

Composition and antioxidant activity of water-soluble oligosaccharides from *Hericiium erinaceus*

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Received September 23, 2013; Accepted June 26, 2014

DOI: 10.3892/mmr.2014.3121

Abstract. Oligosaccharide are carbohydrate molecules, comprising repeating units joined together by glycosidic bonds. In recent years, an increasing number of oligosaccharides have been reported to exhibit various biological activities, including antitumor, immune-stimulation and antioxidation effects. In the present study, crude water-soluble oligosaccharides were extracted from the fruiting bodies of *Hericiium erinaceus* with water and then successively purified by diethylaminoethyl-cellulose 52 and Sephadex G-100 column chromatography, yielding one major oligosaccharide fraction: *Hericiium erinaceus* oligosaccharide (HEO-A). The structural features of HEO-A were investigated by a combination of monosaccharide component analysis by thin layer chromatography, infrared spectroscopy, nuclear magnetic resonance spectroscopy, scanning electron microscopy and high-performance gel permeation chromatography. The results indicated that HEO-A was composed of D-xylose and D-glucose, and the average molecular size was ~1,877 Da. The antioxidant activity of HEO-A was evaluated using three biochemical methods to determine the scavenging activity of HEO-A on 1,1-diphenyl-2-picrylhydrazyl, hydrogen peroxide and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sufonic acid) diammonium radicals. The results indicated that HEO-A may serve as an effective healthcare food and source of natural antioxidant compounds.

Introduction

Oligosaccharides are frequently found as components of glycoproteins or glycolipids; therefore, these molecules are often used as chemical markers, particularly for cell recognition. When oligosaccharides are consumed, any undigested

remnants act as fuel for the intestinal microflora. The effects exerted on the bacterial groups may be either stimulatory or suppressive, depending on the type of oligosaccharide (1,2). Clinical studies have demonstrated that the administration of fructo-oligosaccharides, galacto-oligosaccharides or inulin can enhance the levels of useful intestinal bacteria and simultaneously reduce the number of harmful bacteria (3,4).

The sialyl-Lewis(x) oligosaccharide, identified in a study in 2011 (5), is believed to be the most abundant carbohydrate receptor in the outer coating, or zona pellucida (ZP), of human female ova. This oligosaccharide has been suggested to play a pivotal role in the binding of sperm to the ZP, leading to fertilization. Sialyl-Lewis(x) branches are more abundant on the ova than on any other type of body cell, forming a complete coating. The identification this prominently featuring receptor on the human ova may enhance research into the field of infertility (5).

In numerous organisms, the production of energy for the fueling of biological processes is dependent on oxidation. However, the uncontrolled production of superoxide anion free radicals is involved in the onset of a number of diseases, including cancer, atherosclerosis and degenerative processes associated with aging (6). Thus, it is essential to develop effective and natural antioxidants to protect the human body from free radicals and certain chronic diseases (7).

Hericiium erinaceus is a type of fungi belonging to the *Hericiium* family, which grows in Xiaojin county of Sichuan in China at altitudes of ~3,700 m (8). In the present study, water-soluble oligosaccharides were extracted and purified from the fruiting bodies of *Hericiium erinaceus* using diethylaminoethyl (DEAE)-cellulose and Sephadex G-200 column chromatography. To the best of our knowledge, the present study is the first to characterize the chemical structure of the extracted *Hericiium erinaceus* oligosaccharide (HEO-A). The antioxidant activity of HEO-A was evaluated using three biochemical methods.

Materials and methods

Chemicals. The fruiting bodies of *Hericiium erinaceus* were collected in Xiaojin county of Sichuan, China, and were authenticated by Professor Zhirong Yang (College of Life Sciences, Sichuan University, Chengdu, China). At the same time, a voucher specimen was preserved in the Key Laboratory of Southwest China Wildlife Resources Conservation

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Key words: oligosaccharide, antioxidant activity, *Hericiium erinaceus*

(Nanchong, China). DEAE-cellulose 52 and Sephadex G-200 were purchased from Sigma-Aldrich (Shanghai, China). Monosaccharide standards and Dextran T-500, T-110, T-70, T-40, and T-10, were purchased from Beijing Biodee Biotechnology Co., Ltd. (Beijing, China). All other reagents used were of analytical grade.

Extraction, purity and fractionation of oligosaccharides from *Hericium erinaceus*. Once the fruiting bodies (200 g) of *Hericium erinaceus* were soaked with 95% ethanol (EtOH), the residue was dried and then extracted with boiling water three times (3 h each). Following the concentration, dialysis and centrifugation of the filtrate, three equivalents of 95% EtOH were added to the supernatant to precipitate the crude oligosaccharides (HEO, 21.7 g; recovery, 10.85%). Following deproteinization using the Sevag method (9), LEO (5 g) was purified using a DEAE-cellulose column (Tris-HCl, pH 7.0, 4.5x50 cm, Cl⁻) and eluted stepwise with 0, 0.1, 0.2, 0.3, 0.4, 0.5 and 1.0 M NaCl. The eluate was monitored by the phenol-sulfuric acid method (10). The 0 M NaCl eluate was concentrated, lyophilized and purified on a Sephadex G-200 column (2.6x60 cm). The resulting HEO-A was obtained by the above processes at a yield rate of 0.12% (0.24 g) for the starting material.

Assessment of molecular weight. High-performance gel permeation chromatography was performed to measure the molecular weight of HEO-A (11). The column was calibrated with standard T-series Dextran (T-500, T-110, T-70, T-40 and T-10). Data were processed using the Waters Millennium32 Gel Permeation Chromatography software (Waters Corp. Milford, MA, USA).

Monosaccharide composition analysis. The HEO-A (5.0 mg) was hydrolyzed with 2 M trifluoroacetic acid at 110°C for 6 h by acid-catalyzed hydrolysis (12). Excess acid was removed by co-distillation with methyl alcohol following the completion of hydrolysis. The hydrolysate was used for thin layer chromatography (TLC) analysis as previously described (13) using acetoacetate-pyridine-EtOH-water (8:5:1.5:1) as the developing solvent and a diphenylamine-aniline system [85% phosphoric acid solution (140 ml) containing 8 ml diphenylamine and 8 g aniline] as the developer system.

Polarimetry (ORD), ultraviolet (UV) and infrared (IR) spectroscopic analysis. HEO-A was assessed using UV spectroscopy at wavelengths of 200-600 nm. IR analysis of the HEO sample was obtained by grinding a mixture of oligosaccharide with dry KBr and then pressing into a mold. Spectra were run in the 4,000-400 cm⁻¹ range (14). ORD analysis of the HEO sample was obtained using an HK7_SGW_1 automatic optical polarimeter (Shanghai Jingke Scientific Instrument Co., Ltd., Shanghai, China) at room temperature.

Nuclear magnetic resonance (NMR) experiment. ¹H-NMR and ¹³C-NMR spectra were recorded on a Varian Unity INOVA 400/45 (Varian Medical Systems, Palo Alto, CA, USA) in D₂O with tetramethylsilane as the internal standard (15).

Assessment of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of HEO-A. The DPPH[•] radical

scavenging activity of HEO-A was measured according to the method described by Braca *et al.* (16). The percentage scavenging activity was calculated using the following formula: Scavenging effect (%) = (1-A sample/A control) x100, where A control is the absorbance of the control (DPPH solution without sample) and A sample is the absorbance of the test sample (DPPH solution plus test sample or positive control). Vitamin c (Vc) and butylated hydroxytoluene (BHT) were used as positive controls.

Assessment of the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium (ABTS) radical scavenging activity. To determine the ABTS^{•+} scavenging activity, the assay was performed as described by Auddy *et al.* (14). ABTS^{•+} radicals were produced by reacting ABTS and ammonium persulfate and incubating the mixture at room temperature in the dark for 16 h. A total of 2 ml HEO-A solution at different concentrations and 2 ml ABTS^{•+} radical solution (0.7 mM) were then added. The absorbance was measured immediately at 734 nm. A control reaction was performed without the extract. The percentage scavenging of ABTS^{•+} radicals was calculated as follows: Scavenging effect (%) = [1-(A sample - A sample blank)/A control] x100, where A control was the absorbance of the control group in the ABTS^{•+} radical generation system, A sample was the absorbance of the test group and A sample blank was the absorbance of the sample only. Vc was used as a positive control.

Assessment of the hydroxyl radical scavenging activity. The ability of HEO-A to scavenge hydrogen peroxide was determined according to the method of Smirnoff and Cumbes (17). The percentage scavenging of hydroxyl radicals was calculated as follows: Scavenging effect (%) = [1-(A sample - A sample blank)/A control] x100, where A control was the absorbance of the control group in the hydroxyl radical generation system, A sample was the absorbance of the test group and A sample blank was the absorbance of the samples only. Vc was used as a positive control.

Statistical analysis. All values are expressed as the mean ± standard deviation of three replications. Statistical analyses were performed using the Student's t-test and one-way analysis of variance. Values of P<0.05 were considered to indicate a statistically significant difference.

Results and Discussion

Extraction, purity and composition of oligosaccharides. The crude oligosaccharide was obtained from the fruiting bodies of *Hericium erinaceus* with a yield of 8.7%. Following fractionation using DEAE-cellulose 52 and Sephadex G-200 column chromatography, 180 mg HEO-A was obtained from the 0 M NaCl eluate and detected using the phenol-sulfuric acid assay as a single peak (Figs. 1 and 2). The homogeneity of the oligosaccharide was then elucidated. An absence of absorption at 280 and 260 nm in the UV absorption spectra of HEO-A demonstrated the absence of protein and nucleic acid in this oligosaccharide. In addition, HEO-A exhibited the same optical rotation, [α]_D²⁰ -11.2° (concentration, 0.5 g/100 ml water), in different low concentrations (5, 10 and 15%) of EtOH,

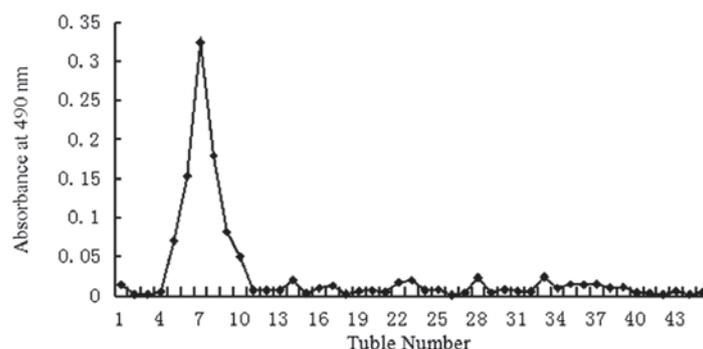


Figure 1. Chromatogram of *Hericium erinaceus* oligosaccharide on a diethylaminoethyl-cellulose 52 column eluted with distilled water and a stepwise gradient of NaCl (0, 0.1, 0.2, 0.3, 0.4 and 0.5 M).

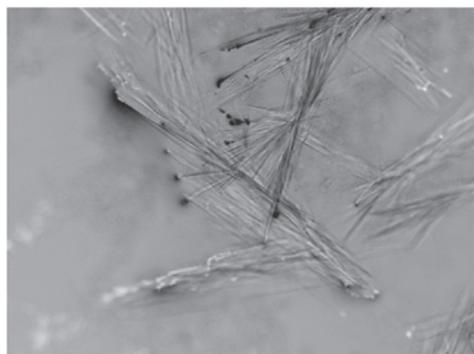


Figure 2. *Hericium erinaceus* oligosaccharide was visualized by scanning electron microscopy. Magnification, x200.

as shown using an HK7-SGW-1 automatic optical polarimeter at room temperature. The weight-average molecular weight of HEO-A was $\sim 1,877$ Da (Fig. 3). The two monosaccharides, D-glucose (D-Glu) and D-xylose (D-Xyl), were identified by analyzing the hydrolysate of HEO-A using TLC.

Structure elucidation of HEO-A. The intensity of bands around $3,422.38\text{ cm}^{-1}$ in the IR spectrum (Fig. 4) was due to the hydroxyl stretching vibration of the oligosaccharide and, as expected, they were broad. The bands in the region of $2,922.70\text{ cm}^{-1}$ were due to a C-H stretching vibration and the bands in the region of $1,648.42\text{ cm}^{-1}$ were due to associated water (18). The strong absorption bands at $1,155.58$ and $1,060.45\text{ cm}^{-1}$ in the range of $1,200\text{--}1,000\text{ cm}^{-1}$ in the IR spectrum suggested that the monosaccharide in HEO-A had a pyranose ring. The strong absorption bands at $1,402.57\text{ cm}^{-1}$ were due to the C-H bending vibration, and the bands in the region of $923.69\text{--}707.59\text{ cm}^{-1}$ were due to the C-H rocking vibration. In addition, the characteristic absorptions at 707.59 cm^{-1} indicated α -configurations existing in the oligosaccharide, which was consistent with the anomeric proton signals at $\delta 5.06$ and 5.07 in the $^1\text{H-NMR}$ (400 MHz) spectrum (Fig. 5). The signals at $\delta 3.0\text{--}3.9$ were the signal peaks of remaining protons, which were mostly formed by a number of overlapping signal peaks. Among them, the signals at $\delta 3.79, 3.78$ and 3.77 were ascribed to the α -H of the methyl group in xylose and the signals at $\delta 3.55$ corresponded with the β -H of the methyl group in xylose. Signals at $\delta 3.53, 3.52$

and 3.45 were the signal peaks of the other hydrogen atoms in xylose. The signals at $\delta 3.65$ and 3.63 were the signal peaks of the hydroxy-methyl group in glucose and the signals at $\delta 3.80$ indicated the hydrogen atom of C-3 of glucose. The hydrogen signal of water was observed at $\delta 4.67$. The $^1\text{H-NMR}$ data were consistent with monosaccharide analysis data. According to previous studies, the resonances in the region of $95\text{--}100$ ppm in the $^{13}\text{C NMR}$ (400 MHz) spectrum of HEO-A were attributed to the anomeric carbon atoms of D-Glu and D-Xyl (Fig. 6) (19).

Determination of the DPPH $^{\cdot}$ radical scavenging activity of HEO-A. Previous studies have shown that oligosaccharides composed of different monosaccharides and with different molecular weights may exhibit different antioxidant activities, particularly in terms of scavenging free radicals (20). As excess free radicals are harmful to human health, the free radical scavenging activity of the extracted oligosaccharide against DPPH $^{\cdot}$, hydroxyl radicals and superoxide anions was evaluated.

The stable DPPH $^{\cdot}$ radical is commonly used to measure the antioxidant activity of a sample (21). Furthermore, the DPPH $^{\cdot}$ assay is a more time-efficient method than other strategies. In the presence of hydrogen-donating antioxidants, the characteristic purple of an alcoholic solution containing DPPH $^{\cdot}$ radicals and unpaired electrons changes to yellow. This discoloration occurs due to the single paired electrons. Thus, the DPPH $^{\cdot}$ radical scavenging activity of HEO-A was determined by measuring the discoloration and absorbance values at 517 nm , and the results were compared with those of BHT. Fig. 7 shows the scavenging activity of the purified oligosaccharide samples on the DPPH $^{\cdot}$ radical. These results showed that the IC_{50} value of HEO-A for eliminating DPPH $^{\cdot}$ radicals was $\sim 12.5\text{ mg/ml}$, which indicated that HEO-A had a notable effect on scavenging DPPH $^{\cdot}$ radicals, particularly when added at high quantities. However, the inhibition ability was lower than that of BHT and Vc.

Assessment of the ABTS $^{\cdot+}$ radical cation scavenging activity of HEO-A. The ABTS $^{\cdot+}$ radical scavenging activity of HEO-A was measured spectrophotometrically at 734 nm . Upon interaction with the extract at various concentrations, the absorbance of the ABTS $^{\cdot+}$ radical cations was decreased dose dependently, and the IC_{50} value of HEO-A was 0.93 mg/ml

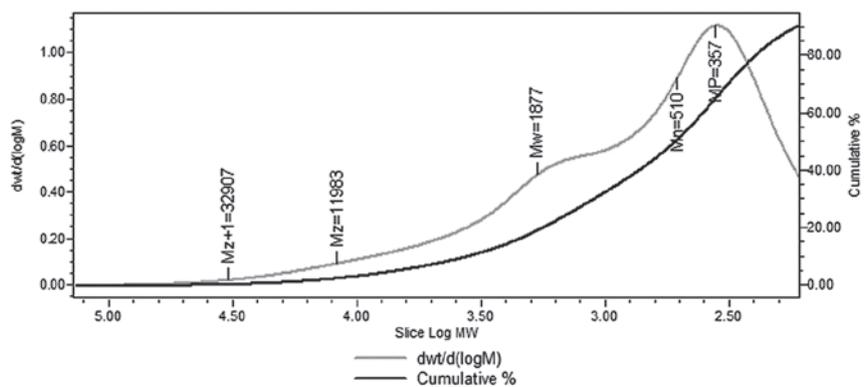


Figure 3. Molecular weight of *Hericium erinaceus* oligosaccharide.

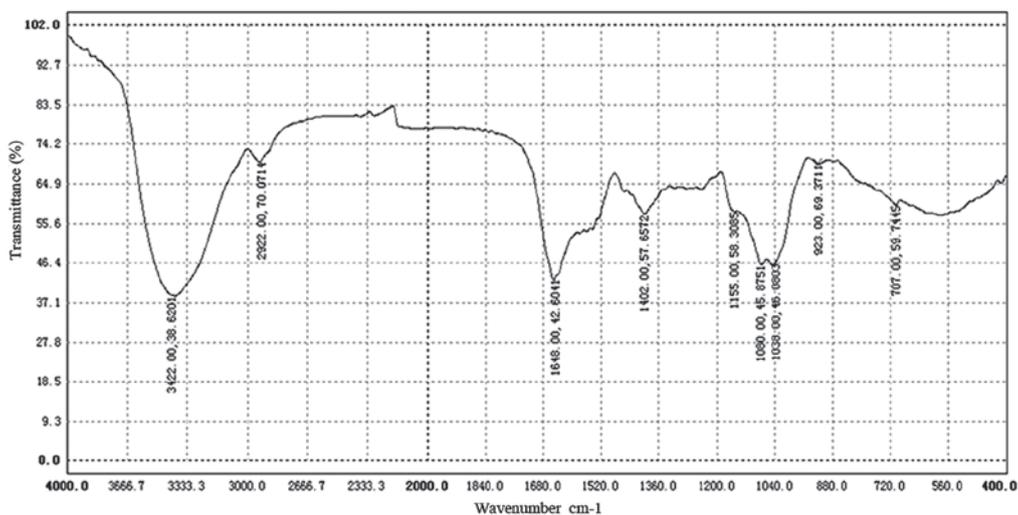


Figure 4. Spectrum of *Hericium erinaceus* oligosaccharide obtained by Fourier transform infrared spectroscopy.

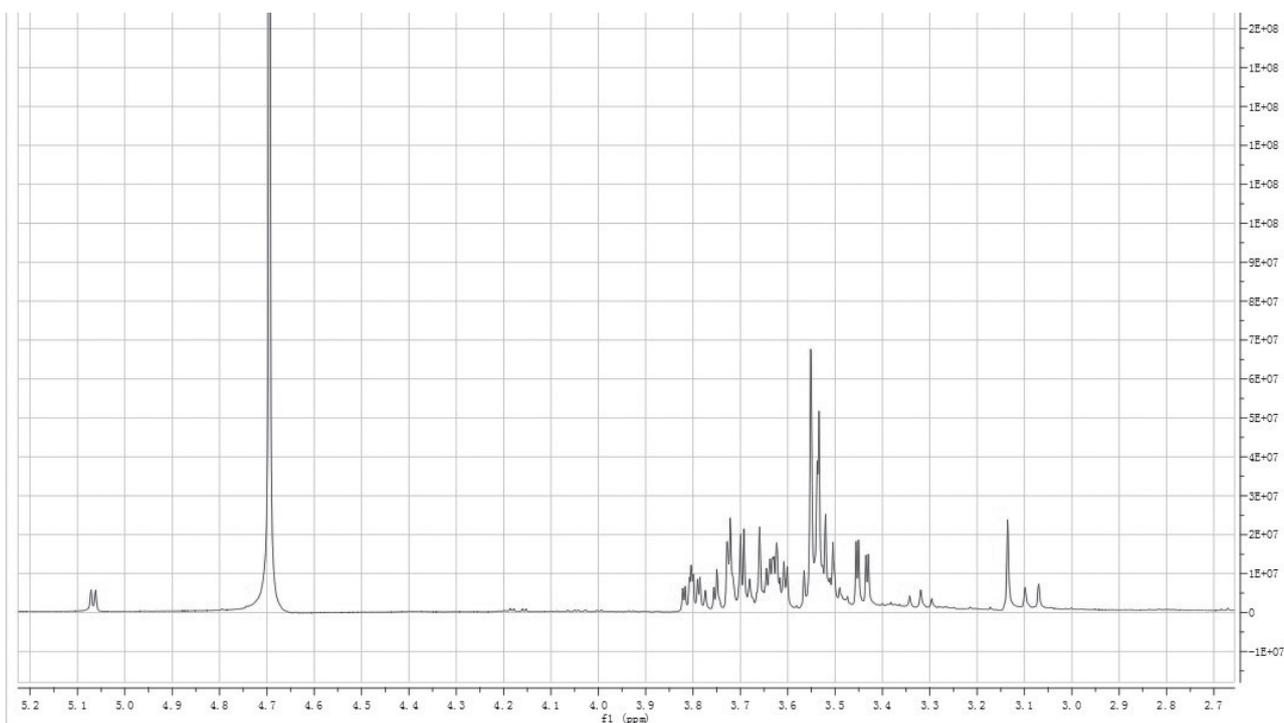


Figure 5. ¹H-nuclear magnetic resonance spectrum of *Hericium erinaceus* oligosaccharide.

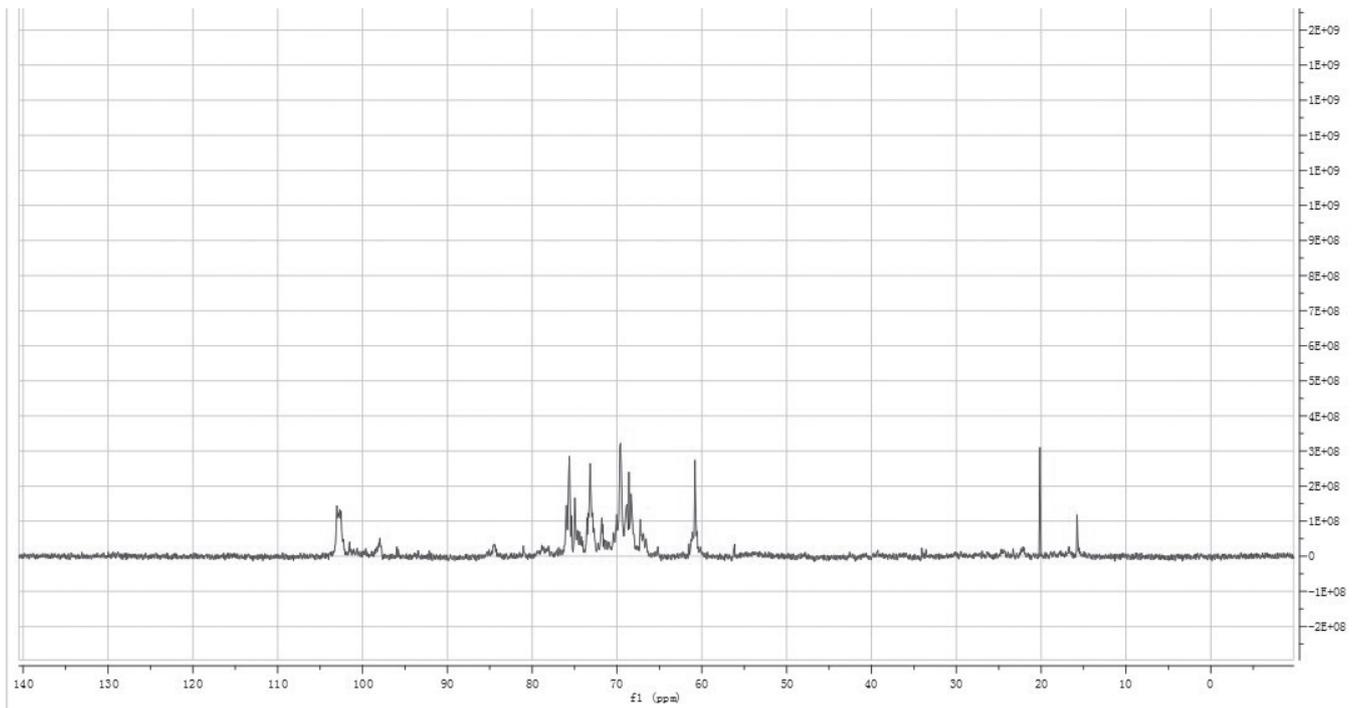


Figure 6. ¹³C-nuclear magnetic resonance spectrum of *Hericium erinaceus* oligosaccharide.

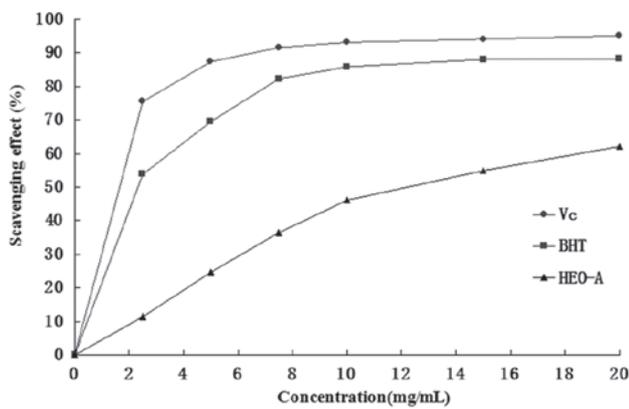


Figure 7. Scavenging effects of HEO-A on 1,1-diphenyl-2-picrylhydrazyl radicals. Vc, vitamin c; BHT, butylated hydroxytoluene; HEO-A, *Hericium erinaceus* oligosaccharide.

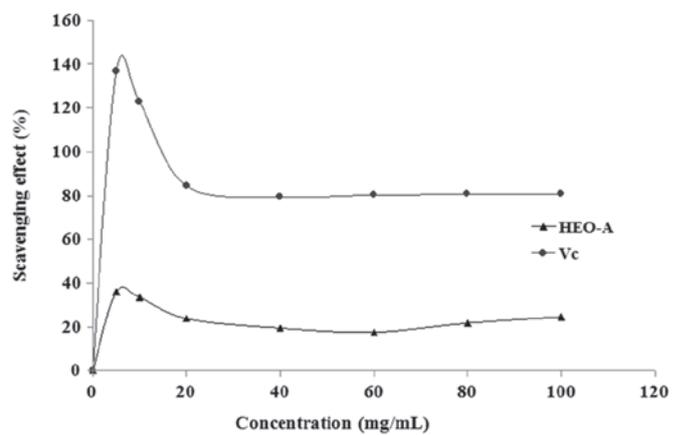


Figure 9. Scavenging effects of HEO-A on hydroxyl radicals. HEO-A, *Hericium erinaceus* oligosaccharide; Vc, vitamin c.

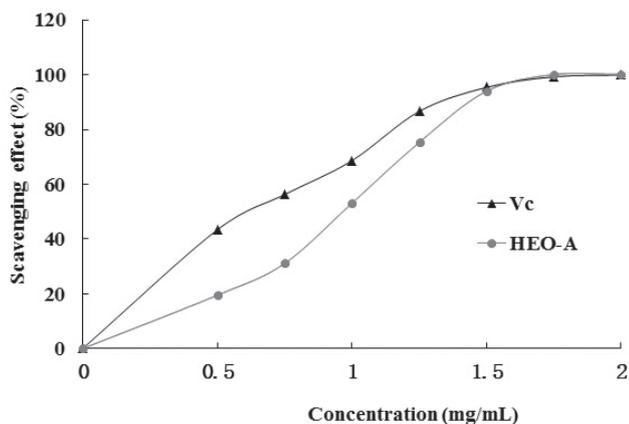


Figure 8. Scavenging effects of HEO-A on 2,2'-azino-bis(3-ethylbenzthiazoline-6-sufonic acid) diammonium radicals. HEO-A, *Hericium erinaceus* oligosaccharide; Vc, vitamin c.

(Fig. 8). However, the scavenging activity of HEO-A was lower than that of Vc.

Assessment of the hydroxyl radical scavenging activity of HEO-A. The hydroxyl radical is one of the most reactive and dangerous reactive oxygen species to human health. The hydroxyl radicals generated through the Fenton reaction in this system were scavenged by HEO-A. Fig. 9 shows the percentage hydroxyl radical scavenging effects of HEO-A at different doses. At the test concentrations, HEO-A exhibited a concentration-dependent scavenging effect on the hydroxyl radicals, which showed that the purified oligosaccharide exhibited weaker hydroxyl radical-scavenging effects than Vc at the same dose.

Conclusion. According to the results above, *Hericium erinaceus* may be introduced as a possible valuable source of

oligosaccharides, which exhibits unique antioxidant properties.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant nos. 31200012 and 31400016), the Application Foundation Project of Sichuan Province (grant no. 2013JY0094), the Science and Technology Support Project of Sichuan Province (grant nos. 2014SZ0020 and 2014FZ0024), The Cultivate Major Projects of Sichuan Province (grant no. 14CZ0016), and the Doctor Startup Foundation Project of China West Normal University (grant nos. 11B019 and 11B020).

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