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# Modulation of antiviral cytokine production and lung protection against influenza virus by the glycosides from *Ligustrum purpurascens*

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**Background: Background:** *Ligustrum purpurascens* has been used as a traditional herb for over 2,000 years in China. This study was design to investigate the modulation of antiviral cytokines and reduction in lung inflammation of virus-infected mice by the glycosides isolated from *Ligustrum purpurascens.* **Methods:** *Ligustrum purpurascens* glycosides (LPG) were isolated from the leaves of *Ligustrum purpurascens*. Proliferation of spleen lymphocytes were investigated after LPG treatment. The in vitro and in vivo cytokine modulation of LPG was studied. Furthermore, the anti-inflammatory and antiviral activities of LPG, with the potential to reduce inflammatory lung disorders, were investigated by influenza A virus infected mice. **Results:** LPG could significantly promote the proliferation, and also could stimulate the production of IFN-γ by spleen lymphocytes in a dose-dependent manner. IFN-γ expression level was increased significantly compared to the control and presented a dose-dependent manner in vitro. Furthermore, LPG inhibit the expression of TNF-α and IL-10, which return to normal level in the cyclophosphamide -induced mice model in vivo. Besides, the histopathological analysis indicated LPG reduced acute lung injury in mice infected with influenza virus. **Conclusion:** This study suggested that LPG could increase the exression of IFN-γ, immunoregulation and decrease lung inflammation of virus-infected mice.
**Key words:** *Ligustrum purpurascens*, Glycosides, Cytokines, Immunoregulation, Antiviral

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***Abbreviations*:**

IL, interleukin; IFNs, interferons; TNFs, tumor necrosis factors; LPG, *Ligustrum purpurascens glycosides*; ACN, acetonitrile; OD, optical density; CTX, cyclophosphamide; WBC, white blood cells; HPLC, high performance liquid chromatography.

***Competing interests*:**

The authors declare that they have no conflict of interest.

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## Background

Zijingnvzhen (*Ligustrum purpurascens* (*L. purpurascens*)) is a species of *Ligustrum* genus (Oleaceae) mainly distributed in Southwestern China [1], which is a raw material of Kudingcha for a folk beverage. *L. purpurascens* has been used for herb medicine over 2,000 years in China, and it has been used medicinally against hepatitis, hyperlipidemia, and antivirus [1]. Immunomodulatory effects of extracts of the leaves of *L. purpurascens* have been confirmed in our previous study [2]. The leaves of *L. purpurascens* as tea is now on the market in China. No toxic effects or drug interactions were reported for the use of *L. purpurascens* [2].

Antiviral cytokines including interleukins (ILs), interferons (IFNs) and tumor necrosis factors (TNFs), can orchestrate antiviral innate immune responses. They played very important functions in immune system, and were initially identified as antiviral [3] or antineoplastic agents [4], and they are now regarded as key role in innate and adaptive inflammatory responses [5].

*L. purpurascens* glycosides (LPG) were found to activate both phagocytosis of macrophages and NK cells [2]. In addition, studies have shown that phenylethanoid glycosides have inhibitory effects on HIV-integrase [6] and 5-lipoxygenase [7], and have the anti-bacterial activities [8]. Compared to other clinical immunomodulators or antiviral drugs, LPG has the advantages of safe use, low production cost, no toxic side-effects. In this research, the effect of LPG has mainly focused on the modulation of antiviral cytokines in vitro and in vivo. The potential of alleviating lung injury by LPG was also studied in the viral infected mice based on the modulation of inflammatory cytokines.

## Methods

### Collection of plant material and extraction

The leaves of *L. purpurascens* were collected from Suijiang county, Yunnan province, China, and were authenticated by Peng Hua (The specimen voucher number: NPCR-Lipu-1, deposited in Shenzhen University).

The dried leaves (1.92 kg) of *L. purpurascens* were extracted with hot ethanol (EtOH) (20 L × 3), and the residue obtained by removal of solvent in vacuo was triturated with $H_2O$ (3 L × 4). The insoluble and aqueous phase was parted, and the aqueous phase was directly subjected to chromatography column on the highly porouspolymer Diaion (HP-20, 1.5 L), eluting with $H_2O$, $H_2O$-EtOH (1:1) and EtOH. The eluates with 50% $H_2O$-EtOH (4 L) were concentrated in vacuo for residues LPG (230.4 g).

### Chemical components of LPG

LPG was prepared in methanol (MeOH, 10 mg/mL), and then filtered for high performance liquid chromatography (HPLC) analysis. The HPLC system (Agilent 1260 Infinity Series, USA) used in this research was equipped with a standard binary pump, auto-sampling, and a multiple-wavelength UV-visible detector. HPLC analyses were performed on an Agilent Microsorb MV100 RP18 column (250 mm × 4.6 mm, 5 μm i.d.), and used ultraviolet absorption at 220 nm. A volume of 10 μL of LPG was analyzed at 25 ℃ using the following gradient: 0–5 min: 5% acetonitrile (ACN), 5.01–40 min: 5–100% ACN, 40.01–48 min: 100 % ACN, 48.01–50 min: 100–5 % ACN, 50.01–52 min, 5% ACN. LPG was then dissolved in methanol and then conducted to semi-HPLC to obtain 9 known compounds.

### Isolation of splenic lymphocytes

Spleen collected under aseptic conditions in 4 mL of lymphocyte separation medium (Dakewe, China), was minced using a pair of scissors and passed through a fine nylon mesh to obtain a homogeneous cell suspension and the erythrocytes were lysed with the medium. Then, transferred the medium to 15 mL of centrifuge tube, covered with 500 μL of RPMI 1640 medium (Hyclone, USA). After centrifugation (800 ×g at room temperature for 10 min), the lymphocytes layer under the 1640 medium was sucked out. The lymphocytes were washed with 10 mL of 1640 medium and resuspended in complete medium (RPMI 1640 supplemented with 10% FCS) (Hyclone, USA). The cell number was counted with a hemocytometer by the trypan blue dye (Sigma, USA) exclusion technique and adjusted to desired concentration for further use. Cell viability exceeded 95%.

### Collection of peritoneal macrophages

A volume of 5 mL of RPMI 1640 was injected into the peritoneal cavity of mice. After 5 min, the medium was taken out and centrifuged at 1800 ×g for 10 min at 4 °C. The cell pellet was re-suspended in RPMI 1640 medium. Macrophages ($3 \times 10^6$ cells) were seeded in 24-well culture plate in a $CO_2$ incubator for 4 h. At the end of incubation period, non-adherent cells were removed and plates were further incubated for 48 h in the presence of different studied reagents. Supernatants were collected after centrifugation and kept at −80 °C for the measurement of cytokines.

### Cell proliferation assay

Cell proliferation was analyzed using a cell count kit (CCK-8, sigma, USA). Lymphocytes isolated from spleen of mouse were seeded at a density of $2 \times 10^4$ cells/well in 96-well microplates. The cells were exposed to different doses of LPG or vehicle for control for 24 h at 37 ℃ in humidified atmosphere air containing 5% $CO_2$. After 24 h incubation, 10 μL of CCK-8 reagent was added, and incubated for 2.5 h at 37

°C. A microplate reader (Biotek, USA) measured optical density (OD) at 450 nm. Each concentration was assayed 3 times and 6 additional controls were used in each test.

**Assay for cytokines by ELISA**

The amount of cytokines in the supernatant or serum was measured using mouse IL-2 (Dakewei, 1210202), IL-10 (Dakewei, DKW12-2100-096), IL-12 (Abcam, ab100699), IL-15 (Abcam, ab100701), TNF-β (BD, 560478), IFN-α (Abcam, ab252352), IFN-β (Abcam, ab252363) and IFN-γ (Dakewei, 2210003) ELISA sets according to the manufacturer's instructions. Cytokine concentrations were calculated using the standard curve obtained for each ELISA plate.

**Immunosuppressive animal model and treatment**

The in vivo study was conducted on male Kunming mice (18–22 g). All mice were purchased from the Animal Supply Center of Guangdong Academy of Medical Science, Guangzhou, China. Animals were divided into 6 groups with 12 animals each group. Cyclophosphamide (CTX)-induced immunosuppressed mice model can be developed by treatment of CTX [9]. Before the treatment, all animals received CTX (80 mg/kg, i.p., Tonghua pharmaceutical, China) on the 1st, 3rd, 5th, 7th, 9th day to establish the immunosuppressive animal model, except group 1 which received normal saline as the normal control. Group 1 (control group) and group 2 (CTX treated control) received the vehicle (sterile water, p.o.) for a period of 10 days. Groups 3 (positive group) were given levamisole (100 mg/kg, p.o., Huanan pharmaceutical, China) daily for 10 days. Groups 4–6 were given the test LPG (200, 400 and 800 mg/kg separately, p.o.) daily for 10 days. On the 10th day, 4 h after the administration of the respective treatment, blood samples were collected for further experiment.

**Measurement of IL-2, IL-10, TNF-α, IFN-α, IFN-β and IFN-γ in mice serum**

Serum was collected 4h after the final oral administration of reagents. IL-2, IL-10, TNF-α, IFN-α, IFN-β and IFN-γ concentration were measured by enzyme-linked immunosorbent assay according to the instructions of the manufacturer.

**Peripheral blood white blood cells count**

On the 10th day after treatments, blood was collected from eyeballs of mice and white blood cells (WBC) were counted by automated blood cell counter (Sysmex, Japan).

**Immune organ indices**

The thymus and spleen were calculated according to the following formula: organ indices (mg/g) = (weight of spleen/body weight).

**Virus challenge and treatments in vivo**

Mouse-adapted influenza virus (A/FM/1/47 H1N1, FM1) was kindly donated by the Institute of Tropical Medicine, Guangzhou University of Chinese Medicine. All tests were performed in class II biosafety cabinets in P2 lab.

Male C57BL/6 mice (20 ± 2 g) were maintained under the control conditions of temperature (22 ± 2 °C) and humidity (50 ± 5%), with a 12 h light-dark cycle for acclimatization. The Animal Care and Use Committee of Shenzhen University approved all experimental animals (SYXK(yue)2018–0002).

Diethyl ether was used for the mice anesthesia. Mice were infected with mouse-adapted H1N1 virus (FM1; $5LD_{50}$ intranasal, 25 μL/nare). Infected mice were divided into 4 groups for treatment (20 mice each): LPG (200, 400 and 800 mg/kg) and positive drug (ribavirin, 100 mg/kg). The mice were orally administrated respectively for 2 weeks. Control and model groups received equivalent amounts of sterile water.

**Histopathology**

After challenge, the animals were sacrificed, the lungs were excised immediately, inflated and fixed with 4% paraformaldehyde. Fixed sections (8 mm) of paraffin-embedded lungs were stained with hematoxylin and eosin staining. Slides were randomized, read blindly, and examined for tissue damage necrosis, and inflammatory cellular infiltration [10].

**Statistical analysis**

All experiments that were performed in triplicate had statistical analysis performed using a t-test or one way analysis of variance (ANOVA) with GraphPad Prism 5.0 (GraphPad Software, California USA). *P*-values of $< 0.05$ were considered statistically significant.

## Results

**Chemical components of LPG**

The chemical profile of LPG was analyzed by HPLC, which was shown in Figure 1, and the major constituents were glycosides (compounds 1 to 9). The known compounds 1–9 were identified as angoroside A (1) [11], acteoside (2) [12, 13], ligupurpuroside A (3) [12, 13], isoacteoside (4) [14], ligurobustoside D (5) [15]), ligupurpuroside D (6) [16], ligurobustoside Q (7) [16], osmanthuside B (8) [12, 13], ligupurpuroside B (9) [17] by comparison of their NMR and MS data with those in the literature. Compounds 1–9 were marked correspondingly in the HPLC chromatogram of LPG (Figure 1).

**Effect of LPG on the splenic lymphocyte proliferation**

LPG on spleenic lymphocyte proliferation was studied by CCK8 assay. Cell viability was significantly increasing as dose dependent at 24 h of exposure at all

LPG concentrations tested (Figure 2). LPG showed non-cytotoxicity to the lymphocyte of mouse.

**Cytokine productivity in vitro**

In vitro induction of cytokines in spleenic lymphocyte after LPG treatment was performed by the previously reported method [2]. LPG showed no effects to induce IFN-α and IFN-β at 25–150 μg/mL. There was no significant difference between various doses of LPG and media group with TNF-β (LPG, 25–150 μg/mL, 140–165 pg/mL; control, 156 pg/mL; $P > 0.05$). While, the level of IL-2 in the supernatant of splenocytes was decreased to 10 pg/mL compared with control of 50 pg/mL by treatment of LPG (25 μg/mL). LPG had significantly decreased concentration of IL–12 to 24 pg/mL by treatment of LPG at 25 μg/mL compared with media control of 230 pg/mL ($P < 0.05$) in vitro. As shown in Figure 3, IFN-γ levels in the cell culture supernatant were significantly increased by the treatments of LPG. LPG alone could stimulate the production of IFN-γ with around 10 times higher than in the control group.

**Effect of LPG on serum cytokine concentration**

To further elucidate the cytokine modulation effect of LPG, cytokine levels in the sera of immunosuppressive mice were determined by ELISA. Initially, we evaluated the in vivo effect of short-term oral administration of LPG on cytokines including IL-2, IL-10, TNF-α, IFN-α, IFN-β and IFN-γ. Ten days after CTX treatment, as shown in Figure 4, the level of TNF-α and IL-10 in model control were increased when treated with CTX as compared with normal control, which suggested the severity of the immunosuppression state. The administration of LPG could decrease the levels of TNF-α and IL-10 when compared with model control to reduce inflammation (Figure 4B and C). No significant changes were observed on IL-2.

Compared with model control, the LPG-treated (200 mg/kg, 400 mg/kg and 800 mg/kg) group secreted less IFN-α, however, significantly down-regulated the production of IFN-α by 30.3%, 29.9% and 48.5% respectively ($P < 0.05$), compared with control mice (Figure 4D). However, levamisole and LPG-treated (400 mg/kg) mice showed significantly higher level of IFN-β than normal mice or model mice (Figure 4E). The data suggested that LPG may interfere with the secretion of cytokines in some specific ways.

**Effect of LPG on immune organ indices in immunosuppressed mice**

The spleen and thymus indices were considered to directly reflect the immune capabilities [18]. Thus, the spleen and thymus indices of the normal and experiment mice were listed in Table 1. Compared with the normal group, the spleen and thymus indices of the CTX-treatment group were significantly decreased. However, when treated with LPG, the relative spleen and thymus indices remarkably increased, which were most obvious in the concentration of 400 and 800 mg/kg treatment group. The results indicated that LPG could alleviate the immune organ injury induced by CTX.

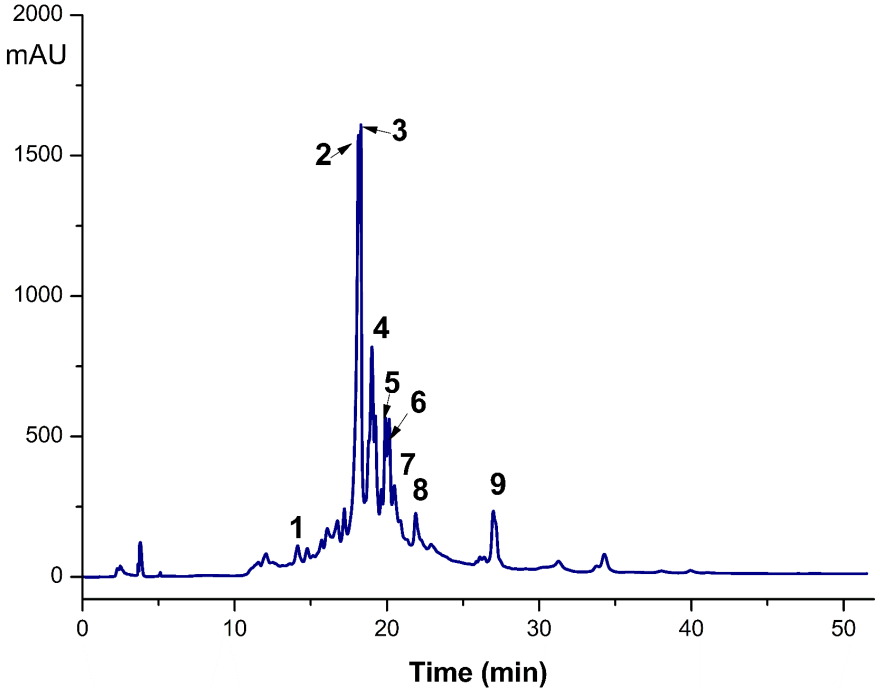


**Figure 1 HPLC-DAD (220 nm) finger printing of the LPG from Ligustrum purpurascens.** Compounds 1–9 were marked correspondingly to the peaks in the HPLC chromatogram. Compounds 1–9: angoroside A (1), acteoside (2), ligupurpuroside A (3), isoacteoside (4), ligurobustoside D (5), ligupurpuroside D (6), ligurobustoside Q (7), osmanthuside B (8), ligupurpuroside B (9). HPLC, high performance liquid chromatography; LPG, *Ligustrum purpurascens glycosides*.

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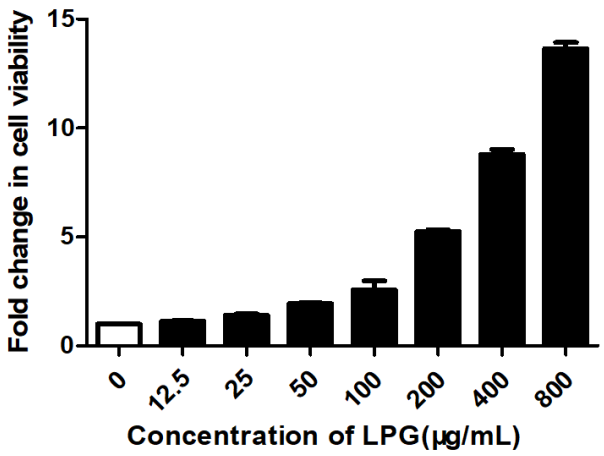


**Figure 2 LPG induced proliferation of lymphocytes in a concentration-dependent.** Cell viability of lymphocytes was determined by CCK8 assay after stimulation with various concentrations of LPG at 24 h. The cell viability was calculated by the following equation: OD450nm sample/OD450nm control (cell treated with PBS). Data are shown as mean ± SD (n = 6). LPG, *Ligustrum purpurascens glycosides*.

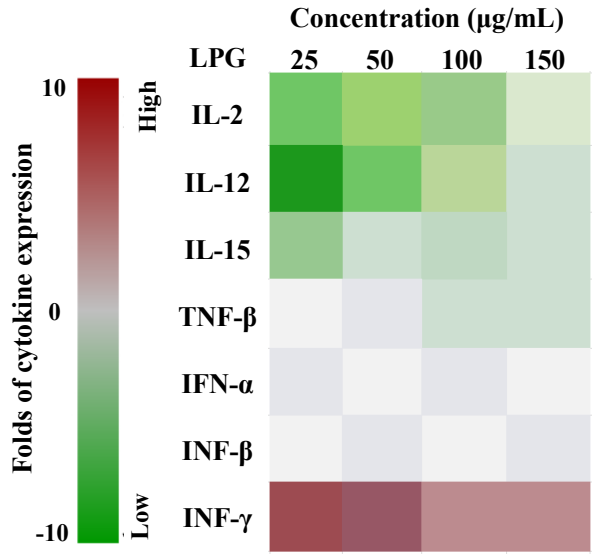


**Figure 3 Heat map displaying cytokine fold changes by LPG at 200 μg/mL for all primary cells assessed.** Up-regulation is represented with deep red shading and down-regulation with green. Expression varies from −10 to 10. LPG, *Ligustrum purpurascens glycosides*.

### Effect of LPG on hematological indices in immunosuppressed mice

The number of WBC is known as an indicator to reflect the body's immune function [19]. As shown in Table 2, compared with the normal group, the numbers of peripheral blood WBC were significantly decreased in the CTX group. When treatment of LPG, the numbers of peripheral blood WBC were significantly increased by all dosage of LPG. Therefore, LPG could ameliorate CTX-induced immunosuppression.

### LPG reduces lung inflammation of influenza virus-infected mice

Infiltration of inflammatory cells into alveolar walls with focal areas of consolidation were observed in the lungs of mice on day 7 post infeiction with influenza virus (Figure 5B). While, the structure of alveolar cells and pulmonary alveolar capillary endothelial were maintained normal.in the control group (Figure 5A). Figure 5B showed that a large number of infiltrating inflammatory cells such as mononuclear cells could be observed in the alveoli space of model group. Ribavirin treatment mice had no inflammation (Figure 5C). Mice recieved with different dosages of LPG had minimal lung mononuclear cell infiltrate in the alveoli at day 7 post infection (Figure5 D–F). Only a few small areas of lung showed inflammatory mononuclear cell infiltration in the alveoli of mice administered with LPG (800 mg/kg). LPG may ameliorate lung lesions in mice with acute respiratory distress syndrome.

## Discussion

The antiviral cytokines, mediating the establishment of antiviral state and active immune cells against infection, divided into 2 types: direct antiviral and indirect antiviral cytokines [3]. Interferons, interleukins and tumor necrosis factors are important antiviral cytokines produced in the innate immune response against virus infection. IFNs are the direct antiviral cytokines as the first line of defense against virus [20, 21]. For instant, IFN-γ has potent antiviral activity against HCV in the subgenomic replicon system [22]. Besides, IFN-γ has the most potent immunomodulatory activity of all the interferons.

In present study, we found that LPG strongly increased the production IFN-γ in vitro. It may indicate that LPG might have immunoregulation and beneficial effects in the treatment of various virus infection diseases. With 48 h culture, expression levels of IL-2, IL-12 and IL-15 were lower than the medium-control. The amount of IFN-γ were raised and no alteration of TNF-β secretion were observed. The up-regulation of IFN-γ was not dependent on the IL-12 way [23]. These data indicate that the stimulated production patterns of cytokines differ from the concentrations of LPG.

Cyclophosphamide, as a chemotherapeutic agent, is widely used in immunosuppressive mice model in drug discovery [24]. We study the regulation of TNF-α, IL-10, IFN-α, IFN-β and INF-γ in immunosuppressive mice serum treated with LPG. The data showed that LPG treatment could increase the amounts of IFN-γ to normal in CTX-induced immunosuppression mice (Figure 4F). The results showed that TNF-α and IL-10 were strongly augmented after the administration of CTX compared to the other cytokine production. When the mice were treated with CTX, with anti-inflammatory properties, IFN-γ were inhibited in the

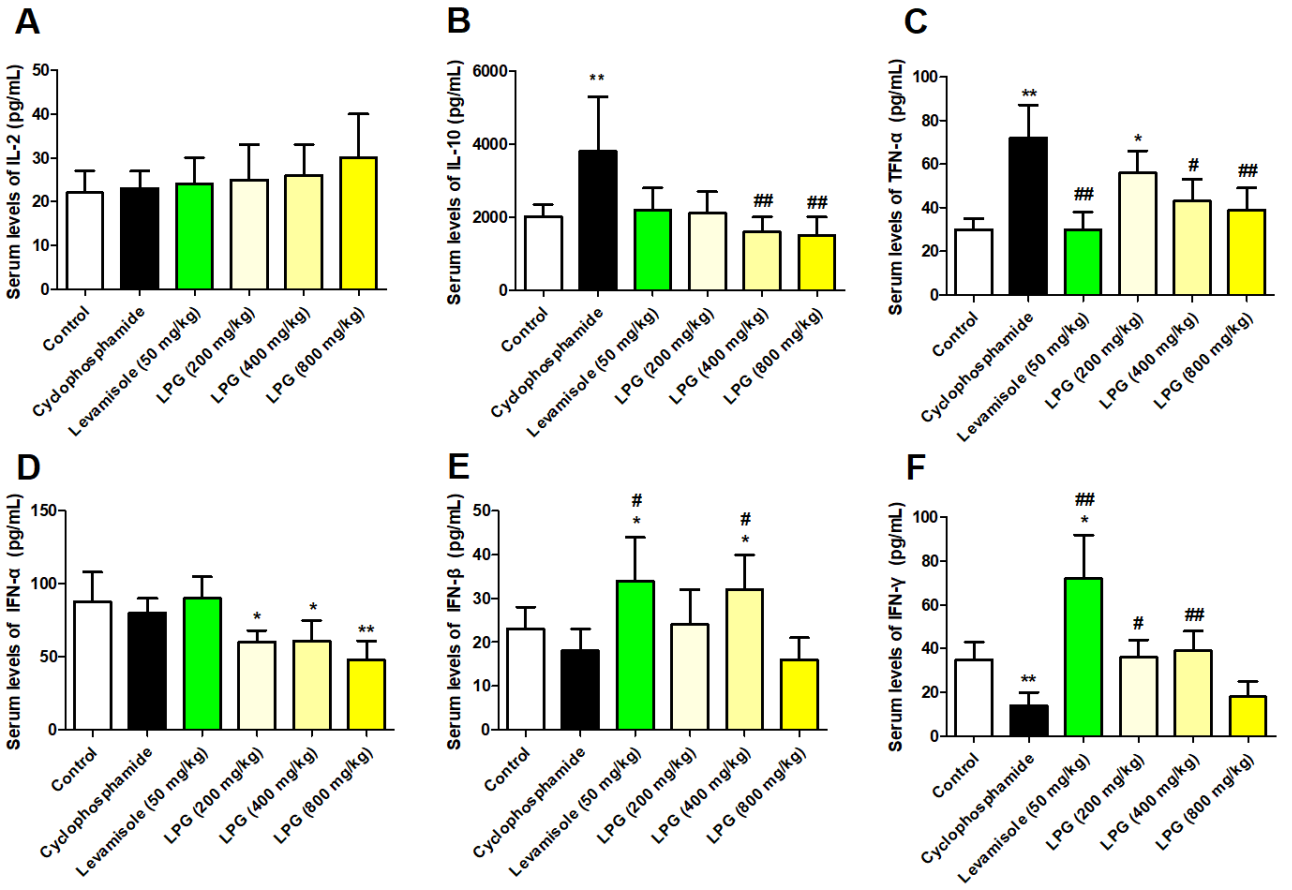


**Figure 4 LPG regulates cytokines (IL-2, IL-10, TNF-α, IFN-α, IFN-β and IFN-γ) production in mouse serum.** The concentration of cytokines in pg/mL were determined using ELISA. Data are mean ± SD of at least five animals. *, $P < 0.05$ and **, $P < 0.01$ compared with control group determined by one-way ANOVA. #, $P < 0.05$ and ##, $P < 0.01$ compared with model group determined by one-way ANOVA. LPG, *Ligustrum purpurascens glycosides*.

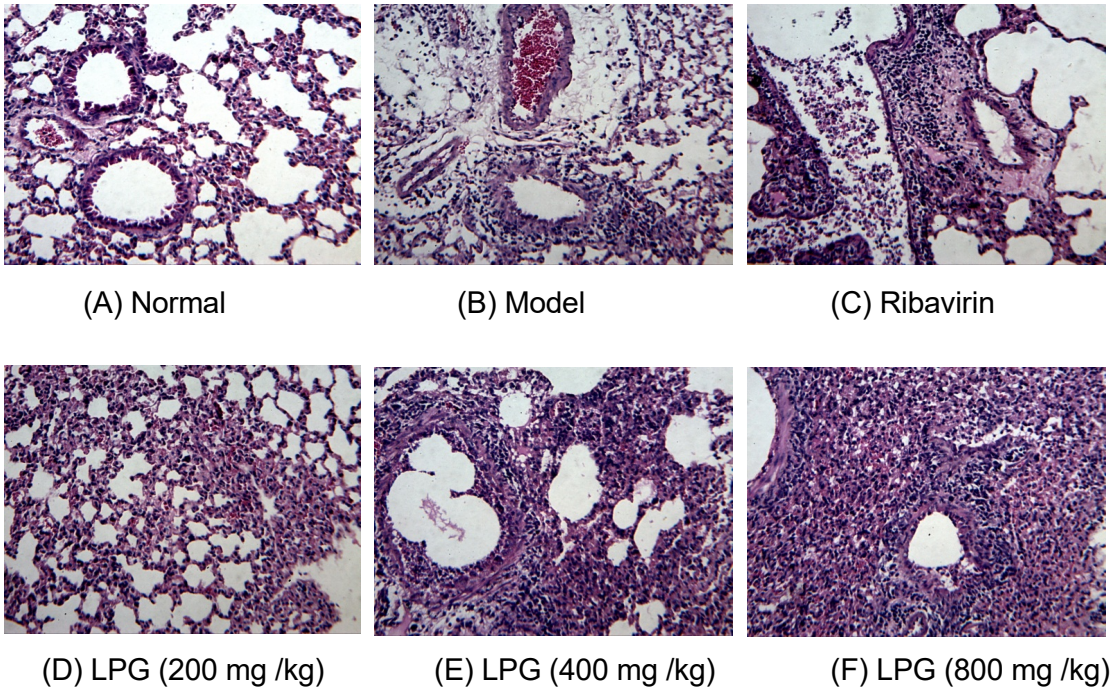


**Figure 5 The images of histopathological change of lungs from a representative animal in treatment group are shown.** Control group lung (A); infected group lung (B); ribavirin group (100 mg/kg) lung (C); LPG group (200 mg/kg, 400 mg/kg and 800 mg/kg) lung (D, E and F). LPG, *Ligustrum purpurascens glycosides*.

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**Table 1 Effects of LPG on immune organ indices in immunosuppressive mice**

| Group | Organ indices（mg/g） | |
|---|---|---|
| | Thymus | Spleen |
| Normal | 1.69 ± 0.68[a] | 3.26 ± 0.32[a] |
| Model (CTX) | 1.25 ± 0.22[b] | 2.02 ± 0.23[b] |
| Positive (levamisole, 100 mg $kg^{-1}$ $day^{-1}$) | 1.73 ± 0.45[a] | 3.03 ± 0.71[a] |
| CTX + LPG (200 mg $kg^{-1}$ $day^{-1}$) | 1.29 ± 0.58[b] | 2.17 ± 0.32[b] |
| CTX + LPG (400 mg $kg^{-1}$ $day^{-1}$) | 1.68 ± 0.34[a] | 3.12 ± 0.41[a] |
| CTX + LPG (800 mg $kg^{-1}$ $day^{-1}$) | 1.72 ± 0.58[a] | 3.20 ± 0.22[a] |

LPG, *Ligustrum purpurascens glycosides*; normal, normal; CTX, cyclophosphamide. Data are presented as means ± SD (n = 12). Different letters for same index among the groups represent significant differences at $P < 0.05$.

**Table 2 Effects of LPG on the number of peripheral blood WBC**

| Group | WBC ( × $10^9$/L) |
|---|---|
| Normal | 2.72 ± 0.68[a] |
| Model (CTX) | 1.48 ± 0.52[b] |
| Positive (levamisole, 100 mg $kg^{-1}$ $day^{-1}$) | 2.83 ± 0.45[a] |
| CTX + LPG (200 mg $kg^{-1}$ $day^{-1}$) | 2.42 ± 0.48[a] |
| CTX + LPG (400 mg $kg^{-1}$ $day^{-1}$) | 2.48 ± 0.35[a] |
| CTX + LPG (800 mg $kg^{-1}$ $day^{-1}$) | 2.73 ± 0.58[a] |

LPG, *Ligustrum purpurascens glycosides*; normal, normal control; CTX, cyclophosphamide; WBC, white blood cell. Data are presented as means ± SD (n = 12). Different letters for same index among the groups represent significant differences at $P < 0.05$.

immunosuppressive mice. The increase of TNF-α and IL-10 may be related to the severity of the immunosuppression state [24].

IL-10 is originally produced by Th2 subset cells, and also known as cytokine synthesis inhibitory factor [25, 26]. In this study, we found that the expression of IL-10 in mouse serum administrated with CTX was very high. Our data are consistent with the fact that IL-10 strongly above the control levels in immunosuppressive state [26]. Another interesting finding is that LPG have an inhibitory effect mostly on TNF-α (dose-dependent in the first 10 days) and a partial effect on IL-10 (reduced significantly only by 800 mg/kg). The ability of LPG to inhibit TNF-α and IL-10 more than the other cytokines, may be due to its capacity to affect mononuclear cells, T and B cells. The data presented here show that LPG are effective at 200 mg/kg on TNF-α production, while for IL-10, higher concentrations are needed. It is possible that LPG inhibits only the pro-inflammatory cytokines and not the anti-inflammatory cytokines.

The activation of splenic lymphocytes in infection may lead not only to the production of the above-mentioned cytokines, but also to the generation of IFN-γ which is reported to be efficient in killing microorganisms [27]. Others reported that IFN-γ from Th1 cells inhibited the development of Th2 cells as well as humoral responses. In this study, LPG increased IFN-γ back to normal levels.

The immune system is made up of entire organs (spleen and thymus), immune cells (lymphocytes and macrophages), and immune molecules (cytokines) [28]. The immune organs work together to help the body maintain homeostasis. However, chemotherapy drugs can damage this balance and cause immune disorder. The spleen and thymus, as the important immune organs, are closely related to cellular immunity and humoral immunity. Therefore, the thymus and spleen indices can reflect the strength of the body's immune function [29]. LPG treatment could alleviate the atrophy of immune organs in immunosuppressed mice, and significantly increased the spleen and thymus indexes in a dose-dependent manner compared with those of CTX treatment group. On the other side, WBC plays an important role in defense system of body, which are part of the immune system. The change of WBC amount is one of diagnostic criterias in immunocompromised diseases and acute infectious diseases [30]. The results in our study revealed that LPG could increased WBC counts of immunocompromised mice treated with CTX. Thus, phenylpropanoid glycoside from *L. purpurascens*, at some extent, have been shown to possess immune-enhancing effects.

In addition, on HE staining of influenza virus infected mice, lesions accompanied inflammatory cell infiltration occurred in the infected group. LPG treatment reduced the inflammation in infected mice. Therefore, LPG may alleviate the development of inflammation.

## Conclusion

Our studies are concerning the antiviral cytokines which are induced by natural constituents from glycosides obtained from *L. purpurascens* and whether enhance the

body's antiviral capability. LPG enhances the innate and adaptive immunity via up-regulating endogenous IFN-γ, which is essential for LPG mediated protection during viral infection. Therefore, this study provided *in vivo* evidences that LPG effectively improved immune function in CTX-treated mice, which implying that LPG could be a suitable functional food additive or a potential source of immunologic adjuvant in antiviral treatment.

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