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Antioxidant and immunological activities of water-soluble polysaccharides from *Aconitum kusnezoffii* Reichb.

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ABSTRACT

Aconitum kusnezoffii Reichb., one of the earliest recorded toxic species of genus *Aconitum*, has been used as traditional Chinese medicine and medicinal diet over the last 2500 years to treat heart failure congestion, neuralgia, rheumatism and gout, etc. In the present paper, four water-soluble polysaccharide fractions isolated from the tubers of *A. kusnezoffii* Reichb. were studied the antioxidant and immunological activities for the first time. *In vitro* antioxidant assays indicated that fraction WKCP-A had noticeable scavenging activities on DPPH radical, hydroxyl radical, superoxide anion, H_2O_2 and self-oxidation of 1,2,3-phentriol, ferrous ion-chelating ability and reducing power. Moreover, the *in vivo* immunological assay exhibited that fractions WKCP-A and WKHP could more significantly enhance splenic lymphocyte proliferation and macrophage phagocytosis than other fractions. Therefore, the water-soluble polysaccharides from *A. kusnezoffii* Reichb., especially WKCP-A, have the potential to be explored as novel natural antioxidants and immunostimulating agents for using in functional foods or medicine.

1. Introduction

Oxidative stress imposed by reactive oxygen species (ROS) may be the direct or indirect cause of tissue damage and many human diseases such as aging, cancer, atherosclerosis and inflammation [1]. The synthetic antioxidants, able to prevent the radical chain reactions of oxidation, present almost unavoidable side effects which might be responsible for liver damage and carcinogenesis [2]. Thus, it is essential to develop natural antioxidants which can protect the human body from free radicals and retard the progress of many chronic diseases. As research being carried out, medicinal plants are proved to be good resources of natural antioxidants [3–7].

Aconitum kusnezoffii Reichb., one of the earliest recorded toxic species of genus *Aconitum*, has been used as traditional Chinese medicine (TCM) to treat heart failure congestion, neuralgia, rheumatism and gout, etc. by homeopaths over the last 2500 years [8–10]. It also has been used as medicinal diet for many years, such as stewing with meat. But eating too much or cooking improperly will make one's poisoning. Up to now, the well-investigated pharmaceutical ingredients in *A. kusnezoffii* Reichb. are a group of small molecular compounds including diterpene alkaloids, isoquinoline alkaloids and amines. However, there is no report on the water-soluble polysaccharides from it to date. In the present study, four water-soluble polysaccharide fractions were isolated from tubers of *A. kusnezoffii* Reichb. Furthermore, their antioxidant and immunological activities were evaluated by *in vitro* or *in vivo* assays.

2. Materials and methods

2.1. Plant materials and chemicals

A. kusnezoffii Reichb. was collected in October, 2008 at Changbai mountain area in Jilin Province, China and kindly identified by Prof. H. Xiao, School of Life Sciences, Northeast Normal University in Changchun, China. A voucher specimen (No. 20081009) was deposited at the School of Life Sciences, Northeast Normal University.

1,1-Diphenyl-2-picrylhydrazy (DPPH), ascorbic acid, deoxyribose, ethylenediamine tetraacetic acid (EDTA), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), dihydronicotineamidadenine dinucleotide (NADH), nitro blue tetrazolium salt (NBT), horseradish peroxidase (HRPase, 300 U/mg), phenazine methosulfate (PMS), ferrozine [3-(2-pyridy)-5,6-bis-(4-phenylsulfonicacid)-1,2,4-triazine, monosodium salt], potassium ferricyanide [$K_3Fe(CN)_6$],

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1,2,3-phentriol, 2,6-di-tert-butyl-4-methylphenol (BHT), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (ConA) and lipopolysaccharide (LPS) were purchased from Sigma Co. (St. Louis, USA), while Sepharose CL-6B and DEAE-Sepharose Fast Flow were from the Pharmacia Co. (Sweden). Lentinan (SFDA License No.: H20067183) was purchased from Nanjing Pharmaceutical Factory Co. Ltd. (China). Medium RPMI-1640 was from Gibco Invitrogen Co. The complete RMPI-1640 medium, used for immunological tests, was supplemented with penicillin 100 IU/mL, streptomycin 100 μg/mL and 10% fetal bovine serum (FBS), pH 7.4. All other reagents used were of analytical grade made in China.

2.2. Animals and treatment

ICR mice (Grade II, 5–6 weeks old) weighing 18–22 g, half male and half female, were purchased from the Center of Experimental Animals of China Medical University and acclimatized for 1 week prior to use (24 ± 1 °C, with humidity of 50 ± 10%, and a 12/12 h light/dark cycle). All the procedures were in strict accordance with the PR China legislation on the use and care of laboratory animals and with the guidelines established by the Center of Experimental Animals of China Medical University and were approved by the university committee for animal experiments.

2.3. Preparation of polysaccharide fractions

The tubers of *A. kusnezoffii* Reichb. were dried at 50 °C for three days, before grinding. The ground material was first exhaustively extracted with 95% ethanol under reflux for 12 h to remove hydrophobic compounds. After filtration through a gauze (100 mesh), the residues were dried at room temperature, and then extracted with cold water (4 °C, 1:20, w/v) three times (6 h for each). The aqueous filtrates were combined and concentrated to small volume, and then 95% ethanol was added to the aqueous filtrates up to 80% to precipitate the polysaccharides which were collected by centrifugation and dried in vacuum. The precipitate was dissolved in water (5%, w/v) and centrifuged to remove the insoluble substances by centrifugation. The supernatant was treated with Sevag reagent (1:4 of n-butanol:chloroform, v/v) to remove free proteins. After removing the remaining Sevag reagent by vacuum evaporation, the water phase was submitted to freeze-thawing process, centrifuged to remove the precipitate and fractionated by DEAE-Sepharose Fast Flow ion-exchange chromatography, eluted with distilled water and 0.5 M NaCl by turns, giving two fractions: WKCP-N and WKCP-A, respectively. Meanwhile, the cold water extracted residues were extracted with hot water (95 °C, 1:20, w/v, 6 h × 3) and fractionated following the above procedure to obtain fractions WKHP-N and WKHP-A. The four fractions WKCP-N, WKCP-A, WKHP-N and WKHP-A were respectively further purified by Sepharose CL-6B gel filtration chromatography to make their molecular weights homogeneous.

2.4. General methods

The total carbohydrate content was determined by phenol–H_2SO_4 method using glucose as standard. All gel filtration chromatography was monitored by assaying carbohydrate content. Uronic acid content was determined by *m*-hydroxydiphenyl colorimetric method, using galacturonic acid as standard [11]. Protein content was determined by the method of Sedmak and Grossberg [12], with Coomassie brilliant blue reagent and bovine serum albumin as the standard. Starch content was determined according to the method of Gur et al. [13], using soluble starch as the standard. Contaminant endotoxin was analyzed by a gel-clot Limulus amebocyte lysate assay [14].

UV–vis absorbance spectra were recorded with a UV–vis spectro-photometer (Model SP-752, China). HPLC was carried out on a Shimadzu 10Avp HPLC system equipped with 10Avp HPLC Pump, SPD-10Avp UV-VIS detector. FT-IR spectra were obtained on a Nicolet 560 FT-IR spectrometer with DTGS detector in a range of 400–4000 cm^{-1}. The samples were measured as a film on KBr discs.

2.5. Determination of homogeneity and molecular mass

The homogeneity and molecular weight distributions were determined by gel filtration chromatography. Sample (5 mg) was dissolved in 0.15 M NaCl (0.5 mL), and applied to a column of Sepharose CL-6B (85× 1.5 cm i.d.), eluting with 0.15 M NaCl at a flow rate of 0.15 mL/min. The gel filtration column was calibrated by standard dextrans (12 kDa, 50 kDa, 150 kDa, 470 kDa and 670 kDa) using linear regression.

2.6. Determination of the monosaccharide component

The monosaccharide component analysis was performed by HPLC method as described by Honda et al. [15] Briefly, sample (2 mg) was first methanolyzed using anhydrous methanol (0.5 mL) containing 2 M HCl at 80 °C for 16 h. Then the methanolyzed products were hydrolyzed with 2 M CF_3COOH (0.5 mL) at 120 °C for 1 h. The hydrolyzed-products monosaccharides were derivatized to be 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives and subsequently analyzed by HPLC on a Shim-pak VP-ODS column (150 mm × 4.6 mm i.d.) with a guard column on a Shimadzu HPLC system and monitored by UV absorbance at 245 nm.

2.7. Determination of antioxidant activity

The scavenging activity of the DPPH free radical was assayed according to the method of Shimada et al. [16]. Hydroxyl radical-scavenging activity was determined as described by Halliwell et al. [17]. The superoxide radical scavenging activity was assayed by the method of Liu et al. in the NADH-NBT-PMS system [18]. The H_2O_2 scavenging activity was assayed by the method of Pick and Mizel [19]. The chelating activity on ferrous ions was measured as described by Dinis et al. [20]. The reducing power was determined referring to the ferric-reducing antioxidant power (FRAP) assay as described by Yuan et al. [21]. The scavenging activity for self-oxidation of 1,2,3-phentriol was evaluated according to the method of Marklund and Marklund [22]. Ascorbic acid, BHT or EDTA was used for comparison. The experimental data were subjected to an analysis of variance for a completely random design.

2.8. Determination of immunological activity

ICR mice were randomly divided into groups of 6 mice each. Polysaccharide fractions were dissolved in physiological saline and administered intraperitoneally (i.p.) into mice at different dosages (50, 100, 200 mg/kg) daily for 10 days. The control group was given physiological saline instead of polysaccharide solution, and lentinan (50 mg/kg) was used to be positive control. The dose volume was 0.2 mL. Lymphocyte proliferation assay was carried out as described by Bao et al. [23]. Macrophage phagocytosis activity was evaluated as described by Chen et al. [24].

2.9. Statistical analysis

All the data were expressed as means ± standard deviation (SD) of six replications. Statistical Product and Service Solutions (SPSS 13.0) was used to calculate IC_{50} values and analyze the variance. One-way ANOVA analysis of variance and Student's *t*-tests were

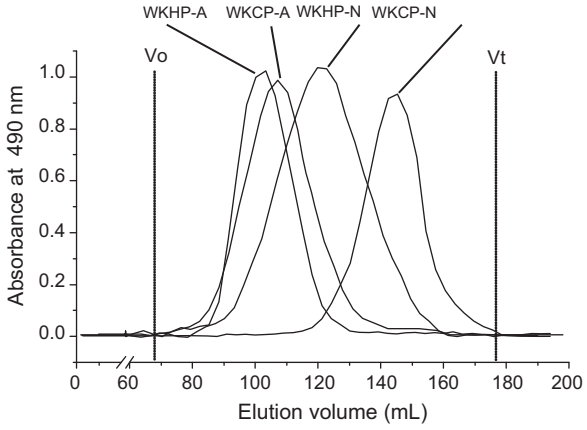

Fig. 1. Sepharose CL-6B elution profiles of water-soluble polysaccharides from *Aconitum kusnezoffii* Reichb.

conducted to identify differences among means. Statistical significance was declared at $P < 0.05$.

3. Results

3.1. Characterization of the polysaccharide fractions

The total carbohydrate, uronic acid, protein, starch contents and yield of each polysaccharide fraction are listed in Table 1, and the monosaccharide composition and molecular weight distribution are shown in Table 2. The endotoxin level in each polysaccharide solution was less than 0.5 EU (endotoxin units)/mL.

The results exhibited that the fractions WKCP-N and WKHP-N were mainly composed of glucans with Mw 9.6 kDa and 113.4 kDa, respectively (Fig. 1), and the absence of starch in them. The characteristic absorbance of polysaccharides at around 1645, 1410 and 1250 cm^{-1} and obvious characteristic peaks of α-D-glucan appeared at around 850 and 925 cm^{-1} indicated that WKCP-N and WKHP-N were mainly α-D-glucans (Fig. 2) [25,26]. While, WKCP-A and WKHP-A contained large amounts of Gal and Ara (Fig. 3), suggesting that these fractions might be composed of arabinans, galactans and/or arabinogalactans [27]. The ratios of Rha/GalUA

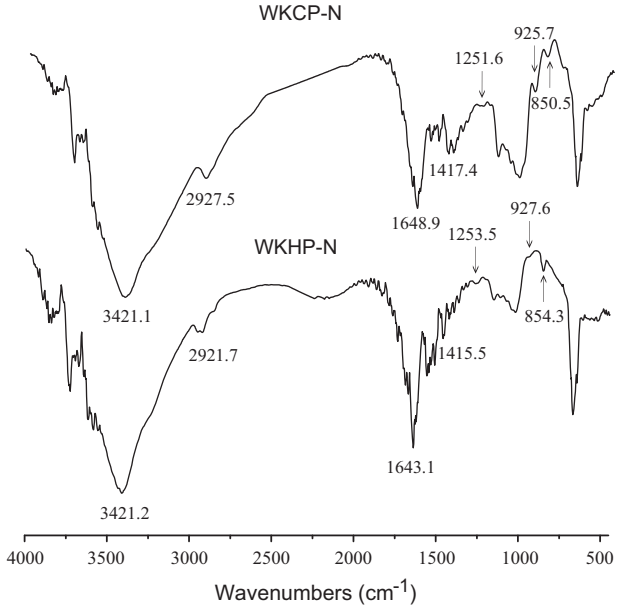

Fig. 2. FT-IR spectra of WKCP-N and WKHP-N from *Aconitum kusnezoffii* Reichb.

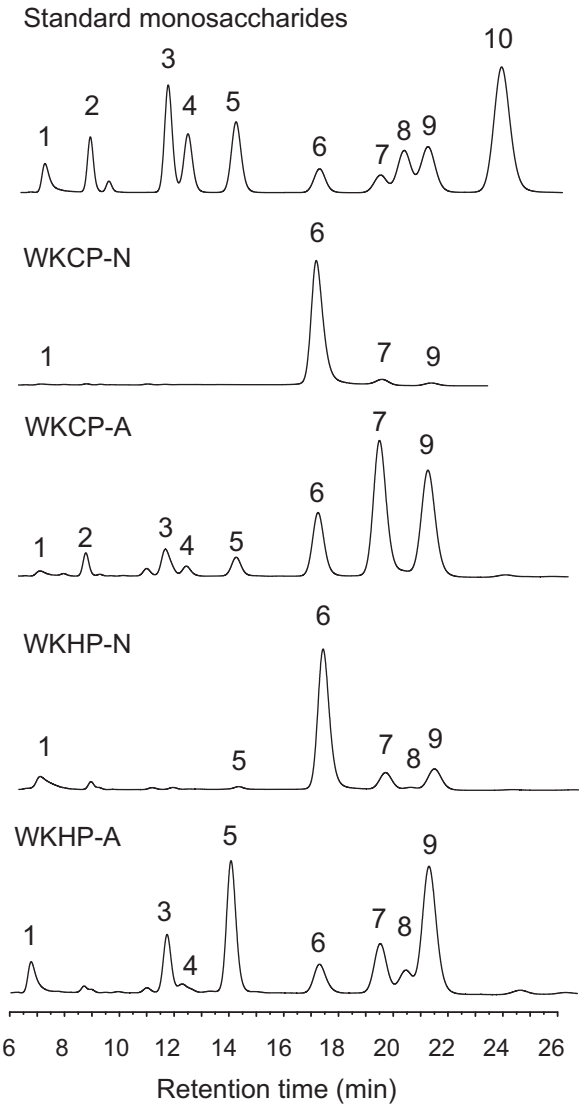

Fig. 3. Monosaccharide compositions of water-soluble polysaccharides from *Aconitum kusnezoffii* Reichb. Each fraction was methanolyzed, hydrolyzed, dried, PMP-labeled and analyzed by HPLC as described in Section 2: 1, PMP; 2, mannose; 3, rhamnose; 4, glucuronic acid; 5, galacturonic acid; 6, glucose; 7, galactose; 8, xylose; 9, arabinose; 10, fucose.

determined for WKCP-A and WKHP-A were 0.86 and 0.37, respectively, which are among the RG-I range from 0.05 to 1.0 defined by Schols and Voragen [28]. This suggested that they might contain RG-I domains. RG-I has been reported to be composed of a-(1,4)-linked D-galacturonic acid and a-(1,2)-linked L-rhamnose, which are alternatively combined with each other in the backbone; and some of the rhamnose residues contained side chains, such as arabinan, galactan and arabinogalactan at 4-O-rhamnose [29]. Low contents of GalUA in WKCP-A indicated that it contained a very low proportion of rhamnogalacturonan regions. The arabinans, galactans and/or arabinogalactans might be associated with RG-I domains in non-covalent form or as side chains of RG-I.

3.2. Antioxidant activities of water-soluble polysaccharides from *A. kusnezoffii Reichb.*

3.2.1. DPPH-radical scavenging activity

The DPPH free radical, a stable free radical, is widely used to evaluate the free radical scavenging ability of natural compounds, and the DPPH radical-scavenging activity was due to

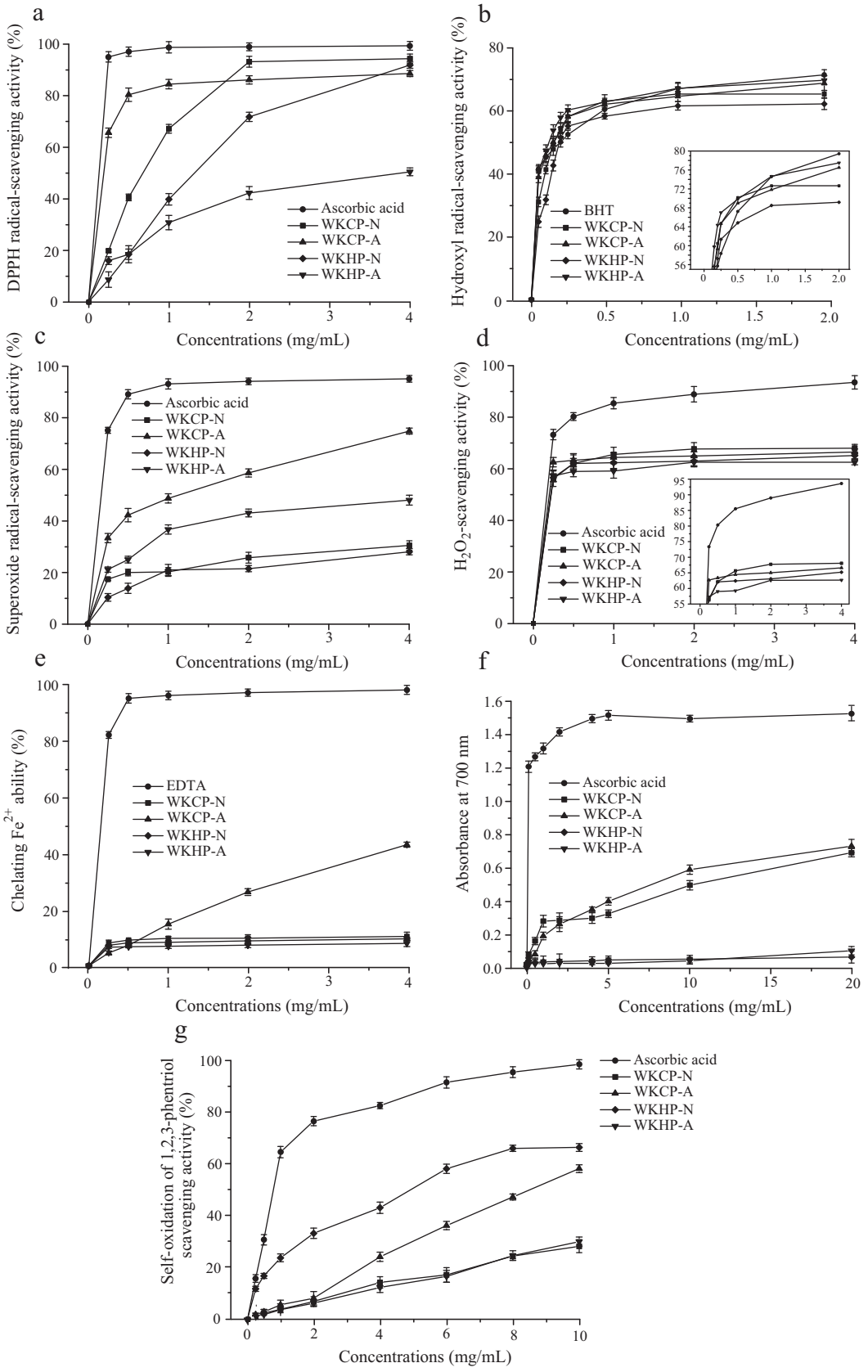

Fig. 4. Antioxidant activity of water-soluble polysaccharides from *Aconitum kusnezoffii* Reichb.: (a) scavenging activity to DPPH-radical; (b) scavenging activity to hydroxyl radical; (c) scavenging activity to superoxide anion; (d) scavenging activity to H_2O_2; (e) chelating activity on ferrous ion; (f) reducing power; (g) scavenging activity to self-oxidation of 1,2,3-phentriol; data are presented as means ± SD ($n = 6$).

Table 1
The yield, total carbohydrate, uronic acid, protein and starch content of each polysaccharide fraction from *Aconitum kusnezoffii* Reichb.

Fraction	Yield[a] (%)	Total carbohydrate content[b] (%)	Uronic acid content[c] (%)	Protein content[d] (%)	Starch content[e] (%)
WKCP-N	0.75	99.9	0	0	0.1
WKCP-A	0.13	99.8	6.1	0.1	0
WKHP-N	0.30	99.9	3.2	0	0.1
WKHP-A	0.34	99.9	29.7	0	0

[a] Yield calculated based on dried materials.
[b] The total carbohydrate content was determined by phenol–H_2SO_4 method using glucose as standard.
[c] Uronic acid content was determined by *m*-hydroxydiphenyl colorimetric method, using galacturonic acid as standard.
[d] Protein content was determined according to the method of Sedmak and Grossberg [12], with Coomassie brilliant blue reagent and bovine serum albumin as the standard.
[e] Starch content was determined according to the method of Gur et al. [13], using soluble starch as the standard.

their hydrogen-donating ability. As shown in Fig. 4a, all the fractions had dose-dependent scavenging activities and IC_{50} values of polysaccharide fractions were 0.61 mg/mL (WKCP-N), 0.04 mg/mL (WKCP-A), 1.08 mg/mL (WKHP-N), and 3.34 mg/mL (WKHP-A), respectively. The scavenging activities of WKCP-N and WKHP-N were closed to that of ascorbic acid at high concentrations, whereas that of ascorbic acid was over 95% at 0.25 mg/mL.

3.2.2. Hydroxyl radical scavenging activity

Hydroxyl radicals, generated by reaction of iron-EDTA complex with H_2O_2 in the presence of ascorbic acid, can yield a pink tint by reacting with deoxyribose and heating with TBA under acid conditions. The yield of the pink tint was used to determine the amount of hydroxyl radicals. As shown in Fig. 4b, all the scavenging effects of polysaccharide fractions on hydroxyl radicals were concentration related, and the scavenging activities of polysaccharide fractions and BHT were all above 68% at dose of 2 mg/mL. The IC_{50} values of tested samples were respectively 0.10 mg/mL (BHT), 0.11 mg/mL (WKCP-N), 0.08 mg/mL (WKCP-A), 0.20 mg/mL (WKHP-N), and 0.06 mg/mL (WKHP-A), suggesting that WKCP-A and WKHP-A were good hydroxyl radical-scavengers.

3.2.3. Superoxide anion scavenging activity

Superoxide anion is one of the precursors of the singlet oxygen and hydroxyl radicals, and indirectly initiates lipid peroxidation. In this study, the superoxide radicals were generated in a PMS/NADH system and assayed by the reduction of NBT. As shown in Fig. 4c, all tested samples reduced superoxide anion radical in a dose-dependent manner. The scavenging activity of WKCP-A was significantly higher than that of other polysaccharide fractions, and its IC_{50} value was 0.90 mg/mL. However, the IC_{50} value of ascorbic acid was 0.03 mg/mL.

3.2.4. H_2O_2 scavenging activity

H_2O_2 is not a free radical, but it plays a radical forming role as an intermediate in the production of more reactive ROS molecules including hypochlorous acid by the action of myeloperoxidase, and $^{\bullet}OH$ via oxidation of transition metals. As shown in Fig. 4d, the H_2O_2 scavenging activities of the four fractions were 62.05–67.45% at doses of 4 mg/mL and showed concentration-dependent manners in low concentrations. The result indicated that all the fractions had good scavenging effects on H_2O_2. However, the H_2O_2-scavenging activities of them were lower than that of ascorbic acid in this study.

3.2.5. Ferrous ion chelating activity

Ferrous ion is an extremely reactive metal and will catalyse oxidative changes in lipids, proteins and other cellular components and the oxidative damage is induced by hydroxyl radicals generated by the Fenton reaction. As shown in Fig. 4e, WKCP-A exhibited significant chelating effect on ferrous ions. The chelating ability of WKCP-A was 43.99% and higher than that of WKCP-N, WKHP-N, WKHP-A by 10.5%, 9.2% and 7.9% at dose of 4 mg/mL, suggesting that it could sequestrate ferrous ions or minimize the concentration of metal in the Fenton reaction. However, the IC_{50} value of WKCP-A (5.80 mg/mL) was significantly lower than that of EDTA (0.02 mg/mL).

3.2.6. Reducing power

The FRAP assay treats the antioxidants as reductants in a redox linked colorimetric reaction, and the value reflects the reducing power of the antioxidants. As shown in Fig. 4f, WKCP-N and WKCP-A had dose-dependent reducing power and displayed $A_{700\,nm}$ 0.672 and $A_{700\,nm}$ 0.712 at 20 mg/mL, respectively, whereas the $A_{700\,nm}$ of ascorbic acid was over 1.4 at 2–20 mg/mL. However, WKHP-N and WKHP-A had weakly reducing power in this study.

3.2.7. Self-oxidation of 1,2,3-phentriol scavenging activity

1,2,3-Phentriol rapidly autoxidizes in alkaline solution and formed intermediate products such as $O_2^{\bullet -}$. The antioxidants can interfere with 1,2,3-phentriol autoxidation by acting as scavengers of $O_2^{\bullet -}$. Therefore, the antioxidant ability can be determined by the scavenging activity of self-oxidation of 1,2,3-phentriol. As shown in Fig. 4g, the 1,2,3-phentriol self-oxidation scavenging capacities of fractions WKCP-N, WKCP-A, WKHP-N and WKHP-A correlated well with increasing concentrations and were 28.51%, 58.57%, 66.78% and 30.29% at the dose of 10 mg/mL, respectively. The IC_{50} values of WKCP-A, WKHP-N were 9.18 mg/mL and 4.15 mg/mL, respectively, whereas that of ascorbic acid was 0.83 mg/mL.

Table 2
The monosaccharide composition and molecular weight distribution of each polysaccharide fraction from *Aconitum kusnezoffii* Reichb.

Fraction	Mw (kDa)	Polydispersity index (Mw/Mn)	Monosaccharide component[a] (%)							
			Man	Rha	GlcUA	GalUA	Glc	Gal	Xyl	Ara
WKCP-N	9.6	1.37					91.95	5.83		2.22
WKCP-A	160.2	1.84	2.90	5.24	1.79	6.06	13.81	38.28		31.92
WKHP-N	113.4	1.45				2.45	73.71	10.00	1.34	12.90
WKHP-A	169.6	1.40		9.37	1.72	25.08	7.39	13.33	5.79	37.33

[a] Sugar-PMP derivatives obtained after methanolysis combined with CF_3COOH hydrolysis followed by PMP precolumn derivation and analyzed by HPLC.

Table 3
Immunomodulatory effects of polysaccharides from *Aconitum kusnezoffii* Reichb. on ConA- or LPS-induced lymphocyte proliferation and macrophage phagocytosis activities of mice *in vivo*.

Group	Dose (mg/kg)	Lymphocyte proliferation activity[a]		Macrophage phagocytosis activity[a]	
		ConA ($A_{570\,nm}$)	LPS ($A_{570\,nm}$)	Phagocytosis index (k)	Phagocytosis coefficient (α)
Control		0.33 ± 0.12	0.29 ± 0.10	0.055 ± 0.007	6.519 ± 0.25
Lentinan	50	0.53 ± 0.07[d]	0.48 ± 0.10[d]	0.075 ± 0.009[d]	8.257 ± 0.31[d]
WKCP-N	50	0.37 ± 0.09[b]	0.36 ± 0.11[c]	0.062 ± 0.007[c]	7.557 ± 0.28[c]
	100	0.43 ± 0.07[d]	0.43 ± 0.09[d]	0.074 ± 0.012[d]	8.196 ± 0.30[d]
	200	0.48 ± 0.11[d]	0.51 ± 0.09[d]	0.077 ± 0.013[d]	8.534 ± 0.27[d]
WKCP-A	50	0.39 ± 0.09[c]	0.43 ± 0.06[d]	0.069 ± 0.008[d]	7.952 ± 0.29[d]
	100	0.46 ± 0.12[d]	0.45 ± 0.09[d]	0.074 ± 0.009[d]	8.392 ± 0.33[d]
	200	0.56 ± 0.07[d]	0.51 ± 0.10[d]	0.080 ± 0.010[d]	9.131 ± 0.27[d]
WKHP-N	50	0.39 ± 0.08[c]	0.41 ± 0.12[d]	0.063 ± 0.006[c]	7.479 ± 0.31[c]
	100	0.45 ± 0.07[d]	0.46 ± 0.08[d]	0.068 ± 0.005[d]	7.817 ± 0.31[d]
	200	0.52 ± 0.13[d]	0.54 ± 0.09[d]	0.074 ± 0.007[d]	8.588 ± 0.34[d]
WKHP-A	50	0.45 ± 0.10[d]	0.43 ± 0.11[d]	0.067 ± 0.009[d]	7.715 ± 0.34[d]
	100	0.51 ± 0.07[d]	0.52 ± 0.09[d]	0.074 ± 0.013[d]	8.537 ± 0.31[d]
	200	0.59 ± 0.09[d]	0.56 ± 0.08[d]	0.083 ± 0.010[d]	9.081 ± 0.32[d]

[a] Results are represented as mean ± SD based on three independent experiments.
[b] $P < 0.05$, significantly different from the control.
[c] $P < 0.01$, significantly different from the control.
[d] $P < 0.001$, significantly different from the control.

3.3. Immunological activities of water-soluble polysaccharides from *A. kusnezoffii Reichb.*

3.3.1. Lymphocyte proliferation activity

Lentinan, a β-(1/3)-D-glucan, is known for its strong immunostimulatory effect, which we used for comparison [30]. The splenic lymphocyte proliferation assay was used to evaluate the general effect on immune cells. The effects of polysaccharide fractions and lentinan on mitogen-stimulated cell proliferation *in vivo* are presented in Table 3, and appeared that treatments with all tested dosages of polysaccharide fractions could significantly increase ConA- or LPS-stimulated cell proliferation compared with the control groups ($P < 0.05$, $P < 0.01$ or $P < 0.001$) in dose-dependent manners. The effects of pectic polysaccharide fractions (WKCP-A and WKHP-A) were better than that of neutral polysaccharide fractions (WKCP-N and WKHP-N). However the lymphocyte proliferation activities of them were slightly lower than that of lentinan in this study.

3.3.2. Macrophage phagocytosis activity

The macrophages are considered the pivotal immunocytes of the host defence. The effect of polysaccharide fractions on macrophage function is presented in Table 3. It was shown that a significant dose-dependent potentiation of the macrophage phagocytosis activity in all of the polysaccharides treated groups was observed as evident by the phagocytosis index ($P < 0.05$, $P < 0.01$ or $P < 0.001$) and phagocytosis coefficient ($P < 0.05$, $P < 0.01$ or $P < 0.001$), when the results were compared with normal mice in the control group. Moreover, the macrophage phagocytosis activity of WKCP-A was close to that of lentinan.

4. Discussion

Since the antioxidant activities of antioxidants have been attributed to various mechanisms, the above seven assays were applied to evaluate the total antioxidant capacities of the four water-soluble polysaccharides [31,32]. All of them presented approximately an identical change in the trend of antioxidant activity, and the results indicated that the water-soluble polysaccharides from *A. kusnezoffii* Reichb., especially the fraction WKCP-A, had good effect on the scavenging free radicals and chelating ferrous ion, and had relatively higher FRAP value, especially at high addition quantity. Moreover, the immunostimulating activity of WKCP-A was also more significant than other polysaccharide fractions, even close to that of lentinan, a positive control.

It has been found that free radicals play a key role related to the degenerative or pathological processes of various diseases, such as cancer, Alzheimer's disease, coronary heart disease, atherosclerosis, neurodegenerative disorders, aging, cataracts, and various inflammations [33–37]. The above assays indicated fraction WKCP-A had good antioxidant and immunostimulatory activities, so it had the potential to eliminate the negative role of radicals which are created during a cascade of oxido-reduction reactions, by UV radiation, toxic compounds, heavy metals, etc. in cells, play an important role in the defence of cellular components or organelles, i.e. lipids, proteins, glycoconjugates, nucleic acids, etc. Therefore, it might be used as a natural antioxidant and immunostimulating agent to protect the human body from free radicals and retard the progress of many chronic diseases.

It was postulated that the presence of anomeric hydrogen in polysaccharide may react with hydroxyl radical to achieve the scavenging activity [38]. In recent studies, the hydroxyl group of polysaccharide was further demonstrated to a significant factor in affecting free radical scavenging activity of polysaccharide [39,40]. Furthermore, the present study showed that the polysaccharide composed of more galactose and with lower molecular weight exhibited more significant antioxidant activity as WKCP-A and WKCP-N exhibited higher scavenging effects than WKHP-A and WKHP-N, respectively. Therefore, we deduced that the content of galactose and molecular weight had great effects on antioxidant capacity of polysaccharide.

5. Conclusion

The above results clearly demonstrated water-soluble polysaccharides from *A. kusnezoffii* Reichb., especially the fraction WKCP-A, were found to have antioxidant potential according to the *in vitro* evaluation of their free radicals and H_2O_2-scavenging activities, reducing power and ferrous ion-chelating ability, and remarkable immunological activities according to the *in vivo* assays of their plenic lymphocyte proliferation activities and macrophage phagocytosis activities for the first time. Therefore, the water-soluble

polysaccharide fraction WKCP-A from *A. kusnezoffii* Reichb. has the potential to be explored as novel natural antioxidant and immunostimulating agent for using in functional foods or medicine.

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