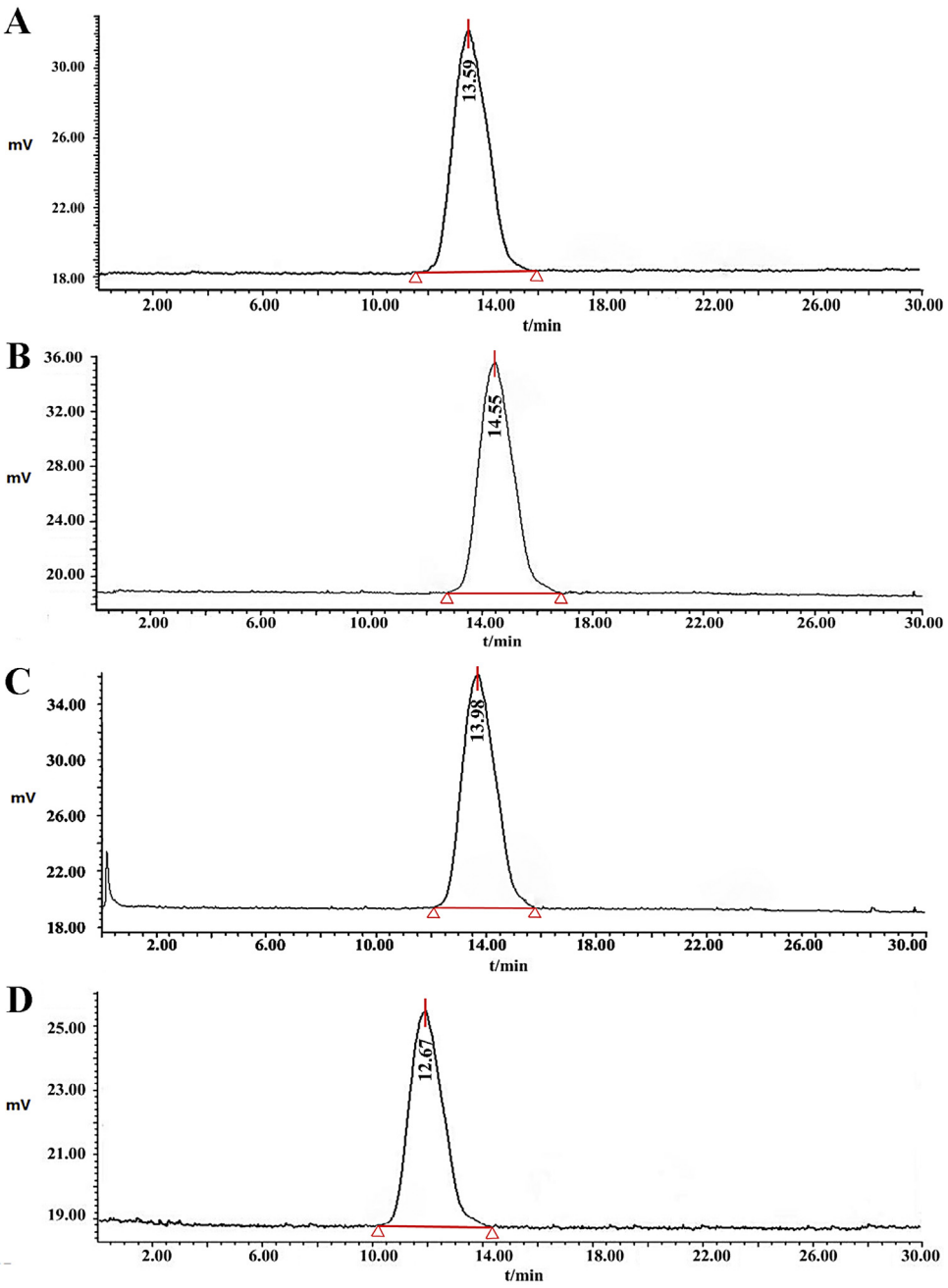

Fig. 2. HPGPC profiles of PDSP-1 (A), PDSP-2 (B), PDSP-3 (C) and PDSP-4 (D).

1954). The peak at 802 cm^{-1} was attributed to D-mannose in pyranose (Barker et al., 1954; Chen, Zhang, Jiang, Mu, & Miao, 2012), and PDSP-3 showed the strongest absorption, indicating more D-mannose.

3.4. Linkage analysis of PDSP fractions

Monosaccharide linkages were found in the four purified PDSP fractions by permethylation of the reduced polymers using

Table 1
Contents of carbohydrate, protein and uronic acid and monosaccharide compositions for four PDSP fractions from seeds of *P. depressa*.

Sample	Molecular weight (kDa)	Carbohydrate (%)	Protein (%)	Uronic acid (%)	Monosaccharide composition (%)								
					Man	Rib	GlcUA	GalUA	Glc	Gal	Xyl	Ara	Fuc
PDSP-1	1698.1	90.7	5.8	19.5	3.3	–[a]	–	22.2	–	–	–	45.8	28.7
PDSP-2	1240.4	89.1	3.5	12.4	2.8	–	–	15.5	–	–	8.4	55.7	17.6
PDSP-3	1495.5	93.1	6.8	10.1	28.1	0.8	3.4	8.1	1.4	4.2	10.1	27.9	16.0
PDSP-4	>2000	91.9	4.7	19.7	11.8	–	4.9	17.7	6.9	2.0	11.6	28.9	16.1

[a] Not detected.

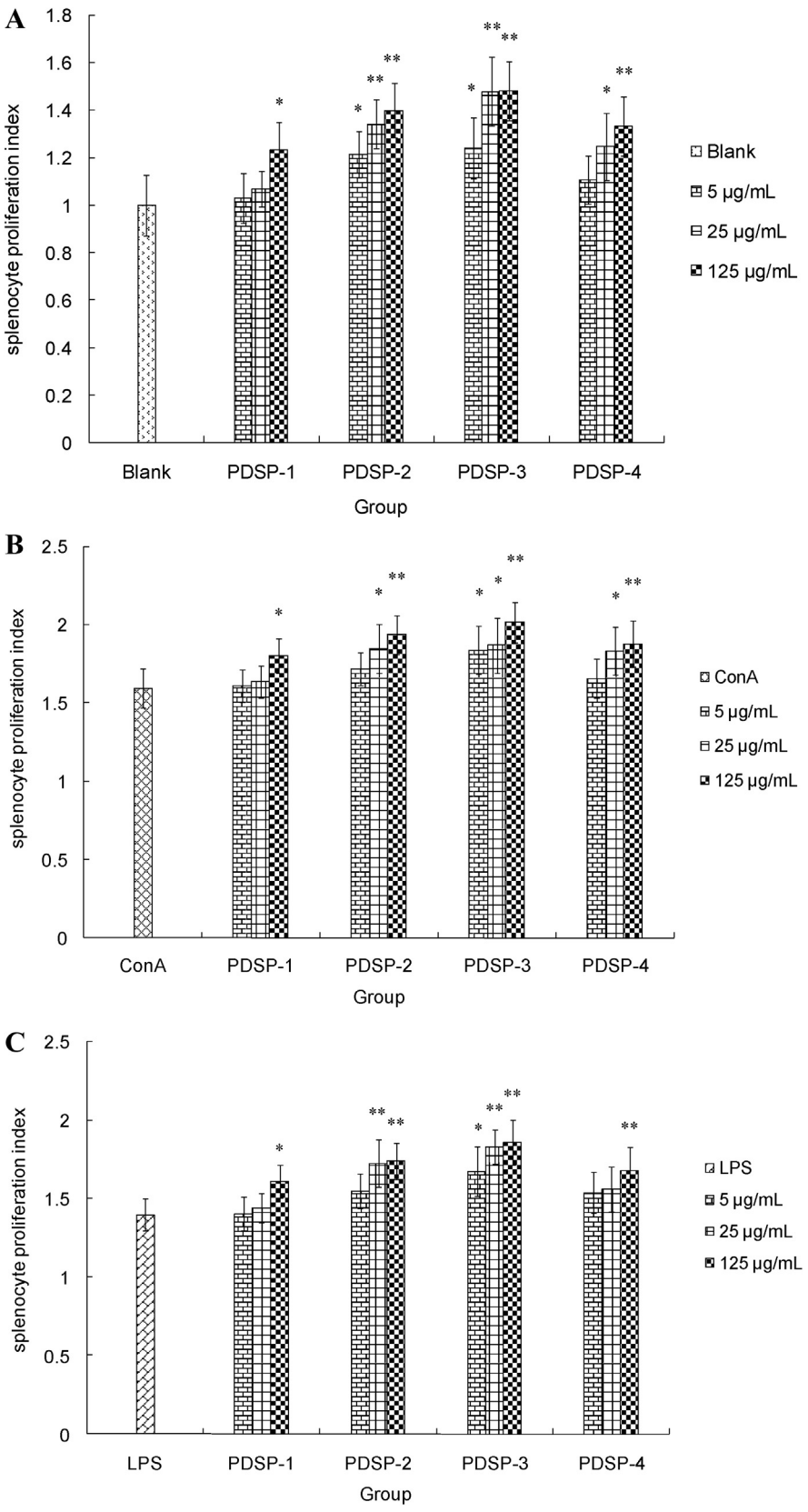

Fig. 3. *In vitro* effects of different concentrations of PDSP fractions on splenocyte proliferation (A), ConA-induced splenocyte proliferation (B), and LPS-induced splenocyte proliferation (C). The results were from three independent experiments and presented as the mean ± SD. * $p < 0.05$, ** $p < 0.01$ *vs.* blank control (A), ConA group (B) and LPS group (C), respectively.

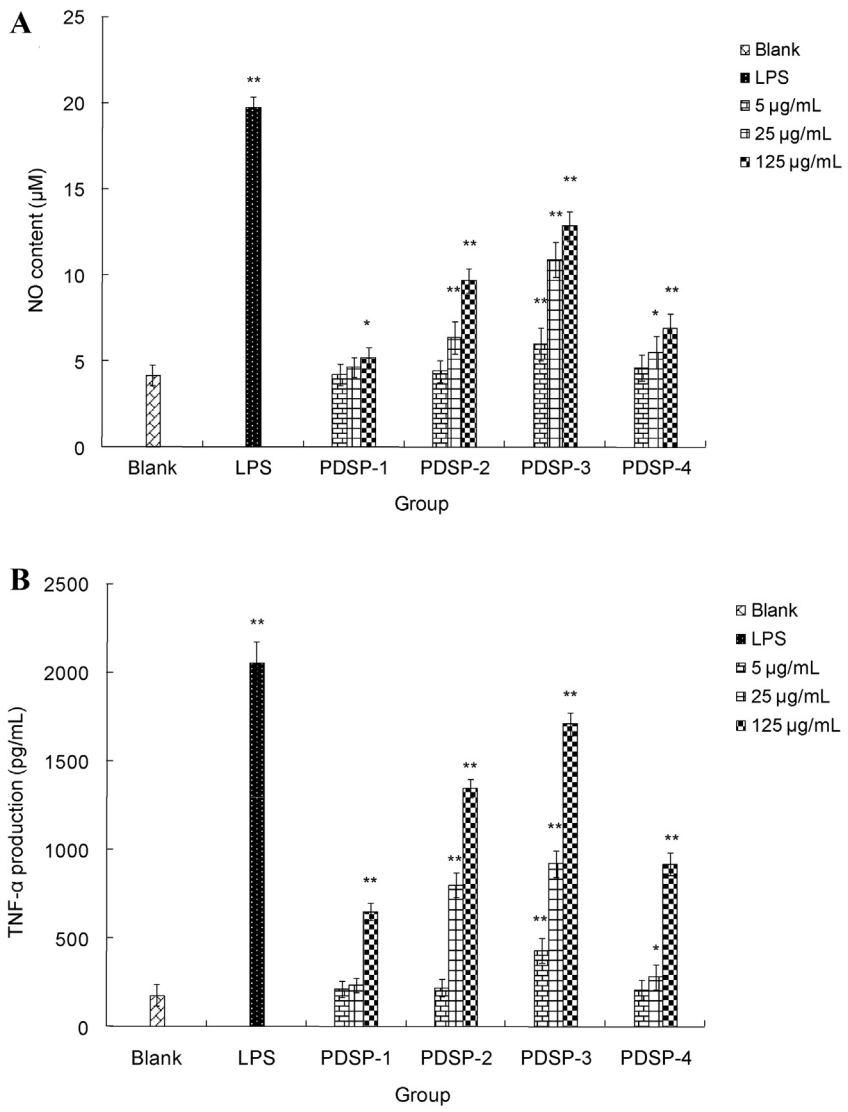

Fig. 4. *In vitro* activation of RAW 264.7 macrophages stimulated with different concentrations of PDSP fractions in terms of NO content (A) and TNF-α production (B). The results were from two independent experiments and presented as the mean ± SD. * $p < 0.05$, ** $p < 0.01$ *vs.* blank control.

GC–MS. The ratios of the linkages were further calculated based on the monosaccharide compositions seen from Table 1, areas of each methylated peak and effective carbon-response factors (Table 2). PDSP-1 may consist of a backbone of 1,3,6-linked Ara*f* and 1,3,4-linked Fuc*p*, and large content (20.1%) of uronic acid appearedmainly as terminally linked Gal*p*A unit. Unlikely to PDSP-1, PDSP-2 presented a linear molecular since around 41.4% of 1,5-linked Ara*f* appeared in the main chain, and only 5.1% of 1,3,4-linked Gal*p*A and 4.6% of 1,2,4-linked Fuc*p* appeared in the branches. PDSP-3 mainly contains 1,3-linked Ara*f* and 1,3-linked Man*p*, in addition to fair amounts of 1,3,4-linked Man*p* and 1-linked Fuc*p*. Interestingly, all fractions were found to contain mannoses as seen by the presence of 1,3-linked Man*p*, but the mannose residues existed in PDSP-3 as 1,3-linked Man*p* (12.3%), 1,3,4-linked Man*p* (10.5%) as well as 1-linked Man*p* (5.3%), most of which were not detected in the other fractions. The results indicated that the sugar residues 1,3-linked Ara*f*, 1,3-linked Man*p* and 1,3,4-linked Man*p* with fair amounts of 1-linked Fuc*p* formed the majority of the polysaccharide chain of PDSP-3. PDSP-4 presented as an acid polysaccharide, but unlikely to PDSP-1, uronic acid appeared in PDSP-4 mainly as 1,3,4-linked Gal*p*A units, and with smaller amounts of 1,4-linked Gal*p*A and 1,4-linked Glc*p*A. Besides, only 1,2-linked Ara*f* and 1,3,5-linked Ara*f* appeared in PDSP-4.

3.5. Immunomodulatory effects of PDSP fractions

Proliferation of splenocytes is an indicator of immunoactivation, being related to immunity improvement of T-lymphocyte or B-lymphocyte (Sarangi, Ghosh, Bhutia, Mallick, & Maiti, 2006; Wang et al., 2012b). Literatures have also reported that combination of the polysaccharide and its receptor on the surface of macrophages may increase the secretion of cytokines, such as NO and TNF-α, which can regulate both cellular and humoral immune responses (Schepetkin and Quinn, 2006). Effects of PDSP-1, PDSP-2, PDSP-3 and PDSP-4 on splenocyte proliferation with mitogens (ConA or LPS) and without mitogens were shown in Fig. 3. They could stimulate proliferation of spleen lymphocytes significantly ($p < 0.05$) with the increase of sample concentration ranging from 5 to 125 μg/mL. In particular, all the concentrations of PDSP-3 significantly increased lymphocyte proliferation, presenting the strongest promoting effect in the four fractions, not only in the presence of ConA or LPS as mitogens for lymphocytes, but also without mitogens (Fig. 3A–C). Furthermore, Supplementary Tables S1 and S2 demonstrated that PDSP-3 improved the splenocytes proliferation activity more noticeably co-treated with LPS than with ConA. As a whole, it was indicated that capacity of PDSP-3 was shown to increase the activate potential of T and B cells. It can be

Table 2
Monosaccharide linkages (mol%) of four PDSP fractions from seeds of *P. depressa* by methylation analysis using GC–MS.

	PDSP-1	PDSP-2	PDSP-3	PDSP-4
1,3-Linked Ara*f*	10.7	–[a]	17.2	11.0
1,3,6-Linked Ara*f*	24.0	–	3.1	–
1,6-Linked Ara*f*	11.2	–	–	–
1,5-Linked Ara*f*	–	41.4	–	–
1-Linked Ara*f*	–	14.9	7.6	–
1,2-Linked Ara*f*	–	–	–	8.0
1,3,5-Linked Ara*f*	–	–	–	10.0
1,3,4-Linked Galp*A*	–	5.1	4.2	12.5
1-Linked Galp*A*	20.1	–	–	–
1,4-Linked Galp*A*	1.9	–	3.9	4.9
1,3-Linked Galp*A*	–	10.6	–	–
1,3-Linked Man*p*	3.2	1.7	12.3	9.8
1,3,4-Linked Man*p*	–	–	10.5	2.0
1-Linked Man*p*	–	–	5.3	–
1,3,4-Linked Fuc*p*	28.7	–	5.8	6.8
1,3-Linked Fuc*p*	–	12.9	–	–
1,2,4-Linked Fuc*p*	–	4.6	–	–
1-Linked Fuc*p*	–	–	10.1	9.1
1,3-Linked Xyl*p*	–	8.2	8.4	6.5
1,2-Linked Xyl*p*	–	–	1.5	5.5
1,4-Linked Glcp*A*	–	–	3.4	4.8
1,3-Linked Gal*p*	–	–	0.2	–
1,3,6-linked Gal*p*	–	–	3.9	–
1-Linked Glc*p*	–	–	1.4	4.3
1,6-Linked Glc*p*	–	–	–	2.6

[a] Not detected.

seen from Fig. 4 that all the isolated PDSP fractions significantly activated macrophage NO content and TNF-α production in a dose-dependent manner. Very interestingly, PDSP-3 possesses strong immunomodulatory potential in both production of NO and TNF-α, leading to the conclusion that PDSP-3 is the most active *P. depressa* polysaccharide fraction to modulate the macrophage function and stimulate the immune system.

3.6. Discussion on structure-activity relationship

Nature-derived polysaccharides play an important role in activating various immune system responses in the host such as splenocyte and macrophage-dependent immune system responses (Lee et al., 2008). Macrophages activated by polysaccharides exert their antitumor action by secreting secondary compounds, such as nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α), by regulating the immune system to process and present antigens (Mackay & Pussell, 1986). From the results, we found that all the isolated PDSP fractions showed significant immunomodulatory effects with molecular weights larger than 1200 kDa, which might be proved by some reports that water-soluble polysaccharide fraction, with molecular weights larger than 100 kDa, exhibited immunoenhancing effects (Wang, Hu, Su, & Lee, 2001). Among all the fractions, PDSP-3 has the highest capacity for promoting the splenocytes proliferation, upregulating TNF-α expression and releasing NO, acting as an immunostimulant through the activation of splenocytes and macrophages. Because of the structural variability of polysaccharides with monosaccharide residues joined to each other by glycosidic linkages, it is highly important to determine the accurate structures, such as their chemical composition, molecular weight, conformation, glycosidic linkage (Yadomae & Ohno, 1996). It is worth noting that PDSP-3 was found to mainly contain arabinose and mannose (Table 1). For a long time, with similar structure of PDSP-3, arabinomannan (Z-100) has been recognized as a classical polysaccharide dictating the immunomodulatory activity (Oka et al., 1999, 2002, 2003, 2004). In addition, the bioactivity of PDSP-3 may also depend on the composition of (1 → 3)–linked mannopyranosyl which existed as a backbone in an acidic heteropolysaccharide (TAPA1) purified from *Tremella aurantialba* stimulating the proliferation of mouse spleen lymphocytes *in vitro* (Du et al., 2009). Besides, PDSP-2 also presented the higher immunomodulatory effects, which may be due to its main composition of 1,5-linked Ara*f* (41.4%) and that certain linkage characteristic was proved to contribute to immunomodulatory activity in some polysaccharides, according to Wang et al. (2007).

4. Conclusions

In this study, four bioactive polysaccharide fractions were isolated and purified from the hot water extract of *P. depressa* by DEAE-52 cellulose and Sephacryl S-400 HR column chromatography. They all exhibited homogeneity with high molecular weight, and many evidences demonstrated that these fractions were bounded with protein, presented as acid heteropolysaccharides. They mainly contained β-type glycosidic linkages, with arabinose, fucose and galacturonic acid except PDSP-3, which contained high content of mannose but low content of galacturonic acid. The results showed that PDSP-1, PDSP-2, PDSP-3 and PDSP-4 could significantly exhibit beneficial stimulating effects not only on splenocyte proliferation but also on NO and TNF-α secretion from RAW 267.4 macrophages *in vitro* in a dose-dependent manner, and PDSP-3 presented the strongest effect. PDSP-3, a high molecular mass homogeneous polysaccharide with an arabinomannan-like composition and the main linkage of 1,3-linked Ara*f* and 1,3-linked man*p*, would be a potent murine splenocyte and macrophage stimulator. The observed differences in biological activities among them were probably due to their structure differences such as monosaccharide compositions, linkage types and molecular weight. These studies indicated that *P. depressa* polysaccharides have immunomodulatory potential for further research. To entirely reveal the structure-activity relationship of this polysaccharide, mechanism studies in terms of signaling pathways on splenocytes and macrophages will be further investigated.

Acknowledgment

Our work was financially supported by Major State Basic Research Development Program (973 Program) of China (2013CB531801).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.05.069.

References

Barker, S. A., Bourne, E. J., Stacey, M., & Whiffen, D. H. (1954). Infra-red spectra of carbohydrates Part I. Some derivatives of D-glucopyranose. *Journal of the Chemical Society*, 171–176.
Bitter, T., & Muir, H. M. (1962). A modified uronic acid carbazole reaction. *Analytical Biochemistry*, *4*, 330–334.
Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254.
Chen, H. S., Tsai, Y. F., Lin, S., Lin, C. C., Khoo, K. H., Lin, C. H., & Wong, C. H. (2004). Studies on the immuno-modulating and anti-tumor activities of *Ganoderma lucidum* (Reishi) polysaccharides. *Bioorganic and Medicinal Chemistry*, *12*, 5595–5601.
Chen, J., Zhang, T., Jiang, B., Mu, W., & Miao, M. (2012). Characterization and antioxidant activity of *Ginkgo biloba* exocarp polysaccharides. *Carbohydrate Polymers*, *87*, 40–45.
Chen, R., Liu, Z., Zhao, J., Chen, R., Meng, F., Zhang, M., & Ge, W. (2011). Antioxidant and immunobiological activity of water-soluble polysaccharide fractions purified from *Acanthopanax senticosu*. *Food Chemistry*, *127*, 434–440.
Chen, Y., Xie, M. Y., Nie, S. P., Li, C., & Wang, Y. X. (2008). Purification, composition analysis and antioxidant activity of a polysaccharide from the fruiting bodies of *Ganoderma atrum*. *Food Chemistry*, *107*, 231–241.

Commision, C. P. (2010). *Pharmacopoeia of the People's Republic of China*. Beijing: China Medical Science Press.
Dai, J., Wu, Y., Chen, S. W., Zhu, S., Yin, H. P., Wang, M., & Tang, J. (2010). Sugar compositional determination of polysaccharides from *Dunaliella salina* by modified RP-HPLC method of precolumn derivatization with 1-phenyl-3-methyl-5-pyrazolone. *Carbohydrate Polymers*, *82*, 629–635.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. T., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.
Du, X., Zhang, J., Yang, Y., Ye, L., Tang, Q., Jia, W., Liu, Y., Zhou, S., Hao, R., Gong, C., & Pan, Y. (2009). Structural elucidation and immuno-stimulating activity of an acidic heteropolysaccharide (TAPA1) from *Tremella aurantialba*. *Carbohydrate Research*, *344*, 672–678.
He, L., Ji, P., Cheng, J., Wang, Y., Qian, H., Li, W., Gong, X., & Wang, Z. (2013). Structural characterization and immunostimulatory activity of a novel protein-bound polysaccharide produced by *Hirsutella sinensis* Liu, Guo, Yu & Zeng. *Food Chemistry*, *141*, 946–953.
He, Y., Liu, C., Chen, Y., Ji, A., Shen, Z., Xi, T., et al. (2007). Isolation and structural characterization of a novel polysaccharide prepared from *Arca subcrenata* Lischke. *Journal of Bioscience and Bioengineering*, *104*, 111–116.
Hirazumi, A., & Furusawa, E. (1999). An immunomodulatory polysaccharide-rich substance from the fruit juice of *Morinda citrifolia* (noni) with antitumour activity. *Phytotherapy Research*, *13*, 380–387.
Hou, J. P., & Jin, Y. (2005). *The healing power of Chinese herbs and medicinal recipes*. New York: Psychology Press.
Hu, J. L., Nie, S. P., Min, F. F., & Xie, M. Y. (2012). Polysaccharide from seeds of *Plantago asiatica* L. increases short-chain fatty acid production and fecal moisture along with lowering pH in mouse colon. *Journal of Agricultural and Food Chemistry*, *60*, 11525–11532.
Huang, D. F., Tang, Y. F., Nie, S. P., Wan, Y., Xie, M. Y., & Xie, X. M. (2009). Effect of phenylethanoid glycosides and polysaccharides from the seed of *Plantago asiatica* L. on the maturation of murine bone marrow-derived dendritic cells. *European Journal of Pharmacology*, *620*, 105–111.
Huang, D. F., Xie, M. Y., Yin, J. Y., Nie, S. P., Tang, Y. F., Xie, X. M., & Zhou, C. (2009). Immunomodulatory activity of the seeds of *Plantago asiatica* L. *Journal of Ethnopharmacology*, *124*, 493–498.
Jung, W. K., Choi, I., Lee, D. Y., Yea, S. S., Choi, Y. H., Kim, M. M., Park, S. G., Seo, S. K., Lee, S. W., Lee, C. M., Park, Y. M., & Choi, I. W. (2008). Caffeic acid phenethyl ester protects mice from lethal endotoxin shock and inhibits lipopolysaccharide-induced cyclooxygenase-2 and inducible nitric oxide synthase expression in RAW 264.7 macrophages via the p38/ERK and NF-κB pathways. *The International Journal of Biochemistry & Cell Biology*, *40*, 2572–2582.
Kim, B. H., Lee, N. K., & Chang, I. M. (2009). Simultaneous analysis of a mixture of iridoids in water extracts of *Plantago asiatica* L. by LC. *Chromatographia*, *69*, 1397–1400.
Kim, J. B., & Carpita, N. C. (1992). Changes in esterification of the uronic-acid groups of cell-wall polysaccharides during elongation of maize coleoptiles. *Plant Physiology*, *98*, 646–653.
Kuang, H., Xia, Y., Yang, B., Wang, Q., & Wang, Y. (2011). Screening and comparison of the immunosuppressive activities of polysaccharides from the stems of *Ephedra sinica* Stapf. *Carbohydrate Polymers*, *83*, 787–795.
Lee, J. S., Synytsya, A., Kim, H. B., Choi, D. J., Lee, S., Lee, J., Kim, W. J., Jang, S., & Park, Y. I. (2013). Purification, characterization and immunomodulating activity of a pectic polysaccharide isolated from Korean mulberry fruit Oddi (*Morus alba* L.). *International Immunopharmacology*, *17*, 858–866.
Lee, J. Y., Kim, J. Y., Lee, Y. G., Rhee, M. H., Hong, E. K., & Cho, J. Y. (2008). Molecular mechanism of macrophage activation by exopolysaccharides from liquid culture of *Lentinus edodes*. *Journal of Microbiology and Biotechnology*, *2*, 355–364.
Li, Q., Yu, N., Wang, Y., Sun, Y., Lu, K., & Guan, W. (2013). Extraction optimization of *Bruguiera gymnorrhiza* polysaccharides with radical scavenging activities. *Carbohydrate Polymers*, *96*, 148–155.
Liu, C., Lin, Q., Gao, Y., Ye, L., Xing, Y., & Xi, T. (2007). Characterization and antitumor activity of a polysaccharide from *Strongylocentrotus nudus* eggs. *Carbohydrate Polymers*, *67*, 313–318.
Liu, H., Fan, Y., Wang, W., Liu, N., Zhang, H., Zhu, Z., & Liu, A. (2012). Polysaccharides from *Lycium barbarum* leaves: Isolation, characterization and splenocyte proliferation activity. *International Journal of Biological Macromolecules*, *51*, 417–422.
Luo, J., Liu, J., Ke, C., Qiao, D., Ye, H., Sun, Y., & Zeng, X. (2009). Optimization of medium composition for the production of exopolysaccharides from *Phellinus baumii* Pilát in submerged culture and the immuno-stimulating activity of exopolysaccharides. *Carbohydrate Polymers*, *78*, 409–415.
Lv, Y., Yang, X., Zhao, Y., Ruan, Y., Yang, Y., & Wang, Z. (2009). Separation and quantification of component monosaccharides of the tea polysaccharides from *Gynostemma pentaphyllum* by HPLC with indirect UV detection. *Food Chemistry*, *112*, 742–746.
Mackay, R. J., & Pussell, S. W. (1986). Protein changes associated with stages of activation of mouse macrophages for tumor cell killing. *The Journal of Immunology*, *137*, 1392–1398.
Miyase, T., Ishino, M., Akahori, C., Ueno, A., Ohkawa, Y., & Tanizawa, H. (1991). Phenylethanoid glycosides from *Plantago asiatica*. *Phytochemistry*, *30*, 2015–2018.
National Pharmacopoeia Committee. (2010). *Chinese pharmacopoeia*. Beijing: China Medical Science Press.
Oka, H., Emori, Y., Ohya, O., Kobayashi, N., Sasaki, H., Tanaka, Y., Hayashi, Y., & Nomoto, K. (1999). An immunomodulatory arabinomannan extracted from *Mycobacterium tuberculosis* Z-100, restores the balance of Th1/Th2 cell responses in tumor bearing mice. *Immunology Letters*, *70*, 109–117.
Oka, H., Sasaki, H., Shiraishi, Y., Emori, Y., Yoshinaga, K., & Takei, M. (2004). Z-100, an immunomodulatory arabinomannan extracted from *Mycobacterium tuberculosis* strain Aoyama B, augments anti-tumor activities of X-ray irradiation against B16 melanoma in association with the improvement of type 1 T cell responses. *Biological and Pharmaceutical Bulletin*, *27*, 82–88.
Oka, H., Shiraishi, Y., Sasaki, H., Yoshinaga, K., Emori, Y., & Takei, M. (2003). Antimetastatic effect of an immunomodulatory arabinomannan extracted from *Mycobacterium tuberculosis* strain Aoyama B, Z-100, through the production of interleukin-12. *Biological and Pharmaceutical Bulletin*, *26*, 1336–1341.
Oka, H., Emori, Y., Sasaki, H., Shiraishi, Y., Yoshinaga, K., & Kurimoto, T. (2002). Antitumor mechanism of Z-100, an immunomodulatory arabinomannan extracted from *Mycobacterium tuberculosis* strain Aoyama B, on pulmonary metastases of B16F10 melanoma: Restoration of helper T cell responses via suppression of glucocorticoid-genesis. *Microbiology and Immunology*, *46*, 343–351.
Peng, Y., Zhang, L., Zeng, F., & Kennedy, J. F. (2005). Structure and antitumor activities of the water-soluble polysaccharides from *Ganoderma tsugae* mycelium. *Carbohydrate Polymers*, *59*, 385–392.
Peng, Y., Zhang, L., Zeng, F., & Xu, Y. (2003). Structure and antitumor activity of extracellular polysaccharides from mycelium. *Carbohydrate Polymers*, *54*, 297–303.
Ravn, H., Nishibe, S., Sasahara, M., & Xuebo, L. (1990). Phenolic compounds from *Plantago asiatica*. *Phytochemistry*, *29*, 3627–3631.
Sarangi, I., Ghosh, D., Bhutia, S. K., Mallick, S. K., & Maiti, T. K. (2006). Anti-tumor and immunomodulating effects of *Pleurotus ostreatus* mycelia-derived proteoglycans. *International Immunopharmacology*, *6*, 1287–1297.
Scientific Database of China Plant Species. (2011). *Scientific Database of China Plant Species*. Available online: ⟨http://db.kib.ac.cn/eflora/default.aspx⟩ (accessed on 23 October 2011)
Schepetkin, I. A., & Quinn, M. T. (2006). Botanical polysaccharides: Macrophage immunomodulation and therapeutic potential. *International Immunopharmacology*, *6*, 317–333.
Sevag, M. G., Lackman, D. B., & Smolens, J. (1938). The isolation of the components of streptococcal nucleoproteins in serologically active form. *Journal of Biological Chemistry*, *124*, 425–436.
Shukla, V., Joshi, G. P., & Rawat, M. S. M. (2010). Lichens as a potential natural source of bioactive compounds: A review. *Phytochemistry Reviews*, *9*, 303–314.
Skjåk-Bræk, G., & Martinsen, A. N. I. T. A. (1991). Applications of some algal polysaccharides in biotechnology. In *Seaweed resources in Europe: Uses and potentials*. New York, NY: Wiley.
Takata, R., Yamamoto, R., Yanai, T., Konno, T., & Okubo, T. (2005). Immunostimulatory effects of a polysaccharide-rich substance with antitumor activity isolated from black currant (*Ribes nigrum* L.). *Bioscience Biotechnology and Biochemistry*, *69*, 2042–2050.
Thakur, M., Connellan, P., Deseo, M. A., Morris, C., & Dixit, V. K. (2011). Immunomodulatory polysaccharide from *Chlorophytum borivilianum* roots. *Evidence-based Complementary and Alternative Medicine (eCAM)*, *8*, 598521.
Thomas, A., Harding, K. G., & Moore, K. (2000). Alginates from wound dressings activate human macrophages to secrete tumour necrosis factor-α. *Biomaterials*, *21*, 1797–1802.
Wakshull, E., Brunke-Reese, D., Lindermuth, J., Fisette, L., Nathans, R. S., Crowley, J. J., Tufts, J. C., Zimmerman, J., Mackin, W., & Adams, D. S. (1999). PGG-glucan, a soluble β-(1,3)-glucan, enhances the oxidative burst response, microbicidal activity, and activates an NF-κB-like factor in human PMN: Evidence for a glycosphingolipid β-(1,3)-glucan receptor. *Immunopharmacology*, *41*, 89–107.
Wang, J. C., Hu, S. H., Su, C. H., & Lee, T. M. (2001). Antitumor and immunoenhancing activities of polysaccharide from culture broth of Hericium spp. *The Kaohsiung Journal of Medical Sciences*, *17*, 461–467.
Wang, M., Zhu, P., Jiang, C., Ma, L., Zhang, Z., & Zeng, X. (2012). Preliminary characterization, antioxidant activity in vitro and hepatoprotective effect on acute alcohol-induced liver injury in mice of polysaccharides from the peduncles of *Hovenia dulcis*. *Food and Chemical Toxicology*, *50*, 2964–2970.
Wang, R., Chen, P., Jia, F., Tang, J., & Ma, F. (2012). Optimization of polysaccharides from *Panax japonicus* C.A. Meyer by RSM and its anti-oxidant activity. *International Journal of Biological Macromolecules*, *50*, 331–336.
Wang, S. C., Shi, S. S., Cui, J., Ding, L. L., Wang, Z. T., & Hu, Z. B. (2007). Isolation and structure analysis of a hetero-polysaccharide from *Angelica sinensis* (Oliv.) Diels. *Chinese Pharmaceutical Journal*, *42*, 1255–1258.
Wang, Z., Cheng, X., Xu, H., Yue, X., Li, H., Zhang, Y., & Chen, D. (2009). Effects of *Bupleurum smithii* var. parvifolium total polysaccharides on immune functions in mice. *Chinese Journal of Clinical Pharmacy*, *4*, 1.
Wang, Z., Luo, D., & Liang, Z. (2004). Structure of polysaccharides from the fruiting body of *Hericium erinaceus* Pers. *Carbohydrate Polymers*, *57*, 241–247.
Wasser, S. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology*, *60*, 258–274.
Wong, K. H., Lai, C. K., & Cheung, P. C. (2011). Immunomodulatory activities of mushroom sclerotial polysaccharides. *Food Hydrocolloids*, *25*, 150–158.
Wu, H., Min, T., Li, X., Li, L., Lai, F., Tang, Y., & Yang, X. (2013). Physicochemical properties and antioxidant activities of acidic polysaccharides from wampee seeds. *International Journal of Biological Macromolecules*, *59*, 90–95.
Xu, H., Zhang, Y., Zhang, J., & Chen, D. (2007). Isolation and characterization of an anti-complementary polysaccharide D3-S1 from the roots of *Bupleurum smithii*. *International Immunopharmacology*, *7*, 175–182.

Yadomae, T., & Ohno, N. (1996). Structure–activity relationship of immunomodulating (1-3)-β-D-glucans. *Recent Res. Dev. Chem. Pharm. Sci.*, *1*, 23–33.

Yan, P. F., Liu, G. Y., Zhao, S. M., Song, L. L., Tan, L. N., Jin, Y. R., & Li, X. W. (2009). Studies on chemical constituents of *Plantago depressa* Willd. *Chinese Pharmaceutical Journal*, *44*, 19–21.

Ye, C. L., & Jiang, C. J. (2011). Optimization of extraction process of crude polysaccharides from *Plantago asiatica* L. by response surface methodology. *Carbohydrate Polymers*, *84*, 495–502.

Ye, C. L., Hu, W. L., & Dai, D. H. (2011). Extraction of polysaccharides and the antioxidant activity from the seeds of *Plantago asiatica* L. *International Journal of Biological Macromolecules*, *49*, 466–470.

Yin, J. Y., Nie, S. P., Zhou, C., Wan, Y., & Xie, M. Y. (2010). Chemical characteristics and antioxidant activities of polysaccharide purified from the seeds of *Plantago asiatica* L. *Journal of the Science of Food and Agriculture*, *90*, 210–217.

Yin, J., Lin, H., Li, J., Wang, Y., Cui, S. W., Nie, S., & Xie, M. (2012). Structural characterization of a highly branched polysaccharide from the seeds of *Plantago asiatica* L. *Carbohydrate Polymers*, *87*, 2416–2424.

Zhang, Z., Lv, G., He, W., Shi, L., Pan, H., & Fan, L. (2013). Effects of extraction methods on the antioxidant activities of polysaccharides obtained from *Flammulina velutipes*. *Carbohydrate Polymers*, *98*, 1524–1531.

Zhao, G., Kan, J., Li, Z., & Chen, Z. (2005). Structural features and immunological activity of a polysaccharide from *Dioscorea opposita* Thunb roots. *Carbohydrate Polymers*, *61*, 125–131.