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Full Length Research Paper

Purification, chemical characterization and *in vitro* antioxidant activities of alkali-extracted polysaccharide fractions isolated from the fruits of *Schisandra chinensis*

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The alkali-extracted crude polysaccharide was extracted from the fruits of *Schisandra chinensis*, and further purified by DEAE-cellulose and Sepharose CL-6B chromatography, giving three polysaccharide fractions coded as ASPS-a-1, ASPS-b-2 and ASPS-b-3. In this study, their chemical and physical characteristics of polysaccharide fractions and antioxidant capacity, including scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, chelating ability, hydroxyl radicals and superoxide radicals, were evaluated. The results showed that ASPS-b-3 exhibited significantly antioxidant activity at a concentration-dependent manner. The alkali-extracted polysaccharide fraction can be developed to be new antioxidant drug.

Key words: *Schisandra chinensis*, polysaccharide, antioxidant activity.

INTRODUCTION

Schisandra chinensis (Turcz.) Baill distributed abundantly in the northeast region of China, Korea and Japan. *S. chinensis* has been used in traditional Chinese medicine (TCM) for thousands of years. The Chinese pin yin name of 'Wu Wei Zi' can be translated to five-taste fruit, giving it a special place in TCM due to the importance of the relationship between taste and herbal action. In particular *S. chinensis* has been developed as an alternative medicine for the treatment of various liver diseases as its capability to protect the liver from injuries, which was useful for acute or chronic liver disease, chemical liver damage and poor liver function, as well as improving the detoxifying ability of the liver (Panossian and Wikman, 2008). In addition, it was also an antioxidant, adaptogenic, nervine tonic and mild antidepressant useful for improving mental and physical performance, endurance and adaptation to stress. Moreover, it was often used for chronic cough and asthma due to its antitussive effect and to assist childbirth because of its oxytocic effects (Lu and Chen, 2009).

The recent abundant evidences suggested that reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and hydroxyl radical, involve in the pathogenesis of various disorders and diseases (Niki, 2010). According to the free-radical theory, the disruption of the delicate balance between generation of reactive oxygen species and antioxidant scavenging systems could eventually lead to serious health problems such as diabetes and Alzheimer's disease (Muller et al., 2007). Herein much more attention has been attracted to develop and utilize effective and natural antioxidants in the maintenance of human health and prevention and retardation of the progress of many chronic diseases induced by ROS (Getoff, 2007).

Current researches on free radicals have confirmed that traditional Chinese medicine rich in antioxidants play an essential role in the prevention of cardiovascular diseases, cancers, neurodegenerative diseases, inflamemation and other free radical induced problems and search for novel type of antioxidants from traditional

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Chinese medicine (TCM) has achieved considerable attention. Polysaccharides as important natural products from traditional Chinese medicine exhibit significant antioxidant activities, which protect cells against the damaging effects of reactive oxygen species, prevention of the chronic and degenerative diseases (Song et al., 2010). In the last decades, the pharmacology and chemistry of *S. chinensis* has been extensively studied and the results of researches show that the polysaccharides of *S. chinensis* are important active component, which have many pharmacological properties including anticancer, anti-inflammation, antiaging and tonic drug. However, there have been seldom reports on free radical scavenging activities of polysaccharide fractions from *S. chinensis*.

In order to fully develop the wild resources and extend the potential use of *S. chinensis* in antioxidant biomedicine, the present study was carried out to investigate antioxidant activities of alkali-extracted polysaccharide fractions from the fruits of *S. chinensis* with *in vitro* antioxidant models, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, superoxide radical scavenging activity, hydroxyl radical scavenging activity and ferrous ion-chelating activity, as well as their chemical and physical characteristics.

MATERIALS AND METHODS

Materials and chemicals

S. chinensis was purchased from a local medicine market and identified according to the identification standard of Pharmacopeia of the People's Republic of China. Sepharose CL-6B was purchased from Amersham Pharmacia Co. (Sweden). 1,1-diphenyl-2-picrylhydrazyl (DPPH), DEAE-cellulose, nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium (NBT), safranine, ferroizine, T-series dextrans, and standard sugars were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemical reagents used were analytical grade.

Isolation and purification of polysaccharide fractions

The air dried fruits of *S. chinensis* were ground and then the powder were extracted with 80% ethanol for 24 h. After filtered, the residues were dried and extracted with distilled water at 90°C for 2 h twice. Then the water unextractable solid was washed, dried and extracted with 0.5 M NaOH solution contained 0.3% (w/w) KBH_4 at room temperature for 2 h. The alkali extract was filtered, centrifuged and neutralized with hydrochloric acid (0.1 M), then concentrated and precipitated with 3 volumes of ethanol. Polysaccharide precipitate was collected by centrifugation, deproteinated by a combination of proteinase and Sevag method (Staub, 1965), and then obtained crude alkaline *S. chinensis* polysaccharides (cASPS).

The cASPS was dissolved in distilled water and then loaded onto DEAE–cellulose column, eluted successively with distilled water and 0.5 M NaCl. Fractions were collected and monitored with the phenol–sulfuric acid method. The two main fractions (ASPS-a and ASPS-b) was collected, dialyzed, lyophilized and were further fractioned on a Sepharose CL-6B column, eluted with 0.15 M NaCl to yield three main fractions, codes as ASPS-a-1, ASPS-b-2 and ASPS-b-3. All the fractions were collected, dialyzed and lyophilized.

Molecular weight determination

Molecular weights of the different polysaccharide fractions were determined by high-performance size-exclusion chromatography (HPSEC). The samples of polysaccharide fractions were dissolved in distilled water, applied to a SHIMADZU HPLC system equipped with a TSK-GEL G3000 PWXL column, eluted with 0.1 mol/L Na_2SO_4 solution and detected by a RID-10A Refractive index detector. Dextran standards with different molecular weights (T-2000, T-70, T-40, T-20, and T-10) were to calibrated the column and establish a standard curve.

Monosaccharide composition analysis

Polysaccharide fractions were hydrolyzed and acetylated according to Lehrfeld (1985). Simply, the samples were hydrolyzed with TFA and then hydrolyzed product was reduced with KBH_4, followed by neutralization with acetic acid. After adding myo-inositol and Na_2CO_3, the residue was concentrated. The reduced products were added with pyridine–propylamine and acetylated with pyridine–acetic anhydride. The acetylated products were analyzed by gas chromatography (GC) and identified and estimated with myo-inositol as the internal standard.

GC was performed on a Agilent 6890 instrument (Agilent Technologies, USA) equipped with HP-5 capillary column (30 m × 0.32 mm × 0.2 μm) and flame-ionization detector (FID) and temperatures programmed from 120 to 250°C at a rate of 8°C /min.

Measurement of carbohydrate and protein contents

Total carbohydrate contents of the polysaccharide fractions were determined by phenol-sulfuric acid colorimetric method (Dubois et al., 1956). Protein contents were quantified according to the Bradford's method (Bradford, 1976). Total uronic acid contents were measured by *m*-hydroxydiphenyl method (Filisetti-Cozzi and Carpita, 1991).

DPPH free radical scavenging activity

Radical scavenging activity against the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was measured by the method of Mavundza et al. (2010) with a minor modification. Samples were dissolved in distilled water at 0 (control), 0.5, 1, 2, 4, and 8 mg/ml. One milliliter samples were mixed with 1 ml DPPH (0.1 mM, in 50% ethanol). After incubated at 25°C for 30 min in the dark, the absorbance was measured at 517 nm. The scavenging activity of DPPH radicals was calculated from the following equation:

Scavenging effect (%) = (1 - *A*sample/*A*control) × 100%.

Metal chelating assay

The ferrous ion chelating ability of all different fractions was investigated according to the method of Lin et al. (2009). Briefly, samples were dissolved in distilled water at 0 (control), 0.5, 1, 2, 4, and 8 mg/ml. The reaction mixture contained 0.1 ml $FeCl_2$ (2 mM), 0.4 ml ferroizine (5 mM) and 1 ml samples of varying concentrations. After shaken well and incubated for 10 min at room temperature, the absorbance of the mixture was measured at 562 nm. The ability of different fractions to chelate ferrous ion was

calculated using the following equation:

Chelating ability (%) = (1−Asample/Acontrol) ×100%

Superoxide radical scavenging assay

Superoxide radicals scavenging activity of alkali-extracted polysaccharide fractions were assayed by the method of photoreduction of NBT (Hakkim et al., 2008) with some modifications. Superoxide anions were generated in a nonenzymatic PMS-NADH system by the oxidation of NADH and assayed by reduction of NBT. Reaction mixtures in a final volume of 3 ml contained the following reagents at final concentration: 60 µM phenazine methosulfate (PMS), 468 µM nicotinamide adenine dinucleotide (NADH), 150 mM nitroblue tetrazolium (NBT) and various concentrations of samples. The mixture reacted at 20°C for 10 min and then the absorbance was measured at 560 nm. Each value was expressed by the mean of triplicate measurements with standard deviation. The capability of scavenging the superoxide radical was calculated using the following equation:

Scavenging effect (%) = (1−Asample/Acontrol) ×100%

Hydroxyl radical scavenging activity

The hydroxyl radical assay was measured by the method of Fenton reaction (Wang et al., 2008) with a minor modification. Briefly, Samples were dissolved in distilled water at 0 (control), 0.5, 1, 2, 4, and 8 mg/ml. The reaction mixture contained 1 ml of safranine (0.36 mM), 0.5 ml of EDTA-Fe (2 mM), 1.5 ml of H_2O_2 (3.0%) and 1 ml samples of varying concentrations. After incubation at room temperature for 20 min, the absorbance of the mixture was measured at 520 nm. Hydroxyl radicals gave a crimson colour, so the absorbance change of the reaction mixture indicated the scavenging ability for hydroxyl radicals. The hydroxyl radical-scavenging activity was expressed as:

Scavenging effect (%) = (1 - Asample/Acontrol) × 100%.

RESULTS AND DISCUSSION

Isolation and purification of polysaccharides

The yield of the alkali-extracted crude polysaccharide from the fruits of *S. chinensis* was 1.1%. After deproteinated, the crude polysaccharide sample (cASPS) was loaded onto the DEAE-cellulose column (Figure 1), de-ionized water was used to elute the unbound component (ASPS-a); and the retained components (ASPS-b) was eluted with 0.5 M NaCl. Then ASPS-a and ASPS-b were loaded onto Sepharose CL-6B column, respectively, eluted with 0.15 M NaCl, and three main fractions (ASPS-a-1, ASPS-b-2 and ASPS-b-3) were separated for further analysis of physicochemical properties and *in vitro* antioxidant activities.

Physicochemical properties and chemical compositions

The total sugar, protein, uronic acid contents, molecular weight and monosaccharides composition of the polysaccharide fractions are summarized in Table 1. The carbohydrate content of ASPS-a-1, ASPS-b-2 and ASPS-b-3 were 97.1, 96.3 and 98.5%, respectively. Based on Bradford method, no protein was detected in all the fractions. Furthermore, the uronic acid contents in ASPS-a-1, ASPS-b-2 and ASPS-b-3 were 7.8, 39.4 and 43.5%, respectively. The average molecular weights of ASPS-a-1, ASPS-b-2 and ASPS-b-3, calculated by HPLC, were 103.3, 61,6 and 34,7 kDa, respectively. According to GC analysis, All the three polysaccharide fractions were composed of arabinose, xylose, glucose and galacturonic acid.

DPPH radical scavenging activity

DPPH radical model is widely used to evaluate anti-oxidant activities, and the principle of scavenging is based on the reduction of the stable DPPH solution (purple) in the presence of antioxidant, leading to the for-mation of non-radical form DPPH-H (yellow). Figure 2 demonstrated DPPH scavenging activity caused by alkali-extracted polysaccharide fractions at different concentrations. In all the three fractions, the scavenging activity increased steadily at the concentration range of 0.5 to 8 mg/ml. ASPS-b-2 and ASPS-b-3 both exhibited significant radical scavenging activities. When the concentration was more than 2 mg/ml, DPPH scavenging activity of ASPS-b-2 and ASPS-b-3 was significantly higher ($P < 0.01$) than that of ASPS-a-1. The DPPH radical scavenging rate of ASPS-a-1, ASPS-b-2 and ASPS-b-3 reached 53.4, 81.5 and 87.2% at 8 mg/ml, respectively. There was no significant difference in scavenging activity between ASPS-b-2 and ASPS-b-3 at the concentration rang of 2 to 8 mg/ml ($P >0.05$). The IC50 values of ASPS-a-1, ASPS-b-2 and ASPS-b-3 were 7.4, 2.9, and 1.8 mg/ml, respectively, revealing that the fra-ction ASPS-b-3 possessed the highest DPPH radical scavenging activity.

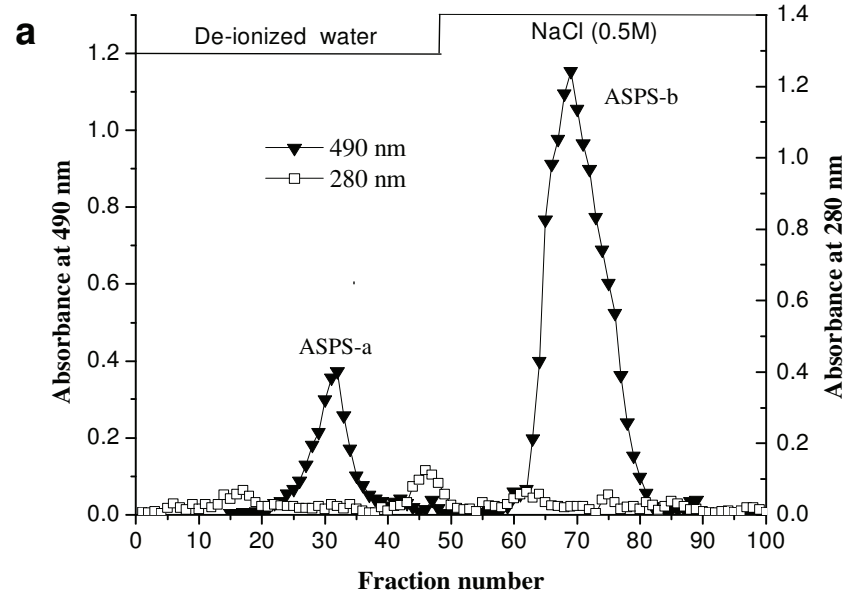
Ferrous ion-chelating effect of polysaccharide fractions

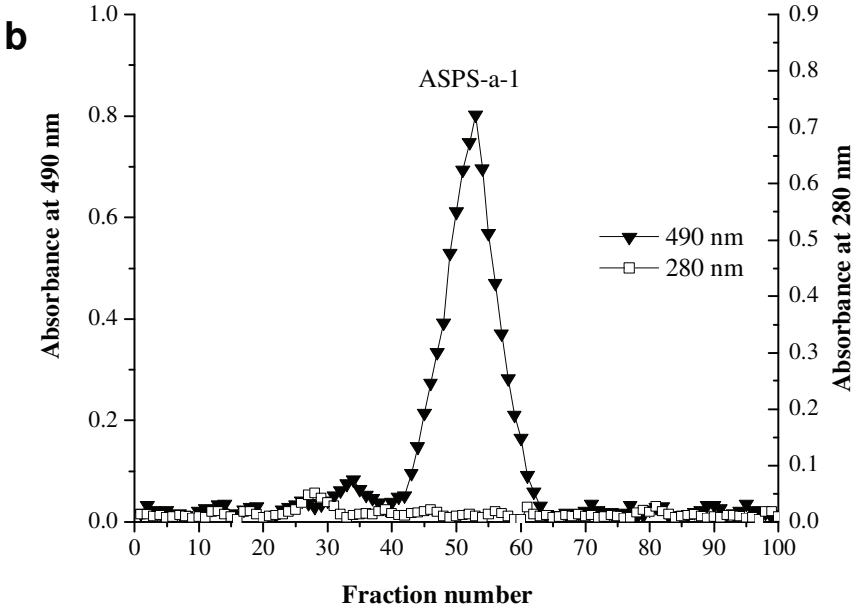
Ferrous ions (Fe^{2+}) can catalyze and induce superoxide anion to form more harmful hydroxyl radicals. Ferrozine can form complexes with Fe^{2+} quantitatively and the complex formation is disrupted with the result that the red color of the complex is decreased in the presence of chelating agents. Therefore, measurement of color reduction allows estimation of the chelating activity. The chelating ability of the samples is shown in Figure 5, ASPS-b-2 and ASPS-B-3 showed an excellent chelating ability. The chelating ability increased significantly ($P < 0.01$) with increasing concentrations from 0.5 to 8.0 mg/ml, and the chelating ability of ASPS-a-1, ASPS-b-2 and ASPS-b-3 at 8.0 mg/ml was 44.5, 72.1 and 73.5%, respectively.

Table 1. Molecular weight and composition of alkali-extracted different polysaccharide fractions from the fruits of *Schisandra chinensis*.

Parameter	ASPS-a-1	ASPS-b-2	ASPS-b-3
Molecular weight	103,300	61,600	34,700
Total sugar (%)	97.1	96.3	98.5
Protein (%)	nd [a]	nd	nd
Uronic acid (%)	7.8%	39.4%	43.5%
Sugar components (mol%)			
Arabinose	22	14	16
Xylose	15	18	9
Glucose	51	33	32
Galacturonic acid	6	36	41

[a] nd: not detect.





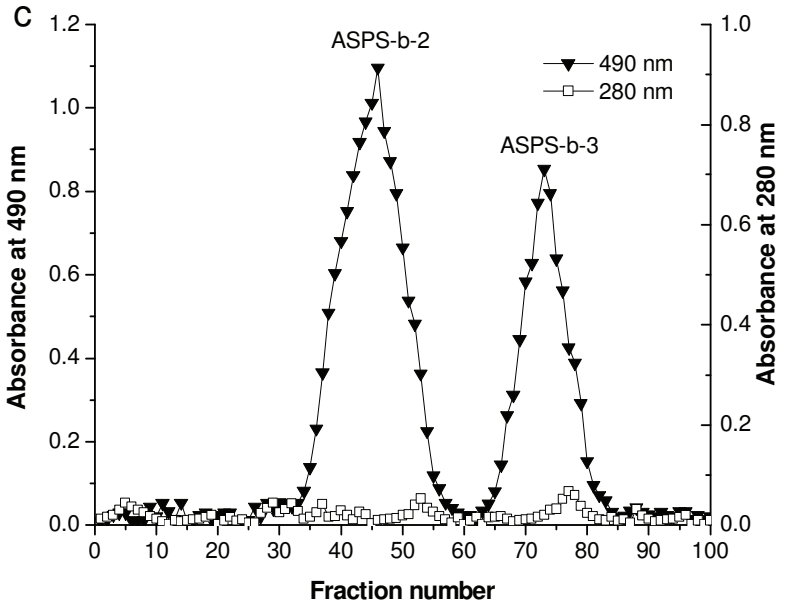


Figure 1. Alkali-extracted polysaccharide fractionations from the fruits of *Schisandra chinensis* puridied by DEAE-cellulose (a) and Sepharose CL-6B (b, c). Crude polysaccharide (cASPS) was loaded onto the DEAE-cellulose column, de-ionized water was used to elute the unbound component (ASPS-a); and the retained components (ASPS-b) was eluted with 0.5 M NaCl. Then ASPS-a and ASPS-b were loaded onto Sepharose CL-6B column, respectively, eluted with 0.15 M NaCl, giving three main fractions (ASPS-a-1, ASPS-b-2 and ASPS-b-3).

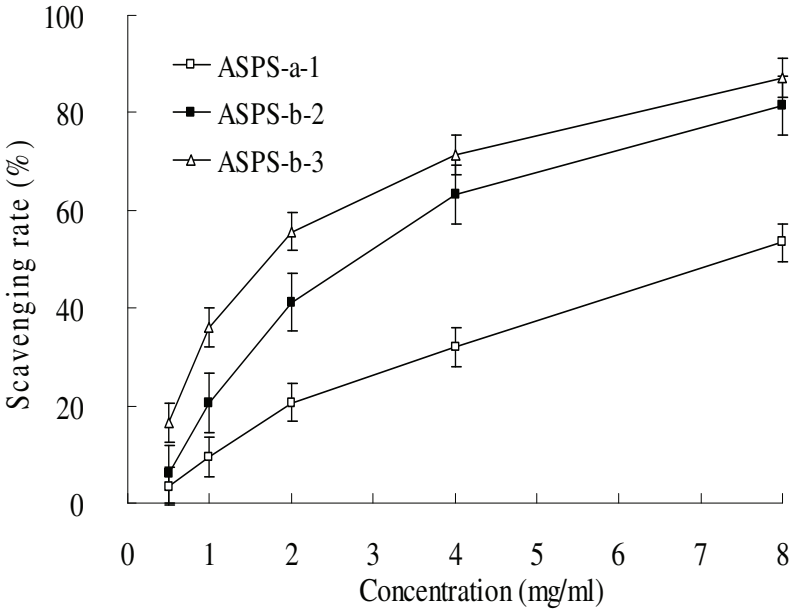


Figure 2. Scavenging rate of alkali-extracted different polysaccharide fractions isolated from the fruits of *Schisandra chinensis* against DPPH radical. Results were presented as means ±S.D (n = 3).

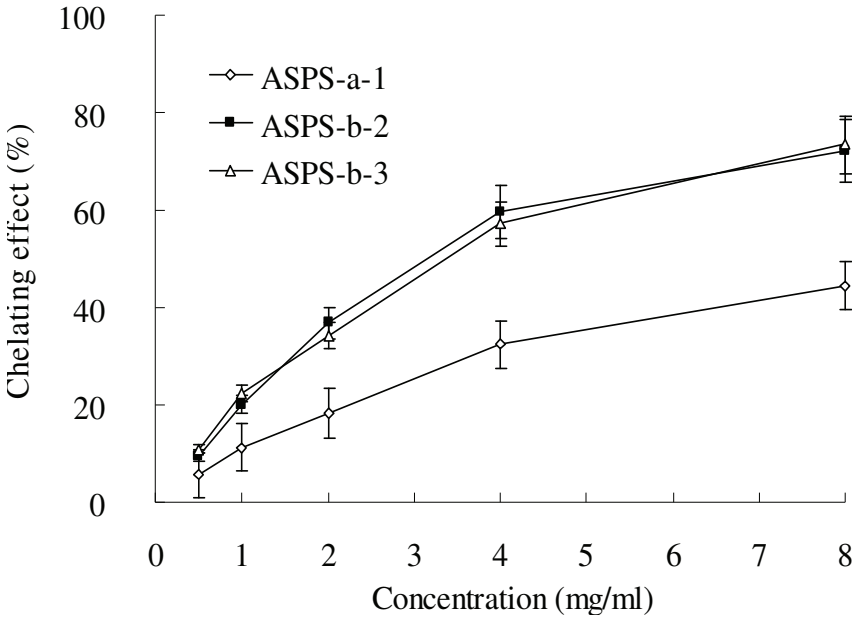


Figure 3. Ferrous ion-chelating ability of alkali-extracted different polysaccharide fractions isolated from the fruits of *Schisandra chinensis*. Results were presented as means ±S.D (n = 3).

Superoxide radical scavenging activity

Superoxide anions are a precursor to active free radicals, although it is not highly reactive, it can produce other ROS such as hydroxyl radical and singlet oxygen, further cause damage in biological macromolecules.

As shown in Figure 3, superoxide scavenging activities of three fractions increased significantly ($P < 0.01$) with increasing concentrations from 0.5 to 8.0 mg/ml, and the superoxide radical scavenging rate of ASPS-a-1, ASPS-b-2 and ASPS-b-3 at 8.0 mg/ml was 22.5, 46.6 and 63.5%, respectively. The IC50 values of ASPS-a-1, ASPS-b-2 and ASPS-b-3 were 17.5, 8.8 and 4.1 mg/ml, respectively. Scavenging activity of ASPS-b-3 was significantly higher ($P < 0.01$) than that of ASPS-a-1 and ASPS-b-2.

Hydroxyl radical scavenging activity

Hydroxyl radicals are considered to be the most reactive oxygen radicals. The hydroxyl radicals scavenging abilities were determined by Fenton reaction. The results were shown in Figure 4. ASPS-a-1, ASPS-b-2 and ASPS-b-3 exhibited distinct scavenging ability, ASPS-b-2 and ASPS-b-3 against hydroxyl radical were better than that of ASPS-a-1. Furthermore, the scavenging ability increased with the increasing concentrations from 0.5 to 8.0 mg/ml. At 8.0 mg/ml, the scavenging rate of ASPS-a-1, ASPS-b-2 and ASPS-b-3 was 22.4, 54.9 and 75.3%, respectively. The IC50 values of ASPS-a-1, ASPS-b-2 and ASPS-b-3 were 18.1, 6.1, and 2.6 mg/ml, respectively. These results showed that ASPS-b-3 had a stronger hydroxyl radical scavenging activity.

Conclusion

As a natural antioxidant compounds, polysaccharides can maintain human healthy, prevent and retard the progresses of many free radical-induced chronic diseases. In this paper, three alkali-extracted polysaccharide fractions (ASPS-a-1, ASPS-b-2 and ASPS-b-3) from the fruits of *S. chinensis* were obtained by DEAE-cellulose and Sepharose CL-6B chromatography. The results of *in vitro* antioxidant models showed that polysaccharides with higher content of uronic acid dose-dependently exhibited more significant antioxidant activity than that with lower or no content of uronic acid in a descending order of ASPS-b-3 (uronic acid = 43.5%), ASPS-b-2 (uronic acid = 39.4%), ASPS-a-1 (uronic acid = 7.8%). One of the mechanisms involved in antioxidant activity may originate from hydrogen atom-donating ability of molecules to oxygen radicals, which would terminate radical chain reactions and convert free radicals to non-harmful products. The electron-withdrawing carboxyl groups substituted in C-5 of sugar residue could activate the hydrogen atom release through

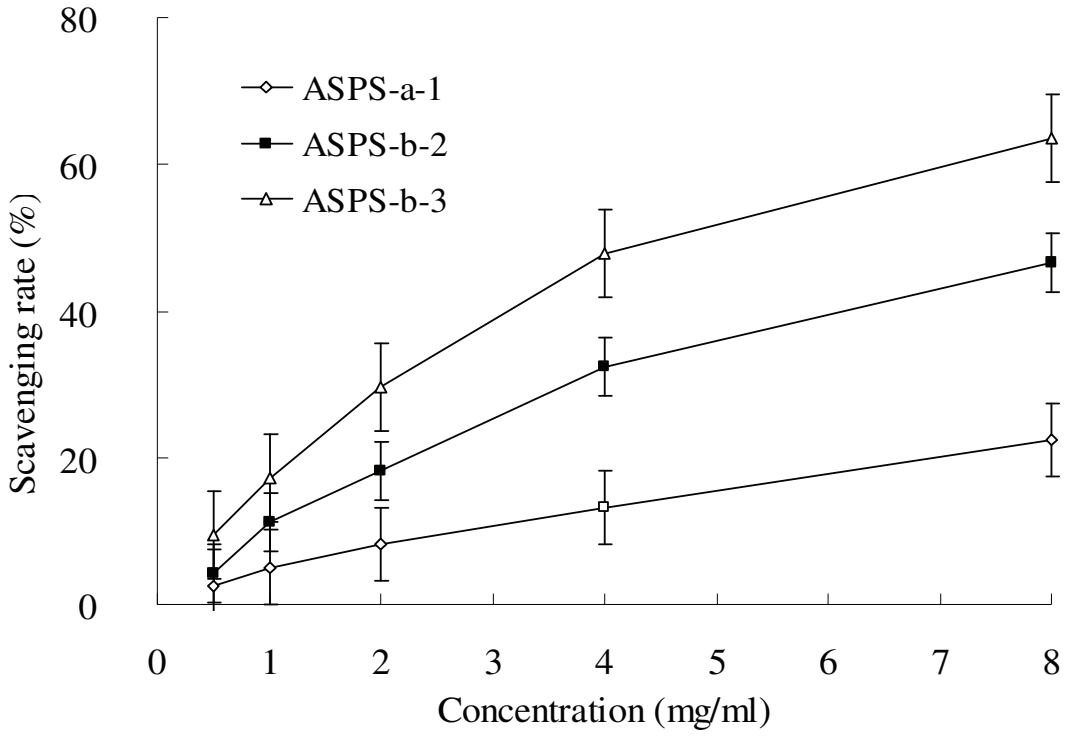


Figure 4. Scavenging rate of alkali-extracted different polysaccharide fractions isolated from the fruits of *Schisandra chinensis* against superoxide radical. Results were presented as means ±S.D (n = 3).

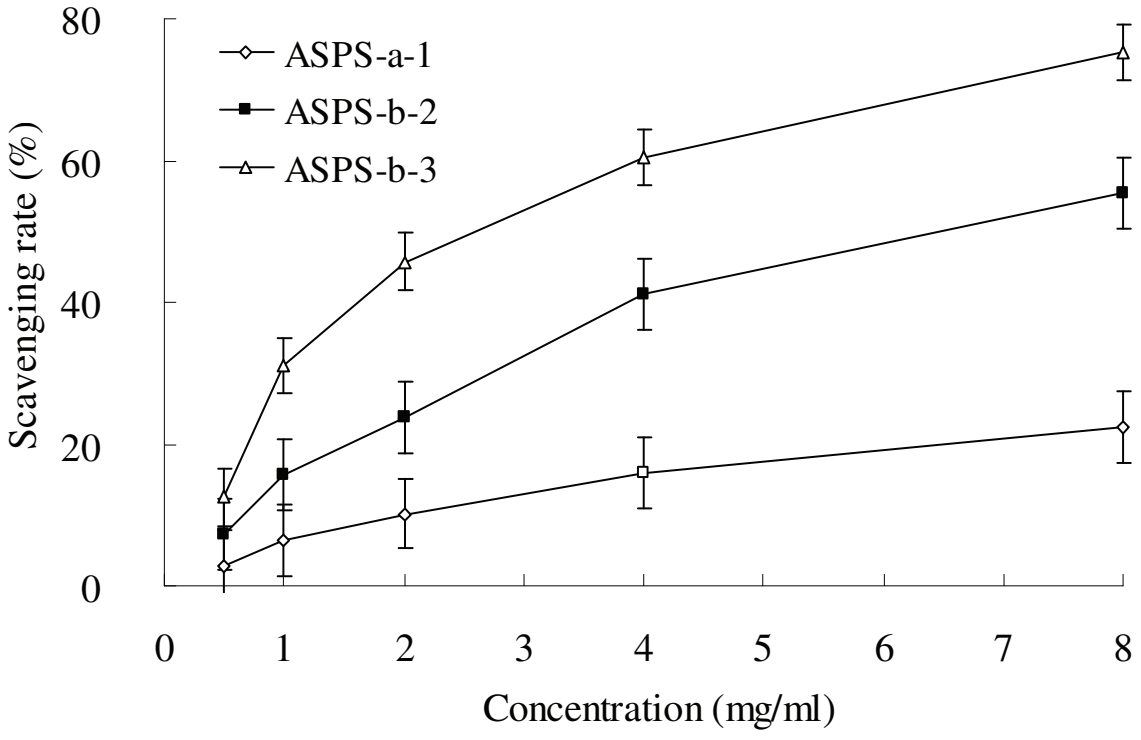


Figure 5. Scavenging rate of alkali-extracted different polysaccharide fractions isolated from the fruits of *Schisandra chinensis* against hydroxyl radical. Results were presented as means ±S.D (n = 3).

field and inductive effects. In addition, it was also reported that the compounds with structures containing functional group of -COOH in a favorable structure–function configuration can show metal chelating activity (Sun and Kennedy, 2010). Based on these studies, we can conclude that ASPS-b-3 will be promising for its application in antioxidant medicinal fields.

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