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Optimized Extraction, Preliminary Characterization, and *In Vitro* Antioxidant Activity of Polysaccharides from *Glycyrrhiza Uralensis* Fisch

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: This study performed optimized extraction, preliminary characterization, and *in vitro* antioxidant activities of polysaccharides from *Glycyrrhiza uralensis* Fisch.

Material/Methods: Three parameters (extraction temperature, ratio of water to raw material, and extraction time) were optimized for yields of *G. uralensis* polysaccharides (GUP) using response surface methodology with Box-Behnken design (BBD). The GUP was purified using DEAE *cellulose* 32-column chromatography. The main fraction obtained from *G. uralensis* Fisch was GUP-II, which was composed of *rhamnose, arabinose, galactose,* and *glucose monosaccharide*, was screened for antioxidant properties using DP Hand hydroxyl radical scavenging assays. In addition, immunological activity of GUP-II was determined by nitric oxide and lymphocyte proliferation assays.

Results: Optimization revealed maximum GUP yields with an extraction temperature of 99°C, water: raw material ratio of 15: 1, and extraction duration of 2 h. GUP-II purified from *G. uralensis* Fisch had good *in vitro* DPPH and hydroxyl radical scavenging abilities. Immunologically, GUP-II significantly stimulated NO production in RAW 264.7 macrophages, and significantly enhanced LPS-induced lymphocyte proliferation.

Conclusions: Extraction of GUP from *G. uralensis* Fisch can be optimized with respect to temperature, extraction period, and ratio of water to material, using response surface methodology. The purified product (GUP-II) possesses excellent antioxidant and immunological activities.

MeSH Keywords: Fungal Polysaccharides • *Glycyrrhiza Uralensis* • Water Purification

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Background

In Chinese traditional medicine, *G. uralensis* Fisch is widely used for the treatment of various diseases and in preparation of tonic medicines containing GUP [1,2]. Mounting evidence confirm that *G. uralensis* Fisch plays an indispensable role in animal health improvement and disease prevention [3]. GUP possesses diverse pharmacological properties, including immune-modulatory, antioxidant, anti-viral, and anti-inflammatory [4–7]. Recently, GUP, as the primary active ingredient in *G. uralensis* Fisch, has drawn much attention, and has been increasingly utilized in animal feed additives, veterinary drugs, and vaccines [8].

It has been suggested that optimization of processing parameters serves important roles in enhancement of desired products. Therefore, groups of statistical experimental designs were investigated in process optimization [9]. Several authors have conducted single-factor optimization in evaluating optimum processing conditions for extraction of GUP under controlled conditions [10,11]. However, single-variable optimization was not only tedious, but also led to misinterpretation of results, especially because it overlooked interaction between different factors [9]. Therefore, researchers prefer to perform multivariable optimizations.

Response surface methodology (RSM) is a collection of statistical techniques, including experiment designing, model building, evaluation of factors, and searching for optimum conditions of factors [12]. Recently, RSM has been used for optimizing extraction conditions for isolation of osthol from *C. monnieri* fruits [13]. Rodríguez-Pérez et al. investigated optimization of microwave-assisted extraction and pressurized liquid extraction of phenolic compounds from *Moringa oleifera* leaves by multi-response surface methodology [14]. However, there are as yet no published reports on optimization of processing parameters for extraction of GUP from *G. uralensis* Fisch using response surface methodology.

Since GUP is associated with diverse pharmacological properties, there is need to identify the fractions with good antioxidant and anti-inflammatory activities for further pharmaceutical studies. DPPH scavenging assay is widely used to examine antioxidant activity since it is sensitive, simple, and rapid [15]. Hydroxyl radical is one of the most powerful oxidative species [16]. The last line of defense barrier in animals is the immune system, which resists inflammation, infection, and cancer cell proliferation. Studies have shown that Chinese herbal GUP showed specific immunological activity via stimulation of immune cells such as lymphocytes and macrophages [17]. Thus, it is necessary to investigate the effect of GUP-II on macrophage activation and lymphocyte proliferation. Lymphocyte proliferation assay is one of the frequently used methods for detecting immune-enhancing activity. Specific immune response refers to the specific T/B lymphocyte antigen stimulation, whose activation, proliferation, and differentiation bring about a series of biological effects, including B cell-mediated immune response as well as T cell- and macrophage-mediated cellular immune response [18].

In the present study, the processing parameters for extraction of GUP from *G. uralensis* Fisch were optimized using Box-Behnken design technique (3 factors and 3 levels) under RSM, and the immunological and antioxidant properties of the purified fraction were determined.

Material and Methods

Materials

Roots of *G. uralensis* Fisch were purchased from Traditional Medicine Company (Shihezi, Xinjiang, China). DEAE *cellulose*-32, *Sephadex* G-100, standard monosaccharides (*D-glucose, D-mannose, L-fucose, D-xylose, D-arabinose, D-galactose, Fructose and L-rhamnose*), dimethyl sulfoxide (DMSO) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich (St. Louis, MO). 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and RAW 264.7 cell line were purchased from Sangon Biotech (Shanghai, China). All other chemicals and analytical-grade solvents were obtained from Wode Chemical Reagent Co. Ltd. (Shihezi, Xinjiang, China).

Extraction of crude GUP

G. uralensis Fisch root powder was soaked in 90% ethanol for 48 h to remove impurities and small lipophilic molecules [19]. Aqueous extraction was performed on the dried residue with respect to designed parameters [20]. The aqueous extract was removed from the pellet by vacuum filtration, followed by supernatant concentration and precipitation with anhydrous ethanol (85% v/v). The precipitate was recovered by centrifugation at 5000× g for 10 min followed by vacuum drying at 45°C to a constant weight. Extraction yield (%) was calculated with the following equation:

$$\text{GUP yield\%} = \text{Crude GUP weight (g)/Raw powder weight (g)} \times 100\%.$$

GUP purification

The crude GUP was purified by DEAE *cellulose*-32 column chromatography via stepwise adding NaCl solutions at different concentrations (0, 0.01 M, 0.05 M, 0.1 M, and 1M). Content of carbohydrate in the elution liquid was determined by anthrone-sulfuric acid method, which produced 5 fractions. The

Table 1. The BBD matrix and the results for extraction yield of crude polysaccharides from GUP.

Run#	A: Extraction temperature (°C)		B: Ratio of water to raw material (v/w)		C: Extraction time (h)		Actual yield (%)	Predicted yield (%)
1	0	(94)	0	(10)	0	(4)	13.66	13.5
2	0	(94)	1	(15)	1	(6)	13.99	14.31
3	−1	(89)	0	(10)	−1	(2)	9.43	9.49
4	1	(99)	−1	(5)	0	(4)	13.21	13.43
5	0	(94)	1	(15)	−1	(2)	14.07	14.23
6	0	(94)	−1	(5)	1	(6)	12.37	12.21
7	0	(94)	0	(10)	0	(4)	12.71	13.50
8	−1	(89)	−1	(5)	0	(4)	7.33	7.59
9	1	(99)	0	(10)	−1	(2)	15.27	15.37
10	1	(99)	1	(15)	0	(4)	16.11	15.85
11	0	(94)	−1	(5)	−1	(2)	9.07	8.75
12	−1	(89)	0	(10)	1	(6)	12.78	12.68
13	0	(94)	0	(10)	0	(4)	13.57	13.50
14	0	(94)	0	(10)	0	(4)	13.89	13.50
15	−1	(89)	1	(15)	0	(4)	12.97	12.75
16	1	(99)	0	(10)	1	(6)	15.81	15.75
17	0	(94)	0	(10)	0	(4)	13.65	13.50

main fraction was further purified through *sephadex* G-100 column and dialyzed, while the other fractions were concentrated and lyophilized for further research.

Optimized calculation process and model fitting

The extraction parameters were optimized based on Design Expert software (Version 8.0.5.0). Box-Behnken design (BBD) was employed to evaluate the combined effects of extraction temperature, ratio of water to raw material, and extraction time. These 3 variables were marked as A, B, and C, respectively. All experiments were carried out in triplicate. Analysis of variance (ANOVA) was determined based on regression coefficients. The significance of each term in the polynomial was statistically analyzed by F-value at $p < 0.05$. Then, regression coefficients were used to make statistical calculation of contour maps and generated dimension from the regression models. The levels and ranges of variables investigated are displayed in Table 1.

Characterization of GUP-II

Monosaccharide composition of GUP-II was analyzed with high-performance anion exchange liquid chromatography (HPAEC) according to the method of Corradini et al. [21].

DPPH free radical scavenging assay

DPPH radical scavenging activity was assayed by the method of Li et al. [22]. Various concentrations of GUP-II solution (0.05, 0.1, 0.2, 0.5, and 1.0 mg/mL) in DMSO were put into different 5-mL tubes containing 2 ml of 0.5 mM DPPH in methanolic DMSO. Absorbance was measured at 519 nm. BHT was used as a positive control. Each assay was repeated 3 times. Scavenging activity was calculated using the formula:

$$\text{Scavenging activity (\%)} = (A_{BHT} - A_{GUP-II})/A_{BHT} \times 100\%.$$

Hydroxyl radical scavenging assay

$FeSO_4$ (2 mL, 1.8 mM) was mixed with 1.5 mL of 1.8 mM salicylate ethanol, 1.5 mL H_2O_2 (0.03%), and 1.0 mL of different concentrations of GUP-II solution (0.05–1.60 mg/mL) in DMSO.
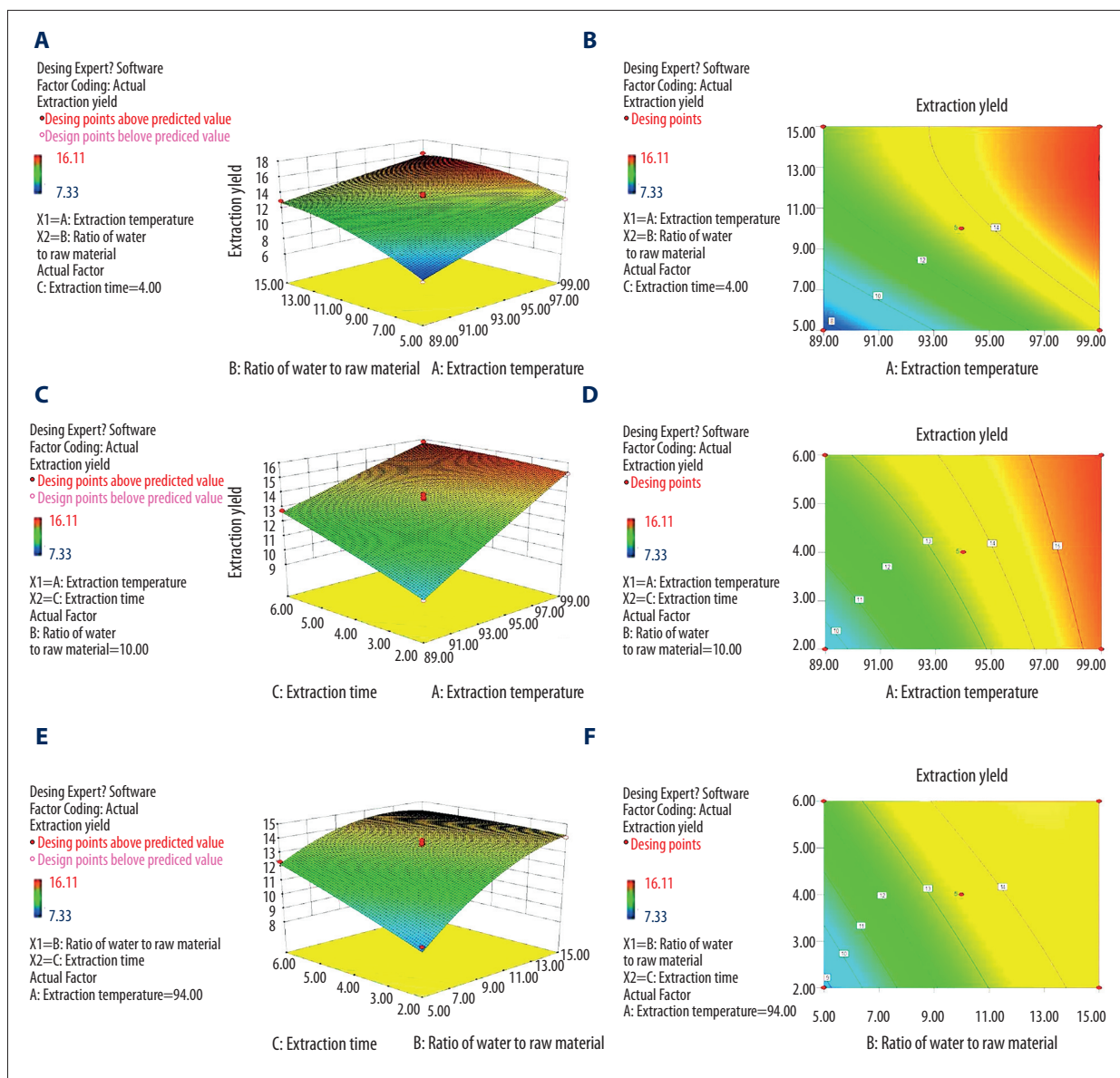
Figure 1. 3D response surface plots (**A, C, E**) and 2D contour plots (**B, D, F**) determined the effects of extraction temperature, ratio of water to raw material, and extraction time, respectively, at 3 different levels on the yield of *G. uralensis* polysaccharide.

The solutions were incubated at 37°C for 30 min, after which absorbance was measured at 510 nm. Vitamin C was used as the positive control. Each was repeated 3 times. Antioxidant activity was calculated with the following equation:

$$\text{Scavenging activity (\%)} = (A_{VC} - A_{GUP-II}) / A_{VC} \times 100\%.$$

Nitric oxide assay

RAW 264.7 cells (1×10^6/well) were seeded in 96-well plates and incubated in media containing different concentrations of GUP-II and 5 μg/mL LPS (positive control) in DMSO at 37°C for various periods, with each period repeated 3 times. Determination

of nitrite in culture medium was done by the Griess reaction, with $NaNO_2$ as standard [23].

Lymphocyte proliferation assay

The effect of GUP-II on lymphocyte proliferation was measured by MTT assay. Spleen cells (2×10^6/mL) were seeded into 96-well plates in the presence of LPS (5 μg/mL). Various concentrations of GUP-II (0, 10, 20, 50, 100, and 200 μg/mL) in DMSO were added, then incubated at 37°C in an incubator supplemented with humidified 5% CO_2 for 48 h. We added 20 μL MTT (5 mg/mL) to each well. After incubation for 4 h, 150 μL DMSO was added to dissolve the formazan crystals, and

Table 2. ANOVA for response surface quadratic model.

Source	Sum of squares	DF	Mean square	F-value	P-value
Model	86.31	9	9.59	49.77	<0.0001
A	40.01	1	40.01	207.62	<0.0001
B	28.73	1	28.73	149.09	<0.0001
C	6.32	1	6.32	32.79	0.0007
AB	1.88	1	1.88	9.74	0.0168
AC	1.97	1	1.97	10.24	0.0151
BC	2.86	1	2.86	14.82	0.0063
A^2	0.022	1	0.022	0.11	0.7472
B^2	4.37	1	4.37	22.70	0.0020
C^2	0.044	1	0.044	0.23	0.6488
Residual	1.35	7	0.19	0.84	0.5403
Lack of fit	0.52	3	0.17		
Pure error	0.83	4	0.21		
Cor total	87.66	16			
R2	0.9846				
Adj R^2	0.9648				
Pred R^2	0.8904				
Adeq R^2	24.542				
C.V.%	3.39				

absorbance was measured at 570 nm. Proliferation percentage was calculated using the following equation:

$$\text{Proliferation (\%)} = (OD_{sample} - OD_{control})/OD_{control} \times 100\%.$$

Statistics

Data are presented as mean ± standard deviation (SD). Regression coefficients were determined by ANOVA followed by *t* test. *P*<0.05 indicates a significant difference.

Results

Results for optimized conditions based on a single-factor test in RSM experiments are shown in Figure 1. Extraction temperature ranged from 89°C to 99°C; the ratio of water to raw material varied from 5: 1 to 15: 1, and extraction time lasted from 2 to 6 h. As shown in Table 1, the yield of crude GUP varied from 7.33% to 16.11%. By means of multiple regression analysis, the relationship between variables and corresponding responses was analyzed using the second-order polynomial equation:

Extraction yield = 13.50 + 2.24A + 1.89B + 0.89C − 0.69AB − 0.70AC − 0.84BC − 0.072A2 − 1.02B2 − 0.10C2.

Table 2 shows results for the model, which was analyzed by ANOVA. Using Design Expert, among all the tested variables, the optimum values for GUP extraction selected were: extraction temperature of 99°C, ratio of water to raw material of 15: 1, and 2 h extraction time. The predicted maximum GUP yield was 16.41%, which corresponded perfectly with actual yield. Extraction temperature, water ratio, and extraction time were the main factors that predominantly affected extraction of GUP (*p*<0.05). The model was consistent with experimental data, with *R²* value of 98.46%, and adjusted *R²* value of 96.48%.

Purification of GUP-II from GUP and analysis by HPAEC

Crude GUP was fractionated by DEAE *cellulose*-32, and 5 fractions were obtained, as shown in Figure 2A. Interestingly, the main fraction was GUP-II, which accounted for 47.66% of GUP. As shown in Figure 2B, GUP-II yielded a single peak and was further purified by sephadex G-100 column. HPAEC results showed that GUP-II was composed of arabinose, rhamnose,
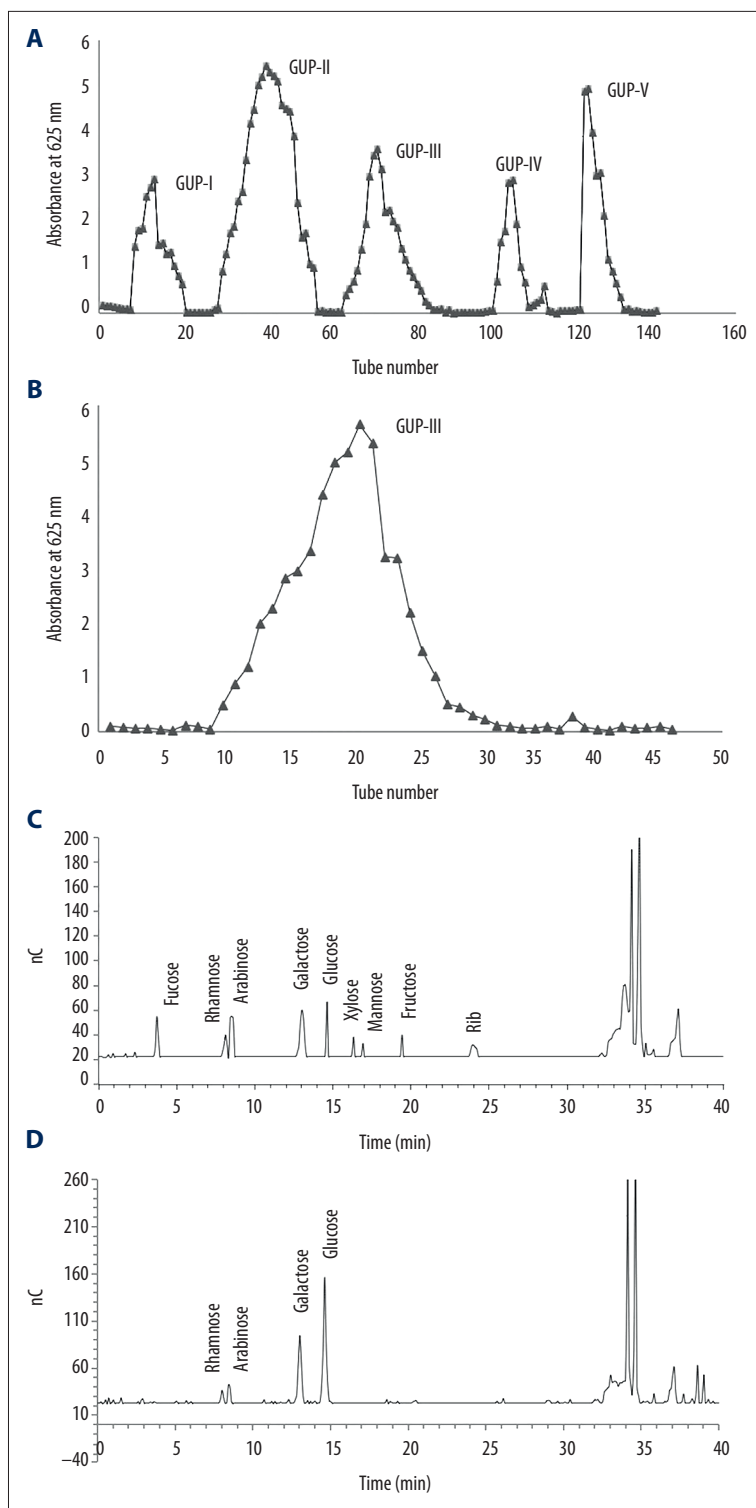
Figure 2. Purification and analysis of GUP-II from GUP. (**A**) GUP-I, GUP-II, GUP-III, GUP-IV and GUP-V were fractionated from the crude GUP by DEAE *cellulose*-32 chromatography. (**B**) GUP-II yielded a single peak, which was further purified by a *Sephadex* G-100 column. (**C, D**) GUP-II was composed of arabinose, rhamnose, galactose, and glucose as evidenced by HPAEC analysis (C – standard control; D – GUP-II).

galactose, glucose, and mannose at molar ratios of 1.08: 1.25: 3.01: 5.85 (Figure 2C, 2D).

Antioxidant properties of GUP-II

As shown in Figure 3, GUP-II showed effective and concentration-dependent DPPH radical scavenging activity, although it was lower than that of BHT (21.7–48.33% for GUP-II, and
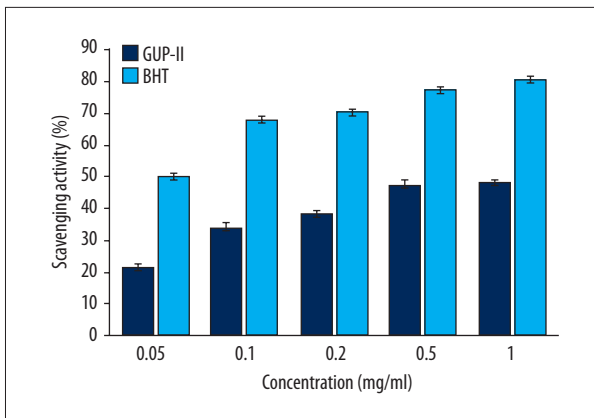
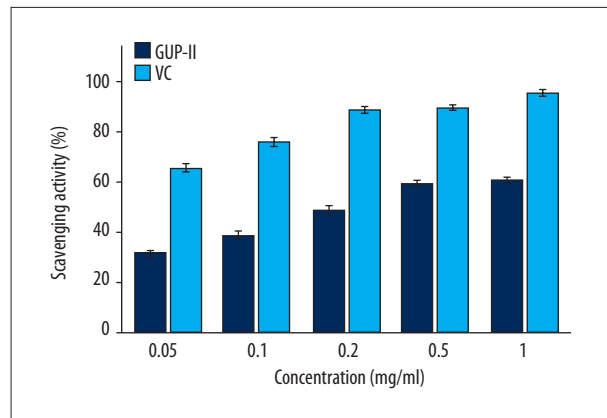**Figure 3.** DPPH scavenging activity of GUP-II and BHT.

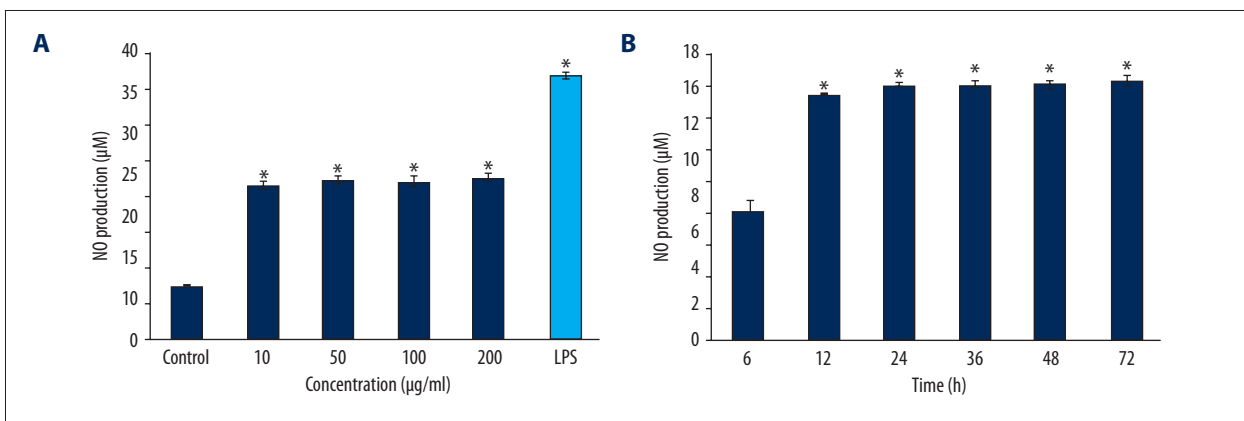**Figure 4.** Hydroxyl radical scavenging activities of GUP-II and vitamin C

Figure 5. Effects of GUP-II on macrophage activation depended on NO production in RAW264.7 cells. (**A**: Different concentrations; **B**: Different time points). * $p<0.05$ compared with control.

50.17–80.55% for BHT at concentrations between 0.05 and 1.0 mg/mL). GUP-II also exhibited effective hydroxyl radical scavenging ability. As shown in Figure 4, the scavenging effects were 31.81%–60.94% and 65.34–95.26% for GUP-II and vitamin C, respectively, at concentrations between 0.05 and 1.0 mg/mL.

GUP-II induced macrophage cell activation

GUP-II effectively induced NO production of RAW 246.7 cells, although this was not significantly dose-dependent (Figure 5). NO production in the GUP-II group after 48 h was nearly 2.5 times of that in the control group ($p<0.05$).

GUP-II promoted the lymphocyte proliferation

As shown in Figure 6, GUP-II at concentrations of 10, 20, 50, 100, and 200 µg/mL significantly up-regulated LPS-induced splenocyte proliferation. The results revealed that only the low concentrations (10 and 20 µg/mL) of GUP-II in combination with LPS showed synergistic immune effects.
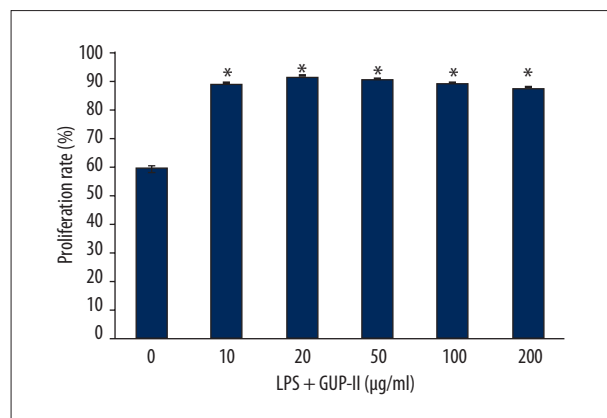

Figure 6. Effect of GUP-II on T and B cell proliferation detected and verified by MTT. * $p<0.05$ compared with control.

Discussion

Results obtained in this study indicated that GUP extraction yield can be optimized and predicted. Three-dimensional response surface and 2D contour map reflected the influence of

variables and interaction among them affecting GUP yield [24]. The optimum extraction conditions are: extraction temperature of 99°C, ratio of water to material of 15: 1, and extraction time of 2 h. Under these conditions, the extraction yield of GUP was in good agreement with the predicted value.

GUP-II exhibited very good concentration-dependent hydroxyl radical scavenging properties and appreciable DPPH radical scavenging ability. NO is considered to be a quantitative indicator of macrophage activation. Related studies have demonstrated that diverse sources of *Glycyrrhizia* polysaccharide activate different types of macrophages [25]. Interestingly, macrophage activation was induced by GUP but not in a concentration-dependent manner. It might be that GUP is acting as a bidirectional immune modulator, thereby modulating and normalizing both high and low levels of immune responses. Recently, it was shown that has antianaphylaxis, non-specific immune function, and steroid-sparing effects, which were neither conclusively immunosuppressant nor immunoenhancing [26]. This is in line with the results obtained in the present study, which suggest that GUP may be an immune regulator with bidirectional functions, inhibiting or enhancing immune activity as a function of specific conditions.

The antioxidant and immunological properties of GUP may be responsible for the observed health benefits of *G. uralensis* Fisch in Chinese herbal medicine. Herbal medicine practitioners in China use *G. uralensis* Fisch for the treatment of various diseases and in the preparation of tonic medicine, in which GUP has been identified as a major component. In this study, the purification of GUP was tedious, and required repeated experiments, which resulted in low yield of GUP. Therefore, extraction of GUP still needs more optimization for future enhanced product yield.

Conclusions

Extraction of GUP from *G. uralensis* Fisch has been optimized with respect to extraction temperature, ratio of water volume to raw material, and the duration of extraction. The major purified fraction GUP-II possesses excellent antioxidant and immunological properties.

Conflict of interest

The authors declare there no conflict of interest.

Acknowledgments

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1790

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