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Extraction, antioxidant and antimicrobial activities of *Epimedium acuminatum* Franch. polysaccharide

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ABSTRACT

Polysaccharides from *Epimedium acuminatum* were extracted by hot water and optimized with response surface methodology. The optimal conditions of the extraction were determined to be the ratio of water to raw material of 29.61, extraction temperature of 85.67 °C and extraction time of 3.57 h. Under these optimal conditions, the yield of polysaccharide was 8.21%, which was well matched with the predictive yield (8.23%). Moreover, three purified fractions (EAP40-1, EAP60-1 and EAP80-2) were obtained for further chemical analysis, antioxidant activity analysis and antimicrobial activity analysis. EAP40-1 with molecular weight of 138,884 Da showed the best radical scavenging activity. Meanwhile, EAP60-1 with molecular weight of 114,667 Da was found to exhibit significant antihemolytic activity and antimicrobial activity.

1. Introduction

Many plant-derived polysaccharides have attracted a great deal of attention recently because of their potent therapeutic properties and relatively nontoxic effects. Some studies have indicated that water-soluble polysaccharide obtained from Chinese traditional herb materials showed efficient bioactivities including immunomodulation, antioxidant, anti-tumor and anticoagulant activities. These resourceful polysaccharides are ideal candidates for extensively research and explored.

Epimedium acuminatum Franch., namely *Yinyanghuo* in Chinese, has been used for modulating immunological function, anti-osteoporosis and anti-aging effects (Ma et al., 2011). The constituents isolated from its leaves consist of flavonoids, icariins and polysaccharides (Fan et al., 2010; Li et al., 2012). Among these compounds, the unique characteristics of polysaccharides have drawn great attention. Crude polysaccharides from *E. acuminatum* were showed to possessed strong DPPH radicals scavenging properties (Cheng et al., 2013). Three water-soluble *Epimedium* polysaccharides could promote proliferation of splenic lymphocytes, activate macrophages and enhance NK cell activity (Chen, Li, Liu, Yang, & Li, 2012). In addition, sulfated modification polysaccharide from *Epimedium* exhibited therapeutic effectiveness in infectious bursal disease (IBDV) (Lu, Wang, Hu, Huang, & Wang, 2008).

Response surface methodology (RSM) is a rapid technique used to evaluate the interactions between multiple parameters and optimize process parameters. In this study, *E. acuminatum* polysaccharide (EAP) was optimized using a five-level, three-variable central composition design (CCD). The physicochemical properties of purified EAP were investigated using different analysis methods, such as Fourier transform infrared spectroscopy (FT-IR), gel permeation chromatography (GPC) and gas chromatography-mass spectrometry (GC–MS). Moreover, antioxidant abilities and antimicrobial activities of EAPs were also investigated *in vitro*.

2. Materials and methods

2.1. Materials and reagents

The materials were collected in October 2012 from Yaan, Sichuan Province, China and identified by Prof. Chunbang Ding, Sichuan Agricultural University, China. The materials were washed, dried, and then were crushed into powder with a powerful mill (FW177, Taisite Instrument Co. Ltd., Tianjin, China). Finally, the powder of the materials was screened through an 80 mesh sieve and stored in a desiccator at room temperature.

2,2-Diphenyl-1-picryl-hydrazy (DPPH) and D-glucose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ascorbic acid

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Table 1
Experimental results of the response surface methodology.

Run	Uncoded levels			Coded levels			Yield (%)[a]
	x_1	x_2	x_3	A	B	C	
1	30.00	70.00	4.00	1.00	−1.00	1.00	7.11
2	25.00	80.00	4.68	0.00	0.00	1.68	7.98
3	25.00	80.00	3.00	0.00	0.00	0.00	7.94
4	25.00	80.00	3.00	0.00	0.00	0.00	7.91
5	25.00	96.82	3.00	0.00	1.68	0.00	7.26
6	25.00	80.00	1.32	0.00	0.00	−1.68	7.19
7	20.00	90.00	2.00	−1.00	1.00	−1.00	6.81
8	20.00	90.00	4.00	−1.00	1.00	1.00	7.42
9	33.41	80.00	3.00	1.68	0.00	0.00	8.01
10	16.59	80.00	3.00	−1.68	0.00	0.00	6.93
11	25.00	80.00	3.00	0.00	0.00	0.00	8.01
12	20.00	70.00	2.00	−1.00	−1.00	−1.00	6.63
13	25.00	80.00	3.00	0.00	0.00	0.00	7.91
14	25.00	80.00	3.00	0.00	0.00	0.00	8.01
15	25.00	80.00	3.00	0.00	0.00	0.00	8.05
16	30.00	90.00	4.00	1.00	1.00	1.00	8.18
17	20.00	70.00	4.00	−1.00	−1.00	1.00	7.04
18	25.00	63.18	3.00	0.00	−1.68	0.00	6.61
19	30.00	70.00	2.00	1.00	−1.00	−1.00	7.18
20	30.00	90.00	2.00	1.00	1.00	−1.00	7.79

[a] Mean of triplicate determination.

(Vc) was purchased from the Sinopharm Chemical Reagent Co. (Beijing, China). All other reagents were analytical grade.

2.2. Hot water extraction

The dried powder was defatted with petroleum ether for 3 h. The extraction process was performed as the previous method (Cheng et al., 2013). Then the pretreated powder (5 g) was extracted by the designed ratio of water to raw material, extraction temperature and extraction time. The effect of ratio of water to raw material (x_1) ranged from 10 to 40, extraction temperature (x_2) ranged from 50 °C to 100 °C and extraction time (x_3) ranged from 1 to 6 h was studied by single factor design. One factor is changed, while the other factors keep constant in each experimental. Then the supernatant was precipitated with four times (v/v) of 95% ethanol aqueous solution at 4 °C. Finally, the crude polysaccharide was obtained by lyophilized. The yield of EAP (%) was calculated as follow equation:

$$\text{Polysaccharide yield}\,(\%) = \frac{A}{B} \times 100$$

where A and B are the weight of crude polysaccharides (g) and *E. acuminatum* powder (g), respectively.

2.3. Experimental design

Based on the single factor test, three independent variables (ratio of liquid to raw material, extraction temperature and extraction time) significantly affected the extraction efficiency and the optimal range of each variable was determined. A central composition design with five-level, three-variable was applied to statistically optimize the extraction process. The code of each factor and its real value are presented in Table 1. For predicting the optimal value of each factor, a second-order polynomial equation is fitted to correlate the relationship between factors and the response. The model equation used for the analysis is given below:

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_i x_i x_j + \Sigma\beta_{ii} x_i^2$$

where Y is predicted yield, x_i and x_j are the coded values of ratio of liquid to raw material, extraction temperature and extraction time, respectively.

The significance in the model was evaluated by analysis of variance (ANOVA) for each response. The accuracy and general ability of the polynomial model could be evaluated by a determination coefficient R^2. In addition, several verification experiments were done according to the optimal conditions.

2.4. Separation and analysis

The crude polysaccharide (EAP) was deproteinized by Sevag's method, and then successively sub-fractionated by gradient concentration of ethanol (40%, 60% and 80%). The precipitates were collected and named as EAP40, EAP60 and EAP80, respectively. They were loaded onto a Sephadex G-100 gel column (2.5 cm × 50 cm) for further purification. The column was eluted with 0.1 mol/L NaCl at a flow rate of 0.2 mL/min. The major fraction was collected and freeze-dried. Total sugars were determined by the phenol-sulfuric acid assay using glucose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1951). Protein was measured by Bradford method using BSA as standard (Bradford, 1976). Uronic acid was assayed by vitriol-carbazole method (Bitter & Muir, 1962). Reducing sugar was measured by Fehling reagent. The average molecular weight (Mw) of EAPs was determined by gel permeation chromatography (GPC) method using dextran of known molecular weight as standard. The flow rate of the mobile phase (0.9% NaCl) was 0.5 mL/min. FT-IR was carried out by the potassium bromide (KBr) pellet method on Fourier Transform Infrared Spectrometer (8400s, Shimadzu, Japan) in the range of 400–4000 cm^{-1}.

2.5. Analysis of monosaccharide composition

Purified fractions (10 mg) were hydrolyzed with 5 mL 3 mol/L trifluoroacetic acid (TFA) at 110 °C for 6 h, reduced by $NaBH_4$, and then followed by acidification with acetic anhydride. The results of alditol acetates were analyzed by GC–MS (QP2010, Shimadzu, Japan) with a fused silica capillary column (0.25 μm × 0.25 mm × 30 m) of RTX 5 ms. The operation conditions of GC–MS were as follows: the temperature of detector and inlet were 280 °C and 250 °C, respectively. The oven temperature program was set changing from 120 °C (standing for 3 min) up to 210 °C (standing for 4 min) at a rate of 3 °C/min. The hydrogen flow rate was 20 mL/min. The ion source temperature was 230 °C and the mass spectra ranged from 40 to 500.

Table 2
ANOVA for response surface quadratic model analysis of variance.

Source	SS	DF	MS	*F*-value	*p*-Value
Model	5.30	9	0.59	84.32	<0.0001[a]
A	1.28	1	1.28	183.01	<0.0001[a]
B	0.81	1	0.81	116.57	<0.0001[a]
C	0.52	1	0.52	74.72	<0.0001[a]
AB	0.16	1	0.16	22.47	0.0008[a]
AC	0.061	1	0.061	8.78	0.0142[b]
BC	0.054	1	0.054	7.80	0.0190[b]
A^2	0.48	1	0.48	68.26	<0.0001[a]
B^2	1.98	1	1.98	284.18	<0.0001[a]
C^2	0.29	1	0.29	41.15	<0.0001[a]
Residual	0.070	10	6.979E−003		
Lack of fit	0.052	5	0.010	2.95	0.1304
Pure error	0.018	5	3.537E−003		
Cor total	5.37	19			

[a] Very significant at <0.01 level.
[b] Significant at <0.05 level.

2.6. Scavenging activity of DPPH radical

The scavenging of DPPH radical was measured according to the method of Cheng et al. (2013). 2 mL samples (0.05–1.0 mg/mL) was added to 2 mL of 0.2 mmol/L DPPH ethanol solution. Absorbance at 517 nm was measured after 30 min incubation at 37 °C.

2.7. Scavenging activity of hydroxyl radical

The scavenging of hydroxyl radical was determined according to Fenton's reaction (Wu, Zhu, Zhang, Yang, & Zhou, 2012). The reaction mixture containing 0.2 mL different samples (0.05–3 mg/mL) was incubated with 1 mL water, 1 mL 1,10-phenanthroline ethanol solution (0.75 mmol/L), 1 mL $FeSO_4$ (0.75 mmol/L) and 1 mL H_2O_2 (0.01%). Absorbance at 520 nm was recorded after 60 min water bath at 37 °C.

2.8. Inhibition of erythrocyte hemolysis

The inhibition of erythrocyte hemolysis was evaluated according to the method previously reported (Yan et al., 2011) with minor modification. The erythrocyte hemolysis was performed with H_2O_2 as free radical initiator. Briefly, 0.25 ml samples (0.1–1 mg/mL) and 1.0 mL of 10 mmol/L H_2O_2 were added in succession to 0.5 mL of 10% (v/v) erythrocyte suspension. The mixture was incubated at 37 °C for 1 h while gentle shaking in dark. Then, the reaction solution was diluted 10 times with phosphate buffered saline (PBS; pH 7.4) and centrifuged at 2000 × *g* for 10 min. The absorbance of the resulting supernatant was measured at 540 nm.

2.9. Inhibition of lipid peroxidation on erythrocyte ghost membrane

The erythrocyte ghost membrane lipid peroxidation was performed according to the method previously reported (Ajila & Prasada Rao, 2008). Samples (0.1–1 mg/mL) were added to 1.0 mL of erythrocyte ghost membrane suspension (200 μg protein) and lipid peroxidation was initialed by adding of 100 μL H_2O_2 (0.2 mmol/L). The mixture was incubated at 37 °C while gentle shaking in dark for 1 h. 2 mL of reaction solution containing 15% (w/v) trichloroacetic acid (TCA), 0.375% (w/v) 2-thiobarbituric acid (TBA) and 0.25 mol/L HCl was then added to the mixture. After boiling at 100 °C for 10 min, the absorbance at 532 nm was recorded.

2.10. Antimicrobial activity

Antimicrobial activity of EAPs was evaluated by the filter disk diffusion plate method with slight modification (Xie et al., 2012). Bacteria (*Escherichia coli* and *Bacillus subtilis*), microzyme (*Saccharomyces cerevisiae*) and fungi (*Aspergillus niger*) were selected as test organisms. After the inoculum solution (100 μL) of each test organism (10^6 CFU/mL) was inoculated on the medium, filters (6 mm in diameter and 1.5 mm thick) containing samples (5 mg/mL) were placed in the center of the medium. Sterilized water was used as a control. Bacterial plates were incubated at 37 °C for 24 h, while microzyme and fungi plates were incubated at 28 °C for 48 h. The antimicrobial activity was measured with calipers and expressed by the diameter (mm) of the inhibition zones.

2.11. Minimal inhibitory concentrations

Minimal inhibitory concentrations (MICs) of samples were carried out as a previous method (He, Yang, Yang, & Yu, 2010). The serial concentration samples (0.125, 0.25, 0.5, 1, 2, 4 mg/mL) were prepared by dissolved in sterilized water. MIC test of samples were carried out as the antibacterial activity tests.

2.12. Statistical analysis

All the experiments were carried out in triplicate and all the data were shown as the means ± standard deviations (SD). Results were evaluated by ANOVA. *p*-Values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Optimization of the extraction process

3.1.1. Statistical analysis and the model fitting

Based on the single-factor investigation results, the coded factor levels and real values of three factors (extraction temperature, extraction time and ratio of liquid to raw material) are presented in Table 1. The results of the responses (yield of polysaccharides) in the factorial design are presented in Table 1. The yield ranged from 6.61% to 8.18%. By applying multiple regression analysis on the experimental data, the response variable and test variables were related by the following second-order polynomial equation:

$$Y = 7.97 + 0.31A + 0.24B + 0.20C + 0.14AB - 0.087AC + 0.083BC - 0.18A^2 - 0.37B^2 - 0.14C^2$$

The fit statistics of extraction yield (Y) for the selected quadratic predictive model is shown in Table 2. The model was highly significant from the evidence of the low *p*-value (<0.0001). The coefficient of determination (R^2) was calculated to be 0.9870 suggesting that

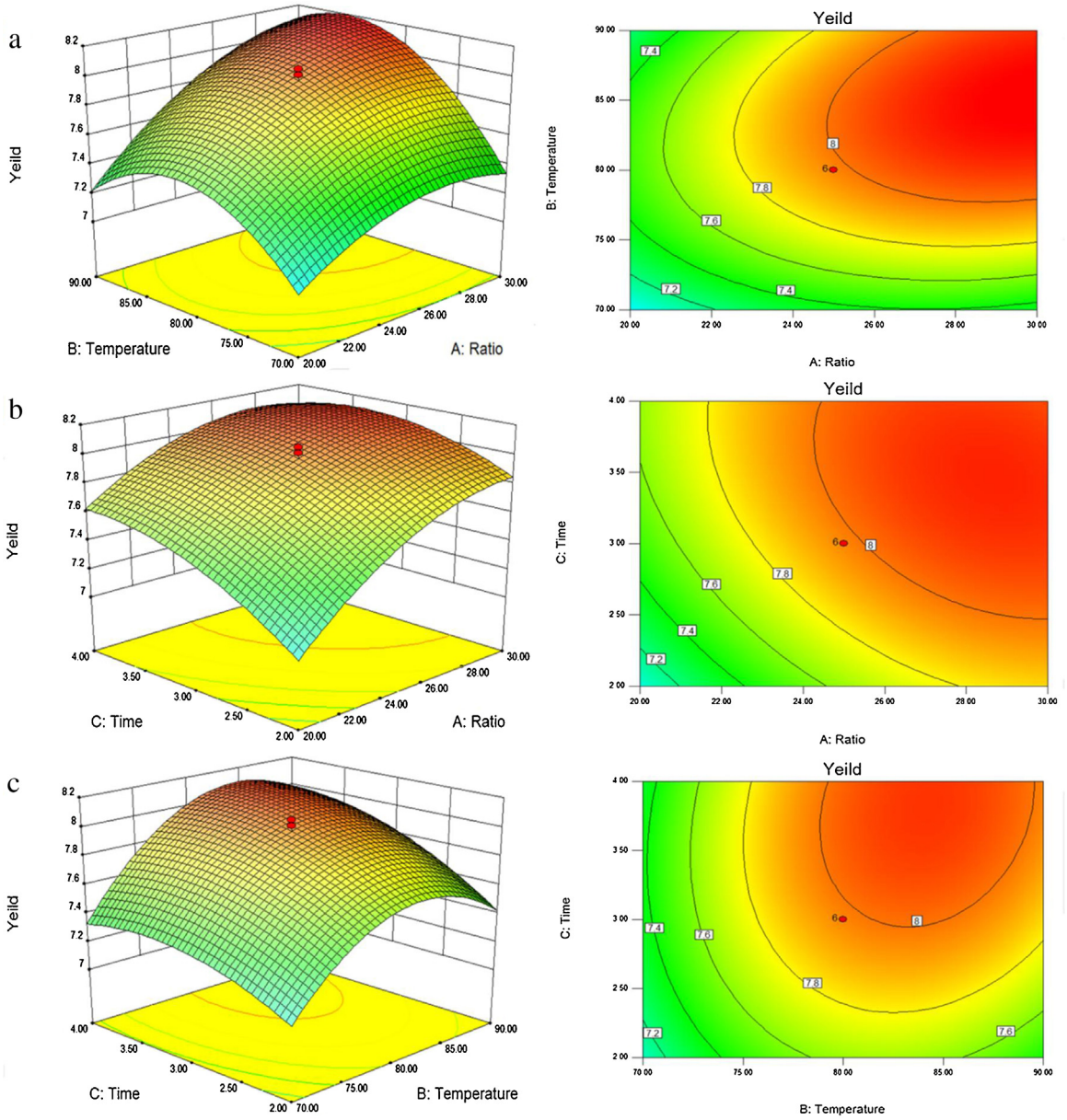

Fig. 1. Response surface plots and contour plots showing the effect of the ratio of water to raw material, extraction temperature and extraction time on the polysaccharide yield.

the yield variation of 98.70% was attributed to the variable factors. The value of the adjusted determination coefficient (adjusted $R^2 = 0.9753$) also confirmed that the model was highly significant. At the same time, a very low value of coefficient of the variation (CV = 1.11%) indicated a high degree of precision and a good deal of reliability of the experimental values.

The 3D response surface plots and contour plots are graphical representations of the regression equation. Three independent response surface plots are shown in Fig. 1. It could be seen that all three variables demonstrated a significant effect on the yield of EAP. Fig. 1a shows the effect of ratio of water to raw material and extraction temperature on the yield of EAP when the extraction time is fixed at level zero. The yield increased with increasing of ratio of water to raw material and extraction temperature from 70 to 83.5 °C, but beyond 83.5 °C, the yield decreased with the increasing of extraction temperature. These results might be attributed to the thermal degradation of EAP at high extraction temperature (Li, Ding, & Ding, 2007). Fig. 1b shows that the ratio of water to raw material has a similar positive effect on the yields when the extraction temperature is fixed. It indicated that the yield increased slowly with the increasing of extraction time from 3.5 to 4 h. As shown in Fig. 1c, a great increase in the yield of EAP occurred when the temperature was increased from 70 to 85 °C. But with further increasing of the temperature, the yield went to slight decrease.

3.1.2. *Verification of results*

The feasibility of the experiment and the suitability of the model equation for predicting the optimal response values were tested by using the selected optimal conditions: extraction time was 3.57 h, extraction temperature was 85.67 °C and ratio of water to raw material was 29.61. The model predicted a maximum response of 8.23%. Under this set combination, the experimental value of yield was 8.21 ± 0.08%. There was no statistically difference between the experimental and predicted values. The result demonstrated the

Table 3
Chemical characteristics of EAPs.

Parameter	EAP40-1	EAP60-1	EAP80-2
Yield (%)	2.14 ± 0.12	1.36 ± 0.17	1.17 ± 0.08
Total sugar (%)	89.59 ± 2.23	92.51 ± 1.45	91.38 ± 1.73
Protein (%)	0.33 ± 0.04	0.48 ± 0.03	0.14 ± 0.06
Uronic acid	+[a]	+	+
Fehling's test	−[b]	−	−
Molecular weight (Da)	138,884	114,667	65,313

[a] Positive.
[b] Negative.

Table 4
Monosaccharide composition of EAPs.

Sample	Monosaccharide composition (relatively mass%)						
	Glucose	Galactose	Mannose	Xylose	Rhamnose	Arabinose	Fructose
EAP40-1	46.79	14.58	36.00	nd[a]	2.63	nd	nd
EAP60-1	43.61	37.67	2.89	10.18	3.37	2.28	nd
EAP80-2	21.64	55.56	6.73	4.46	1.22	nd	10.39

[a] nd, not detected.

validation of the RSM model. Thus, the model can be used to optimize the process of EAP extraction.

3.2. Purification of fractions

The crude polysaccharides from *E. acuminatum* were firstly subfractionated by gradient concentrations of ethanol (40%, 60% and 80%), then they were chromatographed on Sephadex G-100 column. Three peaks were purified from EAP40 and EAP60, and they were named as EAP40-1, EAP40-2, EAP40-3, EAP60-1, EAP60-2 and EAP60-3, respectively. Two peaks obtained from EAP80 were named as EAP80-1 and EAP80-2. The major fractions (EAP40-1, EAP60-1 and EAP80-2) were collected and dried for further investigation (data not shown).

3.3. Characteristics of EAPs

3.3.1. Identification of EAPs

The recovery yields, chemical composition and Mw of EAP40-1, EAP60-1 and EAP80-2 were shown in Table 3. Briefly, the recovery yields of three EAPs were 2.14%, 1.36% and 1.17%, respectively. The contents of total sugar for EAP40-1, EAP60-1 and EAP80-2 were 89.59%, 92.51% and 91.38%, respectively. The protein contents of EAP40-1, EAP60-1 and EAP80-2 were 0.33%, 0.48% and 0.14%, respectively. There were no significant difference in the sugar contents and protein contents among the three polysaccharides. The vitriol-carbazole reaction was plus reaction, proved that EAPs contained uronic acids. Fehling's test indicated that the three polysaccharides did not contain reducing sugar. GPC analysis showed that the Mw of EAPs was declined from 138,884 to 65,313 Da.

3.3.2. FT-IR spectrum

As shown in Fig. 2, the infrared spectrum of EAPs displayed a stretching vibration peak of characteristic of hydroxyl groups at 3300–3500 cm^{-1} and a weak C–H band around 2920 cm^{-1}. The relatively strong absorption peak around 1620 cm^{-1} and 1420 cm^{-1} were attributed to the characteristic of carboxylic group. The peaks at 1000–1200 cm^{-1} suggested the presence of C—O—C and C—O—H link bonds.

3.3.3. Monosaccharide composition

As shown in Table 4, EAP40-1 was mainly consisted of Glucose and Mannose. EAP60-1 was mainly consisted of glucose and galactose. Galactose was the major monosaccharides constructing the backbones of EAP80-2 with molar percent of 55.56%.

3.4. Antioxidant activity

3.4.1. Scavenging activity of DPPH radical

DPPH assay is based on the reduction of DPPH radical in the presence of a hydrogen donating antioxidant, resulting in the formation of a stable non-radical form DPPH-H and color fades up. As shown in Fig. 3a, EAP40-1, EAP60-1 and EAP80-2 showed obvious scavenging effect on DPPH radical in a dose-dependent manner. At the concentrations ranged from 0.2 to 1.0 mg/mL, the scavenging ability on DPPH radical of EAP40-1 was much higher than those of EAP60-1 and EAP80-2. At the concentration of 1 mg/mL, EAP40-1 possessed strong free radical scavenging effects of DPPH radicals (scavenging activity 86.93%), which was significantly higher ($p < 0.01$) than that of EAP60-1 (scavenging activity 76.34%) and EAP80-2 (scavenging activity 57.81%). The IC_{50} values were 0.28, 0.52, and 1.32 mg/mL for EAP40-1, EAP60-1, and EAP80-2. Compared with *Epimedium* leaves polysaccharides, which were isolated by ultrasound complex enzyme assisted extraction, EAPs exhibited higher scavenging activity for DPPH radical at 1 mg/mL (Chen et al., 2012). The result indicated that EAPs had a noticeable effect on scavenging DPPH

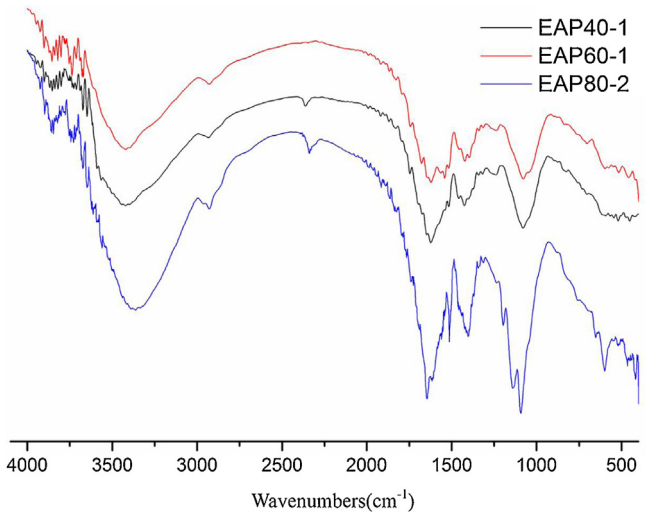

Fig. 2. Infrared spectra of EAP40-1, EAP60-1 and EAP80-2.

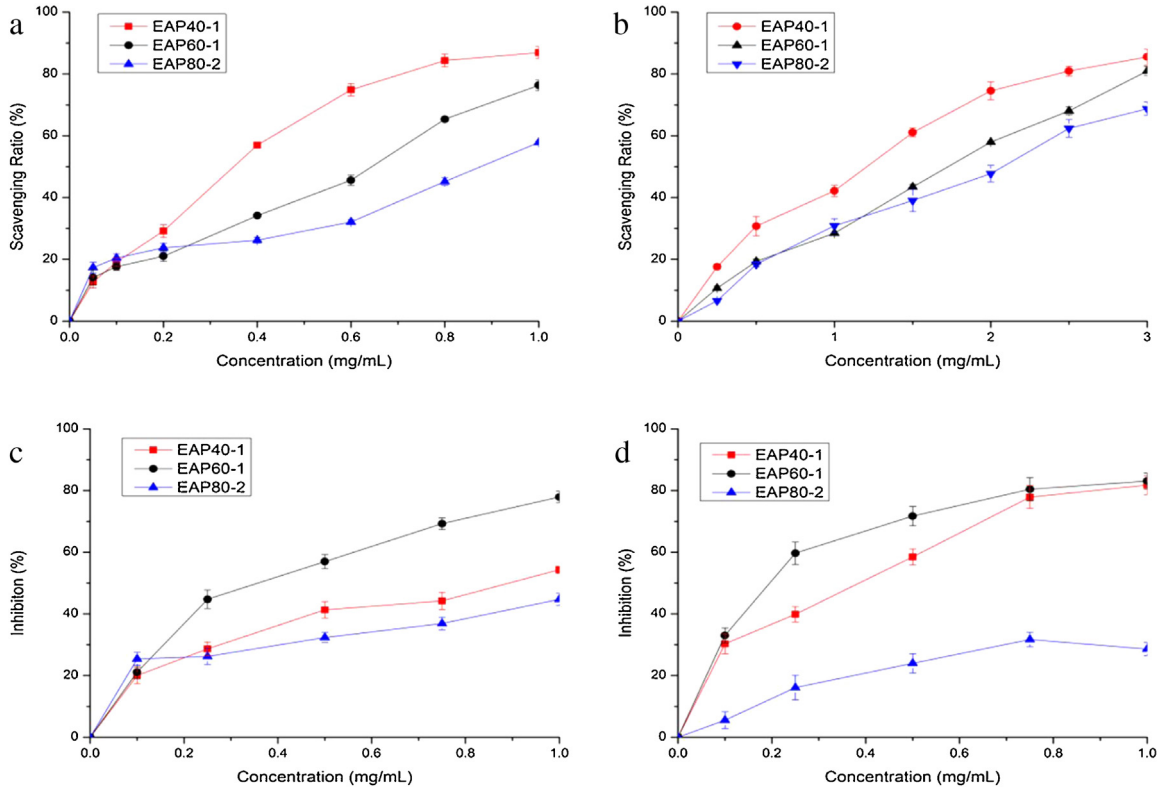

Fig. 3. Effect of EAP40-1, EAP60-1 and EAP80-2 on DPPH radical (a), hydroxyl radical (b), H_2O_2-induced erythrocyte hemolysis (c) and H_2O_2-induced lipid peroxidation (d). Each value is the mean ± SD of triplicate measurements.

radical, especially at low concentration. The possible mechanism of EAPs might be attributed to the supply of hydrogen.

3.4.2. Scavenging activity of hydroxyl radical

The hydroxyl radical is considered to be mainly responsible for oxidative injury of biomolecules. As shown in Fig. 3b, all samples exhibited obvious scavenging activity on hydroxyl radical in a dose-dependent pattern at all concentrations. EAP40-1 had the stronger activity than EAP60-1 and EAP80-2 ($p < 0.05$). At the 3 mg/mL, the scavenging activities of EAP40-1, EAP60-1 and EAP80-2 were 85.52%, 80.97% and 68.73%, respectively. The IC_{50} values were 0.95, 1.49, and 1.84 mg/mL for EAP40-1, EAP60-1, and EAP80-2. The scavenging hydroxyl radical activity was attributed to various mechanisms, such as suppression against hydroxyl radical generation, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging. The possibility of scavenging hydroxyl radical by polysaccharide was that polysaccharide could combine with radical and terminate the radical chain reaction (Chen et al., 2011).

3.4.3. Inhibition of erythrocyte hemolysis

Free radical generated by hydrogen peroxide in the aqueous phase can attack the erythrocytes membranes, leading to hemolysis. Water-soluble antioxidants can scavenge oxygen radicals and protect erythrocytes membranes against hydrogen peroxide-induced hemolysis (Yan et al., 2011). The Fig. 3c depicts that all samples could efficiently protected the erythrocyte membrane from hydrogen peroxide-induced hemolysis in a concentration-dependent manner. The inhibition rate of EAP60-1 was higher than that of EAP40-1 and EAP80-2 at the dosage range of 0.25–1 mg/mL ($p < 0.05$). In particular, the inhibition rate of EAP80-2 was always less than 50% at all concentrations. The IC_{50} values of EAP40-1, EAP60-1, and EAP80-2 were 0.90, 0.34, and 2.92 mg/mL. At the concentration of 1 mg/mL, the percentage of inhibition of EAP60-1 was 77.91%, which was higher than those values of crude polysaccharide from *Tremella mesentrica* and *E. acuminatum* at the same concentration (Cheng et al., 2013; Yan et al., 2011). These results suggested that purified polysaccharide had higher inhibition ability of erythrocyte hemolysis than crude polysaccharide.

3.4.4. Inhibition of lipid peroxidation on erythrocyte ghost membrane

Hydroxyl radical reacts with polyunsaturated fatty acid moieties in biological membrane, leading to the destruction of lipid membranes and produce lipid hydroperoxide. The breakdown of lipid hydroperoxide resulted in formation of malondialdehyde (MDA), which was responsible for DNA damage (Chagas Faustino Alves et al., 2011). The reaction of MDA with TBA was used as a sensitive method for lipid peroxidation investigation. As shown in Fig. 3d, lipid peroxidation was efficiently inhibited by EAP40-1, EAP60-1 and EAP80-2. EAP40-1 and EAP60-1 exhibited a significant concentration-dependent pattern at tested concentration. However, EAP80-2 showed a weak effect and the inhibition rate was less than 50% at all concentrations. Moreover, at the dosage range of 0.75–1 mg/mL, the inhibition rate of EAP80-2 decreased with the concentration increasing. At the 1 mg/mL, the inhibition activities of lipid peroxidation were 81.77%, 83.07% and 28.58% for EAP40-1, EAP60-1 and EAP80-2, respectively. The IC_{50} values of EAP40-1, EAP60-1, and EAP80-2 were 0.28, 0.19, and 2.66 mg/mL.

In vitro antioxidant experiment showed that EAP40-1, EAP60-1 and EAP80-2 exhibited different antioxidant effects. The fractions precipitated by lower concentration of ethanol showed relatively higher radical scavenging activity. For water-soluble polysaccharides from rice bran and *Asparagus officinalis* sub-fractionated by gradient concentrations of ethanol, the similar results were

Table 5
The antimicrobial activity and MICs of EAPs.

Microorganism	Diameters of inhibition zone (mm)			MICs (mg/mL)		
	EAP40-1	EAP60-1	EAP80-2	EAP40-1	EAP60-1	EAP80-2
E. coli	9.07 ± 0.37	11.06 ± 0.41	9.73 ± 0.27	0.5	0.25	0.25
B. subtilis	9.85 ± 0.52	10.16 ± 0.18	9.91 ± 0.33	1	0.25	0.5
S. cerevisiae	11.44 ± 0.20	11.90 ± 0.39	10.09 ± 0.47	0.25	0.25	0.5
A. niger	8.26 ± 0.18	8.72 ± 0.27	7.24 ± 0.44	2	2	4

obtained (Zha et al., 2009; Zhao et al., 2012). Previous studies suggested that the biological activities of polysaccharides were supposed to relate to their molecular weights, monosaccharide compositions and chain conformations (Yang & Zhang, 2009). In this study, the antioxidant activity of EAP40-1 and EAP60-1 with higher Mw were significantly higher than that of EAP80-2 with lower Mw. This result was accordance with Chagas Faustino Alves's work. Polysaccharide from *Hypnea musciformis* with high Mw displayed significant antioxidant action on hydroxyl radical, H_2O_2-induced hemolysis inhibition and lipid peroxidation (Chagas Faustino Alves et al., 2011). Monosaccharide composition analysis indicated that glucose and galactose residues were major composition of EAP60-1 and EAP80-2, and Glucose and Mannose residues were major composition of EAP40-1. The high content of mannose (36.00%) was supposed to relate to the strong DPPH and hydroxyl radical scavenging activity of EAP40-1.

3.5. Antimicrobial activity

Antimicrobial activity of EAPs *in vitro* is showed in Table 5. Of all tested organism, the EAPs were found to be the most effective against microzyme, following by bacteria and *A. niger*. Among three polysaccharides, EAP60-1 showed the best antimicrobial activities against *E. coli*, *B. subtilis*, *S. cerevisiae* and *A. niger* with inhibition zones of 11.06 mm, 10.16 mm, 11.90 mm and 8.72 mm, respectively. The MICs of EAP60-1 were 0.25 (*E. coli*), 0.25 (*B. subtilis*), 0.25 (*S. cerevisiae*) and 2 (*A. niger*) mg/mL (Table 5). As the concentration increasing, the antimicrobial activities of EAPs increased significantly. These results were accordance with previous studies (He et al., 2010; Wang et al., 2007). Water-soluble polysaccharides from *Cyclocarya paliurus* and *Lygodium japonicum* were the most effective against microzymes (Li, Zhou, & Han, 2006; Xie et al., 2012). Significant antibacterial activity was also shown by polysaccharide from spent mushroom substrate (Zhu, Sheng, Yan, Qiao, & Lv, 2012). The mechanisms involved in antimicrobial activity of polysaccharide are worthy further investigation.

4. Conclusions

In this study, RSM was used to determine the main and interaction effects of the variables which were important in the extraction process. The optimal extraction condition was performed as following: extraction time of 3.57 h, extraction temperature 85.67 °C and ratio of liquid to raw material of 29.61. Under the optimized conditions, the experimental yield of EAP was 8.21%, which was closely matched with the predicted yield (8.25%). EAP fractions were obtained by gradient concentration of ethanol precipitation and purified by Sephadex G-100. Among three fractions, EAP40-1 exhibited the highest radical scavenging activity. EAP60-1 showed the best antihemolytic activity and antimicrobial activity. In conclusion, polysaccharide from *E. acuminatum* could be explored as potential natural antioxidant in functional foods and medicine industry.

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