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Extraction, preliminary structural characterization, and antioxidant activities of polysaccharides from *Salvia miltiorrhiza* Bunge

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ABSTRACT

Four polysaccharides were extracted from *Salvia miltiorrhiza* Bunge using hot water, ultrasonic, alkali, and enzyme methods. Preliminary structural characterization was conducted by physicochemical property, Fourier transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM) analyses. Antioxidant activities against 2,2-diphenyl-1-picryl-hydrazyl (DPPH), hydroxyl, and superoxide radicals were also evaluated. The physicochemical property analysis indicated the identicalness of the polysaccharide indices obtained by the hot water and ultrasonic methods. The indices obtained by the alkali and enzyme methods were significantly different. The FTIR spectra revealed the general characteristic absorption peaks of the four polysaccharides. The SEM images demonstrated significant differences in the surface features of the different polysaccharides. The antioxidant activity assay revealed the significant antioxidant activities of three polysaccharides. Overall, the polysaccharides from *S. miltiorrhiza* Bunge may have potential applications in the medical and food industries.

1. Introduction

Salvia miltiorrhiza Bunge is a traditional Chinese medicine used for centuries. This plant belongs to *Salvia* Linn., the largest genus of the Lamiaceae family. It is commonly called "Danshen" because of its bright red skin (Dong, Liu, Liang, & Wang, 2010). In recent decades, *S. miltiorrhiza* has been widely used in clinics in China, Korea, Japan, and other Asian countries for treating various microcirculatory disturbance-related diseases. Such diseases include cardiovascular disease (Xia, Gu, Ansley, Xia, & Yu, 2003), cerebrovascular disease (Zhou, Li, Xu, & Chen, 2011), liver dysfunction (Song et al., 2008), renal deficiency (Kang et al., 2004), and diabetic vascular complication (Jung, Seol, Jeon, Son, & Lee, 2009). There are many traditional Chinese medicine preparations containing *S. miltiorrhiza*, including Fufang Danshen tablets, Compound Danshen dripping pills, Danshen injections, and Xiangdan injections (Zhang, Li, & Wang, 2010). Given its medicinal importance, the demand for *S. miltiorrhiza* has steadily increased in recent years. China alone requires an estimated 80 million kilograms of *S. miltiorrhiza* annually (Hu, Luo, Zhao, & Jiang, 2005; Liu, Wang, Wang, Zou, & Liang, 2011).

The search for natural sources of polysaccharide has intensified over the recent years. The purpose is not only for the prevention and treatment of various diseases caused by oxidative damage, but also for improving the shelf life of food products (Yang, Jiang, et al., 2009; Zhao, Kan, Li, & Chen, 2005). Polysaccharides possess a wide range of biological properties, such as immunomodulation (Zhang et al., 2010), anti-cancer (Jiang, Wang, Liu, Gan, & Zeng, 2011), anti-ageing (Song, Zhang, Zhang, & Wang, 2010), hyperglycemic (Li et al., 2011), and anti- inflammatory (Kang et al., 2011) activities. Plants, particularly those used as traditional Chinese medicines, contain high amounts of natural polysaccharides that have been identified as free radicals or active oxygen scavengers (Wang & Luo, 2007).

To date, studies on *S. miltiorrhiza* mainly focus on low-molecular-weight substances, such as water-soluble phenolic acids and fat-soluble diterpene quinones (Dong, Wan, & Liang, 2010; Jung et al., 2009; Li, Song, Liu, Hu, & Wang, 2009). Less attention is being paid to the extraction, preliminary structural characterization, and antioxidant activities of polysaccharides from *S. miltiorrhiza*, compared with those from other traditional Chinese medicines such as *Polygonum cillinerve* (Cui, Yuan, & Zhang, 2010), *Fructus lycii* (Wang et al., 2002), *Ganoderma lucidum* (Zhu, Chen, & Lin, 2007), and *Achyranthes bidentata* (Xue, Chen, Lu, & Jin, 2009). Therefore,

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in the present study, the extraction, preliminary structural characterization, and antioxidant activities of polysaccharides from *S. miltiorrhiza* are reported.

Polysaccharides from *S. miltiorrhiza* were first extracted using hot water, ultrasonic, alkali, and enzyme methods. The preliminary structural characterization of the four polysaccharides were then conducted via physicochemical property, Fourier transform infrared (FTIR) spectroscopy, and scanning electron microscopy (SEM) analyses. Finally, the antioxidant activities of the four polysaccharides against 2,2-diphenyl-1-picryl-hydrazyl (DPPH), superoxide, and hydroxyl radicals were determined. The main aims of this research are to investigate application value of polysaccharides from *S. miltiorrhiza*.

2. Materials and methods

2.1. Chemicals and reagents

2,2-diphenyl-1-picryl-hydrazyl (DPPH) and phenazin methosulphate (PMS) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Ascorbic acid (Vc), nitro blue tetrazolium (NBT), and nicotinamide adenine dinucleotide-reduced (NADH) were purchased from the Sinopharm Chemical Reagent Co. (Beijing, China). All reagents were analytical grade, or were the highest grade available, and were used without further purification. Ultra-pure water was used throughout the experiments.

2.2. Plant materials

S. miltiorrhiza was obtained from Zhongjiang, Sichuan Province, China in January 2010. The sample was washed and dried to constant weight at 105 °C. Then it was ground into fine powder using a powerful mill (FW177, Taisite Instrument Co., Ltd., Tianjin, China), and screened through an 80 mesh sieve. The materials were stored at room temperature in a desiccator until use.

2.3. Extraction

Dried *S. miltiorrhiza* powder was refluxed with 80% ethanol and petroleum ether for 3 h each to remove small-molecular-weight impurities. The residues were dried at 40 °C for 24 h and extracted. The extractions were based on previously reported methods with some modifications. First was the hot water method (Wang, Wang, Wang, Wang, & Shen, 2006) that involved liquid/solid ratios of 1:20, a temperature 70 °C, and an extraction time of 4 h. Second was the ultrasonic method (Wu, Guo, & Wang, 2007) that involved liquid/solid ratios of 1:20, room temperature, and an extraction time of 40 min. Third was the alkali method (Qian, Chen, Zhang, & Zhang, 2009) that involved liquid/solid ratios of 1:20, room temperature, and an extraction reagent, NaOH (0.5 mol/L). Fourth and last was the enzyme method (Cai, Liu, Liu, Liang, & Liu, 2008) that involved liquid/solid ratios of 1:10, a temperature of 60 °C, 5 g of cellulase, and an extraction time of 2 h. After extraction, the supernatant and sediments were separated by vacuum filtration. The residues were re-extracted three times. All extraction solutions were condensed to about 100 mL. Ethanol (400 mL, 90%, *v*/*v*) was slowly added to the condensed solution, which was left for 12 h at 4 °C. The resulting precipitate was collected, dissolved with 200 mL of water, and subjected to the Sevag method (chloroform:butyl alcohol, 4:1) to remove free proteins. The deproteinized solution was re-precipitated in 90% ethanol four times the solution volume. The precipitate was collected and successively washed with anhydrous ethanol and acetone. After drying, the polysaccharides were obtained and named SMP-1, SMP-2, SMP-3, and SMP-4 according to the respective extraction method.

2.4. Physicochemical property analysis

Physicochemical properties were determined using the following methods: color observation, solubility test, phenol–sulfuric acid test, α-naphthol reaction, iodination reaction, Fehling's test, carbazole reaction, $FeCl_3$ reaction, full wavelength scanning, and Coomassie brilliant blue reaction.

2.5. FTIR analysis

The organic functional groups of the four polysaccharides were identified using an FTIR spectrophotometer (FTIR-8400S, Shimadzu Co., Japan) within 4000–400 cm^{-1} via the KBr pressed-disc method.

2.6. SEM analysis

The four polysaccharides were coated with a thin layer of gold under reduced pressure. They were then examined using a SEM system (JSM-5900LV, JEOL, Japan) at a 25 kV acceleration voltage, as well as image magnifications of 1000× and 3000×.

2.7. DPPH radical scavenging activity

The DPPH radical scavenging activity was determined according to the method described by Yang, Yu, et al. (2009) with some modifications. The four polysaccharides were dissolved in water, yielding a series of sample solutions with different concentrations (0.20, 0.40, 0.80, 1.6, 2.4, 3.2, and 4.0 mg/mL). A sample solution (2 mL) was mixed with 2 mL of 0.20 mM DPPH-ethanol solution, and the absorbance of the sample solution (A_{sample}) was measured at 517 nm against a blank after 60 min. The absorbance of the DPPH-ethanol solution (A_{DPPH}) was also measured at 517 nm. The DPPH radical scavenging capability was calculated using the following equation:

$$\text{DPPH radical scavenging activity}\,(\%) = \left[\frac{A_{DPPH} - A_{sample}}{A_{DPPH}}\right] \times 100$$

2.8. Superoxide radical scavenging activity

Superoxide radical scavenging activity was determined according to the NBT reduction method (Zhang, Lu, Fu, Wang, & Zhang, 2011). The four polysaccharides were dissolved in water, yielding a series of sample solutions with different concentrations (0.020, 0.040, 0.080, 0.16, 0.24, 0.32, and 0.40 mg/mL). The reaction mixture containing 1.5 mL of sample solution, 0.50 mL of 300 μM NBT, 0.50 mL of 468 μM NADH, and 0.50 mL of 60 μM PMS were incubated at 25 °C for 5 min. The absorbance (A_{sample}) was recorded at 560 nm against a blank, wherein the sample was replaced by PBS. Superoxide radical scavenging activity was calculated using the following equation:

$$\text{Superoxide radical scavenging activity}\,(\%) = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] \times 100$$

2.9. Hydroxyl radical scavenging activity

Scavenging activity against hydroxyl radical was determined according to Fenton's reaction (Jiang, Zhang, Liu, Wang, & Fan, 2010). The four polysaccharides were dissolved in water, yielding a series of sample solutions with different concentrations (0.020,

Table 1
Comparison of the physicochemical properties of the four polysaccharides.

Method	SMP-1	SMP-2	SMP-3	SMP-4
Color observation	Brown	Brown	Black	Milky
Solubility test	Water soluble	Water soluble	Alkali soluble	Water soluble
Phenol–sulfuric acid test	(+)[a]	(+)	(+)	(+)
α-Naphthol reaction	(+)	(+)	(+)	(+)
Iodination reaction	(−)[b]	(−)	(−)	(−)
Fehling's test	(−)	(−)	(−)	(−)
Carbazole reaction	(−)	(−)	(−)	(−)
$FeCl_3$ reaction	(−)	(−)	(−)	(−)
Peak at UV 280 nm	(+)	(+)	(+)	(+)
Coomassie brilliant blue reaction	(+)	(+)	(+)	(+)

[a] Positive.
[b] Negative.

0.040, 0.080, 0.16, 0.24, 0.32, and 0.40 mg/mL). A mixture solution was prepared by mixing several solutions into 2 mL of PBS (pH 7.4) in the following order: 1 mL of water, 1 mL of 1,10-phenanthroline ethanol solution (0.75 mM), 1 mL of $FeSO_4$ (0.75 mM), and 1 mL of H_2O_2 (0.01%). The final mixture was incubated for 60 min at 37 °C, and was used as the blank solution. A similar procedure was used to prepare the control solution, wherein 1 mL of water instead of H_2O_2 was added. The absorbance rates of the blank (A_{blank}), control ($A_{control}$), and sample solutions (A_{sample}) were recorded at 510 nm. Superoxide radical scavenging activity was calculated using the following equation:

$$\text{Hydrogen peroxide scavenging activity}\,(\%) = \left[\frac{A_{sample} - A_{blank}}{A_{control} - A_{blank}}\right] \times 100$$

2.10. Statistical analyses

All the experiments were carried out in triplicate, and the data presented are the mean values of these independent experiments. Standard deviation and error bars are shown wherever necessary. All statistical analyses were conducted using Origin.Pro.8.5, where the significances of the effects of and the interactions among the investigated factors with respect to the experimental error were determined.

3. Results and discussion

3.1. Physicochemical property analysis

Table 1 lists the physicochemical properties of the four polysaccharides. The colors of the four polysaccharides extracted by

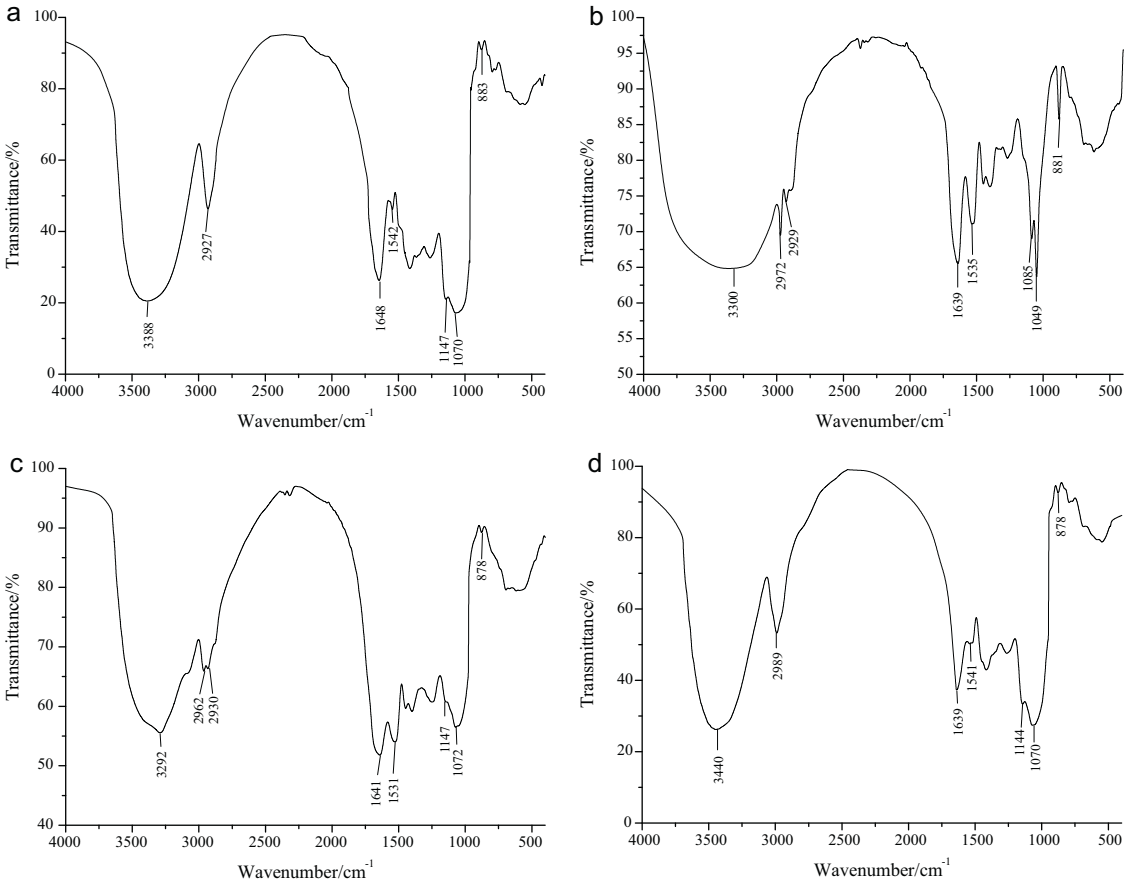

Fig. 1. Infrared spectra of the four polysaccharides. (a) SMP-1, (b) SMP-2, (c) SMP-3, and (d) SMP-4.

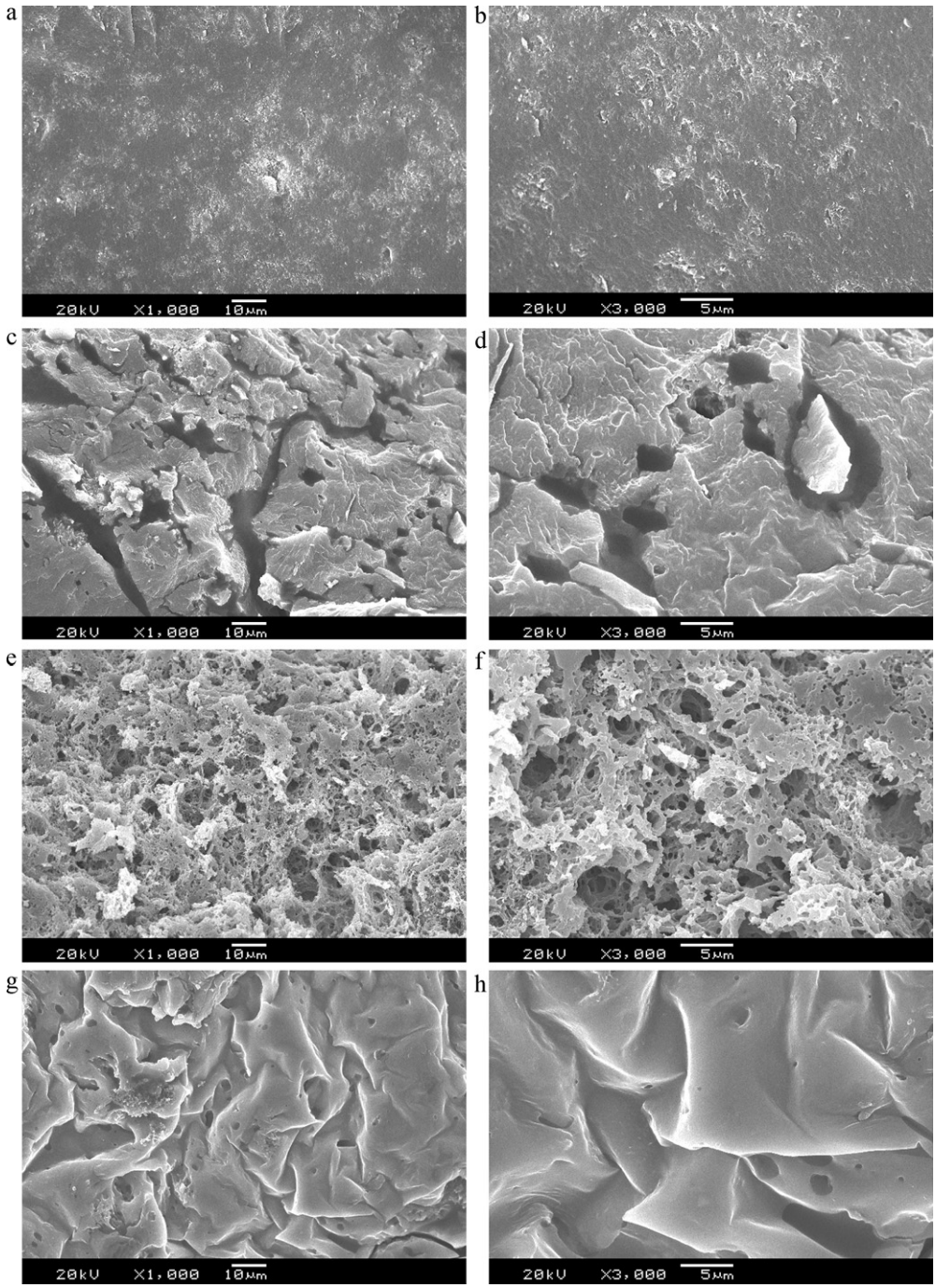

Fig. 2. Scanning electron micrographs of the four polysaccharides. (a) SMP-1 (1000×), (b) SMP-1 (3000×), (c) SMP-2 (1000×), (d) SMP-2 (3000×), (e) SMP-3 (1000×), (f) SMP-3 (3000×), (g) SMP-4 (1000×), and (h) SMP-4 (3000×).

different methods had few differences. SMP-1 and SMP-2 were both brown, whereas SMP-3 and SMP-4 were black and milky, respectively. SMP-3 was alkali soluble, whereas the others were water soluble. The results of the phenol–sulfuric acid, α-naphthol, iodination, Fehling's, carbazole, $FeCl_3$, and Coomassie brilliant blue tests were similar for the four polysaccharides. These similarities indicated that the four extractions were polysaccharides and contained some proteins, but did not contain starch, reducing sugar, uronic acid, or polyphenol. The full wavelength scanning also confirmed that the four polysaccharides had proteins. In conclusion, the basic physicochemical properties of the four polysaccharides were similar.

3.2. FTIR analysis

FTIR spectroscopy is typically used for the qualitative measurement of organic functional groups, especially O–H, N–H, and C=O (Qian et al., 2009). Fig. 1 shows the FTIR spectra of the four polysaccharides from *S. miltiorrhiza*. There was a stretching vibration of O–H and saturated C–H at 3300–3500 and 2929–2989 cm^{-1}, respectively. The absorption bands at nearly 1640 and 1540 cm^{-1} were attributed to amides I and II, indicating that the four polysaccharides had conjugated proteins. The absorption band at 1000–1200 cm^{-1} suggested that the four polysaccharides contained pyrene monomers in their structures. An absorbance at

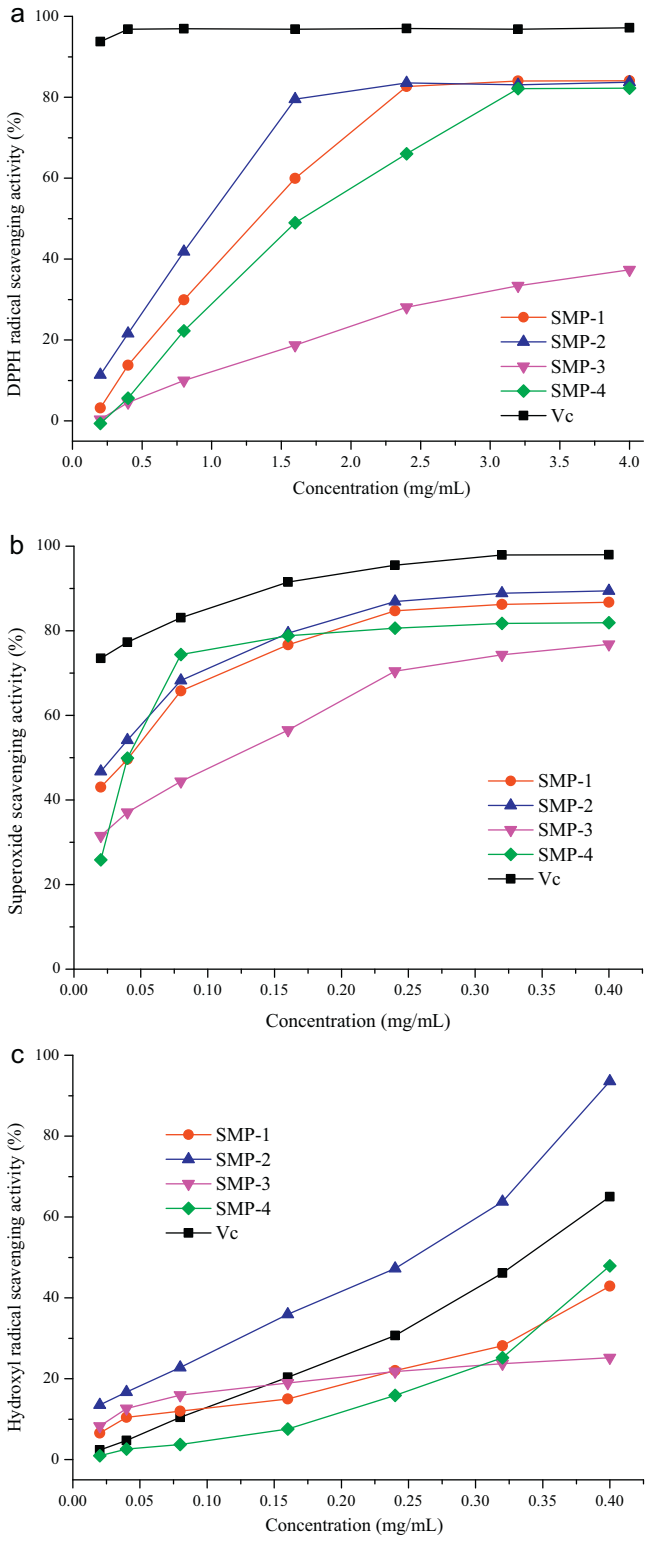

Fig. 3. Scavenging effects on of SMP-1, SMP-2, SMP-3, SMP-4, and Vc against (a) DPPH, (b) superoxide, and (c) hydroxyl radicals.

nearly 880 cm^{-1} strongly suggested the linkage of β-glycosides in the molecular structure of the four polysaccharides.

In conclusion, no significant difference was seen in the FTIR of the four polysaccharides, and FTIR spectroscopy did not clearly distinguish the four polysaccharides from each other.

3.3. SEM analysis

The SEM images of the four polysaccharides are shown in Fig. 2. The results showed that the different extraction methods induced different physical changes. Fig. 2a and b shows that SMP-1 had a flat surface. Fig. 2c and d shows that SMP-2 had a rough surface with characteristic large wrinkles. Fig. 2e and f shows that SMP-3 was sponge-like, and that its surface was very distinct from those of the others. Fig. 2g and h shows that the SMP-4 surface was very smooth, and had distinct pore openings 2–5 μm in diameter. In summary, the polysaccharides from *S. miltiorrhiza* extracted by different methods were qualitatively identified by comparing their micrographs with those of the standards.

3.4. Antioxidant activity

3.4.1. DPPH radical scavenging activity

Fig. 3a shows the scavenging activities of SMP-1, SMP-2, SMP-3, SMP-4, and Vc against the DPPH radical. The scavenging effects of the four polysaccharides were evident at all tested concentrations, and well correlated with increased concentration up to 4.0 mg/mL. SMP-3 had the weakest activity. At 4.0 mg/mL, the scavenging activities of SMP-1, SMP-2, SMP-3, SMP-4, and Vc were 84.0%, 83.7%, 37.3%, 82.2%, and 97.1%, respectively.

3.4.2. Superoxide radical scavenging activity

Fig. 3b shows the scavenging activities of SMP-1, SMP-2, SMP-3, SMP-4, and Vc against the superoxide radical. The scavenging effects of the four polysaccharides were evident at all tested concentrations, and well correlated with increased concentration up to 0.40 mg/mL. SMP-3 had the weakest activity. At 0.40 mg/mL, the scavenging activities of SMP-1, SMP-2, SMP-3, SMP-4, and Vc were 86.7%, 89.4%, 76.8%, 81.9%, and 98.0%, respectively. Obviously, although the four polysaccharides had strong scavenging activities against the superoxide radical, Vc bested them all.

3.4.3. Hydroxyl radical scavenging activity

Fig. 3c shows the scavenging activities of SMP-1, SMP-2, SMP-3, SMP-4, and Vc against the hydroxyl radical. The scavenging effects of the four polysaccharides were evident at all tested concentrations. SMP-2 had a stronger scavenging effect than Vc, and the other three polysaccharides (SMP-1, SMP-3, and SMP-4) had weaker activities than Vc. At 0.40 mg/mL, the scavenging activities of SMP-1, SMP-2, SMP-3, SMP-4, and Vc were 42.8%, 93.5%, 25.2%, 47.9%, and 65.0%, respectively. Obviously, SMP-2 had the strongest scavenging activity against the hydroxyl radical.

4. Conclusions

In the present study, four polysaccharides from *S. miltiorrhiza* were extracted using hot water, ultrasonic, alkali, and enzyme methods. Preliminary structural characterizations were conducted using physicochemical property, FTIR, and SEM analyses. The physicochemical property and FTIR results demonstrated that the basic physicochemical property of the four polysaccharides were similar. The SEM images revealed that different extraction methods led to different stereostructures. Moreover, significant antioxidant activities against DPPH, hydroxyl, and superoxide radicals were possessed by SMP-1, SMP-2, and SMP-4. Hence, these polysaccharides are natural antioxidants, and may be potential functional food ingredients. Further *in vivo* experiments on biological activities of the four polysaccharides are currently in progress.

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