Research Article

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Chemical characteristics and antioxidant activities of polysaccharide purified from the seeds of *Plantago asiatica* L.

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Abstract

BACKGROUND: A water-soluble polysaccharide from the seeds of *Plantago asiatica* L. (*P. asiatica* L. polysaccharide, PLP) was extracted with hot water and purified by gel filtration chromatography. The chemical characteristics of PLP were determined by high-performance gel permeation chromatography (HPGPC) and Fourier transform infrared (FTIR) spectroscopy. In addition, the antioxidant activities of PLP *in vitro* were evaluated using various test systems, including scavenging of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals, scavenging of superoxide radicals generated by 1,2,3-phentriol autoxidation, scavenging of hydroxyl radicals and inhibition of lipid peroxidation.

RESULTS: The molecular weight of PLP was determined by HPGPC to be about 1894 kDa. PLP contained 29.2 g kg^{-1} protein and 145.8 g kg^{-1} uronic acid. The FTIR spectrum of PLP also revealed typical characteristics of a polysaccharide containing protein and uronic acid. Moreover, the results showed that PLP possessed antioxidant activities, but lower than those of ascorbic acid.

CONCLUSION: PLP is an acid protein-bound polysaccharide of high molecular weight, but its structure needs further study. The present results suggest that PLP could potentially be used as a natural antioxidant.

Keywords: *Plantago asiatica* L.; polysaccharide; chemical characteristics; antioxidant activities

INTRODUCTION

It is generally accepted that reactive oxygen species such as superoxide, hydroxyl and peroxyl radicals are generated during oxidative stress. Oxidant by-products of normal metabolism cause extensive damage to DNA, protein and lipid.[1] Free radical mechanisms have been implicated in the pathology of several human diseases, including cancer, atherosclerosis, malaria, rheumatoid arthritis and neurodegenerative diseases.[2] Polysaccharides and their conjugates, used in the food industry and in medicine for a long time, have attracted much attention in recent years owing to their biological activities. Many polysaccharides have antioxidant activities and could potentially be used as natural antioxidants.[3–7]

Plants of the *Plantago* family are used in folk medicine throughout the world.[8] Some have been studied widely, such as *Plantago afra* L., *Plantago psyllium* L., *Plantago ovata* Forsk. (isabgul), *Plantago indica* L. and *Plantago major* L.[9] A petroleum extract of *Plantago asiatica* L. was found to have a pronounced antidepressant effect.[10] A gel extracted from psyllium seed husk promoted laxation as a lubricant in humans.[11] Recently, the *Plantago* family has attracted much attention owing to its antispasmodic,[12] anticomplementary,[13] hyperglycaemia-reducing,[14] nitric oxide-inducing and tumour necrosis factor activities.[15] Our recent studies showed that polysaccharide isolated from the seeds of *P. asiatica* L. could induce maturation of murine dendrite cells[16,17] and promote defaecation.[18] But there have been few reports on its antioxidant effects.

In the present study, polysaccharide isolated and purified from the seeds of *P. asiatica* L. (*P. asiatica* L. polysaccharide, PLP) was screened for its chemical characteristics and antioxidant activities *in vitro*. The antioxidant activities of PLP were evaluated by various tests, including scavenging of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals, scavenging of superoxide radicals generated by 1,2,3-phentriol autoxidation, scavenging of hydroxyl radicals and inhibition of lipid peroxidation, and compared with the abilities of *P. asiatica* L. water extract and *P. asiatica* L. crude polysaccharide to scavenge free radicals. Ascorbic acid was used as a standard control.

MATERIALS AND METHODS

Materials

The seeds of *P. asiatica* L. were purchased from Ji'an, Jiangxi, China and dried in the sun before use.

DPPH was purchased from Sigma Chemical Co. (St Louis, MO, USA). Dextran standards (T-2000, T-70, T-40 and T-10 of molecular weight 2000, 70, 40 and 10 kDa respectively) and Sephacryl™ S-400 HR were obtained from Pharmacia (Uppsala, Sweden). All other

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reagents used were analytical grade and purchased from Shanghai Chemicals and Reagents Co. (Shanghai, China).

Isolation and purification of polysaccharide fraction

The seeds of *P. asiatica* L. (50 g) were defatted with 800 g kg^{-1} ethanol at room temperature for 24 h under stirring, then extracted with 500 mL of doubly distilled water at 100 °C for 2 h. The residue was re-extracted. The combined aqueous extract, a gel of high viscosity, was centrifuged (4800 × *g*, 10 min), prefiltered through a cotton cloth bag and concentrated in a rotary evaporator under reduced pressure at 55 °C to yield *P. asiatica* L. water extract. The filtrate was mixed with 1.5 g L^{-1} papain and heated in water at 60 °C for 2 h. The solution was deproteinised with 1/3 volume of $CHCl_3$/*n*-BuOH (4 : 1 v/v) at least three times according to the Sevag method[19] with some modifications. The resulting aqueous solution was extensively dialysed against doubly distilled water for 72 h and precipitated by adding 4 volumes of anhydrous ethanol at 4 °C for 12 h. After centrifugation the precipitate was washed with anhydrous ethanol, dissolved in water and lyophilised to yield *P. asiatica* L. crude polysaccharide.

The crude polysaccharide (3 mL of 1 mg mL^{-1} solution in 15 mmol L^{-1} NaCl) was applied to a Sephacryl™ S-400 HR column (2.6 cm × 60 cm, molecular weight fractionation range 10–2000 kDa for dextrans) coupled to an ÄKTA™ purifier system (Amersham Pharmacia Biotech, Uppsala, Sweden). The column was eluted with 15 mmol L^{-1} NaCl at a flow rate of 1.5 mL min^{-1}. Fractions of 5 mL were collected and monitored by the phenol/sulfuric acid method.[20] Three tubes of fractions were collected according to the elution curve. The purified *P. asiatica* L. polysaccharide (PLP) was concentrated, dialysed and lyophilised. Gel filtration chromatography in a Waters high-performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA) equipped with a Waters Ultrahydrogel™ linear column (7.8 mm × 300 mm) was used to test the homogeneity of the purified polysaccharide.

Determination of molecular weight

The molecular weight of PLP was determined in a Waters HPLC system (UK6 injector and 515 HPLC pump) equipped with a Waters Ultrahydrogel™ linear column (7.8 mm × 300 mm), a Waters 410 for refractive index (RI) detection (for refractive index (RI) detection) and an ultraviolet (UV) detector and connected in series with a Millennium 32 workstation (Waters, Milford, MA, USA). A sample solution (20 µL of 2 mg mL^{-1} polysaccharide in distilled water) was injected in each run, with distilled water as the mobile phase at a flow rate of 0.6 mL min^{-1}. The molecular weight of PLP was determined by high-performance gel permeation chromatography (HPGPC). Dextran standards and glucose were used to calibrate the column and establish a standard curve.

Fourier transform infrared (FTIR) spectroscopy

IR spectra of PLP were recorded on a Thermo Nicolet 5700 FTIR spectrometer (Thermo Electron, Madison, WI, USA) in the wavenumber range 4000–400 cm^{-1} at a resolution of 4 cm^{-1} with 128 co-added scans, using the KBr disc method.

Analysis of protein and uronic acid contents

Protein content was estimated by photometric assay with bovine serum albumin as a standard.[21] Uronic acid content was determined by measurement of the absorbance at 525 nm using the *m*-hydroxybiphenyl photometric procedure with D-glucuronic acid as a standard.[22]

Scavenging of DPPH radicals

The DPPH radical-scavenging test was performed according to the method of Chen *et al*.[6] with minor modification. A 0.2 mmol L^{-1} solution of DPPH in 950 g kg^{-1} ethanol was prepared daily before UV measurements. Sample solutions (2 mL) of different concentrations were mixed with 2 mL of freshly prepared DPPH solution and 2 mL of 950 g kg^{-1} ethanol. The mixture was shaken vigorously and left to stand for 30 min, after which its absorbance at 525 nm was determined against a blank.

The ability to scavenge DPPH radicals was calculated as follows:

$$\text{scavenging effect (\%)} = [1 - (A_i - A_j)/A_c] \times 100$$

where A_i is the absorbance of the sample mixed with DPPH solution, A_j is the absorbance of the polysaccharide without DPPH solution and A_c is the absorbance of DPPH solution.

Scavenging of superoxide radicals generated by 1,2,3-phentriol autoxidation

This assay was performed using the method of Marklund and Marklund[23] with some modifications. Tris-HCl buffer (pH 8.2, 4.5 mL) was mixed with water (3.2 mL) and sample solutions (1 mL) of different concentrations and incubated in a water bath at 25 °C for 20 min. 1,2,3-Phentriol (0.3 mL, 50 mmol L^{-1}) was then added and the mixture was shaken rapidly. The absorbance of the mixture at 325 nm was recorded every 10 s and a slope was calculated according to the results as absorbance min^{-1}. The ability to scavenge superoxide radicals generated by 1,2,3-phentriol autoxidation was calculated as follows:

$$\text{scavenging effect (\%)} = (1 - \text{slope of polysaccharide/slope of control}) \times 100$$

Scavenging of hydroxyl radicals

The test was performed using the method of Li *et al*.[24] with some modifications. First, 1 mL of 0.4 mol L^{-1} phosphate buffer solution (PBS, pH 7.4), 1 mL of 2.5 mmol L^{-1} 1,10-phenanthroline solution,[25] 1 mL of PLP solution, 1 mL of 2.5 mmol L^{-1} ferrous sulfate ($FeSO_4$) solution and 0.5 mL of 20 mmol L^{-1} hydrogen peroxide (H_2O_2) were mixed in a tube. The mixture was then incubated at 37 °C for 1 h, immediately after which its absorbance at 536 nm was determined. In the control group, samples were replaced by solvent. The ability to scavenge hydroxyl radicals was calculated as follows:

$$\text{scavenging effect (\%)} = [(F_2 - F_1)/(F_0 - F_1)] \times 100$$

where F_1 is the absorbance of the control, F_2 is the absorbance of the polysaccharide and F_0 is the absorbance of the solution without polysaccharide and H_2O_2.

Inhibition of lipid peroxidation

This test was conducted according to the method of Zhang *et al*.[26] with some modifications. Briefly, an equal volume of egg yolk was added to 0.1 mol L^{-1} PBS (pH 7.45). The mixture was stirred magnetically for 10 min and then diluted with 24 volumes of PBS. The yolk homogenate (1 mL), sample (0.5 mL), PBS (1 mL) and 25 mmol L^{-1} $FeSO_4$ (1 mL) were mixed in a tube and shaken at 37 °C for 15 min. The reaction was stopped by the addition of

trichloroacetic acid and the mixture was centrifuged. Then 1 mL of 8 g L^{-1} thiobarbituric acid solution was added to 3 mL of the supernatant. This solution was heated at 10 °C for 10 min, after which its absorbance at 532 nm was measured. The ability to inhibit lipid peroxidation was calculated as follows:

$$\text{inhibitory effect (\%)} = [(B_0 - B)/B_0] \times 100$$

where B_0 is the absorbance of the control and B is the absorbance in the presence of polysaccharide.

Statistical analysis

Data are presented as mean ± standard deviation (SD) of three determinations and were subjected to Student's *t* test. Differences were considered statistically significant at $P < 0.05$, while $P < 0.01$ was considered highly significant. All statistical analyses were performed using SPSS 11.5 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Isolation and purification of polysaccharide

The fractions (5 mL each) separated on the Sephacryl™ S-400 HR column were collected in tubes by the automated fraction collector. Figure 1 shows the chromatograms of the polysaccharide recorded in the ÄKTA™ purifier system with RI detector. Polysaccharide from tubes 9–11 was collected, concentrated, dialysed and lyophilised to yield PLP. PLP was further eluted as a single and symmetrically sharp peak with a molecular weight based on column calibration of 1894 kDa from HPGPC (Figs 2 and 3), in which the protein and sugar peaks appeared at the same time, indicating that PLP was a homogeneous protein-bound polysaccharide, since the Sevag method was repeated several times to remove free protein. The calibration curve prepared with standard dextrans and glucose is shown in Fig. 4. The protein content of PLP was 29.2 g kg^{-1}, which again showed that PLP was a protein-bound polysaccharide. The uronic acid content of PLP was 145.8 g kg^{-1}.

FTIR spectroscopy

There are two types of end carbon–glucoside bonds, α and β, that can be distinguished by IR. In IR spectra the α-type C–H bond has an absorption peak near 844 cm^{-1}, while that of the β-type C–H bond is near 891 cm^{-1}.[27] Fig. 5 shows the FTIR spectrum of PLP. It featured a strong absorption at 1046.1 cm^{-1} due to stretching vibration of the pyranose ring. In the anomeric region (950-700 cm^{-1}) the spectrum exhibited the characteristic absorption at 808.3 cm^{-1} due to the presence of mannose.[28] A characteristic absorption at 899.7 cm^{-1} indicating the β configuration of the sugar units was also observed, but there was no absorption near 844 cm^{-1} corresponding to the α configuration. The absorption at 1612.6 cm^{-1} due to stretching vibration of the carbonyl bond of the amide group and bending vibration of the N–H bond showed the presence of protein, while the absorption band at 1730.2 cm^{-1} indicated the presence of uronic acid. The bands in the region of 3412.9 cm^{-1} were due to hydroxyl stretching vibration of the polysaccharide. The bands in the region of 2931.2 cm^{-1} were due to C–H stretching vibration. These observations further confirmed that PLP was a polysaccharide containing protein and uronic acid.

Scavenging of DPPH radicals

On interacting with DPPH, antioxidants transfer either an electron or a hydrogen atom to DPPH, thus neutralising its free radical character.[29] The DPPH radical-scavenging ability of PLP is shown in Fig. 6. The scavenging effects of water extract, crude polysaccharide, PLP and ascorbic acid on DPPH radicals were 74.3, 50.8, 71.2 and 94.7% respectively at a concentration of 1 mg mL^{-1}. However, the ability to scavenge DPPH radicals did not increase at concentrations above 1 mg mL^{-1}. The reason for this could be that polysaccharide was precipitated, since nearly 50 g kg^{-1} ethanol was present in the solution. The concentration of an antioxidant required to decrease the initial DPPH concentration by 50% (IC_{50}) is a parameter widely used to measure antioxidant activity. The lower the IC_{50} value, the higher is the antioxidant

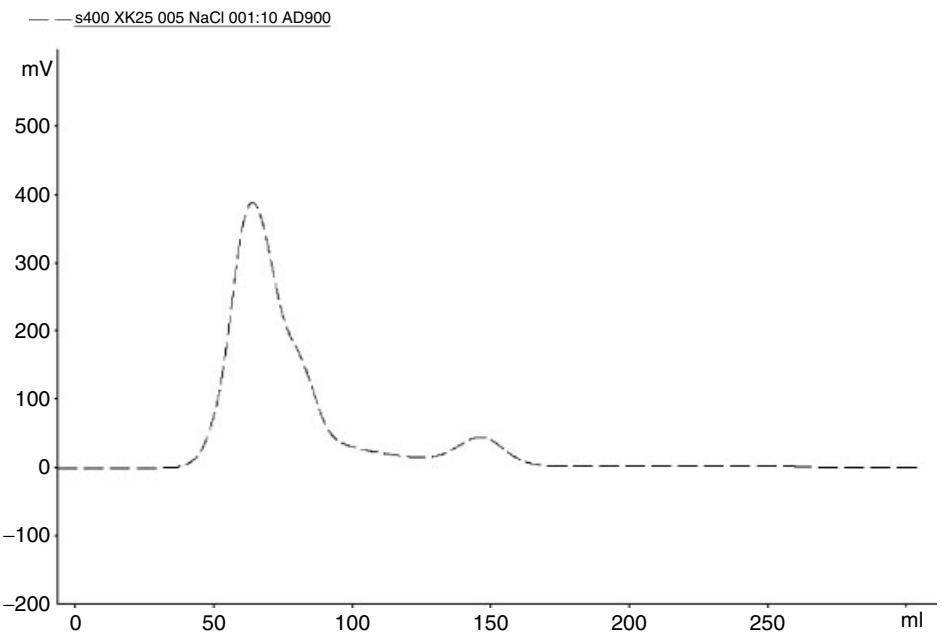

Figure 1. RI chromatogram of polysaccharide purified by ÄKTA purifier system. Eluent: 0.15 mmol L^{-1} NaCl, flow rate: 1.5 mL min^{-1}.

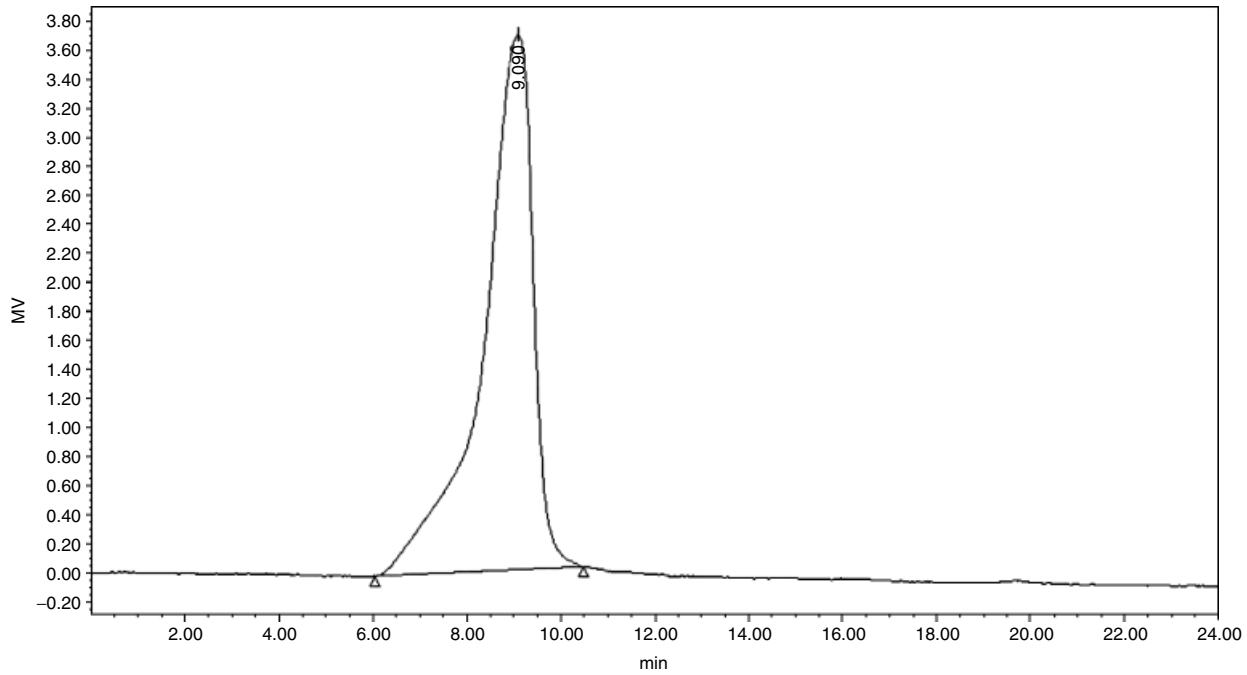

Figure 2. HPGPC chromatogram of PLP with RI detection on Ultrahydrogel™ linear column (7.8 mm × 300 mm) with distilled water at 0.6 mL min^{-1}.

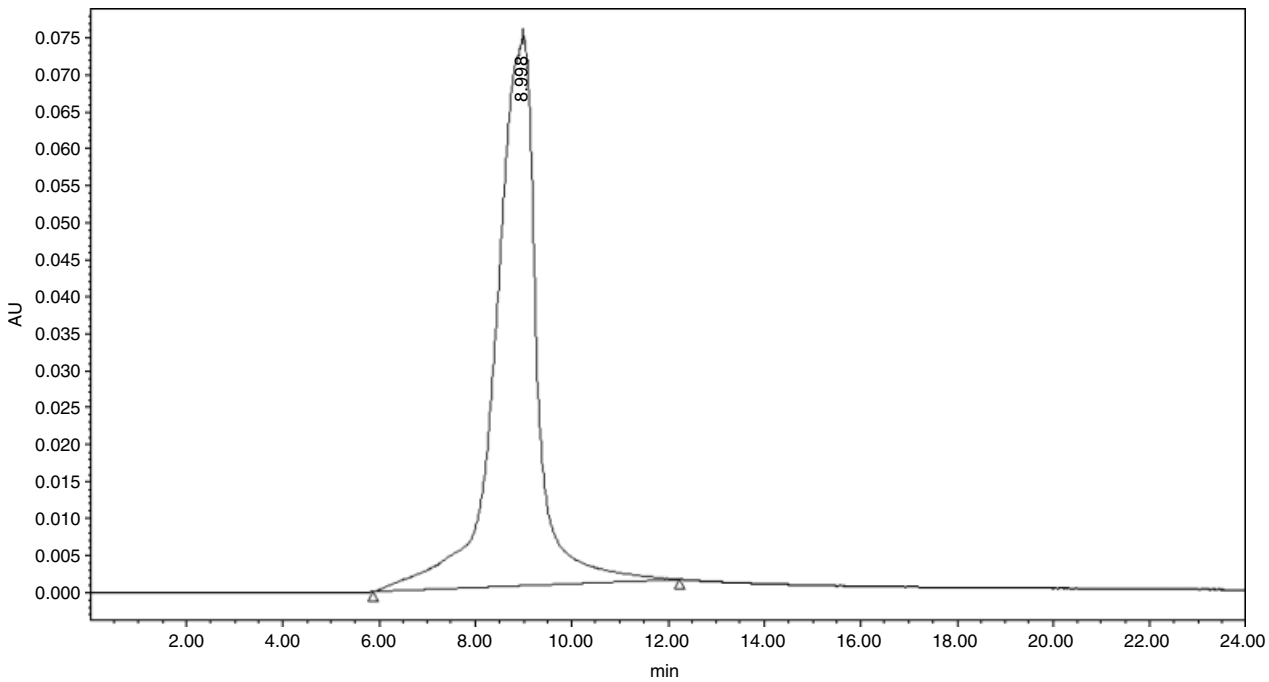

Figure 3. HPGPC chromatogram of PLP with UV detection (280 nm) on Ultrahydrogel™ linear column (7.8 mm × 300 mm) with distilled water at 0.6 mL min^{-1}.

activity. Water extract (IC_{50} 0.42 mg mL^{-1}) exhibited a higher level of radical-scavenging activity than crude polysaccharide (IC_{50} 1.30 mg mL^{-1}), as seen in Table 1. The reason for this could be that the water extract was richer in antioxidant components such as proteins, amino acids, peptides, ascorbic acid, thiamine, nucleotides, tocopherol, organic acids and microelements, which would contribute to its antioxidant properties. However, the ability of PLP to scavenge DPPH radicals (IC_{50} 0.44 mg mL^{-1}) was stronger than that of crude polysaccharide. Further investigation is needed to explain why PLP showed a higher antioxidant effect than crude polysaccharide.

Scavenging of superoxide radicals generated by 1,2,3-phentriol autoxidation

The superoxide radical is one of the precursors of singlet oxygen and the hydroxyl radical. It indirectly initiates lipid

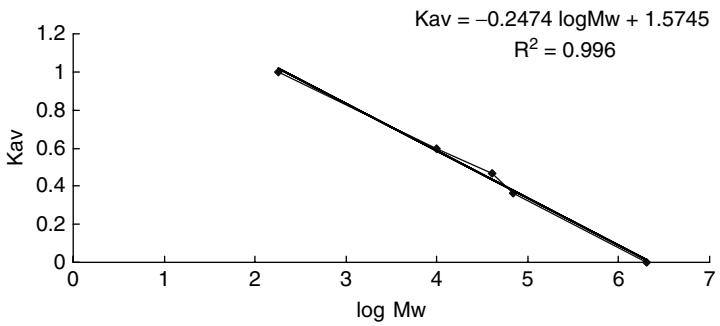

Figure 4. GPC calibration curve of dextran standards (T-2000, T-70, T-40 and T-10) and glucose.

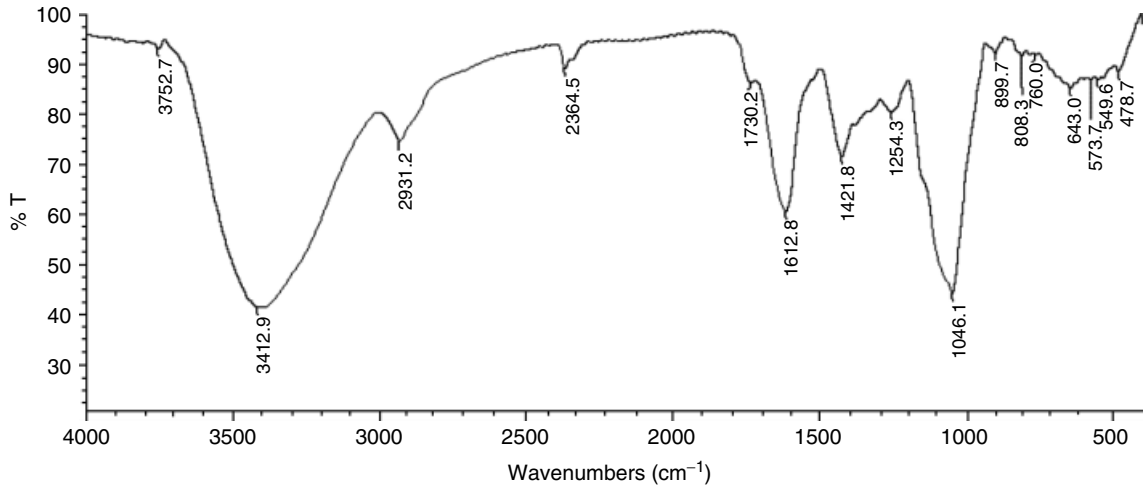

Figure 5. FTIR spectrum of polysaccharide (PLP) from *Plantago asiatica* L.

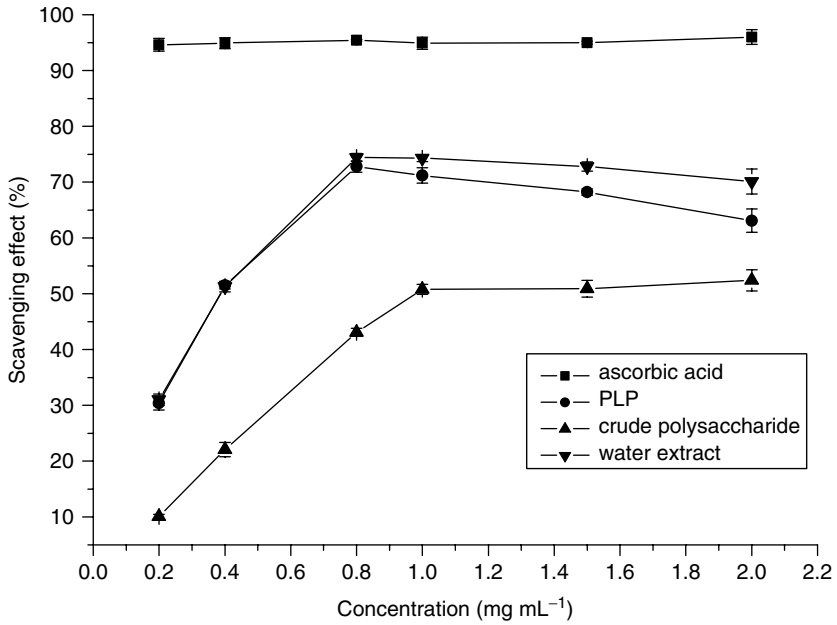

Figure 6. Scavenging effect of PLP on DPPH radicals compared with that of ascorbic acid (standard control), water extract and crude polysaccharide. Data are presented as mean ± SD ($n = 3$).

Table 1. IC_{50} values for antioxidant activity of different samples, calculated according to their ability to scavenge radicals or inhibit lipid peroxidation by 50%

	IC_{50} (mg mL^{-1})			
Test system	PLP	Crude polysaccharide	Water extract	Ascorbic acid
DPPH radical	0.44	1.30	0.42	<0.20
1,2,3-Phentriol	>8	>8	>8	<0.50
Hydroxyl radical	3.46	>4	>4	0.22
Lipid peroxidation	1.00	>2	>2	0.45

peroxidation. Superoxide radicals can be generated by pyrogallol autoxidation.[6,23]

Figure 7 shows the scavenging power of polysaccharide at different concentrations against superoxide radicals generated by 1,2,3-phentriol autoxidation. The scavenging effect of samples increased with increasing concentration but was lower than that of ascorbic acid (~100%). The scavenging power was 23.0% for PLP but only 11.8 and 9.7% for crude polysaccharide and water extract respectively at a concentration of 2 mg mL^{-1}. Compared with water extract and crude polysaccharide, the scavenging power of PLP was higher up to a concentration of 4 mg mL^{-1}. However, there were no statistical differences in scavenging power between PLP, crude polysaccharide and water extract at concentrations above 6 mg mL^{-1} ($P > 0.05$).

It was reported that polysaccharide from *Plantago* species could reduce glycaemic and lipid levels.[30–32] It was also suggested that antioxidant activity of polysaccharide was one of the mechanisms of hypoglycaemic activity.[33] The future challenge is to explore the effect of PLP on superoxide dismutase levels *in vivo* in order to study the role played by polysaccharide from *Plantago* species in the reduction of glycaemic levels by estimation of its antioxidant activity in alloxan-induced hyperglycaemic mice.

Scavenging of hydroxyl radicals

It is known that the hydroxyl radical is a powerful oxidant that can react with nearly all biological molecules such as proteins, lipids and carbohydrates[34] and that oxidative stress can mediate a wide variety of degenerative processes and diseases.[35–37] As seen in Fig. 8, PLP at concentrations above 1 mg mL^{-1} had the ability to scavenge hydroxyl radicals. The scavenging effect increased with increasing concentration. Compared with water extract and crude polysaccharide, the scavenging activity of PLP was the strongest at concentrations between 2 and 4 mg mL^{-1}. The differences in scavenging effect among water extract (17.6%), crude polysaccharide (37.6%) and PLP (63.2%) at a concentration of 4 mg mL^{-1} were highly significant ($P < 0.01$), but they were all weaker than ascorbic acid (99.0%).

Inhibition of lipid peroxidation

Egg yolk lipids undergo rapid non-enzymatic peroxidation when incubated in the presence of $FeSO_4$. Lipid peroxides are likely involved in numerous pathological events, including inflammation, metabolic disorders and cellular aging.[1,38] Fig. 9 presents the results for inhibition of lipid peroxidation by polysaccharide. All samples showed a dose–response relation. At a concentration of 2 mg mL^{-1}, PLP exhibited higher inhibitory activity (58.3%) than crude polysaccharide (30.7%) and water extract (36.6%) ($P < 0.01$), approaching that of the standard control (ascorbic acid). It was reported previously that the ability of polysaccharide isolated and purified from the leaves of *Plantago lanceolata* var. *libor* to inhibit peroxidation of soyabean lecithin liposomes was only 19.1%.[39]

The above results indicated that PLP had good antioxidant abilities and may be the main constituent exerting antioxidant functions in water extract and crude polysaccharide from *P. asiatica* L. The antioxidant mechanism may be due to the supply of hydrogen by PLP, which combines with radicals and itself forms stable radicals to terminate the radical chain reaction. The other

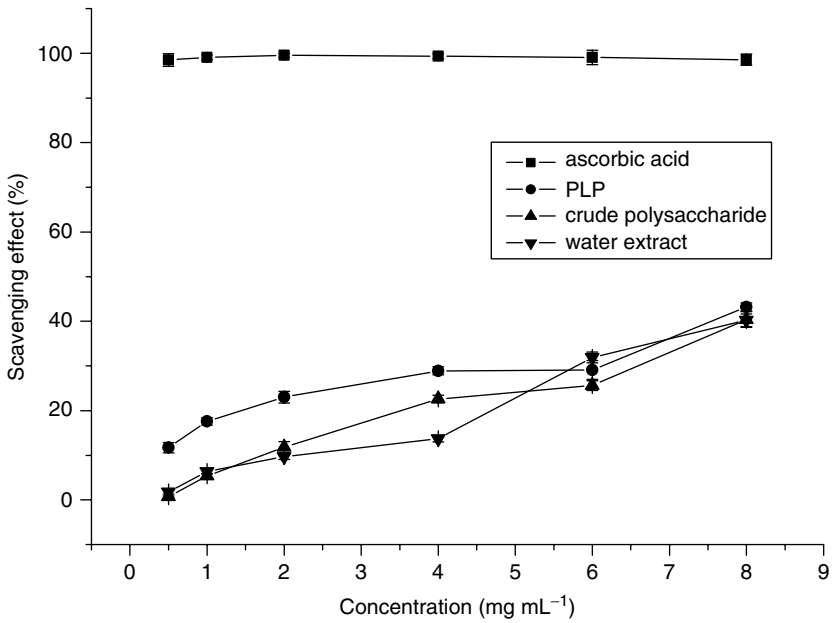

Figure 7. Scavenging effect of PLP on superoxide radicals generated by 1,2,3-phentriol autoxidation compared with that of ascorbic acid (standard control), water extract and crude polysaccharide. Data are presented as mean ± SD ($n = 3$).

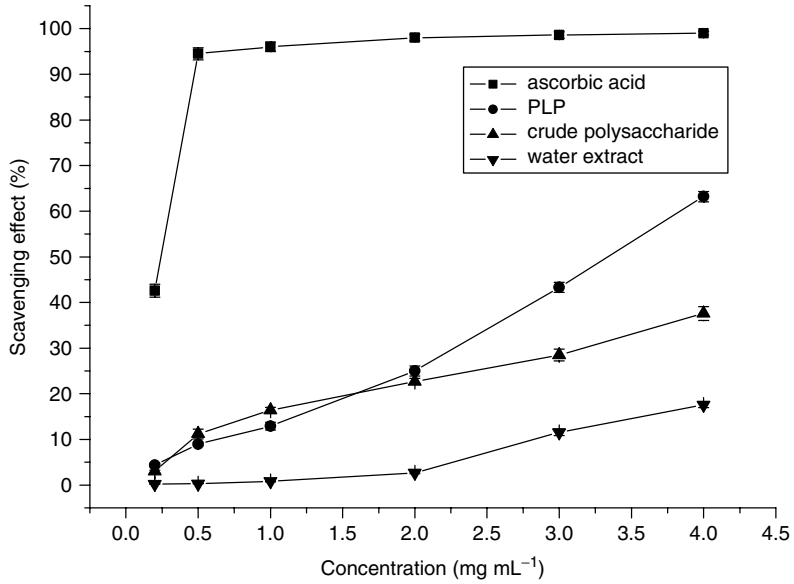

Figure 8. Scavenging effect of PLP on hydroxyl radicals compared with that of ascorbic acid (standard control), water extract and crude polysaccharide. Data are presented as mean ± SD ($n = 3$).

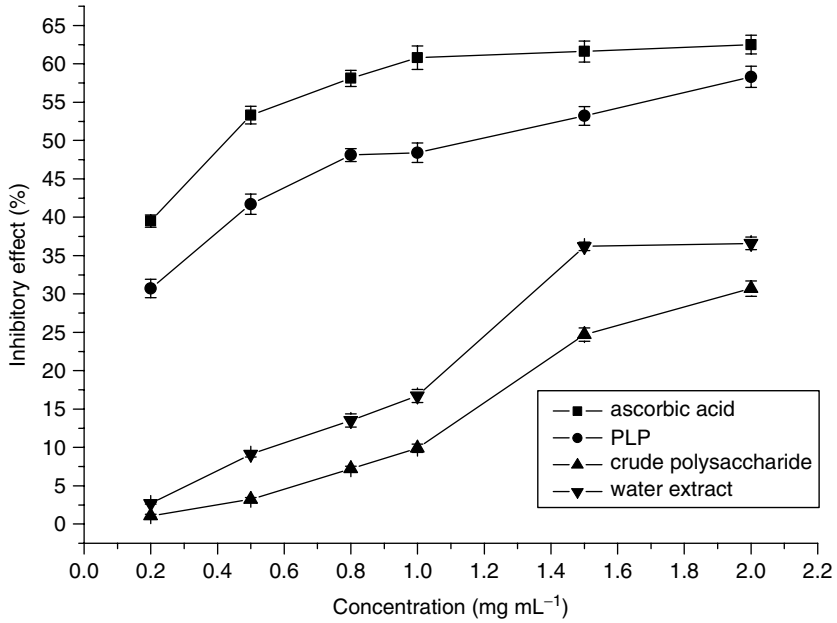

Figure 9. Inhibitory effect of PLP on lipid peroxidation compared with that of ascorbic acid (standard control), water extract and crude polysaccharide. Data are presented as mean ± SD ($n = 3$).

possibility is that PLP combines with radical ions that are necessary for the radical chain reaction, so the reaction is terminated.

CONCLUSION

Sephacryl™ S-400 HR column chromatography coupled with an ÄKTA™ purifier system was used to purify the polysaccharide from *P. asiatica* L.. A main component PLP was obtained that was found to be a homogeneous protein-bound polysaccharide by HPGPC and FTIR, with an average molecular weight of 1894 kDa, a protein content of 29.2 g kg^{-1} and a uronic acid content of 145.8 g kg^{-1}. The antioxidant effects of PLP were evaluated *in vitro* using various test systems. The results showed that PLP had antioxidant activities in all four systems, especially in scavenging DPPH radicals, scavenging hydroxyl radicals and inhibiting lipid peroxidation.

We may rationally conclude that *P. asiatica* L. exerts its curative effect in traditional medicine partly through the antioxidant action of its polysaccharide content. Therefore PLP should be explored further as a novel potential antioxidant.

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