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Antioxidant and immunobiological activity of water-soluble polysaccharide fractions purified from *Acanthopanax senticosu*

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ABSTRACT

A water-soluble polysaccharide obtained from *Acanthopanax senticosus* leaves (ASL), was fractionated by DEAE–Sepharose fast-flow column chromatography, and purified by Sephadex G-75 gel-permeation column chromatography. The characteristics of ASP-2-1 were determined by chemical analysis, high-performance capillary electrophoresis (HPCE), high-performance gel-permeation chromatography (HPGPC). The results show that ASP-2-1 contained 89.47% carbohydrate, 7.45% uronic acid, 2.16% protein and seven kinds of monosaccharides including rhamnose, xylose, glucose, mannose, arabinose, galactose and glucuronic acid in a molar ratio of 7.45:18.63:25.15:0.93:8.35:2.79:5.69, with an average molecular weight of about 14,573 Da. Furthermore, the immunobiological and antioxidant activities, in vitro, of ASP-2-1 were evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and ferric-reducing antioxidant power assay (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH·), superoxide radical ($\cdot O_2^-$) and hydroxyl radical (·OH) free radical-scavenging assay, respectively. The results showed that ASP-2-1 exhibited significantly higher immunomodulatory activities against the lymphocyte proliferation in vitro, pronounced reductive power (FRAP value: 785.1 μM at 0.2 mg/ml), strong hydroxyl radical (89.56% at 1 mg/ml) scavenging activity, moderate superoxide radicals (65.32% at 1 mg/ml) and DPPH radicals (68.9% at 1 mg/ml) scavenging activities. ASP-2-1 should be explored as a novel and potential natural antioxidant and immunostimulating agent for use in functional foods or medicine.

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1. Introduction

In recent years, polysaccharides extracted from plants, animals and microorganisms have attracted increasing attention due to their unique biological activities (Forabosco et al., 2006; Wang, Luo, & Ena, 2007), such as antioxidant activity (Qiao et al., 2009), antitumor activity (Chen, Meng, Liu, Chen, & Zhang, 2010), immunobiological activity (Sun et al., 2008), interferon inducing activity (Kojima, Inaba, Kobayashi, & Kimura, 1996), and anticoagulant (Nagumo & Nishino, 1996). Many studies have shown that polysaccharides can improve the activity of antioxidant enzymes, scavenge free radicals, and inhibit lipid oxidation (Xu et al., 2009a). Among them, the radical scavenging activity is one of the important functional properties for bioactive compounds (Athukorala, Kim, & Jeon, 2006), and the formation of some diseases can be directly induced by free radicals, therefore it is worthwhile to study it.

Acanthopanax senticosu (AS) contains glycoside, polyose, chromocor, organic acids and amino acids (Yi et al., 2001), which has been used for the treatment and prevention of various diseases such as ischaemic heart diseases, hypertension, rheumatism, allergies, chronic bronchitis, diabetes, and tumour (Li & Zhou, 2007; Yi et al., 2001). Recent studies have shown that AS plays an important role in enhancing immunity, antibiosis, and anti-oxidation (Yang, Liu, Zhang, & Zheng, 2004). However, to date, no investigation has been carried out on polysaccharides that may account for the textural properties, immunomodulatory activity and antioxidant activities of AS.

In this study, we report on the extraction, isolation, and further purification of a major polysaccharide of ASL using a DEAE–fast flow Sepharose anion-exchange and a Sephadex G-75 column chromatography. In addition, the characteristics, immunomodulatory activity and antioxidant activities of the major polysaccharides are also investigated.

2. Materials and methods

2.1. Materials and chemicals

Male Kunming mice (8–12 weeks old) were purchased from Pharmacology Experimental Center of Jilin University. All mice

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were kept at the animal facilities under specific pathogen-free condition until used. Sterile food and water were supplied.

The DEAE fast flow Sepharose and Sephadex G-75 resin for chromatography were purchased from Amersham Biosciences. 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), 1-phenyl-3-methyl-5-pyrazolone, D-glucose (Glc), L-rhamnose (Rha), D-arabinose (Ara), D-xylose (Xyl), D-galactose (Gal), D-mannose (Man), D-glucuronic acid (GlcA), ascorbic acid (Vc), Concavalin A (Con A), lipopolysaccharide (LPS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Kaiyang Biochemistry Co. (Shanghai, China). Dextrans of different molecular weights were purchased from Pharmacia Co. Trifluoroacetic acid (TFA) was from Merck (Germany). Medium RPMI-1640 was purchased from Gibco Invitrogen Co. The complete RMPI-1640 medium, used for immunological tests, was supplemented with penicillin 100 IU/ml, streptomycin 100 μg/ml, and 10% newborn bovine serum, pH 7.2. All other reagents were of analytical grade.

2.2. Preparation of crude polysaccharides from Acanthopanax senticosu

The powder of ASL was extracted by 95% ethanol to remove the pigments, fat and inactivate enzymes, extracted with hot distilled water for 4 h at 90 °C three times, and the aqueous extract was concentrated to 30% of the original volume under reduced pressure in a rotary evaporator. Protein was removed with Sevag method (Vilkas & Radjabi-Nassab, 1986). The solution was precipitated with ethanol up to 70%, and kept at 4 °C overnight. Finally, the polysaccharide pellets were obtained by centrifugation, resuspended in appropriate volume of distilled water and dialysed for 2 days against distilled water (cut-off molecular weight 3500 Da). The solution was concentrated, and centrifuged to remove insoluble materials. Finally the supernatant was lyophilised in the freeze–dry apparatus (ALPHA 2-4/LSC, Marin Christ Co., Germany) to produce crude ASP.

2.3. Purification of polysaccharide

2.3.1. Anion-exchange chromatographic method

The crude polysaccharides (100 mg) were dissolved in distilled water and filtered through a membrane (0.45 μm, Nucleopore). Then the solution was applied to a DEAE-Sepharose fast-flow column (1.6 × 20 cm) pre-equilibrated with 20 mM Tris–HCl buffer (pH 7.8). Fractions were prepared in a stepwise elution with increased concentration of NaCl (0.1–0.8 M) solution at a flow rate of 1.0 ml/min, and with collection of 5 ml for each tube. The polysaccharide content in each fraction was detected by phenol–sulphuric acid method (Cuesta, Suarez, Bessio, Ferreira, & Massaldi, 2003). The appropriate fractions were concentrated, dialysed against water, and finally lyophilised.

2.3.2. Gel-filtration chromatography

Size-exclusion chromatography of the purified polysaccharide from AS was performed on Sephadex G-75 (16 × 500 mm), and 0.1 M NaCl was used as eluant. The major polysaccharide fractions were collected with a fraction collector, then dialysed with water, and lyophilised to give a polysaccharide named ASP-2-1, which was used in subsequent analyses.

2.4. Determination of the physical characteristics of the polysaccharide

Total sugar content in the purified polysaccharide was determined by the phenol–sulphuric acid method, using D-glucose as standard. Protein content was also determined by BSA standard curve (Bradford, 1976). Total uronic acid content was determined by photometry with *m*-hydroxybiphenyl at 523 nm (Blumenkrantz & Asboe-Hansen, 1973), using GlcA as the standard.

2.4.1. Molecular weight determination

The homogeneity and molecular weight of ASP-2-1 were determined by high-performance gel-permeation chromatography (HPGPC). The sample solution (20 μl of 0.5%) was applied to Agilent 1100 HPLC system equipped with a Dhpak SB-803 HQ (8.0 × 300 mm), eluted with 0.05 M Na_2SO_4 solution at a flow rate of 0.5 ml/min and detected by a RID-10A refractive index detector. The columns were calibrated with T-series dextran (T-200, T-70, T-40, T-20 and T-10) and glucose as standards. The molecular weight of ASP-2-1 was estimated by the calibration curve made above.

2.4.2. Polysaccharide hydrolysis with acid and monosaccharides PMP derivative

The polysaccharide (10 mg) was hydrolysed with 2 M TFA (10 ml) for 8 h at 100 °C in a sealed glass tube. The excess acid was completely removed at 70 °C by a steady stream of nitrogen, and the hydrolysed products were used for the preparation of its derivative. The reaction was carried out by mixing 10 mg of hydrolysed products and 5 ml 0.5 M NaOH, and then 6 ml 0.5 M PMP-methanol solution were added followed by incubation at 70 °C water bath for 30 min. Subsequently the tube was cooled down to room temperature, and 0.5 M HCl was added to neutralise solution pH to 7. Finally, the solution was extracted with 20 ml $CHCl_3$ twice. The extract was centrifuged (3000 rpm, 15 min), the supernatant was collected and the derivatives of the sugars were performed on HPCE. As references, the following neutral sugars were derived and analysed: Rha, Xyl, Glu, Man, Ara, Gal, and GlcA.

2.4.3. High-performance capillary electrophoresis (HPCE) analysis

HPCE was performed on an uncoated fused-silica capillary tube (50 μm × 70 cm) at 25 °C using 75 mM sodium tetraborate buffer (pH 9.5) as solvent, the absorbance was detected at 254 nm. Before each run, the capillary tube was washed by methanol (5 min), double distilled water (2 min), 0.1 M HCl (5 min), double distilled water (2 min), 0.1 M NaOH (10 min), double distilled water (2 min), and conditioned with the operating buffer for 10 min. The samples to be analysed were injected automatically, using the low pressure injection mode (0.5 psi), in which the sample is pressurised for 5 s. The injection volume can be calculated with the Poiseuille equation, as proposed by the manufacturer, giving an estimated volume of 6 nl/s of injection time. Electrophoresis was performed at 15 kV using normal polarity. Peak areas were recorded and calculated using the Beckman software system.

2.5. Determination of antioxidant activities in vitro of ASP-1

2.5.1. Ferric-reducing antioxidant power (FRAP) assay

The total antioxidant potential of sample was determined using an iron (III) reducing ability of plasma (FRAP) by the method described earlier (Benzie & Strain, 1996). FRAP assay measures the change in absorbance at 593 nm due to the formation of a blue-coloured Fe (II)–tripyridyltriazine compound by the reaction of colourless Fe (III) and electron-donating antioxidants. FRAP reagent was prepared by mixing 10 vol of 300 mM acetate buffer (pH 3.6) with 1 vol of 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) in 40 mM HCl and 1 vol of 20 mM $FeCl_3$. Freshly prepared FRAP reagent (1.5 ml) was warmed to 37 °C, and a reagent blank reading was taken at 593 nm. Subsequently, 50 μl of sample and 150 μl of deionized water were added to the FRAP reagent. The final dilution of the sample in the reaction mixture was 1:34. The sample was run in triplicate. After addition of the sample to the FRAP

reagent, a second reading at 593 nm was performed after 8 min. The initial blank reading with the FRAP reagent alone was subtracted from the final reading of the FRAP reagent with the sample to determine the FRAP value of the sample. A standard curve was prepared using different concentrations (100–1000 μM) of $FeSO_4{\cdot}7H_2O$. The final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 μM $FeSO_4{\cdot}7H_2O$.

2.5.2. Superoxide radical-scavenging assay

Superoxide radical was generated in the system of pyrogallol's autoxidation in an alkalescent condition (Zou, Gui, Zhong, & Zhu, 1986). With some modification in this experiment, the reaction was performed in 5.0 ml of phosphate buffer (50 mM, pH 8.2), which contained 3 mM pyrogallol solution and the samples to be tested at different amounts. The reaction mixture was incubated at 25 °C for 20 min, and the change speed of absorbance (A/min) of the reactive solution was measured at 325 nm, against a blank (water and 50 mM phosphate buffer instead of sample). The percent scavenging activity of superoxide radical was calculated by the following formula:

$$\text{Scavenging activity}\,(\%) = (1 - A_1/A_0) \times 100,$$

where A_0 is the change speed of absorbance of the control group in the superoxide radical generation system and A_1 is the change speed of absorbance of the test sample.

2.5.3. Hydroxyl radical-scavenging assay

Hydroxyl radicals were generated using an innovative method (Smirnoff & Cumbes, 1989). The 3 ml sodium phosphate buffer (150 mM, pH 7.4), which contained 10 mM $FeSO_4$, 2 mM sodium salicylate, 6 mM H_2O_2, and varying concentrations of polysaccharides, were incubated at 37 °C for 1 h. The absorbance was detected at 510 nm, and Vc was used as a positive control in the study. The percent scavenging activity of hydroxyl radicals was calculated as follows:

$$\text{Scavenging activity}\,(\%) = [1 - (A_1 - A_2)/A_0] \times 100,$$

where A_0 is the absorbance of the control group in the hydroxyl radicals generating system (water instead of test sample solution), A_1 the absorbance of the test group and A_2 blank is the absorbance of the samples only (water instead of H_2O_2 solution).

2.5.4. DPPH radical-scavenging assay

The DPPH radical scavenging activity was measured using the method reported by (Li, Zhou, & Han, 2006) with slight modification. Briefly, 0.1 mM solution of DPPH in methanol was prepared and 1.0 ml of this solution was added to 3.0 ml of the polysaccharides of various concentrations (0.01–1.0 mg/ml) in water. The mixture was shaken and incubated at 25 °C for 30 min in the dark, then the absorbance was measured at 517 nm against a blank (water instead of test sample and DPPH· solution). Lower absorbance of the reaction mixture indicates higher free-radical scavenging activity. The percent scavenging activity was calculated by the following formula:

$$\text{Scavenging activity}\,(\%) = [1 - (A_1 - A_2)/A_0] \times 100\%,$$

where A_0 is the absorbance of the control (water instead of test sample solution), A_1 is the absorbance of the sample. A_2 is the absorbance of the sample under identical conditions as A_1 and with water instead of DPPH· solution.

2.6. Immunomodulatoryl activity assay in vitro of ASP-2-1

Male Kunming mice (GradeII, 8–12 weeks old) were killed by cervical dislocation. Spleen collected under aseptic conditions was minced and passed through a sterilized ion mesh (200 mesh) to obtain single spleen cell suspensions. Erythrocytes in the cell mixture were destroyed by the rapid addition of H_2O. Then, the cells were washed thrice with phosphate-buffered saline (PBS) and adjusted to a density of 1×10^6 cells/ml in the RPMI 1640 complete medium. Splenocyte proliferation was assayed as previously described (Wang, Li, Song, & Hu, 2002). Briefly, an aliquot of 100 μl of splenocytes were seeded into 4 wells of a 96-well flat-bottom microtiter plate, thereafter Con A (final concentration 2.0 μg/ml), or LPS (final concentration 4.0 μg/ml), or medium with ASP-2-1 (final concentration 1.0, 5.0, 10.0 and 20.0 μg/ml) were added giving a final volume of 180 μl. Control cells were incubated in a medium containing an equivalent solvent amount without the test materials (Con A or LPS). After incubation at 37 °C in a humid atmosphere with 5% CO_2 for 48 h, 20 μl of MTT solution (5 mg/ml) were added to each well and incubated for another 4 h. The plate was centrifuged on 2000 rpm for 10 min, the supernate was discard, then 100 μl Me_2SO was added per well. Absorbance at 570 nm was measured on an ELISA reader (Tecan, GENios ELIASA Co., Austria). The stimulation index (SI) was calculated based on the following formula:

$$\text{SI}(\%) = (A_{570}\text{ of treated cells} - A_{570}\text{ of control cells})/(A_{570}\text{of control cells}) \times 100.$$

The differences of absorbance between groups with Con A (or LPS) and groups without expressed the ability of splenocyte proliferation.

3. Results and discussion

3.1. Isolation and purification of polysaccharides composition

The water-soluble crude polysaccharide named ASP was obtained from the AS by hot water extraction, ethanol precipitation, de-proteinized by Sevag method, dialysed with water and lyophilised in freeze–dry apparatus. The total yield of ASP was 5.2% (w/w) of the dried material. To obtain high-purity and homogeneous polysaccharide products for stable and functional property, the ASP were further purified by DEAE fast flow Sepharose anion-exchange chromatography. Different fractions were selected based on total carbohydrate elution profile (Fig. 1). The results showed that ASP was mainly composed of three sub-fractions, namely ASP-1, ASP-2 and ASP-3. Each fraction was eluted as a single symmetrical peak, which indicated that ASP-1 (15.93%), ASP-2 and ASP-3 (18.83%) were three homogeneous polysaccharide

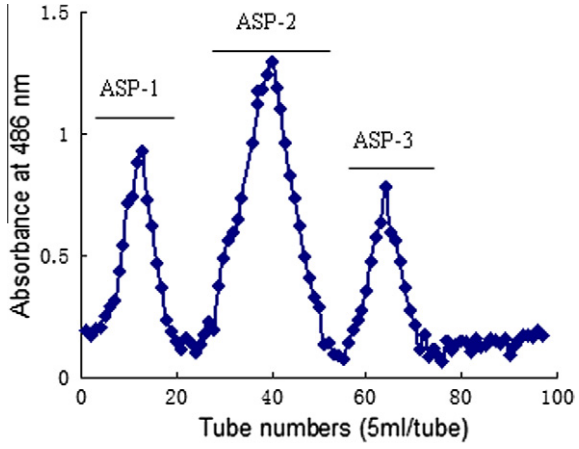

Fig. 1. Elution profiles of crude ASP on DEAE-Sepharose fast flow anion-exchange chromatography column (1.6 × 60 cm) with 0.1–0.8 M NaCl stepwise elute.

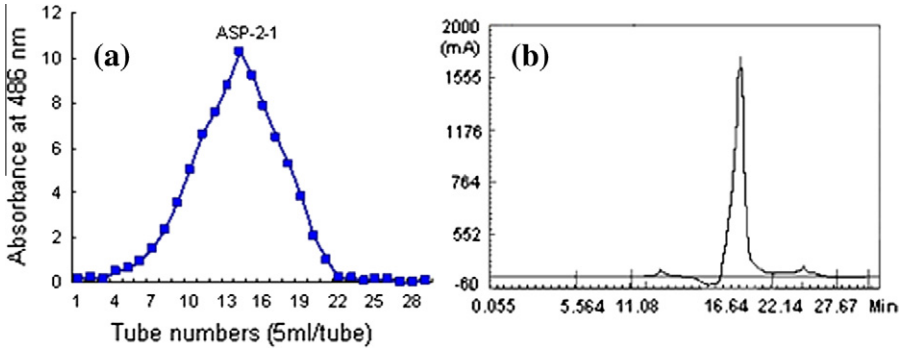

Fig. 2. The profiles of ASP-2 and ASP-2-1. (a) Profile of ASP-2 on Sephadex G75 gel permeation chromatography column (1.6 × 50 cm) with 0.1 M NaCl elute; (b) Profile of SAP-2-1 in HPGPC. The sample was analysed by a Dhpak SB 803 HQ gel filtration column (8.0 × 300 cm) eluted with 0.05 M Na_2SO_4, 0.5 ml/min.

Table 1
Yields, protein contents, sugar contents, and Mw of ASP-2-1.

Sample	Yield[a] (w/w%)	Protein (w/w%)	Uronic acid (mol.%)	Carbohydrate (w/w%)	Mw (Da)	Monosaccharide components (mol.%)[b]						
						Rha	Xyl	Glu	Man	Ara	Gal	GlcA
ASP-2-1	47.33	2.16	7.45	89.47	14,573	7.45	18.63	25.15	0.93	8.35	.2.79	5.69

[a] Percent weights of the ASP.
[b] (mol.%): Quantities of the carboxy-PMP derivative uronic acid are given in mol.%.

sub-fractions and ASP-2 was the predominate fraction with a percent amount of about 47.6% (w/w). To obtain high-purity and homogeneity polysaccharide products for stable functional property, the main fraction of ASP-2 was further purified by Sephadex G-75 gel permeation chromatography based on the molecular size (Fig. 2a). The main fraction was collected, dialysed and lyophilised to obtain a brown purified polysaccharide, named as ASP-2-1. The HPGPC (Fig. 2b) profiles showed a single and symmetrical sharp peak, indicating that ASP-2-1 was a homogeneous polysaccharide with an average molecular weight of about 14,573 Da, according to the calibration curve with standard dextrans. The total sugar content of the polysaccharide was determined to be 89.47%, using

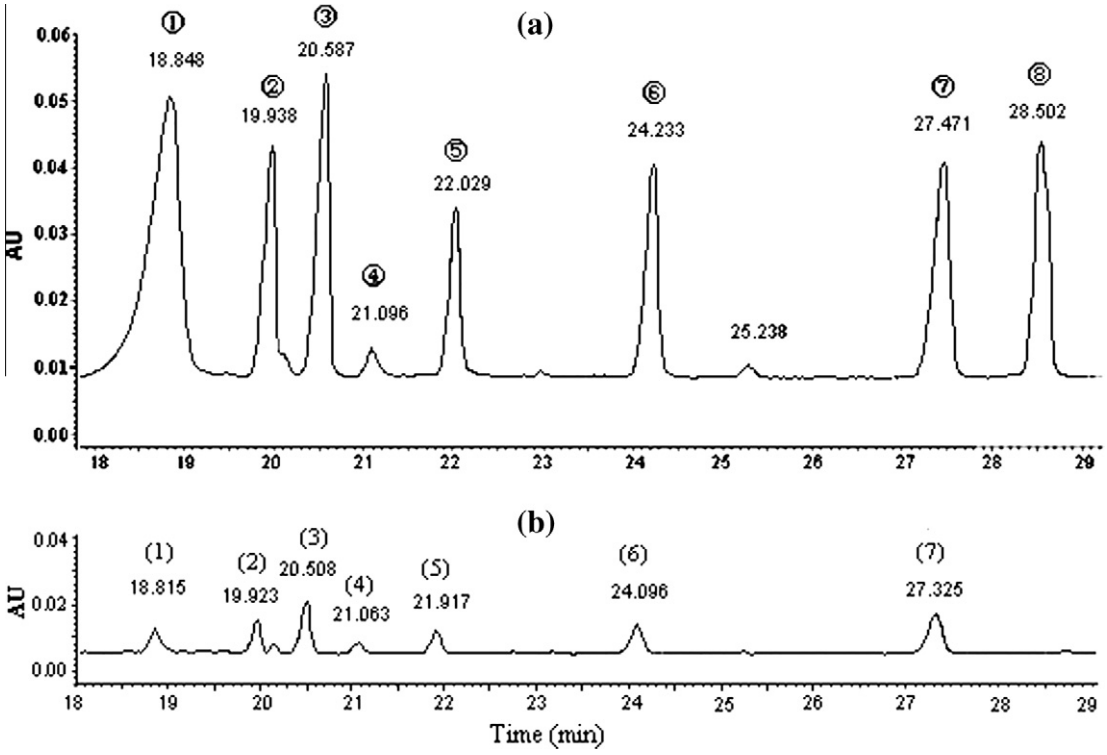

Fig. 3. HPCE electropherogram of monosaccharides (as their PMP derivatives): (A) standard mixture (Peak identity: ①Rha, ②Xyl, ③Glu, ④Man, ⑤Ara, ⑥Gal, ⑦GlcA, ⑧GalA Internal standard); (B) sample (peak identity: (1)Rha, (2)Xyl, (3)Glu, (4)Man, (5)Ara, (6)Gal, (7)GlcA.).

the phenol–sulphuric acid method. The yield, average molecular weight, uronic acid, protein content, and sugar compositions of ASP-2-1 were determined and are shown in Table 1.

3.2. Monosaccharide composition of ASP-2-1

Monosaccharide compositions of ASP-2-1 were determined by the TFA hydrolysis and HPCE analysis method. The results indicated that glucose and xylose were the major monosaccharides constructing the backbones of ASP-2-1. ASP-2-1 was composed of Rha, Xyl, Glu, Man, Ara, Gal and GlcA with molar percent of 7.45%, 18.63%, 25.15%, 0.93%, 8.35%, 2.79% and 5.69%, respectively (Fig. 3).

3.3. Antioxidant activities analysis

3.3.1. Ferric-reducing antioxidant power

The FRAP assay treats the antioxidants contained in the samples as reductants in a redox linked colorimetric reaction, and the value reflects the reducing power of antioxidants. The procedure is relatively simple and easy to standardise. This assay is also commonly used for the routine analysis of single antioxidant and total antioxidant activity of plant extracts (Xu et al., 2009b). The antioxidant potentials of different samples were estimated by their ability to reduce the TPTZ–Fe(III) complex to the TPTZ–Fe(II) complex. The antioxidant capacities of ASP-1, ASP-2-1, ASP-3 and ASP are shown in Fig. 4a and compared with Vc as a control standard. The antioxidant capacities of all the samples correlated well with increasing concentration. The antioxidant capacities of purified fractions ASP-1 and ASP-2-1 were significant higher than that of crude ASP. However, the antioxidant capacity of ASP-3 was weaker than that of crude ASP. The FRAP values of ASP-1, ASP-2-1, ASP-3, ASP and Vc were 724, 785.1, 553, 593.2, and 809 μM, at the concentration of 0.2 mg/ml, respectively. Moreover, ASP-2-1 was found to have higher antioxidant capacities than ASP-1, ASP-3 and ASP, and the antioxidant capacities of ASP-2-1 was close to that of Vc's at 0.2 mg/ml. These results clearly demonstrated that all the samples possessed antioxidant capacities, especially purified fraction of ASP-2-1showed strong antioxidant capacity. The activities of antioxidants have been attributed to various mechanisms, such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Zou et al., 2008). The results also indicate that the purified polysaccharide has a strong antioxidant activity.

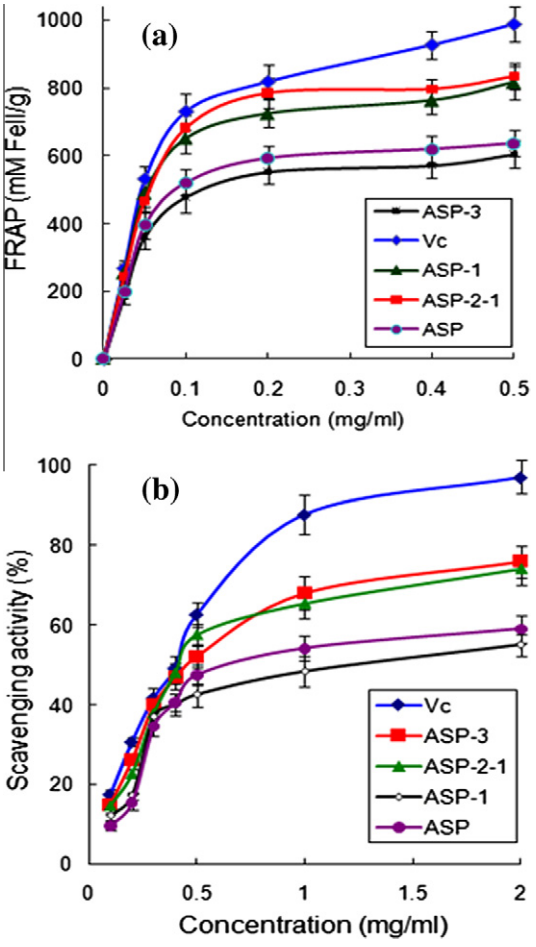

Fig. 4. (a) Ferric-reducing antioxidant power (FRAP) and (b) Scavenging ability on superoxide radicals of ASP-1, ASP-2-1, ASP-3, ASP and Vc. Experimental results were means ± SD of three parallel measurement.

3.3.2. Scavenging effects on superoxide radicals

Superoxide anion is a reductive form of molecular oxygen created by accepting one electron. It is an initial free radical formed from mitochondrial electron transport systems (Bloknina, Virolainen, & Fagerstedt, 2003). Superoxide anion radicals are produced by a number of cellular reactions, including various enzyme systems, such as lipoxygenases, peroxidase, NADPH oxidase, and xanthine oxidase. They play important roles in the formation of other cell-damaging free radicals, such as hydrogen peroxide, hydroxyl radical, and singlet oxygen in living systems. Superoxide radical can be generated by auto oxidation of pyrogallol and it can produce a coloured compound. Due to the colour change from purple to yellow, the absorbance at 320 nm increased when the superoxide anion was scavenged by antioxidant, which can represent the content of superoxide radicals and indicate the antioxidant activity of the sample (Chen, Xie, Nie, Li, & Wang, 2008). As shown in Fig. 4b. The results indicated a concentration-dependent radical scavenging activity at all tested concentrations of all the samples. Especially in relative lower concentration range of 0–0.3 mg/ml, ASP-3 and ASP-2-1 exhibited higher superoxide radical scavenging activity, which was close to that of Vc's. However, in the higher concentration range of 0.5–2.0 mg/ml, the radical scavenging activity of ASP-3 and ASP-2-1 was lower than that of Vc's. At the concentration of l mg/ml, the scavenging activity of the ASP-1, ASP-2-1, ASP-3, ASP and Vc was 48.31%, 65.37%, 68.1%, 54.15% and 85.21%, respectively. Apparently, ASP-3 showed the highest scavenging activity, the scavenging activity was close to that of ASP-2-1, but lower than Vc's, at the tested concentration range (0.1–2.0 mg/ml).It was reported that the scavenging activity of polysaccharides from *Aloe barbadensis* against superoxide radicals was less than 40% at the concentration of 1.0 mg/ml (Liu, Wang, Xu, & Wang, 2007). At the concentration of 10 mg/ml, C-GLP showed good superoxide anions scavenging activities (56%) (Xu et al., 2009). Compared to these results, ASP had a stronger scavenging activity for superoxide radicals. The mechanism of scavenging superoxide anion may be associated with dissociation energy of O–H bond, that is, the higher the number of electron with drawing groups attached to polysaccharide, the weaker the energy of O–H bond. In our experiment, ASP-3 and ASP-2-1 were more effective in scavenging superoxide anion, which should be due to the presence of some types of electrophilic groups like keto or aldehyde in the former, facilitating liberation of hydrogen from O–H bond and thus stabilized superoxide anion (Lin, Wang, Chang, Stephen Inbaraj, & Chen, 2009). Therefore, this ability of polysaccharides from AS to scavenge superoxide may contribute to its significant antioxidant potential.

3.3.3. Scavenging effects on hydroxyl radicals

Hydroxyl radical (·OH) can easily cross cell membranes, readily react with most biomolecules including carbohydrates, proteins, lipids, and DNA in cells, and cause tissue damage or cell death (Yuan, Zhang, Fan, & Yang, 2008). Thus, removing ·OH is important for the protection of living systems. The results of hydroxyl radical scavenging activities of the ASP-1, ASP-2-1, ASP-3, ASP, and Vc were given in Fig. 5a. Among all samples, the scavenging ability on hydroxyl radical decreased in the following order: ASP-2-1 > ASP-3 > ASP-1 > ASP. However, the scavenging activities of all samples were weaker than that of Vc's ($P < 0.05$). The scavenging effects of all samples increased significantly ($P < 0.05$) with the increase of sample concentration ranging from 0.1 to 0.5 mg/ml. After that, the scavenging activity increased slowly with the increase of sample concentration. At a concentration of 1.0 mg/ml, the scavenging activity was 72.6%, 89.56%, 84.5%, 66.9% and 96.85% for the ASP-1, ASP-2-1, ASP-3, ASP and Vc, respectively. The results demonstrated that the polysaccharides from possessed ·OH scavenging activities, especially purified fraction of ASP-2-1 showed the strongest activities. Nevertheless, the scavenging effect of all samples was less effective than Vc. The antioxidant mechanism may be due to the supply of hydrogen by polysaccharide, which combines with radicals and forms a stable radical to terminate the radical chain reaction. The other possibility is that polysaccharide can combine with the radical ions which are necessary for radical chain reaction, and the reaction is terminated. However, the exact mechanism underlying the free-radical scavenging activity exerted by polysaccharides is still not fully understood.

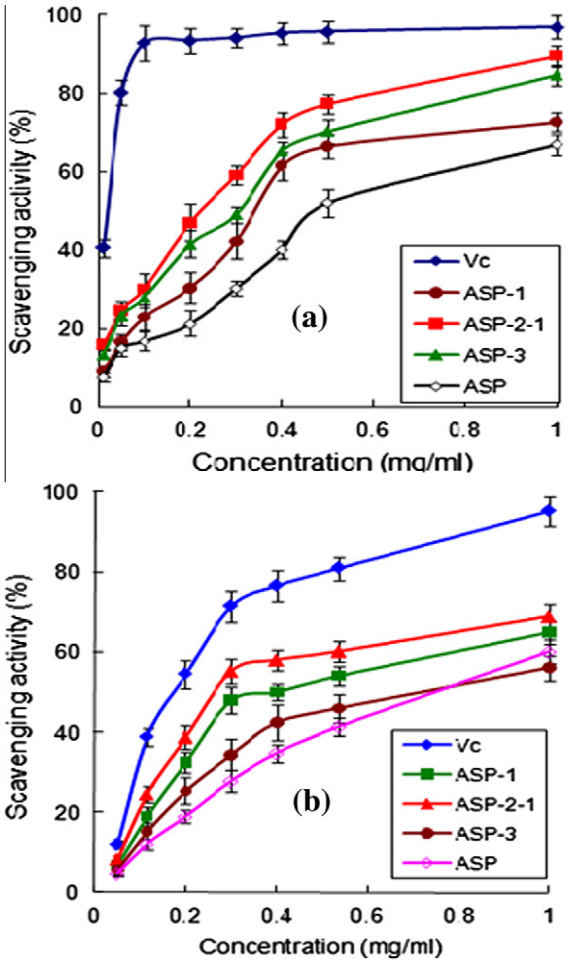

Fig. 5. (a) Scavenging effect on hydroxyl radicals and (b) DPPH radicals of ASP-1, ASP-2-1, ASP-3, ASP and Vc. Each values is the mean ± SD of triplicate measurements.

3.3.4. Scavenging effects on DPPH radicals

DPPH (1,1-diphenyl-2-picrylhydrazyl) is a free-radical compound that has been widely used to determine the free-radical scavenging ability of various samples. The method of scavenging DPPH· is based on the reduction of DPPH· ethanol solution in the presence of a hydrogen donating antioxidant, resulting in the formation of the non-radical form DPPH-H. DPPH· is a stable free radical and can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Therefore, the antioxidant activity of a substance can be expressed as its ability in scavenging the DPPH· free radical. As shown in Fig. 5b. The results indicated that, ASP-1, ASP-2-1, ASP-3 and ASP showed obvious scavenging activity on DPPH radical in a concentration-dependent manner. Furthermore, at relatively low concentration range of 0–0.3 mg/ml, the scavenging activities of ASP-2-1 and ASP-1 increased significantly with increasing concentrations. The scavenging activity of ASP-2-1 was the strongest, followed by ASP-1, ASP-3 and ASP, which was lower than that of Vc's. At 0.5 mg/ml, scavenging activity of ASP-2-1 was around 60.17%, while ASP's scavenging activity was only 41.33%. These results indicate that the purified polysaccharide has a noticeable effect on scavenging DPPH· free radicals. The antioxidant activity of the polysaccharides may be related to monosaccharide component, molecular size, structure and conformation. These monosaccharides in the polysaccharides are reductive agents as they can supply hydrogen, which can combine with radical and form a stable radical to terminate the radical reaction. It has been reported that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds and aromatic amines could reduce and decolourize DPPH· by their hydrogen donating ability (Li, Zhou, & Li, 2007). These results indicate that the ASP-2-1 might act as electron or hydrogen donator to scavenge DPPH·.

3.4. Immunomodulatoryl activity in vitro of ASP-2-1

Lymphocyte proliferation is a crucial event in the activation cascade of both cellular and humoral immune responses. In order to investigate a possible immunomodulatory effect of the polysaccharides, the MTT assay was used to evaluate spleen lymphocyte proliferation induced by Con A or LPS in vitro. As shown in Table. 2. At a lower concentration (1 μg/ml) ASP-2-1 could significantly enhance Con A-induced lymphocyte proliferation ($P < 0.05$) as compared with those of the normal control group. ASP-2-1 exhibited the highest comitogenic activities, at the concentration of 10 μg/ml ($P < 0.001$), but comitogenic effect was not in a concentrations-dependent manner. Similarly, ASP-2-1 showed a significant comitogenic activity on LPS-stimulated lymphocytes ($P < 0.05$), but comitogenic effect was not in a dose-dependent manner, and reached maximum value at the concentration of 10 μg/ml

Table 2
Effects of ASP-2-1 on Con A- and LPS-stimulated splenocyte proliferation the immunised mice[a].

Groups	Concentration (μg/ml)	Con A (A_{570})	LPS (A_{570})
Control	–	0.376 ± 0.018	0.2294 ± 0.009
ASP-2-1	1	0.4045 ± 0.019*	0.2684 ± 0.013*
	5	0.4511 ± 0.016**	0.3161 ± 0.010*
	10	0.5061 ± 0.023***	0.3696 ± 0.012**
	20	0.4482 ± 0.022***	0.3129 ± 0.008*

[a] Values are means ± SD ($n = 10$), Significant differences with control group are designated as.
* $P < 0.05$ compared to normal control.
** $P < 0.01$ compared to normal control.
*** $P < 0.001$ compared to normal control.

($P < 0.01$). In order to avoid false positive of the immunological test of the polysaccharides, contaminant endotoxins, which possess mitogenic activity, were removed from the polysaccharide preparations. Briefly, 1 ml of Affi-Prep Polymyxin Matrix was packed in a Bio-spin column, centrifuged at 200 rpm for 2 min, and then 0.5 ml of ASP-2-1 (10 μg/ml) and LPS (5 μg/ml) was added. After incubating overnight at 4 °C, the effluent was recovered from the column by centrifugation at the same condition. The results indicated that ASP-2-1 could significantly enhance the Con A or LPS-induced lymphocytes proliferation in vitro, and moreover the effect was not due to endotoxin contamination. Thus we can draw a conclusion that ASP-2-1 can be seen as a promising immunopotentiating agent for use in health-care food or medicine, while the mechanism of the immunological activity and structure–function relationship needs further research.

4. Conclusions

According to the results showed above, it was concluded that the water-extracted crude polysaccharide (ASP) from AS was fractionated by DEAE–Sepharose fast-flow column chromatography and the predominated fraction (ASP-2) was further purified by Sephadex G-75 column chromatography. Three major polysaccharide fractions (ASP-1, ASP-2 and ASP-3) were gained, and the predominated fraction (ASP-2) was considered of high purity. Antioxidant test in vitro indicated that ASP-2-1 exhibited a powerful scavenging effect of the superoxide radical, hydroxyl radicals and DPPH radical. Also, the ASP-2-1 showed a higher FRAP value that may be comparable to that of Vc's. ASP-2-1 showed significantly higher immunomodulatory activities against the lymphocyte proliferation in vitro. The results suggest that the water-soluble polysaccharide purified from AS should be explored as a novel and potential natural antioxidant and immunostimulating agent for use in functional foods or medicine.

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