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Structure of an anti-tumor polysaccharide from *Angelica sinensis* (Oliv.) Diels

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Abstract

An arabinoglucan, named APS-1d with a molecular weight of 5.1 kDa determined by high-performance gel-permeation chromatography, was extracted from the roots of *Angelica sinensis* (Oliv.) Diels and further purified by DEAE-Sephadex A-25 and Sephadex G-100 columns. The monosaccharides in the APS-1d, determined by GC, consisted of Glc and Ara in molar ratio of 13.8:1. Using methylation analysis, partial acid hydrolysis, FT-IR, 1D and 2D NMR (H/H-COSY, HSQC, and HMBC) experiments, the structure of APS-1d was elucidated. APS-1d had a backbone composed of 1,4-α-D-glucopyranosyl residues, with branches attached to O-6 of some residues. The branches were composed of 1,6-α-D-Glc*p* residues, and terminated with β-L-arabinofuranose residues. The anti-tumor activities of APS-1d were investigated both in vitro and in vivo. MTT assay revealed that APS-1d significantly inhibited the proliferation of human cervix carcinoma HeLa cells and lung carcinoma A549 cells in vitro. Furthermore, APS-1d inhibited the growth of the tumors on the mice transplanted S180 in a dose dependent manner. The inhibitory rate in mice treated with 100 mg/kg APS-1d reached to 50.7%.

Keywords: *Angelica sinensis*; Polysaccharide; Structure; Anti-tumor

1. Introduction

Chinese Danggui is the root of *Angelica sinensis* (Oliv.) Diels, which is one of the most widely used herbs of all traditional Chinese medicines. While *A. sinensis* has been used historically in gynecology as early as over 2000 years ago (Sarker & Nahar, 2004; Zhao et al., 2003), recent scientific investigations have focused on its cardiovascular, antioxidant, anti-tumor, and immunomodulatory activities (Cheng et al., 2004; Hou et al., 2004; Wang, Ouyang, Liu, Wei, & Yang, 2001; Tsai et al., 2005; Weng, Zhang, Gong, & Xiai, 1987; Wilasrusmee et al., 2002; Wu, Ng, & Lin, 2004; Yim et al., 2000). Low-molecular weight compounds such as essential oil, phenylpropanoids, benzenoids, and coumarins have for a long time been considered as the active principles of this plant (Mei, Tao, & Cui, 1991; Zhao et al., 2003), but they cannot account for all the effects mentioned above.

In recent years, as more and more polysaccharides have been reported to exhibit a variety of biological activities, including anti-tumor (Wasser, 2002), immunostimulation (Wasser, 2002; Yamada, 1994), anti-oxidation (Li et al., 2003; Liu, Ooi, & Chang, 1997), etc., the nonstarchy polysaccharides have emerged as an important class of bioactive natural products. To elucidate the medical mechanism of Chinese Danggui, the polysaccharides have also attracted much attention. The previous study showed that the crude polysaccharide from *A. sinensis* displayed anti-tumor, immunostimulation, blood coagulation, platelet aggregation, and gastrointestinal protection activities (Cho et al., 2000; Choy, Leung, Cho, Wong, & Pang, 1994; Shang et al., 2003; Yang, Jia, Mei, & Shang, 2002;

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Ye, So, Liu, Shin, & Cho, 2003). So, the presence of such compounds could partly be responsible for the clinical effects of this plant.

In the last few years, the structures of several polysaccharides isolated from the other herbs of the same genus, such as *Angelica acutiloba* have been reported (Kiyohara, Yamada, & Otsuka, 1987; Yamada, Kiyohara, Cyong, & Otsuka, 1985; Yamada et al., 1990; Yamada, Kiyohara, & Otsuka, 1984). For example, the water-soluble polysaccharide from *A. acutiloba* was a 1,4-linked α-D-glucan having side chains at O-6 of the glucosyl residues of the main chain (Yamada et al., 1984). However, to our knowledge, few studies on the structural features and linkage composition of polysaccharide from *A. sinensis* have been undertaken. Wang, Ding, Zhu, He, and Fang (2003) reported that polysaccharide from *A. sinensis* was composed of fucose, galactose, glucose, arabinose, rhamnose, and xylose (mole ratio: 1.0:13.6:15.0:8.7:21.3:3.7), which was similar to the results reported by others (Chen, Wang, Xu, Xu, & Chang, 2001). Furthermore, a neutral polysaccharide (ASP1) and two kinds of acidic polysaccharides (ASP2, ASP3) were isolated from *A. sinensis* recently (Sun, Tang, Gu, & Li, 2005). The composition analysis displayed that ASP1 was rich in glucose, galactose, and arabinose. Although these structural investigations shed light on the monosaccharide compositions of polysaccharides from *A. sinensis*, detailed studies of the structures were lacking.

Therefore, we fractionated the polysaccharide from *A. sinensis* and obtained several distinctive polysaccharides. Among them, a low molecular weight arabinoglucan exerted anti-tumor activity. So, the aim of this research was to investigate the complete structure of the unique arabinoglucan and its anti-tumor effects both in vitro and in vivo.

2. Experimental

2.1. Materials

The roots of *A. sinensis* were collected in Minxian County, Gansu Province, China in October 2002 and identified by Professor Niu X.F. in the department of pharmacy, Xi'an Jiaotong University (Xi'an, China), by comparison with a voucher specimen deposited at the herbarium in the department of pharmacy, Xi'an Jiaotong University. The coarse powder of the roots was air-dried in the shade and stored in a well-closed vessel for use.

T-series Dextran, DEAE-Sephadex A-25, Sephadex G-100, and Sephadex G-75 were purchased from Amersham biosciences (Uppsala, Sweden). Trifluoroacetic acid (TFA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI)-1640 medium, phosphate-buffered saline (PBS), and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). All other chemical reagents were analytical reagent grade.

2.2. Extraction and fractionation of polysaccharide

The powdered roots (1.8 kg) of *A. sinensis* (Oliv.) Diels were extracted three times with ethanol (3.6 L) at 80 °C for 3 h, in order to remove the pigments. The residue was decocted three times with water (7.2 L) at 80 °C for 2 h, filtered through the gauze and centrifuged to remove water-insoluble materials. The aqueous extract was concentrated at 50 °C in vacuum and treated with 3 volumes of ethanol for precipitation at 4 °C overnight. The gel-like precipitate was suspended in water and dialyzed against distilled water (exclusion limit 3.5 kDa). The nondialyzable portion was frozen at −20 °C, then thawed and centrifuged again to remove insoluble materials. After the freeze–thaw process was repeated six times, the supernatant was lyophilized and the brown product was obtained (APS-0, yield: 39.6 g).

APS-0 (13.2 g) was dissolved in distilled water, filtered through a 0.65 μm membrane filter and loaded onto a DEAE-Sephadex A-25 column (90 cm × 5 cm). The column was first eluted with distilled water followed by 0.3 M and 0.5 M NaCl, respectively. Fractions of 10 ml were collected and monitored for the presence of carbohydrate using phenol–sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The largest water-eluted fraction was pooled, dialyzed, lyophilized and designated as APS-1 (yield: 4.8 g). APS-1 was further fractionated on a column (100 cm × 5 cm) of Sephadex G-100, eluted with 0.1 M NaCl and separated into four sub-fractions. The forth fraction, which molecular weight was lower than the other fractions, was pooled according to the elution profile. After concentration, the solution was applied onto the column of Sephadex G-100 once more, to get a purified polysaccharide. The relevant fraction was concentrated, dialyzed and lyophilized to get a white powder polysaccharide (APS-1d, 225 mg).

2.3. General methods

Carbohydrate content was measured by the phenol-sulfuric acid method, using D-glucose as the standard (Dubois et al., 1956). Protein was measured by the Bradford (1976) method using bovine serum albumin as standard. The uronic acid content of the sample was measured by spectrophotometry according to the colorimetric method reported by Englyst, Quigley, and Hudson (1994). Optical rotation was measured at 20 °C using a Perkin-Elmer 343 polarimeter. Each sample was analyzed four times by the methods described above. IR spectra (KBr disc) were recorded with a Bruker-Equinox 55 spectrophotometer for detecting functional groups. The homogeneity and molecular weight of the polysaccharide were evaluated and determined by high-performance gel-permeation chromatography (HPGPC) using a Waters Alliance 2690 instrument equipped with a tandem of a Shodex sb-803HQ (Showa Denkko, 8 mm × 30 cm) and a Biosep SEC-S3000 (Phenomenex, 7.8 mm × 30 cm) column, eluted with 0.05 M Na_2SO_4 at a flow rate of 0.8 ml/min. The elution was monitored by Waters Alliance

2414 RI detector and the data were analyzed with Millennium 32 (Waters Alliance) software. The columns were calibrated with standard T-series Dextran T-130, T-80, T-40, T-20, and T-10 (Wei & Fang, 1989).

2.4. Monosaccharide analysis

APS-1d was hydrolyzed with 2 M TFA at 110 °C for 4 h (Honda, Suzuki, Kakehi, Honda, & Takai, 1981). Paper chromatography (PC) and gas chromatography (GC) were used for identification and quantification of the monosaccharide composition. PC was performed on Xinhua (Hangzhou, China) No. 1 paper in the following solvent system: EtOAc-*n*-butyl alcohol–isopropyl alcohol–acetic acid–water–pyridine (20:7:12:7:6:6) and visualized by spraying with phthalic acid reagent and heating at 100 °C for 15 min (Shang et al., 2001). The sugars in the hydrolysate were converted to their alditol acetates as described (Johnes & Albersheim, 1972; Oades, 1967) and analyzed by GC on an Agilent 6890N instrument fitted with FID and equipped with SE-54 column (30 m × 0.32 mm × 0.25 μm) at a temperature program as follows: 160 °C (10 min)–280 °C with a rate of 5 °C/min. The injector and detector heater temperatures were 250 and 300 °C, respectively. The rate of N_2 carrier gas was 1.2 ml/min.

2.5. Methylation analysis

Methylation of APS-1d was carried out three times using the method described early (Needs & Selvandran, 1993). The methylated polysaccharide was examined by IR spectrometry. Complete methylation was confirmed by the lack of a hydroxyl peak. The methylated products were hydrolyzed, reduced, acetylated as described by Sweet, Shapiro, and Albersheim (1975). The resulting alditol acetates were subjected to GC and GC–MS analysis. Analyses were performed on an Agilent 6890N GC interfaced with an Agilent 5973N mass selective detector at 70 eV ionization energy. The GC column was a HP-1 (30 m × 0.2 mm × 0.33 μm) at a temperature program from 140 to 280 °C with a rate of 3 °C /min. The rate of helium carrier gas was at a rate of 3.0 ml/min. The quantification for molar ratio for each sugar was calibrated using the peak area and response factor of the FID in GC.

2.6. Nuclear magnetic resonance (NMR) spectroscopy

The polysaccharide (20 mg) was dissolved in D_2O (0.5 ml). The ^{13}C NMR, ^{13}C DEPT (distortionless enhancement by polarization transfer) and ^{1}H NMR were recorded at 30 °C with a Bruker Avance 500 MHz spectrometer (Germany). The ^{13}C NMR spectrum was recorded using a Bruker 5 mm broadband probe at 125.76 MHz, and the chemical shifts were expressed in ppm relative to the resonance of the internal standard, TMS. The DEPT experiment was carried out using a polarization transfer pulse of 135. Two-dimensional ^{1}H–^{1}H COSY (homonuclear shift correlation) spectrum was recorded in the phase-sensitive mode. Spectral width was 1707 Hz in both dimensions. In the ^{1}H–^{13}C heteronuclear single quantum coherence (HSQC) experiment, echo/antiecho gradient selection with decoupling was used. Spectral widths were 1707 and 11,261 Hz for the proton and the carbon dimensions, respectively. The heteronuclear multiple quantum coherence (HMBC) experiment was recorded using gradient pulses for selection. The repetition time was 2.1 s.

2.7. Partial acid hydrolysis

Partial acid hydrolysis of APS-1d (80 mg) was performed as described by Ishurd and Kennedy (2005) using 0.2 mol/L TFA at 100 °C for 2 h. The mixture was evaporated to dryness using methanol, and the residue was dissolved with a small amount of water, placed in a dialysis tubing (molecular weight cut-off of 2 kDa) and dialyzed against distilled water (3× 2000 ml). The dialyzable fraction was concentrated, hydrolyzed and its monosaccharide composition was analyzed by PC. The nondialyzate was concentrated and purified by chromatography on Sephadex G-75 (90 × 1.6 cm) column to give a pure fragment HAPS-1d (40 mg) as a single and symmetric peak eluted with distilled water. To establish the sequence of linkages in HAPS-1d, it was subjected to monosaccharide composition analysis and methylation analysis as described above.

2.8. Cell lines and animals

Human cervix carcinoma HeLa cells and human lung carcinoma A549 cells were obtained from Xi'an Cell Engineering Center (Xi'an, China). HeLa and A549 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 mg/L) (Hsu, Kuo, & Lin, 2004; Tomatsu, Ohnishi-Kameyama, & Shibamoto, 2003) in a humidified 5% CO_2 atmosphere at 37 °C.

S180 tumor cells were maintained in peritoneal cavities of ICR mice obtained from Shannxi Academy of Traditional Chinese Medicine (SATCM) (Shannxi, China). Male ICR mice, weighed 20.0 ± 2.0 g, purchased from SATCM, were housed five per plastic cages with wood chip bedding in an animal room with a 12 h light and 12 h dark cycle at room temperature (25 ± 2 °C) and allowed free access to standard laboratory diet (purchased from the Laboratory Animal Center of the Forth Military Medical University). The animal experiments were conducted according to the 'Guidelines for Animal Experimentation' of the Forth Military Medical University.

2.9. Cell proliferation assay

The proliferation of HeLa and A549 cells was determined using the colorimetric MTT assay as described previously (Mosmann, 1983). Briefly, cells were seeded at a density of 3×10^3 cells/well in a 100 µl volume of the medium in 96-well plates and allowed to attach 24 h. The dosages of APS-1d on the selected cell lines were in the range of 3–100 µg/ml while the negative controls were treated with the medium only. MTT (5 g/L) 20 µl was added 48 h later. After incubated at 37 °C for 4 h, the supernatant was aspirated, and 150 µl DMSO was added to each well. Absorbance was measured at 570 nm by a 96-well microplate reader (Mode 680, Bio-Rad, Tokyo, Japan).

2.10. Assay of anti-tumor activity in vivo

S180 tumor cells (3×10^6) were implanted subcutaneously into right hind groin of the ICR mice (Zhou et al., 2004). Mice were randomly divided into five groups ($n = 10$). One day after inoculation, APS-1d was dissolved in distilled water and administered intraperitoneally (i.p.) to the mice at the doses of 20, 50, and 100 mg/kg. Positive and negative controls were set for comparison. The positive control was given with 0.2 ml cyclophosphamide (20 mg/kg) and negative one with physiological saline instead of the test solution. Animals were sacrificed after 2 weeks. The body weights were measured. Tumors and spleens were excised and the tumor inhibitory ratio were calculated by following formula: Inhibition ratio (%) $= [(A - B)/A] \times 100$, where A and B were the average tumor weights of the negative control and treated groups, respectively.

2.11. Statistical analysis

Data were expressed as means ± SD. Data in all the bioassays were statistically evaluated by Student's *t* test and $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Isolation and purification of polysaccharide

The crude polysaccharide, APS-0, was obtained by precipitation with ethanol and dialysis from the water extract of the roots of *A. sinensis*. APS-0 which contained 86% of neutral carbohydrate and 4.1% of protein was fractionated on a DEAE-Sephadex A-25 anion-exchange column. The unabsorbed fraction (APS-1) obtained in water eluate, was fractioned by gel filtration on Sephadex G-100 column to get four fractions as shown in Fig. 1. As APS-1d displayed significant

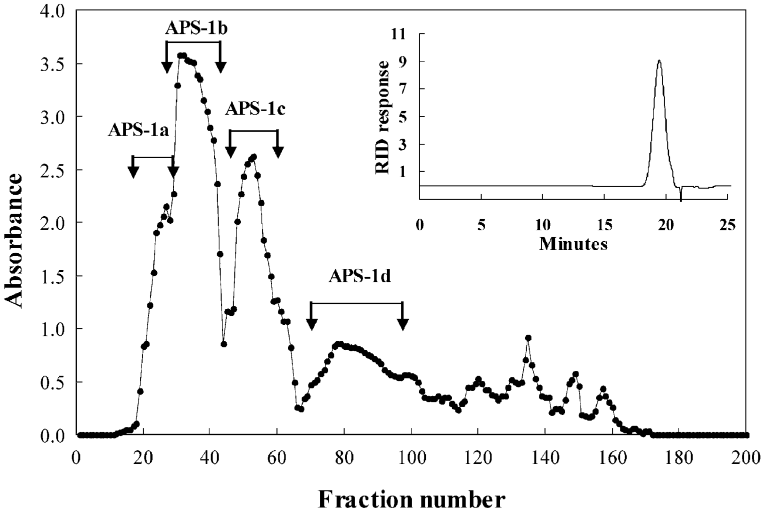


Fig. 1. Isolation and purification of the APS-1d on a column of Sephadex G-100. The inserted graph showed the elution profile of APS-1d on HPSEC using a RI detector.

Table 1
The results of methylation analysis of APS-1d and HAPS-1d

Methylated sugar	Retention time (min)	Molar ratios		Mass fragments (*m*/*z*)	Linkage type
		APS-1d	HAPS-1d		
2,3,5-Me_3-Ara	8.73	1.07		43,73,87,101,117,129,161,189	β-Ara*f*-(1→
2,3,4,6-Me_4-Glc*p*	11.00	1.10	1	43,71,87,101,113,117,129,145,161	α-Glc*p*-(1→
2,3,6-Me_3-Glc*p*	13.68	7.18	94.08	43,87,99,113,117,129,161,191,203,233	→4)-α-Glc*p*-(1→
2,3,4-Me_3-Glc*p*	14.19	4.97		43,87,101,117,129,143,161,173,189,203,233	→6)-α-Glc*p*-(1→
2,3-Me_2-Glc*p*	16.59	1		43,85,101,117,142,159,187,201,207,261	→4,6)-α-Glc*p*-(1→

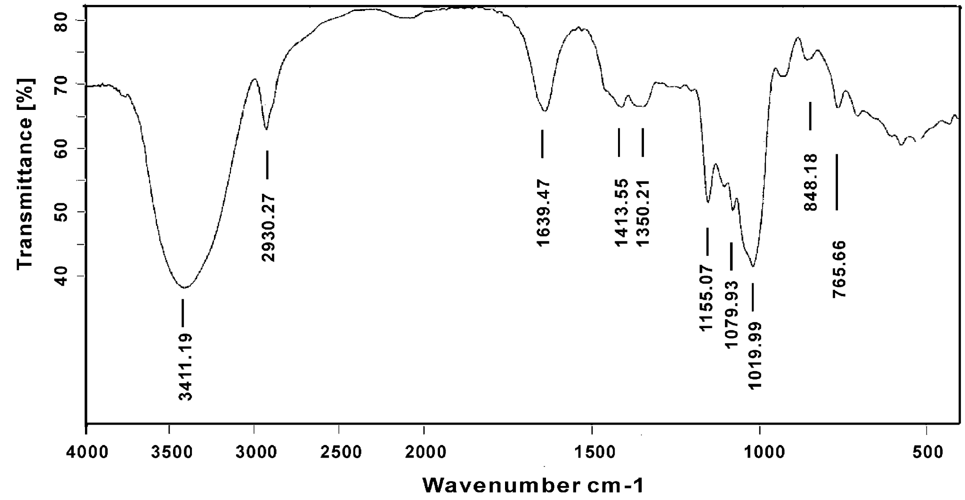


Fig. 2. FT-IR spectrum of APS-1d.

anti-tumor activity among four fractions, it was further purified by gel filtration and gave a homogeneous polysaccharide, which was confirmed by HPSEC. The molecular weight of APS-1d was estimated to be 5.1 kDa from the calibration curve in reference to standard dextrans. APS-1d contained 91.5% of carbohydrate, 0.5% of uronic acid and only trace (less than 2%) of protein. The component sugars of APS-1d were determined to

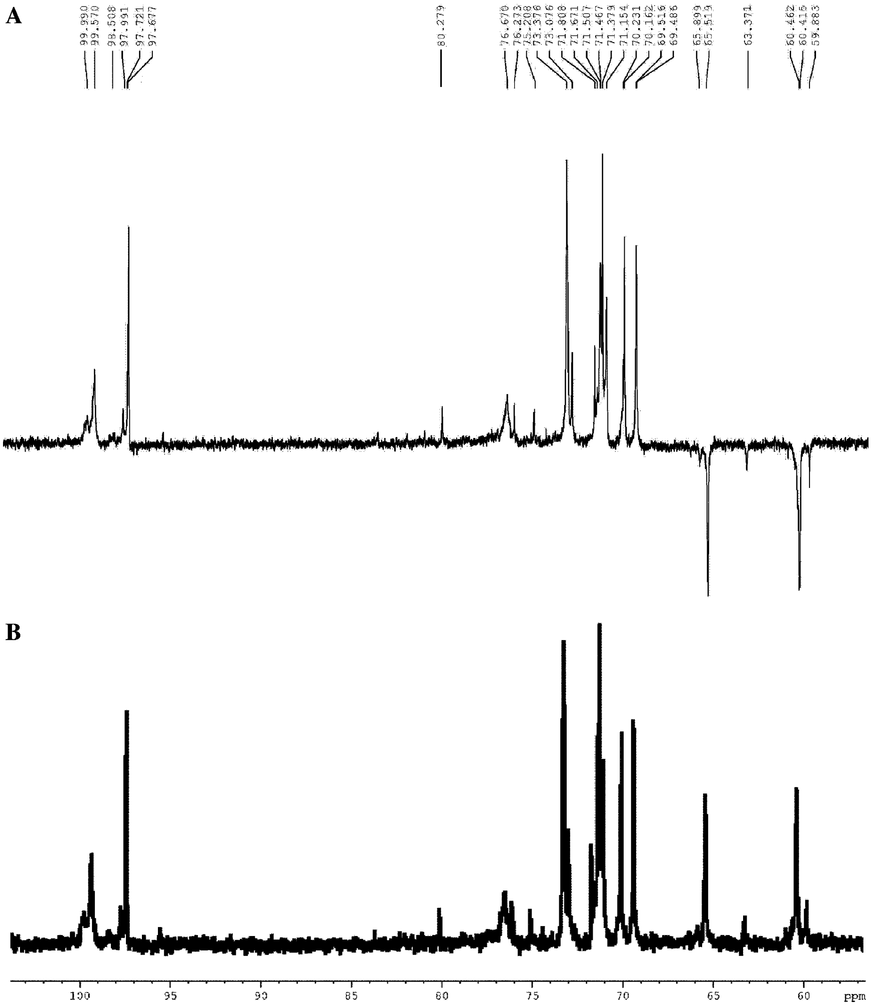


Fig. 3. ^{13}C NMR and DEPT spectra of APS-1d. (A) DEPT, (B) ^{13}C NMR.

Table 2
Chemical shifts of resonances in the ^{13}C and ^{1}H NMR spectra of APS-1d

Sugar residues	Chemical shifts (δ, ppm)					
	C1/H1	C2/H2	C3/H3	C4/H4	C5/H5;H5′	C6/H6;H6′
β-Ara*f*-(1→	100.0/5.33	76.2/4.11	75.2/4.01	80.3/3.87	59.9/3.70;3.61	
α-Glc*p*-(1→	98.0/4.90	71.8/3.62	73.1/3.62	69.5/3.35	70.2/3.62	63.4/3.70;3.60
→4)-α-Glc*p*-(1→	99.6/5.33	71.2/3.55	73.4/3.89	76.7/3.57	71.5/3.62	60.4/3.78;3.76
→6)-α-Glc*p*-(1→	97.7/4.90	71.2/3.48	73.1/3.62	69.5/3.44	70.2/3.83	65.5/3.90;3.67
→4,6)-α-Glc*p*-(1→	98.5/4.90	71.2/3.55	73.1/3.70	76.7/3.57	70.2/3.94	65.9/3.90;3.67

be glucose and arabinose with a molar ratio of 13.8:1. Moreover, the positive value of optical rotation ($[\alpha]_D^{20}$ +45.9 (*c* 0.2, H_2O)) suggested the dominating presence of α-form glycosidic linkages in APS-1d.

3.2. Determination of the structure of APS-1d

A modified methylation analysis (Needs & Selvandran, 1993) of the APS-1d gave five homogeneous peaks on the HP-1 column. Combined with the corresponding MS spectra, they were identified as 2,3,5-Me_3-Ara, 2,3,4,6-Me_4-Glc*p*, 2,3,6-Me_3-Glc*p*, 2,3,4-Me_3-Glc*p*, and 2,3-Me_2-Glc*p*, in the molar ratio of 1:1:7:5:1 (Table 1). The analysis of the methylated sugars was conducted by GC–MS of their alditol acetates (Sweet et al., 1975). So they were identified as terminal arabinose, terminal, 1,4-linked, 1,6-linked, and 1,4,6-linked glucose, respectively. A relatively good agreement was found between molar ratios of methylated alditol acetates and that of their parent sugars measured by direct analysis. Methylation analysis displayed around 6.7% of glucose was branched, and all of the arabinose residues were present as terminal arabinose. Furthermore, the proportion of terminal glucose units was 7.2% indicating a degree of polymerization about 2, according to the mean molecular weight (5.1 kDa) of APS-1d.

The FT-IR spectrum of APS-1d was shown in Fig. 2. The band in the region of 3411.19 cm^{-1} was due to the hydroxyl stretching vibration of the polysaccharide. The band in the region of 2930.27 cm^{-1} was due to C–H stretching vibration and the band in the region of 1639.47 cm^{-1} was owing to associated water. The positive specific rotation and the characteristic absorption at 848.18 cm^{-1} in the IR spectrum indicated α-configuration of D-glucan (Barker, Bourne, Stacey, & Whiffen, 1954).

The ^{13}C NMR spectrum of APS-1d showed in Fig. 3. Based on the data available in the literature, it was possible to identify that the resonances in the region of 97–100 ppm were attributed to the anomeric carbon atoms of glucopyranose (Glc*p*) and arabofuranose (Ara*f*), respectively. Two main peaks at δ 99.6 and 97.7 ppm corresponded to C-1 of the 1,4-D-Glc*p* and 1,6-D-Glc*p* residues, respectively. The signals at δ 98.5 and 98.0 ppm correspond to C-1 of 1,4,6-D-Glc*p*, and T-D-Glc*p* residues, respectively (Funane et al., 2001; Seymour, Knapp, & Bishop, 1976; Tylianakis, Spyros, Dais, Taravel, & Perico, 1999; Uzochukwu, Balogh, Loefler, & Ngoddy, 2002; Wang,

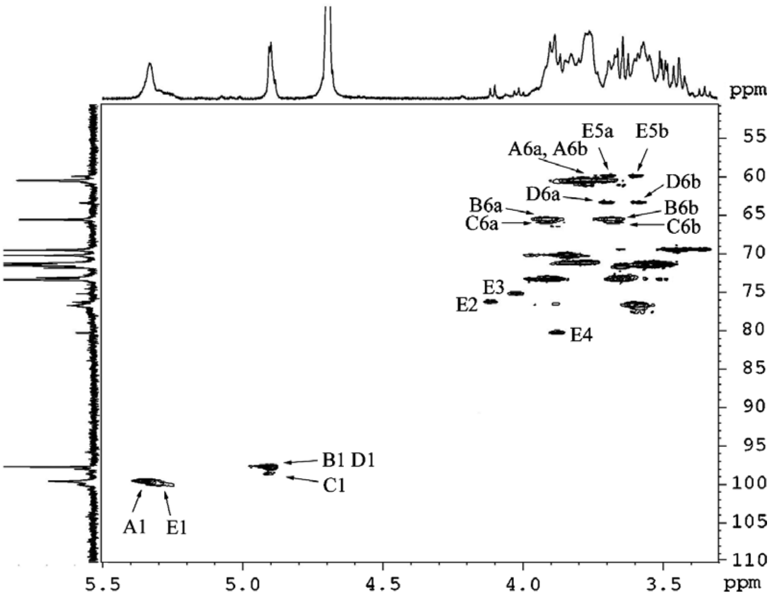


Fig. 4. 500 MHz HSQC spectrum of APS-1d in D_2O solutions at 30 °C. (A) →4)-α-Glc*p*-(1→, (B) →6)-α-Glc*p*-(1→, (C) →4,6)-α-Glc*p*-(1→, (D) α-Glc*p*-(1→, and (E) β-Ara*f*-(1→.

Peng, Huang, Peng, & Tian, 2001). The appearance of the respective carbon signals indicated that the Glc*p* moieties both were the α-anomeric configuration (Uzochukwu et al., 2002). While the resonance due to C-1 of T-Ara*f* was observed at 100.0 ppm which revealed a β-anomeric configuration (Cardoso, Silva, & Coimbra, 2002; Ryden, Colquhoun, & Selvendran, 1989; Swamy & Salimath, 1991). The configurations were confirmed by the ^{1}H NMR spectrum, which displayed signals for five anomeric protons at 5.33 (H-1 of the 1,4-D-Glc*p* and T-Ara*f*) and 4.90 ppm (H-1 of the 1,6-D-Glc*p*, T-D-Glc*p*, and 1,4,6-D-Glc*p*). Furthermore, the signals in the high field of 59–66 ppm were investigated by DEPT 135 (Fig. 3). In this experiment, the methylene carbons show opposite amplitude to the methyl and the methyne carbons. So the well-defined signals at 59.9, 60.4, 63.4, 65.5 and 65.9, could be attributed to C-5 of T-L-Ara*f*, C-6 of 1,4-D-Glc*p*, T-D-Glc*p*, 1,6-D-Glc*p*, and 1,4,6-D-Glc*p*, respectively, referred to the previous reports (Cardoso et al., 2002; Wang et al., 2001). The carbon signal at δ 65.5 ppm should be C-6 of the 1,6-D-Glc*p*, which was

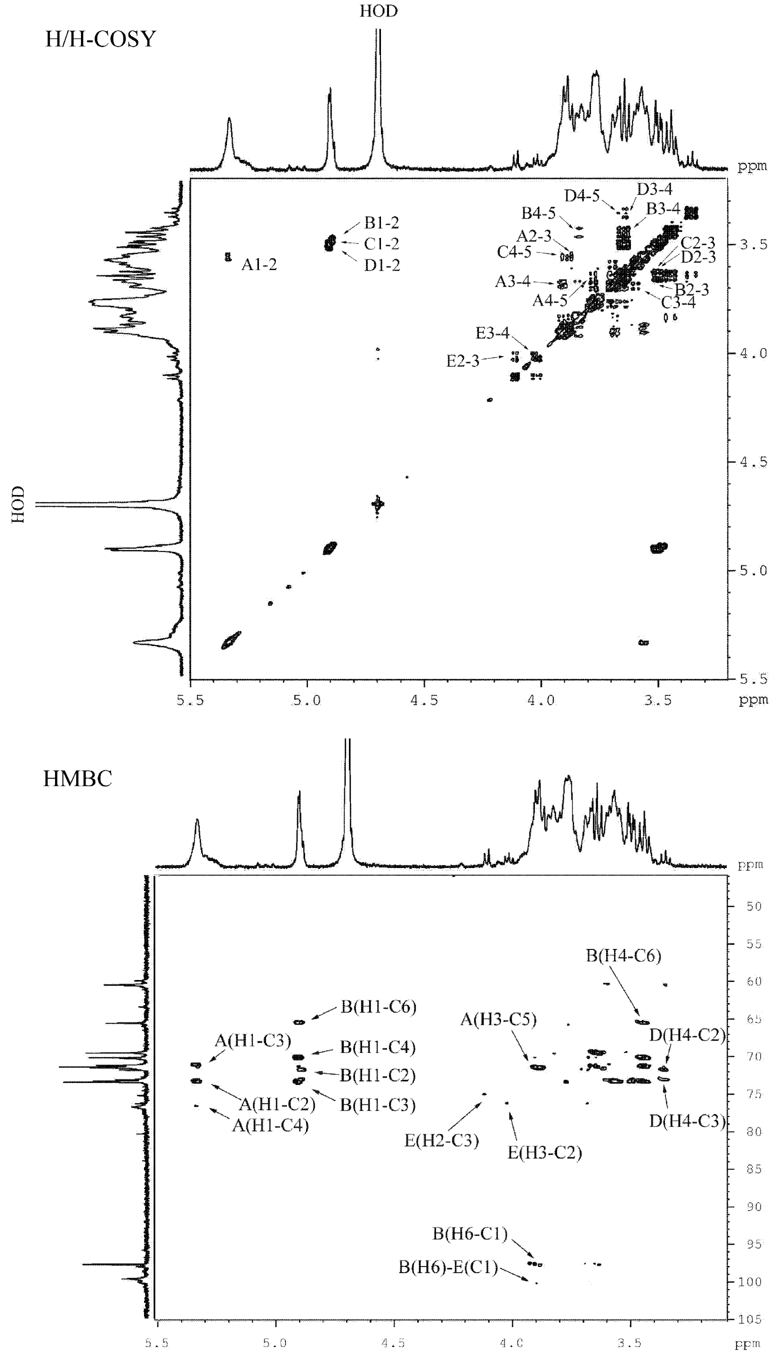


Fig. 5. 500 MHz H/ H-COSY and HMBC spectra of APS-1d in D$_2$O solutions at 30 °C. (A) →4)-α-Glc*p*-(1→, (B) →6)-α-Glc*p*-(1→, (C) →4,6)-α-Glc*p*-(1→, (D) α-Glc*p*-(1→, and (E) β-Ara*f*-(1→.

shifted about 4 ppm downfield compared with the resonance of standard methyl glycoside due to the effect of glycosylation (Seymour, Knapp, Bishop, & Jeans, 1979). Similarly, the C-4 signal at 76.7 ppm of 1,4-D-Glc*p* and 1,4,6-D-Glc*p* appeared 5.25 ppm downfield compared with that of the standard methyl glycoside (Agarwal, 1992).

Other signals in ^{13}C NMR and ^{1}H NMR spectra, which were summarized in Table 2, were assigned on the basis of the correlation of H-H-COSY, HSQC, and HMBC experiments and referred to the previous reports (Cardoso et al., 2002; Seymour et al., 1976; Tylianakis et al., 1999; Uzochukwu et al., 2002; Wang et al., 2001). The HSQC spectrum of APS-1d was shown in Fig. 4. There were several pairs of H-6–C-6 cross peaks presenting in the high field. These H-6 resonances appeared as doublets due to coupling each other. The C-6 signals of 1,4-D-Glc*p*, 1,6-D-Glc*p*, 1,4,6-D-Glc*p*, and T-D-Glc*p* were correlated with the resonances at $\delta_{\text{H-6}}$ 3.78 and 3.76, 3.90 and 3.67, 3.90 and 3.67, 3.70 and 3.60, respectively. Similarly, The C-5 signal of T-L-Ara*f* was correlated with the resonances at $\delta_{\text{H-5}}$ 3.70 and 3.61.

From the COSY spectrum (Fig. 5), it was possible to correlate H-1 of 1,6-D-Glc*p* (δ 4.90) with H-2 (δ 3.48), H-2 with H-3 (δ 3.62), H-3 with H-4 (δ 3.44), and H-4 with H-5 (δ 3.83). These results and the analysis of the HSQC spectrum could help to assign C-2, C-3, C-4 and C-5 of 1,6-D-Glc*p* to $\delta_{\text{C-2}}$ 71.2, $\delta_{\text{C-3}}$ 73.1, $\delta_{\text{C-4}}$ 69.5 and $\delta_{\text{C-5}}$ 70.2, respectively. C-2, C-3, C-4, and C-5 of other residues were assigned by a similar procedure. δ 71.2, δ 73.4, δ 76.7, and δ 71.5 were due to C-2, C-3, C-4, and C-5 of 1,4-D-Glc*p*. However, clear assignments of the H-2s of T-D-Glc*p* and 1,4,6-D-Glc*p* were unobtainable due to a high degree of overlap between the H-2,3. Referred to the reports mentioned above (Seymour et al., 1976; Wang et al., 2001), the C-2 signals of T-D-Glc*p* and 1,4,6-D-Glc*p* were correlated with the resonances at δ 71.8 and δ 71.2. So C-3, C-4, and C-5 of T-D-Glc*p* were assigned to δ 73.1, δ 69.5, and δ 70.2, C-3, C-4, and C-5 of 1,4,6-D-Glc*p* were assigned to δ 73.1, δ 76.7, and δ 70.2, respectively, according to the cross peaks in the COSY and HSQC spectra. Furthermore, as there was no clear H-2–C-1 cross peak of T-L-Ara*f* in the COSY spectrum, the signal at δ 4.11 was assigned to H-2 according to the literature (Cardoso et al., 2002). With the assistant of HSQC spectrum, cross peaks of H-2 of T-L-Ara*f* with H-3 (δ 4.01) and H-3 with H-4 (δ 3.87) could found in the COSY spectrum.

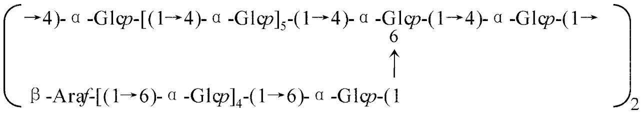
To deduce more information about the structure of APS-1d and to confirm the assignments made from HSQC and COSY spectra, a HMBC spectrum was recorded (Fig. 5). In the HMBC spectrum, cross peaks H-1–C-6 (linkages of two 1,6-D-Glc*p* residues), H-1–C-4 (linkages of two 1,4-D-Glc*p* residues), and similarly, H-6–C-1 (linkages of 1,6-D-Glc*p* and T-L-Ara*f* residue) were identified. Moreover, there was no cross peak between H-1 of 1,4-D-Glc*p* and C-6 of 1,6-D-Glc*p* or cross peak between H-1 of 1,6-D-Glc*p* and C-4 of 1,4-D-Glc*p* founded. These results did not support the occurrence of the direct linkage of 1,4-D-Glc*p* to 1,6-D-Glc*p*, but suggested that the T-β-L-Ara*f* should be linked directly to a 1,6-D-Glc*p* residue. The cross peaks at $\delta_{\text{H-1}}$ 5.33 and the ^{13}C resonances at δ 70–74 in HMBC spectrum allowed their identification as C-3 (δ 73.4) and C-2 (δ 71.2) of 1,4-D-Glc*p* residues. These, along with the additional intra-residue correlations observed between the resolved H-2s of each residue and their respective C-1 and C-3 resonances, or the resolved H-3s of each residue and their respective C-2 and C-4 resonances, served to confirm the assignments within each glucosyl residue.

APS-1d was partially hydrolyzed with 0.2 mol/L TFA, the major dialyzable fraction contained glucose and arabinose, indicating that they were present at outer chains. The nondialyzable fraction was further fractionated by Sephadex G-75 column and a pure fraction HAPS-1d with a molecular weight of 2.8 kDa was obtained. HAPS-1d was per-methylated and analyzed on GC–MS. The result (Table 1) showed all the Ara residues and 1,6-Glc*p* were lost, while the proportion of 1,4-Glc*p* residues increased, compared with methylation analysis of APS-1d. It suggested that 1,6-Glc*p* existed as side chain and attached directly to the 1,4-Glc*p* main chain. These results were in agreement with the data above.

Since the molecular weight of APS-1d was 5.1 kDa, the possible structure was shown in Fig. 6.

3.3. Anti-tumor activity of APS-1d

The anti-tumor activity of the polysaccharide was usually believed to be a consequence of the stimulation of the cell-mediated immune response (Ooi & Liu, 2000). For instance, immunostimulatory activities were found in the



$$\left(\begin{array}{l}\rightarrow 4)\text{-}\alpha\text{-Glc}p\text{-}[(1\rightarrow 4)\text{-}\alpha\text{-Glc}p]_5\text{-}(1\rightarrow 4)\text{-}\alpha\text{-}\underset{\substack{6\\ \uparrow}}{\text{Glc}p}\text{-}(1\rightarrow 4)\text{-}\alpha\text{-Glc}p\text{-}(1\rightarrow \\ \beta\text{-Ara}f\text{-}[(1\rightarrow 6)\text{-}\alpha\text{-Glc}p]_4\text{-}(1\rightarrow 6)\text{-}\alpha\text{-Glc}p\text{-}(1\end{array}\right)_2$$

Fig. 6. Predicted structure of APS-1d.

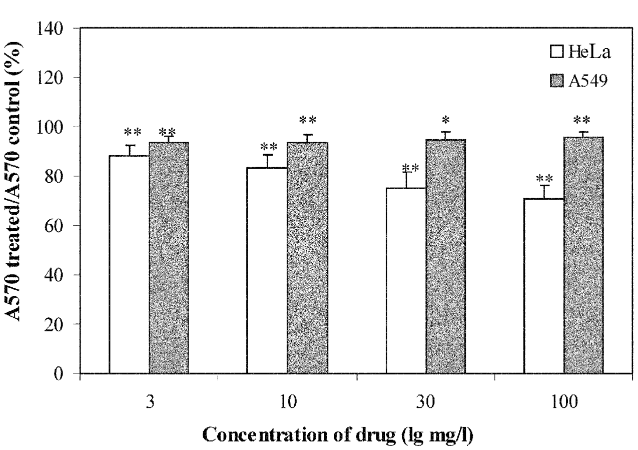


Fig. 7. The MTT assay of HeLa and A549 cells induced with different concentrations of APS-1d. Significant differences from negative control group were evaluated using Student's *t* test: $*P < 0.05$, $**P < 0.01$.

Table 3
Anti-tumor activities of APS-1d on S180 tumor

Sample	Dose (mg/kg)	Increase of body weight (g)	Spleen weight (g)	Tumor weight (g)	Inhibitory rate of tumor (%)
Negative control		4.22 ± 0.98	0.18 ± 0.03	1.42 ± 0.43	
Positive control	20	1.10 ± 0.94	0.09 ± 0.03	0.45 ± 0.20	68.31
	20	4.04 ± 2.42^{d}	0.20 ± 0.04^{ad}	0.78 ± 0.26^{bd}	45.07
APS-1d	50	5.83 ± 1.74^{ad}	0.21 ± 0.04^{ad}	0.71 ± 0.19^{bd}	50.00
	100	6.92 ± 1.31^{bd}	0.21 ± 0.03^{bd}	0.70 ± 0.24^{bc}	50.70

Significant differences from negative control group were evaluated using Student's *t* test: $^{a}P < 0.05$, $^{b}P < 0.01$. Significant differences from positive control group (spleen weight): $^{c}P < 0.05$, $^{d}P < 0.01$.

polysaccharides from *Panax ginseng*, *Ganoderma lucidum*, *Coriolus versicolor*, etc., which suggested that immunostimulatory effects might be the main mechanism of polysaccharides' anti-tumor activities (Shin et al., 2002; Cao & Lin, 2004; Ho, Konerding, Gaumann, Groth, & Liu, 2004). But some polysaccharides, such as polysaccharides from *Phellinus linteus* (Li et al., 2004) and *Cordyceps sinensis* (Chen, Shiao, Lee, & Wang, 1997), could directly inhibit the proliferation of cancer cell in vitro. In this study, we investigated the anti-tumor activities of APS-1d against two kinds of human solid cancer cell lines, HeLa and A549 in vitro. Fig. 7 showed the effects of the polysaccharide on the growth of HeLa and A549 cells. At the concentrations from 3 to 100 μg/ml, APS-1d significantly inhibited the proliferation of HeLa cells ($P < 0.01$) and the effects were in a concentration-dependent manner. At the highest concentration of 100 μg/ml, APS-1d had the inhibition ratio of 23.0 ± 5.5%. However, although APS-1d also had significant suppressing activity on A549 cells, there was no clear relationship between the concentrations and the effects. The highest inhibition ratio on A549 cells was 6.6 ± 3.6% between four dosages, which was far lower than the ratio (17.0 ± 5.8%) on HeLa cells at the same concentration (10 μg/ml). From above, APS-1d displayed significant anti-tumor activity, especially in human HeLa cells.

To confirm the anti-tumor activity of APS-1d in vivo, we used the mice transplanted S180 to evaluate the effects and the results were summarized in Table 3. APS-1d could inhibit the growth of the tumors ($P < 0.01$) in a dose-dependent manner. The inhibitory rate in mice treated with 100 mg/kg APS-1d was 50.7%, being the highest in the three doses. Furthermore, during the experiments, the appetite, activity and coat luster of each animal in APS-1d groups were better than the mice treated with cyclophosphamide. On the 14th day, the average increased body weight of negative control mice was 4.22 g, whereas the weight of mice in APS-1d group at dose of 100 mg/kg was 6.92 g. The increased body weights of the most test groups were significantly greater than those in the cyclophosphamide group ($P < 0.01$), even the negative control mice ($P < 0.05$). The average weights of the spleens in test groups were significantly greater than that in the cyclophosphamide mice ($P < 0.01$), and even that of the negative control mice ($P < 0.05$), indicating that APS-1d could increase the weights of immune organs. These results suggested that activating immune responses in the host might be one of the mechanisms of anti-tumor activity of APS-1d, as many anti-tumor polysaccharides found in the world (Ooi & Liu, 2000; Wasser, 2002).

4. Conclusion

APS-1d, first isolated from the roots of *A. sinensis*, was a heteropolysaccharide, having a backbone composed of 1,4-α-D-Glc*p*, with branches attached to O-6 of some residues. The branches were composed of 1,6-α-D-Glc*p* residues, and terminated with β-L-Ara*f* residues. APS-1d exhibited significant anti-tumor activates both in vitro and in vivo. The structural and pharmacological results obtained might help enlarge the knowledge of structural correlation to anti-tumor effects of polysaccharides.

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References

Agarwal, P. K. (1992). NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. *Phytochemistry, 31*, 3307–3330.

Barker, S. A., Bourne, E. J., Stacey, M., & Whiffen, D. H. (1954). Infrared spectra of carbohydrates. Part I. Some derivatives of D-glucopyranose. *Journal of the Chemical Society*, 171–176.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Cao, Q. Z., & Lin, Z. B. (2004). Antitumor and anti-angiogenic activity of *Ganoderma lucidum* polysaccharides peptide. *Acta Pharmacologica Sinica, 25*, 833–838.

Cardoso, S. M., Silva, A. M. S., & Coimbra, M. A. (2002). Structural characterisation of the olive pomace pectic polysaccharide arabinan side chains. *Carbohydrate Research, 337*, 917–924.

Chen, R., Wang, H., Xu, H., Xu, G., & Chang, L. (2001). Isolation, purification and determination of polysaccharides X-C-3-III and X-C-3-IV from *Angelica sinensis* (Oliv) Diels. *Zhong Yao Cai, 24*(1), 36–37.

Chen, Y. J., Shiao, M. S., Lee, S. S., & Wang, S. Y. (1997). Effect of *Cordyceps sinensis* on the proliferation and differentiation of human leukemic U937 cells. *Life Sciences, 60*, 2349–2359.

Cheng, Y. L., Chang, W. L., Lee, S. C., Liu, Y. G., Chen, C. J., Lin, S. Z., et al. (2004). Acetone extract of *Angelica sinensis* inhibits proliferation of human cancer cells via inducing cell cycle arrest and apoptosis. *Life Sciences, 75*(13), 1579–1594.

Cho, C. H., Mei, Q. B., Shang, P., Lee, S. S., So, H. L., Guo, X., et al. (2000). Study of the gastrointestinal protective effects of polysaccharides from *Angelica sinensis* in rats. *Planta Medica, 66*, 348–351.

Choy, Y. M., Leung, K. N., Cho, C. S., Wong, C. K., & Pang, P. K. (1994). Immunopharmacological studies of low molecular weight polysaccharide from *Angelica sinensis*. *American Journal of Chinese Medicine, 22*, 137–145.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimeteric method for determination of sugars and related substances. *Analytical Chemistry, 28*, 350–356.

Englyst, H. N., Quigley, M. E., & Hudson, G. J. (1994). Determination of dietary fibre as non-starch polysaccharides with gas–liquid chromatographic, high-performance liquid chromatographic or spectrophotometric measurement of constituent sugars. *Analyst, 119*, 1497–1509.

Funane, K., Ishii, T., Matsushita, M., Hori, K., Mizuno, K., Takahara, H., et al. (2001). Water-soluble and water-insoluble glucans produced by *Escherichia coli* recombinant dextransucrases from *Leuconostoc mesenteroides* NRRL B-512F. *Carbohydrate Research, 334*, 19–25.

Ho, J. C., Konerding, M. A., Gaumann, A., Groth, M., & Liu, W. K. (2004). Fungal polysaccharopeptide inhibits tumor angiogenesis and tumor growth in mice. *Life Sciences, 75*, 1343–1356.

Honda, S., Suzuki, S., Kakehi, K., Honda, A., & Takai, T. (1981). Analysis of the monosaccharide compositions of total non-dialyzable urinary glycoconjugates by the dithioacetal method. *Journal of Chromatography, 226*, 341–350.

Hou, Y. Z., Zhao, G. R., Yang, J., Yuan, Y. J., Zhu, G. G., & Hiltunen, R. (2004). Protective effect of *Ligusticum chuanxiong* and *Angelica sinensis* on endothelial cell damage induced by hydrogen peroxide. *Life Sciences, 75*(14), 1775–1786.

Hsu, Y. L., Kuo, P. L., & Lin, C. C. (2004). Proliferative inhibition, cell-cycle dysregulation, and induction of apoptosis by ursolic acid in human non-small cell lung cancer A549 cells. *Life Sciences, 75*, 2303–2316.

Ishurd, O., & Kennedy, J. F. (2005). The anti-cancer activity of polysaccharide prepared from Libyan dates (*Phoenix dactylifera* L. *Carbohydrate Polymers, 59*, 531–535.

Johnes, T. M., & Albersheim, P. (1972). A gas chromatographic method for the determination of aldose and uronic acid constituents of plant cell wall polysaccharides. *Plant Physiology, 49*, 926–936.

Kiyohara, H., Yamada, H., & Otsuka, Y. (1987). Unit structure of the anti-complementary arabinogalactan from *Angelica acutiloba* Kitagawa. *Carbohydrate Research, 167*, 221–237.

Li, G., Kim, D. H., Kim, T. D., Park, B. J., Park, H. D., Park, J. I., et al. (2004). Protein-bound polysaccharide from *Phellinus linteus* induces G2/M phase arrest and apoptosis in SW480 human colon cancer cells. *Cancer Letters, 216*, 175–181.

Li, S. P., Zhao, K. J., Ji, Z. N., Song, Z. H., Dong, T. T., Lo, C. K., et al. (2003). A polysaccharide isolated from *Cordyceps sinensis*, a traditional Chinese medicine, protects PC12 cells against hydrogen peroxide-induced injury. *Life Sciences, 73*(19), 2503–2513.

Liu, F., Ooi, V. E. C., & Chang, S. T. (1997). Free radical scavenging activities of mushroom polysaccharide extracts. *Life Sciences, 60*, 763–771.

Mei, Q. B., Tao, J. Y., & Cui, B. (1991). Advances in the pharmacological studies of radix *Angelica sinensis* (Oliv) Diels (Chinese Danggui). *Chinese Medical Journal, 104*, 776–781.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*, 55–63.

Needs, P. W., & Selvandran, R. R. (1993). Avoiding oxidative degradation during sodium hydroxide/methyl iodide-mediated carbohydrate methylation in dimethyl sulfoxide. *Carbohydrate Research, 245*, 1–10.

Oades, J. M. (1967). Gas-liquid chromatography of alditol acetates and its application to the analysis of sugars in complex hydrolysates. *Journal of Chromatography, 28*, 246–252.

Ooi, V. E., & Liu, F. (2000). Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. *Current Medicinal Chemistry, 7*, 715–729.

Ryden, P., Colquhoun, I. J., & Selvendran, R. R. (1989). Investigation of structural features of the pectic polysaccharides of onion by ^{13}C-n.m.r. spectroscopy. *Carbohydrate Research, 185*, 233–237.

Sarker, S. D., & Nahar, L. (2004). Natural medicine: The genus *Angelica*. *Current Medicinal Chemistry, 11*, 1479–1500.

Seymour, F. R., Knapp, R. D., & Bishop, S. H. (1976). Determination of the structure of dextran by ^{13}C nuclear magnetic resonance spectroscopy. *Carbohydrate Research, 51*, 171–194.

Seymour, F. R., Knapp, R. D., Bishop, S. H., & Jeans, A. (1979). High temperature enhancement of ^{13}C-N.M.R. chemical-shifts of unusual dextrans, and correlation with methylation structural analysis. *Carbohydrate Research, 68*, 123–140.

Shang, P., Qian, A. R., Yang, T. H., Jia, M., Mei, Q. B., Cho, C. H., et al. (2003). Experimental study of anti-tumor effects of polysaccharides from *Angelica sinensis*. *World Journal of Gastroenterology, 9*, 1963–1967.

Shang, P., Yang, T. H., Jia, M., Mei, Q. B., Zhao, W. M., Cao, Z. X., et al. (2001). Purification and analysis of polysaccharides of *Angelica sinensis* (Oliv) Diels. *Journal of the Fourth Military Medical University, 22*, 1311–1314.

Shin, J. Y., Song, J. Y., Yun, Y. S., Yang, H. O., Rhee, D. K., & Pyo, S. (2002). Immunostimulating effects of acidic polysaccharides extract of *Panax ginseng* on macrophage function. *Immunopharmacology and Immunotoxicology, 24*, 469–482.

Sun, Y., Tang, J., Gu, X., & Li, D. (2005). Water-soluble polysaccharides from *Angelica sinensis* (Oliv.) Diels: Preparation, characterization and bioactivity. *International Journal of Biological Macromolecules, 36*(5), 283–289.

Swamy, N. R., & Salimath, P. V. (1991). Arabinans from *Cajanus cajan* cotyledon. *Phytochemistry, 30*, 263–265.

Sweet, D. P., Shapiro, R. H., & Albersheim, P. (1975). Quantitative analysis by various g.l.c response-factor theories for partially methylated and partially ethylated alditol acetates. *Carbohydrate Research, 40*, 217–225.

Tomatsu, M., Ohnishi-Kameyama, M., & Shibamoto, N. (2003). Aralin, a new cytotoxic protein from *Aralia elata*, inducing apoptosis in human cancer cells. *Cancer Letter, 199*, 19–25.

Tsai, N. M., Linm, S. Z., Lee, C. C., Chen, S. P., Su, H. C., Chang, W. L., et al. (2005). The antitumor effects of *Angelica sinensis* on malignant brain tumors in vitro and in vivo. *Clinical Cancer Research, 11*(9), 3475–3484.

Tylianakis, M., Spyros, A., Dais, P., Taravel, F. R., & Perico, A. (1999). NMR study of the rotational dynamics of linear homopolysaccharides in dilute solutions as a function of linkage position and stereochemistry. *Carbohydrate Research, 315*, 16–34.

Uzochukwu, S., Balogh, E., Loefler, R. T., & Ngoddy, P. O. (2002). Structural analysis by ^{13}C-nuclear magnetic resonance spectroscopy of glucan extracted from natural palm wine. *Food Chemistry, 76*, 287–291.

Wang, B. H., Ouyang, J. P., Liu, Y. M., Wei, L., & Yang, J. W. (2001). Protective effect of Angelica on ECV(304) from injury induced by hyperlipidemic serum in vitro. *Sheng Li Xue Bao, 53*(3), 240–243.

Wang, Q., Ding, F., Zhu, N., He, P., & Fang, Y. (2003). Determination of the compositions of polysaccharides from Chinese herbs by capillary zone electrophoresis with amperometric detection. *Biomedical Chromatography, 17*(7), 483–488.

Wang, Z., Peng, X., Huang, L., Peng, Z., & Tian, G. (2001). Structure elucidation of glycan of a glycoconjugate SPPA-1 isolated from *Spirulina Platrnsis*. *Yao Xue Xue Bao, 36*(5), 356–359.

Wasser, S. P. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology, 60*, 258–274.

Wei, Y. A., & Fang, J. N. (1989). Determination of purity and molecular weight of polysaccharides by high performance gel permeation chromatography. *Yao Xue Xue Bao, 24*, 532–536.

Weng, X. C., Zhang, P., Gong, S. S., & Xiai, S. W. (1987). Effect of immuno-modulating agents on murine IL-2 production. *Immunological Investigations, 16*(2), 79–86.

Wilasrusmee, C., Siddiqui, J., Bruch, D., Wilasrusmee, S., Kittur, S., & Kittur, D. S. (2002). In vitro immunomodulatory effects of herbal products. *American Surgeon, 68*(10), 860–864.

Wu, S. J., Ng, L. T., & Lin, C. C. (2004). Antioxidant activities of some common ingredients of traditional chinese medicine, *Angelica sinensis*, *Lycium barbarum* and *Poria cocos*. *Phytotherapy Research, 18*(12), 1008–1012.

Yamada, H. (1994). Pectic polysaccharides from Chinese herbs: Structure and biological activity. *Carbohydrate Polymers, 25*, 269–276.

Yamada, H., Kiyohara, H., Cyong, J. C., & Otsuka, Y. (1985). Studies on polysaccharides from *Angelica acutiloba*-IV. Characterization of an anti-complementary arabinogalactan from the roots of *Angelica acutiloba* Kitagawa. *Molecular Immunology, 22*, 295–304.

Yamada, H., Komiyama, K., Kiyohara, H., Cyong, J. C., Hirakawa, Y., & Otsuka, Y. (1990). Structural characterization and antitumor activity of a pectic polysaccharide from the roots of *Angelica acutiloba*. *Planta Medica, 5*, 182–186.

Yamada, H., Kiyohara, H., & Otsuka, Y. (1984). Characterization of a water-soluble glucan from *Angelica acutiloba*. *Phytochemistry, 23*, 587–590.

Yang, T., Jia, M., Mei, Q., & Shang, P. (2002). Effects of Angelica polysaccharide on blood coagulation and platelet aggregation. *Zhong Yao Cai, 25*, 344–345.

Ye, Y. N., So, H. L., Liu, E. S., Shin, V. Y., & Cho, C. H. (2003). Effect of polysaccharides from *Angelica sinensis* on gastric ulcer healing. *Life Sciences, 72*, 925–932.

Yim, T. K., Wu, W. K., Pak, W. F., Mak, D. H., Liang, S. M., & Ko, K. M. (2000). Myocardial protection against ischaemia-reperfusion injury by a Polygonum multiflorum extract supplemented ‘Dang-Gui decoction for enriching blood’, a compound formulation, ex vivo. *Phytotherapy Research, 14*(3), 195–199.

Zhao, K. J., Dong, T. T., Tu, P. F., Song, Z. H., Lo, C. K., & Tsim, K. W. (2003). Molecular genetic and chemical assessment of radix Angelica (Danggui) in China. *Journal of Agricultural and Food Chemistry, 51*, 2576–2583.

Zhou, G., Sun, Y., Xin, H., Zhang, Y., Li, Z., & Xu, Z. (2004). In vivo antitumor and immunomodulation activities of different molecular weight lambda-carrageenans from *Chondrus ocellatus*. *Pharmacological Research, 50*, 47–53.