Carbohydrate Polymers 83 (2011) 787–795

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Screening and comparison of the immunosuppressive activities of polysaccharides from the stems of *Ephedra sinica* Stapf

Haixue Kuang*, Yonggang Xia, Bingyou Yang, Qiuhong Wang, Yanhong Wang

Key Laboratory of Chinese Materia Medica (Heilongjiang University of Chinese Medicine), Ministry of Education, Harbin 150040, China

ARTICLE INFO

Article history:
Received 4 April 2010
Received in revised form 19 August 2010
Accepted 23 August 2010
Available online 27 August 2010

Keywords:
Ephedra sinica Stapf
Mahuang
Polysaccharide
Immunosuppressive effects
Chemical composition

ABSTRACT

Three polysaccharide fractions A, B and C (PA, PB and PC) were acquired from the stems of *Ephedra sinica* Stapf by sequential extraction with cold water, hot water, and 1.0 M NaOH, respectively. Immunosuppressive activities of PA, PB and PC were investigated by carbon clearance test, delayed-type hypersensitivity reaction and humoral immune response in vivo. Then PB was further isolated to afford four purified polysaccharides (ESP-B1, ESP-B2, ESP-B3 and ESP-B4) by various ion exchange and gel-filtration chromatography. Meanwhile, the purified polysaccharides were subjected to composition analysis and valuated for mice splenocyte proliferation activity in vitro. Among four purified polysaccharides, ESP-B4 has the highest inhibitory effect on splenocyte proliferation and its branches are extremely important for the expression of the inhibitory effect. The results in our study indicate that PB and ESP-B4 have therapeutic potential for the treatment of autoimmune and atopic diseases due to their immunosuppressive effects.

1. Introduction

The discovery of highly effective immunosuppressive agents has greatly contributed to the progress of organ transplantation (Sigal & Dumont, 1992). Meanwhile, some immunosuppressants, including steroids, penicillamine and methotrexate, have been shown to be useful for the treatment of various autoimmune and atopic diseases, however, severe side effects have often been observed (Leaker & Cairns, 1994). Thus, it is of great significance to search for a novel class of immunosuppressive compounds with low toxicity and few side effects.

Ephedra sinica Stapf is the main botanical origin of the Chinese herbal drug Mahuang or Ephedra herb, which has been used for more than 5000 years and distributed mainly in Shanxi, Jilin, Liaoning and Inner Mongolia provinces of China (Jiangsu New Medical College, 1986). It is contained in various herbal preparations and has been utilized for respiratory, antitussive, central nervous system stimulant, antipyretic and anti-inflammatory (Jiang, Lv, & Wang, 2007; Trujillo & Sorenson, 2003). In particular, the stimulant effects of Mahuang are linked to presence in aerial parts of the plant of six alkaloids: ephedrine, pseudoephedrine, methylephedrine, methylpseudo-ephedrine, norephedrine and norpseudoephedrine (Ganzera, Lanser, & Stuppner, 2005). In April 2004 the Food and Drug Administration prohibited the sale of *Ephedra* containing dietary supplements in the US (U.S. Food and Drug Administration, 2004). Using these products for longer periods of time (e.g. for weight loss) can cause serious adverse health outcomes (heart ailments, strokes); alkaloids are responsible for these side effects (Dunnick, Kissling, Gerken, Vallant, & Nyska, 2007; Marchei, Pellegrini, Pacifici, Zuccaro, & Pichini, 2006), which greatly limits the application of this medicinal plant.

Though ephedrine alkaloids have for a long time been considered as the pharmacologically active ingredients of *E. sinica* for treatment of various diseases and symptoms, they cannot account for all the effects mentioned above and the water-soluble polysaccharides may be one of the main bioactive constituents of *E. sinica* (Cheng, Zhu, & Xu, 2001; Ding, Shi, Cui, Wang, & Wang, 2006; Konno, Mizuno, & Hikino, 1985). In fact, the roles of water-soluble polysaccharides from TCM in biological processes have been studied with increasing attention over the past recent years because of their broad spectrum of therapeutic properties and relatively low toxicity (Chen, Zhang, & Zhang, 2007; Fang & Ding, 2007; Schepetkin & Quinn, 2006). Indeed, immunomodulation, anti-tumor, anti-virus, anti-complementary, anticoagulant hypoglycaemic, anti-inflammatory and antioxidation bioactivities have been presented by many polysaccharides extracted from medicinal fungi and plants (Chen et al., 2007; Fang & Ding, 2007; Hsieh et al., 2008; Liu, Wang, Xu, & Wang, 2007; Schepetkin & Quinn, 2006; Yu et al., 2007). However, there are few scientific reports available in the literature on the isolation, purification, and bioactivity determination of polysaccharides from *E. sinica*, especially detailed studies of the immunosuppressive effects and

* Corresponding author. Tel.: +86 45182193001; fax: +86 45182110803.
E-mail address: hxkuang@hotmail.com (H. Kuang).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.08.056

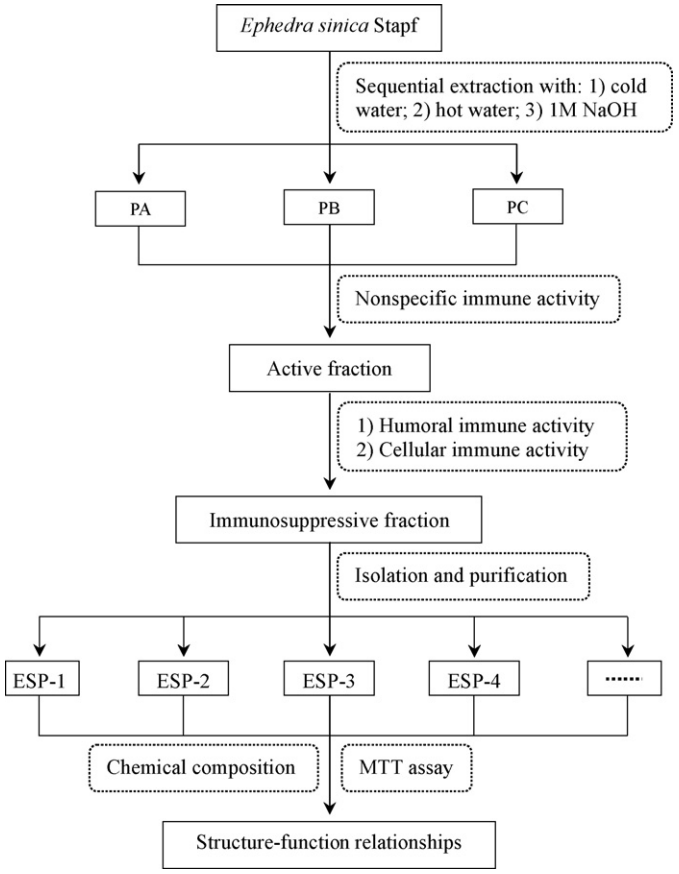

Fig. 1. The strategy for screening and comparison of immunosuppressive activities of polysaccharides from the stems of *Ephedra sinica* Stapf.

chemical composition until now. Therefore, the purpose of the present study was to screen and compare immunosuppressive effect of polysaccharide fractions from the stems of *E. sinica* under a series of activity-guided bioassays and then active polysaccharide fraction was further isolated, purified and analyzed the chemical composition. Our experimental protocols are shown as in Fig. 1.

2. Experimental

2.1. Materials and reagents

The dry stems of *E. sinica* were collected in March 2007 from Datong of Shanxi Province, China and identified by Prof. Zhenyue Wang of Heilongjiang University of Chinese Medicine. The voucher specimen (20070016) was deposited at Herbarium of Heilongjiang University of Chinese Medicine, Harbin, P.R. China.

RPMI 1640 medium, fetal calf serum (FCS) and dimethyl sulfoxide (DMSO) were from Gibco, Grand Island, NY, USA. 2,4-Dinitrofluorobenzene (DNFB) was from PharMingen products (SanDiego, CA). D-Mannose (Man), L-rhamnose (Rha), D-glucose (Glc), D-galactose (Gal), L-arabinose (Ara), D-xylose (Xyl), D-glucuronic acid (GlcUA), D-galacturonic acid (GalUA), sulfuric acid (H_2SO_4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and concanavalin A (Con A) were purchased from Sigma (St. Louis, USA). 1-Phenyl-3-methyl-5-pyrazolone (PMP), purchased from Beijing Reagent Plant (Beijing, China), was recrystallized twice from chromatographic grade methanol before use. DEAE-Sepharose Fast Flow, Sephacryl S-400 HR, Sephacryl S-300 HR and Sephacryl S-100 HR were from the Pharmacia Co. (Sweden). DEAE-52 was purchased from Whatman International Ltd. (Maidstone, Kent, UK). All other chemicals were of the highest grade available.

2.2. Animals

Male BALB/c mice, aged 6–8 weeks (18–22 g) were obtained from Experimental Animal Center of Heilongjiang University of Chinese Medicine (Harbin, China). They were maintained with free access to pellet food and water in plastic cages at 21 ± 2 °C, 60 ± 5 humidity, and kept on a 12 h light/dark cycle. Animal welfare and experimental procedures were carried out strictly in accordance with the guide for the care and use of laboratory animals (The Ministry of Science and Technology of China, 2006) and the related ethical regulations of our university. All efforts were made to minimize animal's suffering and to reduce the number of animals used.

2.3. Isolation of crude polysaccharides from E. sinica

The dry stems of *E. sinica* were ground to powders, and submitted to sequential extractions as follows: dry powders (1.0 kg) were extracted 3 times with 10 vol of 95% ethanol under reflux for 3 h each time to remove lipids. The residue was dried in air and then extracted 3 times with 10 vol of distilled water for 24 h (each time) at 4 °C. The combined aqueous extracts were filtered, concentrated 10-fold, and 95% EtOH added to final concentration of 80%. The precipitate was dissolved in 600 mL of water and deproteinated 15 times with 200 mL of 5:1 chloroform-*n*-butanol as described by Staub (1965). The resulting aqueous fraction was extensively dialyzed (cut-off M_w 3500 Da) against tap water for 48 h and distilled water for 48 h and precipitated again by adding a 5-fold volume of ethanol. After centrifugation, the precipitate was washed with anhydrous ethanol and then dissolved in water and lyophilised to yield the crude polysaccharide A (8.5 g) was collected by centrifugation (3000 rpm, 10 min, 20 °C). A similar procedure was used with 10 vol of hot water (100 °C, 3 h, 3×) and 1 M sodium hydroxide solutions (4 °C, 12 h, 3×) for above the residue after cold water extraction. The fractions obtained were labeled crude polysaccharides B (35.7 g) and C (12.5 g).

Crude polysaccharide B (6 g) was dissolved in distilled H_2O and passed through two series connected resin columns (Amberlite FPA90-Cl (Cl^- form) and Amberlite IRC-84 (H^+ form)) eluting with distilled H_2O and 1.0 M NaCl to yield fractions Fr. B1 (2.3 g) and Fr. B2 (2.2 g), respectively. Fr. B1 (2.3 g) was chromatographed over DEAE-cellulose 52 eluting with distilled water, 0.2 M NaCl and 0.4 M NaCl to yield subfractions Fr. I, Fr. II and Fr. III, respectively (Fig. 2A). Fr. I (290.0 mg) was further purified by gel-permeation chromatography on a high resolution Sephacryl S-100 eluting with distilled water to afford ESP-B1 (200.0 mg) (Fig. 3A). Fr. II (800.0 mg) was chromatographed over Sephacryl S-300 eluting with 0.1 M NaCl to afford ESP-B2 (650 mg) (Fig. 3B). Fr. III (0.35 g) was chromatographed over DEAE-Sepharose F. F eluting with 0.2 M NaCl to afford ESP-B3 (120.0 mg) (Fig. 3C). Fr. B2 (1.0 g) was applied to a DEAE-cellulose 52 column with 1.0 M NaCl to afford Fr. IV (Fig. 2B), which was further purified by gel-permeation chromatography on a high resolution Sephacryl S-400 eluting with distilled water to afford ESP-B4 (880 mg) (Fig. 3D). Polysaccharides in the eluted fractions were detected using the phenol–sulfuric acid method and all NaCl elute fractions in column chromatography were dialyzed against distilled water for 48 h (cut-off M_w 3500 Da) and then freeze-dried.

2.4. Identification on purity of polysaccharides and molecular weight determination

The molecular mass of the polysaccharide (5 mg/mL) was determined by high performance liquid chromatography (HPLC),

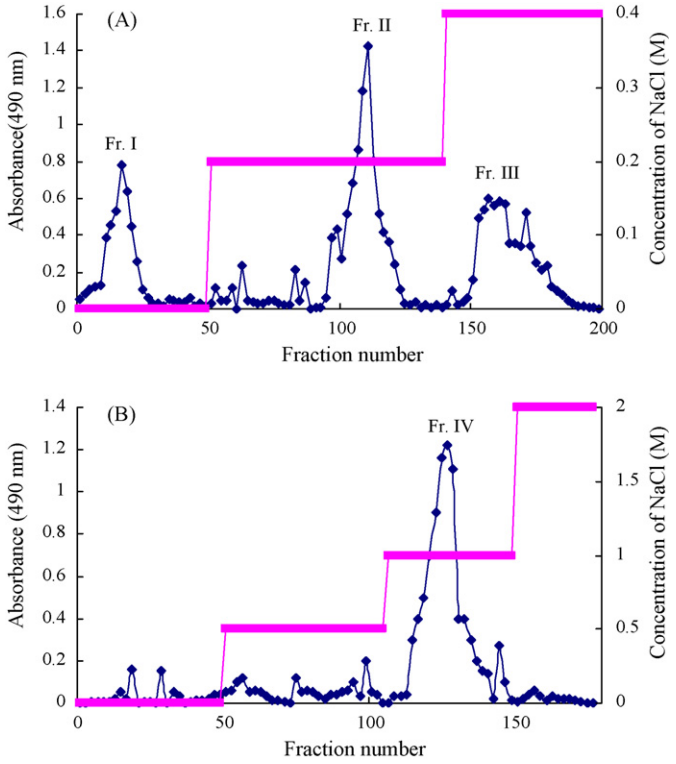

Fig. 2. (A) Chromatography of eluted crude polysaccharide fraction (Fr. B1) on DEAE-cellulose column. Fr. I eluted with distilled water, Fr. II eluted with 0.2 M NaCl, Fr. III eluted with 0.4 M NaCl. (B) Chromatography of eluted crude polysaccharide fraction (Fr. B2) on DEAE-cellulose column. Fr. IV eluted with 1 M NaCl.

using Waters 2695 HPLC and Alltech ELSD 2000 detector. The separation was carried out on a Shodex sugar KS-805 column (8.0 mm × 300 mm, 17 μm) coupled with a Shodex KS-G guard column (6 mm × 50 mm, 7 μm). The Dextran standards (T10, T40, T70, T500, T2000) were used for the calibration curve. The isocratic elution was employed using water with 0.5 mL/min at 30 °C and the injection volume was 10 μl. While the drift tube temperature for ELSD was set at 116 °C, the nitrogen flow rate was 3.3 L/min for the determination of polysaccharides. Their purities were over 98% by HPLC analysis. Total carbohydrate contents in purified samples were determined by phenol–sulfuric acid colorimetric method using glucose as the standard. Proteins in the polysaccharides were detected by the method of UV absorption on a TU-1800PC spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., China).

2.5. Analysis of monosaccharide composition

Monosaccharide composition was analyzed according to the following procedure: each polysaccharide sample (20 mg) was dissolved in 2 mL of 2.0 M H_2SO_4 in an ampoule (5 mL). The ampoule was sealed under a nitrogen atmosphere and kept in 110 °C to hydrolyze the polysaccharide into component monosaccharides for 6 h, then cooled to room temperature and neutralized with 2 mL of 4.0 M sodium hydroxide. The reaction mixture was diluted to 5 mL with deionized water and was centrifugalized at 1000 rpm for 5 min. Then the supernatant was ready for the following experiments.

PMP derivatization of monosaccharides was carried out as described previously with proper modification (Andersen, Bjergegaard, Mller, Srensen, & Srensen, 2003; Lv et al., 2009). 200 μl of individual standard monosaccharide, or mix standard monosaccharide solutions, or the hydrolyzed polysaccharide sam-

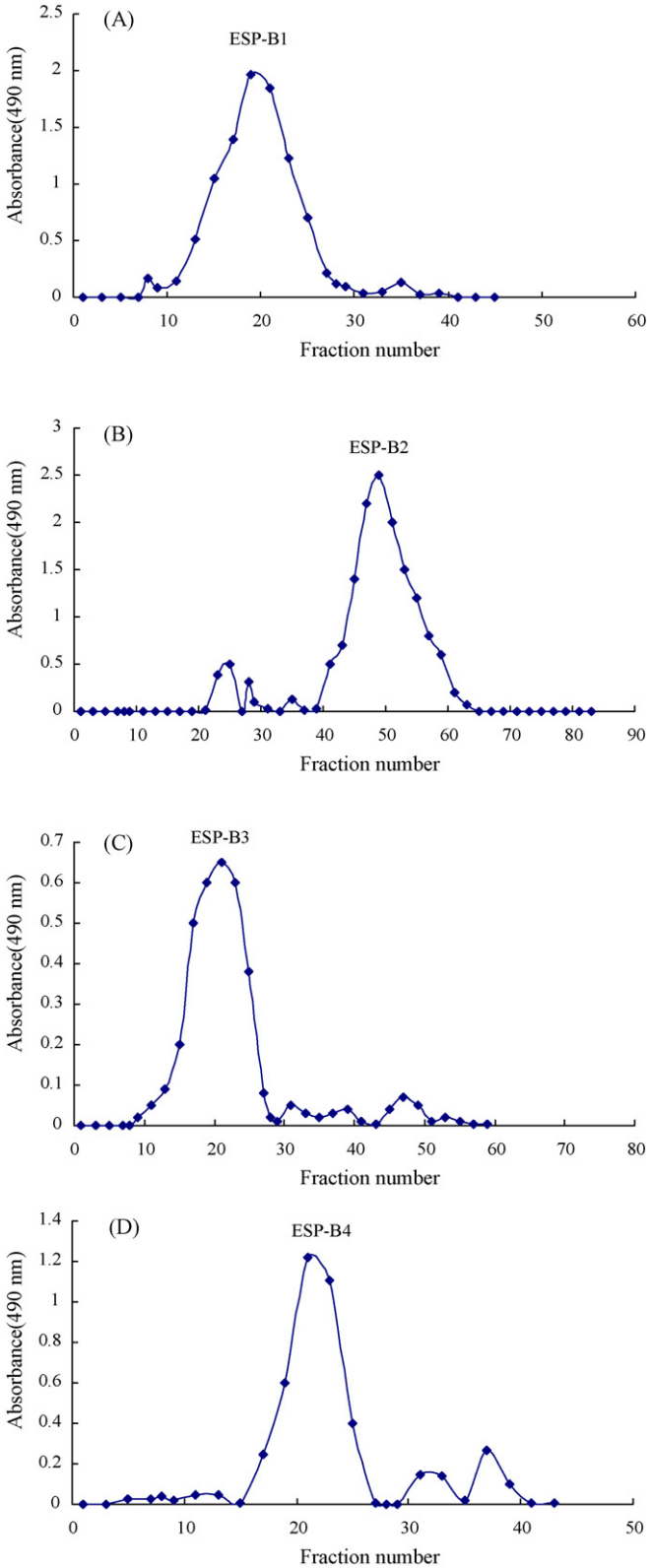

Fig. 3. (A) Sephacryl S-100 HR column chromatogram of ESP-B1; (B) sephacryl S-300 HR column chromatogram of ESP-B2; (C) DEAE-Sepharose F. F column chromatogram of ESP-B3; and (D) sephacryl S-400 HR column chromatogram of ESP-B4.

ples were placed in the 2.0 mL centrifuge tubes, respectively, then 0.5 M methanol solution (100 μl) of PMP and 0.3 M aqueous sodium hydroxide (100 μl) were added to each. Each mixture was allowed to react for 30 min at 70 °C water bath, then cooled to room temperature and neutralized with 100 μl of 0.3 M HCl. The resulting solution was performed on liquid–liquid extraction with same volume of isoamyl acetate (two times) and chloroform (one time), respectively. After being shaken vigorously and centrifuged, the organic phase was carefully discarded to remove the excess reagents. Then the aqueous layer was filtered through a 0.45 μm membrane and diluted with water before HPCE analysis.

The analysis of PMP-labeled monosaccharides was carried out on a P/ACE MDQ capillary electrophoresis instrument (Beckman Coulter, Fullerton, CA, USA). An integrated P/ACE 32 Karat Station (software version 4.0) was used to perform the data collection and to control the operational variables of the system. Separation was carried out in an unmodified fused silica capillary (48.5 cm × 50 μm i.d., effective length 40 cm) with direct UV monitoring using a photodiode array detector at wavelength 254 nm including 35 mM borate at pH 10.02, capillary temperature 25 °C and applied voltage 20 kV. The molar ratio of the component monosaccharides is calculated as follows. The correction factor is shown in the equation: $f_{i/n} = (m_i/A_i)/(m_n/A_n)$, where A_i and A_n are the values of their peak areas in the standard monosaccharide, respectively. m_i and m_n are the values of their weights of the standard monosaccharide, respectively. The molar ratio value is shown in the equation: $R_{i/n} = f_{i/n} \times (A'_i/A'_n)$, where A'_i/A'_n is the ratio value of peak area for the component monosaccharide of tested samples and $f_{i/n}$ is the correction factor.

2.6. Partial acid hydrolysis of ESP-B4

ESP-B4 (100 mg) was hydrolyzed with 0.1 M TFA (3 mL) for 6 h at 100 °C, and TFA was removed in vacuum by addition of methanol repeatedly. The hydrolysis sample was dialyzed with distilled water for 48 h in a dialysis sack (cut-off M_w 3500 Da), and then diluted the solution in the sack with ethanol. After hydrolysis, the precipitate (a) and supernatant in the sack (b) and the fraction out of sack (c) were dried, respectively, and then ESP-B4-a was used for MTT experiment.

2.7. Carbon clearance test

Male BALB/c mice were randomly divided into five groups (n = 10/group) as follows: control group, oral gavage with 0.9% saline solution at a single volume of 0.2 mL/each daily for 7 days; standard drug cyclophosphamide (cy), at a single dose of 30 mg/kg on the 1st, 3rd and 5th day; three polysaccharide groups, oral gavage with same volume of polysaccharide solutions as blank control at a single dose of 150.0 mg/kg daily for 7 days. At the end of 7 days, the mice were injected with carbon ink suspension (10 μl/g body weight) through tail vein. Blood samples were drawn (in EDTA solution 5 μl), from the retro-orbital vein, at intervals of 2 (T_1) and 6 (T_2) min, a 25 μl sample was mixed with 0.1% sodium carbonate solution (2 mL) and the absorbance measured at 660 nm. Carbon clearance index (K) and phagocytic index (α) were calculated by using following formula: $K = (\log OD_2 - \log OD_6)/(T_2 - T_1)$ and $\alpha = (K^{1/3} \times \text{body wt of animal})/(\text{liver wt} + \text{spleen wt})$ (Jayathirtha & Mishra, 2004; Yang et al., 2007). Where OD_2 is the log absorbance of blood at 2 min; OD_6 is log absorbance of blood at 6 min; T_2 is the last time point of blood collection; T_1 is the first time point of blood collection.

2.8. 2,4-Dinitrofluorobenzene (DNFB) induce DTH reaction

DTH assay was carried out as previously described (Feng et al., 2002; Makare, Bodhankar, & Rangari, 2001; Zhou et al., 2005). Briefly, male BALB/c mice were randomly divided into six groups (n = 10/group) as follows: negative control and model group, oral gavage with 0.9% saline solution at a single volume of 0.2 mL/each daily for 7 days; standard drug dexamethasone (DXM), at a single daily dose of 10 mg/kg on the 1st, 3rd and 5th day; three concentrations of PB groups, oral gavage with same volume of polysaccharide solutions as blank control at different doses of 26, 52 and 156 mg/kg daily for 7 days, respectively. The hair was removed from the abdomen of each mouse to achieve the scope (1 cm × 2 cm) so that the shaved abdomen was fully exposed. Mice were initially sensitized by uniformly painting 20 μl 5% DNFB dissolved in acetone–olive oil (4:1) on each shaved abdomen on days 1 and 2. Only 20 μl solvent (acetone:olive oil = 4:1) without DNFB was uniformly painted at the shaved abdomen as negative control. These mice were referred to as unsensitized, challenged group. Five days after the second sensitization, all mice were challenged with 10 μl 5% DNFB on both sides of their right ear. Ear swelling was expressed as the difference between the weight of the left and right ear patches obtained from 8-mm punches 48 h after challenge. Mice were killed after the last medicine administration and their spleens and thymus were rapidly removed and weighed. The punches were obtained in a blinded manner. The swelling rate was calculated by using following formula: swelling rate = ear swelling/left ear wt.

2.9. Serum hemolysin analysis

The serum hemolysin was analyzed by the method described in reference (Wen, Zhang, Liu, Guo, & Wang, 2006). Male BALB/c mice were randomly divided into five groups (n = 8/group) as follows: blank control and model groups, oral gavage with 0.9% saline solution at a single volume of 0.2 mL/each daily for 14 days; three concentrations of PB groups, oral gavage with same volume of polysaccharide solutions as blank control at different doses of 52.3, 156.9 and 313.8 mg/kg daily for 14 days, respectively. On the 8th day of administered with PB, the mice except for the blank control group were sensitized by injection of 0.2 mL SRBC (10%, v/v). After 6 days, mice were anesthetised with pentobarbital. The blood was collected from the retro-orbital venous plexus of the mice and serum was separated by centrifugation at 2000 rpm for 10 min. The serum was diluted with normal saline to 300-fold. Serum (1 mL), 0.5 mL SRBC (10%, v/v), and 0.5 mL complement (serum mixture from ten Guinea pig and diluted with saline by 1:10 before use) were added into the tubes by turns. The mixture was incubated at 37 °C for 30 min and terminated on ice. The reaction mixture was centrifuged at 2000 rpm for 10 min and the supernatant (1 mL) was separated. Take the Supernatant 1 mL and Kathmandu's reagent 3 mL together as sample reagent by mixing uniformly and standing for 10 min. The blank control (without mice serum) was also synchronously prepared. The absorbance of blank control and the sample solutions was recorded at 540 nm. Determination of serum hemolysin was expressed as the half value of hemolysis (HC_{50}). The HC_{50} value is shown in the equation: HC_{50} = (sample optical density/SRBC hemolysis, when half of the optical density value) × dilution factor.

2.10. Cell viability assay

Isolated splenocytes (100 μl/well) were cultured in 96-well tissue culture plates for 48 h in the presence or absence of four concentrations of compounds separately. MTT was added after 48 h at a final concentration of 500 μg/mL and incubated for 4 h. After

Table 1
The results of composition analysis of four purified polysaccharides from PB.

Preparation	Molar ratios (mol%)							
	Xyl	Ara	Glc	Rha	Man	Gal	GlcUA	GalUA
ESP-B1	–	90.0	6.0	–	3.0	1.0	–	–
ESP-B2	4.7	21.8	3.2	10.3	16.3	13.4	4.0	26.3
ESP-B3	3.6	9.7	4.2	19.7	8.4	17.8	4.2	32.4
ESP-B4	1.5	6.8	1.5	3.0	1.5	8.3	2.3	75.2

removal of MTT, the formazan precipitate was solubilized in DMSO (100 μl/well) and measured on an ELISA reader at the absorbance wavelength of 570 nm. The experiment was repeated three times.

2.11. Concanavalin A (Con A)-induced mouse splenocyte proliferation

Mouse splenocyte proliferation was assayed by MTT method as previously described (Hakki, Hallquist, Friedman, & Pross, 2000). Splenocytes were seeded into a 96-well flat-bottom microtiter plate at 1×10^8 cells/mL in 100 μl of complete medium. Serial drug dilutions were prepared in medium immediately prior to each assay. Thereafter, 100 μl aliquots of serial dilution of each test compound were added (parallel triplicate wells were set), and then the cells were incubated in the absence or presence of Concanavalin A (Con A, final concn. 5 μg/mL) for 44 h in the humidified 5% CO_2 incubator at 37 °C. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) in phosphate buffered saline (PBS) at 5 mg/mL (10 μl) was added to each well, and plates were incubated at 37 °C for 4 h and the formazan crystals formed were dissolved through addition of 100 μl of DMSO/well. The absorption of the samples was measured using an ELISA reader (Uniscan Titertec) at a wavelength of 570 nm. The blank consisted of 100 mL of RPMI 1640 medium (Sigma), 10 mL of MTT stock, and 100 mL of DMSO.

2.12. Statistical analysis

Data were expressed as the mean standard deviation of the means (S.D.) and statistical analysis was performed by paired samples *t*-test using SPSS 16.0 to evaluate the significance of differences between groups.

3. Results

3.1. Isolation, purification and chemical composition of the polysaccharides

Crude polysaccharides A, B, and C were isolated from the stems of *E. sinica* by sequential extraction with cold water, hot water, and 1 M potassium hydroxide solutions followed by ethanol precipitation and dialyzed (cut-off M_w 3500 Da) and then freeze-dried, with yields of 0.85%, 3.57% and 1.25% of the plant raw material, respectively, and the yields of four purified polysaccharides were 3.3%, 10.8%, 2.0% and 14.7% for ESP-B1, ESP-B2, ESP-B3 and ESP-B4 of the parent crude polysaccharide B by ion exchange and gel-filtration chromatography, respectively (Figs. 2 and 3). The four polysaccharides appeared as only a single and symmetrical sharp peak in high performance gel-permeation chromatography by HPLC-ELSD with Shodex sugar KS-805 column. The average molecular weights were ca. 1.4×10^5 Da and $>2000 \times 10^6$ Da for ESP-B1 and other three polysaccharides, respectively, by reference to the calibration curve ($y = -2.2303\ x + 31.68$, $r^2 = 0.9936$) made from a Dextran T-series standard of known molecular weight (T10, T40 T70, T500, T2000). These polysaccharides showed negative Fehling's reagent and iodine–potassium iodide reactions, indicating that they did not contain reducing sugar and starch-type polysaccharide (Yang, Zhao, & Lv, 2008). All four polysaccharides had negative responses to the Bradford test and no absorption at 280 nm in the UV spectrum, indicating the absence of protein.

Taking ESP-B3 for example (Fig. 4A), PMP derivatives of the component monosaccharides released from four purified polysaccharides could be still perfectly baseline separated from each other and the component monosaccharides could be identified by comparing with the chromatogram of the mixture of standard monosaccharides (Fig. 4B). ESP-B1 was composed of Ara, Glc, Man and Gal and their corresponding molar percentages (mol%) were 90.0%, 6.0%, 3.0% and 1.0%, respectively. ESP-B2, ESP-B3 and ESP-B4 were all composed of Xyl, Ara, Glc, Rha, Man, Gal, GlcUA and GalUA and their corresponding molar percentages (mol%) were 4.7%, 21.8%, 3.2%, 10.3%, 16.3%, 13.4%, 4.0% and 26.3% for ESP-B2, respectively, 3.6%, 9.7%, 4.2%, 19.7%, 8.4%, 17.8%, 4.2% and 32.4% for ESP-B3, respectively, and 1.5%, 6.8%, 1.5%, 3.0%, 1.5%, 8.3%, 2.3% and 75.2% for ESP-B4, respectively (Table 1). However, the composition and the ratio of these monosaccharides greatly differed from one another. Xyl, Rha, GlcUA and GalUA were found in ESP-B2, ESP-B3 and ESP-B4, but not present in ESP-B1. In addition, there were significant differences in the average molecular weights, as well as the contents of Ara and GalUA between ESP-B1 and ESP-B4. ESP-B2, ESP-B3 and ESP-B4 were acidic hetero-polysaccharides with the GalUA contents being 26.3%, 32.4% and 75.2%, respectively. ESP-B1 was not detectable for uronic acid, suggesting it belonged to neutral polysaccharides. The Ara contents in four polysaccharides increased in the order of ESP-B4, ESP-B3, ESP-B2 and ESP-B1, with being 6.8%, 9.7%, 21.8% and 90.0%, respectively.

3.2. Carbon clearance test in mice

Mahuang crude polysaccharides A, B and C showed carbon clearance indexes as 0.029 ± 0.004, 0.023 ± 0.006, and 0.025 ± 0.004, respectively, and phagocytic indexes as 4.758 ± 0.512, 4.211 ± 0.564, and 4.325 ± 0.412 with a single daily dose of 150 mg/kg, respectively. The carbon clearance index and phagocytic indexes of the control group were 0.033 ± 0.003 and 4.971 ± 0.342, respectively. The carbon clearance and phagocytic index of PB were significantly different compared with those of control group ($P < 0.01$) (Fig. 5).

3.3. DNFB-induced delayed-type hypersensitivity (DTH) reaction in mice

The inhibitory effect of PB on DNFB-induced DTH was illustrated in Fig. 6. The ear swelling was significantly increased in model group compared with control group ($P < 0.01$), indicating that DNFB-induced DTH model had been made successfully. The ear swelling was significantly decreased in mice treated with the high dose of PB (156 mg/kg) and standard drug DXM compared with model group ($P < 0.05$) (Fig. 6A), respectively. Furthermore, to determine effect of PB on spleen and thymus index of mice, the spleen and thymus index of mice were quantified. As shown in Fig. 6B and C, the spleen and thymus index of mice were 0.0421 and

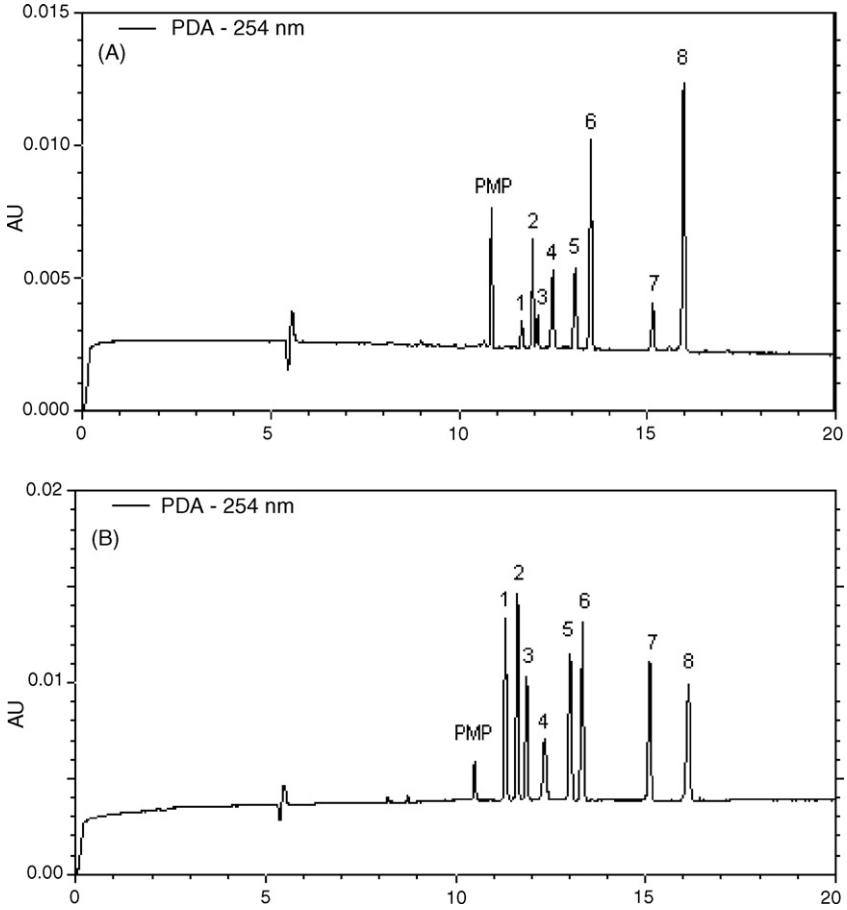

Fig. 4. (A) Electropherograms of PMP derivatives of monosaccharides in ESP-B3; (B) electropherograms of PMP derivatives of 8 standard monosaccharides.

0.02011 in control group, respectively, while a significant increase of spleen and thymus index of mice was observed in model group compared with the control group ($P < 0.05$). However, the spleen and thymus index were significantly lower in DXM and high dose of PB groups than those of model group ($P < 0.01$), respectively.

3.4. Effect of PB on humoral immune response

Effect of the PB on humoral immunity response is shown in Fig. 7. The HC_{50} was significantly increased in model group compared with control group ($P < 0.01$), indicating that the model caused by SRBC has been made successfully. The PB with high dose (313.8 mg/kg) could significantly decrease HC_{50} compared with model group ($P < 0.01$), suggesting a markedly inhibitory action of the PB on humoral immune response.

3.5. Effect of polysaccharides on the splenocyte proliferation of mouse

The colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for cell proliferation was then carried out to evaluate Con A-stimulated mouse splenocyte in the presence of various concentrations of ESP-B1, ESP-B2, ESP-B3 and ESP-B4. In comparison with control group (without treatment of samples), the inhibitory effects of three purified acid heteropolysaccharides on the proliferation of mouse splenocyte were higher with the dose increasing (Table 2). However, neutral ESP-B1 with high dose of 250 μg/mL stimulated cell proliferation of mouse splenocytes ($P < 0.01$) (Table 2). In addition, any effect on the proliferation of splenocytesin was not observed for ESP-B4-a.

It should be noted that four purified polysaccharides at the concentrations mentioned above did not affect the splenocyte's viability by MTT uptake assay (data not shown). These results further elucidated that the immunosuppressive activities of three acidic polysaccharides have no cytotoxicities at the concentrations up to 250 μg/mL.

Table 2
Effect of the polysaccharides from PB on Con A proliferation of mouse splenocytes in vitro. The backbone of ESP-B4 (the mild hydrolysis product) was named as ESP-B4-a.

Preparation	Concentrations (μg/mL)	Con A (A_{570})
Control	–	0.31 ± 0.01
ESP-B1	10.0	0.32 ± 0.02
	50.0	0.36 ± 0.01
	250.0	0.43 ± 0.01*
ESP-B2	10.0	0.31 ± 0.01
	50.0	0.28 ± 0.02
	250.0	0.25 ± 0.01*
ESP-B3	10.0	0.31 ± 0.01
	50.0	0.26 ± 0.02*
	250.0	0.24 ± 0.01**
ESP-B4	10.0	0.30 ± 0.01
	50.0	0.25 ± 0.02*
	250.0	0.20 ± 0.01**
ESP-B4-a	10.0	0.32 ± 0.01
	50.0	0.34 ± 0.02
	250.0	0.31 ± 0.01

* $P < 0.05$ when compared with the control group.
** $P < 0.01$ when compared with the control group.

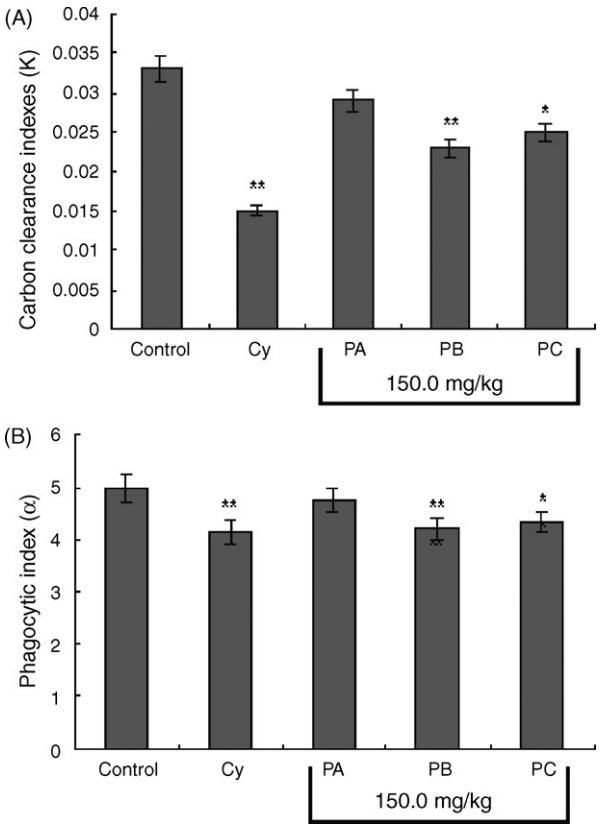

Fig. 5. Effect of PA, PB and PC at a dose of 150 mg/kg on carbon clearance index (A) and phagocytic index (B), significant differences were evaluated using paired samples *t*-test compared with control group $^{*}P < 0.05$ and $^{**}P < 0.01$.

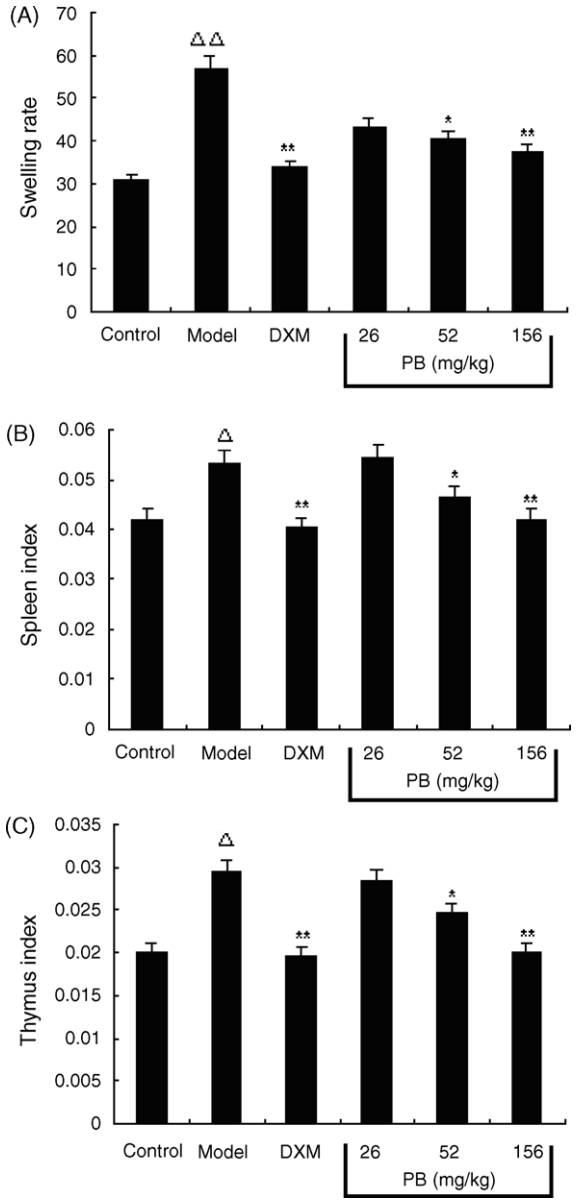

Fig. 6. Effect of PB at different concentrations on swelling rate (A), spleen index (B) and thymus index (C). Significant differences with control group were evaluated using paired samples *t*-test $^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$. Significant differences with model group were evaluated using paired samples *t*-test $^{*}P < 0.05$ and $^{**}P < 0.01$.

4. Discussion

Patients suffered from autoimmune diseases, allergies and organ transplantations are usually treated with immunosuppressive drugs. However, the risks of systemic immunosuppression are increasing with the use of immunosuppressive drugs (Leaker & Cairns, 1994). Thus the finding of lead compounds is of great interest. So far, only a few polysaccharides with immunosuppressive effects have been isolated from herbal plants and culture bacterium such as *Tripterygium wilfordii*, *Aeromonas cuviue*, *Klebsiella oxytoca* and *Pseudomonas aeruginosa*. (Luk, Tam, Fan, & Koo, 2000; Ohmori et al., 1998; Sugihara, Oiso, Matsumoto, & Ohmori, 2001; Vikström, 2003). In the present study, PB was found to possess significant inhibitory activity against carbon clearance test, DTH reaction and humoral immune response in mice. Then four purified polysaccharides were isolated from PB, among which ESP-B4 showed the strongest immunosuppressive activity in vitro test.

Phagocytosis is the process by certain body cells, collectively known as phagocytes, ingests and removes microorganisms, malignant cells, inorganic particles and tissue debris (Miller, Ludke, Peacock, & Tomar, 1991). The faster removal of carbon particles has been correlated with the enhanced phagocytic activity. The phagocytic activity of the reticulo-endothelium system was measured by the removal of carbon particles from the blood circulation (Shukla et al., 2009). The results obtained in the present study indicated that PB can obviously inhibit the phagocytic activity of the macrophages as evidenced by the decrease in the carbon clearance and phagocytic index (Fig. 5).

Cell-mediated immunity (CMI) involves effector mechanisms carried out by T-lymphocytes and their products (lymphokines). CMI responses are critical to defend against infectious organisms, infection of foreign grafts, tumor immunity and delayed-type hypersensitivity reactions (Miller et al., 1991). DNFB-induced DTH reaction is a Th1 cell-mediated pathologic response involved with T cell activation and the production of many cytokines (Grabbe & Schwarz, 1998; Kobayashi, Kaneda, & Kasama, 2001). The mechanism behind this elevated DTH during the CMI responses could be due to sensitized T-lymphocytes. When challenged by the antigen, they are converted to lymphoblasts and secrete a variety of molecules including proinflammatory lymphokines, attracting more scavenger cells to the site of reaction (Delves & Roitt, 1998). The infiltrating cells are probably immobilized to promote defensive (inflammatory) reaction (Fulzele, Satturwar, Joshi, & Dorle, 2003). The results showed that PB (middle and high) in a dose dependent manner significantly inhibited T cell functions in a T cell-mediated DTH model (Fig. 6). Ear swelling in DTH is primarily the result of in vivo functions of antigen specific CD4+ T cell response (Grabbe & Schwarz, 1998; Kobayashi et al., 2001).

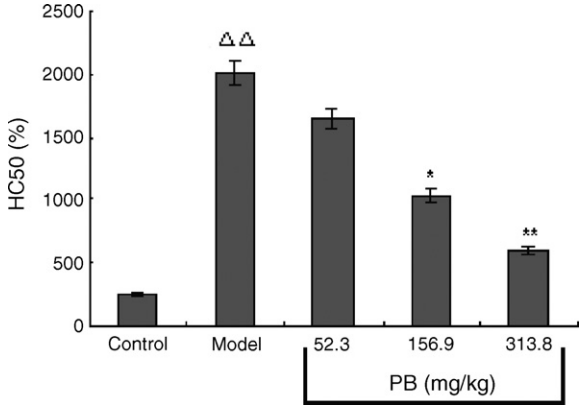

Fig. 7. Effects of PB at different concentrations on HC_{50}. Each bar in this figure represents the mean ± SD of eight animals. Significant differences with control group were evaluated using paired samples *t*-test $^{\triangle\triangle}P < 0.01$. $^{*}P < 0.05$ and $^{**}P < 0.01$ when compared with the model group.

Antibody molecules, a product of B lymphocytes and plasma cells, are central to humoral immune responses, IgG and IgM are the major immunoglobulins which are involved in the complement activation, opsonization, neutralization of toxins, etc. (Miller et al., 1991). Serum hemolysin level reflects the intensity of humoral immune function. Some reports have pointed out that some botanical polysaccharides can increase humoral immune response by activation of B cells (e.g. polysaccharides from *Tinospora cordifolia*) and production of specific IgA, IgM, and IgG (e.g. polysaccharides from *Astragalus membranaceus*) (Yang et al., 2009). In this study, we observed that the PB can also decrease the serum hemolysin level, but the mechanism still needs to be studied (Fig. 7).

Up to now, many of those herbal plants have been universally made into medicines, such as *Lentinus edodes* polysaccharide and *Tuckahoe* polysaccharide (Chen, 1997). Based on the sugar composition analysis, many international researches suggested that polysaccharides of medicinal plants composed of glucan were known to stimulate the immune system and have perfect anticancer effects. Moreover, polysaccharides of plants consisting of Gal, Glc, GalA and Rha are also said to have powerful anti-inflammation, anti-bacteria and anti-virus properties, such as *Althaea officinalis* var. *Rhobusta* polysaccharide (Peter, Jozef, & Alzbeta, 1987). However, there have been few reports on the heteropolysaccharide consisting of GalUA, GlcUA, Xyl, Ara, Glc, Rha, Man and Gal. Therefore it is necessary to study the relationship between the structure of the polysaccharide and its pharmacological activities. Furthermore, it has been reported that many factors affected the activities of polysaccharides, such as monosaccharide composition, molecular mass, glycosyl residues, and chain conformation (Bao, Liu, Fang, & Li, 2001; Hua, Zhang, Fu, Chen, & Chan, 2004; Huang, Jin, Zhang, Cheung, & Kennedy, 2007). At the mean time, much controversy surrounds the biochemical and molecular principles of those bioactive polysaccharides (Xu, Yao, Sun, & Wu, 2009). Some researchers reported the complete structure and high molecular weight of those polysaccharides were highly desired for their observed immunomodulatory action, while other investigation showed the activity was dependent upon their basic structure-oligosaccharide unit (Ning et al., 2003; Yan et al., 2003).

ESP-B4 was mainly consisted of the acid sugar GalUA and GlcUA (77.5%) with small amounts of the neutral sugar Xyl, Ara, Glc, Rha, Man and Gal (22.5%). ESP-B2 and ESP-B3 were mainly consisted of the neutral sugar Xyl, Ara, Glc, Rha, Man and Gal with small amounts of the acidic sugar GalUA and GlcUA. ESP-B1 without the uronic acid groups was mainly consisted of Ara (90%). For ESP-B1 at high dose, the increase in splenocytesin proliferation stimulation was observed. However, ESP-B4 exhibited the higher immunosuppressive effects than ESP-B2 and ESP-B3, and the higher immunosuppressive effect for ESP-B4 might be attributed to the higher GalA contents. The material retained in the sack after dialyzing of the partial acid hydrolyzate was named as ESP-B4-a, and it had the highest molecular size of the three fractions, its structure was proposed closest with ESP-B4. Immunobiological activity assay showed ESP-B4-a has nothing to do with the splenocyte proliferation in vitro (Table 2). Therefore, we can draw a conclusion that the branches are extremely important in biological activities of ESP-B4, and further studies are needed to elucidate the structure–function relationships and mechanisms responsible for its immunosuppressive activities.

5. Conclusion

Finally, based on the results we observed, PB and ESP-B4 have therapeutic potential and could be served as a novel safe immunosuppressive candidate. This finding extended our understanding of the effects of *E. sinica* and its polysaccharide components. In order to promote our research and the development of PB and ESP-B4 and establish the therapeutic potential for the prevention and treatment of autoimmune diseases, further investigation of the mechanism of immunosuppressive action will be carried out in our later work.

Acknowledgements

Our work was supported by the Major State Basic Research Development Program of China (973 Program 2006CB504708), State Key Creative New Drug Project of 12th Five-year Plan of China (2011ZX11102), National Natural Science Foundation of China (No. 30973870) and Heilongjiang Research Based Innovation for Graduate Student (YJSCX2009-171HLJ).

References

Andersen, K. E., Bjergegaard, C., Mller, P., Srensen, J. C., & Srensen, H. (2003). High performance capillary electrophoresis with indirect UV detection for determination of α-galactosides in leguminose and brassicaceae. *Journal of Agricultural and Food Chemistry*, *51*, 6391–6397.

Bao, X. F., Liu, C. P., Fang, J. N., & Li, X. Y. (2001). Structural and immunological studies of a major polysaccharide from spores of *Ganoderma lucidum* (Fr.) Karst. *Carbohydrate Research*, *332*, 67–74.

Chen, H. L. (1997). *Function and structure of glycobiology*. Shanghai: Shanghai Medical University Publishing Company. (pp. 336–339).

Chen, X., Zhang, Y., & Zhang, J. B. (2007). Advances in research on the plant polysaccharides. *Chinese Journal of New Drugs*, *13*, 1000–1004.

Cheng, R. M., Zhu, G. X., & Xu, Z. R. (2001). Effect of different extracts from Ephedra on cell immunity. *Journal of Nanjing TCM University*, *17*, 234–236.

Delves, P. J., & Roitt, I. M. (1998). *Encyclopedia of immunology*. London: Academic Press.

Ding, L. L., Shi, S. S., Cui, J., Wang, S. C., & Wang, Z. T. (2006). Advances in research of chemical constituents and pharmacological activities of Ephedra. *China Journal of Chinese Materia Medica*, *31*, 1661–1664.

Dunnick, J. K., Kissling, G., Gerken, D. K., Vallant, M. A., & Nyska, A. (2007). Cardiotoxicity of Ma Huang/caffeine or ephedrine/caffeine in a rodent model system. *Toxicologic Pathology*, *35*, 657–664.

Fang, J. N., & Ding, K. (2007). Bioactivities, isolation and purification methods of polysaccharide. *Chinese Journal of Natural Medicine*, *5*, 338–347.

Feng, Y. H., Zhou, W. L., Wu, Q. L., Li, X. Y., Zhao, W. M., & Zou, J. P. (2002). Low dose of resveratrol enhanced immune response of mice. *Acta Pharmacologica Sinica*, *23*, 893–897.

Fulzele, S. V., Satturwar, P. M., Joshi, S. B., & Dorle, A. K. (2003). Study of the immunomodulatory activity of *Haridradi ghrita* in rats. *Indian Journal of Pharmacology*, *35*, 51–54.

Ganzera, M., Lanser, C., & Stuppner, H. (2005). Simultaneous determination of *Ephedra sinica* and *Citrus aurantium* var. *amara* alkaloids by ion-pair chromatography. *Talanta*, *66*, 889–894.

Grabbe, S., & Schwarz, T. (1998). Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity. *Immunology Today*, *19*, 37–44.

Hakki, A., Hallquist, N., Friedman, H., & Pross, S. (2000). Differential impact of nicotine on cellular proliferation and cytokine production by LPS-stimulated murine splenocytes. *International Journal of Immunopharmacology*, *22*, 403–410.

Hsieh, Y. S. Y., Cheng, C., Liao, S. K. S., Liao, S. F., Hung, W. T., Yang, W. B., et al. (2008). Structure and bioactivity of the polysaccharides in medicinal plant *Dendrobium huoshanense*. *Bioorganic Medicinal Chemistry*, *16*, 6054–6068.

Hua, Y. F., Zhang, M., Fu, C. X., Chen, Z. H., & Chan, G. Y. S. (2004). Structural characterization of a 2-O-acetylglucomannan from *Dendrobium officinale* stem. *Carbohydrate Research*, *339*, 2219–2224.

Huang, Q. L., Jin, Y., Zhang, L. N., Cheung, P. C. K., & Kennedy, J. F. (2007). Structure, molecular size and antitumor activities of polysaccharides from *Poria cocos* mycelia produced in fermenter. *Carbohydrate Polymers*, *70*, 324–333.

Jayathirtha, M. G., & Mishra, S. H. (2004). Preliminary immunomodulatory activities of methanol extracts of *Eclipta alba* and *Centella asiatica*. *Phytomedicine*, *11*, 361–365.

Jiang, T. F., Lv, Z. H., & Wang, Y. H. (2007). Chiral separation of ephedrine alkaloids from *Ephedra sinica* extract and its medicinal preparation using cyclodextrin-modified capillary zone electrophoresis. *Chinese Journal of Analytical Chemistry*, *62*, 85–89.

Jiangsu New Medical College. (1986). *Dictionary of Chinese materia medica*. Shanghai: Shanghai People's Press. (pp. 2221–2224).

Kobayashi, K., Kaneda, K., & Kasama, T. (2001). Immunopathogenesis of delayed-type hypersensitivity. *Microscopy Research Technique*, *53*, 241–245.

Konno, C., Mizuno, T., & Hikino, H. (1985). Isolation and hypoglycemic activity of ephedrans A, B, C, D and E, glycans of *Ephedra distachya* herbs. *Planta Medica*, *51*, 162–163.

Leaker, B., & Cairns, H. S. (1994). Clinical aspects of cyclosporin nephrotoxicity. *British Journal of Hospital Medicine*, *52*, 529–534.

Liu, C. H., Wang, C. H., Xu, Z. L., & Wang, Y. (2007). Isolation, chemical characterization and antioxidant activities of two polysaccharides from the gel and the skin of *Aloe barbadensis Miller* irrigated with sea water. *Process Biochemistry*, *42*, 661–670.

Luk, J. M., Tam, P., Fan, S. T., & Koo, M. W. L. (2000). Immunosuppressive effects of *Tripterygium wilfordii* polysaccharide on LPS-stimulated human monocytes. *Transplantation Proceedings*, *32*, 2013–2015.

Lv, Y., Yang, X. B., Zhao, Y., Ruan, Y., Yang, Y., & Wang, Z. 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.

Makare, N., Bodhankar, S., & Rangari, V. (2001). Immunomodulatory activity of alcoholic extract of *Mangifera indica* L. in mice. *Journal of Ethnopharmacology*, *78*, 133–137.

Marchei, E., Pellegrini, M., Pacifici, R., Zuccaro, P., & Pichini, S. (2006). A rapid and simple procedure for the determination of ephedrine alkaloids in dietary supplements by gas chromatography–mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, *41*, 1633–1641.

Miller, L. E., Ludke, H. R., Peacock, J. E., & Tomar, R. H. (1991). *Manual of laboratory immunology*. London: Lea and Febiger. (pp. 1–18).

Ning, J., Zhang, W., Yi, Y., Yang, G., Wu, Z., & Yi, J. (2003). Synthesis of β-(1→6)-branched β-(1→3) glucohexose and its analogues containing an α-(1→3) linked bond with antitumor activity. *Bioorganic and Medical Chemistry*, *11*, 2193–2203.

Ohmori, H., Raitoh, M., Harano, M., Oda, T., Hikida, M., Sugihara, R., et al. (1998). Search for immunosuppressive substances produced in the culture of *Enteric bacteria* isolated from marine fish. *Journal of Fermentation and Bioengineering*, *85*, 117–119.

Peter, C., Jozef, R., & Alzbeta, K. (1987). Polysaccharide from the roots of the Marsh Mallow (*Althaea officinalis* L. var. Rhobusta): Structural features of an acidic polysaccharide. *Carbohydrate Research*, *164*, 443–452.

Schepetkin, I. A., & Quinn, M. T. (2006). Botanical polysaccharides: Macrophage immunomodulation and therapeutic potential. *International Immunopharmacology*, *6*, 317–333.

Shukla, S., Mehta, A., John, J., Mehta, P., Vyas, S. P., & Shukla, S. (2009). Immunomodulatory activities of the ethanolic extract of *Caesalpinia bonducella* seeds. *Journal of Ethnopharmacology*, *125*, 252–256.

Sigal, N. H., & Dumont, F. J. (1992). Cyclosporin A, FK-506, and rapamycin: Pharmacologic probes of lymphocyte signal transduction. *Annual Review Immunology*, *10*, 519–560.

Staub, A. M. (1965). Removeal of protein-sevag method. *Methods in Carbohydrate Chemistry*, *5*, 5–6.

Sugihara, R., Oiso, Y., Matsumoto, Y., & Ohmori, H. (2001). Production of an immunosuppressive polysaccharide, AZ9, in the culture of *Klebsiella oxytoca* strain TNM3. *Journal of Fermentation and Bioengineering*, *92*, 485–487.

Trujillo, W. A., & Sorenson, W. R. (2003). Determination of ephedrine alkaloids in human urine and plasma by liquid chromatography/tandem mass spectrometry: Collaborative study. *Journal of AOAC International*, *86*, 643–656.

U.S. Food and Drug Administration. (2004). *Final rule declaring dietary supplements containing ephedrine alkaloids adulterated because they present unreasonable risk* (pp. 6788–6854).

Vikström, E. V. (2003). Components with potential immunosuppressive activity in lipopolysaccharide of laboratory *Pseudomonas aeruginosa* strains. *Immunology Letters*, *85*, 29–33.

Wen, X. M., Zhang, Y. L., Liu, X. M., Guo, S. X., & Wang, H. (2006). Immune responses in mice to arecoline mediated by lymphocyte muscarinic acetylcholine receptor. *Cell Biology International*, *30*, 1048–1053.

Xu, H. S., Yao, L., Sun, H. X., & Wu, Y. W. (2009). Chemical composition and antitumor activity of different polysaccharides from the roots of *Actinidia eriantha*. *Carbohydrate Polymers*, *78*, 316–322.

Yan, J., Zong, H., Shen, A., Chen, S., Yin, X., & Shen, X. (2003). The β-(1→6)-branched β-(1→3) glucohexaose and its analogues containing an α-(1→3) linked bond have similar stimulatory effects on the mouse spleen as lentinan. *International Immunopharmacology*, *3*, 1861–1871.

Yang, X. B., Zhao, Y., & Lv, Y. (2008). In vivo macrophage activation and physicochemical property of the different polysaccharide fractions purified from *Angelica sinensis*. *Carbohydrate Polymers*, *71*, 372–379.

Yang, X. M., Yu, W., Qu, Z. P., Ma, H. L., Liu, W. M., & Ji, X. L. (2009). Antioxidant and immunity activity of water extract and crude polysaccharide from *Ficus carica* L. Fruit. *Plant Foods Human Nutrition*, *64*, 167–173.

Yang, Y., Liu, W. S., Han, B. Q., Wang, C. H., Fu, C. W., Liu, B., et al. (2007). The antioxidant and immunostimulating properties of glucosamine. *International Immunopharmacology*, *7*, 29–35.

Yu, R. M., Yang, W., Song, L. Y., Yan, C. Y., Zhang, Z., & Zhao, Y. (2007). Structural characterization and antioxidant activity of a polysaccharide from the fruiting bodies of cultured *Cordyceps militaris*. *Carbohydrate Polymers*, *70*, 430–436.

Zhou, R., Zhang, F., He, P. L., Zhou, W. L., Wu, Q. L., Xu, J. Y., et al. (2005). (5R)-5-hydroxytriptolide (LLDT-8), a novel triptolide analog mediates immunosuppressive effects in vitro and in vivo. *International Immunopharmacology*, *5*, 1895–1903.