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Antioxidant activity of *Astragalus* polysaccharides and antitumour activity of the polysaccharides and siRNA

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ABSTRACT

In this study, *Astragalus* polysaccharides was analysed using high performance liquid chromatography (HPLC) and Fourier transform infrared (FT-IR). Compared with retention time of six standard compounds in HPLC analyses, we found that the polysaccharides were composed of glucose. The absorption bands at 1639 and 1538 cm^{-1} were attributed to the stretching vibration of the C–O bond of carboxyl group. The absorption bands at 1035 and 956 cm^{-1} suggested that the extract contained pyrene monomer in its structure. The antioxidant activity of *Astragalus* polysaccharides was evaluated by various antioxidant assays. *Astragalus* polysaccharides showed strong antioxidant activity in all the tested methods. The in vitro antioxidant capacity of *Astragalus* polysaccharides was significantly correlated with its content. In addition to the antioxidant activity of the polysaccharides, it still showed strong antitumour activity. Then, CD40 gene siRNA plasmid (Psilencer1. 0-U6-P I3K-siRNA) was constructed. Antitumour activity of siRNA was evaluated. Among these samples, *Astragalus* polysaccharides exhibited more antitumour potency than siRNA. Mechanism of antitumour activity of *Astragalus* polysaccharides and siRNA may be explained by decreasing CD40 in cells.

1. Introduction

Oxidative stress, induced by oxygen radicals, is believed to be a primary factor in various degenerative diseases, such as cancer (Muramatsu et al., 1995; Zhao, Zhang & Li, 2009), atherosclerosis (Steinberg, Parthasarathy, Carew, Khoo, & Witztum, 1989), gastric ulcer (Das, Bandyopadhyay, Bhattacharjee, & Banerjee, 1997; Adesina et al., 2009), and other conditions (Oliver, Ahn, Moerman, Goldstein, & Stadtmaan, 1987). Many antioxidant compounds, naturally occurring from plant sources, have been identified as a free radical or active oxygen scavengers (Zheng & Wang, 2001; Wang et al., 2009; Mocanu et al., 2009). Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity (Ito, Fukushima, Hasegawa, Shibata, & Ogiso, 1983).

Astragalus membranaceus (Fisch.) Bunge (AM), Maxim of the Leguminosae family, is a traditional Chinese medicinal herb originated in Northern China. The dried root of AM, Huangqi, contains 2′4′-dihydroxy-5,6-dimethoxyisoflavone, kumatakenin, choline, betaine, polysaccharides, saponins, glucuronic acid, sucrose, amino acids, traces of folic acid and astraisoflavanin (Bensky & Gamble, 1993; Ma, Shi, Duan, Dong, & Tsim, 2002; Wu & Chen, 2004). Huangqi is the Chinese name for the root of AM. Huangqi, which was found to be effective in treating a wide variety of diseases, has been extensively used as a tonic to enhance the body's defense system (Liu, Yang, & Du, 2004; Yin et al., 2004). Evidences have indicated the importance of AM polysaccharide fractions in the modulation of immune functions both in human and experimental animals (Chen, Shen, Wang, Zhai, & Liu, 1981). Small interfering RNA (siRNA) is a powerful tool for controlling cellular processes of gene silencing at a posttranscriptional level due to high sequence-specific inhibition efficiency (Novina & Sharp, 2004). There have been numerous studies using siRNAs for down-regulations of certain proteins in areas of functional genomics and genomic therapeutics (Dorsett & Tuschl, 2004; Ryther, Flynt, Phillips, & Patton, 2005; Schiffelers, Woodle & Scaria, 2004). Although, siRNAs can be potential therapeutic agents for various genetic-related diseases including cancer, their therapeutic applications are still limited because of inherent instability against nucleases and poor intracellular uptake (Raymond et al., 2004; Thazha et al., 2005).

In the present study, we extracted water-soluble polysaccharides from *Astragalus*. Analysis of antioxidant and antitumour activities of the *Astragalus* polysaccharides was performed.

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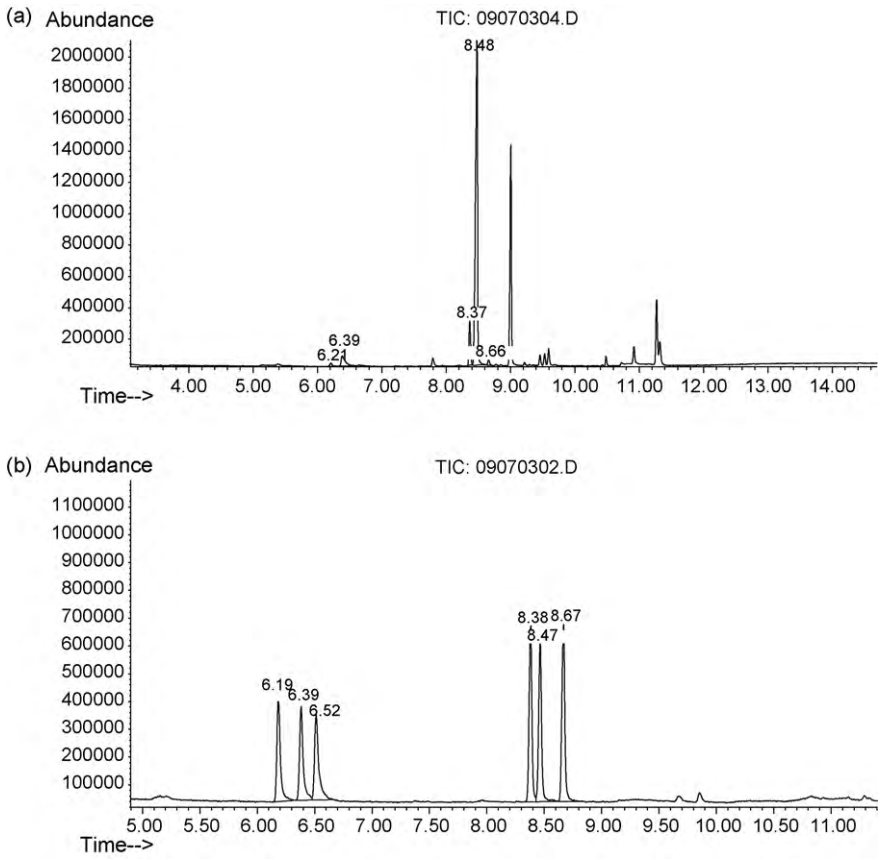

Fig. 1. (a) HPLC of *Astragalus* polysaccharides; (b) HPLC of standard sample.

2. Materials and methods

2.1. HPLC analysis

Astragalus polysaccharides were hydrolyzed with 2 M H_2SO_4 for 5–6 h at 121 °C in sealed glass test tube. After complete hydrolysis, content was neutralized with $BeCO_3$ and filtered. Monosaccharide composition of the hydrolysate was determined by HPLC (Waters Alliance, 2996-seperation module) using Supelco gel 610H column (30 cm × 7.8 mm) and RI (2414) detector with flow rate 0.4 mL/min at temperature 30 °C and mobile phase, 0.17% H_3PO_4 in water. The relative proportion of the peak area was calculated to estimate the monomer composition.

2.2. IR

Infrared spectra (IR) were also used to identify the polysaccharides compounds. The infrared spectra (450–4000 cm^{-1}) of all the subfractions (EA1–EA7) were recorded in potassium bromide (KBr) disks with a Fourier transform IR spectrophotometer (Bio-Rad FTS-135). One milligram of dry sample was mixed with 100 mg of dry KBr, and the mixture was pressed into a disk for spectrum recording.

2.3. Superoxide anion scavenging activity

Measurement of superoxide anion scavenging activity was based on the method described by Liu, Ooi, and Chang (1997) with slight modification. Superoxide radicals were generated in a PMS-NADH system by oxidation of reduced form of nicotinamide-adenine dinucleotid (NADH) and assayed by the reduction of nitro blue tetrazolium (NBT). In these experiments, the superoxide radicals were generated in 3 mL of Tris–HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (50 μM) solution, 1 mL of NADH (78 μM) solution and varying concentrations of sample solution in Tris–HCl buffer. The reaction was initiated by adding 1 mL of PMS solution (10 μM) to the mixture. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm was measured against blank samples. In the essential control, NADH was substituted with Tris–HCl buffer. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\%\,\text{Inhibition} = \left(\frac{1 - A_1}{A_0}\right) \times 100\%,$$

where A_0 is the absorbance of the control and A_1 is the absorbance of samples.

2.4. OH^- scavenging activity in vitro

OH^- was generated by the Fenton reaction and detected by its ability to hydroxylate salicylic acid as described by Smirnoff and Cumbes (1988). The reaction mixture, total volume of 3 mL, contained 150 mM potassium phosphate buffer (pH 7.4), 0.26 mM ascorbic acid, 0.15 mM $FeSO_4$, 0.6 mM H_2O_2, 2 mM Na salicylate and varying concentrations of sample. The OH^- scavenging activity of sample was assessed according to its ability to compete with salicylate for OH^-.

2.5. Antitumour activity of polysaccharides and siRNA

CD40 gene siRNA plasmid (Psilencer1. 0-U6-P I3K-siRNA) was constructed. A preliminary experiment was carried out to inves-

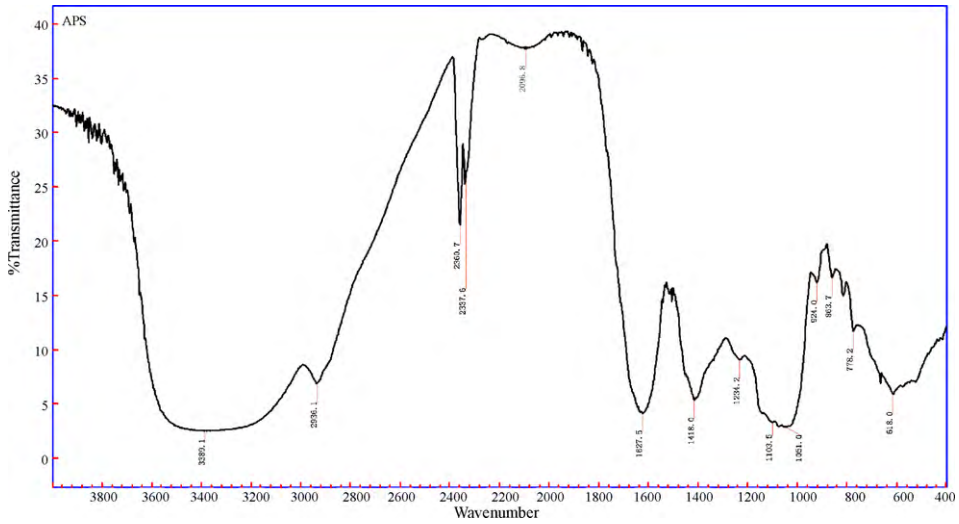

Fig. 2. Infrared spectroscopy of *Astragalus* polysaccharides.

tigate the antitumour activity of *Astragalus* polysaccharides and siRNA in vivo. Male mice (2 weeks, 24–30 g) were inoculated subcutaneously with viable B16F10 cells (10^5 cells/0.1 mL). Tumours were allowed to establish (area approx. 70 mm^2; product of two orthogonal diameters), and a single treatment (siRNA) at a dose of nM administered by intraperitoneal injection. Another 5 animals were orally treated with *Astragalus* polysaccharides (100 mg/kg body weight) for 1 week. Tumour size was monitored.

3. Result and discussion

3.1. Preparative HPLC separation

The HPLC analyses of the polysaccharides showed one main peak component, detected with an ELSD system (Fig. 1a). The preparative HPLC separations of standard samples finally yielded five compounds corresponding to peaks 1, 2, 3, 4 and 5 (Fig. 1b). From the comparison of retention time of the six standard compounds in HPLC analyses, we found that the polysaccharides were composed of glucose. That mean that glucan appeared as the major compound in the polysaccharides extract.

3.2. IR spectrum

Fig. 2 showed the IR spectrum of the polysaccharides extract. The absorption band at 2360.7 and 2337.6 cm^{-1} was assigned to the hydroxyl (OH) group. The absorption bands at 1639 and 1538 cm^{-1} were attributed to the stretching vibration of the C–O bond of carboxyl group. The absorption band at 1035 and 956 cm^{-1} suggested that the extract contained pyrene monomer in its structure.

The band at 850.81 cm^{-1} was ascribed to α-type glycosidic linkages in the polysaccharide (Barker, Bourne, Stacey, & Whiffen, 1954). The bands at 850.81 and 915.56 cm^{-1} were characteristic of (1 → 4)-α-glucan (Li et al., 2008).

3.3. Superoxide anion radical-scavenging activity

Overproduction of superoxide anion radical has long been known as the starting point of ROS/RNS accumulation in cells, contributing to redox imbalance and associated harmful physiological consequences (Pervaiz & Clement, 2007). One simple, rapid and low-cost method for evaluating superoxide anion radical-scavenging activity of extracts is based on the triad NADH/reduced phenazine methosulfate (PMS)/dioxygen as a source of superoxide anion radical. Reduction of nitro blue tetrazolium (NBT), as a superoxide anion radical-scavenging compound, results in a stable formazan. Fig. 3 showed superoxide anion radical-scavenging activity of the polysaccharides. The polysaccharides revealed modest scavenging activity of superoxide anion radical, monitored as a significant inhibition of the NBT reduction index. It is well known that superoxide radical itself is not a "super" redox agent, but is a key upstream source of highly oxidising derivatives, such as hydroxyl radicals and reactive nitrogen species (Radi, Peluffo, Alvarez, Naviliat, & Cayota, 2001).

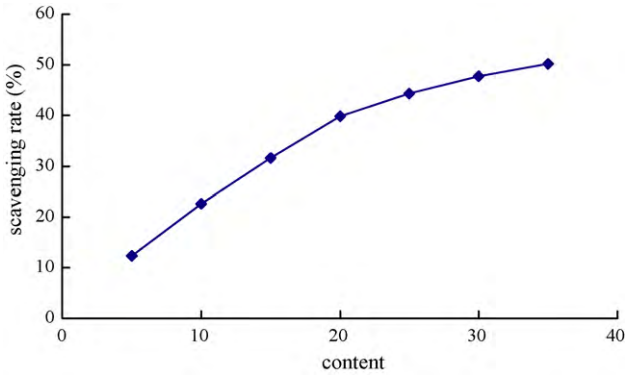

Fig. 3. Superoxide anion radical-scavenging activity of *Astragalus* polysaccharides.

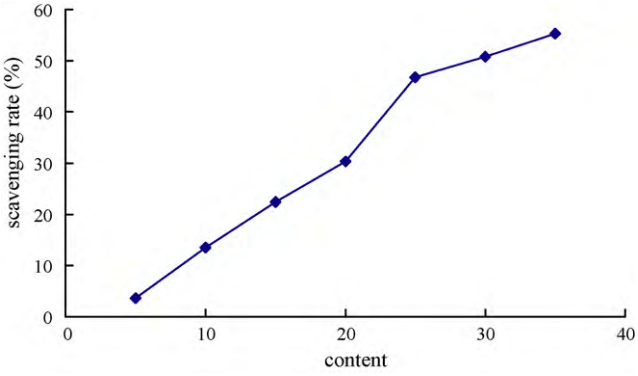

Fig. 4. Hydroxyl radical-scavenging activity of *Astragalus* polysaccharides.

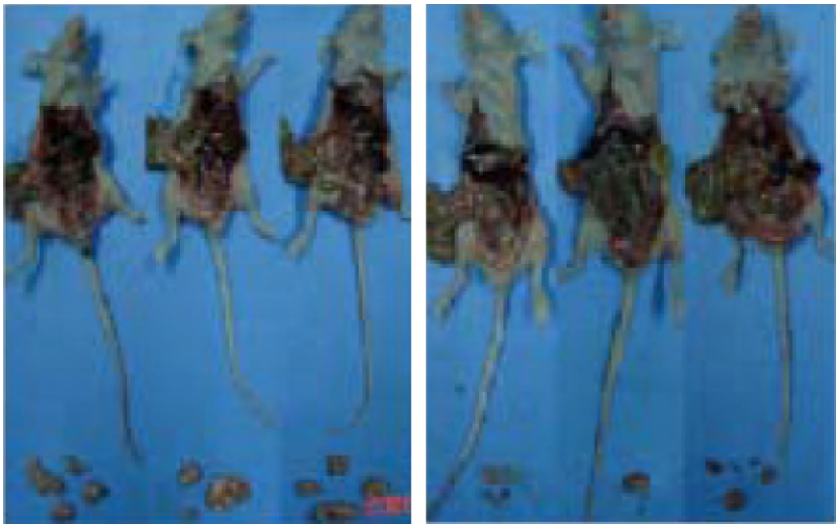

Fig. 5. Antitumour activity of *Astragalus* polysaccharides and siRNA.

3.4. Hydroxyl radical scavenging activity

Hydroxyl radicals generated in Fe^{2+}/H_2O_2 system is trapped by 5,5-dimethyl-1-pyrroline N-oxide (DMPO) forming spin adduct which could be detected by electron spin resonance (ESR) spectrometer. The ESR spectrum is inhibited by the presence of hydroxyl radical scavengers, which compete with DMPO for hydroxyl radicals (Gao, Huang, Yang, & Xu, 1999). As illustrated in Fig. 4, the radical scavenging activity of polysaccharides increased with the increasing content.

3.5. Antitumour activity of Astragalus polysaccharides and siRNA

Applying the standard criteria for antitumour activity (T/C < 50% and SGD > 1.0) antitumour activity was achieved in viable B16F10 cells. Antitumour activity of *Astragalus* polysaccharides was observed in tumour cells. Moderate to high antitumour activity was observed with increasing doses of siRNA treatment (Fig. 5). We speculated that *Astragalus* polysaccharides and siRNA displayed strong antitumour activity by decreasing CD40 in cells according to our previous work (Li et al., 2009).

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