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Extraction, characterization and antioxidant activities of polysaccharides from *E. corneum gigeriae galli*

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ABSTRACT

In the present study, optimization of enzyme-assisted extraction, characterization and antioxidant activities *in vitro* of polysaccharides from *Endothelium corneum gigeriae galli* (PEGG) were investigated. It was found that the optimum extraction conditions were determined as follows: extraction temperature 87.0 °C, extraction time 177.0 min, enzyme concentration 1.65%, enzymatic hydrolysis time 141.0 min, liquid-to-solid ratio 20, enzymatic hydrolysis temperature 55 °C and enzymatic hydrolysis pH 3.6. Under these conditions, the experimental yield of polysaccharides was 5.08%. In addition, PEGG had a relatively high sulfate radical content. PEGG was composed of rhamnose, fucose, mannose, glucose and galactose, with molar percentages of 13.1, 4.5, 72.8 and 9.6%, respectively. The average molecular weight was 83 kDa. And there were infrared characteristic absorption peaks of polysaccharides in the FT-IR spectroscopy of PEGG. For antioxidant activities *in vitro*, PEGG showed possessed strong hydroxyl radical scavenging, Fe^{2+} chelating and lipid peroxidation inhibitory activities.

1. Introduction

Endothelium corneum gigeriae galli (EGG) is the dried inner wall of the *Gallus gallus domesticus* Brisson and has been used as traditional Chinese animal medicines for centuries (Zhang, Li, Jiang, Li, & Zhou, 1997). It has been reported that EGG has strong effects in promoting blood circulation, removing blood stasis, relieving stagnated food, strengthening the spleen and stomach and can be used for the treatment of diarrhea, dyspepsia, infantile malnutrition and mammary gland proliferation (Luo & Hu, 2008; Zhang, Meng, Zhang, Ou, & Liu, 2011). In addition, EGG could also be used to treat spermatorrhea, diabetes, renal calculus, frequent urination and oral ulcers resulted from chemotherapy (Li, 2012; Li, Sun, & Lv, 2002; Yuan & Yuan, 2008). It has been demonstrated that EGG is rich in protein, amino acid and polysaccharide that may contribute to the biological functions, such as anti-tumor, anti-radiation anti-inflammation, anti-coagulation and immunological enhancement (Kariya et al., 2004; Machiah, Girish, & Gowda, 2006; Wang et al., 2006; Xu, Wu, Mei, & Xu, 2004). However, to the best of our knowledge, there is limited literature on the extraction, characterization and biological activities of PEGG. Therefore, we report here the extraction, preliminary characterization and antioxidant activities *in vitro* of PEGG.

Enzyme-assisted extraction offers many advantages such as high extraction yield, lower investment costs and energy requirements compared to the hot-water extraction method and may be an effective and advisable technique for the extraction of polysaccharides (Nyam, Tan, Lai, Long, & Man, 2009; Yin, You, & Jiang, 2011). Therefore, enzyme-assisted extraction was used to produce PEGG. The pre-experiment firstly screened out the enzyme-assisted extraction parameters having significant effect on PECG production using a fractional factorial design. Then, we optimized the extraction conditions for PEGG production by using central composite design (CCD), one of response surface methodology (RSM). Since RSM is a powerful technique for testing multiple variables and widely used to optimize the extraction of polysaccharides (Guo, Zou, & Sun, 2010; Qiao et al., 2009; Sun, Liu, & Kennedy, 2010). Furthermore, It was characterized PEGG by chemical analysis, ultraviolet spectroscopy (UV), high performance liquid chromatography

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(HPLC), gas chromatography (GC) and Fourier transform-infrared spectroscopy (FT-IR). Finally, the antioxidant activities *in vitro* of PEGG were investigated by determining the hydroxyl radical scavenging activity, Fe^{2+} chelating activity and lipid peroxidation inhibitory activity.

2. Materials and methods

2.1. Materials

E. corneum gigeriae galli was purchased from the Traditional Chinese Medicine Market (Nanyang, China). Compound enzyme (42 U/mg) was obtained from Novozymes Investment Co. Ltd. (Beijing, China). All other reagents were of analytical grade.

2.2. Preparation of PEGG

The extraction of PEGG was performed according to previously reported methods with some modifications (Jiang, Wang, Liu, Gan, & Zeng, 2011). Briefly, fresh *E. corneum gigeriae galli* was collected and washed carefully with cold water. After removing some impurities, the flesh was crushed by a high speed disintegrator. The homogenate was defatted with petroleum ether (70–80 °C) and was kept in 90% of ethanol (v/v) for two weeks to remove lipids and some colored materials. Then, the collected flesh was air-dried at 50 °C. The defatted powder (1 g) was hydrolyzed by compound enzymes at the given concentration and liquid-to-solid ratio in a designed temperature, time and pH. After the enzymatic treatment, the samples were extracted by hot water in a designed extraction temperature and extraction time. After the treatment, the mixture was centrifuged at 5000 rpm for 20 min, and the insoluble residue was treated in the same extraction temperature and extraction time. The supernatants were collected, concentrated to a proper volume by using a vacuum rotary evaporator, deproteinated by the method of Sevag (Sevag, Lackman, & Smolens, 1938) and mixed with three times volume of absolute ethanol. The mixture was stirred vigorously and then kept overnight at 4 °C. The precipitate was collected by centrifugation at 5000 rpm for 20 min and air-drying at 50 °C to a constant weight, affording PEGG.

2.3. Determination of polysaccharides yield

The carbohydrate content of PEGG was measured by phenol sulfuric acid method using glucose as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The percentage PEGG extraction yield (%) was calculated with the formula below:

$$Y\ (\%) = 100\% \times \frac{w_1}{w_0} \tag{1}$$

where w_1 was the polysaccharide weight of extraction (g), and w_0 represented dried sample weight (g).

2.4. Optimization procedure and experimental design

2.4.1. Fractional factorial design (FFD)

The present study was aimed at screening of the important variable factors with respect to their main effects on the yield of PEGG by FFD. Based on FFD, each variable was examined in three levels: −1 for low level; +1 for high level and 0 for zero level. Seven variable factors considered for the design were enzymatic hydrolysis temperature, enzyme concentration, liquid-to-solid ratio, enzymatic hydrolysis time, enzymatic hydrolysis pH, extraction temperature and extraction time. The variables were screened in 19 experimental designs. All experiments were carried out in triplicate and the averages of the PEGG yield were taken as response. Table 1 shows the variables and their levels used in the experimental design, and represents the design matrix and the experimental results. The FFD design was based on the first order polynomial model:

$$Y = \beta_0 + \beta_i X_i$$

where Y is the response (PEGG yield), β_0 is the model intercept, β_i is the linear regression coefficient, and X_i is the level of the independent variable. This model does not describe interaction among variables and it is used to screen and evaluate the important variables that influence the response. The statistical analysis of the data was done by using Design Expert software of version 7.0 (Stat-Ease Inc., Minneapolis, USA). Variables with very low p-values ($p < 0.05$) were considered to have greater impact on yield of PEGG.

2.4.2. Central composite design (CCD)

The levels of the significant variables and the interaction between variables, which influence the PEGG extraction yield, were further analyzed and optimized by CCD methodology. The independent variables chosen in the FFD experiment are studied at five different levels (−2, −1, 0, 1, 2). The variables and their coded levels used for the study are shown in Table 2. The CCD plan consisted of 26 trials, all the experiments were done in triplicate and the average of PEGG extraction yield obtained was taken as the dependent variable. For statistical calculation, the variables were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \tag{2}$$

where x_i is the coded value of an independent variable, X_i is the actual value of an independent variable, X_0 is the actual value of an independent variable at center point, and ΔX_i is step change value of an independent variable.

For predicting the optimal point, a second-order polynomial function was fitted to correlate the relationship between the independent variables and the response. The quadratic equation for the independent variables was expressed as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \tag{3}$$

where Y is the predicted response (yield of PEGG), β_0, β_i, β_{ii} and β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, X_i and X_j are the independent variables ($i \neq j$).

2.5. Analysis of polysaccharides characterization

2.5.1. Analysis of contents of total sugars, sulfate, protein and uronic acid

The carbohydrate content in PEGG was determined by phenol–sulfuric acid method using glucose as the standard. The content of uronic acid was determined according to a *m*-hydroxydiphenyl colorimetric method by using D-glucuronic acid as the standard (Blumenkrantz & Asboe-Hansen, 1973). The content of sulfate radical was determined according to the reported method (Doigson & Price, 1962). The protein content was determined by the method described by Bradford (Wang et al., 2004), and bovine serum albumin was used as the standard.

2.5.2. Determination of monosaccharide composition of PEGG

The monosaccharide composition of PEGG was determined using aldononitrile acetate precolumn-derivatization gas chromatography (GC) method with slight modification (Guerrant & Moss, 1984). Briefly, the polysaccharide sample (5.0 mg) was hydrolyzed with 4 ml trifluoroacetic acid (TFA, 2 M) at 120 °C in an oven for 2 h, and the excess TFA was removed by evaporation at a temperature of 40 °C. Then, the hydrolyzate was repeatedly co-concentrated with methanol to dryness and acetylated by the

Table 1
Results of two-level fractional factorial design of PEGG yield.

Run	Extraction temperature (°C)	Extraction time (min)	Liquid-to-solid ratio	Enzymatic hydrolysis temperature (°C)	Enzymatic hydrolysis pH	Enzyme concentration (%)	Enzymatic hydrolysis time (min)	Yield (%)
1	−1 (65)	−1 (120)	1 (30)	−1 (50)	1 (4.0)	1 (3)	1 (180)	3.06
2	−1 (65)	1 (170)	−1 (10)	−1 (50)	1 (4.0)	1 (3)	−1 (60)	5.07
3	1 (95)	1 (170)	1 (30)	−1 (50)	1 (4.0)	−1 (1)	−1 (60)	2.41
4	1 (95)	−1 (120)	−1 (10)	1 (60)	1 (4.0)	1 (3)	−1 (60)	2.26
5	−1 (65)	1 (170)	−1 (10)	1 (60)	1 (4.0)	−1 (1)	1 (180)	2.89
6	−1 (65)	1 (170)	1 (30)	−1 (50)	−1 (3.2)	−1 (1)	1 (180)	5.09
7	1 (95)	−1 (120)	−1 (10)	−1 (50)	1 (4.0)	−1 (1)	1 (180)	3.26
8	1 (95)	1 (170)	−1 (10)	−1 (50)	−1 (3.2)	1 (3)	1 (180)	3.44
9	0 (80)	0 (145)	0 (20)	0 (55)	0 (3.6)	0 (2)	0 (120)	3.41
10	1 (95)	−1 (120)	1 (30)	−1 (50)	−1 (3.2)	1 (3)	−1 (60)	2.88
11	−1 (65)	−1 (120)	−1 (10)	1 (60)	−1 (3.2)	1 (3)	1 (180)	4.57
12	−1 (65)	−1 (120)	−1 (10)	−1 (50)	−1 (3.2)	−1 (1)	−1 (60)	3.86
13	1 (95)	−1 (120)	1 (30)	1 (60)	−1 (3.2)	−1 (1)	1 (180)	2.76
14	−1 (65)	1 (170)	1 (30)	1 (60)	−1 (3.2)	1 (3)	−1 (60)	3.94
15	0 (80)	0 (145)	0 (20)	0 (55)	0 (3.6)	0 (2)	0 (120)	3.64
16	0 (80)	0 (145)	0 (20)	0 (55)	0 (3.6)	0 (2)	0 (120)	3.53
17	1 (95)	1 (170)	1 (30)	1 (60)	1 (4.0)	1 (3)	1 (180)	4.88
18	−1 (65)	−1 (120)	1 (30)	1 (60)	1 (4.0)	−1 (1)	−1 (60)	5.51
19	1 (95)	1 (170)	−1 (10)	1 (60)	−1 (3.2)	−1 (1)	−1 (60)	3.22

addition of a mixture of methanol, pyridine and acetic anhydride. The monosaccharide standards including rhamnose, arabinose, fucose, xylose, mannose, glucose and galactose were acetylated in the same way. Finally, the acetylated samples were analyzed by a 7890N GC (Agilent Technologies, Santa Clara, CA, USA) equipped with flame ionization detector and a HP-5 fused silica capillary column (30 m × 0.32 mm × 0.25 mm). The oven temperature was maintained at 120 °C for 3 min, and then increased gradually to 210 °C at a rate of 3 °C/min. The temperatures of detector and injector were set at 280 °C and 250 °C, respectively. The flow rates of N_2, H_2 and air were 25, 30 and 400 ml/min, respectively.

2.5.3. Determination of molecular weight

The molecular weight determination of PEGG was performed using a size-exclusion HPLC chromatography instrument (Agilent 1100, USA) equipped with a refractive index detector (RID). All samples (10.0 mg) were dissolved in distilled water (1.0 ml), passed through a 0.45 μm filter and applied to a gel-filtration chromatographic column of TSK-GEL G3000SW$_{xl}$ (7.5 mm × 300 mm, Tosoh Corp., Japan). The column was maintained at a temperature of 25 °C and eluted with 0.1 M Na_2SO_4 solution in PBS buffer (0.01 M, pH 6.8) at a flow rate of 0.8 ml/min (Xiong et al., 2013). For preparing molecular weight calibration curve, P-5, P-10, P-20, P-100, P-200, P-400 and P-800 (Showa Denko K.K., Japan) were used as the molecular weight references.

2.5.4. UV and FT-IR spectrometric analysis

UV spectra of PEGG was recorded with a UV-2450 Spectrophotometer (Shimadzu Co., Kyoto, Japan). FT-IR spectra of PEGG was recorded with a Nicolet 6700 FT-IR Spectrometer (Thermo Co., USA)

Table 2
Central composition design with four independent variables for the extraction of PEGG and the response values (yield of PEGG).

Run	Extraction temperature (°C)		Extraction time (min)		Enzymes concentration (%)		Enzymatic hydrolysis time (min)		Yield (%)
	X_1	Code x_1	X_2	Code x_2	X_6	Code x_6	X_7	Code x_7	
1	87.5	1	232.5	1	2.5	1	90.0	−1	3.96
2	72.5	−1	232.5	1	2.5	1	150.0	1	3.16
3	72.5	−1	157.5	−1	2.5	1	90.0	−1	2.75
4	87.5	1	232.5	1	1.5	−1	150.0	1	4.67
5	80.0	0	195.0	0	2.0	0	180.0	2	4.18
6	80.0	0	270.0	2	2.0	0	120.0	0	3.28
7	72.5	−1	157.5	−1	1.5	−1	90.0	−1	2.84
8	72.5	−1	232.5	1	2.5	1	180.0	2	2.58
9	80.0	0	120.0	−2	2.0	0	120.0	0	2.56
10	95.0	2	195.0	0	2.0	0	120.0	0	5.02
11	87.5	1	232.5	1	2.5	1	150.0	1	4.49
12	72.5	−1	157.5	−1	1.5	−1	150.0	1	2.87
13	87.5	1	157.5	−1	1.5	−1	150.0	1	4.85
14	87.5	1	157.5	−1	2.5	1	150.0	1	4.51
15	80.0	0	195.0	0	1.0	−2	120.0	0	3.35
16	80.0	0	195.0	0	2.0	0	60.0	−2	2.09
17	80.0	0	195.0	0	2.0	0	120.0	0	4.89
18	80.0	0	195.0	0	2.0	0	120.0	0	4.56
19	72.5	−1	157.5	−1	2.5	1	150.0	1	3.29
20	65.0	−2	195.0	0	2.0	0	120.0	0	3.42
21	87.5	1	157.5	−1	1.5	−1	90.0	−1	3.16
22	87.5	1	157.5	−1	2.5	1	90.0	-1	2.34
23	87.5	1	232.5	1	1.5	−1	90.0	−1	3.19
24	72.5	−1	232.5	1	1.5	−1	150.0	1	2.44
25	72.5	−1	232.5	1	1.5	−1	90.0	−1	3.05
26	80.0	0	195.0	0	3.0	2	120.0	0	4.31

using KBr disks method. Briefly, samples were dried at 35–44 °C in vacuum over P_2O_5 for 48 h, ground with potassium bromide (KBr) powder and then pressed into pellet for FT-IR spectral measurement in the frequency range of 4000–400 cm^{-1}.

2.6. Antioxidant activities in vitro of PEGG

2.6.1. Assay of hydroxyl radical scavenging activity

Briefly, 1 ml sample solution was added to 2 ml of sodium phosphate buffer (150 mM, pH 7.4) containing 10 mM $FeSO_4$, 2 mM sodium salicylate and 6 mM H_2O_2. The mixture was incubated at 37 °C for 30 min and the absorbance of the solution was detected at 510 nm (Chun et al., 2007). The hydroxyl radical scavenging activity was calculated according to the formula below:

$$\text{Hydroxyl radical scavenging activity}\,(\%) = 100 \times \frac{A_0 - A_1 + A_2}{A_0} \quad (4)$$

where A_0 is the absorbance of control sample, A_1 is the absorbance in the presence of tested samples, and A_2 is the absorbance of tested sample without sodium salicylate solution. The ascorbic acid was used as positive control.

2.6.2. Assay of Fe^{2+} chelating activity

The Fe^{2+} chelating activity of PEGG was determined according to the method reported by (Liu et al., 2010). 1 ml polysaccharide sample was added to 0.05 ml of ferrous chloride ($FeCl_2$) solution (2 mM), 0.2 ml of ferrozine solution (5 mM), and 2.75 ml of water. The mixture was shaken well and incubated for 10 min at room temperature, and then the absorbance of the mixture was determined at 562 nm. The Fe^{2+} chelating activity was calculated by the following formula:

$$\text{Fe}^{2+}\text{ chelating activity}\,(\%) = 100 \times \frac{A_0 - A_1 + A_2}{A_0} \quad (5)$$

where A_0 is the absorbance of control sample (water instead of sample), A_1 is the absorbance in the presence of tested samples, and A_2 is the absorbance of the sample only (water instead of $FeCl_2$ solution). Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was used as positive control.

2.6.3. Assay of lipid peroxidation inhibitory activity

The lipid peroxidation inhibitory activity of PEGG was determined by thiobarbituric acid-reactive-substances (TBARS) assay using mouse liver homogenate as the lipid rich media with some modification (Yen & Hsieh, 1998). Briefly, 1.0 ml of 1% (w/v) mouse liver homogenate was mixed with 1.0 ml sample solution, then 0.05 ml of $FeCl_2$ (0.5 mM) and H_2O_2 (0.5 mM) were added to initiate lipid peroxidation, which was carried out in a 37 °C water bath for 30 min. Reaction was terminated by adding 1.5 ml of trichloroacetic acid (TCA) (20%, w/v) and 1.5 ml of thiobarbituric acid (TBA) solution (0.8%, w/v). The resulting mixture was vortexed and heated in a boiling water bath for 10 min. After centrifugation at 4000 rpm for 10 min, the TCA–TBA phase was removed and the absorbance of the upper layer was recorded at 532 nm. The inhibition effect on lipid peroxidation was calculated according to the formula below:

$$\text{Lipid peroxidation inhibitory activity}\,(\%) = 100 \times \frac{A_0 - A_1 + A_2}{A_0} \quad (6)$$

where A_0 is the absorbance of control sample (water instead of sample), A_1 is the absorbance in the presence of tested samples, and A_2 is the absorbance of the sample only (water instead of liver homogenate). The ascorbic acid was used as positive control.

2.7. Statistical analysis

Analysis of the experimental design and data was carried out using Design Expert software of version 7.0 (Stat-Ease Inc., Minneapolis, USA). Analysis of variance (ANOVA) was carried out and the fitness of the polynomial model equation was expressed as the coefficient of determination R^2. The significances of the regression coefficients were tested by F-test. One-way ANOVA was performed using the SPSS 13.0 for windows (SPSS, Chicago, IL, USA). p-values of less than 0.05 were regarded as significant.

3. Results and discussion

3.1. Screening of parameters using FFD

By employing multiple regression analysis on the experimental data, the predicted response Y for PEGG yield can be obtained by the following second-order polynomial equation:

$$\begin{aligned} Y = {} & 3.69 - 0.56x_1 + 0.17x_2 + 0.12x_3 + 0.06x_4 - 0.03x_5 + 0.07x_6 \\ & + 0.05x_7 + 0.18x_1x_2 - 0.03x_1x_3 + 0.08x_1x_4 + 0.09x_1x_5 \\ & + 0.16x_1x_6 + 0.40x_1x_7 - 0.19x_2x_4 + 0.62x_1x_2x_4 \end{aligned} \quad (7)$$

where Y represents the yield of PEGG, x_1, x_2, x_3, x_4, x_5, x_6, x_7 are the variables of extraction temperature (°C), extraction time (min), liquid-to-solid ratio, enzymatic hydrolysis temperature (°C), enzymatic hydrolysis pH, enzyme concentration and enzymatic hydrolysis time (min), respectively.

Statistical analysis of the responses and the effects of the selected variables were done and the results are shown in Table 3. The F- and p-value for the model were found to be 81.45 and 0.012, respectively, which implied that the model was significant. The determination coefficient R^2 of the model was 0.9706, indicating that 97.06% of the variability in the response could be explained by the model. The curvature F-value and p-value (5.13 and 0.1473, respectively) implied that the curvature in the design space was not significant relative to the noise. Among the variables screened, extraction temperature and extraction time were determined as the most significant variables influencing PEGG yield. Enzyme concentration, enzymatic hydrolysis time, liquid-to-solid, enzymatic hydrolysis temperature and enzymatic hydrolysis pH in PEGG yield did not result in significant variation at 5% level. From the p-values of each model term, it could be concluded that three interactive terms (X_1X_2, X_1X_5, X_1X_6 and $X_1X_2X_4$) were also significant at 5% level. Thus, the four variables, extraction temperature, extraction time, enzyme concentration and enzymatic hydrolysis time were selected and used for further optimization by RSM. Liquid-to-solid ratio, enzymatic hydrolysis temperature and enzymatic hydrolysis pH in the following CCD experiments were set at their middle levels of 20, 55 °C and 3.6, respectively.

3.2. Central composite design and response surface analysis

RSM is a useful mathematical and statistical technique for the modeling analysis of problems in which a response of interest is influenced by several variables. The central composite design is the standard RSM and allows estimating the second order polynomial of the relationships between the independent variables and the dependent variable and gives information about the interaction between independent variables in relation to the dependent variable (Tan, Ahmad, & Hameed, 2008). In this study, a five-level, four-variable central composite design with 24 runs and two replications of the center points was selected to determine the extraction temperature, extraction time, enzyme concentration and enzymatic hydrolysis time for the maximum production of

Table 3
Statistical analysis of fractional factorial design for screening the significant variables.

Source	Sum of squares	DF	Mean square	*F*-value	*p*-Value
Model	16.168	15	1.078	81.451	0.0122[a]
Curvature	0.071	1	0.071	5.329	0.1473[b]
Pure error	0.026	2	0.013		
Corrected total	16.265	18			
X_1	4.928	1	4.928	372.423	0.0027[a]
X_2	0.483	1	0.483	36.501	0.0263[a]
X_3	0.240	1	0.240	18.144	0.0509[b]
X_4	0.058	1	0.058	4.353	0.1722[b]
X_5	0.011	1	0.011	0.833	0.4577[b]
X_6	0.076	1	0.076	5.715	0.1393[b]
X_7	0.040	1	0.040	3.023	0.2242[b]
X_1X_2	0.490	1	0.490	37.028	0.0260[a]
X_1X_3	0.013	1	0.013	0.999	0.4228[b]
X_1X_4	0.106	1	0.106	7.982	0.1058[b]
X_1X_5	0.130	1	0.130	9.793	0.0887[b]
X_1X_6	0.397	1	0.397	29.992	0.0318[a]
X_1X_7	2.512	1	2.512	189.841	0.0052[a]
X_2X_4	0.608	1	0.608	45.975	0.0211[a]
$X_1X_2X_4$	6.076	1	6.076	459.161	0.0022[a]

$R^2 = 0.9706$, adjusted $R^2 = 0.9331$.
[a] 5% significance level.
[b] Not significant relative to the pure error.

PEGG. By applying multiple regression analysis on the experimental data, the second-order polynomial equation was established and all terms regardless of their significance were included in the following equation:

$$Y = 4.63 + 0.41x_1 + 0.16x_2 + 0.15x_6 + 0.41x_7 + 0.02x_1x_2 - 0.17x_1x_6 + 0.39x_1x_7 + 0.20x_2x_6 - 0.21x_2x_7 + 0.02x_6x_7 - 0.01x_1^2 - 0.44x_2^2 - 0.21x_6^2 - 0.36x_7^2 \quad (8)$$

where Y represents the yield of PEGG, x_1, x_2, x_6, x_7 represent extraction temperature, extraction time, enzyme concentration and enzymatic hydrolysis time, respectively.

A summary of the analysis of variance (ANOVA) for the fitted quadratic polynomial model of extraction yields of polysaccharides are listed in Table 4. The Fisher *F*-test [$F(14,15) = 15.43$] with a very low probability value ($p < 0.0001$) demonstrate a high significance for the regression model. The value of the determination coefficient ($R^2 = 0.9515$) showed a good fit to the model and indicated that only 5% of the total variations was not explained by the model. The value of 1.689 for lack-of-fit implied that the lack-of-fit was insignificant relative to the pure error due to noise, indicating the model equation was adequate to predict the extraction yield of PEGG within the range of experimental variables. The coefficient of the variation (C.V.%) and value of adjusted determination coefficient was 8.43 and 0.8899, respectively, which indicated a high degree of precision of reliability of the experimental values and a high degree of correlation between the observed and predicted values.

The coefficient estimates of model equation, along with the corresponding *p*-values, were presented in Table 4. The *p*-values are used as a tool to check the significance of each coefficient. When the *p*-value is less than 0.05, the corresponding coefficient is significant. This implied that the first order main effects of extraction temperature, extraction time, enzyme concentration and enzymatic hydrolysis time on the extraction yield of PEGG were highly significant as was evident from their respective *p*-values ($px_1 < 0.0001$, $px_2 < 0.0001$, $px_6 = 0.0247$, $px_7 = 0.0413$) and with their second order main effects ($px_2{}^2 < 0.0001$, $px_6{}^2 = 0.012$, $px_7{}^2 = 0.0001$). The interaction effects of extraction temperature

Table 4
Variance analysis of response surface quadratic model for the extraction of PEGG.

Source	Sum of squares	DF	Mean square	*F*-value	*p*-Value
Model	19.122	14	1.366	15.427	0.0001[a]
Lack of fit	0.919	10	0.092	1.689	0.5406[b]
Pure error	0.054	1	0.054		
Corrected total	20.096	25			
X_1	3.674	1	3.674	41.503	0.0001[a]
X_2	0.598	1	0.598	6.754	0.0247[a]
X_6	0.472	1	0.472	5.335	0.0413[a]
X_7	3.825	1	3.825	43.205	0.0001[a]
$X_1{}^2$	0.239	1	0.239	2.696	0.1288[b]
$X_2{}^2$	3.517	1	3.517	39.724	0.0001[a]
$X_6{}^2$	0.818	1	0.818	9.243	0.0112[a]
$X_7{}^2$	2.856	1	2.856	32.254	0.0001[a]
X_1X_2	0.008	1	0.008	0.092	0.7673[b]
X_1X_6	0.412	1	0.412	4.653	0.0540[b]
X_1X_7	2.288	1	2.288	25.847	0.0004[a]
X_2X_6	0.583	1	0.583	6.590	0.0262[a]
X_2X_7	0.654	1	0.654	7.392	0.0200[a]
X_6X_7	0.008	1	0.008	0.092	0.7672[b]

$R^2 = 0.9515$, adjusted $R^2 = 0.8899$, C.V.% = 0.843.
[a] 5% significance level.
[b] Not significant relative to the pure error.

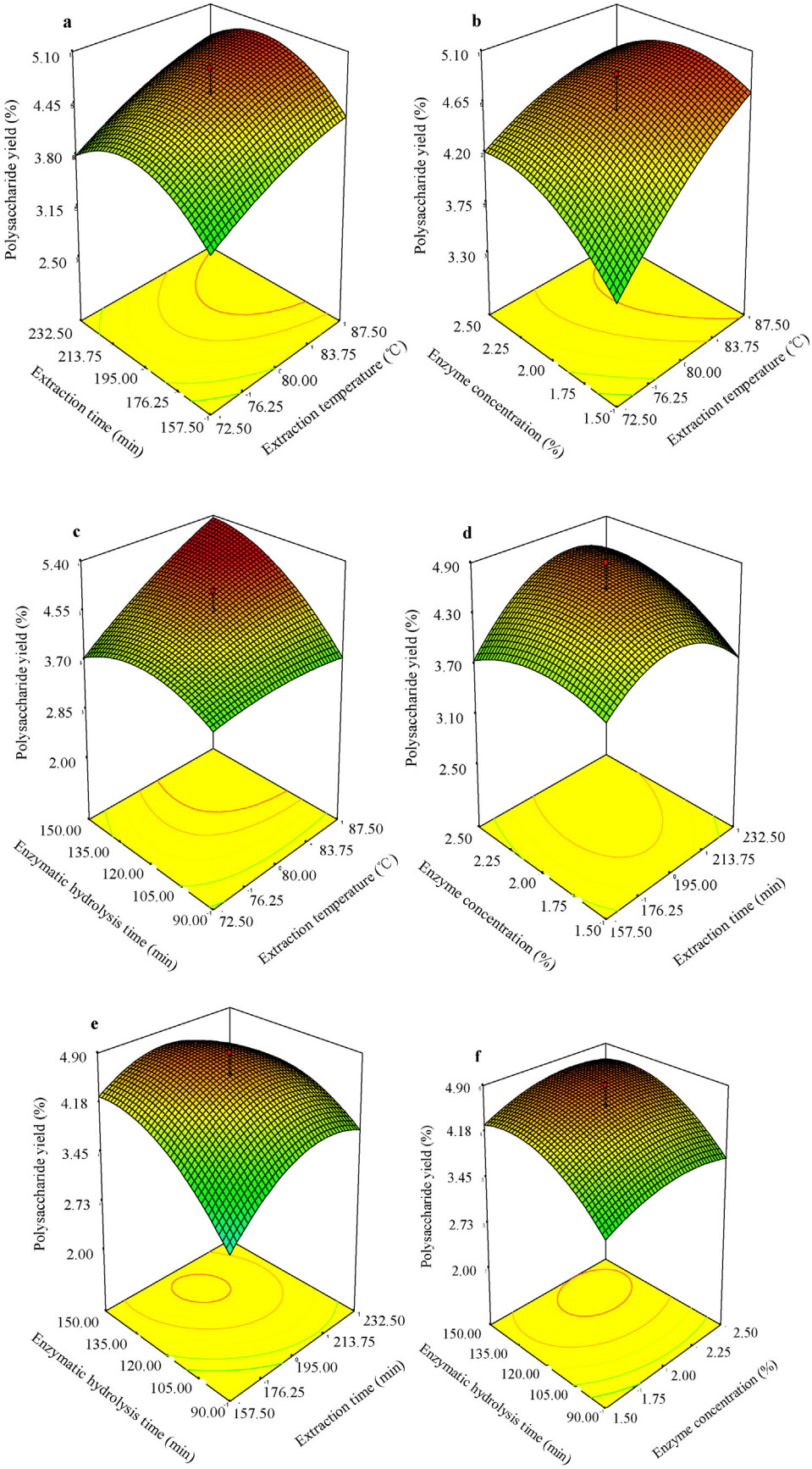

Fig. 1. Response surface plots (a, b, c, d, e and f) showing the effects of extraction temperature, extraction time, enzyme concentration, enzymatic hydrolysis time and their mutual effects on extraction yield of PEGG.

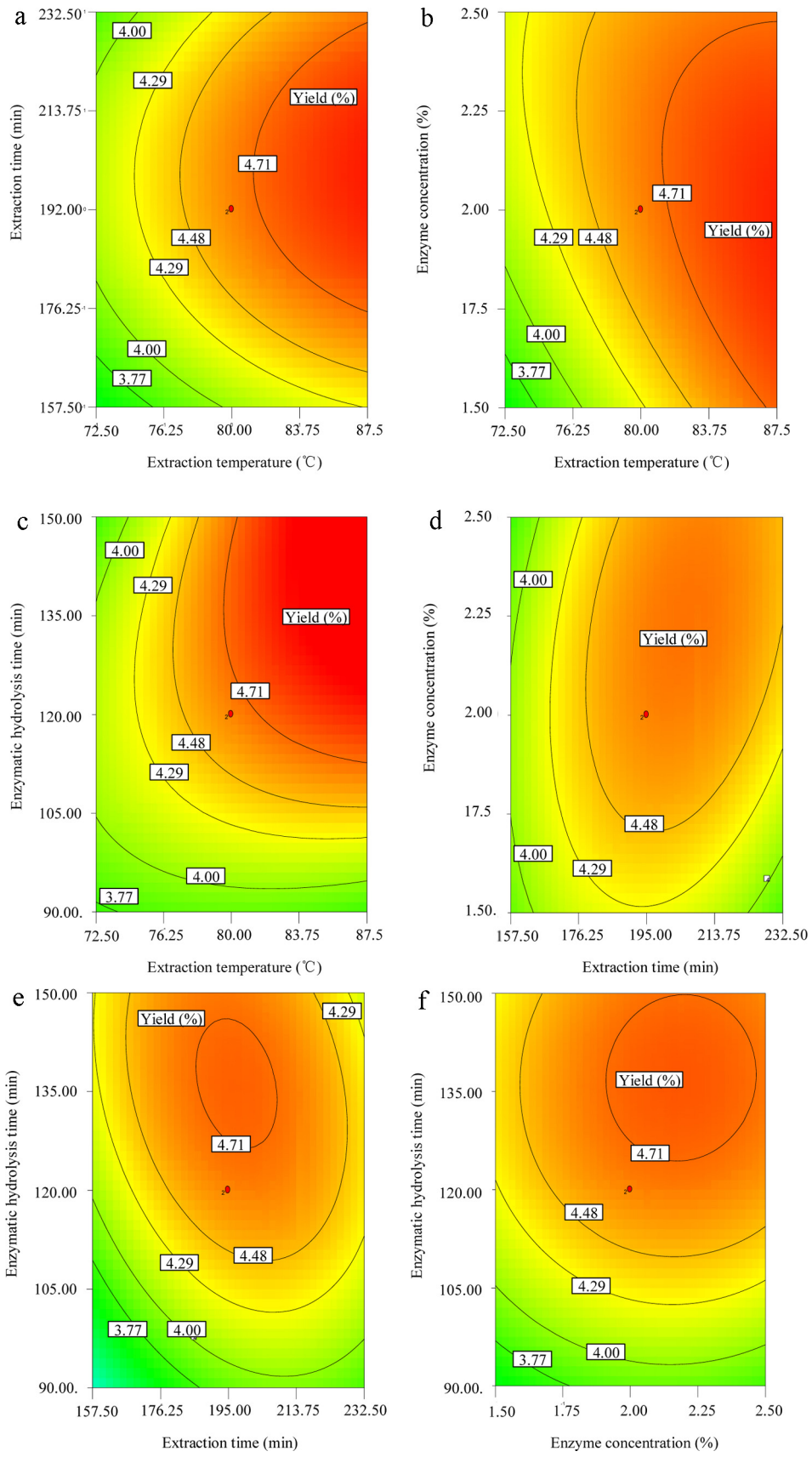

Fig. 2. Contour plots (a, b, c, d, e and f) showing the effects of extraction temperature, extraction time, enzyme concentration, enzymatic hydrolysis time and their mutual effects on extraction yield of PEGG.

and enzymatic hydrolysis time, extraction time and enzyme concentration, extraction time and enzymatic hydrolysis time were also highly significant with very low probability values ($p = 0.0004$, 0.0260, 0.0200, respectively). On the other hand, smaller the p-value is, more significant the corresponding coefficient is (Muralidhar, Chirumamila, Marchant, & Nigam, 2001). Data in Table 4 implied that extraction temperature and extraction time were the most significant parameters influencing the extraction yield of PEGG followed by enzyme concentration and enzymatic hydrolysis time.

The fitted response surface plots and contour plots for the model were generated by the Stat-Ease Design-Expert software in order to better understand the interactions of the variables. The shape of the contour plots indicates whether the mutual interactions between variables are significant or not. A circular contour plot indicates that the interaction between related variables is negligible, while an elliptical contour plot indicates that the interaction between related variables is significant. The response surface plots and contour plots as shown in Figs. 1 and 2 were generated using Design-Expert, which depicted the interactions between two variables by keeping the other variables at their zero levels for PEGG production. It is evident that these three-dimensional plots and their corresponding contour plots provided a visual interpretation of the interaction between two variables and facilitated the location of optimum experimental conditions.

By employing the software Design-Expert, the solved optimum values of the tested variables were extraction temperature 87.3 °C, extraction time 177.28 min, liquid-to-solid ratio 20, enzymatic hydrolysis temperature 55 °C, enzymatic hydrolysis pH 3.6, enzyme concentration 1.65%, enzymatic hydrolysis time 141.01 min. Under the optimal conditions, the maximum predicted yield of PEGG was 5.14%. Taking account of the operating convenience, the optimal parameters were determined as following: extraction temperature 87.0 °C, extraction time 177.0 min, liquid-to-solid ratio 20, enzymatic hydrolysis temperature 55 °C, enzymatic hydrolysis pH 3.6, enzyme concentration 1.65%, enzymatic hydrolysis time 141.0 min. To ensure the predicted result was not biased toward the practical value, experimental rechecking was performed using the optimal conditions. A mean value of 5.08 ± 0.38 ($n = 4$), obtained from real experiments, demonstrated the validation of the RSM model. The good correlation between experimental and predicted values confirmed that the response model was accurate and adequate for the production of PEGG.

3.3. Characterization of PEGG

In the present study, PEGG was prepared through a series procedure of hot water extraction based on the optimal extraction conditions, centrifugation, ethanol precipitation and drying. Then, PEGG was preliminary characterized by chemical analysis, UV, HPLC, GC and FT-IR. As results, the contents of carbohydrate, protein and uronic acid in PEGG were determined to be 72.6, 1.31 and 1.64%, respectively. Notably, PEGG had relatively high content of sulfate radical (3.47%).

The UV spectra of PEGG was shown in Fig. 3a. Significant absorption at 260–280 nm was found in the UV spectrum, indicating the possible presence of protein. The results are in accordance with the analytic result of protein content.

For determination of homogeneity and molecular weight of PEGG, an Agilent 1100 HPLC system was used. As shown in Fig. 3b, the HPLC profile of each purified fraction had a single and symmetrical narrow peak, which indicated that PEGG was homogeneous polysaccharides. In addition, the average molecular weights of PEGG was estimated to be 83 kDa, respectively.

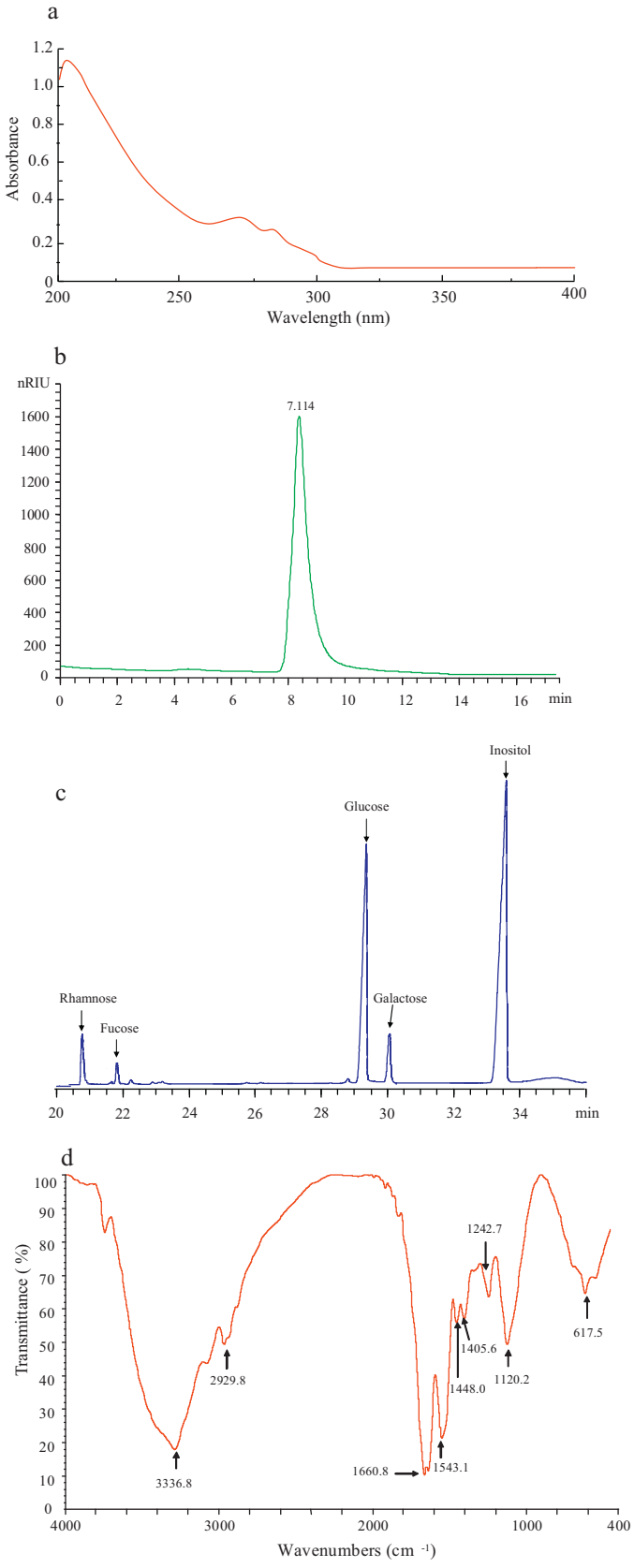

Fig. 3. UV spectra (a), HPLC profiles (b), FT-IR spectra (c) and GC chromatograms of monosaccharid (d) of PEGG.

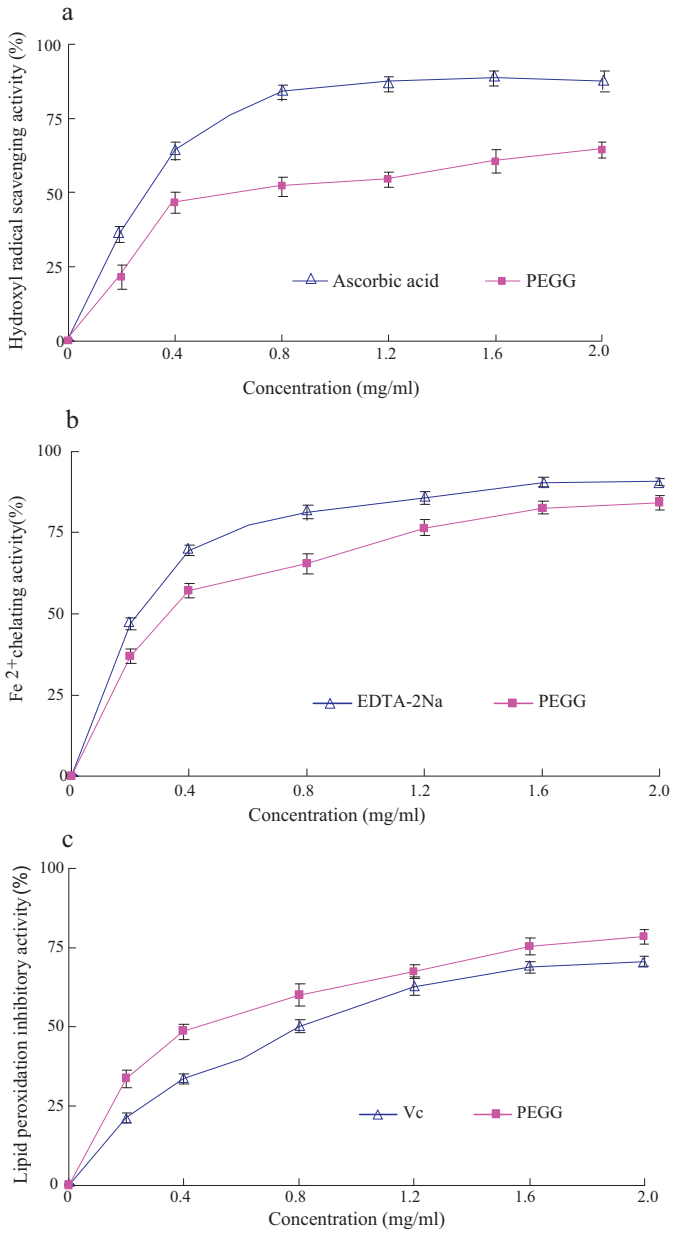

Fig. 4. Hydroxyl radical scavenging activity (a), Fe^{2+} chelating activity (b) and lipid peroxidation inhibitory activity (c) of PEGG.

As shown in Fig. 3c, PEGG was composed of rhamnose, fucose, glucose and galactose in a molar percent of 13.1, 4.5, 72.8 and 9.6%, respectively. Other sugars, such as mannose, arabinose and xylose were not found in PEGG.

FT-IR spectroscopy of PEGG is shown in Fig. 3d. A strong and broad absorption peak at 3336.8 cm^{-1} for O—H stretching vibrations, a peak at 2929.8 cm^{-1} for C—H stretching vibrations, and a strong extensive absorption in the region of 900–1200 cm^{-1} for coupled C—O and C—C stretching and C—OH bending vibrations were observed in PEGG, indicating the characteristic absorptions of polysaccharides (Liu et al., 2008). Furthermore, an asymmetrical stretching peak at 1660.8 cm^{-1} and a weak symmetrical stretching peak near 1405.6 cm^{-1} indicated that there were carboxyl groups in PEGG (Mao, Li, Gu, Fang, & Xing, 2004). In addition, the absorption at 1242.7 cm^{-1} was related to S=O stretching vibration of the sulfate group.

3.4. Antioxidant activities in vitro of PEGG

3.4.1. Scavenging activity on hydroxyl radical of PEGG

Hydroxyl radical is one of the most reactive free radicals and can react with all bio-macromolecules in living cells (Huang, Ou, & Ronald, 2005; Sun et al., 2009). The results of hydroxyl radical scavenging activity of PEGG and ascorbic acid are shown in Fig. 4a. The hydroxyl radical scavenging activity of PEGG and ascorbic acid increased with increasing concentrations. And the hydroxyl radical scavenging rate of PEGG and ascorbic acid at 2.0 mg/ml was 64.5% and 86.8%, respectively. The IC_{50} value of PEGG and ascorbic acid for eliminating hydroxyl radical was about 0.80 and 0.35 mg/ml, respectively, which indicated that both the PEGG and ascorbic acid had significant hydroxyl radical scavenging activity.

3.4.2. Fe^{2+} chelating activity of PEGG

Some transition metals, including Fe^{2+}, Cu^{+}, Pb^{2+}, and Co^{2+}, can trigger process of free radical reaction to magnify the cellular damage. Among these transition metals, Fe^{2+} is known as the most powerful prooxidant due to its high reactivity. And it can stimulate lipid peroxidation by generating hydroxyl radicals through Fenton reaction (Sun & Kennedy, 2010). Therefore, Fe^{2+} chelating is extremely important to antioxidant work. As shown in Fig. 4b, the Fe^{2+} chelating activity increased from 36.7% to 84.1%, when the concentration of PEGG increased from 0.2 to 2.0 mg/ml. The IC_{50} of PEGG and EDTA-2Na were 0.35 and 0.20 mg/ml, respectively. The results indicated that PEGG had significant Fe^{2+} chelating activity.

3.4.3. Lipid peroxidation inhibitory activity of PEGG

Lipid peroxidation is a common consequence of free radical-mediated chain reactions. Its end-products can damage DNA directly or indirectly (Zhu et al., 2004). In the present study, $FeCl_2$–H_2O_2 system was used to induce lipid peroxidation in mouse liver homogenate (Liu et al., 2009). As shown in Fig. 4c, PEGG and ascorbic acid exerted concentration-dependent lipid peroxidation inhibitory activity. The lipid peroxidation inhibitory activity of PEGG and ascorbic acid at 2.0 mg/ml was 70.8% and 78.5%, respectively. The IC_{50} value of PEGG and ascorbic acid for lipid peroxidation inhibitory activity was about 0.4 and 0.8 mg/ml, respectively. The results indicated that PEGG had significant lipid peroxidation inhibitory activity.

4. Conclusions

In this study, optimization of enzyme-assisted extraction, preliminary characterization and antioxidant activities *in vitro* of polysaccharides from *E. corneum gigeriae galli* were investigated. Firstly, the enzyme-assisted extraction of polysaccharides of technology was performed for the polysaccharides extraction from PEGG. And FFD and CCD were employed to optimize the parameters for the extraction of PEGG. *As a result*, the maximum yield of PEGG (5.14%) was obtained under the following conditions: extraction temperature 87.3 °C, extraction time 177.28 min, enzyme concentration 1.65%, enzymatic hydrolysis time 141.01 min. Under the optimal conditions of extraction temperature 87.0 °C, extraction time 177.0 min, enzyme concentration 1.65%, enzymatic hydrolysis time 141.0 min, liquid-to-solid ratio 20, enzymatic hydrolysis temperature 55 °C and enzymatic hydrolysis pH 3.6, an actual experimental yield of 5.08 ± 0.38% was obtained. There was no difference at significant level of 0.05 between the experimental and predicted values. The results suggested that the regression model was accurate and adequate for the extraction of PEGG. Then, PEGG was characterized by chemical analysis, UV, HPLC, GC and FT-IR. The results demonstrated that PEGG contained protein and uronic acid

and had relatively high content of sulfate radical. PEGG was composed of rhamnose, fucose, mannose, glucose and galactose with a molar percent of 13.1, 4.5, 72.8 and 9.6%, respectively. The average molecular weight was 83 kDa. And there was infrared characteristic absorption peak of polysaccharides in the FT-IR spectroscopy of PEGG. Finally, the antioxidant activities *in vitro* of PEGG were evaluated. The results demonstrated that PEGG possessed strong hydroxyl radical scavenging activity, Fe^{2+} chelating activity and lipid peroxidation inhibitory activity. Further work on the isolation, purification, and antioxidant activities *in vivo* of PEGG is in progress.

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