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Extraction optimization and biological properties of a polysaccharide isolated from *Gleoestereum incarnatum*

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ABSTRACT

Extraction was optimized of polysaccharides from *Gleoestereum incarnatum* (GIP). The three parameters, extraction temperature, extraction time and the ratio of water to raw material, were optimized using the Box–Behnken design. As a result, the optimal extraction conditions were: extraction temperature 87.5 °C, extraction time 1 h and the ratio of water to raw material of 39.7 mL/g, where the highest yield of polysaccharide (13.18%) was obtained. GIP-II was the main fraction purified form GIP. GIP-II was composed of galactose, glucose, xylose, and mannose, with glucose was the predominant monosaccharide. GIP-II exhibited strong scavenging activities against DPPH and hydroxyl radials *in vitro*, as well as a strong inhibitory effect on the growth of HepG2 cells. The overall findings indicated that GIP-II is worthy of further exploration for its potential applications in antitumor drugs or health foods.

1. Introduction

Mushrooms have been known to be an important source of nutritional diet and medicine for thousands of years. Extensive studies have revealed that many species of mushrooms are good for improving human health and preventing diseases (Yang et al., 2009). Polysaccharides isolated from mushrooms have been correlated with multiple pharmacological properties, such as antioxidant (Wang, Zhang, Wang, & Wang, 2013), immunomodulatory (Zhao, Kan, Li, & Chen, 2005), reducing blood lipid (Yu et al., 2013), antidiabetic (Yang, Hsu, Lin, Hsu, & Chen, 2012) and antitumor activities (Sun, Wang, & Zhou, 2012).

Gloeostereum incarnatum is a species of fungi in the family Cyphellaceae. This is a monotypic genus containing the single species *G. incarnatum*, an edible mushroom native to China. Polysaccharides from the mycelium of *G. incarnatum* possess a wide range of biological properties, such as antioxidant (Wang, Zhang, Liu, Zhang, & Hou, 2012b), immunomodulation (Weng, Weng, & Qiu, 2009), anti-inflammatory (Zhang & Li, 1999) and antibacterial activities (Zhang, Zhu, Mu, Liu, & Hou, 2012a).

To the best of our knowledge, there are no reports available in the literature regarding the optimization of extraction of polysaccharides from the fruiting bodies of *G. incarnatum* (GIP) using response surface methodology (RSM). It is therefore interesting and attractive to extract them and investigate their biological activities. In this study, the extraction parameters of GIP were optimized using a three-level, three variable Box–Behnken design (BBD). GIP was then purified and the antioxidant and antitumor activities of purified polysaccharide from *G. incarnatum* were evaluated *in vitro*.

2. Materials and methods

2.1. Materials

The fruiting body of *G. incarnatum* was obtained from the Institute of mushroom of Yutai, Shandong, China. Standard monosaccharides (L-fucose, L-rhamnose, D-mannose, D-xylose, D-glucose, D-galactose, Fructose and D-arabinose), 1,1-diphenyl-2-picrylhydrazyl (DPPH), trifluoroacetic acids (TFA), DEAE cellulose-52, Sephadex G-100 and dimethyl sulfoxide (DMSO) were all purchased from Sigma–Aldrich. Cancer cell line HepG2 (human hepatoma) was obtained from the Cell Bank, Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). DMEM medium and fetal bovine serum (FBS) were obtained from Thermo scientific (Hyclone). 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and 5-fluorouracil (5-Fu) were obtained from Sangon Biotech. All other chemicals and solvents were obtained from Huadong Chemical Reagent Co. Ltd. (Hangzhou, China) at the highest commercial grade.

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2.2. Preparation of crude GIP

The fruiting body of *G. incarnatum* was dried at 60 °C in an oven for 24 h and then powdered for this study. Then, the powder was pretreated twice with 90% ethanol to remove oligosaccharides, some colored materials and small molecules. Finally, the organic solvent was volatilized to obtain a pretreated dry powder. The dried powder (5.0 g) was extracted in a HH-6 water bath (Guohua Wiring Company, Shanghai, China) with distilled water using designed parameters. The suspension was centrifuged (5000 × *g*, 10 min) and the insoluble residue was treated again twice as described above. The supernatants were collected and concentrated to a proper volume using a vacuum rotary evaporator. The supernatant was precipitated by the addition of anhydrous ethanol to a final concentration of 75% (v/v). The precipitate was collected by centrifugation (5000 × *g*, 10 min) and air-dried at 50 °C to a constant weight, yielding crude GIP. The GIP was then weighted with a balance and the percentage GIP yield (%) is calculated as follows:

$$\text{GIP yield } (\%) = \frac{m_0}{m} \times 100, \quad (1)$$

where m_0 (g) is the dried GIP weight and m (g) is the dried raw material weight.

2.3. Purification of GIP

The crude polysaccharide was purified by chromatography on a DEAE cellulose-52 column. After the GIP solution was applied to the column of DEAE-52, the column was eluted by the stepwise addition of NaCl solutions of different concentrations (0, 0.1 M, 0.2 M, 0.4 M NaCl). Eluate was collected automatically and the carbohydrate content was determined by the phenol–sulfuric acid method (Dubosi, Gilles, Hamilton, Rebers, & Smith, 1956). As a result, three fractions were obtained and the main fraction (the second fraction) was dialyzed and further purified through a column of Sephadex G-100. The purified fraction was concentrated, dialyzed and lyophilized for further study.

2.4. Optimization experimental design

Design Expert software (Version 8.0.6) was used to analyze the experimental data. A three-level, three-factor BBD was employed to evaluate the combined effect of three independent variables: extraction temperature, ratio of water to raw material and extraction time coded as *A*, *B* and *C*, respectively. The complete design consisted of 17 combinations including five replicates of the center point. The ranges and levels of variables investigated in the current study are given in Table 1a. All trials were performed in triplicate and the averages of polysaccharide yields were taken as response of this system.

Analysis of variance (ANOVA) tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The statistical significance for each term in the polynomial was determined by computing the *F*-value at a probability *P* of 0.05. The regression coefficients were then used to make statistical calculations and generate contour maps from the regression models.

2.5. Preliminary characterization of GIP-II

Monosaccharide composition analysis was conducted according to Yang, Zhang, Tang, and Pan (2005). The average molecular weight (Mw) of GIP-II was determined using a gel permeation chromatography (GPC) method according to the reported method (Fu, Tian, Cai, Liu, & Li, 2007).

2.6. Assay for antioxidant activity

2.6.1. DPPH radical scavenging activity

The method used to measure free radical scavenging capability was adapted from Shimada, Fujikawa, Yahara, and Nakamura (1992). DPPH (1 mL of 0.325 mM in methanolic dimethyl sulfoxide) was added to 1 mL of various concentrations of the polysaccharides (0.05–1.60 mg/mL) in 5 mL test tubes. The mixture was shaken vigorously and kept at room temperature for 30 min. The absorbance of the resulting solution was recorded at 517 nm. BHA was used as a reference material. Scavenging activity was calculated as follows:

$$\text{Scavenging activity } (\%) = \left(\frac{A_0 - A_1}{A_0}\right) \times 100, \quad (2)$$

where A_0 is the absorbance of the control (DPPH solution with no sample) and A_1 is the absorbance of the tested sample (DPPH solution with either sample or positive control).

2.6.2. Hydroxyl radical scavenging assay

Hydroxyl radical-scavenging activity was measured according to Smirnoff and Cumbes's work (1989). For this, $FeSO_4$ (0.5 mL of 1.5 mM) was mixed with 0.35 mL H_2O_2 (6 mM), 0.15 mL sodium salicylate (20 mM) and 1.0 mL polysaccharide (0.05–1.60 mg/mL), and then incubated at 37 °C for 1 h. The absorbance of the hydroxylated salicylate complex was measured at 562 nm. BHA was used as the positive control. The antioxidant activity was calculated with the following equation:

$$\text{Scavenging effect } (\%) = \left(\frac{A_0 - A_1}{A_0}\right) \times 100, \quad (3)$$

where A_0 is the absorbance of the solvent control, and A_1 is the absorbance of the test (sample or BHA).

2.7. Assay for antitumor activity

The cancer cell line HepG2 was maintained in DMEM medium supplemented with 10% (v/v) FBS with 5% CO_2 at 37 °C. Culture broths were substituted with fresh medium every 2 or 3 days. Effect of GIP-II on HepG2 cell proliferation was evaluated as described by MTT assay (Gan, Ma, Jiang, Xu, & Zeng, 2011). Briefly, cells were seeded in 96-well plates at 1×10^5 cells/well containing 100 μL culture medium per well and permitted to adhere for 24 h. GIP-II solutions (100 μL) with different concentrations (0.05–3.2 mg/mL in DMEM medium) and 5-Fu (25 μg/mL) were added to each well. After incubation for 72 h, the medium was removed and 100 μL MTT (0.5 mg/mL in DMEM without FBS) was added. After further incubation at 37 °C for 4 h, the supernatant was aspirated and 150 μL DMSO was added to each well. The absorbance was measured spectrophotometrically at 570 nm with a microtiter plate reader. 5-Fu (25 μM/mL) was used as the positive control. Absorbance was measured at 570 nm. Growth inhibition rate was calculated as follows:

$$\text{Growth inhibition rate } (\%) = \left(\frac{A_{\text{control}} - A_{\text{experiment}}}{A_{\text{control}}}\right) \times 100 \quad (4)$$

2.8. Statistical analysis

All the experiments were carried out in triplicate and data were expressed as mean ± standard deviation. Statistical analysis was performed with ANOVA followed by Student's *t*-test. A level of $P < 0.05$ was taken as statistically significant.

Table 1a
The Box–Behnken design matrix and the results for extraction yield of crude polysaccharides from *Gleoestereum incarnatum*.

Run numbers	*A*: Extraction temperature (°C)	*B*: Ratio of water to material(mL/g)	*C*: extraction time (h)	Response (%)	Predict response (%)
1	0(80)	−1(20)	−1(1)	8.98	8.69
2	−1(70)	0(30)	1(2)	12.24	9.96
3	−1(70)	1(40)	0(1.5)	10.53	10.63
4	1(90)	0(30)	1(2)	10.82	11.04
5	0(80)	0(30)	0(1.5)	12.14	12.1
6	0(80)	0(30)	0(1.5)	12.01	12.09
7	−1(70)	−1(20)	0(1.5)	8.18	8.7
8	−1(70)	0(30)	−1(1)	9.62	9.4
9	0(80)	1(40)	1(2)	10.72	11.01
10	0(80)	1(40)	1(2)	12.64	12.76
11	1(90)	−1(20)	0(1.5)	10.06	9.96
12	0(80)	0(30)	0(1.5)	12.08	12.09
13	0(80)	−1(20)	1(2)	11.76	11.64
14	0(80)	0(30)	0(1.5)	12.16	12.19
15	0(80)	0(30)	0(1.5)	12.09	12.1
16	1(90)	1(40)	0(1.5)	11.98	11.46
17	1(90)	0(30)	−1(1)	11.89	12.29

Table 1b
ANOVA for response surface quadratic model.

Variables	Sum of squares	DF	Mean square	*F* value	*P*-value
Model	26.05	9	2.89	17.14	0.0006
A	2.18	1	2.18	12.93	0.0088
B	5.93	1	5.93	35.13	0.0006
C	0.726	1	0.73	4.298	0.0768
AB	0.046	1	0.046	0.274	0.617
AC	3.4	1	3.404	20.153	0.0028
BC	5.525	1	5.522	32.696	0.0007
A^2	3.377	1	3.377	19.99	0.0029
B^2	4.321	1	4.32	25.581	0.0015
C^2	0.0142	1	0.014	0.0839	0.7805
Residual	1.1823	7	0.1689		
Lack of fit	1.1686	3	0.3895	113.5689	0.0003
Pure error	0.0137	4	0.00343		
Cor total	27.235	16			
R^2	0.957				
Adj R^2	0.9007				
Pred R^2	0.3127				
Adeq precision	12.92				
CV%	3.679				

3. Results and discussion

3.1. Fitting the process models

Based on preliminary single-factor experiments, the following conditions were adopted in the RSM experiments: extraction temperature, 70–90 °C; ratio of water to raw material, 20–40 and extraction time 1–2 h (data not shown).

As seen in Table 1a, results showed that the yield of polysaccharides ranged from 8.18 to 12.64%. Multiple regression analysis was performed on the experimental data and the relationship of the response variable and test variables was given by the following second-order polynomial equation:

$$Y\ (\text{yield}) = 12.10 + 0.52A + 0.86B + 0.30C - 0.11AB - 0.92AC - 0.12BC - 0.90A^2 - 1.01B^2 - 0.06C^2$$

Analysis of variance (ANOVA) results for the model are given in Table 1b. Extraction temperature and ratio of water to raw material exhibited significant impact on the extraction yield of GIP (*P*-values less than 0.05), while the effects of extraction time failed to reach statistical significance. The quadratic term of extraction temperature and ratio of water to raw material indicated that the two variables had a larger effect.

The model fits well with the experimental data, which was approved by the high values of determination coefficient R^2 (95.7%) and the adjusted determination coefficient Adj. R^2 (90.07%). The low coefficient value of the variation (CV = 3.679%) clearly suggested that a high degree of precision and a good deal of reliability of the experimental values. This result implied that polysaccharides extraction results could be analyzed and predicted by the model.

The effects of variables and their interactions on the yield of polysaccharides are illustrated by the 3D response surfaces and the 2D contour plots. Results from Fig. 1 further confirmed ANOVA findings. During GIP extraction, the temperature and water to solid ratio are more important. Therefore, the best combinations of process variables for response functions are obtained. Using Design-Expert, the optimum values for the tested variables for extraction of GIP were: an extraction temperature of 87.5 °C, an extraction time of 1 h and a ratio of water to raw material of 39.7 mL/g. the maximum predicted extraction yield of GIP was 13.26%, which corresponded well with the actual yield (13.18 ± 0.13%, $n = 3$). These results suggested that the model designed in this study was valid.

3.2. Purification of GIP

As shown in Fig. 2a, GIP was fractionated by DEAE-52 cellulose column chromatography to obtain three fractions. The second fraction was the main fraction, representing 65.25% of GIP. This fraction was further purified on a Sephadex G-100 column to obtained GIP-II. As shown in Fig. 2b, GIP-II yielded a single peak.

3.3. Preliminary characterization of GIP-II

HPAEC analysis showed that GIP-II was composed of galactose, glucose, xylose, and mannose at molar ratios of 1: 4.25: 1.14: 1.85. Glucose was the predominant monosaccharide (Fig. 3). The linearity of the method was calibrated using dextran standards of different molecular weights and the average Mw of GIP-II was about 42.87 kDa (data not shown).

3.4. Antioxidant activity of GIP-II

3.4.1. DPPH scavenging effect

The DPPH scavenging assay is widely used to test antioxidant activity because it is simple, rapid and sensitive (Benvenuti, Pellati, Melegari, & Bertelli, 2004). The observed DPPH scavenging activity of GIP-II is exhibited in Fig. 4a. The DPPH scavenging activity increased with increasing concentrations of GIP-II and BHA was stronger than that of GIP-II at every concentration point. At 0.8 mg/mL, the scavenging activities of GIP-II and BHA were 68.51

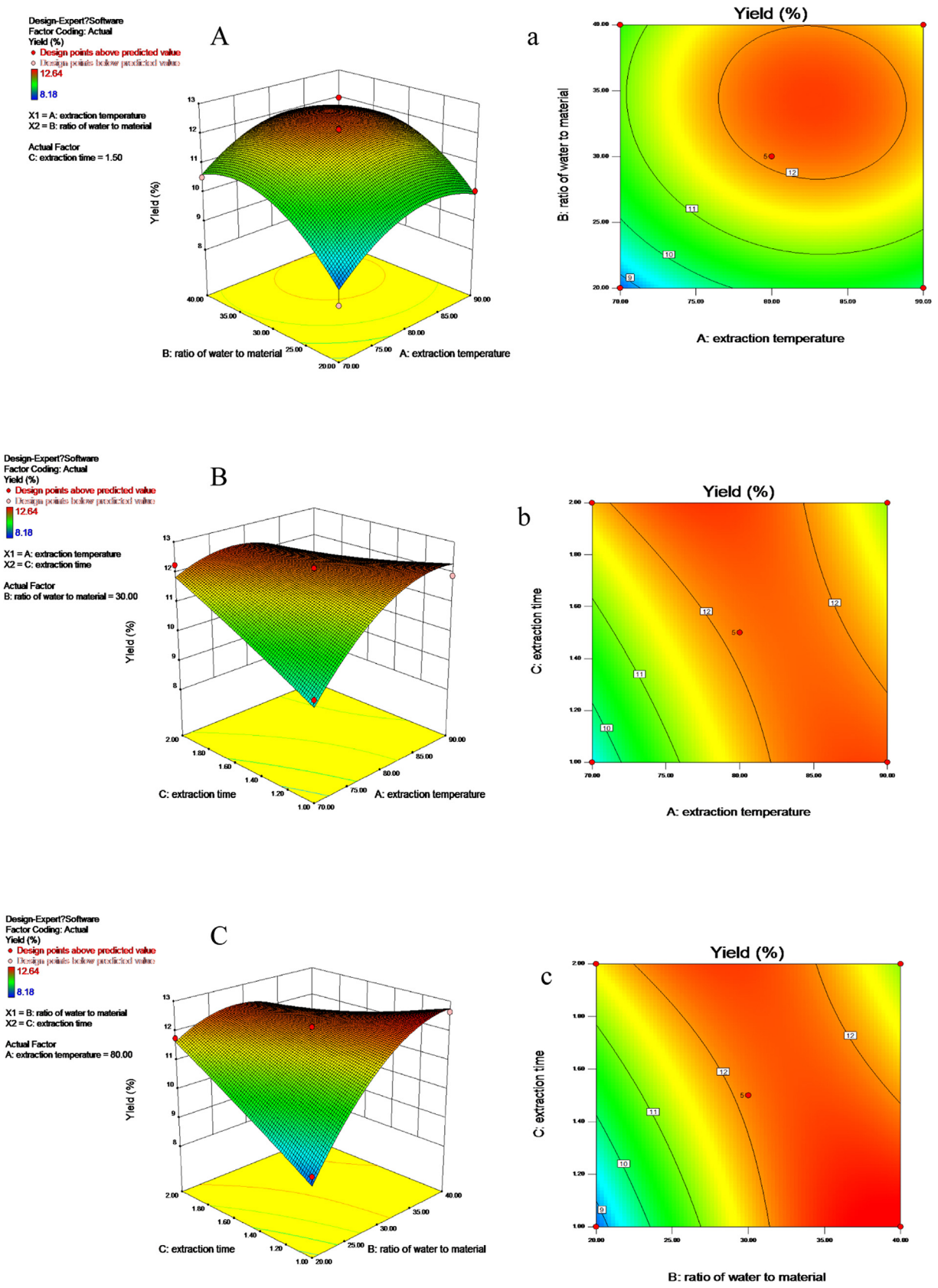

Fig. 1. 3D response surface plots (A–C) and 2D contour plots (a–c) showing the effects of temperature, time and ratio of water to materials on the yield of *Gleoestereum incarnatum* polysaccharide.

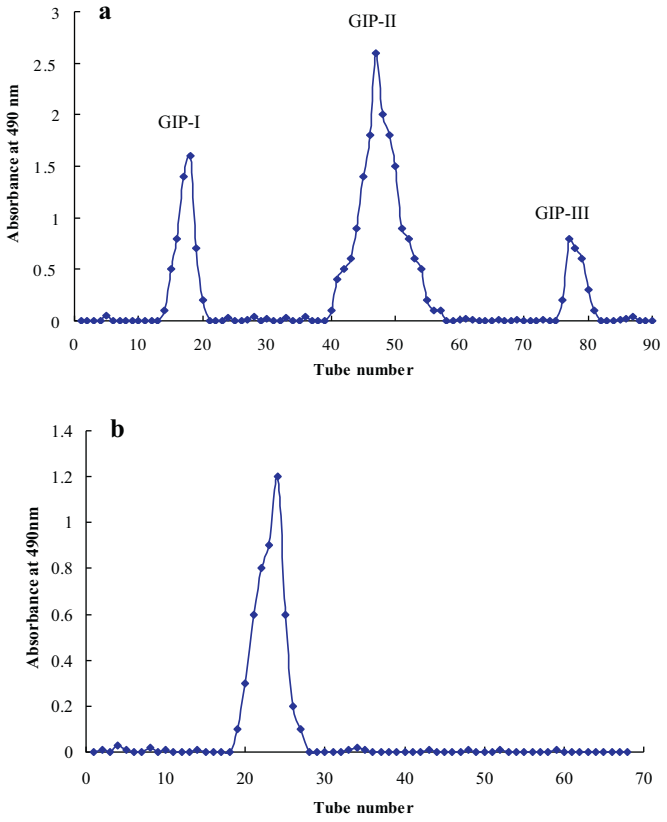

Fig. 2. Polysaccharides from crude *Gleoestereum incarnatum* extract were isolated by DEAE cellulose-52 chromatography (a). The main fraction (GIP-II) was fractionated by Sephadex G-100 column. A single peak was designated as GIP-II (b).

and 94.26%, respectively. These results indicate that GIP-II is a DPPH scavenger.

3.4.2. Hydroxyl radical scavenging activity

Fig. 4b shows the scavenging activities of GIP-II and BHA against hydroxyl radicals. Both GIP-II and BHA showed obvious scavenging activity on hydroxyl radicals in a concentration-dependent manner. At concentrations between 0.05 and 1.60 mg/mL, the scavenging effect was 17.56–56.23% for GIP-II and 37.31–92.38% for BHA. The hydroxyl radical is one of the most powerful oxidative species (Halliwell, Murcia, Chirico, & Aruoma, 1995). Therefore, the removal of hydroxyl radicals of GIP-II is important for antioxidant defense in cell or food systems.

3.5. Antitumor activity of GIP-II

The antiproliferative activity of GIP-II on HepG2 cells *in vitro* was investigated. As shown in Fig. 4c, GIP-II presented an obvious inhibition effect on HepG2 at all concentrations tested. At concentrations between 0.05 and 3.2 mg/mL, the inhibition rates of GIP-II were 26.55–69.37%. For 5-Fu, the inhibition rate was 68.32% at a concentration of 0.025 mg/mL. These results indicated that GIP-II had a potent anti-proliferation effect on HepG2 cells. However, the detailed antitumor mechanisms of GIP-II need to be further studied.

Polysaccharides with antioxidant and antitumor activities have been reported by many researchers (Jin, 2012; Zhang et al., 2012b; Zhao et al., 2012). The biological activity of polysaccharides might depend on their molecular weight, configuration and chemical composition and so on (Tao, Zhang, & Zhang, 2009; Zaidman, Yassin, Mahajna, &Wasser, 2005; Zou, Zhang, Yao, Niu, & Gao, 2010). Studies have shown that polysaccharides extracted from various mushrooms have significant anti-proliferative activities on HepG2 cells because of the specific conformation and high affinity for HepG2 cell receptors (Li et al., 2012; Wang et al., 2012a). Research suggests that the physicochemical properties of mushroom-derived polysaccharides are favorable for collision and

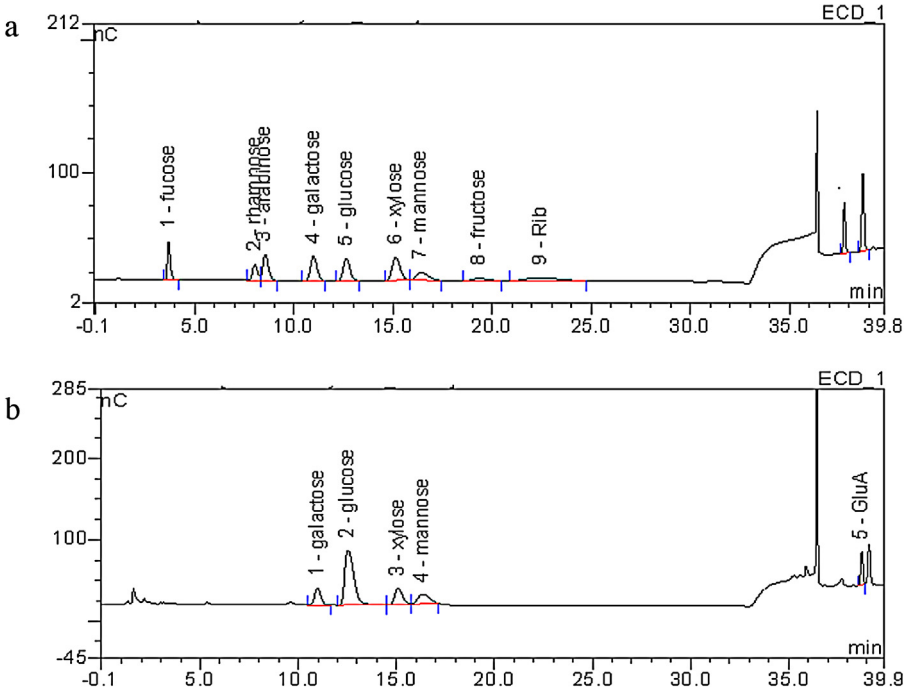

Fig. 3. (a) High-performance anion-exchange chromatography of standard monosaccharides. (b) High-performance anion-exchange chromatography of monosaccharide composition of polysaccharide (GIP-II) purified from *Gleoestereum incarnatum*.

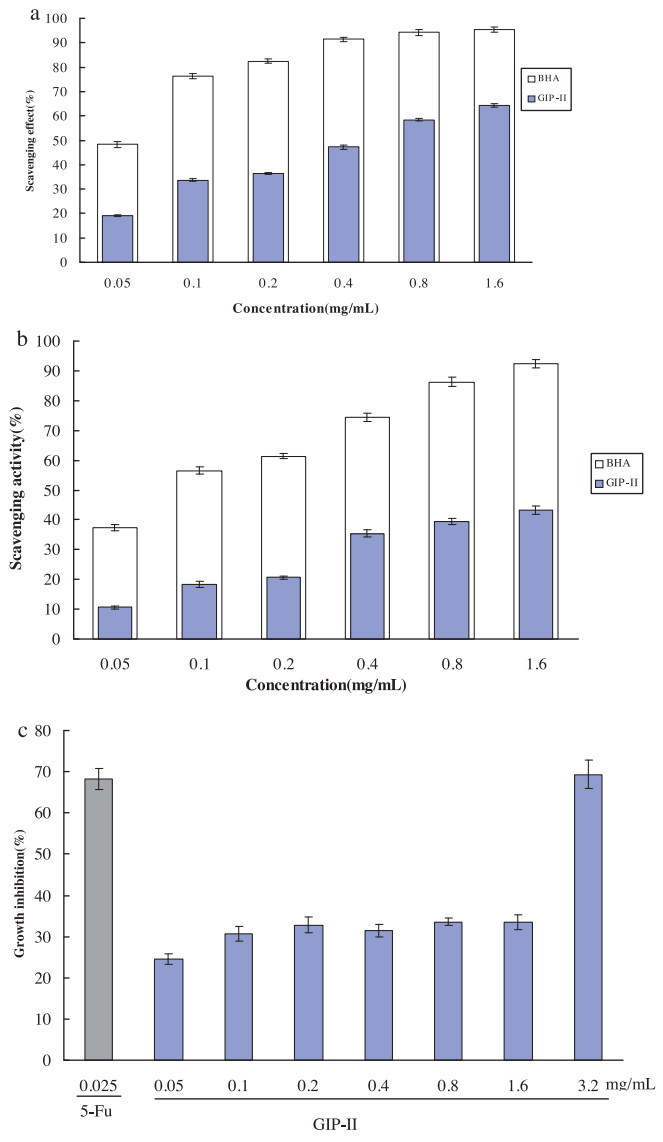

Fig. 4. Antioxidant and antitumor activities of the purified polysaccharide (GIP-II) from *Gleoestereum incarnatum*. (a) DPPH radical scavenging activity; (b) hydroxyl radial scavenging activity; (c) growth inhibitory effect on HepG2 cells treated for 72 h.

binding with the surface receptors of tumor cells (Ren, Reynisson, Perera, & Hemar, 2013).

It has been reported that the levels of anti-oxidation and reactive oxygen species are correlated with the generation and malignant transformation of cancer cells (Leng, liu, & Chen, 2005). Therefore, polysaccharides could potentially inhibit cell growth by enhancing levels of antioxidation and the clearance of ROS in cancer cells (Jiang, Wang, Liu, Gan, & Zeng, 2011). Consequently, the higher anticancer activity of GIP-II may be partly due to its higher scavenging activity against free radicals in cancer cells. This result is supported by recent research studies (Shao, Chen, & Sun, 2014; Xin et al., 2012). Furthermore, results show that both acute and chronic toxicity of GIP in animals is very low (Li, 2002). Taken together, these results showed that GIP-II is a natural polymer with antioxidant and antitumor activities worthy of further investigation.

4. Conclusion

In the present study, RSM was used to optimize an extraction process for GIP. The optimal conditions were: extraction temperature 87.5 °C, extraction time 1 h and a ratio of water to raw material of 39.7 mL/g. Under these conditions, a GIP yield of 13.18% was obtained. GIP-II was the main fraction purified from GIP. GIP-II was composed of galactose, glucose, xylose, and mannose, with glucose was the predominant monosaccharide. GIP-II exhibited strong scavenging activities on DPPH and hydroxyl radials *in vitro*, as well as a strong inhibitory effect on the growth of HepG2 cells. The overall findings indicate that GIP-II should be a promising natural polymer with antioxidant and antitumor activities.

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