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Chemical structure and immunity activity of *Liquiritia glycyrrhiza* heteropolysaccharide in animal

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ABSTRACT

Liquiritia glycyrrhiza heteropolysaccharide was extracted and analyzed using high-performance gel permeation chromatography (HPGPC) and Fourier transform infrared spectroscopy (FTIR). HPGPC showed that the weight-average molecular weight (Mw) of *L. glycyrrhiza* heteropolysaccharide was 2.936×10^5 Da. A typical infrared spectrum of *L. glycyrrhiza* heteropolysaccharide showed strong absorbances at about 3282, 2921, 1608, 1410 and 990 cm^{-1} and small ones at about 2853, 1708, 1515, 1280, 1241, and 1103 cm^{-1}. Ovarian cancer is a cancerous growth arising from different parts of the ovary. This study was designed to determine the effect of *L. glycyrrhiza* heteropolysaccharide on immunity function in animal suffering from ovarian cancer. In the in vivo assay, model control animals displayed a decrease in immunity activities. After 30 days of treatment, *L. glycyrrhiza* heteropolysaccharide-treated group showed a marked increase in immunity indexes (spleen and thymus index, IgA, IgG, IgM, IL-2, IFN-α and TNF-α levels) in comparison with the model control group. It can be concluded that *L. glycyrrhiza* heteropolysaccharide may improve immunity function in animal suffering from ovarian cancer.

1. Introduction

Licorice, the dry roots of *Glycyrrhiza glabra L.* (*Fabaceae*), is considered one of the oldest and most widely used herbal drugs around the world, being present in most pharmacopoeias of eastern and western countries. It has been traditionally used for respiratory, gastrointestinal, cardiovascular, genitourinary, eye and skin disorders, and for its antiviral effects (Fiore, Eisenhut, Ragazzi, Zanchin, & Armanini, 2005). The antiulcerogenic action of licorice, and its consumption as a food ingredient, has also been reported (Isbrucker & Burdock, 2006). Recently, the ethanol extract of roasted licorice was shown to inhibit lipopolysaccharide (LPS)-induced inflammatory responses in murine macrophages (Kim et al., 2006). *Glycyrrhizin* is the principal compound in *licorice* (4–10%) and was reported to inhibit LPS/D-galactosamine-induced liver injury (Yoshida et al., 2007) and to alleviate asthmatic features in mice (Ram et al., 2006). However, *glycyrrhizin* is converted, by human intestinal bacteria, to *glycyrrhetic acid* (Hattori, Sakamoto, Kobashi, & Namba, 1983) and *glycyrrhetic acid* has been reported to be a cause of severe hypertension and hypocalcemia (Asl & Hosseinzadeh, 2008; van Uum, 2005). Thus, we previously prepared a hexane/ethanol (90:10, v:v) extract of *G. uralensis* (HEGU), which did not harbour measurable quantities of *glycyrrhizin* (Choi et al., 2008).

Ovarian cancer is the leading cause of death from gynecologic cancer and is the 4th leading cause of cancer death overall among women in the United States. In 2005, it is estimated that it was diagnosed in 22,220 women and claimed 16,210 lives (Jemal et al., 2005). Advanced epithelial ovarian cancer is currently treated by cytoreductive surgery combined with chemotherapy (Pignata et al., 2011). The effectiveness of combination regimens of antineoplastic agents such as cisplatin, carboplatin, Taxol, and doxorubicin is low, however, and systemic administration of chemotherapeutic drugs may produce severe toxic side effects (Kaye, 2008; Markman et al., 2004). Natural medicine has been applicated in therapy of cancers for less toxic side-effects and positive therapeutic effect.

Therefore, in this work we studied the effect of *Liquiritia glycyrrhiza* heteropolysaccharide extract on immunity activities in mice with ovarian cancer.

2. Materials and methods

2.1. Material

L. glycyrrhiza was collected from a drug store in Zhenzhou city, China. Heteropolysaccharide were extracted from *L. glycyrrhiza*.

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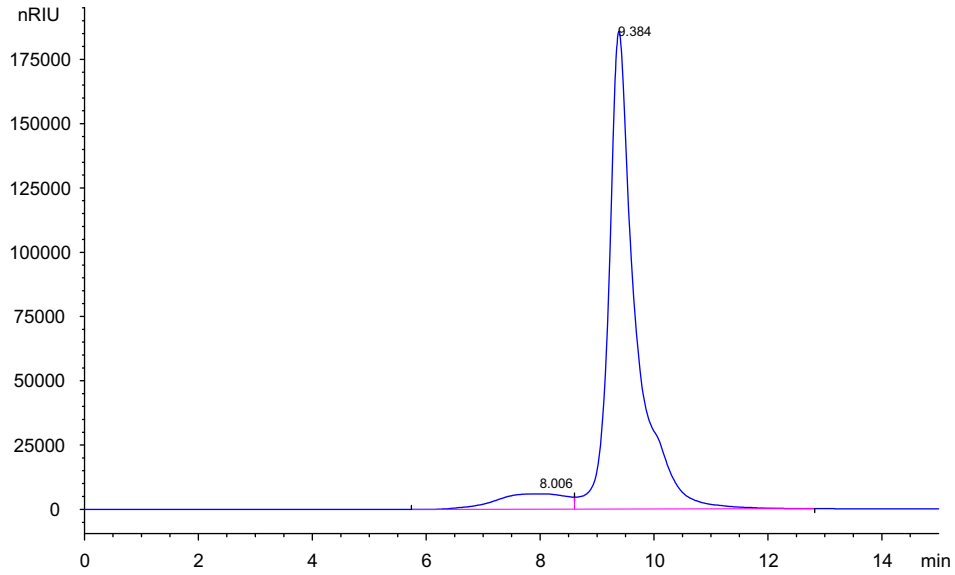

Fig. 1. The average molecular weights (Mw) can be determined using HPGPC.

2.2. High-performance gel permeation chromatography

Samples for HPGPC analysis were filtered through a syringe filter (MetaChem nylon, 0.2 μm, 13 mm, Torrance, CA, USA) and injected using an autosampler (Waters 717, Milford, MA, USA) into the connected columns (TSK Guard PWXL 6.0 × 40 mm and TSK G4000 PWXL 7.8 × 300 mm, Tosoh, Tokyo, Japan), with the column oven set at 40 °C. The eluting solvent (10% methanol) was filtered (Millipore HV 0.45 μm, Bedford, MA, USA), degassed (Waters in-line degasser, Milford, MA, USA), and then delivered by the HPLC pumping system (Waters 600 System Controller, Milford, MA, USA) at a flow rate of 0.3 ml/min. The eluent was monitored at 280 nm with a photodiode array detector (Waters 996, Milford, MA, USA).

A standard curve of glucan was obtained with a series of standard compound at varying concentrations. Triplicate analyses were conducted and the mean values were obtained. The linear regression equation of the standard curve was determined. HPGPC showed that the weight-average molecular weight (Mw) of *L. glycyrrhiza* heteropolysaccharide was 2.936×10^5 Da (Fig. 1).

2.3. Cell lines and culture

OVCAR-3 (human ovarian adenocarcinoma) cell line from Chinese Academy of medical science were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 100 mg/ml of streptomycin, 100 U/ml of penicillin. Cells were maintained in a humidified atmosphere of 5% carbon dioxide and 90% air at 37 °C.

2.4. Establishment of animals model and manipulation of tissue samples

Animal experiments were performed under the National Institutes of Health Animal Care and Use guidelines and were approved by Zhenzhou university Institutional Animal Care Committee. Animals were fed with a standard laboratory diet and water ad libitum. Standard laboratory diet was shown in Table 1. A total of 40 female nude mice (5 weeks old) were each subcutaneously injected with OVCAR-3 cells (1×10^7) in 0.3 ml of PBS, in the neck using a 23 gauge needle. After ovarian cancer model was established, animals were randomly divided into four groups (10 in each group). In addition, 10 untreated nude animal served as control.

Normal control and model control animal received the standard diet. Medicine-treatment animals were fed basic diet containing *L. glycyrrhiza* heteropolysaccharide (0.3%, 0.6% and 0.9%) per day, respectively.

On completion of 30 days experimental period, blood samples were collected via the ear vein using 10 ml tubes with heparin. The samples were then centrifuged at 1500 × *g* in a refrigerated centrifuge (Beckman Model TJ-6) at 4 °C for 10 min and harvested plasma stored at −20 °C until assayed for the contents of IgM, IgG, IgA, IL-2, IFN-α and TNF-α. Then, animals per group were killed. The spleen and thymus were sampled and stored at −20 °C for analysis.

2.5. Biochemical analysis

The dot ELISA kit was also used to assess the changes in the levels of immunoglobulin G (IgG), IgA and IgM.

Measurement of Interleukin 2 (IL-2) and TNF-α level was performed with the immunoassay kits.

TNF-α concentrations in serum were assayed with specific enzyme-linked immunoabsorbant assay (ELISA) kits purchased from R&D Systems (Abingdon, Oxon, UK) with a sensitivity of 5 ng/ml.

Table 1
Composition of the control diet (g/kg).

Diet ingredient (g/kg diet)	Standard diet	Diet I	Diet II	Diet III
Casein	220	210	200	190
DL-Methionine	9	9	9	9
Wheat starch	493	483	473	463
Sucrose	157	157	147	142
Liquiritia glycyrrhiza	30	60	90	
Cellulose	70	60	60	60
Mineral mix[a]	41	41	41	36
Vitamin mix[b]	10	10	10	10

[a] This mineral mix provided the following nutrients (g/kg of dry diet): Ca, 4; K, 2.4; Na, 1.6; Mg, 0.4; Fe, 0.12; elements (traces): Mn, 0.032; Cu, 0.005; Zn, 0.018; Co, 0.00004; I, 0.00002, completed to 40,000 with cellulose.

[b] Vitamin mixture contained (mg/kg of diet) the following: retinol, 12; cholecalciferol, 0.125; thiamine, 40; riboflavin, 30; pantothenic acid, 140; pyridoxine, 20; inositol, 300; cyano-cobalamin, 0.1; menadione, 80; nicotinic acid, 200; choline, 2720; folic acid, 10; p-aminobenzoic acid, 100; biotin, 0.6; sufficient starch to bring to 20 g (per kg of diet).

Table 2
Spleen index and thymus index.

Group	Spleen index	Thymus index
Control	2.68 ± 0.14	0.98 ± 0.06
OC Model	2.43 ± 0.09##	0.76 ± 0.05##
Diet I	2.57 ± 0.09*	0.87 ± 0.09
Diet II	2.66 ± 0.09*	0.94 ± 0.11*
Diet III	2.72 ± 1.31**	1.05 ± 0.09**

$P < 0.01$, OC model vs control.
* $P < 0.05$.
** $P < 0.01$, treatment groups vs OC model.

Table 3
Serum LgA, LgG and LgM levels.

Group	IgM (mg/L)	IgA (mg/L)	IgG (mg/L)
Control	262.5 ± 10.4	120.4 ± 7.3	1406.3 ± 12.8
OC Model	247.31 ± 10.3##	98.81 ± 4.3##	1267.1 ± 14.5##
Diet I	253.3 ± 8.6	104.1 ± 7.1	1285.7 ± 10.3*
Diet II	261.1 ± 11.2*	110.6 ± 8.2*	1307.1 ± 15.6*
Diet III	286.1 ± 13.7**	127.2 ± 6.9**	1462.8 ± 11.8**

$P < 0.01$, OC model vs control.
* $P < 0.05$.
** $P < 0.01$, treatment groups vs OC model.

2.6. Infrared spectroscopy

To record the infrared spectra in the same conditions as in the part one of this series of papers (Sekkal et al., 1993), the infrared microspectrometry method was applied. Likewise, spectra of very small amounts of cuticles were recorded, while they could never be obtained by the use of the usual sampling methods (films or KBr discs). All of the spectra were recorded in the 4000–600 cm^{-1} spectral range at a resolution of 2 cm^{-1}, 200 scans were accumulated for each sample. The powders were deposited on a BaF_2 window.

2.7. Statistical analysis

All the data were statistically analyzed by one-way ANOVA (SPSS, 1999). Differences among treatments were separated by Duncan's multiple range tests. Differences were considered significant at $P < 0.05$.

3. Result and discussion

The results demonstrated that spleen and thymus indexes in model control mice ($P < 0.01$) were significantly reduced compared to the control group (Table 2). Mice feeding with heteropolysaccharide extract from *L. glycyrrhiza* had significantly higher ($P < 0.01$) spleen and thymus indexes than those in the model control group after 30 days of treatment (Table 2).

In order to explore the effects of *L. glycyrrhiza* heteropolysaccharide on the immunity activities in all mice, the levels of serum IgA, IgG and IgM were evaluated according to appropriate methods reported previously. The results were shown in Table 3. These results showed that the levels of serum LgA, LgG and LgM were increased significantly ($P < 0.05$) by three doses of *L. glycyrrhiza* heteropolysaccharide, as compared to model control group.

Interleukin-2 (IL-2) is an interleukin, a type of cytokine immune system signaling molecule, which is a leukocytotrophic hormone that is instrumental in the body's natural response to microbial infection and in discriminating between foreign (non-self) and self. IL-2 mediates its effects by binding to IL-2 receptors, which are expressed by lymphocytes, the cells that are responsible for immunity.

The use of immunoadjuvants, such as α-interferon, in treatment of epithelial ovarian carcinomas is based on the presence of ovarian tumor antigens, data from animal studies, studies of immunological capacities of cancer patients, and information regarding therapy interactions (Bast et al., 1983; Knapp & Berkowitz, 1977; Mitchell & Kanhorn, 1979). Gresser evaluated α-interferon against a series of ascites tumors in different strains of mice and found that daily treatments inhibited tumor growth and prolonged survival (Gresser, 1977). Optimal effects were achieved when α-interferon was administered intraperitoneally, rather than intravenously. The appearance of a potential survival difference with no progression-free survival difference is likely to be a chance occurrence, given the small sample sizes overall; however, there is a precedent for high dose α-interferon being associated with survival prolongation in the adjuvant setting in post-surgical patients with a deep primary or regionally metastatic melanoma (Kirkwood et al., 1996).

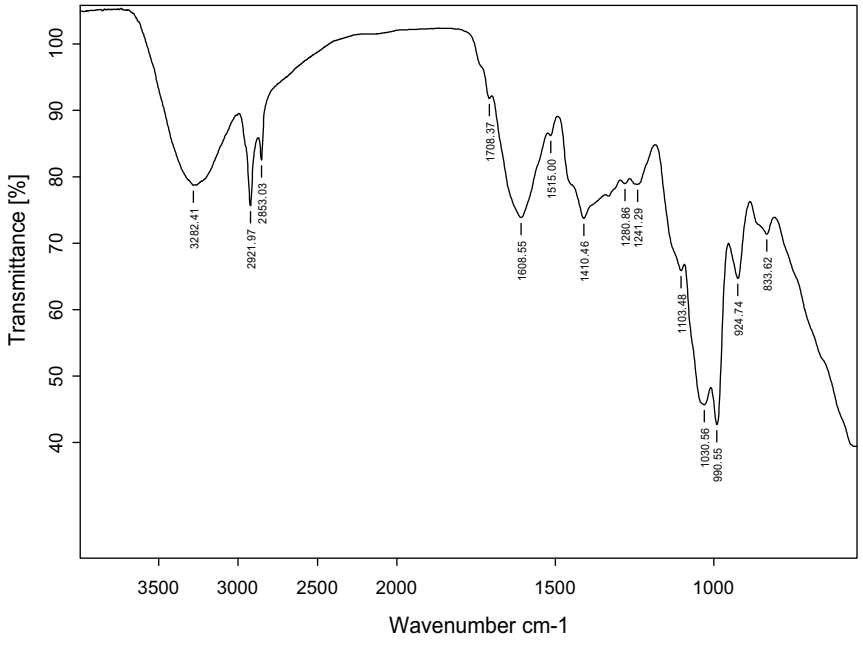

Fig. 2. Infrared spectrum.

Table 4
Serum IL-2, IFN-α and TNF-α.

Group	IL-2	IFN-α	TNF-α
Control	0.41 ± 0.02	50.31 ± 1.65	190.53 ± 3.04
OC model	0.29 ± 0.03##	37.52 ± 1.42##	208.47 ± 10.53##
Diet I	0.33 ± 0.03	44.49 ± 3.06*	197.41 ± 10.03
Diet II	0.45 ± 0.04**	47.21 ± 2.55**	188.31 ± 11.41*
Diet III	0.49 ± 0.03**	59.32 ± 3.61**	180.22 ± 10.02**

$P < 0.01$, OC model vs control.
* $P < 0.05$.
** $P < 0.01$, treatment groups vs OC model.

The changes in the serum IL-2, IFN-α and TNF-α levels were summarized in Table 4. The administration of *L. glycyrrhiza* heteropolysaccharide significantly enhanced the levels of serum IL-2, IFN-α, but reduced TNF-α level and the effect was more pronounced at high dose (0.9%).

TNF-α is a cytokine which is highly expressed in ovarian cancer patients and is known to promote the cancer cell proliferation (Nash et al., 1999). Furthermore, TNF-α is able to induce various cytokines and cytokine receptors having diverse physiological effects on proliferation, invasion, angiogenesis, and immune avoidance in ovarian cancer cells (Kulbe et al., 2005; Nash et al., 1999). The regulatory effect of *L. glycyrrhiza* heteropolysaccharide on TNF-α level in ovarian cancer mice implies that the *L. glycyrrhiza* heteropolysaccharide could modulate improper expression profile of cytokines in the ovarian cancer cells.

Spectra were recorded in the transmission mode between 4000 and 400 cm^{-1} at a resolution of 4 cm^{-1} and analyzed after Fourier transform. A typical infrared spectrum of *L. glycyrrhiza* heteropolysaccharide showed strong absorbances at about 3282, 2921, 1608, 1410 and 990 cm^{-1} and small ones at about 2853, 1708, 1515, 1280, 1241, and 1103 cm^{-1} (Fig. 2). Absorption at 3282 cm^{-1} indicated the presence of OH group. Absorption at 1708 cm^{-1} indicated the presence of C=O group. Carboxylate groups showed two bands: an asymmetrical stretching band near 1708 cm^{-1} and a weaker symmetric stretching band near 1400 cm^{-1}. Two intensive bands at 1410 and 1241 cm^{-1} corresponded to a C–O stretch and C–H or OH bending. The 1241–1000 cm^{-1} region was dominated by sugar ring vibrations overlapping with stretching vibrations of (C–OH) side groups. β-Pyran ring appeared at 924 cm^{-1}.

In conclusion, a typical infrared spectrum of *L. glycyrrhiza* heteropolysaccharide showed strong absorbances at about 3282, 2921, 1608, 1410 and 990 cm^{-1} and small ones at about 2853, 1708, 1515, 1280, 1241, and 1103 cm^{-1}. The 1241–1000 cm^{-1} region was dominated by sugar ring vibrations overlapping with stretching vibrations of (C–OH) side groups. β-Pyran ring appeared at 924 cm^{-1}. We have demonstrated that *L. glycyrrhiza* heteropolysaccharide can enhance serum IgG, IgM, IgA, IL-2, IFN-α levels and reduced TNF-α level. This study has also indicated that *L. glycyrrhiza* heteropolysaccharide may improve immunity activity in nude mice suffering from ovarian cancer.

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