Structural Characterization of Water-Soluble Polysaccharides from Sophora flavescensAit. and Their Anti-Inflammatory Evaluation Based on NO Release

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Abstract

Background: The dried roots of *Sophora flavescens* Ait. are traditionally used as Sophora Flavescens (Kushen in Chinese) to treat inflammatory diseases. It is traditionally served as a decoction, and polysaccharides represent one the major chemical constituents of this decoction. How about the structure of *S. flavescens* polysaccharides and whether they have anti-inflammatory activity should be uncovered.

Methods: The purified polysaccharides were isolated through a combination of ion-exchange chromatography on DEAE 650 M and gel filtration on Superdex G-200 from hot water extract of *S. flavescens.* Structure was characterized by chemical derivatization as well as HPLC, FT-IR, GC-MS and NMR technologies. The preliminary *in vitro* antiinflammation activity was tested on RAW 264.7 cells upon NO release inhibition.

Results: In this study, four polysaccharides, namely, SFNP-1, SFNP-2, SFAP-1, and SFAP-2, were isolated from *S. flavescens*. Results showed that both SFNP-1 and SFNP-2 contained (1→4)-linked glucans with small amounts of side chains at the O-4 position of the backbone chain residues. The two acidic polysaccharides (i.e.,SFAP-1 and SFAP-2) were identified to be pectin-type polysaccharides mainly containing a homo-galacturanan backbone consisting of *α*-(1→4)-linked GalA*p* and methyl-esterified *α*-(1→4)-linked GalA*p* residues at a ratio ofapproximately1:1. The bioactivity test revealed that the four purified polysaccharides have no cytotoxicity on RAW264.7andthat SFNP-1 and SFNP-2 show significant stimulating activity. Although the decoction of *S. flavescens* has been traditionally used as an anti-inflammatory agent, NO release inhibition results showed thatSFAP-1 and SFAP-2, as the major polysaccharides of SFCP, do not have significant anti-inflammatory effects.

Conclusions: This result suggests that the anti-inflammatory effect of the decoction of *S. flavescens* may depend on the presence of alkaloids and not the polysaccharides it contains.

1. Background

Inflammation is a common and important basic pathological process. Traumatic infection of body surfaces and most common and frequently occurring diseases of various organs (e.g., pneumonia, hepatitis, and nephritis) may be classified as inflammatory diseases[1]. Anti-inflammatory drugs have become the second class of drugs, second only to anti-infective drugs. Adrenocorticoid hormones and nonsteroidal anti-inflammatory drugs are widely used in the clinical setting, but they have been reported to induce a number of adverse reactions. Many plant materials traditionally used in inflammatory diseases have been explored in view of the immunomodulating activity of polysaccharides. In the current study, the anti-inflammatory effects of *Sophora flavescens* Ait. (*S. flavescens*) are investigated.

S. flavescens belongs to the Leguminosae family and is widely distributed all over China. Its dried roots are traditionally used as Sophora Flavescens(Kushen in Chinese) to treat inflammatory diseases, fever, gastrointestinal bleeding, pubic swelling and itching, skin pruritus, acute dysentery and eczema, and other

diseases [2]. Modern pharmacological studies have shown that *S. flavescens* has pharmacological properties, such as anti-inflammation, antitumor, analgesia, antiallergy, antipathogen, antiarrhythmia, antimyocardial ischemia, and antidiarrhea effects [3–8]. Research reportsthat the main material bases for the pharmacological action of *S. flavescens* are alkaloids and flavonoids[9, 10]. Further studies showed various chemical constituents, including phenolic acid, triterpenoid saponins, polysaccharides, lignans, and a small amount of phenylpropanoids[11], with significant biological activities. Most current reports focus on the alkaloids and flavonoids in *S. flavescens* [10]. *S. flavescens*is traditionally served as a decoction, and polysaccharides represent one the major chemical constituents of this decoction. As a substance found widely in plants, polysaccharides have unique pharmacological and biological properties, including antitumor, immunity regulation, antioxidation, antivirus, and especially, antiinflammation activities as reviewed elsewhere [12–15]. However, few reports are available on the study of *S. flavescens* polysaccharides. Thus, how about the structure of *S. flavescens* polysaccharides and whether they have anti-inflammatory activity must be uncovered. In the present work, the crude polysaccharide fraction and homogeneous polysaccharides were isolated from *S. flavescens* and structurally characterized. Then, the anti-inflammatory activities of these polysaccharides were evaluated by using the RAW264.7 cell line.

2. Materials And Methods

2.1 Materials and chemicals

The dried roots of *S. Flavescens* were purchased from the local market in Anguo, Hebei province, China. The dextrans (MW 1100, 670, 410, 270, 150, 80, 50, 12, and 5 kD)and monosaccharide standards (L-arabinose, D-xylose, L-rhamnose, L-fucose, D-mannose, D-glucose,and D-galactose)were purchased from Sigma and domestic companies, Milli-Q water was prepared by SCM-80, and ion exchange resin (Toyopearl DEAE-650M) was obtained from Tosoh, Japan. Sepharose 6 B and Sephacryl S-300 HR were obtained from GE Health Care (Amersham Biosciences AB, Uppsala, Sweden). RPMI 1640 medium was obtained from GIBCO (Grand Island, NY, USA) and supplemented with 10% fetal bovine serum (FBS, Hy Clone Laboratories, Logan, Utah, USA). Other chemicals were obtained from Sigma-Aldrich Co., LLC. A PL aquagel-OH mixed-H (7.5 mm × 300 mm, 8 μm) column was purchased from Agilent (USA).

Vacuum concentration was performed on an N1100 rotary evaporator equipped with an OSB-2100 oil bath pot (Shanghai Ailang Instrument Co., Ltd.). High-performance gel permeation chromatography (HPGPC) was conducted by using an Agilent 1200 system coupled to an evaporative light-scattering detector (ELSD). Gas chromatography–mass spectroscopy (GC–MS) was performed on an Agilent GCMS-5975systemusing helium as the carrier gas. Fractions obtained from column chromatography were collected and monitored via thephenol–H_2SO_4 method and UV absorbance at 280 nm to monitor proteins.

2.2 Extraction, isolation, and purification of polysaccharides

The dried roots of *S. flavescens* (500 g) were pulverized and defatted twice using 95% EtOH for 12 h. The residues were extracted thrice with hot H_2O for 3 h under reflux. After filtration through defatted cotton, concentration in vacuo and centrifugation were performed at 3000 rpm for 20 min to remove the residues. The supernatant was concentrated and precipitated with 80% EtOH (final concentration) at 4 °C overnight. The precipitate was collected by centrifugation and washed with ethanol. The solid was dissolved in distilled H_2O and centrifuged. The supernatant was deproteinated four times via the Sevage method. Finally, the solution was lyophilized in a vacuum freeze dryer to obtain the crude polysaccharide (SFCP, 5.2%).

As shown in Fig. 1, SFCP (26.0 g) was suspended in 80 mL of H_2O, stirred at room temperature, and centrifuged at 5000 rpm for 15 min to remove insoluble portions (0.80%). The supernatant (10 mL) was subjected to anion-exchange chromatography on a Toyopearl DEAE-650M column (5.0 i.d.×30 cm) and eluted with the following: distilled H_2O; 0.5 M, 1.0 M and 2.0 M NaCl; and 0.2 M NaOH. This process yielded two major fractions, namely, SFN (12.4%; eluted with distilled H_2O), and SFA (38.8%; eluted with 0.5 M NaCl). Fractions of 20 mL were collected and monitored at 480 nm via the phenol–H_2SO_4 method and at 280 nm by UV absorbance spectroscopy.

The H_2O-eluted fraction SFN (500 mg) was loaded on a Sepharose 6 B column (5.0 i.d.×100 cm) and eluted with 0.1 M NaCl to provide two fractions, namely, SFN-1, SFN-2, SFN-3, and SFN-4. SFN-1 and SFN-4, as the major two fractions, were purified once more on a Sephacryl S-300 HR column (2.2 i.d.×100 cm) to provide the purified polysaccharides SFNP-1 (17.2%) and SFNP-2 (37.6%) based on the fraction SFN. Fractions of 6 mL were collected and monitored at 480 nm via the phenol–H_2SO_4 method and at 280 nm by UV absorbance spectroscopy.

As the major part of acidic polysaccharides, the 0.5 M NaCl-eluted fraction SFA (3.0 g) was subjected again to anion-exchange chromatography on DEAE 650 M column (5.0 i.d.×30 cm) and eluted with 0.1 M, 0.2 M, 0.3 M, and 0.5 M NaCl. This process yielded two major fractions, namely, SFA1 (28.8%; eluted with 0.1 M NaCl), and SFA2 (29.3%; eluted with 0.2 M NaCl). The two major acidic fractions SFA1 and SFA2 were separately subjected to gel filtration chromatography on a Sepharose 6B column and eluted with 0.1 M NaCl solution to give the major fractions SFA1B and SFA2C, respectively. Fractions of 15 mL were collected and monitored at 480 nm via the phenol–H_2SO_4 method and at 280 nm by UV absorbance spectroscopy. After dialysis (cut-off, 7 kD) and concentration of SFA1B and SFA2C, the fractions were purified by gel filtration on a Sephacryl S-300 HR column to give the purified polysaccharides SFAP-1 (293 mg) and SFAP-2 (300 mg).

2.3 Estimation of homogeneity and apparent molecular weight

The molecular weight distributions of SFAP-1 and SFAP-2 were determined by HPGPC on an Agilent 1200 system equipped with an ELSD detector. Samples (5 mg/mL, 10 μL) were applied to a PL aquagel-OH mixed-H column (7.5 mm × 300 mm, 8 μm) and eluted with 0.1 M $NaNO_3$ at 0.6 mL/min with the column

temperature maintained at 35 °C. Commercially available dextrans (MW 1100, 670, 410, 270, 150, 80, 50, 12, and 5 kD) were used as standard molecular markers. Molecular weights were estimated by referring to a calibration curve made from the series of dextran standards.

2.4 Colorimetric analysis

The contents of total carbohydrates and uronic acid were determined via the phenol–H_2SO_4[16] and *m*-hydroxydiphenyl [17] methods, respectively. Galactose(Gal)and galacturonic acid(GalA)were used as standards. ABio-Rad protein analysis kit was used to analyze the protein content on the basis of BSA.

2.5 Reduction of SFAP-1 and SFAP-2

The uronic acid residues of SFAP-1 and SFAP-2 were reduced to their corresponding neutral sugars before structural analysis according to a previously reported method [18]. Briefly, $NaBH_4$was used to reduce carboxyl esters in imidazole buffer to form 6,6-dithiosaccharides. After reduction, free uronic acid was activated by 1-cyclohexyl-3-(2-morpholine ethyl) carbodiimide and reduced by $NaBH_4$. The resulting products obtained from SFAP-1 and SFAP-2were named SFAP-1R and SFAP-2R, respectively.

2.6 Analysis of monosaccharide composition

The monosaccharide composition of the polysaccharides was analyzed by GC–MS as described previously [19]. SFNP-1 and SFNP-2 were directly hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h according to the routine method of complete hydrolysis for neutral polysaccharides. Equal amounts of native and reduced samples of the acidic polysaccharides (SFAP-1 and SFAP-2) were hydrolyzed as described above. After removal of TFA under nitrogen gas, the hydrolysates were converted into alditol acetates and then subjected to GC–MS using a fused silica capillary column (HP-5 MS, 30 mm × 0.25 mm i.d., 0.25 μm, Agilent). The injection and detector temperatures were maintained at 240 °C. The oven temperature was programmed to increase from 160 °C to 190 °C at a rate of 2 °C/min and then to 240 °C at a rate of 5 °C /min. Then, the temperature was kept at 240 °C for 5 min. Helium was used as the carrier gas.

2.7 Methylation analysis

The neutral polysaccharides SFNP-1 and SFNP-2 were directly applied to methylation analysis according to Ciucanu's method [20]. Thecarboxyl residues of the acidic polysaccharides SFAP-1 and SFAP-2were reduced prior to methylation analysis. Then, equal amounts of native and reduced SFAP-1 and SFAP-2 were methylated according to Ciucanu's method [20]. The methylated products were hydrolyzed, reduced, and acetylated to form partially methylated alditol acetates. The partially methylated alditol acetates were analyzed by GC–MS using an HP-5 MS fused silica capillary column (30 m × 0.32 mm i.d., J&W Scientific Inc., CA, USA). The compounds corresponding to each peak were identified by interpretation of their characteristic mass spectra and retention relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol. The molar ratio of each residue was calculated byusing peak areas.

2.8 Infrared and NMR spectroscopies of polysaccharides

Fourier transform infrared spectra were recorded on a Nicolet Nagna-IR 550 spectrophotometer in the 4000–400 cm^{-1}region using KBr tablets.

NMR spectra were recorded by using a Varian INOVA 300 NMR spectrophotometer (Varian, Palo Alto, CA, USA). Each sample (30 mg) was dissolved in D_2O (99.8 Atom% Deuterium, Schweres Wasser, USA), and all spectra were recorded at 30 °C. HDO was used as the internal standard.

2.9 Cell line and culture medium

The murine macrophage cell line RAW 264.7 was purchased from the American Type Culture Collection (Manssas, VA, USA) and grown in RPMI 1640 medium containing 10% FBS and penicillin (100 IU/mL).

2.10 Cell viability assay

The cytotoxicity of the polysaccharides was evaluated. Briefly, macrophages were seeded at a density of $5{\cdot}10^4$ cells/mL in 96-well plates and cultured overnight. Samples (SFCP, SFN, SFA, SFNP-1, SFNP-2, SFAP-1, and SFAP-2) were added to the wells at final concentrations of 0, 1.6, 8, 40, 200, 500, and 1000 μg/mL. After 24 h of incubation at 37 °C in a humidified atmosphere containing 5% CO_2, 30 μL of amixed solution of 3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxylphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) reagent (20:1 v/v) was added to each well. After 1 h of incubation, absorbance was recorded at 490 nm usinga microplate reader (SpectraMax 190, Molecular Devices Corporation, Sunnyvale, CA, USA) and then transformed into a macrophage viability ratio for comparison.

2.11 NO production assay

Nitrite concentration, which can be used as a measure of NO production, was assayed by the standard Griess method. Briefly, cells were seeded in a 96-well plate at a cell density of 1×10^5cells/mL (100 μL) and cultured for 24 h. One hundred microliters of lipopolysaccharide (LPS, 1 μg/mL) was used to replace the original medium. After 1 h of inoculation, 100 μL of the purified acidic polysaccharides SFAP-1 and SFAP-2(final concentrations of 0, 100, 500, and 1000 μg/mL and of0, 0.1, 1, 10, and 100 μg/mL, respectively) was used to replace the LPS. The cells were further incubated for 40 h at 37 °C in a humidified atmosphere containing 5% CO_2. Then, 30 μL of a mixed solution of MTS and PMS reagent (20:1 v/v) was added to each well. After 1 h of incubation, the absorbance was recorded at 540 nm using a microplate reader. Nitrite concentration was calculated from a $NaNO_2$ standard curve.

2.12 Statistical analysis

All experiments described were repeated thrice, and results are expressed as the mean ± SD of triplicate analyses. Statistical significance was analyzed by one-way ANOVA using SPSS 16.0 software. *P* values less than 0.05 were considered statistically significant.

3. Results And Discussion

3.1 Isolation, homogeneity, and estimation of apparent molecular weight of the polysaccharides

The crude polysaccharide fraction SFCP (yield, 5.2% of the dried materials) was obtained from *S. flavescens* by hot water extraction, 80% EtOH precipitation, and dialysis (cutoff, 7 kD). As shown in Fig. 1, SFCP was fractionated into a neutral polysaccharide fraction (SFN) and several acidic polysaccharide fractions (SFAs) by ion-exchange chromatography. SFA, which was eluted with 0.5 M NaCl, dominated the crude fraction. HPGPC (Fig. 2a) showed that the molecular weight distribution of the SFAP fractions is consistent with that of SFCP; this finding suggests that the *S. flavescens* crude polysaccharide is mainly composed of SFA. SFN made up a small part of SFCP and was distributed in the lower-molecular weight region of the latter. Two neutral polysaccharides (i.e., SFNP-1 and SFNP-2) were purified from the fraction SFN by gel filtration on Sepharose 6 B and Sephacryl S-300 HR columns. Two acidic polysaccharides (i.e., SFAP-1 and SFAP-2) were obtained from the major acidic fraction SFA by ion-exchange and gel filtration chromatography. The four polysaccharides obtained were all purified as colorless powders and eluted as single symmetrically sharp peaks on the corresponding HPGPC chromatograms (Fig. 2b); these findings suggest their homogeneous properties. The apparent molecular weights of the polysaccharides were estimated from the dextran standard curve and are summarized in Table 1.

Table 1
Chemical (%, w/w) and sugar composition (%, Area) of purified polysaccharides from *Sophora flavescens* Ait.

Polysaccharides	SFNP-1	SFNP-2	SFAP-1	SFAP-2
Apparent molecular weight (× 10^3)	41.4	8.0	87.7	104.6
Chemical composition				
Total sugar content (%)[a]	99.4	96.5	90.0	78.9
Protein content(%)[b]	1.2	1.7	0.5	1.2
Uronic acid content(%)[c]	-	-	84.2	92.5
Sugar composition				
Rha	-	-	tr.[d]	4.6
Ara	18.0	13.4	8.6	6.0
Xyl	2.7	tr.	-	-
Glc	54.6	76.9	-	-
Gal	24.7	8.8	2.0	1.5
GalA	-	-	89.4	92.6

[a] Determined by phenol-sulfuric acid-H_2SO_4 method;[b] Determined by Bio-Rad protein assay kit;[c] Determined by m-hydroxydiphenyl method; [d] the amount less than 1.0%.

3.2 Structural characterization of the polysaccharides

The contents of total carbohydrate, uronic acid, and protein were determined by colorimetric analyses. As summarized in Table 1, the four polysaccharides contained large amounts of carbohydrates and small amounts of proteins (< 2%). No uronic acid was detected in SFNP-1 and SFNP-2, which suggests that these polysaccharides are neutral polysaccharides. By contrast, large amounts of uronic acids were determined in SFAP-1 and SFAP-2, which suggests their acidic property.

The monosaccharide compositions of the purified polysaccharides were determined as alditol acetates by GC–MS. SFNP-1 and SFNP-2 were mainly composed of glucose, small amounts of arabinose(Ara) and Gal, and small amounts of xylose. Before detecting the monosaccharide composition of the acidic polysaccharides, the -COOH groups of uronic acid residues were first reduced to -CH_2OH. It is concluded that the three polysaccharide samples are completely reduced as suggested from the disappearance of the aborsbance band around at 1750 cm^{-1} in the FT-IR spectrum(Fig. 3).Monosaccharide composition

analysis showed that a large amount of GalA and small amounts of Gal and Ara are present in SFAP-1 and SFAP-2, thus supporting the uronic acid content results. Rhamnose (Rha) was also detected in small amounts (4.6%) in SFAP-2but in trace amounts in SFAP-1. The presence of predominantly composed of GalA residues, with minor amounts of Ara, Gal, and Rha residues, suggesting their pectin-type feature [21]. The GalA glycosyl residues were calculated from the increase in amount of Gal residues in the reduced polysaccharides compared with that in native polysaccharides.

The glycosyl-linkage composition of the two neutral polysaccharides was analyzed by methylation and GC–MS determination. As shown in Fig. 4, (1→4)-linked Glc*p* residues dominated the glycosyl residues in SFNP-1 and SFNP-2, thus suggesting the presence of (1→4)-linked glucans. Small amounts of terminally linked (t-) and (1→4, 6)-linked Glc*p* residues were observed, thus suggesting a small amount of branching at the O-4 position of the backbone chain residues. In addition, small amounts of t- and (1→5)-linked Ara*f* and t-, (1→3)-, (1→4)-, and (1→3,6)-linked Gal*p* residues were determined, as summarized in Table 2. These glycosyl residues may reflect the presence of impurities.

Table 2
The linkage analysis of SFNP-1 and SFNP-2 determined by methylation and GC-MS analyses.

Glycosyl residues	Substituted position	SFNP-1 (mol %)	SFNP-2 (mol %)
Ara*f*			
	T [a] -	3.8	3.7
	1,5-	9.8	5.4
Xyl*p*			
	1,4-	3.1	Tr. [b]
Gal*p*			
	T-	3.4	-
	1,3-	2.1	-
	1,4-	2.1	2.6
	1,3,6-	4.8	-
Glc*p*			
	T-	5.1	3.0
	1,4-	62.0	80.8
	1,4,6-	3.7	4.6

[a] terminally linked; [b] trace amount.

The linkages of GalA residues are usually deduced from increases in Gal residues compared with those in the native form. Therefore, equal amounts of SFAP-1 and SFAP-1R andofSFAP-2 and SFAP-2R were subjected to methylation analysis. When the PMAA derivatives were applied to GC–MS analysis, the PMAAs from native polysaccharides were not detected; this finding maybe due to the predominant presence of GalA, which is resistant to TFA hydrolysis and, therefore, lost during the post-treatment steps, as reported previously [22]. The results suggest that the amount of Gal residues in reduced form could serve as GalA residues in native form SFAP-1 or SFAP-2. According to this deduction, GalA predominantly exists as (1 → 4)-linked GalA*p*, and small amounts of GalA occur as terminally linked GalA residues (t-GalA*p*). Small amounts of terminally linked, (1→5)-, and (1→3, 5)-linked arabinosyl residues were also observed. Thus, SFAP-1 and SFAP-2 were deduced to be typical pectin-type polysaccharides containing a homo-galacturonan backbone due to their dominant feature of a linear chain of (1 → 4)-linked GalA units (smooth region)[23].

To further interpretation of the backbone structure of SFAP-1 and SFAP-2, 1D- and 2D-NMR spectra were recorded to examine the backbone structures of SFAP-1 and SFAP-2. As shown in Fig. 5, the ^{1}H- and ^{13}C-NMR spectra of SFAP-1 and SFAP-2 are highly similar, thus suggesting comparable structural characteristics, consistent with the results of methylation analysis. Thus, only the structure of SFAP-1 was analyzed here.

In the ^{1}H-NMR spectrum of SFAP-1 (Fig. 5a), the strong signal at δ3.75 ppm is derived from the esterified methyl groups of GalA, as deduced from the correlation between δ3.75 and δ170.7 ppm in the HMBC spectra and comparison with the reported values [19, 22]. The signal at δ52.8 ppm could be assigned to the esterified methyl groups of GalA, and signals at δ174.6 and δ170.7 ppm were attributed to the carboxyl groups of GalA (residue A) and methyl esterified GalA (residue B), respectively [22]. In the HSQC spectrum, the three signals at δ5.02, δ5.04, and δ4.90 ppm were respectively assigned to H-1 of residues A, B, and C in the ^{1}H-NMR spectrum and corresponded to the carbon signals at δ100.1, δ100.5, and δ101.0 ppm in the ^{13}C-NMR spectrum (Fig. 5b). In the high-field region, four major signals at δ3.67 (67.9), δ4.00 (68.4), δ4.41 (78.5), and δ5.10 (70.5) ppm were respectively assigned to H-2 (C-2), H-3 (C-3), H-4 (C-4), and H-5 (C-5) of the major residue B According to the HSQC spectrum and literature data [22–25], residue A is α-(1→4)-linked D-GalA. The strong signal at δ174.6 ppm, which is assigned to the carboxyl group (C-6) of residue A, supports this deduction. Moreover, according to the HSQC spectrum, among the signals obtained, H-5 appeared to overlap with the HDO signal (Fig. 5c). The anomeric signal at δ4.90 (100.0) ppm and the signal at δ174.6 ppm were assigned to terminally linked D-GalA resulting from residue C on the basis of the HSQC and literature data [22–25]. The chemical shifts of H-2, H-3, H-4, and H-5 of residues A and C were deduced from the HSQC spectrum, as shown in Table 3. Weak signals(δ1.32/19.6 ppm) appearing in the high-field region of the ^{1}H- and ^{13}C-NMR spectra were assigned to H6 and C6 of the Rha residue, thus suggesting the presence of a small amount of Rha. Although the correlation signals of residues A, B, and C were not observed in the HMBC spectrum (not shown here), the results of methylation analysis and the reference NMR data imply that SFAP-1 is a galacturonan predominantly composed of highly methyl-esterified α-(1→4)-linked GalA residues. The degree of methyl-esterification (DM) was estimated to be 57.4% on the basis of the reported method [26]. The weak signal at δ2.02 ppm suggests that the backbone sugar may also be substituted at the O-2 and/or O-3 positions by small amounts of acetyl groups, similar to the structure of previously reported pectin-type polysaccharides [26]. Taking the results together, SFAP-1 was deduced to be a native pectin-type polysaccharide containing a homo-galacturanan backbone consisting of α-(1→4)-linked D-GalA*p* and methyl-esterified α-(1→4)-linked D-GalA*p* residues at a ratio ofapproximately1:1.

Table 3

^{1}H-, ^{13}C-NMR chemical shift data (δ ppm) of the major sugar residues in main chain of SFAP-1.

Residues	C1/H1	C2/H2	C3/H3	C4/H4	C5/H5	C6	OCH_3
A [a]	99.1	67.9	68.4	77.9	71.0	174.6	
	5.02	3.67	4.00	4.38	4.79		
B [b]	99.6	67.9	68.4	78.5	70.5	170.7	52.8
	5.04	3.67	4.00	4.41	5.10		3.75
C [c]	100.0	67.9	68.4	79.0	71.0	174.6	
	4.90	3.67	4.00	4.64	4.79		

[a] the residue of (1→4)-*α*-GalA*p*; [b] the residue of methyl-esterified (1→4)-*α*-GalA*p*; [c] the residue of terminally linked *α*-GalA*p*.

Based on its similarity to SFAP-1, SFAP-2 mainly contains a homo-galacturonan composed of highly methyl-esterified and partially acetylated α-(1→4)-linked GalA residues. The DM of this polysaccharide was estimated to be 55.2%.

3.3 Cytotoxicity and anti-inflammatory activities of the polysaccharides

In this experiment, the cytotoxicity of the sugar-containing parts of *S. flavescens* was tested. As shown in Fig. 6, the crude polysaccharide fraction SFCP and other fractions (SFN and SFA) exhibited cytotoxicity at concentrations higher than 200 μg/mL. After purification, neither SFNP-1 and SFNP-2 isolated from SFN nor SFAP-1 and SFAP-2 isolated from SFA showed any cytotoxicity.

Inflammation is a natural biological response to injury or infection in the human body. Inhibition of the production of inflammatory mediators, such as NO, and inflammatory cytokines, such as tumor necrosis factor α,interleukin 6, and interleukin 1*β*, serves as a key mechanism in controlling inflammation [27]. In this study, the inflammatory mediator NO was used to test of the anti-inflammation activity of the extracted polysaccharides. LPS, as an endotoxin from Gram-negative bacteria, can induce macrophages to release the inflammatory mediator NO and proinflammatory factors. Therefore, LPS was employed in the present work to stimulate RAW264.7 cells and build an experimentally inflammatory model in vitro.

The inflammatory cell model was constructed by stimulating macrophages with LPS to release NO; then the samples at the concentrations of 100, 500, and 1000 μg/mL were added. Because the significant cell proliferation activity of neutral polysaccharides, here, only acidic polysaccharides, i.e., SFAP-1 and SFAP-2 were applied to evaluate anti-inflammation effects. The results are shown in Fig. 7a.The purified acidic polysaccharides SFAP-1 and SFAP-2 did not present any inhibitory effect on NO release. On the contrary,

they significantly stimulated NO production in a dose-dependent manner compared with not only the control group ($P < 0.001$) but also the LPS group at high-concentrations (100, 500, 1000 μg/mL). Some polysaccharides have been reported to show anti-inflammation activity at low concentrations. For instance, a polysaccharide from *Moringa oleifera* roots, MRP-1, exhibits anti-inflammation activity by suppressing the release of NO when applied at concentrations of 25, 50, and 100 μg/mL[28]. The *Apios americana* Medikus tuber polysaccharide ATP-1 suppresses NO release inLPS-induced RAW264.7 cells when applied at concentrations of50, 100, and 150 μg/mL [29]. Thus, the anti-inflammatory activities of low concentrations ofSFAP-1 and SFAP-2 toward NO production in LPS-induced RAW264.7 cells were evaluated. As shown in Fig. 7b, neither of the polysaccharides significantly inhibited NO production compared with the LPS group. In summary, SFAP-1 and SFAP-2 cannot inhibit NO release in LPS-induced RAW264.7 cells regardless of the applied concentration. This finding suggests that SFAP-1 and SFAP-2 do not possess anti-inflammatory activity. Our results demonstrate that the polysaccharides of the decoction of *S. flavescens* may not be the major effective substances with anti-inflammatory activity.

4. Conclusion

Four polysaccharides, namely, SFNP-1, SFNP-2, SFAP-1, and SFAP-2, were isolated from *S. flavescens*. Structural analyses revealed that SFNP-1 and SFNP-2 contain (1→4)-linked glucans with small amounts of side chains at the O-4 position of the backbone chain residues. Small amounts of t- and (1→5)-linked Ara*f* and t-, (1→3)-, (1→4)-, and (1→3, 6)-linked Gal*p* residues were also found. The two acidic polysaccharides, SFAP-1 and SFAP-2, were identified as pectin-type polysaccharides mainly containing a homo-galacturanan backbone consisting of *α*-(1→4)-linked GalA*p* and methyl-esterified *α*-(1→4)-linked GalA*p* residues at a ratio of approximately 1:1. This study is the first to report a purified pectin-type polysaccharide from *S. flavescens*. The bioactivity test revealed that the four purified polysaccharides have no cytotoxicity to RAW264.7 and that SFNP-1 and SFNP-2 show significant stimulating activity. Although the decoction of *S. flavescens* has been traditionally used as an anti-inflammatory agent, NO release inhibition results indicated thatSFAP-1 and SFAP-2, as major polysaccharides of SFCP, do not have significant anti-inflammatory effects. This result suggests that the anti-inflammatory effect of the decoction of *S. flavescens* may depend on the presence of alkaloids and not the polysaccharides it contains.

Abbreviations

SF: *Sophora flavescens*; SFNP: *Sophora flavescens* neutral polysaccharide; SFAP: *Sophora flavescens* acid polysaccharide; NMR: Nuclear Magnetic Resonance; HPGPC: High Performance Gel Permeation Chromatography; FT-IR: Fourier Transform Infrared; GC-MS: Gas Chromatography-Mass Spectrometry; HPLC: High Performance Liquid Chromatography; ELSD: Evaporative Light Scattering Detector; TFA: Trifluoroacetic Acid; PMS: Phenazine methosulfate; MTS: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; LPS: lipopolysaccharide; Glc: Glucose; Gal: Galactose; GalA: Galactose Acid; Rha: Rhamnose; FBS: Fetal bovine serum.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors critically reviewed the content of the manuscript. The consent for publication was obtained from all authors.

Availability of data and materials

The data used in this study can be obtained from the corresponding author upon reasonable request.

Competing interests

The author declares that there is no conflict of interest with the publication of this article.

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Authors' contributions

CXD, HY and JD conceived and designed the research; YXL, KGL, SLJ, and YSC per-formed the experiments; YXL, and KGL analyzed the data; YXL and CXD wrote the manuscript. All authors read and approved the final manuscript.

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Figures

Fig. 1

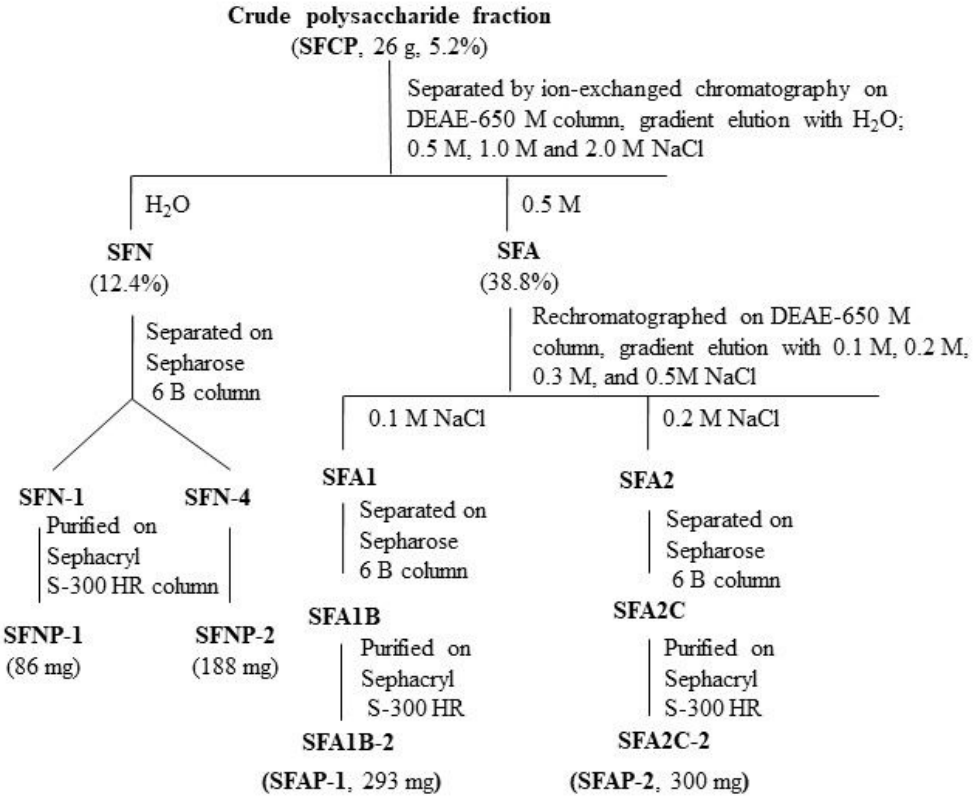

Figure 1

Purification ofSFNP-1, SFNP-2, SFAP-1 and SFAP-2 by the combination of ion-exchange and gel permeation chromatography.

Fig. 2

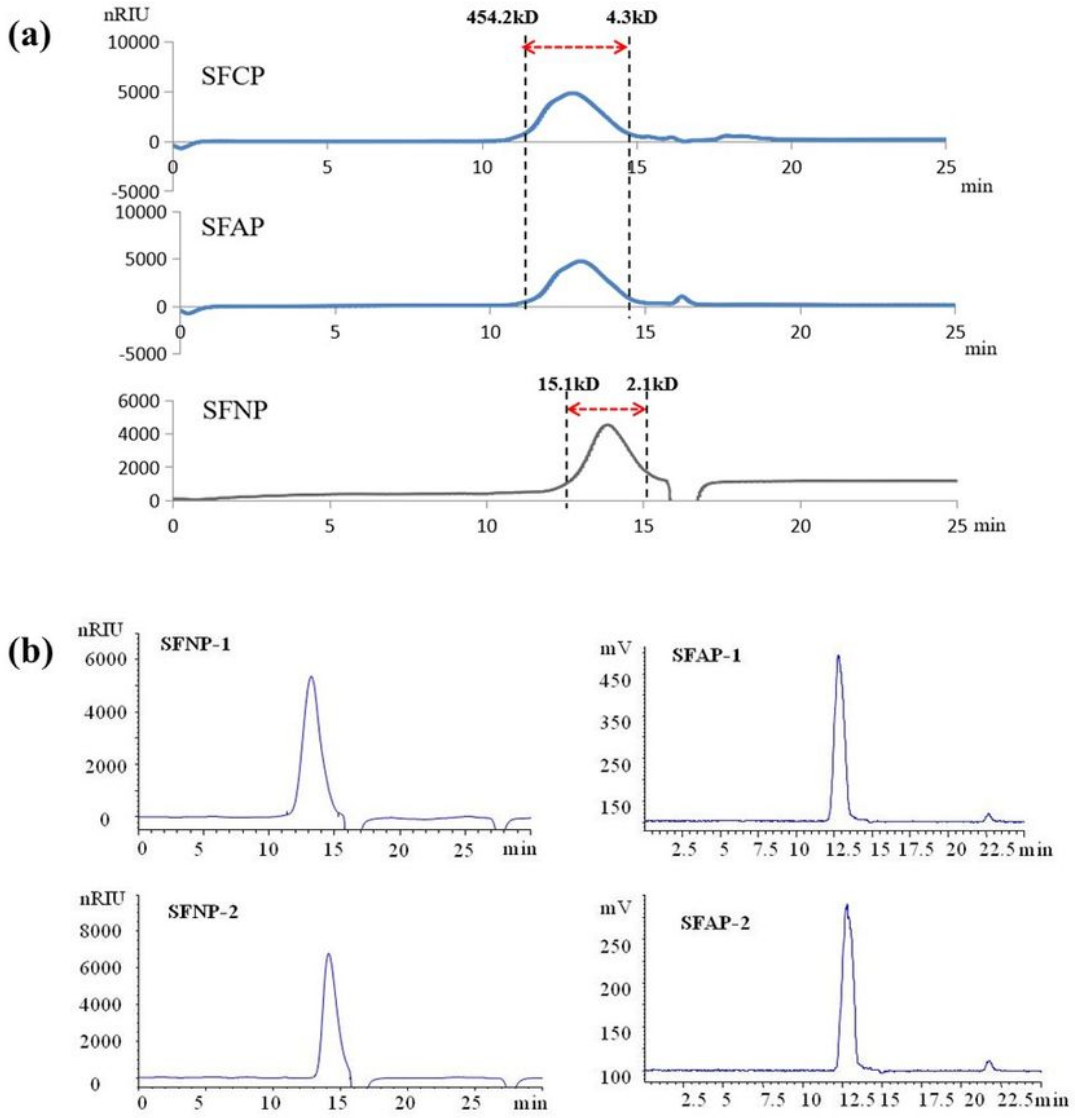

Figure 2

Molecular weight distribution of SFCP, SFN, and SFA (a) and purified polysaccharides (b) on high performance gel permeation chromatogram (HPGPC). The samples were applied to PL aquagel-OH MIXED-H column (7.5 mm ×300 mm, 8 μm) and eluted with 0.1M $NaNO_3$ at 0.6 mL/min with column temperature maintained at 35oC. Commercially available T-series dextrans were used as standard molecular markers.

Fig. 3

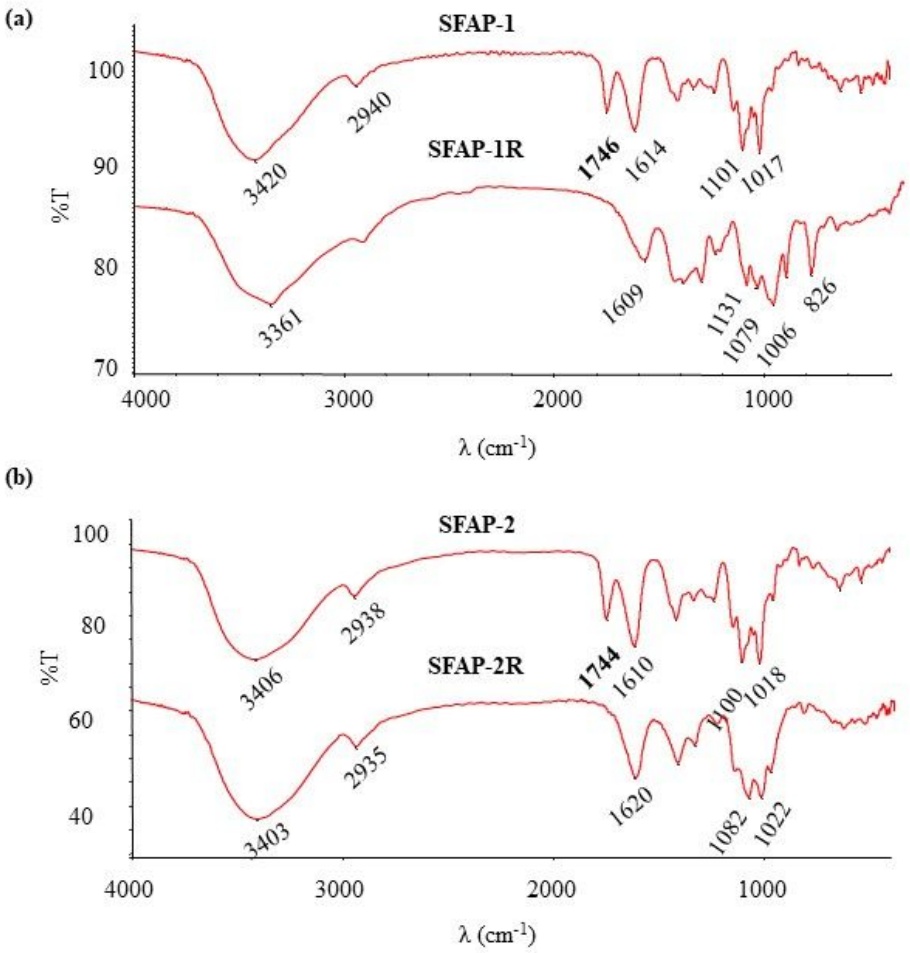

Figure 3

FT-IR spectra of the native and reduced polysaccharides (a, SFAP-1 and SFAP-1R; b, SFAP-2 and SFAP-2R) recorded at room temperature.

Fig. 4

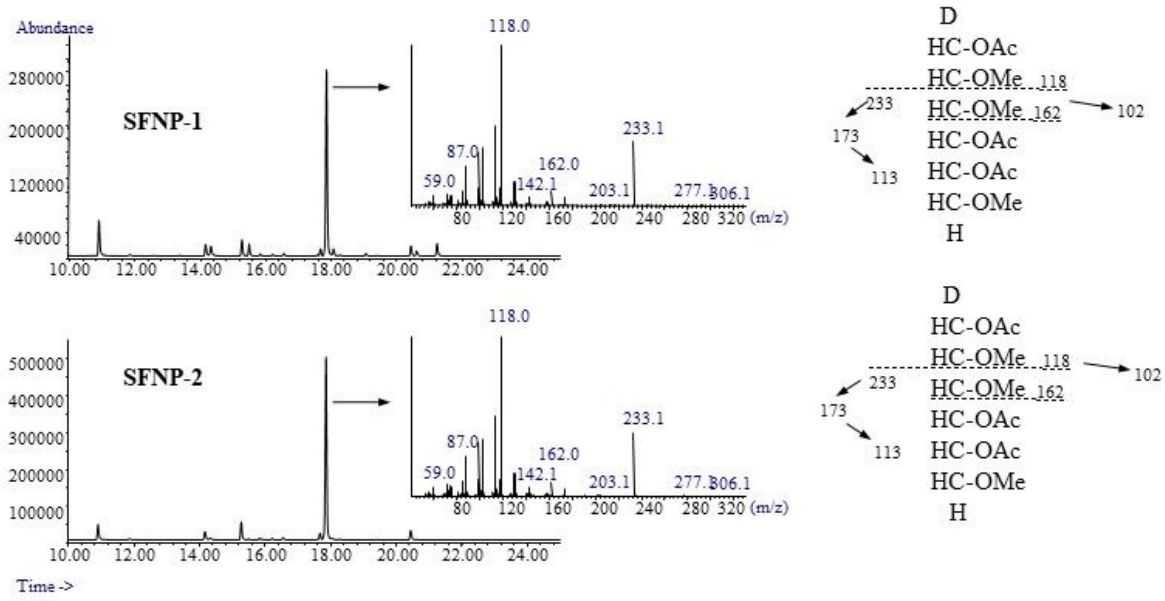

Figure 4

The acetylated derivatives were analyzed on GC-MS using a HP-5 MS fused silica capillary column (30 m × 0.25 mm, 0.25 µm, Agilent Technologies Inc.). The temperature program was set starting at 160°C followed at a rate of 2°C /min to 200°C, then to 240°C at a rate of 4°C /min, and the injector temperature was kept at 250°C.

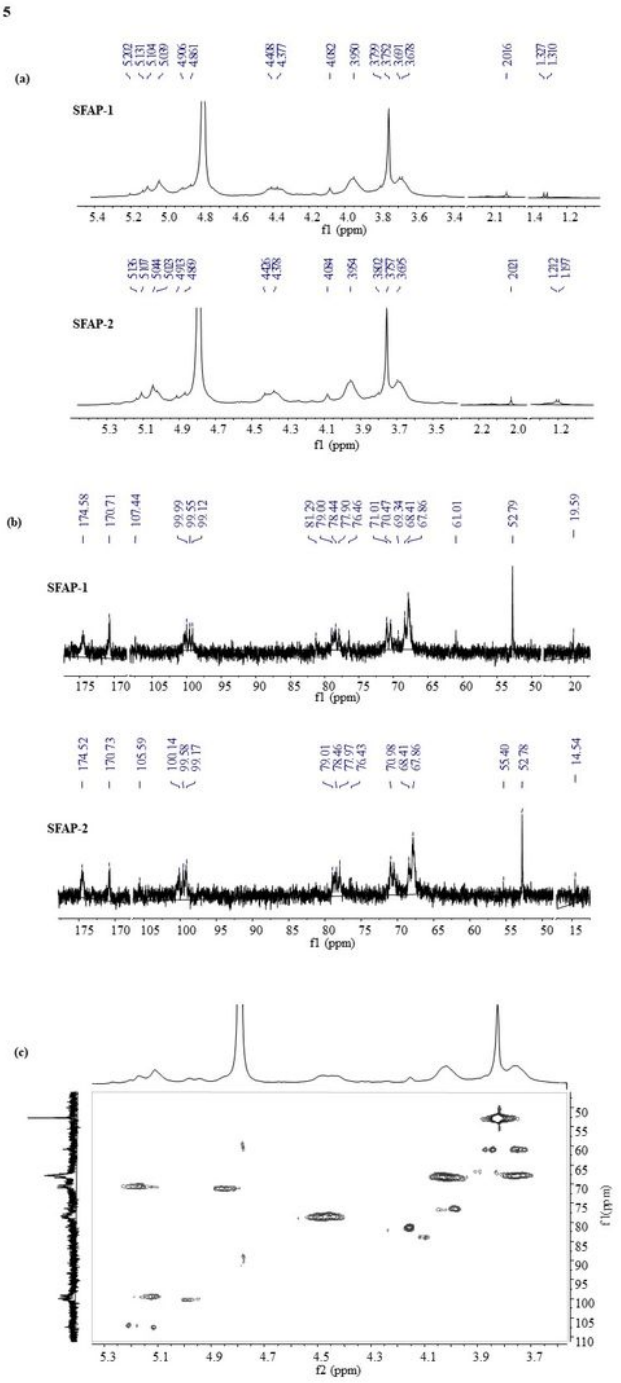

Figure 5

The 1H-NMR spectra (a) and 13C-NMR spectra (b) of the purified polysaccharides SFAP-1and SFAP-2 and HSQC (c) of SFAP-2 recorded at 30oC.

Fig. 6

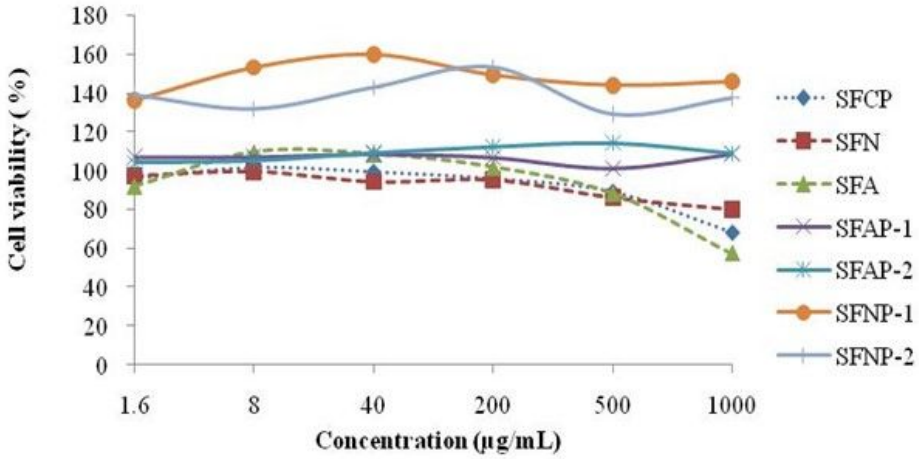

Figure 6

The cytotoxic evaluation of SFCP, SFN, and purified polysaccharides (SFNP-1, SFNP-2, SFAP-1 and SFAP-2) on RAW264.7 cells.

Fig. 7

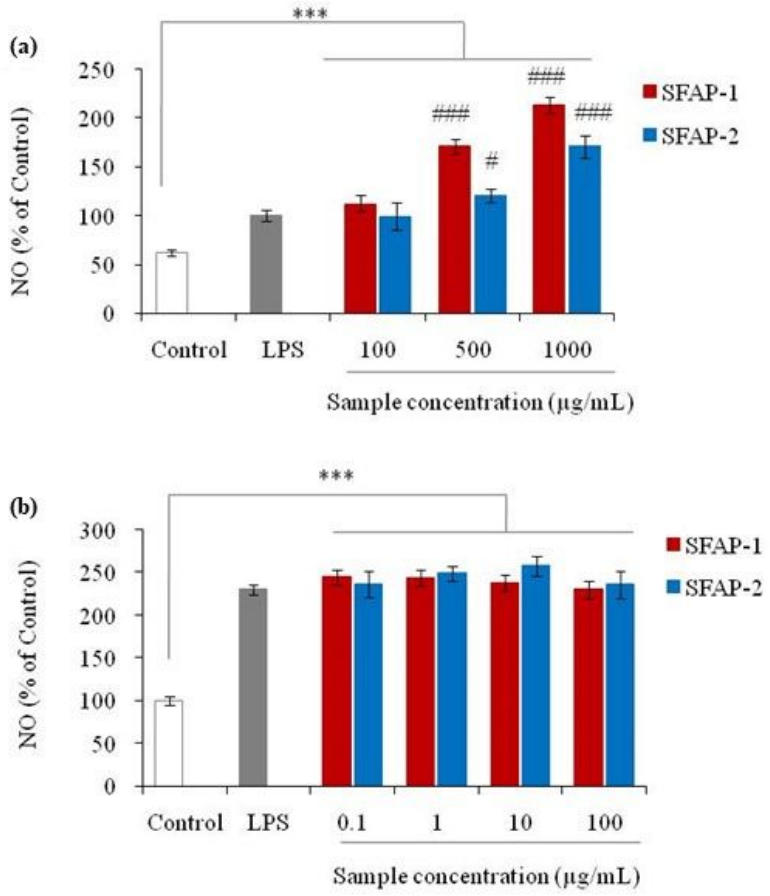

Figure 7

NO-release inhibitory effect of SFAP-1 and SFAP-2 on LPS-induced RAW264.7 cells at the high (a) and low (b) concentrations. Error bars indicate standard deviation of the means. Data are expressed as mean±SD of triplicates. * indicates a statistical significance compared with control group (***P< 0.01); # indicates a statistical significance compared with cell model group (#P< 0.05; ###P< 0.001)