

Structural elucidation and *in vitro* antioxidant activities of a new heteropolysaccharide from *Litchi chinensis*

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Summary

Two polysaccharides, LCP70S-1 and LCP70W, were isolated from the pulp tissues of *Litchi chinensis* by anion-exchange chromatography and gel-filtration chromatography, while the structure of LCP70S-1 was elucidated and its physico-chemical properties was analyzed. The results demonstrated that LCP70S-1 is composed of L-rhamnose, L-arabinose and D-galactose in the ratio of 1.06:6.39:4.21, and the main chain of the heteropolysaccharide possess (1→3,6)-linked galactopyranosyl branches at O-6. The three branches consist of (1→3)-linked rhamnopyranosyl residues, (1→3,6)-linked galactopyranosyl and (1→5)-linked arabinopyranosyl residues, and terminated with (1→)-linked arabinopyranosyl residues, respectively. The two polysaccharides were further evaluated with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities and their reducing power *in vitro*. The results showed that LCP70W and LCP70S-1 possessed significant antioxidant activities, especially for LCP70S-1. As such, LCP70S-1 could potentially serve as an antioxidant and would value further study for human healthcare.

Keywords: *Litchi chinensis*, heteropolysaccharide, structural elucidation, antioxidant activity

1. Introduction

Natural polysaccharides from different sources have long been studied in medicine and pharmaceuticals and has been the core of intense research for the understanding and utilization of bioactive polysaccharides in recent decades (1). In fact some of these polysaccharides have already made their way to the market as antitumor, immunostimulating or prophylactic drugs (2). More importantly, polysaccharides are promising radical scavengers for the prevention of oxidative stress in living systems owing to their biocompatibility and non-toxicity (3).

Finding natural, effective and safe antioxidants is

important for healthcare to protect mankind from the attack of free radicals and retard the initiation of many chronic diseases (4). Published data have indicated that some plant polysaccharides have strong antioxidant activities and therefore could be developed into supplements or even therapeutic agents (5,6). In addition, polysaccharides extracted from *Auricularia auricular* have been regarded as potential health-promoting functional food provided that they possess strong radical scavenging ability (7).

Litchi (*Litchi chinensis*), a tropical to subtropical fruit originally from China, is cultivated all over the world in warm climates. Litchi fruit pericarp tissues contain various polysaccharides and proteins in high quantity. Previous investigation by our research group has demonstrated that the polysaccharide from pulp tissues of *L. chinensis* shows strong antioxidant activity which values further research and development as potential antioxidants and health foods (8). Recently, Kong has reported that a fraction of water-soluble polysaccharide coded as LFP3 from litchi pulp exhibited higher antioxidant activity (9). Meanwhile, another novel water-soluble fraction LCP50W has been

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reported to contain immunomodulatory activity through promoting the proliferation of mouse splenocytes and enhanced cytotoxicity towards NK cells (10). Although several bioactive polysaccharide fractions from *Litchi chinensis* have been reported, there are still other fractions value further investigation for food and pharmaceutical industries.

In the current research, crude polysaccharides were extracted from *L. chinensis* to obtain the polysaccharide fraction (LCP70). LCP70 was further purified and two homogenous heteropolysaccharides (LCP70S-1 and LCP70W) were isolated. The apparent molecular weight and monosaccharide composition of the polysaccharides were analyzed. The representative polysaccharide LCP70S-1 was selected to determine its polymer chain structures with physico-chemical and instrumental analyses, such as Fourier-transform infrared spectra (FTIR), GC-MS, ^1H NMR and ^{13}C NMR spectra which provided systematically valuable and fundamental information on the structure and chain conformation of the polysaccharide. In particular, the *in vitro* antioxidant activities of LCP70S-1 were also evaluated by three methods.

2. Materials and Methods

2.1. Plant materials

The pulp tissues of *Litchi chinensis* were purchased from Guangzhou Qingping Chinese Medicinal Materials Market, China, and identified by Dr. R.M. Yu, Jinan University, China.

2.2. Experimental reagents and materials

DEAE-52 cellulose and Sephadex G-100 were obtained from Whatman Ltd. Sephacryl S-300 HR was received from Amersham Biosciences. Ascorbic acid (Vitamin C, Vc), hydrogen peroxide (H_2O_2) and ferrous sulfate (FeSO_4) were obtained from Guangzhou Chemical Reagent Company. All other analytical grade reagents were obtained from Sigma and used as received.

2.3. Extraction, isolation and purification of polysaccharides

The pulp tissues of *L. chinensis* (300 g) was defatted with ethanol for 8 h and extracted three times with hot water (85°C). The water extract was filtered, concentrated under vacuum and centrifuged at 5,031 g for 15 min. The supernatant was concentrated to one fourth of its original volume and treated with 1 volume of ethanol for precipitation at 4°C . The precipitate was collected and the supernatant was concentrated to a quarter of its original volume for the treatment with 3 volumes of 95% ethanol for further precipitation at 4°C overnight. The precipitate was collected by centrifugation and washed three times with 95%

ethanol, anhydrous ethanol and acetone respectively. The resulting precipitate was dialyzed using a cellulose membrane (molecular weight cut off 3500, purchased from Lvniao, China) against distilled water for two successive days. The retained fraction was concentrated and lyophilized. One gram of dried precipitate 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 (11). The crude polysaccharide was dissolved in distilled water and was fractionated on a DEAE-cellulose-52 column (2.6×40 cm, from Whatman) equilibrated by distilled water, with a linear gradient from 0 to 0.5 M NaCl at a flow rate of 0.5 mL/min. All of the fractions were assayed for carbohydrate content by the phenol-sulfuric acid method (12), and two sharp peaks were pooled, dialyzed, concentrated, and further purified by a Sephadex G-100 column (1.6×70 cm, from Whatman) eluting with distilled water at a flow rate of 0.3 mL/min. The polysaccharide fractions were collected, dialyzed, and lyophilized.

Consequently, two polysaccharides, LCP70W and LCP70S-1, were obtained. LCP70S-1 was used in the subsequent studies on its structure and bioactivity.

2.4. Analyses of chemical composition

Total sugar content was determined by the phenol-sulfuric acid colorimetric method using glucose as the standard (12). Sulfate content was measured according to the literature (13). Uronic acid content was evaluated by the carbazole-sulfuric acid method using glucuronic acid as the standard (14). The homogeneity and molecular weight of LCP70S-1 were determined 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 LCP70S-1 was plotted in the same graph, and the molecular weight was measured (15). The monosaccharide composition was analyzed by high-performance anion exchange chromatography (HPAEC) after hydrolyzation and UV detection, coupled with pulsed amperometric detection (PAD), equipped with a Carbo PAC $^{\text{TM}}$ PA10 (2.0×250 mm) column. The hydrolysate (1 mg) was dissolved in pure water (1 mL). The solution (25 mL) was used for the ionic-chromatographic analysis by HPAEC-PAD of Dionex ICS-2500 system, eluted with a mixture of water and 200 mM NaOH in the volume ratio of 92:8 (16).

2.5. Spectroscopic analysis

Optical rotations were recorded with a Jasco P-1020 polarimeter. IR spectra were recorded with a Tensor 27 Bruker instrument with KBr pellets. The sample was

ground with KBr powder and then pressed into pellets for FTIR measurement at 4,000-500 cm^{-1} . ^{13}C NMR spectra were recorded with a Bruker 500 instrument and the sample was dissolved in D_2O (17).

2.6. Partial acid hydrolysis

LCP70S-1 (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 removing TFA by co-distillation with MeOH (1 mL \times 3), the fraction was evaporated to dryness (fraction 1). The fraction in the sack was evaporated, and then hydrolyzed with 0.5 M TFA. The hydrolysate was again 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.

2.7. 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 microliters of the solution were withdrawn at 6 h intervals under the dark at 4°C, then diluted to 5 mL with distilled water and measured in a spectrophotometer at 223 nm (18). Consumption of HIO_4 was determined by a spectrophotometric method (19), and formic acid production was measured by titration with 0.061 M NaOH. The nondialysate was concentrated and reduced with NaBH_4 (30 mg) for 24 h at room temperature, and neutralized to pH 6.0 with 0.1 M HOAc. After dialyzed and concentrated, the mixture product was hydrolyzed with 2 M TFA (4 mL) at 100°C for 8 h and tested by HAPEC-PAD analysis.

2.8. Methylation analysis

Polysaccharide was dried at 30°C under vacuum overnight and was methylated by the method of Hakomori (20). The polysaccharide was dispersed in 3 mL of dimethyl sulfoxide and stirred until dissolved after bubbling with N_2 , then 15 mL of solid anhydrous NaH and 1.5 mL of methyl iodide were added, and the solution was allowed to react in the dark for 2 h. The reaction was terminated by the addition of water, and the permethylated polysaccharide was extracted with chloroform. The permethylated polysaccharide was then further 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 evaporated to dryness. The methylated sugars were reduced with NaBH_4 , and then acetylated with acetic anhydride. Alditol acetates were analyzed by GC-MS (21). GC-MS was conducted with a Hewlett Packard 5895 instrument, using a fused-silica capillary column

(30 \times 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.

2.9. In vitro antioxidant activity assay

2.9.1. Determination of DPPH radical-scavenging activity

DPPH radical-scavenging activity was determined using the method described by Hua with some modifications (22). Briefly, DPPH ethanol solution (190 μL , freshly prepared at a concentration of 0.2 mM) was added to 10 μL of polysaccharide solution of gradient concentrations (0, 25, 50, 100, 200, 400, 800, and 1,600 $\mu\text{g}/\text{mL}$) 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 DPPH radical was calculated by using the following equation:

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

where A_0 was the absorbance of control (without sample) and A_1 was the absorbance of sample (23).

2.9.2. Determination of hydroxyl radical-scavenging activity

The scavenging activity for hydroxyl radicals was measured with Fenton reaction. The 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 following equation:

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

where A_0 was the absorbance of the control (blank, without H_2O_2), A_1 was the absorbance in the absence of sample, and A_2 was the absorbance in the presence of sample (16).

2.9.3. Reducing power assay

The reducing power was evaluated according to the method of Sheng (24). Different concentrations of each sample (0.1-20 mg/mL , 2.5 mL) were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and 5 mL of potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] (1%, w/v). The mixture was incubated at 50°C for 20 min. After 5 mL of trichloroacetic acid (10%, w/v) was added to terminate the reaction, the mixture was centrifuged at 5,000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl_3 (0.5 mL, 0.1%), and the absorbance was measured at 700 nm against a blank. Vitamin C and α -tocopherol were used for comparison.

2.9.4. Statistical analysis

All bioassay results were expressed as means \pm S.D. The experimental data were subjected to an analysis of variance for a completely random design.

3. Results and discussion

3.1. Extraction, isolation and purification of polysaccharides

Crude polysaccharide (3.54 g) was obtained from pulp tissues *L. chinensis* with hot water extraction followed by ethanol precipitation. After purification with DEAE-cellulose 52 and Sephadex G-100 column, the profiles of LCP70S-1 and LCP70W appeared as single and symmetrical sharp peaks (Figure 1), which were detected by the phenol-sulfuric acid assay. The yield of LCP70S-1 and LCP70W from crude polysaccharides was 22.4% and 12.6%, respectively. LCP70S-1 did not have any sulfate esters. The uronic acid contents of LCP70S-1 and LCP70W were below the detection limit. The average molecular weight of LCP70S-1 and LCP70W were determined to be as 11.7 kDa and 5.3 kDa respectively, by gel permeation chromatography (GPC). Calibration was performed with dextran molecular weight standards (dextran Mw: 5×10^6 , 2×10^5 , 7×10^4 , 1×10^4 , and 3×10^3 ; Pharmacia). HPAEC-PAD analysis showed that LCP70W was composed of glucose, and LCP70S-1 was composed of rhamnose, arabinose, and galactose in a molar ratio of 1.06:6.39:4.21.

3.2. Structural elucidation of LCP70S-1

The total carbohydrate content of LCP70S-1 was measured to be 98.2% (w/w) and m-hydroxybiphenyl colorimetric test for uronic acid was shown to be negative. The optical rotation of LCP70S-1 was

$$[\alpha]_D^{20} = -35^\circ \text{ (c 1.0, 23}^\circ\text{C, H}_2\text{O)}.$$

The composition of LCP70S-1 is presented in Table 1.

Fractions 1-3 obtained through partial acid hydrolysis were subjected to HPAEC-PAD analysis and the results were shown in Table 1. Fraction 3, the precipitate in the sack, possesses the biggest size among the three fractions. Only galactose could be detected in fraction 3, implying that galactose could be the backbone of the structure of LCP70S-1. The analysis results of fractions 1 and 2 showed that the branched structure of LCP70S-1 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 L-rhamnose but no L-arabinose or D-galactose

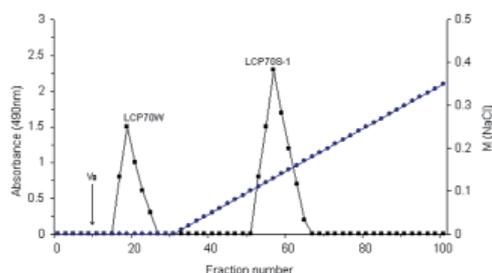


Figure 1. Profile of LCP70 in DEAE-cellulose 52 column chromatography. Flow rate, 0.5 mL/min. Fraction size, 1 mL/min. Void volume, 10 mL.

Table 1. HPAEC-PAD analysis of acid hydrolysis and Smith degradation products from LCP70S-1

Items	Molar ratios		
	Rhamnose	Rabinose	Galactose
LCP70S-1	1.06	6.39	4.21
Fraction 1	0.03	1.00	0.06
Fraction 2	0.68	1.87	0.91
Fraction 3	n.d.	n.d.	1.00
Smith degradation	1.00	n.d.	n.d.

n.d., not detected.

in the oxidation products. It could therefore be inferred that linkages of galactose were (1 \rightarrow), (1 \rightarrow 2), (1 \rightarrow 6), (1 \rightarrow 2,6), (1 \rightarrow 4), and (1 \rightarrow 4,6), which could be oxidized to produce glycerol and erythritol. The presence of L-rhamnose revealed that some residues of L-rhamnose were (1 \rightarrow 3)-linked, (1 \rightarrow 2,3)-linked, (1 \rightarrow 2,4)-linked, (1 \rightarrow 3,4)-linked or (1 \rightarrow 2,3,4)-linked, which could not be oxidized, respectively (25,26). The fully methylated LCP70S-1 was hydrolyzed with acid and analyzed by GC-MS. The results showed the presence of four components, namely 2,3,5-Me₃-Ara, 2,4-Me₂-Rha, 2,3-Me₂-Ara, 2,4-Me₂-Gal in a relative molar ratio of 4.23:0.78:2.17:3.84 (Table 2a). Based on the standard data in the CCRC Spectral Database for partially methylated alditol acetates (PMAA's), the linkage of L-rhamnose and D-galactose were deduced as (1 \rightarrow 3), (1 \rightarrow 3,6) while the linkages of L-arabinose were deduced as (1 \rightarrow) and (1 \rightarrow 5). The results showed a good correlation between terminal and branching points. In addition, these molar ratios also agreed with the overall monosaccharide composition of LCP70S-1 described above.

3.3. IR spectroscopy and NMR analysis

The infrared spectra of the *L. chinensis* LCP70W and LCP70S-1 polysaccharides revealed their major functional groups and the chemical bonds (Figure 2a). The broad peaks at 3,404, 3,436 cm⁻¹ were caused by the stretch vibration of O-H. The bands at 2925-2928 cm⁻¹ were attributed to the stretch vibration of C-H, and the signals at 1,635 and 1,641 cm⁻¹ were due to the bound water. No signal corresponding to sulfate esters was found in FTIR spectra of the two polysaccharides.

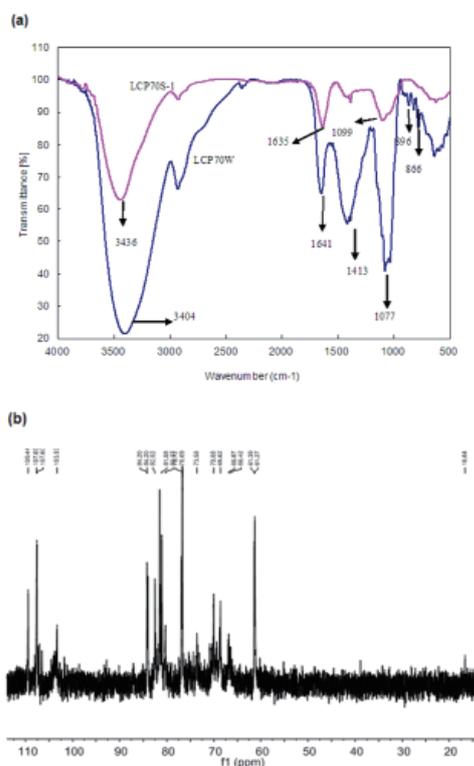
Table 2. GC-MS analysis of partially methylated alditol acetates (PMAA) from LCP70S-1(a) and Assignment of ¹³C NMR chemical shifts for LCP70S-1(b)

(a)

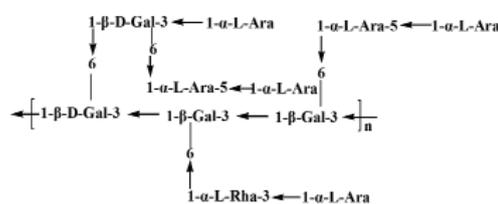
Methylation sugar	Molar ratios	Mass fragments (m/z)	Retention time (min)	Linkage type
2,3,5-Me3-Ara	4.23	43, 45, 71, 87, 101, 117, 129, 161	13.00	T→
2,4-Me2-Rha	0.78	43, 59, 69, 75, 85, 101, 117, 129	14.17	1→3
2,3-Me2-Ara	2.17	43, 87, 101, 117, 129, 189	15.18	1→5
2,4-Me2-Gal	3.84	43, 71, 101, 129, 161, 189, 233	18.97	1→3,6

(b)

Sugar residue	Chemical shifts (ppm)						
	C1	C2	C3	C4	C5	C6	CH ₃
α-L-Ara(1→	107.69	76.69	80.27	70.36	68.62		
→3)-α-L-Rha(1→	103.33	70.08	81.08	73.58	69.40		16.64
→5)-α-L-Ara(1→	109.44	82.52	80.35	82.52	66.87		
→3,6)-β-D-Gal(1→	107.60	70.11	84.20	68.80	76.73	61.27	

**Figure 2. FTIR (a) and ¹³C NMR (b) spectra of LCP70S-1.**

In addition, the peaks at 1,077 and 1,099 cm^{-1} were caused by the change of angle vibration of O-H. The characteristic absorption at 896 cm^{-1} indicated the presence of β -type glycosidic linkage. There was no absorption at 1,740 cm^{-1} , indicating the lack of uronic acid in the polysaccharide structure (27). The spectrum of ¹³C NMR of LCP70S-1 was shown in Figure 2b. According to the literatures (28,29) the resonances in the region of 103-109 ppm in ¹³C NMR were attributed to the anomeric carbon atoms of L-rhamnose (Rhap), D-galactose (Galp) and L-arabinose (Arap). The peaks at 103.33 ppm corresponded to C-1 of (1→3)-linked α -L-Rha units, 107.60 ppm corresponded to C-1 of (1→3,6)-linked β -D-Gal units, 107.69 ppm

**Figure 3. Predicted structure for LCP70S-1.**

corresponded to C-1 of (1→)-linked α -L-Ara units, and 109.44 ppm corresponded to C-1 of (1→5)-linked α -L-Ara units, respectively. The result also indicated that the backbone was composed of (1→3,6)-linked β -D-galactopyranosyl residues which branches at O-6. The assignment of the carbon atoms signals was shown in Table 2b.

Based on the results of HPAEC-PAD, GC-MS and ¹³C NMR, the backbone of LCP70S-1 should contain (1→3)-linked galactopyranosyl with branches at O-6. The three branches consisted of (1→3)-linked rhamnopyranosyl residues, (1→3,6)-linked galactopyranosyl and (1→5)-linked arabinofuranosyl residues, and each of them was terminated with a (1→)-linked arabinopyranosyl residues. From the aforementioned results, the repeating structural unit of LCP70S-1 could be illustrated as shown in Figure 3.

3.4. *In vitro* antioxidant activities

3.4.1. Scavenging activity of polysaccharides for DPPH radicals

DPPH assay has been widely used to evaluate free radical scavenging activity of antioxidants by monitoring their ability to scavenge the stable free radical from DPPH molecule. As shown in Figure 4A, LCP70S-1, LCP70W and vitamin C reacted directly with and quenched DPPH radicals to different degrees with increased activities at higher concentrations. The maximum value of LCP70S-1

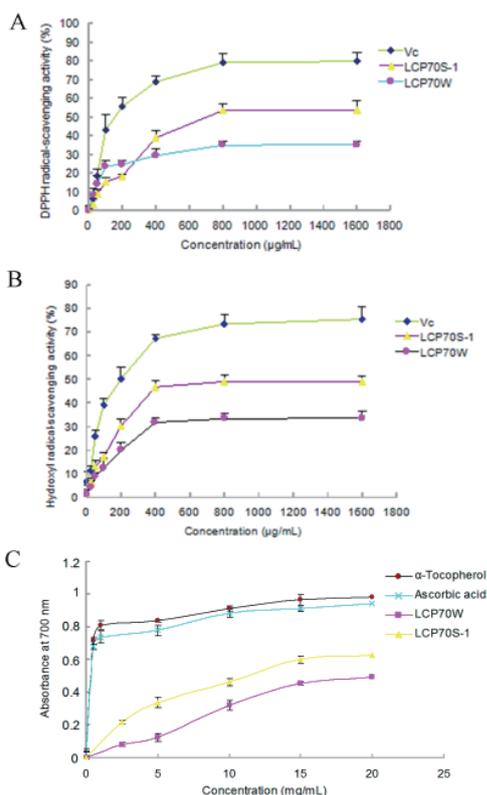


Figure 4. DPPH radical-scavenging activity (A), hydroxyl radical-scavenging activity (B) and reducing power (C) of LCP70S-1 and LCP70W. Values are means \pm S.D. of three separate experiments.

and LCP70W reached up to 67.4% and 44.1% of that of Vc, respectively.

3.4.2. Scavenging activity of polysaccharides for hydroxyl radicals

Hydroxyl radical is very reactive and can be generated in biological cells through the Fenton reaction. As shown in Figure 4B, LCP70S-1, LCP70W and Vc exhibited concentration-dependent scavenging activities against hydroxyl radicals generated in a Fenton reaction system. The maximum value of LCP70S-1 and LCP70W reached to 65.0% and 44.6% of that of Vc, respectively.

3.4.3. Reducing power

The reducing properties were generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free-radical chain through donating a hydrogen atom. Figure 4C depicted the reducing power of tested samples. Higher absorbance value means stronger reducing power of samples. In this assay, the reducing power of the tested polysaccharides steadily increased with increasing sample concentration. The reducing abilities of LCP70S-1 and LCP70W at 20.0 mg/mL were 0.63 and 0.47, respectively, which were weaker than those of α -tocopherol and Vc. Our data showed that the reducing power of the polysaccharides

probably played a role in the antioxidant ability of *L. chinensis*.

In conclusion, this paper described a novel water-soluble polysaccharide (LCP70S-1) isolated from the pulp tissues of *L. chinensis* contained predominantly three monosaccharides, which were found out to be L-rhamnose, L-arabinose and D-galactose. *In vitro* antioxidant activity assays showed that this natural polysaccharide possesses free radical scavenging ability. We further illustrated that natural polysaccharide possesses free radical scavenging ability which could possibly attributed to the curative effect of *L. chinensis* as traditional Chinese medicine. Further investigation on LCP70S-1 could be launched to explore its potential as a potential antioxidant for human healthcare.

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