

# Radical Scavenging and Low - Density Lipoprotein Cholesterol Oxidation Inhibitory Effects of Polysaccharides Extracted from Chinese Yam, Pumpkin, and Shiitake

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**Abstract** The inhibitory effects of three polysaccharides from Chinese yam (*Dioscorea opposita*), pumpkin (*Cucurbita pepo L. var. Kintoga Mak*), and shiitake (*Lentinula edodes*) on the oxidation of low-density lipoprotein cholesterol (LDL) were studied. The molecular weights of the Chinese yam polysaccharide (CP), pumpkin polysaccharide (PP), and shiitake polysaccharide (SP) were 17318 Da, 16619 Da and 14977 Da, respectively. The results of GC-MS showed that all three polysaccharides had mannose and glucose in their monosaccharide composition. FTIR and NMR showed that the three polysaccharides had typical characteristic peaks. The three polysaccharides demonstrated potent *in vitro* antioxidant activity, among which CP was the best, with  $IC_{50}$  values for DPPH, superoxide, and hydroxyl radical removal ability of 1.06, 1.06, and 0.98 mg/mL, respectively. All three polysaccharides effectively delayed the formation of conjugated diene (CD) in LDL oxidation induced by  $CuSO_4$  and significantly inhibited the production of thiobarbituric acid reactive substances (TBARS). At a concentration of 1.0  $\mu$ g/mL, CP, PP, and SP inhibited TBARS production at rates of 16.28%, 19.38%, and 26.36% of the  $CuSO_4$  group, respectively ( $P > 0.05$ ). The electrophoretic patterns of LDL glycated products showed that PP strongly inhibited the production of glycated products. The present study may provide useful information for understanding the inhibitory effects of polysaccharides on LDL oxidation.

**Keywords:** polysaccharides, antioxidant, LDL, oxidation, glycation

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## 1. Introduction

Cardiovascular diseases are the leading cause of mortality worldwide, and atherosclerosis is the main contributor to these diseases. Atherosclerosis is characterized by the formation of plaques that develop in the artery wall [1]. In the early stages of this disease, native low-density lipoprotein cholesterol (LDL) enters the arterial subendothelial intima where it accumulates and becomes oxidized, leading to the formation of plaques [2]. Therefore, LDL is a significant risk factor for atherosclerotic vascular disease. LDL glycation contributes to the development and progression of atherosclerosis [3]. Glycation of LDL occurs mainly due to the reaction of glucose or fructose with the free amino groups of lysine, in which LDL is rich.

This reaction causes the generation of a schiff base, amadori products, and eventually advanced glycation end products (AGEs) [3], which are strongly related to the development of diabetes - associated cardiovascular diseases [4]. Oxidation and glycation of LDL are presumed to be interdependent, therefore, atherosclerosis can be prevented by inhibiting LDL oxidation and glycation processes. In fact, clinically available lipid - lowering agents (e.g., statins and ezetimibe) and antidiabetic agents (e.g., metformin) have many side effects. Furthermore, their inhibitory effects on LDL oxidation and glycation are not fully understood. Therefore, more investigation is required to discover new LDL oxidation and glycation inhibitors. In previous studies, several natural compounds including guggulsterone, cineole, and sethylcysteine were found to significantly inhibit LDL oxidation and glycation [5].

Polysaccharides are chain polymers formed by dehydration of monosaccharides to linear or branching glycosidic bonds. Many studies have shown that polysaccharides extracted from plants have many biological functions, such as participating in the immune regulation process of organisms, lowering blood sugar, lowering blood lipids, resisting fatigue, and anti-aging. Chinese yam, pumpkin, and shiitake are popular with consumers because they are rich in polysaccharides and many other functional components. In diabetic mice, the addition of crude yam polysaccharides to their diet has a significant hypoglycemic effect, mostly mediated by increasing insulin secretion, thereby repairing the damaged  $\beta$ -cell and eliminating excessive free radicals [6]. In recent years, it has been found that water-soluble polysaccharides isolated from pumpkin fruits have various antioxidant activities [7]. Polysaccharides extracted from several types of mushroom have also been reported to exhibit significant antioxidant activities based on various *in vitro* and *in vivo* assays [8]. However, no one has yet studied whether polysaccharides have an impact on the oxidation and glycation process of LDL.

In this study, Chinese yam, pumpkin, and shiitake polysaccharides were extracted, purified, and characterized by gas chromatography-mass spectrometry (GC-MS), Fourier transform infrared spectroscopy (FTIR), and nuclear magnetic resonance (NMR) and investigated for antioxidant activity against DPPH, superoxide, and hydroxyl radicals. Finally, their inhibition of LDL oxidation and glycation were evaluated.

## 2. Methods and Materials

### 2.1. Materials

Chinese yam (*Dioscorea opposita*), pumpkin (*Cucurbita pepo* L. var. *Kintoga Mak*), and shiitake (*Lentinula edodes*) were purchased from a local vegetable market near the Beijing Agricultural University (Beijing, China). Monosaccharide standards, including rhamnose, fucose, arabinose, xylose, mannose, glucose, and galactose, were purchased from the Sigma Chemicals Company (St. Louis, USA). Acetic acid, acetic anhydride, sodium borohydride, chloroform, and trifluoroacetic acid were purchased from the China National Pharmaceutical Group Co., Ltd. (Sinopharm). All of the other chemicals were of analytical reagent grade and were purchased from the Beijing Changhua Zhicheng Technology Company Limited (Beijing, China).

### 2.2. Preparation of Polysaccharides

All samples were dried at 60°C for 48 h, and then ground in a high-speed disintegrator and passed through a 40-mesh sieve. Polysaccharides were extracted using the method described by Chen [9]. Briefly, the sample was extracted with distilled water (1:20, w/v, g/mL) for 1 h at 70°C, followed by centrifugation at 10000 rpm for 10 min, and the supernatant was concentrated to 25% of the original volume using a rotary evaporator under a vacuum at 60°C. Then the concentrated supernatant was precipitated by adding a four-fold volume of absolute

ethanol. After standing for 24 h at 4°C, the precipitate was separated and deproteinated using the Sevag method (chloroform-butyl alcohol, 4:1, v/v). Finally, the polysaccharide was dialyzed (cut-off of 8000 Da) to remove small molecular substances, and the crude polysaccharide was vacuum-dried. The polysaccharide fractions from Chinese yam, pumpkin, and shiitake were named CP, PP, and SP respectively.

Total sugar and protein contents were determined using the phenol-sulfuric acid method [10] and the Lowry method [11], respectively. The ash content was assayed by GB 5009.4-2016 (Chinese State Standard). The uronic acid was analyzed using the sulfuric acid carbazole method [12].

### 2.3. Determination of Polysaccharide Molecular Weight

The homogeneity and molecular weight of polysaccharide were identified by high-performance gel permeation chromatography (HPGPC) on a Shimadzu LC-10A system equipped with a KS805-804-802 column (7.8 mm × 300 mm). The determination procedure was carried out according to the method in the literature [13]. A sample solution (5 mg/mL; 20  $\mu$ L) was injected for each run, and eluted with double-distilled water at 40°C at a flow rate of 0.8 mL/min. The homogeneity of polysaccharide was judged according to the peak shape of the HPGPC chromatogram. Dextran standards with different molecular weights (Mw 1152, 5220, 11600, 23800, 48600, 80900, 148000, 273000, 409800, 667800 Da) were used for data analyses.

### 2.4. Analyses of Monosaccharide Composition

GC-MS was used for identification and quantification of monosaccharides in each fraction. The polysaccharide (2 mg) was hydrolyzed with trifluoroacetic acid (TFA, 2 M, 2 mL) at 110°C for 90 min and evaporated using a rotary evaporator [14]. After hydrolysis, the hydrolysate was neutralized with dilute acetic acid and dried at 110°C after adding 2 mL double-distilled water. Subsequently, the product was reduced with NaBH<sub>4</sub> (100 mg) and acetylated with 1 mL acetic anhydride at 100°C for 1 h. The residue was concentrated by adding 3 mL toluene, which was repeated four to five times to remove the excess acetic anhydride. Finally, the acetylated product was dissolved in 3 mL chloroform and transferred to a separating funnel. After a small amount of distilled water was added and fully shaken, the supernatant was removed, and the chloroform layer was dried with an appropriate amount of anhydrous sodium sulfate at a constant volume of 10 mL. The acetylation products were analyzed by Shimadzu GCMS-QP 2010 gas chromatography-mass spectrometry. GC-MS conditions: RXI-5 SIL MS column (30 m × 0.25 mm × 0.25  $\mu$ m). The column temperature was programmed from 120°C to 250°C at 3°C/min, and kept at 250°C for 5 min. Helium (purity, 99.999%) was used as a carrier gas at a constant flow rate of 1 mL/min. The temperature of the detector and inlet were both 250°C. Monosaccharide standards (rhamnose, fucose, arabinose, xylose, mannose, glucose, and galactose) were prepared by the same procedure for quantifying the monosaccharide

content based on the corresponding peak areas and response factors.

## 2.5. FTIR Analyses

For the FTIR analyses [14], 2 mg polysaccharide was mixed with 200 mg KBr powder, ground, and pressed. A blank control was prepared using KBr powder pressed into tablets. FTIR spectra of the polysaccharides were recorded at a frequency range of 400 - 4000  $\text{cm}^{-1}$  using an FTIR650 (Tianjin Gangdong, Tianjin).

## 2.6. NMR Spectroscopy

20 mg polysaccharide was weighed and dissolved in 0.5 mL  $\text{D}_2\text{O}$ . The polysaccharide was homogenized by oscillation and then transferred to the NMR tube.  $^1\text{H}$  - NMR (600 MHz) and  $^{13}\text{C}$  - NMR (150 MHz) were measured and analyzed on an NMR at 23°C [15].

## 2.7. DPPH Radical Scavenging

The DPPH radical scavenging activity of polysaccharides was determined using the method of [14] with minor modifications. The reactants, 0.1 mL polysaccharides (0.4, 0.8, 1.2, 1.6, or 2.0 mg/mL), and 3.9 mL DPPH - methanol solution (0.1 mM) were mixed. After 30 min incubation at 37°C, the absorbance was measured at 517 nm using distilled water as the control. Vc (Vitamin C) was used as a positive control. The DPPH radical scavenging rate was determined using the following equation:

$$\begin{aligned} \text{DPPH radical scavenging rate (\%)} \\ = [1 - (A_1 - A_2 / A_0)] \times 100 \end{aligned}$$

where  $A_0$  is the absorbance of the control (distilled water instead of sample),  $A_1$  is the absorbance of the sample, and  $A_2$  is the absorbance of the sample only (ethanol instead of reaction solution).

## 2.8. Superoxide Radical Scavenging

The superoxide radical scavenging activity was investigated as described by Wang [14] with minor modifications. First, 0.5 mL sample (0.4, 0.8, 1.2, 1.6, or 2.0 mg/mL) was added to 1.5 mL 50 mM Tris - HCl buffer solution (pH = 8.0) and the reaction solution was incubated at 30°C for 20 min. Cooled to room temperature, the sample was mixed with a 1.5 mL 7 mM pyrogallol solution. After 3 min, the reaction system was quickly terminated by adding 0.5 mL HCl. Vc was used as a positive control. The absorption value was determined at 320 nm. The superoxide radical scavenging activity was calculated using the following equation:

$$\begin{aligned} \text{Superoxide radical scavenging rate (\%)} \\ = [(A_0 - A_1) / A_0] \times 100 \end{aligned}$$

where  $A_0$  is the absorbance of the control (distilled water instead of sample) and  $A_1$  is the absorbance of the sample.

## 2.9. Hydroxyl Radical Scavenging

The hydroxyl radical scavenging activity was investigated as described by Chen [9] with minor modifications. The polysaccharide sample was dissolved in 10 mL distilled water at the concentration of 0.4, 0.8, 1.2, 1.6, or 2.0 mg/mL. First, 1.0 mL sample solution was mixed with 1.0 mL 9 mM  $\text{FeSO}_4$  and 1.0 mL 9 mM  $\text{H}_2\text{O}_2$  in 20 mL clamped test tubes. After incubation at room temperature for 10 min, 1.0 mL 9 mM salicylic acid-ethanol solution was added to the solution. The reaction solution was incubated at 37°C for another 30 min. After cooling to room temperature, the reaction solution was centrifuged at 3000 rpm for 10 min. The absorption value  $A_X$  of the supernatant was measured at 510 nm. The light absorption value  $A_{X0}$  was measured using isovolumetric distilled water instead of salicylic acid as a sample control group; the equivalent volume of distilled water instead of polysaccharide was used as a blank control group, and the light absorption value was  $A_0$ . Vc was used as a positive control. Hydroxyl radical scavenging activity was calculated using the following equation:

$$\begin{aligned} \text{Hydroxyl radical scavenging rate (\%)} \\ = \left\{ [A_0 - (A_X - A_{X0})] / A_0 \right\} \times 100 \end{aligned}$$

## 2.10. Calculation of $IC_{50}$ Value of Semi Clear Concentration

The  $IC_{50}$  values were calculated as described by Sun [16]. According to the curves of DPPH, superoxide and hydroxyl radicals with PPY concentration, the  $IC_{50}$  values of three radicals were calculated by probit regression method in SPSS 16.0.

## 2.11. Preparation of LDL

This study was approved by the Ethics Committee of Beijing Agricultural University. LDL extraction was conducted as described by Cai [17] with minor modifications. A volume of 1 L fresh porcine blood was taken and 3.8% sodium citrate was added at a volume ratio of 1:9. The plasma was separated by centrifugation at 3000 rpm for 10 min at 4°C. Then 300 mL fresh plasma was fully mixed with 3 L sediment (64 mM sodium citrate solution, in which the heparin sodium concentration is 50,000 U/L, the pH was adjusted to 5.04 with 5 M HCl). The reaction solution was incubated at 37°C for 15 min then separated by centrifugation at 3000 rpm/min for 10 min at 4°C to obtain the precipitate. Finally, 1500 mL solution (64 mM sodium citrate solution, the pH was adjusted to 5.11 with 5 M HCl) was used to wash the precipitate, then the precipitate was separated and dissolved in 600 mL PBS (pH = 7.4, containing 160 g/L NaCl, 8.1 mM  $\text{Na}_2\text{HPO}_4$ , 1.5 mM  $\text{KH}_2\text{PO}_4$ , and 2.7 mM KCl) and incubated at 37°C to completely dissolve the LDL precipitate. The isolated LDL was submitted to exhaustive dialysis with 10 mM PBS (pH 7.4) for 24 h at 4°C. After dialysis, the isolated LDL was stored at -20°C for less than 2 weeks. Protein quantification of the isolated LDL was performed according to the method described by Lowry *et al.* [11] and adjusted it to 400 mg/L as a backup.

## 2.12. Conjugated Dienes Formation

The production of conjugated diene (CD), the primary product of LDL lipid peroxidation, and the inhibition effects of each sample were determined using the method described by Santos *et al.* [18] with slightly modified. Briefly, LDL samples (400 µg protein/L) were incubated in a medium of PBS (10 mM, pH 7.4) and polysaccharide (1.0 µg/mL) for 5 min at 37°C. CuSO<sub>4</sub> (final concentration 10 µM) was added to the medium for oxidation. In assays, aliquots from the reaction medium were removed at different time points to evaluate CD formation, which was monitored spectrophotometrically based on changes in the absorbance at 234 nm. Absorbance was measured every 20 min (0 - 240 min). In the blank group, the same volume of methanol solution was used instead of the sample solution, and the same volume of deionized water was used instead of CuSO<sub>4</sub>.

## 2.13. Production of Thiobarbituric Acid Reactive Substances

The end products from lipid peroxidation were evaluated by measuring the levels of thiobarbituric acid reactive substances (TBARS), as discussed by Santos *et al.* [18] with slight modifications. The experimental group was established as follows: the LDL samples (400 µg protein/L) were incubated in a medium phosphate buffer (10 mM, pH 7.4) and different concentrations of polysaccharide (0.2 - 1.0 µg/mL) and CuSO<sub>4</sub> (final concentration 10 µM) were added to the medium for oxidation; distilled water of equal volume was used instead of the sample as the oxygen-promoting control group; and the sample solution was replaced with CuSO<sub>4</sub> and distilled water of equal volume for the blank group. All reaction systems were incubated at 37°C for 2 h. The color reaction of TBARS was carried out by adding 50 µL 1% EDTA-Na<sub>2</sub>, 2 mL 15% trichloroacetic acid, and 2 mL 0.76% 2 - thiobarbituric acid to 1 mL the mixture. The TBARS content was measured via spectrophotometry at 532 nm after 35 min in a boiling water bath, which then was rapidly cooled to room temperature and centrifuged at 4000 r/min for 10 min at 4°C.

## 2.14. AGEs Formation by LDL Glycation

A non - enzymatic glycation incubation system for LDL was established according to the method of Santos [18]. LDL (400 µg protein/mL) was incubated with glucose (final 500 mM) for 48 h at 37°C in a medium containing PBS (10 mM, pH 7.4) in the presence or absence of aminoguanidine (AG, an anti-glycative agent used as a positive control, 10 mM) or polysaccharide (1.0 mg/mL). Afterwards, the solutions were dialyzed for 24 h at 4°C in 2 L 10 mM PBS (pH 7.4). A reaction solution of distilled water and LDL was used as the blank group.

## 2.15. Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed on an 0.5% agarose gel in TAE buffer (40 mM Tris, 40 mM acetic acid, and 1 mM EDTA) using the electrophoretic system discussed by Kim [10] with slightly modifications. To

samples containing native LDL, glyLDL, and glyLDL treated with polysaccharides or AG, 0.5 mL 0.05% bromophenol blue staining solution was added. After mixing, the supernatant was centrifuged at 2000 r/min for 5 min. The supernatant was sampled at 15 µL per comb hole and electrophoresis was performed at 120 V and 400 mA for 30 min. Coomassie brilliant blue was dyed and decolorized with glacial acetic acid-ethanol solution. A gel imaging system was used for collection.

## 2.16. Statistical Analyses

All results are expressed as mean ± standard deviation (SD). Data were analyzed by one - way analysis of variance (ANOVA) using SPSS 16.0. The significance level was set at P < 0.05.

## 3. Results and Discussion

### 3.1. Chemical Composition of Polysaccharides

The total sugar, protein, and ash content of CP, PP, and SP are shown in Table 1. There were significant differences in the chemical composition (dry basis) of the three polysaccharides. The total sugar content of the three polysaccharides was above 50%, indicating that the extracts were relatively pure and were appropriate for structural analyses. The protein contents were still high after deproteinization using Sevage reagent (chloroform - butyl alcohol, 4:1, v/v), with that of PP being 19.96 ± 0.81%. Chen *et al.* [9] found that the protein content of polysaccharides was more than 10% after deproteinization using the TCA method. These results indicate that the proteins in polysaccharides are not easy to remove, and may also covalently interact with the polysaccharide. The ash contents of CP and PP were high, indicating that these polysaccharides contained minerals. All three polysaccharide contained uronic acid between 19% and 30%.

**Table 1. Chemical composition (% , w/w, dry basis,) of CP, PP and SP**

Sample	Total sugar	Protein	Ash content	Uronic acid
CP	56.44 ± 1.03 a	5.89 ± 0.43 c	8.51 ± 0.14 f	30.81 ± 1.14 i
PP	54.15 ± 0.86 a	19.96 ± 0.81 d	5.28 ± 0.16 g	22.85 ± 1.26 j
SP	50.36 ± 0.76 b	10.17 ± 0.36 e	0.46 ± 0.16 h	19.28 ± 0.56 k

All experiments were performed at least in duplicate, and analyses of all samples were run in triplicate. The results are presented as means of three determinations ± standard deviation (SD). Values in the same column represented by different letters differ significantly (P < 0.05).

### 3.2. Molecular Weights of Polysaccharides

The molecular weight spectra of the polysaccharide fractions are shown in Figure 1A. The main peaks were eluted between 40 min and 45 min from HPGPC. CP consisted of six polysaccharide fractions with molecular weights of 933,742 Da (Rt = 33.865 min), 148,878 Da (Rt = 37.848 min), 39,233 Da (Rt = 40.741 min), 17,318 Da (Rt = 42.515 min), 1414 Da (Rt = 47.95 min), and 30 Da (Rt = 56.325 min), respectively. This result is different

from the data reported by Yang *et al.* [19], who reported a single polysaccharide peak with a molecular weight of 16,619 Da after hot water extraction with sequential Sevege deproteinization and Sephadex G - 100 size - exclusion chromatography. PP was mainly composed of three fractions with molecular weights of 119,769 Da (Rt = 38.32 min), 37,535 Da (Rt = 40.837 min), and 17,804 Da (Rt = 42.455 min), which agrees with the results reported by Lu [20]. SP was mainly composed of two fractions with molecular weights of 681,853 Da (Rt = 34.547 min) and 14,977 Da (Rt = 42.83 min), indicating that relatively pure polysaccharide was obtained. Wang *et al.* [14] also reported polysaccharides with similar MWs from shiitake.

### 3.3. Monosaccharide Composition of Polysaccharides

The monosaccharide compositions of the polysaccharides were analyzed by GC (Figure 1B). CP was mainly composed of seven monosaccharides, i.e., rhamnose, fucose, arabinose, xylose, mannose, glucose, and galactose at a ratio of 2.7: 1.0: 8.2: 0.9: 42.2: 12.8: 32.2, among which mannose, glucose, and galactose were the main ones. Yang *et al.* [19] reported that the purified polysaccharide from Chinese yam was composed of glucose and galactose at a ratio of 1.52: 1. PP was mainly composed of five monosaccharides, including rhamnose, arabinose, mannose, glucose, and galactose at a ratio of 8.4: 43.7: 1.0: 9.3: 34.3, with arabinose and galactose being the richest. These results are consistent with those of Zhang *et al.* [21]. The monosaccharide composition of SP was the simplest, mainly consisting of mannose and glucose at a ratio of 3.5: 1.0. These results coincide with those of Zhang *et al.* [22]. Mannose, glucose, and galactose were the most abundant.

### 3.4. FTIR Spectra of Three Polysaccharides

The infrared spectra of the polysaccharides are shown in Figure 1C. The polysaccharides exhibited typical FTIR spectra. The wide and strong absorption peaks at 3300 - 3400  $\text{cm}^{-1}$  showed the presence of intermolecular and intramolecular hydrogen bonds and the characteristics of hydroxyl bending vibrations. This is the characteristic peak of sugars. A weak absorption peak was observed at around 2900  $\text{cm}^{-1}$ , which was the C - H stretching vibration of polysaccharides [23], indicating that all three substances are polysaccharides. The peak near 1650  $\text{cm}^{-1}$  was the absorption peak of the carbonyl C = O stretching vibration, indicating that the sugar chain contains a carbonyl group [24]. At 1410 - 1200  $\text{cm}^{-1}$  was the absorption peak caused by C - H angular vibration. The peak at 1371  $\text{cm}^{-1}$  was due to the symmetrical angle-change vibration of  $\text{CH}_3$ , indicating that the  $\text{CH}_3$  group exists in SP.

Absorptions at 1741  $\text{cm}^{-1}$  and 1743  $\text{cm}^{-1}$  were seen in CP and PP, indicating the C = O stretching vibration of uronic acid, and PP also exhibiting C - O stretching vibrations at 1417  $\text{cm}^{-1}$  [25]. These results indicate that the sugar chain in CP and PP contains carboxyl - COOH, and thus uronic acids [26]. Absorption peaks in the 1200 - 1000  $\text{cm}^{-1}$  range, caused by two types of C - O stretching vibrations, indicated that the polysaccharides had

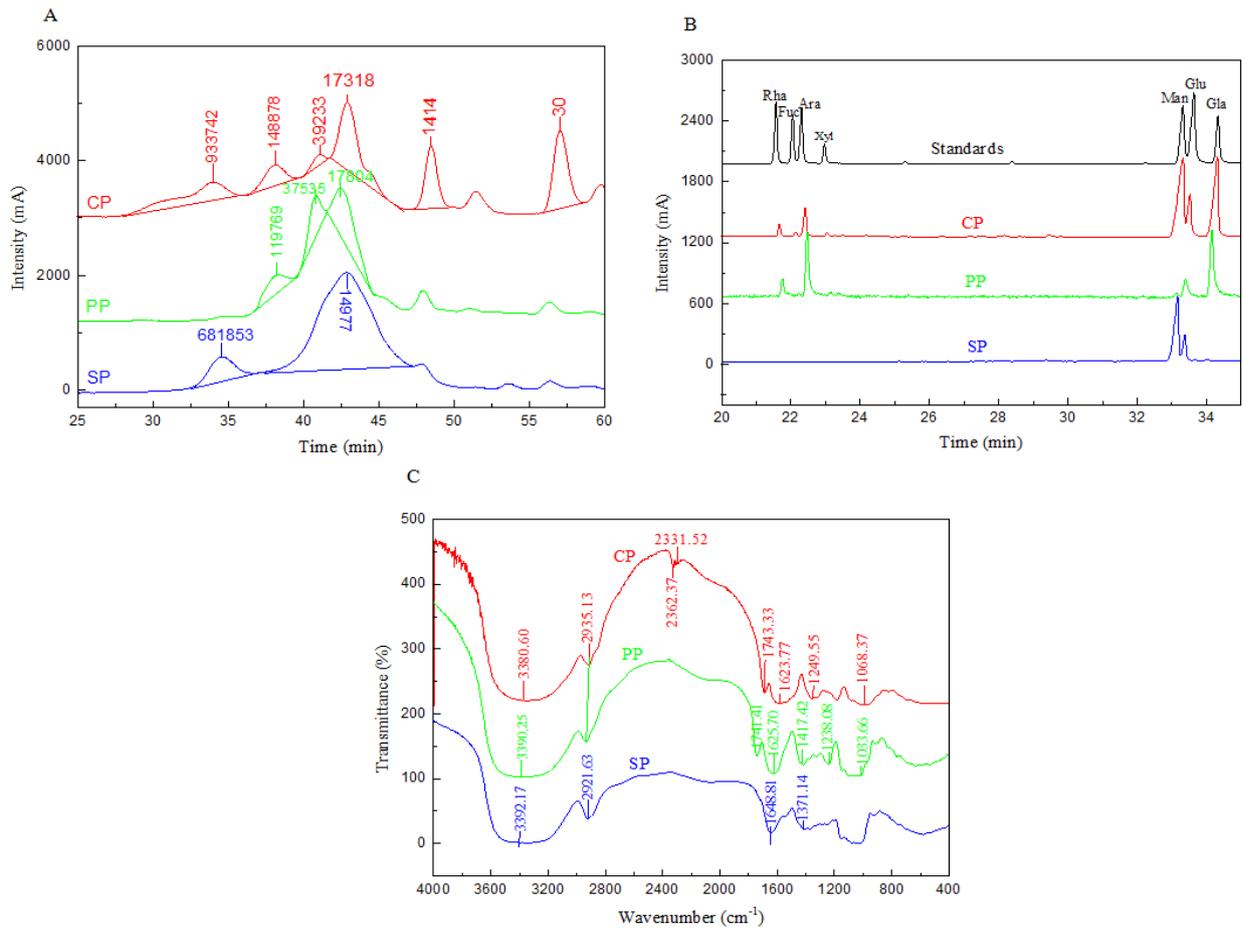
pyranose rings. The absorption band was mainly the stretching vibration of the side branches of the ring overlapping C - OH and the vibration of the C - O - C glycosidic bond [27]. We also found that both CP (1068.37 $\text{cm}^{-1}$ ) and PP (1033.66 $\text{cm}^{-1}$ ) contained pyranose rings.

### 3.5. NMR Spectra of Three Polysaccharides

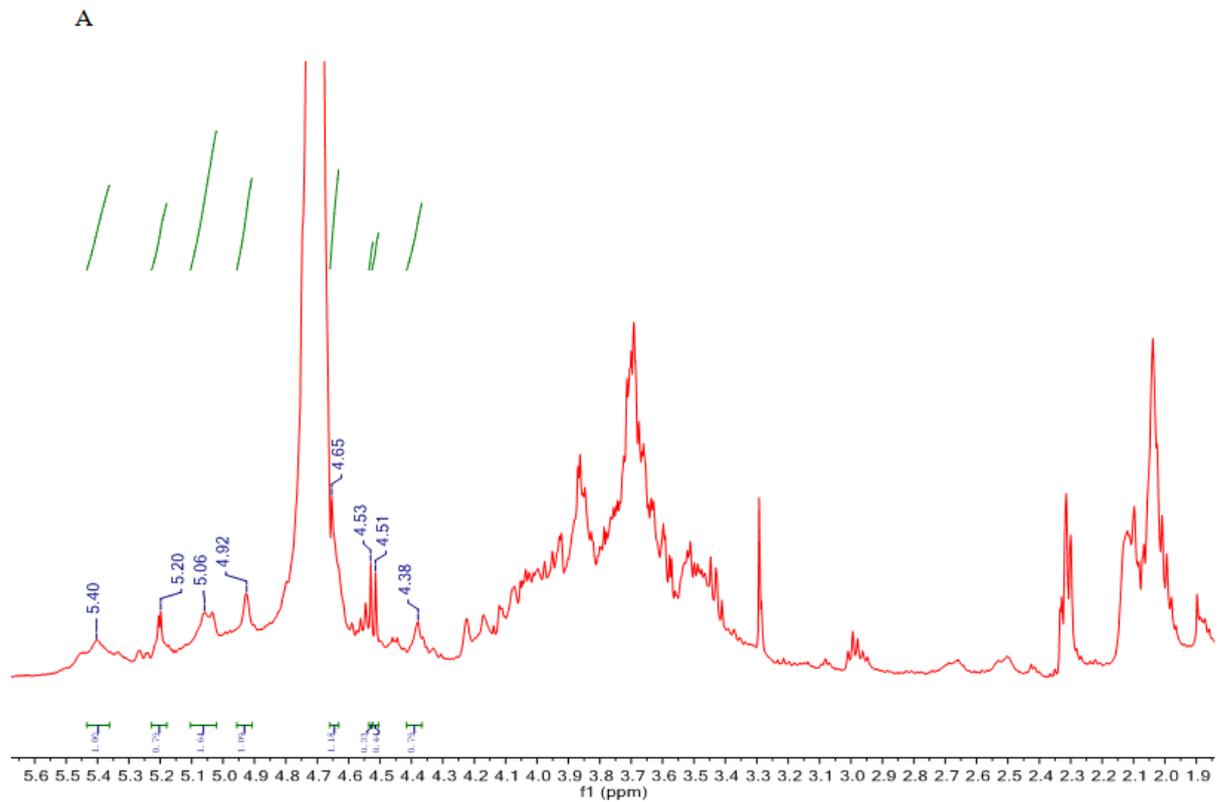
Figure 2A shows the  $^1\text{H}$  NMR spectrum of CP. The peaks of the anomeric hydrogens of CP were at the chemical shifts of 5.40 ppm, 5.20 ppm, 5.06 ppm, 4.92 ppm, 4.65 ppm, 4.53 ppm, 4.51 ppm, 4.38 ppm, 2.31 ppm, and 2.04 ppm. It can be seen that CP contained galactose (4.38 ppm, 4.51 ppm, 4.53 ppm), mannose (4.65 ppm, 4.92 ppm, 5.20 ppm, 5.40 ppm), glucose (4.65 ppm, 5.06 ppm), and xylose (4.65 ppm) [28]. The peak area ratio of xylose, mannose, glucose, and galactose was 1.0: 3.4: 2.3: 1.3, consistent with the monosaccharide composition found from the GC results. In the  $^{13}\text{C}$  NMR spectra of CP (Figure 2B), the peaks of CP were at 189.87 ppm, 174.47 ppm, 172.84 ppm, 129.37 ppm, 104.31 ppm, 101.50 ppm, 98.06 ppm, 96.45 ppm, 92.29 ppm, 33.12 ppm, 26.78 ppm, and 20.43 ppm. At 160-180 ppm the peaks must be assigned to the carboxylic carbon nuclei of the glycuronic acid or its methyl esters. The peak at 129 ppm is at far lower field than the anomeric region. This signal is originated from  $^{13}\text{C}$  of aromatic ring or conjugated dienes. The main peaks of the anomeric carbons were chemical shifts 104.31 ppm, 101.50 ppm, 98.06 ppm, 96.45 ppm, and 92.29 ppm [29]. The resonance region of carbon (C2, C3, C4, C5, C6) on other sites of the sugar ring were found at 60.8 - 75.2 ppm while 104.31 ppm was the characteristic absorption peak of glucose.

Figure 2C shows the  $^1\text{H}$  NMR spectra of PP, with the shift of anomeric hydrogens present at 6.05 ppm, 5.25 ppm, 5.18 ppm, 5.08 ppm, 5.05 ppm, 4.97 ppm, 4.89 ppm, 4.76 ppm, 4.66 ppm, 4.53 ppm, 4.43 ppm, and 4.34 ppm. Clearly, galactose (4.43 ppm, 4.89 ppm), mannose (4.66 ppm, 5.08 ppm), arabinose (5.25 ppm), glucose (4.76 ppm, 4.97 ppm, 5.18 ppm), and glucopyranoside methyl glycoside (4.34 ppm, 5.05 ppm) were present. The peak area ratio of arabinose, mannose, glucose, and galactose was 1.0: 8.5: 7.4: 6.9. By observing the carbon spectrum (Figure 2D), we can conclude that glucuronic acid [30] (170.86 ppm), glucose (99.47 ppm), galactose (109.22 ppm), and fucose (16.58) were present. The anomeric carbon nuclei absorb at the region 92-112 ppm.

From the  $^1\text{H}$  NMR of SP (Figure 2E), we can see that the peaks were at 5.33 ppm, 5.17 ppm, 5.16 ppm, 4.93 ppm, 4.58 ppm, and 4.57 ppm. Mannose (4.93 ppm, 5.17 ppm) and glucose (5.16 ppm) were clearly observed. The peak area ratio of mannose and glucose was 3.4: 1.0. This conclusion is consistent with the monosaccharide composition results, indicating that the mannose content in SP is much higher than the glucose content. The signal peaks of chemical shifts 3.0 - 4.3 ppm were glycol cyclic proton signals. As shown in Figure 2F, NMR signals were mainly concentrated between 60 ppm and 96 ppm. The main peaks of carbon signals were chemical shifts 95.90 ppm and 92.08 ppm, indicating that the polysaccharide was mainly composed of two monosaccharides, consistent with the monosaccharide determination results.



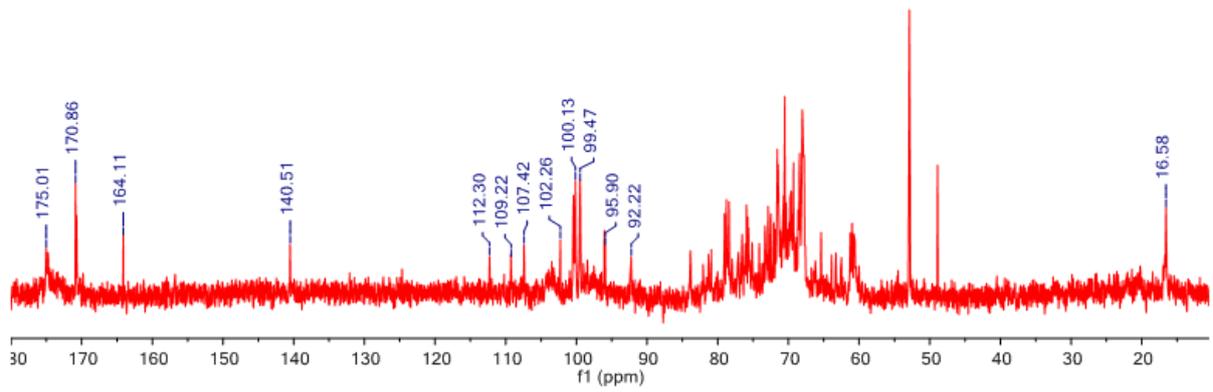
**Figure 1.** (A) Molecular weight spectra of the polysaccharides; (B) Monosaccharide compositions of polysaccharides based on GC - MS spectra; (C) FTIR spectra of polysaccharides (Chen *et al*)



**Figure 2.** (A) NMR spectra of CP, PP, and SP (Chen *et al*)



D



E

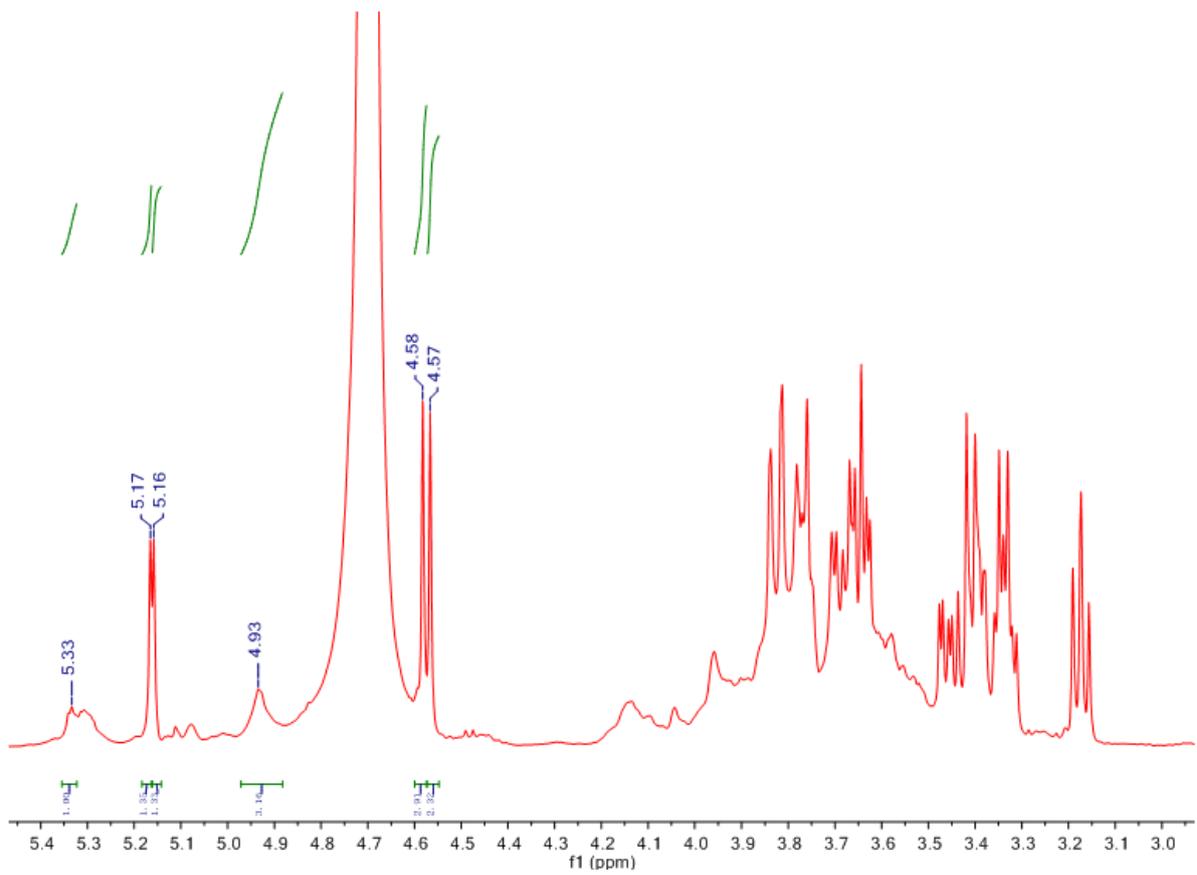


Figure 2D-E. NMR spectra of CP, PP, and SP (Chen *et al*)

F

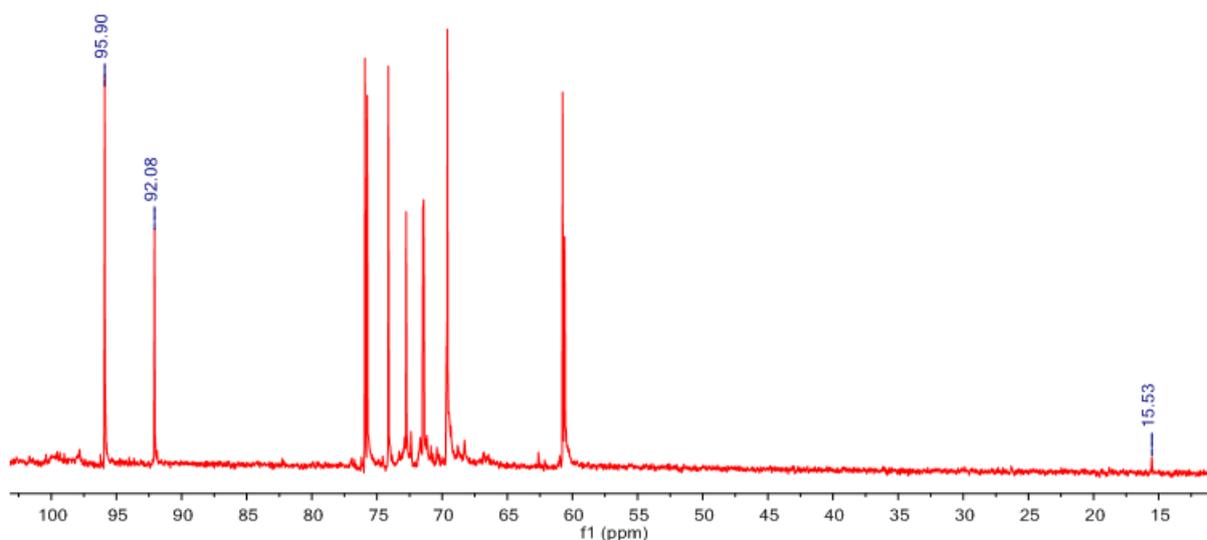


Figure 2F. NMR spectra of CP, PP, and SP (Chen *et al*)

### 3.6. DPPH Radical Scavenging Activity

The DPPH radical scavenging is based on the hydrogen - donating ability of antioxidants. The results of the DPPH radical scavenging activity of the three polysaccharides are shown in Figure 3A. Vc, a positive control, had good antioxidant properties, which increased rapidly at 0 - 0.4 mg/mL, and then leveled off. The antioxidant activities of the polysaccharides were lower than Vc at the same concentration, but they were concentration - dependent across a concentration range of 0 - 2.0 mg/mL. The  $IC_{50}$  values of CP, PP, and SP were 1.06, 1.23, and 1.35 mg/mL, respectively. When the polysaccharide concentration was 2.0 mg/mL, the CP, PP, and SP maximum scavenging rates were  $79.0 \pm 0.2\%$ ,  $74.3 \pm 0.3\%$ , and  $71.0 \pm 0.2\%$ , respectively. When the concentration increased from 0.8 to 2.0 mg/mL, CP had a significantly higher scavenging effect on DPPH radicals than the other polysaccharides ( $P < 0.05$ ). However, the difference between the DPPH radical suppression of PP and SP was nonsignificant at all concentrations ( $P > 0.05$ ).

### 3.7. Superoxide Radical Scavenging Activity

The superoxide radical scavenging activities of polysaccharides are shown in Figure 3B. Vc had the same trend of superoxide radical suppression as the DPPH radical, in a concentration-dependent manner at low concentrations of 0 - 0.4 mg/mL. The scavenging rates of CP were highest among all three polysaccharides with an  $IC_{50}$  value of 1.06 mg/mL. The  $IC_{50}$  values of PP and SP

were 1.21 mg/mL and 1.27 mg/mL, respectively. At a concentration of 2.0 mg/mL, the scavenging activities of CP, PP, and SP reached their highest values, at  $76.0 \pm 2.8\%$ ,  $71.3 \pm 0.1\%$ , and  $68.0 \pm 0.4\%$ , respectively. PP and SP exhibited no significant difference in scavenging activity against superoxide radicals ( $P < 0.05$ ).

### 3.8. Hydroxyl Radical Suppression

The hydroxyl radical scavenging activities of the three polysaccharides are shown in Figure 3C. It is clear that Vc was the strongest as a positive control. The activities of the three polysaccharides maintained an almost linear concentration-dependent growth. The  $IC_{50}$  values of CP, PP, and SP were 0.98, 1.25, and 1.45 mg/mL, respectively. When the concentration of polysaccharide was between 0.4 mg/mL to 1.6 mg/mL, the clearance rate of CP was significantly ( $P < 0.05$ ) higher than those of both SP and PP. At a concentration of 2.0 mg/mL, the scavenging activities of CP, PP, and SP were  $65.0 \pm 0.4\%$ ,  $61.3 \pm 0.2\%$ , and  $58.0 \pm 0.3\%$ , respectively; these values were not significantly different ( $P > 0.05$ ). These results indicate that the three polysaccharides contained high radical scavenging activity, and among them CP was the most potent.

### 3.9. Inhibition of CDs Formation

The formation of CDs is an important indicator of dynamic changes in LDL lipid modification during the delay, proliferation, and early degradation stages [17].

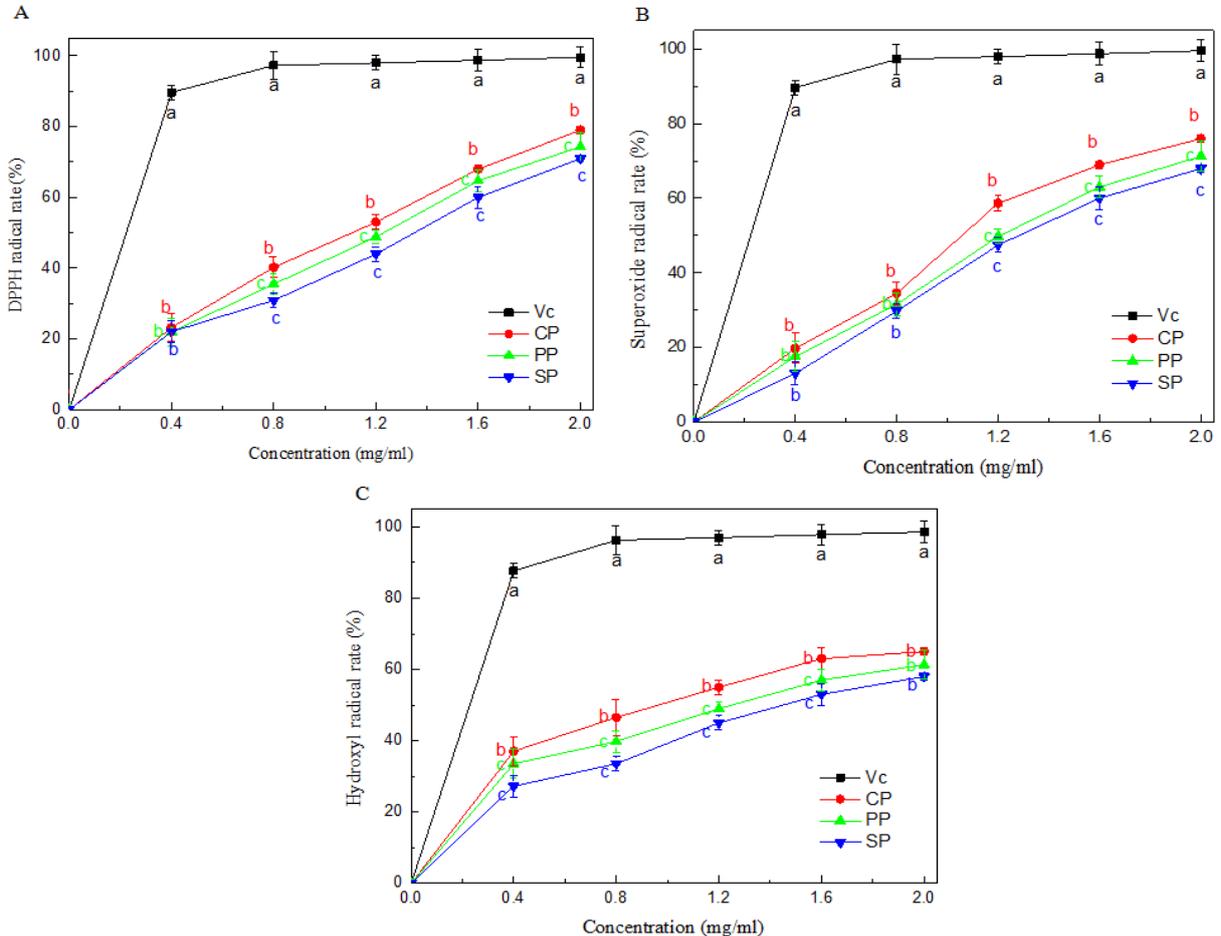
Based on the above radical scavenging activity results, we further studied whether polysaccharides could have inhibited the oxidation of LDL. Figure 4A shows the changes in the absorption values (CD formation) of the control and experimental groups over time. There were significant differences in the absorption values of the five reaction systems over the whole observation period ( $P < 0.05$ ). The absorbance changes in the blank control and polysaccharide groups were lower than those in the oxygen-promoting control group, indicating that the polysaccharides effectively delayed the formation of CD in LDL oxidation induced by  $\text{CuSO}_4$ . When the reaction was conducted for 40 min, the degree of change in the absorption values of the control group, the oxygen-promoting group, CP, PP, and SP were 0.018, 0.102, 0.038, 0.068, and 0.087, respectively. When the reaction was conducted for 240 min, the values for the last four groups were 0.724, 0.438, 0.489, and 0.532, respectively. These results indicate that polysaccharides significantly reduce the LDL oxidation rate. Among these groups, CP had the most obvious effect on reducing LDL oxidation rate, consistent with the radical scavenging activities of the polysaccharides. One explanation for this may be that polysaccharides could remove the hydrogen peroxide radicals generated from the LDL-induced weak oxidation modification process, inhibit the lipid peroxidation caused by the free radical chain reaction and the formation of CDs, and finally inhibit the weak oxidation of LDL [31].

LDL is a kind of lipoprotein particle that carries cholesterol into peripheral tissue cells. It can be oxidized

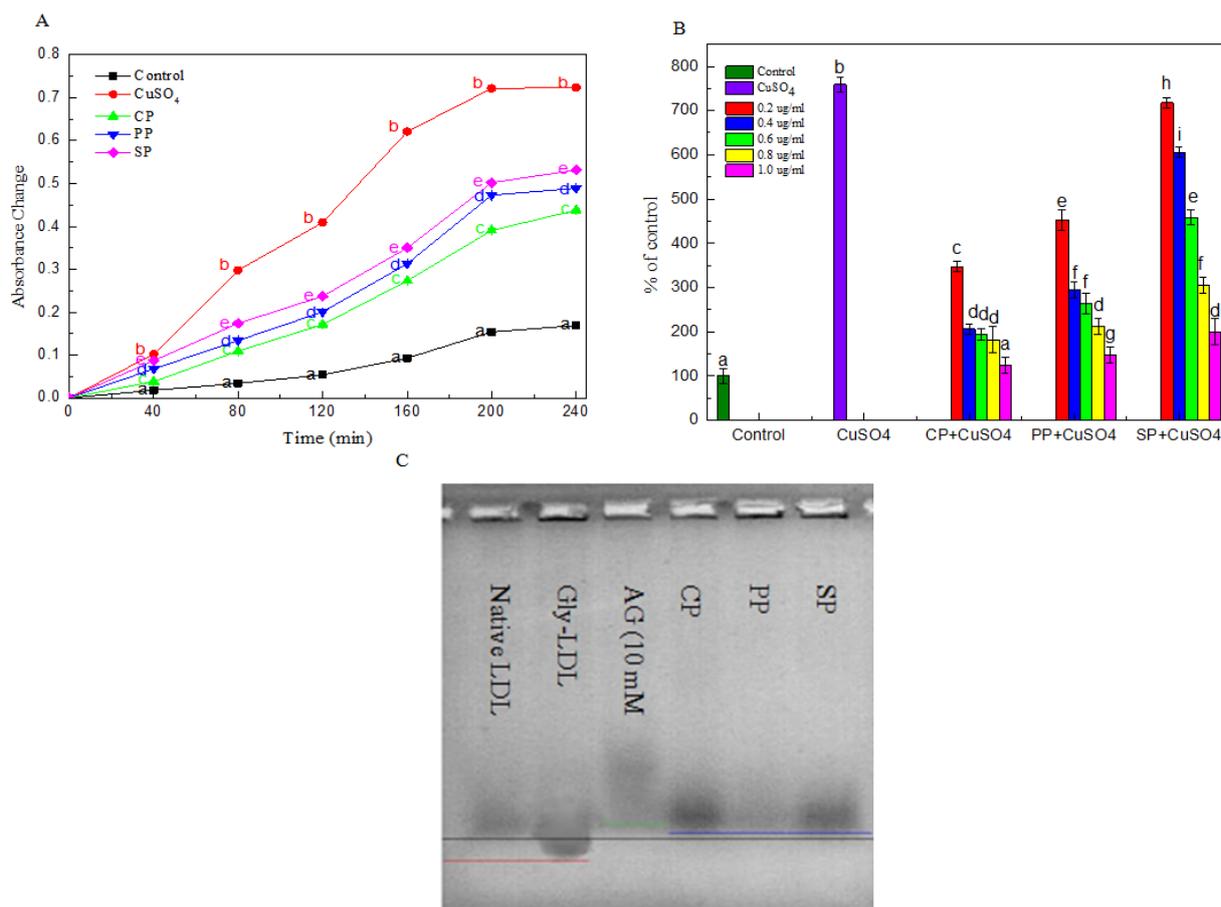
to ox - LDL. When LDL, particularly ox - LDL, is excessive, the cholesterol it carries accumulates on the arterial wall, which can cause arteriosclerosis. The role of polysaccharides in the prevention of LDL oxidation has not been reported to date; our results show that polysaccharides can inhibit the oxidation rate of CDs, which has important theoretical significance for the prevention of atherosclerosis.

### 3.10. Inhibition of TBARS Production

As LDL lipid peroxidation progresses, the LDL oxidation modification enters the degradation phase and leads to the degradation of fatty acids hydroperoxides produced during the proliferation phase, forming a large number of toxic aldehydes. These aldehydes subsequently react with TBA to produce pink materials with a characteristic absorption peak at 532 nm [32]. We further determined the delayed effects of polysaccharides on LDL lipid peroxidation modification during the degradation stage by measuring TBARS production (Figure 4B). CP exhibited the best inhibitory effect at a concentration of 1.0  $\mu\text{g/mL}$ , and the TBARS productions for CP, PP, and SP were 16.28%, 19.38%, and 26.36% of the  $\text{CuSO}_4$  group, respectively ( $P > 0.05$ ). Overall, the inhibitory effects of the three polysaccharides increased with increasing concentration. These results suggest that polysaccharides can further inhibit the oxidation of LDL by inhibiting the production of TBARS.



**Figure 3.** Scavenging percentage of polysaccharides to A) DPPH, B) superoxide, and C) hydroxyl radicals (Chen *et al*)



**Figure 4.** Inhibitory effects of three polysaccharides on A) CD formation, B) TBARS production, and C) LDL glycation evaluated by agarose gel electrophoresis (Chen *et al*)

### 3.11. Inhibition of LDL Glycation

LDL is an important carrier of cholesterol in the body. When blood sugar levels in the human body are too high, the side chain amino group in the LDL protein is prone to a glycation reaction with glucose. During the early, middle, and late stages of glycation, stable final glycation products are produced. At the end of glycation, a high degree of glycated LDL can accumulate in the human body, which is a complication of diabetes. Therefore, the effects of polysaccharides on the glycation of LDL was further studied to explore whether polysaccharides can inhibit the oxidation of LDL in multiple ways.

As shown in Figure 4C, glyLDL exhibited the longest electrophoretic migration distance (5.2 cm), and AG had the shortest. Compared to glyLDL, the migration distances of the HDL treated with the three polysaccharide groups were all 4.7 cm, indicating that the polysaccharides inhibited LDL glycation. The bands of PP were thinner (less dark and thick) than those of CP and SP, indicating that PP had the strongest inhibitory effect on LDL glycation. This may be related to the lower glucose content of PP among the three polysaccharides.

Many studies have shown that polysaccharides have strong antioxidant properties. In this study, CP, PP, and SP showed strong antioxidant activity and also suppressed the oxidation and glycation of LDL. CP and PP were the most potent, which we attribute to the glucuronic acid contained in these two polysaccharides. A previous study showed that the antioxidant activity of polysaccharides

was associated with uronic acid content. CP also had the strongest antioxidant activity among the three polysaccharides, which could be explained by the total sugar content. The glycation of LDL is caused by the reaction of side chain amino groups of LDL with glucose. Gel electrophoresis analyses showed that PP produced less glycation products, indicating that it could more efficiently inhibit the glycation reaction of LDL, which might originate from the low glucose content of PP.

## 4. Conclusion

This study demonstrated that CP, PP, and SP had strong radical scavenging activities and potently inhibited the formation of LDL CDs and subsequent TBARS, as well as LDL glycation. This study is the first to demonstrate the potent inhibitory effects of polysaccharides on LDL oxidation and glycation, which is associated with some chronic diseases, including atherosclerosis and diabetes. Hence, some Chinese traditional foods such as Chinese yam, pumpkin, and shiitake could provide a rich source of functional polysaccharides.

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## Statement of Competing Interests

The authors have declared no conflict of interest.

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