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Structural characterization and antioxidant activities of polysaccharides extracted from *Epimedium acuminatum*

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ABSTRACT

The polysaccharides were extracted from *Epimedium acuminatum* by hot water extraction, ultrasonic-assisted extraction, enzyme extraction, and microwave-assisted extraction. The physicochemical properties of *Epimedium* polysaccharides were then determined by chemical composition analysis, Fourier transform infrared (FT-IR) spectroscopy, and scanning electron microscopy (SEM) analysis. Further, the antioxidant activities were studied *via* different methods, including DPPH assay, ABTS assay, FRAP assay and AAPH-induced erythrocyte hemolysis assay. Results showed that the physicochemical properties of different polysaccharides were similar. Antioxidant assay indicated that four polysaccharides exhibited significant antioxidant activities in a dose-dependent manner. The antioxidant activities of the polysaccharides which obtained by hot water extraction were higher than those of other polysaccharides. Overall, *E. acuminatum* polysaccharides might be used as potential natural antioxidant.

1. Introduction

Epimedium acuminatum Franch., a traditional Chinese herb, belongs to *Epimedium* which includes 52 species in the Berberidaceae family. The aerial parts of the plants are commonly used as tonic, aphrodisiac and antiheumatic in China, Japan and Korea for more than 2000 years (Li et al., 2012). *Epimedium* and its active compounds possess pharmacological action, such as modulating immunological function, anti-osteoporosis, anti-tumor and anti-aging effects, which have been shown in clinical practices, *in vivo* and *in vitro* experiments (Ma et al., 2011). Polysaccharides are one of the most important effective ingredients in *Epimedium* (Kovačević, Čolić, Backović, & Došlov-Kokoruš, 2006). In previous reports, *Epimedium* polysaccharides exhibit therapeutic effectiveness in infectious bursal disease (IBDV), Newcastle disease virus (NDV) and immune enhancement (Chu, Yan, Li, & Hu, 2006; Fan et al., 2010; Kong, Hu, Rui, Wang, & Li, 2004; Lu, Wang, Hu, Huang, & Wang, 2008; Sun, Hu, Wang, Zhang, & Liu, 2006).

Polysaccharides, which are widely distributed in animals, plants, and microorganisms, possess many biological activities, such as anti-coagulant, antiviral, anti-tumor, immunomodulation, and anti-inflammatory (Chen et al., 2011; Ge, Duan, Fang, Zhang, & Wang, 2009; Li & Zhou, 2007). During the past few decades, polysaccharides from traditional Chinese medicine have drawn significant attention to their potent function in food and medical industry for strong reactive oxygen species (ROS) scavenging ability. As we know, uncontrolled production of ROS may cause oxidative damage of macromolecules, which is associated with cancer, diabetes mellitus, and neurodegenerative and inflammatory diseases (Barry & Gutteridge, 1984). The use of synthetic antioxidants, such as BHA, BHT, and TBHQ, are restricted because these antioxidants have been suspected of causing liver damage and carcinogenic (Guyton et al., 1991; Lin & Tang, 2007). However, natural sources of polysaccharides can be explored as potential antioxidants with low cytotoxicity (Ramarathnam, Osawa, Ochi, & Kawakishi, 1995). Several researches have reported that polysaccharides from plant showed strong antioxidant ability. It is reported that water-soluble polysaccharides obtained from *Acanthopanax senticosu* exhibit strong free radical scavenging activity (Chen et al., 2011). Water-soluble polysaccharides extracted and purified from litchi indicate strong superoxide radical and hydroxyl radical scavenging ability (Kong et al., 2010).

The most commonly used method for polysaccharides extraction is hot water extraction. The yield of hot water extraction largely depends on extraction time and temperature (Bendjeddou, Lalaoui, & Satta, 2003). In order to increase the yield, other new methods are employed to extract polysaccharides, such as ultrasonic-assisted extraction (Yan et al., 2011) and microwave-assisted extraction (Wang et al., 2010). The better extraction efficiency by ultrasonic treatment, enzyme treatment, and microwave treatment is mainly attributed to the mechanical effects or catalytic action, which may influence the structure of polysaccharides.

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In this paper, we extracted four polysaccharides from *E. acuminatum via* the method of hot water extraction, ultrasonic-assisted extraction, enzyme extraction, and microwave-assisted extraction. The preliminary structural characterization and antioxidant activities of four polysaccharides were estimated by chemical composition analysis, FT-IR, SEM and antioxidant assays, which include DPPH assay, ABTS assay, FRAP assay and AAPH-induced erythrocyte hemolysis assay. The aim of this research was to investigate the influence of different extraction methods on the physicochemical properties and antioxidant activities of polysaccharides from *E. acuminatum*.

2. Materials and methods

2.1. Materials and reagents

Materials were collected from Yaan, Sichuan Province, China in September, 2011 and identified by Prof. Chunbang Ding, Sichuan Agricultural University, China. The materials were washed thoroughly with water, dried at 70 °C, pulverized in a powerful mill (FW177, Taisite Instrument Co., Ltd., Tianjin, China), and screened through an 80 mesh sieve. The powder of the materials was stored in a desiccator at room temperature.

2,2-Diphenyl-1-picryl-hydrazy (DPPH), 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH), 2,4,6-tripyridyl-s-trazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), D-glucose, and 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ascorbic acid (Vc) was purchased from the Sinopharm Chemical Reagent Co. (Beijing, China). All reagents were analytical grade.

2.2. Extraction

The dried *E. acuminatum* powder was defatted with 80% petroleum ether for 3 h to remove colored ingredients, and small molecular impurities. Then, the residues were dried and extracted by different previously reported methods with some modifications (Rodriguez-Jasso, Mussatto, Pastrana, Aguilar, & Teixeira, 2011; Wu, Zhu, Zhang, Yang, & Zhou, 2012). The polysaccharides extracted from *E. acuminatum* by hot water extraction, microwave-assisted extraction, enzyme extraction, and ultrasonic-assisted extraction were designated as EAP-H, EAP-U, EAP-E, and EAP-M, respectively. EAP-H was extracted with hot water in a ratio (material to water) of 1:10 at 70 °C for 1 h. EAP-U was extracted by the ultrasonic-assisted method in a ratio (material to water) of 1:10 with a power of 250 W in an ultrasonic bath (KQ-400GKDV, Kunshan Ultrasonic Instrument Co., Ltd., China). The extraction process was performed for 30 min. EAP-E was extracted by the enzyme method in a ratio (material to water) of 1:10 with cellulase at 60 °C for 1 h. EAP-M was extracted by the microwave-assisted method in a ratio (material to water) of 1:10 for 1 h. The extraction process was performed in an oven model MDS-2000 (CEM Corporation, Matthews, NC). All extraction solutions were condensed to 100 mL and precipitated with four times the volume of 90% ethanol solution for 12 h at 4 °C. Precipitates were solubilized in deionized water, deproteinized by Sevag solution (chloroform:butyl alcohol, 4:1), and dried to obtain EAP-H, EAP-U, EAP-E, and EAP-M.

2.3. Chemical composition analysis

The carbohydrate content was analyzed by phenol–sulfuric acid colorimetric method using D-glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1951). The uronic acid content was measured by vitriol-carbazole method using glucuronic acid as the standard (Bitter & Muir, 1962). The protein compounds content was estimated by the Coomassie Brilliant Blue reaction using bovine serum albumin as the standard (Bradford, 1976).

2.4. FT-IR spectroscopy

The FT-IR spectrum of EAP was carried out using a Shimadzu 8400S spectrophotometer (Japan). The polysaccharides were incorporated into KBr powder and then pressed into a 1.0 mm disk. The results were recorded in the frequency range of 4000–500 cm^{-1} (Ganesh, Joo, Choi, Koo, & Chang, 2004).

2.5. Scanning electron microscope

The polysaccharides were coated with gold and examined with a scanning electron microscope system (JSM-7500, JEOL, Japan) under high vacuum condition at an accelerating voltage of 5 kV, as well as image magnifications of 1000× and 3000×.

2.6. Antioxidant activity

2.6.1. Scavenging activity of DPPH radical

The scavenging activity of EAP on DPPH radical was measured by the method described by Shimada, Fujikawa, Yahara, and Nakamura (1992) with some modifications. Polysaccharide samples were dissovled in distilled water to form sample solution in final concentrations of 0.1, 0.5, 1, 2, 3, and 4 mg/mL, respectively. 2 mL of the sample solution was mixed with 2 mL of 0.2 mmol/L DPPH ethanol solution. The reaction solution was incubated for 60 min at room temperature, and the absorbance of the mixture was measured at 517 nm using the spectrophotometer (UV-1750, Shimadzu). The scavenging activity on DPPH radical was calculated by the following equation.

$$\text{Scavenging activity}(\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

where A_{control} is the absorbance of the DPPH radical solution without sample and A_{sample} is the absorbance of the DPPH radical solution with tested samples.

2.6.2. Scavenging activity of ABTS radical

The ABTS radical scavenging activity was carried out by the method described by Re et al. (1999) with some modifications. ABTS radical solution was produced by mixing ABTS aqueous solution (final concentration 7 mmol/L) with potassium persulphate (final concentration 2.45 mmol/L), and the mixture was incubated in the dark at room temperature for 16 h. After incubation, the ABTS radical solution was diluted with PBS (pH 7.0) to an absorbance of 0.70 (±0.02) at 734 nm. The polysaccharide samples were dissolved in distilled water to form sample solution in final concentrations of 0.1, 1, 2, 3, 4, and 5 mg/mL, respectively. The sample solution was added to ABTS radical solution in a ratio of 1:20, and the mixture solution was incubated for 6 min at room temperature. The absorbance was measured at 734 nm. The scavenging activity on ABTS radical was calculated by the following formula.

$$\text{Scavenging activity}(\%) = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100$$

where A_{control} is the absorbance of the ABTS radical solution without sample and A_{sample} is the absorbance of the ABTS radical solution with tested samples.

2.6.3. FRAP assay

The FRAP assay was measured by the method described by Benzie and Strain (1996) with some modifications. FRAP reagent

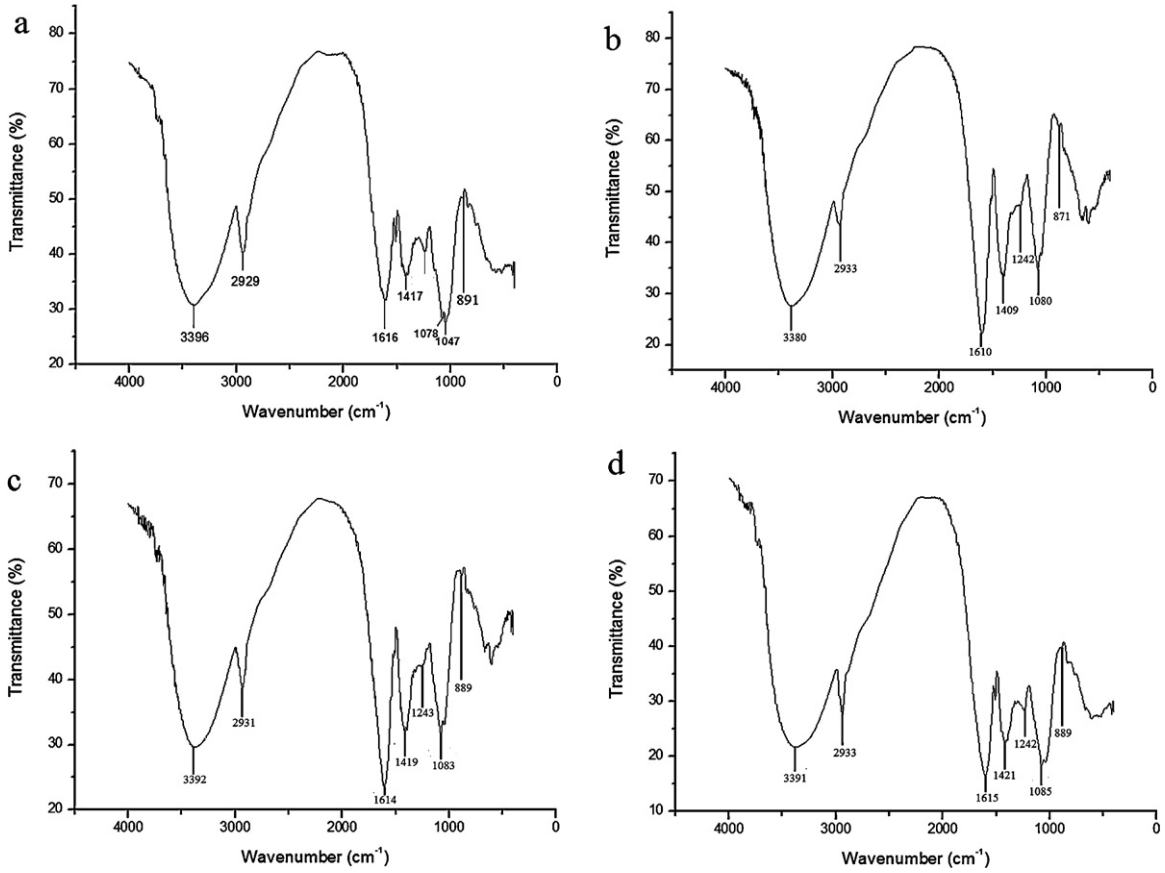

Fig. 1. (a) Infrared spectra of polysaccharides: (a) EAP-H; (b) EAP-U; (c) EAP-E; (d) EAP-M.

was prepared by mixing 300 mmol/L Acetate buffer (pH 3.6) with 10 mmol/L 2,4,6-tripyridyl-s-trazine (TPTZ) and 20 mmol/L $FeCl_3$ in a 10:1:1 ratio. Polysaccharide samples were dissolved in distilled water to form sample solution in final concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, and 1 mg/mL, respectively. 20 μL sample solution was added to 500 μL FRAP reagent, and the mixture was heated to 37 °C. After 8 min, the absorbance of mixture solution was measured at 593 nm. A standard curve was prepared by $FeSO_4{\cdot}7H_2O$ solution with several concentrations (100–1000 μM). The final results were expressed as the concentrations of $FeSO_4{\cdot}7H_2O$ with equivalent antioxidant activity.

2.6.4. Erythrocyte hemolysis

Blood samples were collected from a healthy volunteer. The 10% erythrocyte suspension from blood samples was obtained by the method of Malpezzi and Freitas (1991). AAPH-induced erythrocyte hemolysis assay was carried out by the method of Miki, Tamai, Mino, Yamamoto, and Niki (1987) with some modifications. The reaction was initialed by mixing 0.5 mL of 200 mmol/L AAPH solutions to 0.5 mL of 10% erythrocyte suspension. Then, 0.5 mL of 5 mg/mL sample solution was added to the mixture. The reaction solution was incubated at 37 °C for 1 h. After incubation, the mixture was diluted with 4 mL physiological saline and centrifuged at 3500 g for 10 min. The absorbance of the supernatant was measured at 540 nm. The percentage of inhibition was calculated by the following equation.

$$\text{Hemolysis}(\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

where A_{control} is the absorbance of the reaction solution without sample and A_{sample} is absorbance of the reaction solution with the tested samples.

2.7. Statistical analysis

All the experiments were carried out in triplicate, and the data were shown in means ± standard deviation (SD) and evaluated by one-way analysis of variance (ANOVA) with Duncan multiple range tests. The p values were set at $p < 0.05$ to assess the statistically significant. All statistical analysis was carried out with SPSS 12.0.

3. Results and discussion

3.1. Chemical compositions

The chemical compositions of EAP-H, EAP-U, EAP-E, and EAP-M are shown in Table 1. The carbohydrate contents were similar, and there was no significant difference in the protein contents among four polysaccharides. But the uronic acid contents were significant difference followed the order of EAP-M, EAP-H, EAP-U, and EAP-E. The difference may be related the selective type of extraction method. In conclusion, the chemical compositions of four polysaccharides were similar.

Table 1
Duncan[a] for chemical compositions of EAP-H, EAP-U, EAP-E, and EAP-M.

Samples	EAP-H	EAP-U	EAP-E	EAP-M
Carbohydrate (%)	73.84 ± 2.35[a]	74.09 ± 1.71[a]	73.21 ± 1.23[a]	74.59 ± 1.54[a]
Protein (%)	2.42 ± 0.28[a]	1.94 ± 0.14[a]	2.55 ± 0.39[a]	2.03 ± 0.14[a]
Uronic acid (%)	8.65 ± 0.25[ab]	7.93 ± 0.16[b]	4.31 ± 0.60[c]	8.93 ± 0.24[a]

Each value is expressed as mean means ± stand deviation ($n = 3$). Means with different letters within a row are significantly different ($p < 0.05$).

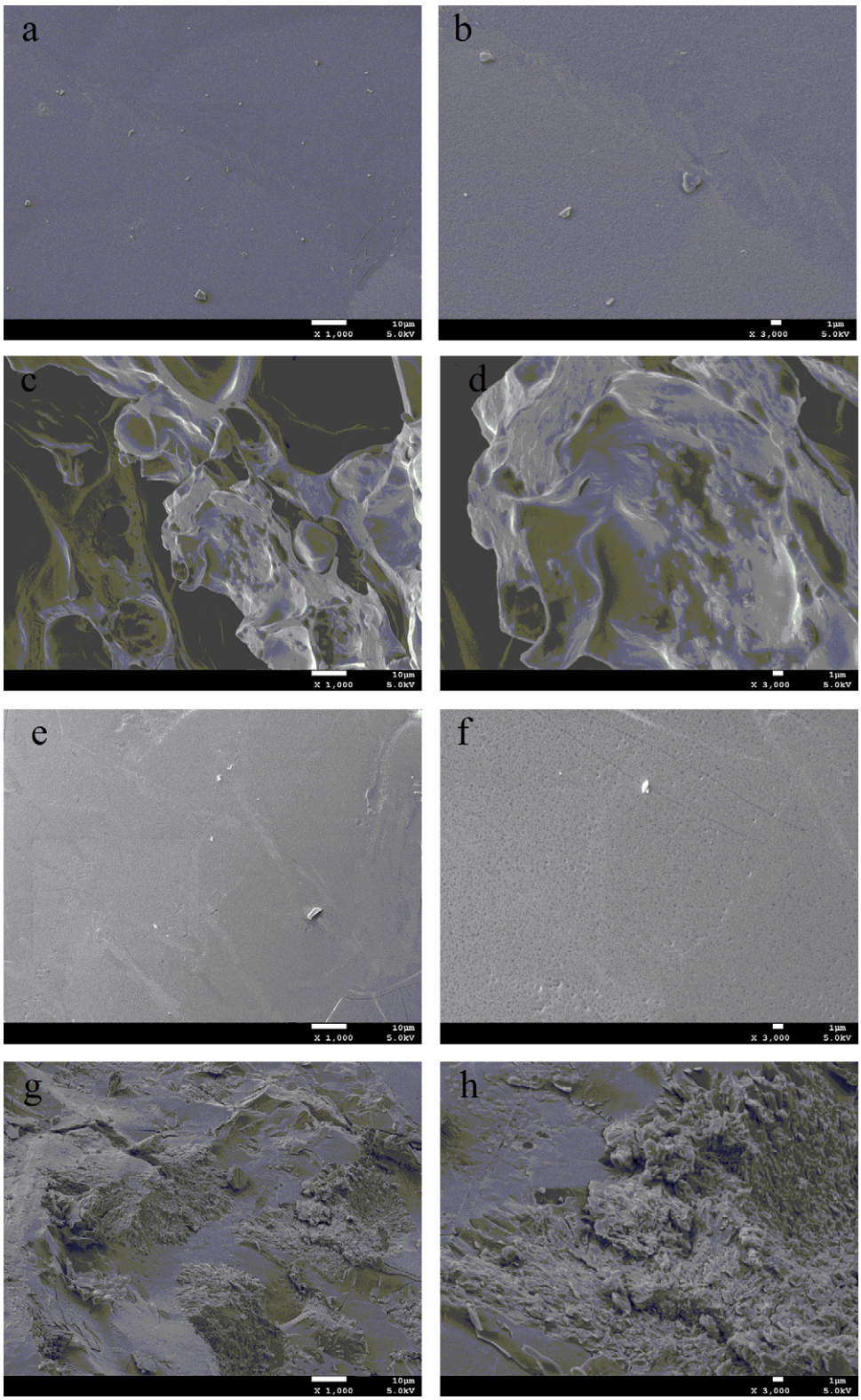

Fig. 2. (a) Scanning electron micrographs of the five polysaccharides: (a) EAP-H (1000×); (b) EAP-H (3000×); (c) EAP-U (1000×); (d) EAP-U (3000×); (e) EAP-E (1000×); (f) EAP-E (3000×); (g) EAP-M (1000×); (h) EAP-M (3000×).

3.2. FT-IR analysis

FT-IR spectra of EAP-H, EAP-U, EAP-E, and EAP-M are shown in Fig. 1. The broad bands at 3380–3400 cm^{-1} were the characteristic peaks of hydrogen bonded O—H stretching vibration and the signals at 2929–2933 cm^{-1} indicated C—H stretching vibration. The signals at 1620 cm^{-1} and 1420 cm^{-1} were attribute to asymmetric and symmetric stretching of carboxylate anions group (C=O). Protein structure could also be presented by the absorption around 1243 cm^{-1}. The absorption bands between 1100 cm^{-1} and 1000 cm^{-1} were attribute to the characteristic of C—O—C glycosidic bond vibrations and ring vibrations overlapped with stretching

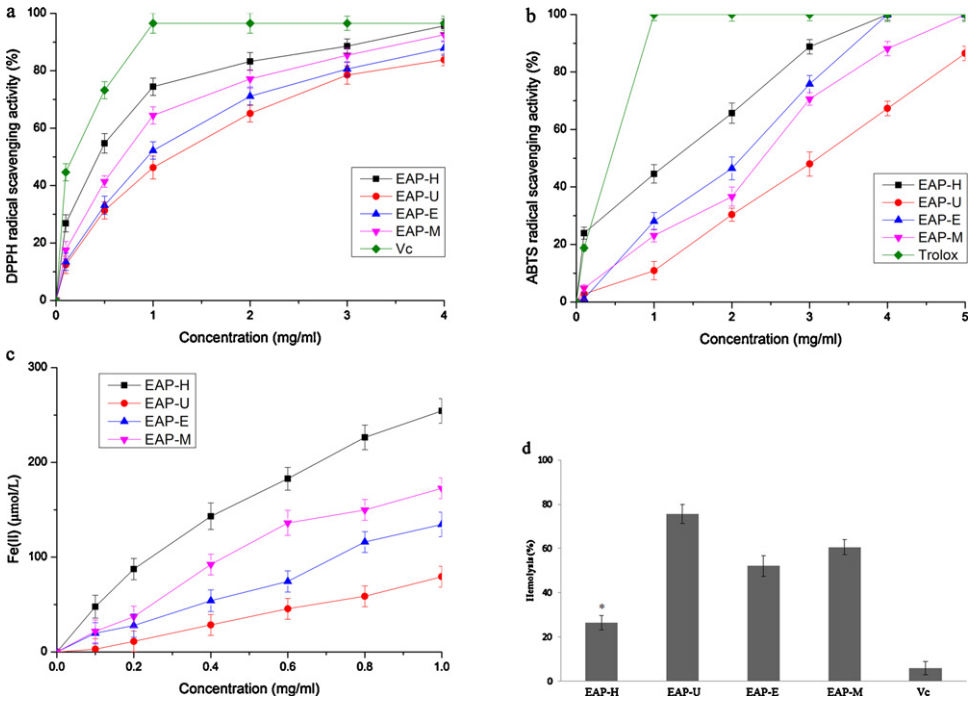

Fig. 3. (a) DPPH radical scavenging activities of EAP-H, EAP-U, EAP-E, EAP-M, and Vc. (b) ABTS radical scavenging activities of EAP-H, EAP-U, EAP-E, EAP-M and Trolox. (c) Reducing power of EAP-H, EAP-U, EAP-E, and EAP-M. (d) AAPH radical induced hemolysis with EAP-H, EAP-U, EAP-E, EAP-M, and Vc. (a–d) Each value is the mean ± SD of triplicate measurements.

vibrations of side group C—O—H link bonds. EAP-H, EAP-E, and EAP-M displayed the absorption near 890 cm^{-1}, which was due to the linkage of β-glycosides. The absorption band at 871 cm^{-1} suggested the linkage of α-glycosides in the molecular structure of EAP-U.

3.3. SEM analysis

SEM images provide visual evidences of the difference morphological changes of EAPs. As shown in Fig. 2, EAP-H (Fig. 2a and b) had a flat and smooth surface. EAP-U (Fig. 2c and d) was distinctive from others. The surface was observed as rough stone with large cave. The structural changes were caused by ultrasonic cavitation. The surface of EAP-E (Fig. 2e and f) appeared to be a flat and smooth surface with pores openings. For the EAP-M (Fig. 2g and h), the surface appeared to be rough with characteristic large wrinkles, this was different from the works of Rodriguez-Jasso (Rodriguez-Jasso et al., 2011), in which it was reported that polysaccharides had a rough flat with many cavities. The different surface topography of the polysaccharides was probably caused by the changes of physicochemical properties and the selective type of extraction method.

3.4. Antioxidant activity

3.4.1. Scavenging activity of DPPH radical

As shown in Fig. 3a, EAP-H, EAP-U, EAP-E, EAP-M and Trolox possessed DPPH radical scavenging activities in a concentration-dependent manner, and the abilities were followed by Vc, EAP-H, EAP-M, EAP-E, and EAP-U. At 2 mg/mL, the scavenging activities were 83.23%, 65.15%, 71.14% and 77.16% for the EAP-H, EAP-U, EAP-E, and EAP-M, respectively. The IC_{50} values were 0.59, 1.30, 1.12, and 0.8 mg/mL for EAP-H, EAP-U, EAP-E, and EAP-M. In summary, *E. acuminatum* polysaccharides might act as electron or hydrogen donator to scavenge DPPH radical, and EAP-H showed significantly higher ability on DPPH radical scavenging activities than EAP-M, EAP-E and EAP-U.

3.4.2. Scavenging activity of ABTS radical

As shown in Fig. 3b, EAP-H, EAP-U, EAP-E, EAP-M and Trolox possessed ABTS radical scavenging activities in a dose-dependent manner, and the abilities were followed by Trolox, EAP-H, EAP-E, EAP-M, and EAP-U. At 2 mg/mL, the scavenging activities were 65.68%, 30.36%, 46.49%, and 36.63% for EAP-H, EAP-U, EAP-E, and EAP-M, respectively. The IC_{50} values of EAP-H, EAP-U, EAP-E, and EAP-M were 1.53, 2.94, 1.74, and 2.25 mg/mL. Obviously, ABTS radical scavenging activities of EAP-H were significantly higher than that of EAP-U, EAP-E, and EAP-M.

3.4.3. FRAP

FRAP assay was often used to evaluated the antioxidant ability of polysaccharides (Fan, Li, Deng, & Ai, 2012). It utilizes the procedure that a colorless Fe(III) reacts with electron-donating antioxidants to generate a colored Fe(II)–tripyridyltriazine form. As shown in Fig. 3c, all polysaccharides possessed reducing power in the order of EAP-H, EAP-M, EAP-E, and EAP-U. The FRAP values of EAP-H, EAP-U, EAP-E, and EAP-M were 162.76, 45.55, 74.33 and 136.2 μmol/L at the concentration of 0.6 mg/mL, respectively. Obviously, ferric reducing power of EAP-H was significantly higher than that of EAP-U, EAP-E, and EAP-M.

3.4.4. Erythrocyte hemolysis

AAPH-induced hemolysis assay was a common model to evaluate oxidative membrane damage and the protective effects of antioxidants (Miki et al., 1987; Niki et al., 1988). It is initialed by the procedure that AAPH radicals react with Fe(II) in erythrocyte. As shown in Fig. 3d, EAP-H exhibited higher ability of hemolysis inhibition than EAP-M, EAP-E, and EAP-U. Our data on AAPH-induced hemolysis assay indicated that polysaccharides could act as antioxidant to reduce the formation of free radical mediated by AAPH.

4. Conclusions

In the present study, the polysaccharides from *E. acuminatum* were obtained by hot water extraction, ultrasonic-assisted extraction, enzyme extraction, and microwave-assisted extraction. The carbohydrate content, protein content, and FT-IR spectra of EAP-H, EAP-U, EAP-E, and EAP-M were similar, and the uronic acid content was significantly different. The SEM images revealed that different extraction methods led to different surface. Moreover, *E. acuminatum* polysaccharides showed significant free radical scavenging activities in a dose-dependent manner, and EAP-H showed significantly higher scavenging activity on DPPH assay, ABTS assay, FRAP assay, and the AAPH-induced erythrocyte hemolysis assay than EAP-U, EAP-E, and EAP-M.

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References

Barry, H., & Gutteridge, J. M. (1984). Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochemical Journal*, *219*(1), 1–14.

Bendjeddou, D., Lalaoui, K., & Satta, D. (2003). Immunostimulating activity of the hot water-soluble polysaccharide extracts of *Anacyclus pyrethrum*, *Alpinia galanga* and *Citrullus colocynthis*. *Journal of Ethnopharmacol*, *88*(2–3), 155–160.

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: The FRAP assay. *Analytical Biochemistry*, *239*(1), 70–76.

Bitter, T., & Muir, H. M. (1962). A modified uronic acid carbazole reaction. *Analytical Biochemistry*, *4*, 330–334.

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.

Chen, R. Z., Liu, Z. Q., Zhao, J. M., Chen, R. P., Meng, F. L., Zhang, M., et al. (2011). Antioxidant and immunobiological activity of water-soluble polysaccharide fractions purified from *Acanthopanax senticosu*. *Food Chemistry*, *127*(2), 434–440.

Chu, Y. F., Yan, X. M., Li, X. R., & Hu, Y. L. (2006). Effect of chinese herbal medicinal ingredients on IL-2 mRNA levels of T lymphocytes in mice measured using semiquantification RT-PCR. *Agricultural Sciences in China*, *5*(11), 873–878.

Dubois, M., Gilles, K., Hamilton, J. K., Rebers, P. A., & Smith, F. (1951). A colorimetric method for the determination of sugars. *Nature*, *168*(4265), 167.

Fan, L. P., Li, J. W., Deng, K. Q., & Ai, L. Z. (2012). Effects of drying methods on the antioxidant activities of polysaccharides extracted from *Ganoderma lucidum*. *Carbohydrate Polymers*, *87*(2), 1849–1854.

Fan, Y. P., Hu, Y. L., Wang, D. Y., Guo, Z. H., Zhao, X. N., Guo, L. W., et al. (2010). Epimedium polysaccharide and propolis flavone can synergistically stimulate lymphocyte proliferation in vitro and enhance the immune responses to ND vaccine in chickens. *International Journal of Biological Macromolecules*, *47*(2), 87–92.

Ganesh, K. C., Joo, H. S., Choi, J. W., Koo, Y. M., & Chang, C. S. (2004). Purification and characterization of an extracellular polysaccharide from haloalkalophilic *Bacillus* sp. I-450. *Enzyme and Microbial Technology*, *34*(7), 673–681.

Ge, Y., Duan, Y. F., Fang, G. Z., Zhang, Y., & Wang, S. (2009). Polysaccharides from fruit calyx of *Physalis alkekengi* var. *francheti*: Isolation, purification, structural features and antioxidant activities. *Carbohydrate Polymers*, *77*(2), 188–193.

Guyton, K. Z., Bhan, P., Kuppusamy, P., Zweier, J. L., Trush, M. A., & Kensler, T. W. (1991). Free radical-derived quinone methide mediates skin tumor promotion by butylated hydroxytoluene hydroperoxide: Expanded role for electrophiles in multistage carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(3), 946–950.

Kong, F. L., Zhang, M. W., Kuang, R. B., Yu, S. J., Chi, J. W., & Wei, Z. C. (2010). Antioxidant activities of different fractions of polysaccharide purified from pulp tissue of litchi (*Litchi chinensis* Sonn.). *Carbohydrate Polymers*, *81*(3), 612–616.

Kong, X. F., Hu, Y. L., Rui, R., Wang, D. Y., & Li, X. R. (2004). Effects of Chinese herbal medicinal ingredients on peripheral lymphocyte proliferation and serum antibody titer after vaccination in chicken. *International Immunopharmacology*, *4*(7), 975–982.

Kovačević, N., Čolić, M., Backović, A., & Došlov-Kokoruš, Z. (2006). Immunomodulatory effects of the methanolic extract of Epimedium alpinum in vitro. *Fitoterapia*, *77*(7–8), 561–567.

Li, H. F., Guan, X. Y., Yang, W. Z., Liu, K. D., Ye, M., Sun, C., et al. (2012). Antioxidant flavonoids from *Epimedium wushanense*. *Fitoterapia*, *83*(1), 44–48.

Li, X. L., & Zhou, A. G. (2007). Evaluation of the antioxidant effects of polysaccharides extracted from *Lycium barbarum*. *Medicinal Chemistry Research*, *15*(9), 471–482.

Lin, J. Y., & Tang, C. Y. (2007). Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation. *Food Chemistry*, *101*(1), 140–147.

Lu, Y., Wang, D. Y., Hu, Y. L., Huang, X. Y., & Wang, J. M. (2008). Sulfated modification of epimedium polysaccharide and effects of the modifiers on cellular infectivity of IBDV. *Carbohydrate Polymers*, *71*(2), 180–186.

Ma, H. P., He, X. R., Yang, Y., Li, M. X., Hao, D. J., & Jia, Z. P. (2011). The genus *Epimedium*: An ethnopharmacological and phytochemical review. *Journal of Ethnopharmacology*, *134*(3), 519–541.

Malpezzi, E. L., & Freitas, J. C. (1991). Hemolytic activity of the nematocyst venom from the sea anemone *Bunodosoma caissarum*. *Brazilian Journal of Medical and Biological Research*, *24*(12), 1245–1249.

Miki, M., Tamai, H., Mino, M., Yamamoto, Y., & Niki, E. (1987). Free-radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-tocopherol. *Archives of Biochemistry and Biophysics*, *258*(2), 373–380.

Niki, E., Komuro, E., Takahashi, M., Urano, S., Ito, E., & Terao, K. (1988). Oxidative hemolysis of erythrocytes and its inhibition by free radical scavengers. *The Journal of Biological Chemistry*, *263*(36), 19809–19814.

Ramarathnam, N., Osawa, T., Ochi, H., & Kawakishi, S. (1995). The contribution of plant food antioxidants to human health. *Trends in Food Science & Technology*, *6*(3), 75–82.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved abts radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*(9–10), 1231–1237.

Rodriguez-Jasso, R. M., Mussatto, S. I., Pastrana, L., Aguilar, C. N., & Teixeira, J. A. (2011). Microwave-assisted extraction of sulfated polysaccharides (fucoidan) from brown seaweed. *Carbohydrate Polymers*, *86*(3), 1137–1144.

Shimada, K., Fujikawa, K., Yahara, K., & Nakamura, T. (1992). Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *Journal of Agricultural and Food Chemistry*, *40*(6), 945–948.

Sun, J. L., Hu, Y. L., Wang, D. Y., Zhang, B. K., & Liu, J. G. (2006). Immunologic enhancement of compound Chinese herbal medicinal ingredients and their efficacy comparison with compound Chinese herbal medicines. *Vaccine*, *24*(13), 2343–2348.

Wang, J. L., Zhang, J., Zhao, B. T., Wang, X. F., Wu, Y. Q., & Yao, J. (2010). A comparison study on microwave-assisted extraction of *Potentilla anserina* L. polysaccharides with conventional method: Molecule weight and antioxidant activities evaluation. *Carbohydrate Polymers*, *80*(1), 84–93.

Wu, W. L., Zhu, Y. T., Zhang, L., Yang, R. W., & Zhou, Y. H. (2012). Extraction, preliminary structural characterization, and antioxidant activities of polysaccharides from Salvia miltiorrhiza Bunge. *Carbohydrate Polymers*, *87*(2), 1348–1353.

Yan, Y. L., Yu, C. H., Chen, J., Li, X. X., Wang, W., & Li, S. Q. (2011). Ultrasonic-assisted extraction optimized by response surface methodology, chemical composition and antioxidant activity of polysaccharides from *Tremella mesenterica*. *Carbohydrate Polymers*, *83*(1), 217–224.