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Structure Characterization and Antioxidant Activity of a Novel Polysaccharide Isolated from Pulp Tissues of *Litchi chinensis*

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ABSTRACT: A novel polysaccharide (LCP50S-2) with antioxidant activity was isolated from *Litchi chinensis* Sonn. The structure of LCP50S-2 was elucidated on the basis of physicochemical and instrumental analyses, and its average molecular weight was determined by gel permeation chromatography to be 2.19×10^2 kDa. The backbone of LCP50S-2 was composed of (1→3)-linked β-L-rhamnopyranosyl residues, (1→4)-linked α-D-xylopyranosyl residues, (1→4)-linked β-D-glucopyranosyl residues, and (1→4)-linked α-D-glucopyranosyl residues which branched at O-6. The two branches consisted of α-L-arabinopyranosyl residues and (1→6)-linked β-D-galactopyranosyl residues terminated with α-L-arabinopyranosyl residues, respectively. In the in vitro antioxidant assay, LCP50S-2 was found to possess DPPH radical-scavenging activity and hydroxyl radical-scavenging activity with IC_{50} values of 220 and 266 μg/mL, respectively.

KEYWORDS: polysaccharide of *Litchi chinensis*, structure characterization, HPAEC-PAD analysis, antioxidant activity

INTRODUCTION

Oxidation is an essential process for all living organisms for the production of energy necessary for biological processes.[1,2] However, the uncontrolled production of oxygen-derived free radicals is responsible for many diseases such as cancer, rheumatoid arthritis, and atherosclerosis, as well as degenerative processes associated with aging.[3] It has also been reported that reactive oxygen species (ROS) are capable of damaging DNA, which has been associated with cancer, coronary heart diseases, and many other age-related health problems.[4] Thus, there has been increasing interest in finding natural, effective, and safe antioxidants to protect the human body from free radicals and prevent the onset of chronic diseases.[5,6] Pharmacological antioxidant activities are generally achieved in two ways, one by scavenging ROS and the other by inhibiting the generation of ROS. Synthetic antioxidants are currently being used for industrial processing to preserve food quality. Published data indicate that some plant polysaccharides have strong antioxidant activities and could be developed into drugs.[7−9] The antioxidants present in plants are of great interest as potential protective agents against oxidative damage. For instance, polysaccharides extracted from fruit bodies of *Auricularia auricular* were shown to possess antioxidant properties by their free radical-scavenging abilities and could be developed into health food.[10]

Litchi (*Litchi chinensis* Sonn.), a tropical fruit native to China, is cultivated in many parts of the world having a warm climate.[11−16] Litchi fruit pericarp tissues contain a large number of polysaccharides and proteins. Kong et al. reported that three fractions of water-soluble polysaccharide fractions, coded LFP1, LFP2, and LFP3, were isolated and purified from litchi pulp. The antioxidant activities of these fractions were investigated using various in vitro assay systems. Results indicated that LFP3 showed the highest antioxidant activity among the three fractions of polysaccharides and could be explored as a novel potential antioxidant.[2,12] However, to date, little is known about the fine structural elucidation and antioxidant activities of litchi pulp polysaccharides.

Detailed characterizations of these polysaccharides are critical to a better understanding of the structural and functional properties of these substances for future nutritional and therapeutic applications. Here, we present the first detailed study of the polysaccharide structure of *L. chinensis* pulp tissues. In this study, we report the extraction, purification, and detailed structures of a novel polysaccharide (LCP50S-2) from the pulp tissues of *L. chinensis* using DEAE-52 cellulose column chromatography and DEAE-Sepharose Fast Flow column chromatography, as well as physicochemical properties and instrumental analyses. In particular, the in vitro antioxidant activity of LCP50S-2 was also assayed and established.

MATERIALS AND METHODS

Plant Material. Pulp tissues (*L. chinensis* Sonn. cv. Nuomici) were obtained from Guangzhou Qingping Chinese Medicinal Materials Market, Guangzhou, China. The material was identified by Professor R. M. Yu, College of Pharmacy, Jinan University, China.

Experimental Reagents and Materials. DEAE-52 cellulose and DEAE-Sepharose Fast Flow were obtained from Whatman Ltd. Sephacryl S-300 HR was obtained from Amersham Biosciences. Ascorbic acid (vitamin C, Vc), hydrogen peroxide (H_2O_2), ferrous sulfate ($FeSO_4$), and brilliant green were obtained from Guangzhou Chemical Reagent Co., Guangzhou, China. All other reagents were obtained from Sigma Chemical Co. All reagents were of analytical grade.

Extraction, Isolation, and Purification of LCP50S-2. The pulp tissues of *L. chinensis* (300 g) were defatted with ethanol for 8 h and extracted three times with hot water (80 °C), each time for 3 h.

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The water extract was filtered, concentrated under vacuum, and centrifuged at 5000 rpm for 15 min. The supernatant was concentrated to a fourth of its original volume and treated with 1 volume of ethanol for precipitation at 4 °C. The precipitate was obtained by centrifugation and lyophilization. Dried precipitate (1 g) was dissolved in 20 mL of distilled water, and 5 mL of Sevag reagent (chloroform/*n*-butanol, v/v = 4:1) was added three times to degrade any protein component.[17] After removal of the Sevag reagent, the solution was decolorized with 30% H_2O_2 and then dialyzed against tap water and distilled water, each for 48 h. The resulting polysaccharide solution was concentrated and lyophilized.

Ion-exchange chromatography and gel filtration column chromatography were used for the isolation of the polysaccharide. Each sample (160 mg) was dissolved in distilled water and then centrifuged. The supernatant was injected to a column of DEAE-cellulose-52 (2.6 × 40 cm) equilibrated with distilled water.

After sample loading, the column was eluted with a linear gradient solution of aqueous NaCl (0−0.5 M) at a flow rate of 0.5 mL/min, and this process was monitored by the phenol−sulfuric acid method.[18] The fractions were further purified by anion-exchange chromatography on a column of DEAE-Sepharose Fast Flow (2.6 × 40 cm), eluting with a linear gradient from 0 to 0.5 M NaCl at a flow rate of 0.8 mL/min.

As a result, two polysaccharides, namely, LCP50S-1 and LCP50S-2, were obtained. LCP50S-2, which had $[\alpha]_D^{20}$ = +119° (*c* 1.0, water), was used in the subsequent studies on its structure and bioactivity.

Analyses of LCP50S-2. The sugar content of LCP50S-2 was determined according to the phenol−sulfuric acid colormetric method using glucose as standard.[18] Optical rotation was recorded with a Jasco P-1020 polarimeter. IR spectra were recorded with a Tensor 27 Bruker instrument with KBr pellets. The sample (1 mg) was ground with KBr powder and then pressed into pallets for Fourier transform infrared spectra (FTIR) measurement in the frequency range of 4000−500 cm^{-1}. ^{13}C NMR spectra were recorded with a Bruker 500 instrument, and 40 mg of polysaccharide was dissolved in 0.5 mL of 99.98% D_2O.[19] Chemical shifts are expressed in parts per million (ppm). High-performance anion-exchange chromatography (HPAEC) was analyzed on a Dionex ICS-2500 system, coupled with pulsed amperometric detec tion (PAD), equipped with a Carbo PAC PA10 (2.0 × 250 mm) column. GC-MS was conducted with a Hewlett-Packard 5895 instrument, using a fused-silica capillary column (30 × 25 mm) coated with a 0.2 mm film of DB-5. The ionization potential was 70 eV, and the temperature of the ion source was 220 °C.

Determination of Molecular Weight. The homogeneity and molecular weight of LCP50S-2 were evaluated by gel permeation chromatography (GPC) on a Sephacryl S-300HR column (1.6 × 70 cm) with standard dextrans (T-4, T-7, T-10, T-70, T-200, and blue dextran) and glucose. The elution volumes were plotted against the logarithm of their respective molecular weights. The elution volume of LCP50S-2 was plotted in the same graph, and the molecular weight was measured.[20]

Analysis of Monosaccharide Composition. LCP50S-2 (5 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA, 2 mL) at 100 °C in a sealed tube for 8 h. Excess acid was removed by evaporation on a water bath at a temperature of 40 °C and codistilled with MeOH after the hydrolysis was completed. The monosaccharide content was measured by HPAEC-PAD. The hydrolysate (1 mg) was dissolved in pure water (1 mL). The solution (25 mL) was used for the ionic chromatography analysis by HPAEC-PAD on the Dionex ICS-2500 system, eluted with a mixture of water and 200 mM NaOH in the volume ratio of 92:8.[21]

Partial Acid Hydrolysis. LCP50S-2 (15 mg) was hydrolyzed with 0.05 M TFA for 6 h at 100 °C and dialyzed with distilled water for 48 h. The fraction out of the sack was collected. After excess TFA in the fraction was removed by codistillation with MeOH (1 mL × 3), the fraction was evaporated to dryness (fraction 1). The fraction in the sack was dried by evaporation and then hydrolyzed with 0.5 M TFA. The hydrolysate was dialyzed, and the fraction out of the sack (fraction 2) and the fraction in the sack (fraction 3) were collected, respectively. Fractions 1−3 were hydrolyzed with 2 M TFA and tested by HPAEC-PAD.

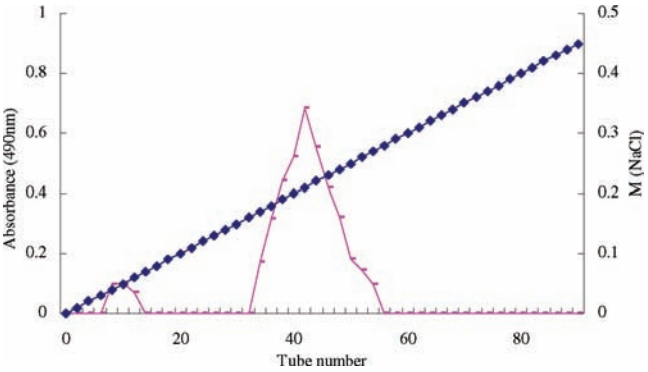

Figure 1. Profile of LCP50 in DEAE-cellulose 52 column chromatography.

Periodate Oxidation−Smith Degradation. The polysaccharide (15 mg) was swelled in 5 mL of distilled water, and then 25 mL of 15 mM $NaIO_4$ was added into the solution. Thirty milliliters of the solution was drawn at 6 h intervals in the dark at 4 °C, then diluted to 5 mL with distilled water, and measured in a spectrophotometer at 223 nm.[22] Consumption of HIO_4 was determined by a spectrophotometric method,[23] and formic acid production was measured by titration with 0.061 M NaOH. The nondialysate was concentrated, reduced with $NaBH_4$ (30 mg) for 24 h at room temperature, and neutralized to pH 6.0 with 0.1 M HOAc. After dialysis and concentration, the mixture product was hydrolyzed with 2 M TFA (4 mL) at 100 °C for 8 h and tested by HPAEC-PAD analysis.

Methylation Analysis. LCP50S-2 (5 mg) was methylated according to the method of Hakomori.[24] The methylated polysaccharide was treated with 90% formic acid (3 mL) for 10 h at 100 °C in a sealed tube. After removal of the formic acid, the residues were heated with 2 M TFA (2 mL) under the above conditions and the hydrolysate was concentrated to dryness. The methylated sugars were reduced with $NaBH_4$ and then acetylated with acetic anhydride. Alditol acetates were analyzed by GC-MS.[25]

Determination of DPPH Radical-Scavenging Activity of LCP50S-2. DPPH ethanol solution (190 μL, freshly prepared at a concentration of 0.2 mM) was added to 10 μL of LCP50S-2 solution of gradient concentrations (0, 25, 50, 100, 200, 400, and 800 μg/mL) in water. The absorbance was measured at 517 nm after 30 min. The lower absorbance of the reaction mixture indicated the higher free radical-scavenging activity. The capability to scavenge the DPPH radical was calculated by using the equation

$$\text{scavenging ability } (\%) = [(A_0 - A_1)/A_0] \times 100\%$$

where A_0 is the absorbance of control (without sample) and A_1 is the absorbance in the presence of sample.[26]

Determination of Hydroxyl Radical-Scavenging Activity of LCP50S-2. The scavenging activity for hydroxyl radicals was measured by Fenton reaction. Reaction was started by adding H_2O_2 and incubating at room temperature for 60 min. The absorbance of the mixture at 510 nm was measured. The hydroxyl radical-scavenging activity was calculated according to the equation

$$\text{scavenging ability } (\%) = [(A_2 - A_1)/(A_0 - A_1)] \times 100\%$$

where A_0 is the absorbance of the control (blank, without H_2O_2), A_1 is the absorbance in the absence of sample, and A_2 is the absorbance in the presence of sample.[21]

Statistical Analysis. Values were expressed as means ± SD of three separate experiments.

RESULTS AND DISCUSSION

Isolation, Purification, and Composition of LCP50S-2. LCP50, a crude polysaccharide (538 mg), was obtained from the pulp tissues of *L. chinensis* by hot water extraction followed by ethanol precipitation. After purification with DEAE-cellulose 52 and DEAE-Sepharose Fast Flow columns, profiles LCP50S-1 and LCP50S-2 were obtained from the NaCl elution (Figure 1), which were detected by the phenol−sulfuric acid assay. The yields of LCP50S-1 and LCP50S-2 from crude polysaccharide were 4.6 and 42.4%, respectively.

LCP50S-2 was a white to pale yellow loose powder, odorless, and freely soluble in water. The average molecular weight of the polysaccharide was determined to be 2.19×10^2 kDa by GPC technique on a Sephacryl S-300 HR column. Calibration was performed with dextran molecular weight standards (dextran M_w: 5×10^6, 2×10^5, 7×10^4, 1×10^4, and 3×10^3, Pharmacia).

Total carbohydrate content was measured to be 94% (w/w), and *m*-hydroxybiphenyl colorimetric test was shown to be negative. The optical rotation of LCP50S-2 was $[\alpha]_D^{20} = +119°$ (c 1.0, H_2O). The composition of LCP50S-2 is presented in Table 1. Five monosaccharides, L-Rha, L-Ara, D-Gal, D-Glu, and D-Xyl, were identified in the hydrolysate of LCP50S-2, and their ratio was 1.00:7.30:3.06:15.60:8.18 by HPAEC-PAD analysis.

Structural Characterization of LCP50S-2. Fractions 1−3 were obtained through partial acid hydrolysis. Each fraction was subjected to HPAEC-PAD analysis, and the results are shown in Table 1. Fraction 3, the precipitate in the sack, possesses the biggest size among the three fractions. The component of

Table 1. Results of HPAEC-PAD Analysis for LCP50S-2 Degradation Products

	molar ratios				
	L-rhamnose	L-arabinose	D-galactose	D-glucose	D-xylose
LCP50S-2	1.00	7.30	3.06	15.60	8.18
fraction 1	0.02	1.00	0.07	nd[a]	nd
fraction 2	nd	nd	1.00	0.18	0.17
fraction 3	1.00	nd	nd	15.40	7.95
Smith degradation	1.00	nd	nd	nd	nd

[a] nd, not detected.

Table 2. Results of Methylation Analysis of LCP50S-2

methylation sugar	molar ratio	mass fragments (m/z)	retention time (min)	linkage type
2,3,5-Me_3-Ara	7.01	43, 45, 71, 87, 101, 117, 129, 161	12.60	T→
2,3-Me_2-Xyl	8.23	43, 101, 117, 189	14.34	1→4
2,4-Me_2-Rha	1.00	43, 117, 131, 189	15.71	1→3
2,3,4-Me_3-Gal	2.89	43, 71, 101, 129, 161, 189, 233	17.74	1→6
2,3,6-Me_3-Glc	8.35	43, 87, 101, 113, 117, 161, 233	17.95	1→4
2,3-Me_2-Glc	6.75	43, 101, 117, 261	18.13	1→4,6

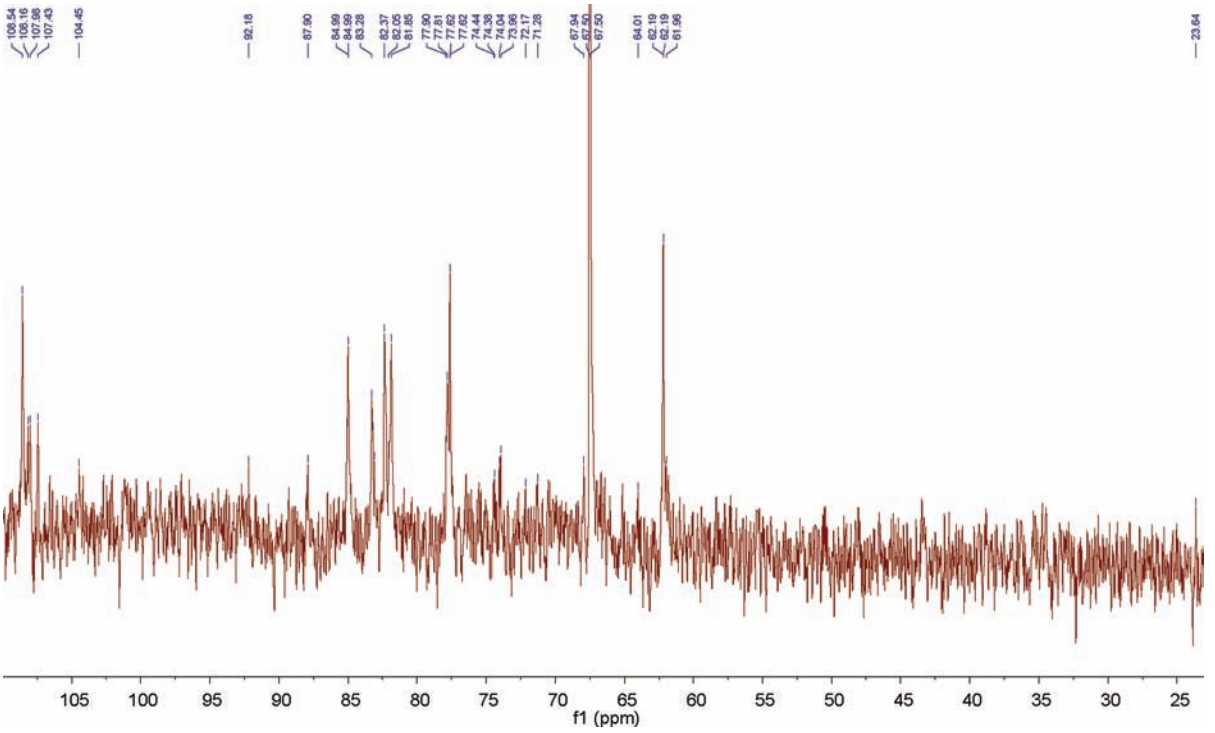

Figure 2. ^{13}C NMR spectrum of LCP50S-2.

fraction 3, of which the molar ratio of L-rhamnose to D-glucose to D-xylose was 1:15.40:7.95, indicated that L-rhamnose, D-glucose, and D-xylose could be the backbone of the structure of LCP50S-2, and D-galactose may be close to this backbone.

The analysis results of fractions 1 and 2 showed that the branched structure of LCP50S-2 was composed of L-arabinose and D-galactose and terminated with L-arabinose. The periodate-oxidized products were fully hydrolyzed and analyzed by HPAEC-PAD after periodate oxidation. The results shown in Table 1 demonstrated that there was no L-arabinose, D-galactose, D-glucose, or D-xylose in the oxidation products. From this it could be inferred that linkages of rhamnose and galactose were (1→), (1→2), (1→6), (1→2,6), (1→4), and (1→4,6), which might be oxidized to produce glycerol and erythritol. The presence of L-rhamnose revealed that some residues of L-rhamnose were (1→3)-linked, (1→2,3)-linked, (1→2,4)-linked, (1→3,4)-linked, or (1→2,3,4)-linked, which could not be oxidized, respectively.[27,28] The fully methylated LCP50S-2 was hydrolyzed with acid and analyzed by GC-MS. The results showed the presence of six components, namely, 2,3,5-Me_3-Ara, 2,3-Me_2-Xyl, 2,4-Me_2-Rha, 2,3,4-Me_3-Gal, 2,3,6-Me_3-Glc, and 2,3-Me_2-Glc in a molar ratio of 7.01:8.23:1:2.89:8.35:6.75 (Table 2). On the basis of the standard data in the CCRC Spectral Database for PMAA's, the linkages of L-rhamnose, D-xylose, L-arabinose, and D-galactose were deduced as (1→3), (1→4), (1→), and (1→6), whereas the linkages of D-glucose were deduced as (1→4) and (1→4,6). This result showed good correlation between terminal and branched residues. In addition, these molar ratios also agreed with the overall monosaccharide composition of LCP50S-2 described above.

The infrared spectrum of LCP50S-2 showed absorption bands at 3335, 2940, 1604, 1095, and 1012 cm^{-1}. The absorption bands at 894 cm^{-1} indicated that LCP50S-2 contained a β-type glycosidic linkages in its structure. There was no absorption at 1740 cm^{-1}, indicating the absence of uronic acid in the polysaccharide structure.[29] The spectrum of ^{13}C NMR of LCP50S-2 is shown in Figure 2. According to the literature,[30,31] the resonances in the region of 98−109 ppm in ^{13}C NMR were attributed to the anomeric carbon atoms of D-xylose (Xylp), L-rhamnose (Rhap), D-glucose (Glcp), D-galactose (Galp), and L-arabinose (Arap). The peaks at 92.2 ppm corresponded to C-1 of (1→4)-linked α-D-Xyl units, 108.0 ppm corresponded to C-1 of (1→4)-linked α-D-Glc units, 108.5 ppm corresponded to C-1 of (1→)-linked α-L-Ara units, 108.2 ppm corresponded to C-1 of (1→3)-linked β-L-Rha units, 104.5 ppm corresponded to C-1 of (1→6)-linked β-D-Gal units, and 107.4 ppm corresponded to C-1 of (1→4,6)-linked α-D-glucopyranosyl units, respectively. The result also indicated that the backbone was composed of (1→4)-linked α-D-glucopyranosyl residues, which branch at O-6. The assignment of the carbon atom signals is shown in Table 3.

Table 3. Assignment of ^{13}C NMR Chemical Shifts of LCP50S-2

	chemical shifts (ppm)						
sugar residue	C1	C2	C3	C4	C5	C6	CH_3
α-L-Ara(1→	108.5	82.4	77.6	87.9	64.0		
→4)-α-D-Xyl(1→	92.2	72.2	71.3	77.6	62.2		
→3)-β-L-Rha(1→	108.2	77.9	85.0	77.8	74.4		23.6
→6)-β-D-Gal(1→	104.5	77.6	74.0	77.6	77.9	68.0	
→4)-β-D-Glc(1→	108.0	77.6	77.6	82.4	74.0	62.0	
→4,6)-α-D-Glc(1→	107.4	77.8	77.9	87.9	74.0	74.0	

On the basis of the results of HPAEC-PAD, GC-MS, and ^{13}C NMR, the backbone of LCP50S-2 should contain (1→3)-linked glucose, (1→4)-linked glucose, (1→4)-linked xylose, and (1→3)-linked rhamnose. The repeating unit of (1→4)-linked glucose branches at O-6. The branches might contain (1→6)-linked galactose and (1→6)-linked arabinose. From the aforementioned results, the repeating structural unit of LCP50S-2 could be as shown in Figure 3.

Scavenging Activity of LCP50S-2 for DPPH Radicals. The DPPH molecule is a stable free radical that has been widely used to evaluate the radical-scavenging ability of antioxidants. The free radical-scavenging activities of LCP50S-2 were assayed by using DPPH. As shown in Figure 4, both Vc and LCP50S-2 reacted with and quenched DPPH radicals to different degrees with increased activities at higher concentrations. The IC_{50} values of LCP50S-2 and Vc were 220 and 65 μg/mL, respectively. The maximum value of LCP50S-2 reached 75.6% that of Vc.

Scavenging Activity of LCP50S-2 for Hydroxyl Radicals. The hydroxyl radical is very reactive and can be generated in biological cells through the Fenton reaction. Figure 5 shows that both Vc and LCP50S-2 exhibited concentration-dependent scavenging activities against hydroxyl radicals generated in a Fenton reaction system. The IC_{50} values of Vc and LCP50S-2 were 115 and 266 μg/mL, respectively. The maximum value of LCP50S-2 reached 73.9% that of Vc.

In conclusion, the results obtained in the present study clearly demonstrate that a novel water-soluble polysaccharide (LCP50S-2) isolated from the pulp tissues of *L. chinensis* contains predominantly five monosaccharides. It consists of L-rhamnose, L-arabinose,

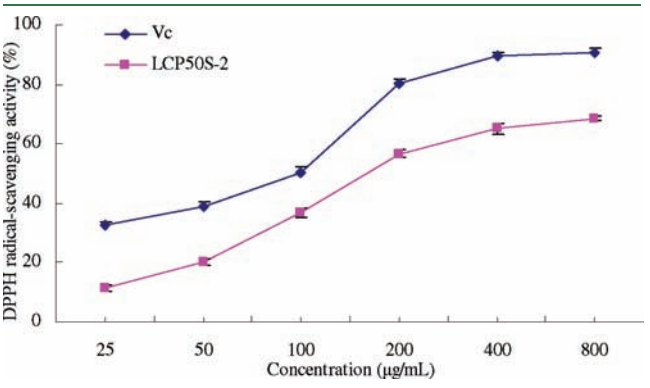

Figure 4. DPPH radical-scavenging activity of LCP50S-2 and Vc. Values are means ± SD of three separate experiments.

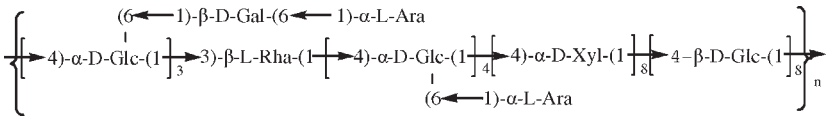

Figure 3. Predicted structure of LCP50S-2.

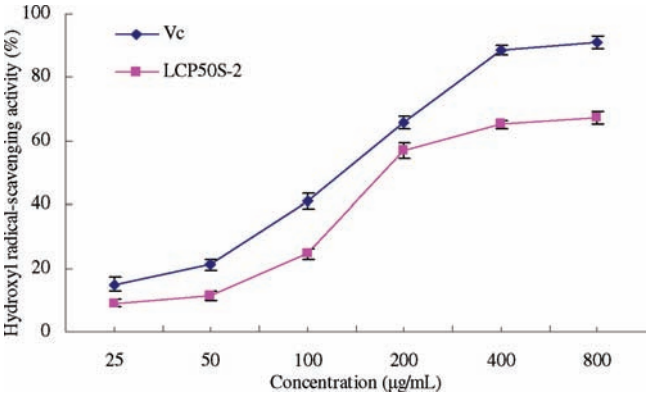

Figure 5. Hydroxyl radical-scavenging activity of LCP50S-2 and Vc. Values are means ± SD of three separate experiments.

D-galactose, D-glucose, and D-xylose. Antioxidation tests performed in vitro showed that this natural polysaccharide possesses radical-scavenging activity. According to the results above, consuming about 200 g of litchi pulp tissue would amount to a similar antioxidant effect as ingesting the recommended daily dose of vitamin C, assuming that all of the polysaccharide is bioavailable. Further studies on other activities of the polysaccharide are in progress.

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Author Contributions

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