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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