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Chinese Journal of Natural Medicines 2019, **17**(10): 0792–0800
Chinese Journal of Natural Medicines

Purification, structure characterization and antioxidant activity of polysaccharides from *Saposhnikovia divaricata*

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Available online 20 Oct., 2019

[ABSTRACT] Polysaccharide from traditional Chinese herb, *Saposhnikovia divaricata* (Turcz.) Schischk. (SD) was extracted, fractionated and characterized in this work. Four fractions were prepared. Their molecular weight, monosaccharide compositions, linkage modes and structural properties were characterized with SEC-MALS-RI, HPAEC-PAD, GC-MS and NMR. SDP1 was assigned as a 1, 4-*α*-glucan with small amount of O-6 linked branches. SDP2 contained a big amount of the 1, 4-*α*-glucan and a small amount of arabinogalactan, while SDP3 possessed relatively lower amount of the 1, 4-*α*-glucan and a big amount of the arabinogalactan. SDP4 was defined as a pectic arabinogalactan. Four fractions showed antioxidant activities in both molecular and cellular levels and their activity was ranked as SDP4 ≈ SDP3＞SDP2＞SDP1. The 1, 4-*α*-glucan in SDP1 had the weakest, while SDP3 and SDP4 showed similar and the highest antioxidant activity. The arabinogalactan was the major component of both SDP3 and SDP4, which significantly contributed to the antioxidant activity of SDP.

[KEY WORDS] *Saposhnikovia divaricata*; Polysaccharides; Arabinogalactan; Antioxidant activity

[CLC Number] R284.1, R965 **[Document code]** A **[Article ID]** 2095-6975(2019)10-0792-09

Introduction

Root of *Saposhnikovia divaricata* (Turcz.) Schischk. (SD), a traditional Chinese herbal medicine called “Fangfeng”, is commonly used to treat rheumatism, arthralgia, headaches, stroke, fever, cold, etc [1-2]. Modern pharmacological researches on the extracts of SD have proved its various activities, such as analgesic, anti-convulsant, anti-cancer, anti-inflammatory, anticoagulant and antipyretic activities etc [3-5]. However, most of these works focus on the structure and activity researches of small molecular substances extracted from SD, such as chromones [6-7], coumarins [8] and panaxynol [9-10]. The SD polysaccharides (SDP) exist in the traditional decoction achieved few attentions. Existing studies show that the SDP possess the effects of immuno-regulation, anti-oxidant and anti-tumor activity *in vivo* [11]. However, structure of SDP is still ambiguous which limited our understanding of the structure-activity relationship of SDP. A few studies about SDP showed that it is mainly composed of arabinose (Ara), galactose (Gal), glucose (Glc) and galacturonic acid (GalA). Shimizu and Tomoda extract a major acidic polysaccharide from SD which has the backbone of 1, 4-*α*-galacturonan bearing 1, 5-*α*-arabino-3, 6-*β*-galactan side chains [11]. Moreover, recent literature report the type Ⅱ arabinogalactan existed in SDP, consisting of alternating 1, 3-*β*-Gal*p* and 1, 6-*β*-Gal*p* as the main chains and substituting by 1, 5-*α*-Ara*f* residues to different extent on the O-3 and/or O-4 of 1, 6-*β*-Gal*p* [12].

Structure of polysaccharide extracted from same origin is usually reported differently. It could be resulted from different preparation and purification processes. It also could be derived from the different analytical methods [13-14]. In this study, crude SDP was extracted from SD with boiling water. It was fractionated with DEAE-Sepharose. The molecular weights (MW) and MW distributions of all those fractions were determined by size exclusion chromatography linked to multiple

[Received on] 07-Aug.-2019
[Research funding] This work was supported by the National Natural Science Foundation of China (Nos. 81473179 and 81673388), and the funding for Jiangsu Key Laboratory of Translational Research and Therapy for Neuro-Psycho-Diseases (No. BM2013003).
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These authors have no conflict of interest to declare.

angle laser scattering and refractive index detectors (SEC-MALS-RI). Their sugar compositions were analyzed with high performance anion exchange chromatography linked to pulsed amperometric detector (HPAEC-PAD) after complete hydrolysis. Their linkage information was detected with gas chromatography and mass spectrometry (GC-MS). Their structures were profiled with ^{1}H, ^{13}C and heteronuclear single quantum coherence (HSQC) NMR. Furthermore, the antioxidant activity of each SDP fraction was measured by ABTS kit and investigated on RAW 264.7 macrophage cell line. These results would provide comprehensive information about the SDP structure and valuable clue about antioxidant bioactivity of SDP.

Materials and Methods

Materials

Root of SD was purchased from a traditional medicine store in China (LeiYunShang, Suzhou, China) and its growth place was Heilongjiang. Trypsin from bovine pancreas was purchased from Aladdin (Shanghai, China). DEAE-Sepharose Fast Flow was purchased from the GE Healthcare Ltd. (Uppsala, Sweden). Arabinose (Ara), rhamnose (Rha), xylose (Xyl), fucose (Fuc), fructose (Fru), glucosamine hydrochloride (GlcN), galactosamine hydrochloride (GalN), mannose (Man), galactose (Gal), glucose (Glc), glucuronic acid (GlcA) and galacturonic acid (GalA) were purchased from Sigma. 2, 2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) was obtained from TCI. BCA protein content assay kits and Cell Counting Kit-8 (CCK-8) were obtained from Beyotime Institute of Biotechnology. Murine macrophage cell line RAW264.7 cells were purchased from American Type Culture Collection (Rockville, Md., USA). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific. High-purity water (resistivity ≥ 18.2 MΩ × cm, 25 °C) was used throughout the whole LC analysis. All other chemicals and reagents were of analytical reagent (AR) grade.

Experiments

The purification and fractionation of SDP

Root of SD (20 g) was smashed and added into 1 L water. The mixture had been boiled with refluxing for 2 h before cooling down to room temperature. The supernatant was collected after filtration and precipitated with ethanol to final volume ratio at 70%. The crude SDP was collected with filtration and dried with vacuum dryer.

The crude polysaccharides (2 g) were dissolved in 200 mL pH 8.0 PBS buffer. The mixture was incubated at 37 °C for 2 h after adding 20 mg trypsin. The protein digestion was stopped by sitting the container into the boiling water bath for 20 min. The denatured proteins were removed by centrifuge at 3000 r·min^{-1} for 10 min. The supernatant was concentrated and precipitated with 80 % (*V*/*V*) ethanol at 4 °C. After that, the precipitates were re-dissolved in water and deproteinized with one-fifth volume of Sevage solution (chloroform–butyl = 5 : 1, *V*/*V*) for 3 times. The upper layer was collected and dialyzed against DI water (cut-off 3500 Da) to remove the small molecules and pigments. The aqueous solution was then collected from the dialysis bag and lyophilized for further fractionation.

The purified SDP was then separated by weak anion exchange chromatography. The purified SDP (100 mg) was dissolved in 1 mL H_2O, loaded on a DEAE Sepharose (Fast Flow, FF) column (2.5 cm × 30 cm), and eluted with step gradient under the flow rate of 10 mL·min^{-1}. Eluent A was water, eluent B was 1 mol·L^{-1} NaCl. The step gradient was set up as 0% B for 7.5 min, 5% B for 7.5 min, 10% B for 10 min, 20% B for 10 min and 30% B for 15 min (Fig. 1). The eluents were collected as 5 mL/tube. The amount of carbohydrate in each tube was detected by the phenol-sulfuric acid assay [15]. The colorimetric intensity was plotted as a function of retention time as in Fig. 1.

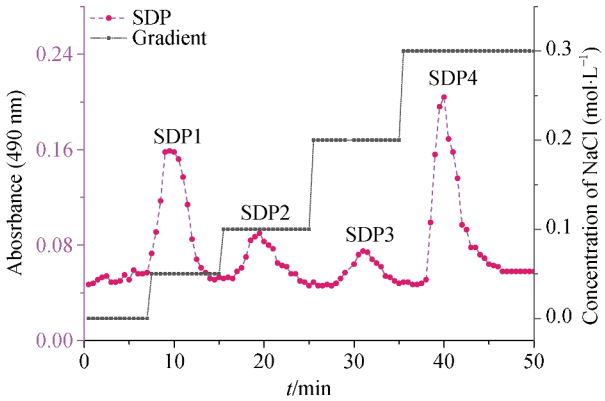

Fig. 1 Elution curve of SDP separated by DEAE-Sepharose FF column

Monosaccharide analysis

The monosaccharide composition of each SDP fraction was identified and quantified by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) after each sample was decomposed. The hydrolysis was performed with 1 mol·L^{-1} trifluoroacetic acid (TFA) at 100 °C for 12 h. Each hydrolysate was evaporated to remove TFA, dissolved in ultrapure water and filtered through a 0.22 μm filter before the analysis. Monosaccharide compositions were analyzed using a Metrohm 850 profession system equipped with a dual pump and PAD. The analysis was performed onto a Carbopac PA1 column (4 mm × 250 mm, Dionex, Sunnyvale, CA) at 30 °C with a flow rate of 1 mL·min^{-1}. 15 mmol·L^{-1} NaOH was used as Mobile phase A, while 150 mmol·L^{-1} NaOAc in 15 mmol·L^{-1} NaOH solution was employed as mobile phase B. Isocratic mobile phase A for 10 min followed by a 30 min gradient from 0% to 100% of mobile phase B was applied for the separation [16]. Neutral sugar standards (Fuc, Rha, Ara, Gal, Glc, Xyl, Man, Fru), amino sugar standards (GalN, GlcN) and uronic acid standards

(GalA, GlcA) were used for identification and quantification of monosaccharides composition of SDP fractions.

Molecular weight determination by SEC-MALS-RI

The homogeneity and molecular weight distribution were analyzed using an Agilent 1260 HPLC system linked to an 18-angles MALS (Wyatt, USA) and a refractive index (RI) detector (Agilent, USA). The dn/dc value was set at 0.138 mL·g^{-1}. The separation was performed on an ACQUITY UPLC@BEH125 SEC column (1.7 μm, 4.6 mm × 300 mm, Waters, USA) with an isocratic gradient (80 mmol·L^{-1} CH_3COONH_4) at a flow rate of 0.1 mL·min^{-1} and 25 °C. The collected data were processed with the ASTRA 6.1 software.

Glycosidic linkage position analysis with GC-MS

The glyosidic linkage position of each fraction was analyzed following the previously described method with slightly modification [17]. About 1 mg of each polysaccharide were suspended in 1 mL DMSO/NaOH and methylated with CH_3I. After successive hydrolysis with 2.4 mol·L^{-1} trifluoroacetic acid (TFA) at 105 °C for 6 h, the sugars were completely reduced with sodium borodeuteride ($NaBD_4$) and acetylated with ethyl acetate (Ac_2O). These partially methylated alditol acetates (PMAAs) were identified and semi-quantified on a gas chromatography/mass (GC-MS) spectrometer (Agilent GM-MS 7890B) fitted with an Agilent J&W capillary column (30 m × 250 μm × 0.25 μm). These PMAAs were separated from a temperature ramp of 5 °C·min^{-1} from 100 to 220 °C with a hold of 5min at the upper temperature. Mass spectra of these PMAAs were compared to the standard MS spectra in Complex Carbohydrate Structural Database of Complex Carbohydrate Research Center (http://www.ccrc.uga.edu/) for identification.

NMR analysis

The ^{1}H, ^{13}C and HSQC NMR spectra of each polysaccharide were acquired using a Bruker ASCEND^{TM} 600 spectrometer (Bruker Instruments, Inc., Bil-lerica, MA, USA). The ^{1}H NMR spectrum (16 scans) was recorded by the frequency of 600 MHz, while the ^{13}C NMR analysis (10240 scans) was acquired on 150 MHz at 298 K. The polysaccharide sample (50 mg) was dissolved in 1.5 mL D_2O (> 99.96 Atom % Deuterium, Sima-Aldrich) and centrifuged at 12 000 r·min^{-1} for 15 min and transferred to an NMR tube before the analysis. The chemical shifts of ^{1}H and ^{13}C NMR were expressed in ppm using TSP-d_4 (d ≥ 98 %) as an internal standard. All the data was processed with MestReNova software of version 6.1.1.2.2.4.

ABTS radical scavenging activity

The ABTS radical scavenging effect of the each polysaccharide was investigated following the previous method [18]. Proper amounts of each sample were precisely weighed and dissolved into water to afford solutions with various concentrations (0.1, 0.5, 1.0, 5.0, 10.0 and 20.0 mg·mL^{-1}). ABTS working fluid (250 μL, 3.7 mmol·L^{-1} ABTS in 1.3 mmol·L^{-1} $K_2S_2O_8$) was added to each 50 μL polysaccharide solution and incubated at room temperature away from light for 2 h before they were measured at 734 nm (Tecan, i-control, Swiss). In this study, isopyknic water served as blank control. The ABTS inhibition (%) was calculated by using the following equation:

$$\text{ABTS inhibition (\%)} = (A_0 - A)/A_0 \times 100 \quad (1)$$

where A_0 was the absorbance of the blank control and A was the absorbance of the sample (0.1–20.0 mg·mL^{-1}).

Protective activity of SDP fractions on H_2O_2-induced RAW264.7 cells

Murine macrophage cell line RAW264.7 was incubated in DMEM basic (1×) medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in an incubator with humidified atmosphere of 5% CO_2 at 37 °C. RAW264.7 cells were seeded in 96-well plates at a density of 3 × 10^5 cells per well in 100 μL culture medium. There are five wells in each group. After incubation at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h, the cultured cells were treated with samples dissolved in the serum-free medium at the final concentration of 1, 10 and 100 μg·mL^{-1}. The isopyknic serum-free medium was added into five wells as a blank group. After additional 12 h culture, diluted H_2O_2 solution was added into each well affording 0.8 mmol·L^{-1} H_2O_2 in the final solution. Meanwhile, the isopyknic culture medium was added into another five wells, but without H_2O_2 treatment as a negative control group. The cells were incubated for another 12 h followed by adding 10 μL of CCK-8 regent into each well. After incubation of additional 1.5 h, the absorbance was measured at 450 nm by a microplate reader (Tecan, i-control, Swiss) and the cell survival ratio was calculated using the following equation:

$$\text{Cell survival rate (\%)} = (A_2 - A_0)/(A_1 - A_0) \times 100 \quad (2)$$

where A_1 was the value of the absorbance of the blank group without H_2O_2 treatment; A_2 were mean values of negative and SDP groups with H_2O_2 treatment; A_0 was the absorbance of the wells corresponding to the blank, negative control and test samples just without cells, respectively.

Results

The fractionation of SDP

The purified SDP was fractionated by DEAE Sepharose FF and four fractions were obtained as shown in Fig. 1. The carbohydrate in each tube was measured by phenol-sulfuric acid assay. The colorimetric intensity in each tube was plotted as a function of retention time. Four fractions were labeled as SDP1 to SDP4, and their yields are measured as 27.4%, 10.1%, 7.0% and 41.2%, respectively, by weight. The total yields of these four fractions are not 100% as the wastage in the processes of collection, desalting and evaporation. No protein of SDP1 and SDP2 was detected by BCA analysis, while lower than 1.00% protein was detected in SDP3 and SDP4.

Monosaccharide composition analysis with HPAEC-PAD

HPAEC-PAD is becoming a popular method to analyze the monosaccharaide composition as its high resolution, sensitivity, specificity and easy sample treatment. In this work, the four fractions (SDP1-SDP4) were hydrolyzed before analysis with HPAEC-PAD, and monosaccharide standards

were used to calibrate the retention time and PAD responses. The HPAEC-PAD chromatogram of monosaccharide standards was shown in Fig. 2A. The chromatograms of SDP1 to SDP4 were shown in Figs. 2B–2E. Dominant Glc was observed in SDP1 (98 mol%). Major Glc (70 mol%), small amounts of Ara and Gal (both of them are 14%) and a little Xyl were observed in SDP2. 20% Glc, 32% Ara, 45% Gal and a little of Xyl were observed in SDP3. Only 5% Glc was left in SDP4, but 33% Ara, 37% Gal and 20% GalA were observed in SDP4. All monosaccharide compositions were listed in Table 1.

Based on the results of monosaccharide composition analysis, SDP1 was composed of glucan and SDP2 was mainly consisted with glucan and mixed with few arabinogalactan. On the contrary, arabinogalactan occupied the main component of SDP3. Moreover, SDP4 was composed of arabinogalactan and polygalacturonic acid. They were eluted from DEAE orderly.

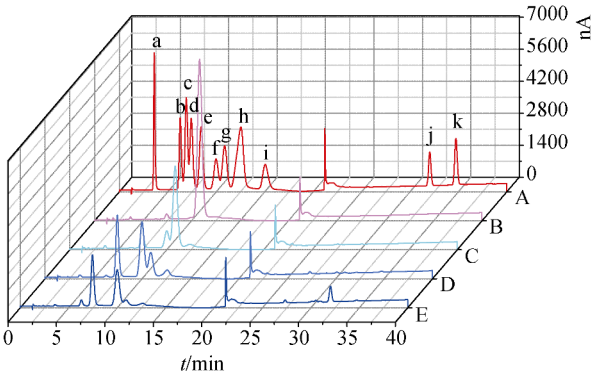

Fig. 2 HPAEC-PAD chromatograms of 11 standard monosaccharides (A) and the hydrolysates of SDP1-SDP4 (B–E) (a-Fuc; b-Rha; c-GalN; d-Ara; e-GlcN; f-Gal; g-Glc; h-Xyl; i-Fru; j-GalA; k-GlcA)

Table 1 The MWs, polydispersity and monosaccharide compositions of SDP fractions

Fraction	Protein content (%)	Molecular weight/distribution		Monosaccharides composition (%)					
		Mw (kDa)	Polydispersity (Mw/Mn)	Rha	Ara	Gal	Glc	Xyl	GalA
SDP1	–	12	1	–	2	–	98	–	–
SDP2	–	13	1	–	14	14	70	2	–
SDP3	0.55	201	1	–	32	45	20	3	–
SDP4	0.86	197	1	3	33	37	5	2	20

(–): not detected

Molecular weight determination with SEC-MALS-RI

The MWs of those fractions were analyzed using SEC-MALS-RI and summarized in Table 1. The RI chromatograms of those fractions were shown as single peaks (Fig. 3) and their MWs were 12, 13, 201 and 197 kDa, respectively. We successfully separated differently molecular weight polysaccharides from SD considering the low polydispersity index (Mw/Mn, close to 1)

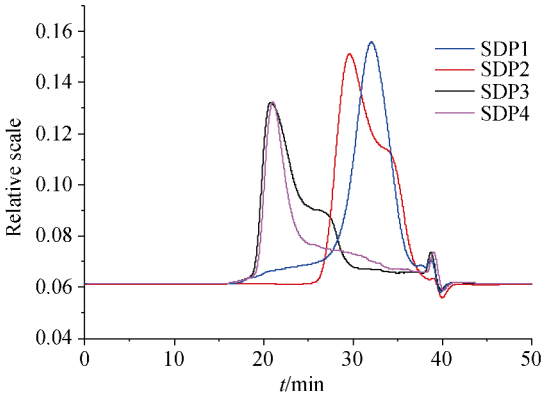

Fig. 3 The RI chromatograms of SDPs analysed by SEC-MALS-RI

Glycosidic linkage position analysis with GC-MS

GC-MS analysis of partially methylated alditol acetates (PMAAs) is a universal way to elucidate the linkage modes of polysaccharide. In this method, the free hydroxyl groups of polysaccharide were fully methylated with CH_3I before complete hydrolysis. The hydrolysis exposed the hydroxyl groups on the glycosidic linkages. Subsequently, the semiacetal of each hydrolyzed monosaccharide was reduced to corresponding alditol. The remained hydroxyl groups were finally acetylated and formed PMAAs. PMAAs with different methylation or acetylation positions generated from different sugars or differently linked sugars were resolved by GC and distinguished by MS/MS.

The extracted ion chromatograms (EICs) of SDP1-SDP4 were shown in Fig. 4. Dominant 1, 4 linked Glc*p* was observed in SDP1 and some branched Glc residues were also observed, including 1, 3, 4 and 1, 4, 6 linked Glc*p*. The linkage modes of Glc in SDP2 are similar to those observed in SDP1. Small amount of Ara and Gal were observed including 1, 5/1, 3, 5 linked Ara*f* and 1, 4/1, 6 linked Gal*p*. The linkage modes of Glc in SDP3 are also similar to those in SDP1 and SDP2, but more branches were observed, including 1, 3, 6 and 1, 3, 4, 6 linked Glc*p*. The linkage modes of Ara and Gal in SDP3 were similar to those observed in SDP2, but with much higher amount. No Glc was observed in methylation analysis of SDP4 mainly because of the complex and violent sample pretreatment causing the missing signals of 5% Glc only existed. A big amount of 1, 3 and 1, 3, 5 linked Ara*f* were observed in SDP4 with. More 1, 3, 5 linked Ara*f* suggests more Ara branches in SDP4. Both 1, 4 and 1, 6 linked Gal*p*

were also observed in SDP4. The linkage information on GalA was not included in this experiment. All linkage information about SDP1-SDP4 was listed in Table 2.

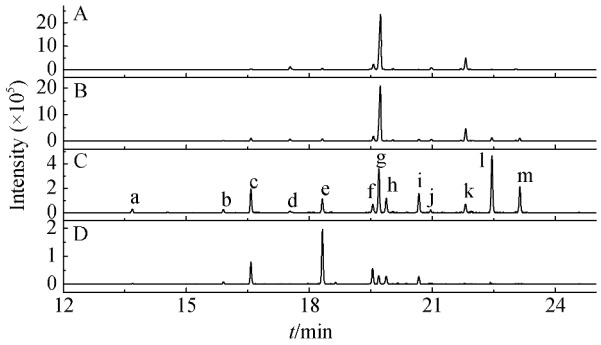

Fig. 4 Extracted ion chromatograms (EICs, *m/z* 118.10) of SDPs. From top to bottom is SDP1, SDP2, SDP3 and SDP4. a: 2, 3, 5-Me$_3$-Ara; b: 2, 5-Me$_2$-Ara; c: 2, 3-Me$_2$-Ara; d: 2, 3, 4, 6-Me$_4$-Glc; e: 2-Me$_1$-Ara; f: 2, 3, 6-Me$_3$-Gal; g: 2, 3, 6-Me$_3$-Glc; h: 2, 3, 6-Me$_3$-Gal; i: 2, 3, 4-Me$_3$-Gal; j: 2, 6-Me$_2$-Glc; k: 2, 3-Me$_2$-Glc; l: 2, 4-Me$_2$-Glc; m: 2-Me$_1$-Glc

Structural analysis with NMR

The structural characteristics of those four fractions were further investigated by NMR analysis. The ^{1}H and ^{13}C spectra were shown in Fig. 5 and the corresponding HSQC spectra of SDPs were not shown. Like the ^{1}H NMR spectra of most natural polysaccharide, the chemical shifts of the SDPs appeared in a narrow region from 3.1 to 5.5 ppm in their ^{1}H NMR spectra [19].

Compared with literature reports, the anomeric hydrogen of *α*-linked Glc residues existed in SDP1 could be assigned as 5.41 ppm and the corresponding anomeric carbon signal was identified at 102.54 ppm (Fig. 5A) [20]. The chemical shifts at 3.85–3.97, 3.85–3.83 and 3.63–3.67 ppm were assigned as the other hydrogens on the sugar ring (H3/5, H6a/b and H2/4, respectively) which demonstrated the existence of 1, 4-*α*-Glc*p* (A) [21]. Considering the result of monosaccharide, SDP1 can unambiguously be identified as a 1, 4-*α*-glucan. No significant signals of glucan branches were observed in the ^{1}H and ^{13}C NMR spectra of SDP1.

Similarly, Fig. 5B revealed that the NMR signals of the 1, 4-*α*-glucan are still dominant in SDP2. Three weak signals of anomeric hydrogens corresponding to terminal Ara, middle Ara and middle Gal were observed at 5.26, 5.07 and 4.53 ppm, respectively, suggesting small amounts of *α*-araban with branches [22] and *β*-galactan presented in SDP2 with a big amount of the 1, 4-*α*-glucan.

Fig. 5C, representing the ^{1}H and ^{13}C NMR spectra of SDP3, demonstrated that the signals of branched α-araban and *β*-galactan increased, while the signals of the 1, 4-*α*-glucan decreased significantly. The H1-5 signals of 1, 5-*α*-Ara*f* (B) were assigned at 5.07, 4.09, 4.03, 4, 19 and 3.94 ppm according to previous publications [23] and their corressbonding carbon signals were assigned at 110.29, 86.98, 79.69, 84.28 and 69.39 ppm, suggesting this fraction contains 1, 5-*α*-araban. The signal of Gal H1 at 4.53 ppm suggests the *β* configuration of these residues [24]. The downfield shifts at 3.68, 3.95 and 3.76ppm were assigned to the H3, H4 and H6 of Gal residues, indicating the presence of 1, 4-*β*-Gal*p* (C).

Table 2 The glyosidic linkage modes of SDP1-SDP4 analyzed with the GC-MS

	t_R/min	Methylated sugars	Linkage pattern	Relative abundance (%)
	17.532	2, 3, 4, 6-Me$_4$-Glc	Glc*p*-(1→	3.47
	19.739	2, 3, 6-Me$_3$-Glc	→4)-Glc*p*-(1→	79.72
SDP1	20.983	2, 6-Me$_2$-Glc	→3, 4)-Glc*p*-(1→	2.45
	21.816	2, 6-Me$_2$-Glc	→4, 6)-Glc*p*-(1→	13.68
	23.037	2-Me$_1$-Glc	→3, 4, 6)-Glc*p*-(1→	0.69
		Ara*f*	Total Ara	4.72
	16.577	2, 3-Me$_2$-Ara	→5)-Ara*f*-(1→	2.77
	18.320	2-Me$_1$-Ara	→3, 5)-Ara*f*-(1→	1.95
		Gal*p*	Total Gal	6.85
	19.563	2, 3, 6-Me$_3$-Gal	→4)-Gal*p*-(1→	5.25
	20.676	2, 3, 4-Me$_3$-Gal	→6)-Gal*p*-(1→	1.60
SDP2		Glc*p*	Total Glc	88.44
	17.532	2, 3, 4, 6-Me$_4$-Glc	Glc*p*-(1→	2.01
	19.732	2, 3, 6-Me$_3$-Glc	→4)-Glc*p*-(1→	66.10
	20.983	2, 6-Me$_2$-Glc	→3, 4)-Glc*p*-(1→	1.69
	21.816	2, 3-Me$_2$-Glc	→4, 6)-Glc*p*-(1→	12.60
	22.453	2, 4-Me$_2$-Glc	→3, 6)-Glc*p*-(1→	3.37
	23.137	2-Me$_1$-Glc	→3, 4, 6)-Glc*p*-(1→	2.67

Continued

	t_R/min	Methylated sugars	Linkage pattern	Relative abundance (%)
		Ara*f*	Total Ara	18.71
	13.687	2, 3, 5-Me_3-Ara	Ara*f*-(1→	1.07
	15.907	2, 5-Me_2-Ara	→3)-Ara*f*-(1→	1.23
	16.576	2, 3-Me_2-Ara	→5)-Ara*f*-(1→	10.50
	18.322	2-Me_1-Ara	→3, 5)-Ara*f*-(1→	5.91
		Gal*p*	Total Gal	18.85
	19.546	2, 3, 6-Me_3-Gal	→4)-Gal*p*-(1→	3.79
SDP3	19.878	2, 4, 6-Me_3-Gal	→3)-Gal*p*-(1→	6.46
	20.676	2, 3, 4-Me_3-Gal	→6)-Gal*p*-(1→	8.60
		Glc*p*	Total Glc	62.44
	17.531	2, 3, 4, 6-Me_4-Glc	Glc*p*-(1→	0.45
	19.695	2, 3, 6-Me_3-Glc	→4)-Glc*p*-(1→	19.75
	21.812	2, 3-Me_2-Glc	→4, 6)-Glc*p*-(1→	3.97
	22.459	2, 4-Me_2-Glc	→3, 6)-Glc*p*-(1→	26.37
	23.140	2-Me_1-Glc	→3, 4, 6)-Glc*p*-(1→	11.90
		Ara*f*	Total Ara	72.24
	13.687	2, 3, 5-Me_3-Ara	Ara*f*-(1→	0.26
	15.905	2, 5-Me_2-Ara	→3)-Ara*f*-(1→	1.50
	16.577	2, 3-Me_2-Ara	→5)-Ara*f*-(1→	19.43
SDP4	18.322	2-Me_1-Ara	→3, 5)-Ara*f*-(1→	51.05
		Gal*p*	Total Gal	27.75
	19.546	2, 3, 6-Me_3-Gal	→4)-Gal*p*-(1→	13.62
	19.878	2, 4, 6-Me_3-Gal	→3)-Gal*p*-(1→	7.79
	20.676	2, 3, 4-Me_3-Gal	→6)-Gal*p*-(1→	6.34

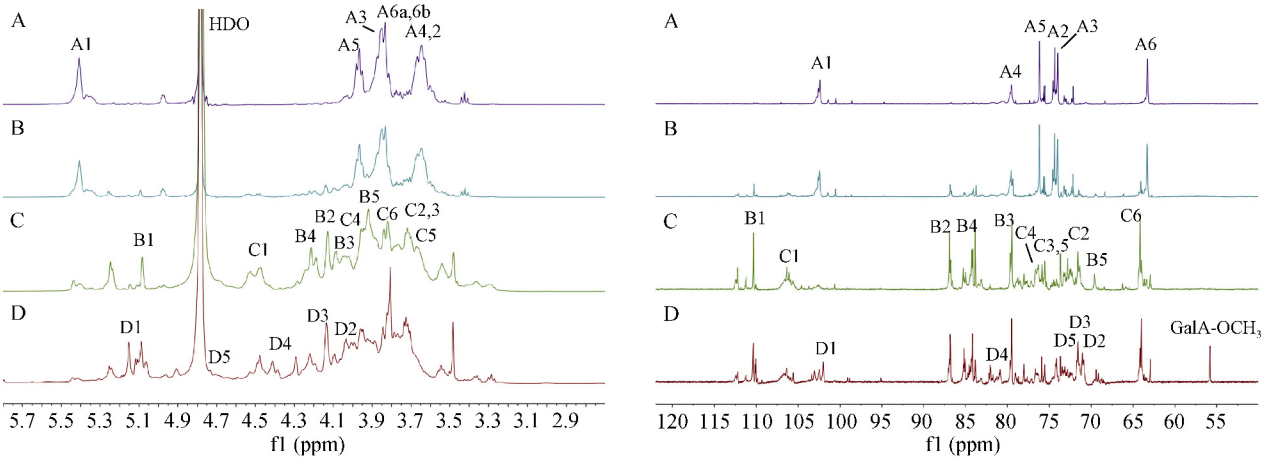

Fig. 5 The ^{1}H and ^{13}C NMR spectra (from top to down) of A: SDP1, B: SDP2, C: SDP3 and D: SDP4. Signals indicated by A, B, C and D refer to 1, 4-*α*-Glc*p*, 1, 5-*α*-Ara*f*, 1, 4-*β*-Gal*p* and 1, 4-*α*-Gal*p*A, respectively

The ^{1}H and ^{13}C NMR spectra of SDP4 were presented in Fig. 5D. No significant glucan signals were observed. Araban and galactan signals were also observed. Moreover, signals corresponding to H1-H5 of GalA were assigned at 5.14, 3.98, 4.13, 4.41 and 4.73 ppm, respectively, suggesting *α*-1, 4-linked Gal*p*A (D) presented in SDP4 [25-27]. In addition, a signal of methylation was observed at 3.48 ppm which was generated by partially methylated Gal*p*AMe. Based on literature report and HSQC spectra, all the chemicals shifts of ^{1}H and ^{13}C NMR spectra of residues from SDPs were assigned and concluded in Table 3.

Table 3 Assignments of ^{1}H and ^{13}C NMR spectra for SDPs

Linkage	Unit	H1-C1	H2-C2	H3-C3	H4-C4	H5-C5	H6-C6
1, 4-*α*-Glc*p*	A	5.41/102.54	3.63/74.47	3.85/74.08	3.67/79.72	3.97/76.25	3.85–3.83/63.40
1, 5-*α*-Ara*f*	B	5.07/110.29	4.09/86.98	4.03/79.69	4.19/84.28	3.94/69.39	
1, 4-*β*-Gal*p*	C	4.53/105.98	3.75/71.12	3.68/75.58	3.95/76.41	3.67/75.57	3.76/64.10
1, 4-*α*-Gal*p*A	D	5.14/102.27	3.98/71.33	4.13/71.35	4.41/80.97	4.73/74.32	– /173.88

(–): not detected

Antioxidant activity of SDPs

The ABTS radicals scavenging activities of SDPs

The effects of SDPs on scavenging ABTS radicals were investigated. The ABTS radicals scavenging rates were plotted as a function of the concentrations of SDPs and shown in Fig. 6. All these four fractions showed concentration-dependent scavenging activity against ABTS radicals. The ABTS scavenging rate of SDP3 and SDP4 are significantly higher than that of SDP2 only at the concentrations above 5 mg·mL^{-1}. The ABTS scavenging rate of SDP1 was the lowest. The scavenging rates of SDPs are 17.75%, 37.37%, 80.93% and 75.89% at 20.0 mg·mL^{-1}, respectively.

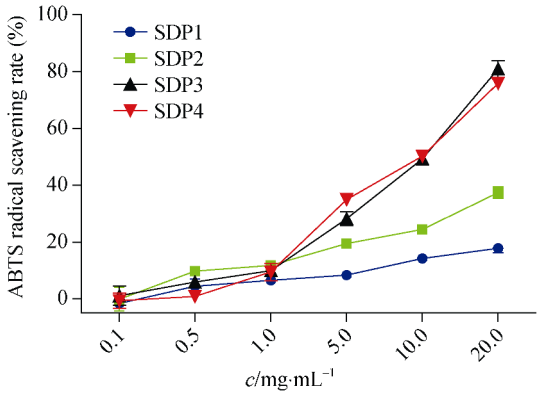

Fig. 6 ABTS radicals scavenging rate of SDPs in the concentration between 0.1 to 20.0 mg·mL^{-1} (mean ± SEM, *n* = 3)

Protective activity of SDP fractions on H_2O_2-induced RAW264.7 cells

We induced oxidative stress on RAW264.7 cells with H_2O_2. Incubation of RAW264.7 cells with certain concentration of H_2O_2 could increase intracellular reactive oxygen species (ROS) and cause a degree of apoptosis [28]. Pre-incubation of RAW264.7 cells with SDPs at different concentrations prior to H_2O_2 treatment could protect the cells from oxidant injury. The results were shown in Fig. 7. The viability of RAW264.7 in blank group with H_2O_2 treatment (H_2O_2 group) was decreased significantly to ~50%. All four fractions showed protective activity trends. No significant protective activity was observed from SDP1 until its concentration was increased to 10 μg·mL^{-1}, and the viability of the cells incubated with 100 μg·mL^{-1} SDP1 was still lower than those in the negative control groups. Significant protective activity of SDP2 was shown at its concentration reached 10 μg·mL^{-1}, and the cell viability was comparable to those of normal cells without H_2O_2 treatment in the negative control group when the concentration of SDP2 is 100 μg·mL^{-1}. The cells were protected by SDP3 and SDP4 very well. The cell viability did not decrease comparing to normal cells without H_2O_2 treatment in negative control when they were protected by 1 μg·mL^{-1} SDP3 or SDP4 and treated by H_2O_2 afterwards. When the concentrations of SDP3 and SDP4 were increased to 10 and 100 μg·mL^{-1} the cells were proliferated to about 150%.

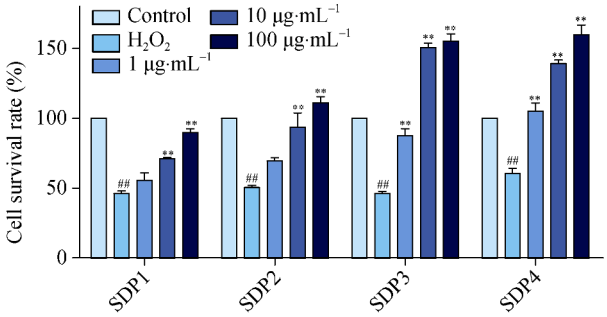

Fig. 7 The cell survival rate of RAW264.7 under protection of SDPs. The values are presented as mean ± SEM, *n* = 5, $^{\#\#}P < 0.01$ *vs* the control group, $^{**}P < 0.01$ *vs* the H_2O_2 group

Discussion and Conclusions

In this work, polysaccharide was extracted from traditional Chinese herb, *Saposhnikovia divaricata* (SD), and it was fractionated with DEAE. The molecular weight, monosaccharide compositions, linkage modes and structural properties of the four fractions were characterized with SEC-MALS-RI, HPAEC-PAD, GC-MS and ^{1}H and ^{13}C NMR. SDP1 was assigned as a 1, 4-*α*-glucan with small amount of O-6 linked branches, unambiguously. Based on the monosaccharide composition analysis, SDP2 still contained a big amount of the 1, 4-*α*-glucan presented in SDP1 and a small amount of arabinogalactan, but SDP3 contained relatively lower amount of the 1, 4-*α*-glucan and a big amount of the arabinogalactan. According to the results from GC-MS analysis, the arabinogalactan involved 1, 5-*α*-linked araban backbone with O-3 substituted 1, 5-Ara*f* branches and different linked galactans. NMR analysis revealed the mainly existed 1, 5-*α*-Ara*f* and 1, 4-*β*-Gal*p* which further demonstrate that it is a typical arabinogalactan [29]. Based on monosaccharide compositional analysis with HPAEC-PAD, SDP4 was an arabinogalactan combined with polygalacturonic acid. GC-MS analysis demonstrated that SDP4 contained higher branched

araban and 1, 4-*β*-linked galactan. Meanwhile NMR analysis told us the component of polygalacturonic acid was 1, 4-*α*-linked and some of GalA residues were methylated, just like pectin. Thus, SDP4 could be defined as a pectic arabinogalactan which the main chain was 1, 4-*β*-Gal*p* and the side chains were 1, 5-*α*-Ara*f* residues combined with 1, 4-*α*-linked polygalacturonic acid.

All these four fractions showed antioxidant activities in both molecular and cellular levels. They showed ABTS radical scavenging activities and the degree of antioxidant activity was ranked as SDP4 ≈ SDP3 > SDP2 > SDP1. The four fractions could also protect RAW264.7 cells from H_2O_2-induced cellular damage and apoptosis. Based on the cell viability test, their antioxidant activity were also ranked as SDP4 ≈ SDP3 > SDP2 > SDP1. Moreover, SDP3 and SDP4 proliferated RAW264.7 significantly when the higher concentrations were used, suggesting their potential function about proliferation of macrophage beyond or based antioxidant induced cellular protection. The results concluded from antioxidant activity of SDPs further showed that the molecular weight distribution of polysaccharides could affect its biological activities to some extent which were in accordance with literature reports [30-31]. Based on the relationship between structural properties of these four fractions and their primary activity test, SDP1 which was the 1, 4-*α*-glucan in SD had weakest antioxidant activity and showed the lowest activity. Accordingly, the high antioxidant activity of SDP3 should not come from the 1, 4-*α*-glucan portion, which was about 20% based on monosaccharide composition analysis. Meanwhile, SDP4 showed similar and the highest antioxidant activity in both molecular and cellular levels. It contained about 80% arabinogalactan and 20% pectin (monosaccharide composition). Thus, it implies that arabinogalactan in SD is the major active component contributed to the antioxidant activity of SDP. This conclusion was also supported by other literature reports that arabinogalactan extracted from herbal plants usually possess significant bioactivities [32-33]. Moreover, the existed pectin in SDP4 could also be a main reason for its significant antioxidant activity considering the other studies of bioactivities on pectin [34-35].

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Cite this article as: MENG Yao, YI Lin, CHEN Lei, HAO Jie, LI Du-Xin, XUE Jie, XU Nai-Yu, ZHANG Zhen-Qing. Purification, structure characterization and antioxidant activity of polysaccharides from *Saposhnikovia divaricata* [J]. *Chin J Nat Med*, 2019, **17**(10): 792-800.

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