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Purification, characterisation and protective effects of polysaccharides from alfalfa on hepatocytes

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ABSTRACT

The objective of this study was to determine the preliminary characteristics and protective effects of alfalfa polysaccharides (APS) on hepatocytes *in vitro*. The crude APS was purified by DEAE-cellulose and Sephadex G-100 chromatography, resulting in the four purified fractions: APS-1, APS-2, APS-3 and APS-4. The results indicated that APS-3 had higher carbohydrate and uronic acid contents and that APS-4 had a more complicated monosaccharide composition compared to the other purified fractions. The average molecular weights of APS-1, APS-2, APS-3 and APS-4 were 48,536, 6,221, 66,559 and 13,076 Da, respectively. Furthermore, APS (crude and its purified fractions) restored the activities of antioxidant enzymes and increased the total antioxidant capacity of hepatocytes subjected to H_2O_2-induced oxidative stress. Furthermore, APS treatment counteracted the increases in lactic dehydrogenase and malonaldehyde in the culture supernatant. These results clearly demonstrate that APS possesses a protective effect against oxidative injury in hepatocytes.

1. Introduction

Polysaccharides are an important macromolecular polymer widely found in plants, microorganisms and animals, and these compounds have been the focus of high-level research because of their potential biological and pharmacological activities, including antioxidant activity (Ge et al., 2013; Wang, Dong, & Tong, 2013) and protective effects against liver injury induced by various chemicals (Jiang et al., 2013; Qin, Huang, & Xu, 2002; Shi, Zhang, & Yang, 2013). The liver plays a key role in regulating various physiochemical processes of the body, including synthesis and the biotransformation and detoxification of xenobiotics and endogenous substances (Yang, Yang, Guo, Jiao, & Zhao, 2013). Oxidative stress may cause liver damage, which is associated with metabolic dysfunction, ranging from the transient elevation of liver enzymes to steatosis, chronic hepatitis, liver cirrhosis and even hepatocellular carcinoma (Shankar, Manavalan, Venkappayya, & Raj, 2008; Vuda et al., 2012). Alfalfa polysaccharides (APS) have antioxidant (Liu, Dong, Tong, & Zhang, 2010; Liu, Dong, Tong, Xu, & Zhang, 2011; Wang et al., 2013), lipid-lowering (Deng, Dong, Tong, Xie, & Zhang, 2012; Dong et al., 2007), anti-tumour (Zhang & Feng, 2006) and immunostimulatory effects (Liu, Dong, Tong, Xu, et al., 2010; Rong & Tong, 2007; Yang et al., 2010). Recently, we used enzyme-assisted technology rather than hot-water extraction to extract alfalfa polysaccharides (Wang et al., 2013), and found that the polysaccharides extracted by this method possessed good antioxidant activities, such as scavenging activity for hydroxyl radicals and DPPH radicals. However, to the best of our knowledge, there were relatively few reports on the purification and characterisation of APS. Moreover, these studies did not evaluate the protective effects of APS on hepatocytes *in vitro*. Therefore, based on our previous work, we purified crude APS (CAPS) using DEAE-cellulose and Sephadex G-100 chromatography. Additionally, gel-permeation chromatography (GPC) and high-performance capillary electrophoresis (HPCE) were used to characterise the purified polysaccharides. The protective effects of APS (crude and purified fractions) on hepatocytes isolated from the livers of laying hens were then assessed using an *in vitro* H_2O_2-induced oxidative stress model.

2. Materials and methods

2.1. Materials and reagents

CAPS was prepared from alfalfa according to our previously reported method (Wang et al., 2013). Assay kits for total protein, superoxide dismutase (SOD), catalase (CAT), total

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antioxidative capacity (TAOC), lactic dehydrogenase (LDH), and malonaldehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). DEAE-cellulose was obtained from Shanghai Hengxing Chemical Reagent Co. (Shanghai, China). Dextrans of various molecular weights, monosaccharide standards, including xylose, arabinose, glucose, rhamnose, galactose, glucuronic acid and galacturonic acid, Sephadex G-100, D-glucose, phenol, m-hydroxybiphenyl, 1-pheny-3-methyl-5-pyrazolone (PMP) and galacturonic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China).

2.2. Purification of CAPS

CAPS (500 mg) was dissolved in distilled water, loaded onto a DEAE-cellulose column (6 cm × 30 cm) and eluted with distilled water and a gradient solution (0.25 M, 0.5 M $NaHCO_3$ and 0.1 M NaOH) at a flow of 1 mL/min. The sugar content of the fractions was assayed using the phenol-sulphuric acid method (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). The two main fractions were combined separately, concentrated, dialysed (molecular weight cut off: 1000 Da) against distilled water for 72 h, and then lyophilised at −40 °C. Each fraction was dissolved in distilled water, and then further purified over a Sephadex G-100 (2 cm × 120 cm) gel filtration column eluted with 0.1 M NaCl at 0.3 mL/min. Four purified polysaccharides fractions, APS-1, APS-2, APS-3 and APS-4, were obtained, dialysed, and lyophilised at −40 °C for further study.

2.3. Characterisation of APS-1, APS-2, APS-3 and APS-4

2.3.1. Carbohydrate, uronic acid and protein content

The sugar contents of APS-1, APS-2, APS-3 and APS-4 were measured using the phenol-sulphuric acid method (Balavigneswarana, Kumara, Packiaraja, Veeraraja, & Prakash, 2013; DuBois et al., 1956), and D-glucose was used to construct the standard curve. The uronic acid content of the purified samples was determined colorimetrically with m-hydroxydiphenyl (Blumenkranta & Asboe-Hansen, 1973) using galacturonic acid to construct the standard curve. The protein content of the purified fractions was determined with the BCA Protein Assay Kit (Nanjing, Jiangsu, China).

2.3.2. Monosaccharide composition analysis using HPCE

The monosaccharide compositions of APS-1, APS-2, APS-3 and APS-4 were analysed by HPCE (Jie, Fu, Hong, & Hua, 2011; Maccari, Tripodi, & Volpi, 2004). Xylose, arabinose, glucose, rhamnose, galactose, glucuronic acid and galacturonic acid were used as the monosaccharide standards. The monosaccharides were derived with PMP after the polysaccharides were degraded with trifluoroacetic acid. The derived monosaccharides were detected under the optimised conditions of 50 mmol/L sodium tetraborate buffer at pH = 10.57, a fused silica capillary column (50 μm × 60 cm), a separation voltage of 15 kV, a detection wavelength of 245 nm, and an injection time of 10 s at 26 °C.

2.3.3. Molecular weight determination by GPC

The molecular weights of APS-1, APS-2, APS-3 and APS-4 were determined by GPC (Lai, Wen, Lin Li, & Li, 2010; Zhu et al., 2013). The purified polysaccharides were dissolved in 0.5 mL 0.1 mol/L Na_2SO_4 (final concentration 10 mg/mL) and characterised by GPC using a Waters 600 instrument (USA) equipped with a TSKgel G-4000 PW × L (7.8 mm × 300 mm) gel column and a Waters 2414 Refractive Index Detector (Waters, USA). Dextrans with various molecular weights (6000, 1.04×10^4, 4.0×10^4, 6.6×10^4 and 2.7×10^4 Da) were used as the standard to calibrate the column and fit the regression curve. The samples (20 μL) were injected with a Waters 717 Plus Autosampler into the gel column, and then eluted with 0.1 mol/L Na_2SO_4 at 35 °C and a flow rate of 0.6 mL/min. The molecular weight (Mw) was calculated from the calibration curve. All samples were filtered through a 0.22 μm pore diameter membrane (Millipore, USA) prior to analysis.

2.4. Assay for the protective effects of APS on hepatocytes

2.4.1. Hepatocyte preparation

Hepatocytes from laying hens were used for this experiment and were isolated using a two-stage collagenase perfusion method (Zhou, Dong, Tong, & Xu, 2012). Briefly, laying hens were injected with heparin sodium under the wing vein, followed by administration of 50 mg/kg pentobarbital sodium intraperitoneally. The tubal vein, pancreaticoduodenal vein, and posterior mesenteric vein in the distal end were ligated prior to liver perfusion. The culture conditions were identical to the normal hepatocyte culture conditions (Zhou, Dong, Tong, Xu, & Wang, 2012). The medium was William's medium E supplemented with an antibiotic mixture of penicillin (100 U/mL), streptomycin (100 μg/mL), and foetal calf serum (10%). The hepatocytes were cultured in a humidified incubator at 37 °C with 5% CO_2 and were plated in 6-well flat-bottomed microplates (Corning Incorporated, USA) at a density of 1×10^6 cells.

2.4.2. Microculture tetrazolium (MTT) assay

The range of non-toxic dose concentrations for APS was determined using the MTT assay with some modifications (Qi et al., 2013; Qin et al., 2002). Briefly, 4×10^5 cells/mL was seeded in 96-well culture plates and cultured overnight. The culture medium was then completely replaced with new medium containing 0, 250, 500, 1000, or 1500 μg/mL APS and the cells were incubated for an additional 6 h. Following this incubation, 20 μL of an MTT solution (5.0 mg/mL) was added to each well. After 4 h, the supernatant was aspirated prior to adding 150 μL of DMSO to the culture to terminate the reaction. The absorbance of each well was measured at 490 nm after shaking the plates at room temperature for 10 min. The percentage of surviving cells was calculated as follows:

$$\text{The ratio of cell survival}\,(\%) = \frac{A_1}{A_0} \times 100\%$$

where A_0 is the absorbance of cells without APS and A_1 is the absorbance of cells with APS.

2.4.3. Lipid peroxidation assay

After pretreatment with APS-1, APS-2, APS-3 and APS-4 for 6 h at final concentrations of 0, 200, 400, 600, 800, and 1000 μg/mL, the hepatocytes were incubated with H_2O_2 (4 mmol/L) for 2 h (Qi et al., 2013) to induce oxidative stress. The culture medium was collected and centrifuged to assay for the lipid peroxidation product MDA in the supernatant according to the manufacturer's instructions (Nanjing Jiancheng Institute of Bioengineering, Nanjing, Jiangsu, China).

2.4.4. Cytotoxicity assay of hepatocytes

The oxidative stress model of hepatocytes was established as described previously. The hepatocytes were also pretreated with APS-1, APS-2, APS-3 and APS-4 for 6 h at the same concentrations described in Section 2.4.3, and the LDH levels in the cell-free culture supernatants were assayed according to the manufacturer's instructions (Nanjing, Jiangsu, China).

2.4.5. Antioxidant enzyme activities assay

The hepatocytes were pretreated with APS prior to oxidative stress treatment. For the experimental cell suspensions, the cells

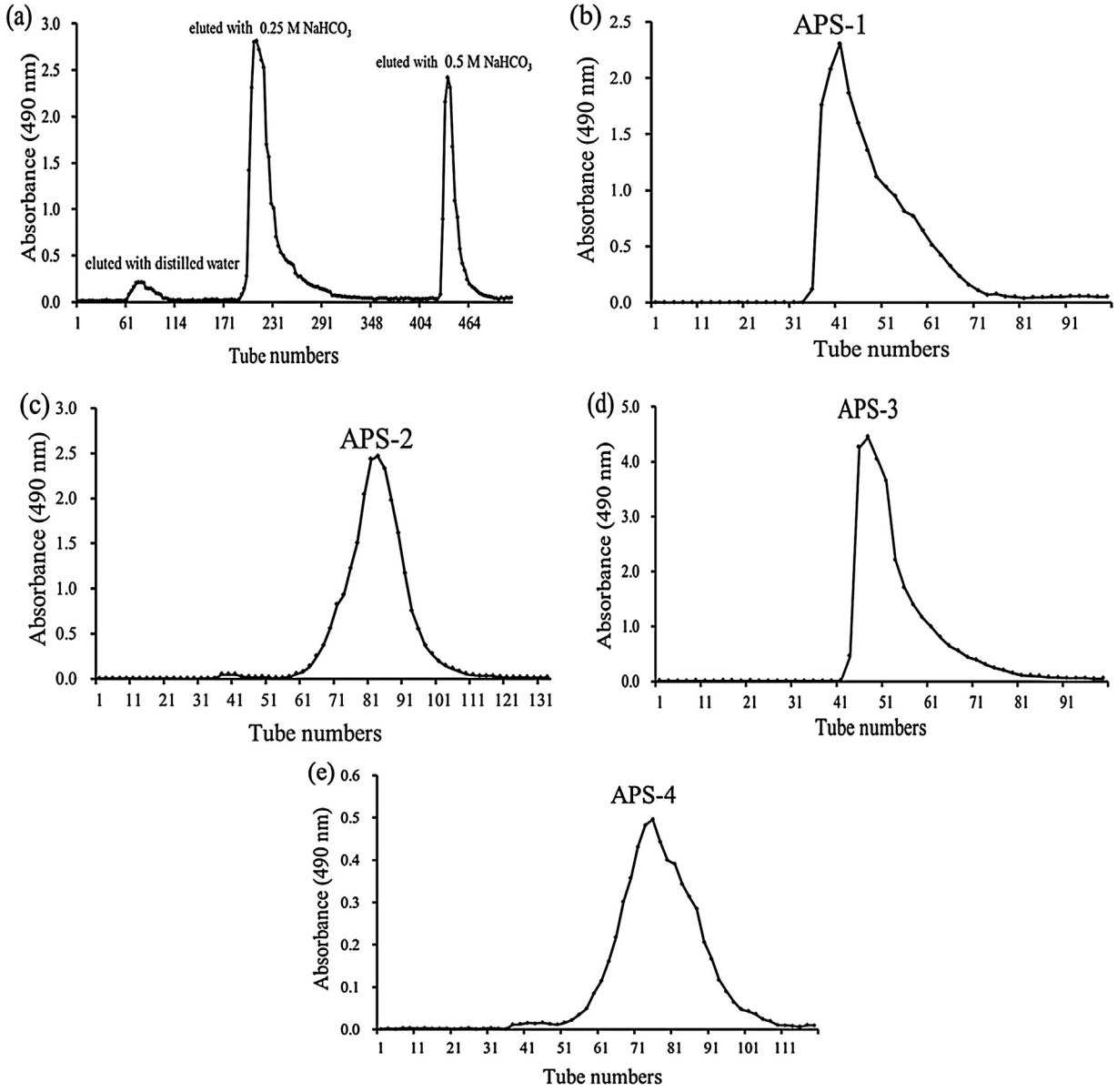

Fig. 1. Elution profile of the crude APS on a DEAE-cellulose anion-exchange column (a), and elution curve of the APS fractions APS-1, APS-2, APS-3 and APS-4 from a Sephadex G-100 gel filtration column (b–e).

were harvested using cell scrapers with PBS buffer of pH 7.4, and centrifuged for 10 min at 1000×*g*. The supernatant was discarded and the cell pellet was then resuspended in the same buffer. The activities of superoxide dismutase (SOD), catalase (CAT), and total antioxidative capacity (TAOC) in the hepatocytes were measured using commercially available kits (Nanjing, Jiangsu, China), and the activities are expressed as U/mg protein. The total protein content in the cells was measured using a BCA Protein Assay Kit (Nanjing, Jiangsu, China).

2.5. Statistical analysis

The data were expressed as the means ± standard deviations ($n = 3$) and were subjected to one-way analysis of variance (ANOVA). The means were compared using Duncan's multiple-range tests with the SAS 9.2 software (SAS Institute Inc., Cary, NC, USA). Significant differences were defined as a value of $P < 0.05$.

3. Results and discussion

3.1. Purification and characterisation of APS-1, APS-2, APS-3 and APS-4

CAPS was obtained using the optimal enzyme-assisted extraction conditions (Wang et al., 2013). CAPS was first separated and purified over an anion-exchange column of DEAE-cellulose. As shown in Fig. 1a, two primary independent elution peaks were observed. The two main fractions were collected, concentrated, dialysed and lyophilised, and then further purified over a gel filtration column of Sephadex G-100. Each fraction generated two elution peaks, which were collected and loaded onto a Sephadex G-100 gel filtration column to increase the purity of each fraction. Finally, APS-1, APS-2, APS-3 and APS-4 were obtained as shown in Fig. 1b–e.

Table 1 shows the carbohydrate, uronic acid, and protein contents, and the monosaccharide compositions of APS-1, APS-2,

Table 1
The carbohydrate, protein, and uronic acid contents and the monosaccharide compositions of APS-1, APS-2, APS-3 and APS-4.

Item	APS-1	APS-2	APS-3	APS-4
Carbohydrate (%)	80.23 ± 1.63	82.92 ± 1.24	92.88 ± 1.28	80.00 ± 2.15
Protein (%)	2.67 ± 0.77	1.62 ± 0.24	0.52 ± 0.40	1.19 ± 0.38
Uronic acid (%)	37.84 ± 1.55	47.68 ± 1.50	52.09 ± 0.82	47.30 ± 0.75
Sugar components (molar ratio)				
Xylose	–[a]	1.00	1.00	1.00
Arabinose	4.88	7.34	1.77	2.35
Glucose	–	–	–	3.78
Rhamnose	–	4.98	1.82	3.05
Galactose	13.53	9.75	4.65	2.74
Glucuronic acid	–	3.25	–	1.72
Galacturonic acid	1.00	24.60	24.60	11.45

[a] Undetected.

APS-3 and APS-4. Notably, the carbohydrate and uronic acid content of APS-3 was the highest of the four fractions because APS-3 was eluted with 0.5 M $NaHCO_3$ from the DEAE-cellulose anion-exchange column. The HPCE analysis shows that APS-1 is composed of arabinose, galactose and galacturonic acid at a molar ratio of 4.88:13.53:1.00, and the molar ratio of the monosaccharide composition of APS-2 is as follows: xylose, arabinose, rhamnose, galactose, glucuronic acid and galacturonic acid at 1.00:7.34:4.98:9.75:3.25:24.60. Similarly, APS-3 consists of xylose, arabinose, rhamnose, galactose, galacturonic acid at a molar ratio of 1.00:1.77:1.82:4.65:24.60. The monosaccharide composition of APS-4 is more complex than APS-1, APS-2 or APS-3. Specifically, seven monosaccharides, including xylose, arabinose, glucose, rhamnose, galactose, glucuronic acid and galacturonic acid were identified in APS-4 at a molar ratio of 1.00:2.35:3.78:3.05:2.74:1.72:11.45. Zhao, Zhang, Ren, Zhang, and Yang (1993) reported that alfalfa polysaccharides obtained using hot-water extraction consist of glucose, mannose, rhamnose, galacturonic acid and an unknown monosaccharide detected by thin layer chromatography (TLC); however, the molar ratio of monosaccharides was not determined. The differences may be resulted from different sources of alfalfa, different extraction technologies and analysis methods for the polysaccharides.

The results of the GPC analysis showed that the average molecular weight (Mw) of APS-1, APS-2, APS-3 and APS-4 were approximately 48,536, 6,221, 66,559 and 13,076 Da, respectively. The average molecular weight (Mw) of APS-3 was the highest and the APS-2 was the lowest among the four fractions. The molecular weight results were somewhat different from a previous study (Zhao, Zhang, Ren, Zhang, & Yang, 1993), in which the average molecular weight of the alfalfa polysaccharides ranged from 20 kDa to 60 kDa, which is higher than that of APS-2 and APS-4. One reason may be that the enzymes cellulase, papain and pectase (Wang et al., 2013) that were added during extraction process can hydrolyse the plant well wall to increase the solubility of polysaccharides. Moreover, cellulase may cause the partial degradation of polysaccharides (Chen et al., 2014).

3.2. Protective effects of alfalfa polysaccharides (APS) on hepatocytes

3.2.1. Cytotoxicity of APS

The results of this experiment (Fig. 2) show that APS at up to 1000 μg/mL, had no toxic effect on the primary hepatocyte cultures from laying hens. Therefore, concentrations of APS less than 1000 μg/mL are suitable for the *in vitro* experiments.

3.2.2. Effects of APS on MDA and LDH levels in the culture supernatant

The effects of APS on the MDA and LDH levels in cell-free culture supernatant are shown in Tables 2–6. Compared to the control group, there were significant increases in the MDA and LDH levels ($P < 0.05$) in the H_2O_2-treated group (model group), which indicated that the hepatocyte H_2O_2-induced oxidative stress model was successful. The elevated levels of MDA and LDH in the supernatants of the groups treated with APS decreased in a dose-dependent manner (Table 2–6). Moreover, the groups treated with CAPS, APS-3 and APS-4 at all concentrations showed significantly decreased MDA ($P < 0.05$). However, there was no significant difference in the LDH level between the model group and the groups treated with 1000 μg/mL APS, which may indicate that high concentrations of APS have a negative effect on hepatocytes. This result is also consistent with the cytotoxicity evaluation of APS (Fig. 2). The enzymatic activity of LDH is a sensitive indicator of hepatocyte damage, which may lead to leakage of the enzyme from the cells into the culture medium (Shi et al., 2013). MDA, as a primary product of lipid peroxidation, is always used as an indicator of lipid peroxidation. Therefore, lower LDH and MDA levels indicate that there is less cells injury, lipid peroxidation and weaker oxidative stress (Bagchi, Bagchi, Hassoun, & Stohs, 1995). The above results suggest that the APS can decrease the LDH and MDA levels in the culture supernatant depending on the concentration, thereby protecting the integrity of the cellular membranes and normal oxidative status.

3.2.3. Effect of APS on the levels of SOD, CAT and TAOC of hepatocytes

As shown in Tables 2–6, the hepatocytes in the model group had significant decreases ($P < 0.05$) in the antioxidant enzymes activities of SOD and CAT, and lower levels of TAOC were obtained compared to the control group. The results confirmed that the hepatocyte oxidative stress model was successfully established in this study. The group treated with APS had significantly enhanced activities of SOD and CAT ($P < 0.05$), as well as increased levels of TAOC in the hepatocytes. Moreover, APS-3 treatment caused higher

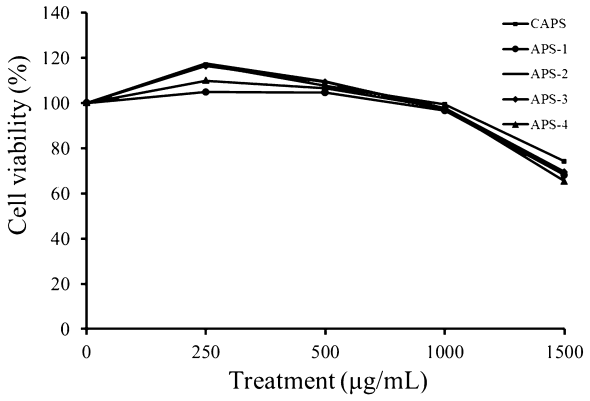

Fig. 2. Cytotoxicity of APS on hepatocytes using an MTT assay.

Table 2
Effects of CAPS on the activities of SOD, CAT and TAOC in hepatocytes, and the levels of LDH and MDA in the culture supernatant.

Treatment	SOD (U/mg protein)	CAT (U/mg protein)	TAOC (U/mg protein)	LDH (U/L)	MDA (nmol/mL)
Control	156.88 ± 6.93^{ab}	46.39 ± 2.39^{a}	1.18 ± 0.04^{ab}	1467.40 ± 105.26^{bc}	0.89 ± 0.06^{d}
H_2O_2	134.74 ± 11.25^{c}	30.39 ± 5.80^{c}	0.69 ± 0.11^{d}	1677.38 ± 99.09^{a}	1.32 ± 0.04^{a}
CAPS (μg/mL)					
200	161.15 ± 11.39^{ab}	35.51 ± 1.96^{bc}	1.29 ± 0.15^{a}	1533.00 ± 106.44^{abc}	1.14 ± 0.11^{b}
400	168.70 ± 8.60^{a}	42.78 ± 4.20^{ab}	1.15 ± 0.09^{ab}	1691.22 ± 68.12^{a}	0.99 ± 0.12^{cd}
600	163.02 ± 11.12^{ab}	42.94 ± 2.42^{ab}	1.06 ± 0.05^{bc}	1559.16 ± 88.02^{abc}	0.91 ± 0.05^{cd}
800	160.70 ± 11.17^{ab}	45.96 ± 7.20^{a}	0.96 ± 0.05^{c}	1455.11 ± 128.64^{c}	1.04 ± 0.07^{bc}
1000	147.57 ± 13.00^{bc}	35.43 ± 2.39^{bc}	0.90 ± 0.14^{c}	1648.72 ± 93.05^{ab}	1.02 ± 0.03^{bcd}

The values are presented as the mean ± SD (n = 3), and the values not sharing a common superscript letter denote significant differences (P < 0.05).

Table 3
Effects of APS-1 on the activities of SOD, CAT and TAOC in hepatocytes, and the levels of LDH and MDA in the culture supernatant.

Treatment	SOD (U/mg protein)	CAT (U/mg protein)	TAOC (U/mg protein)	LDH (U/L)	MDA (nmol/mL)
Control	156.88 ± 6.93^{a}	46.39 ± 2.39^{a}	1.18 ± 0.04^{a}	1467.40 ± 105.26^{c}	0.89 ± 0.06^{d}
H_2O_2	134.74 ± 11.25^{b}	30.39 ± 5.80^{b}	0.69 ± 0.11^{c}	1677.38 ± 99.09^{a}	1.32 ± 0.04^{a}
APS-1 (μg/mL)					
200	158.54 ± 7.11^{a}	46.17 ± 1.63^{a}	1.05 ± 0.13^{ab}	1546.64 ± 35.45^{bc}	1.19 ± 0.08^{ab}
400	169.04 ± 6.87^{a}	47.22 ± 1.50^{a}	1.07 ± 0.15^{ab}	1644.13 ± 12.73^{ab}	1.17 ± 0.12^{ab}
600	167.20 ± 13.72^{a}	46.13 ± 2.50^{a}	1.06 ± 0.08^{ab}	1607.74 ± 35.70^{ab}	1.09 ± 0.17^{bc}
800	168.20 ± 5.79^{a}	46.70 ± 5.05^{a}	1.05 ± 0.11^{ab}	1666.36 ± 14.55^{a}	0.91 ± 0.06^{cd}
1000	158.47 ± 17.67^{a}	41.61 ± 3.14^{a}	0.97 ± 0.03^{b}	1633.16 ± 45.57^{ab}	1.22 ± 0.16^{ab}

The values are presented as the mean ± SD (n = 3), and values not sharing a common superscript letter denote significant differences (P < 0.05).

Table 4
Effects of APS-2 on the activities of SOD, CAT and TAOC in hepatocytes, and the levels of LDH and MDA in the culture supernatant.

Treatment	SOD (U/mg protein)	CAT (U/mg protein)	TAOC (U/mg protein)	LDH (U/L)	MDA (nmol/mL)
Control	156.88 ± 6.93^{a}	46.39 ± 2.39^{a}	1.18 ± 0.04^{a}	1467.40 ± 105.26^{c}	0.89 ± 0.06^{bc}
H_2O_2	134.74 ± 11.25^{b}	30.39 ± 5.80^{c}	0.69 ± 0.11^{c}	1677.38 ± 99.09^{a}	1.32 ± 0.04^{a}
APS-2 (μg/mL)					
200	149.88 ± 4.25^{ab}	36.77 ± 5.02^{bc}	1.07 ± 0.06^{ab}	1545.45 ± 51.42^{bc}	1.09 ± 0.12^{abc}
400	167.30 ± 14.14^{a}	43.46 ± 3.29^{ab}	1.11 ± 0.03^{ab}	1607.64 ± 40.01^{ab}	1.12 ± 0.15^{ab}
600	169.23 ± 13.28^{a}	41.89 ± 1.95^{ab}	1.11 ± 0.04^{ab}	1598.20 ± 38.63^{ab}	0.86 ± 0.14^{c}
800	166.95 ± 7.54^{a}	48.23 ± 3.98^{a}	1.07 ± 0.09^{ab}	1608.24 ± 50.73^{ab}	1.17 ± 0.17^{a}
1000	160.47 ± 13.61^{a}	46.52 ± 3.33^{a}	1.03 ± 0.10^{b}	1566.81 ± 43.48^{abc}	1.09 ± 0.17^{abc}

The values were presented as the mean ± SD (n = 3), and values not sharing a common superscript letter denote significant differences (P < 0.05).

Table 5
Effects of APS-3 on the activities of SOD, CAT and TAOC in hepatocytes, and the levels of LDH and MDA in the culture supernatant.

Treatment	SOD (U/mg protein)	CAT (U/mg protein)	TAOC (U/mg protein)	LDH (U/L)	MDA (nmol/mL)
Control	156.88 ± 6.93^{ab}	46.39 ± 2.39^{ab}	1.18 ± 0.04^{a}	1467.40 ± 105.26^{c}	0.89 ± 0.06^{d}
H_2O_2	134.74 ± 11.25^{b}	30.39 ± 5.80^{c}	0.69 ± 0.11^{b}	1677.38 ± 99.09^{ab}	1.32 ± 0.04^{a}
APS-3 (μg/mL)					
200	165.38 ± 16.02^{a}	44.16 ± 4.76^{ab}	1.34 ± 0.18^{a}	1393.44 ± 81.11^{c}	1.12 ± 0.15^{bc}
400	170.91 ± 17.77^{a}	47.89 ± 2.11^{a}	1.31 ± 0.11^{a}	1498.24 ± 62.77^{bc}	1.14 ± 0.09^{b}
600	170.95 ± 6.28^{a}	48.81 ± 1.92^{a}	1.01 ± 0.10^{a}	1527.06 ± 37.30^{abc}	0.96 ± 0.08^{cd}
800	172.00 ± 7.98^{a}	42.76 ± 5.13^{ab}	1.26 ± 0.33^{a}	1396.51 ± 64.55^{c}	0.96 ± 0.09^{cd}
1000	171.02 ± 21.54^{a}	39.77 ± 4.80^{b}	1.23 ± 0.23^{a}	1687.70 ± 173.71^{a}	1.07 ± 0.08^{bc}

The values are presented as the mean ± SD (n = 3), and values not sharing a common superscript letter denote significant differences (P < 0.05).

Table 6
Effects of APS-4 on the activities of SOD, CAT and TAOC in hepatocytes, and the levels of LDH and MDA in the culture supernatant.

Treatment	SOD (U/mg protein)	CAT (U/mg protein)	TAOC (U/mg protein)	LDH (U/L)	MDA (nmol/mL)
Control	156.88 ± 6.93^{ab}	46.39 ± 2.39^{a}	1.18 ± 0.04^{bc}	1467.40 ± 105.26^{cd}	0.89 ± 0.06^{d}
H_2O_2	134.74 ± 11.25^{b}	30.39 ± 5.80^{b}	0.69 ± 0.11^{d}	1677.38 ± 99.09^{a}	1.32 ± 0.04^{a}
APS-4 (μg/mL)					
200	166.32 ± 17.20^{a}	49.98 ± 5.19^{a}	1.32 ± 0.03^{a}	1514.88 ± 31.76^{bcd}	1.12 ± 0.08^{bc}
400	174.89 ± 9.36^{a}	44.08 ± 3.81^{a}	1.36 ± 0.06^{a}	1611.82 ± 33.35^{ab}	0.99 ± 0.12^{cd}
600	167.87 ± 14.86^{a}	47.30 ± 3.91^{a}	1.27 ± 0.09^{ab}	1377.39 ± 85.52^{d}	1.17 ± 0.10^{b}
800	173.19 ± 12.16^{a}	46.30 ± 1.56^{a}	1.14 ± 0.11^{bc}	1451.88 ± 78.43^{cd}	0.99 ± 0.08^{cd}
1000	160.44 ± 20.93^{a}	43.13 ± 2.96^{a}	1.07 ± 0.04^{c}	1590.50 ± 61.17^{abc}	1.02 ± 0.07^{bcd}

The values are presented as the mean ± SD (n = 3), and values not sharing a common superscript letter denote significant differences (P < 0.05).

TAOC levels in the cells compared to the other purified polysaccharides, whereas APS-4 had more robust effects on the SOD and CAT activities than CAPS, APS-1, APS-2 and APS-3 at 200 μg/mL. The bioactivities of polysaccharides can be affected by many factors, such as extraction method, average molecular weight and monosaccharide composition (Olafsdottir & Ingólfsdottir, 2001). Polysaccharides with a high content of uronic acid exhibit relatively stronger antioxidant activity (Chen, Zhang, & Xie, 2004; Rao & Muralikrishna, 2006). In this study, we found that APS-3 had a higher content of uronic acid than CAPS, APS-1 and APS-2, APS-4 (Table 1). Besides, the average molecular weight of APS-3 was the highest among the four polysaccharide fractions. Therefore, this result also indicates that the structural properties of APS contribute to bioactivity.

Oxidative stress is caused by a decrease in antioxidant defences and an increase in peroxide production (Wang et al., 2012). SOD and CAT are the major antioxidant enzymes and the primary defence system against the reactive oxygen species (ROS) generated during oxidative stress (Inal, Kanbak, & Sunal, 2001). SOD is the only enzyme that disrupts $O_2^{\bullet}$ radicals and exists in all cells, with high amounts present in erythrocytes (Qiao et al., 2009). The primary role of CAT is to abolish the H_2O_2 generated by free radicals and prevent the formation of $OH^{\bullet}$ radicals (Urso & Clarkson, 2003). TAOC reflects the non-enzymatic antioxidant capacity against various reactive oxygen and nitrogen radicals, and generally, low TAOC indicates oxidative stress or increased susceptibility to oxidative damage (Young, 2001). The results obtained in this study suggest that APS may overcome the oxidant injury induced by H_2O_2 by increasing the enzymatic activities of SOD and CAT and the non-enzymatic antioxidant capacity (TAOC) of hepatocytes *in vitro*, thereby exerting a protective effect on hepatocytes.

4. Conclusions

In the present study, four purified alfalfa polysaccharide fractions, APS-1, APS-2, APS-3 and APS-4, were successfully isolated using DEAE-cellulose anion-exchange chromatography and Sephadex G-100 size-exclusion chromatography. The preliminary characteristics including the carbohydrate, protein, and uronic acid contents; the average molecular weight; and the monosaccharide composition of APS-1, APS-2, APS-3 and APS-4, were determined. APS-3 had the highest carbohydrate and uronic acid contents and average molecular weight of the four purified fractions and CAPS. The monosaccharide composition of APS-4 was the most complex. Based on the hepatocyte H_2O_2-induced oxidative stress model *in vitro*, we demonstrated that the four purified polysaccharide fractions and CAPS had a significant protective effect against hepatotoxicity, as evidenced by higher activities of SOD and CAT and a higher level of TAOC in the cells, as well lower levels of LDH and MDA in the culture supernatant compared to the model group. The results also suggest that the monosaccharide compositions (uronic acid content) and average molecular weight may influence the antioxidant activities. Thus, alfalfa may be used as an easily accessible source for natural polysaccharides extracted using enzyme-assisted technology. Further studies are necessary to evaluate the bioactivities of the polysaccharides extracted by this enzyme-assisted method *in vivo* and to utilise alfalfa as a source of potential natural antioxidants in either the feed/food industry or the pharmaceutical and medical industries.

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