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The *in vitro* immunomodulatory activity of a polysaccharide isolated from *Kadsura marmorata*

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ABSTRACT

Kadsura marmorata has been used in traditional Chinese medicine as one of the major ingredients for the treatment of bronchial asthma, eczema, and acute and chronic infection due to either viruses or bacteria. The purpose of this study was to determine the immunomodulatory activities of the *Kadsura* polysaccharide (KPS) that was isolated and purified from the fruit of *K. marmorata*, designated as KPSIII and has a molecular weight of 130,406. The immunomodulatory effects of KPSIII were evaluated *in vitro* on chicken lymphocytes and macrophages. The results indicated that KPSIII could stimulate the cell proliferation of chicken T-lymphocytes, B-lymphocytes, and peritoneal macrophages and could significantly enhance cytokine secretion. Moreover, KPSIII regulated cytokine expression in T-lymphocytes and peritoneal macrophages. This study suggests that KPSIII has potential effects on regulating the immune system and might be an immunotherapeutic agent in treating various immunity-related diseases in chickens.

1. Introduction

Polysaccharides are becoming increasingly important as sources of pharmacotherapeutics, either as herbal drugs for the treatment of chronic diseases or as raw materials from which compounds with particular biological activities are isolated. More than 60% of newly approved anti-cancer and anti-HIV drugs are derived from natural sources (Cragg, Newman, & Snader, 1997; Zhang & Zhang, 1994), including *Kadsura* plants. *Kadsura marmorata* (*K. marmorata*) is a natural species belongs to genus *Kadsura*, subfamily Schisandraceae within the family Magnoliaceae of order Illiciales (Bi, 2003; Greuter et al., 2000). The *K. marmorata* is found in the Palawan region of the Philippines (Bi, Lin, Liu, & Zhao, 2002) and the southeastern and northwest regions of the Yunnan and Guizhou provinces in China. Though it is not a well-known herb within the *Kadsura* genus, *K. marmorata* has been widely used in traditional Chinese medicine as one of the major ingredients for the treatment of bronchial asthma, eczema, and acute and chronic infections (both viral and bacterial) (Li, 2004).

The *Schisandra chinensis* polysaccharides (SCP) were reported as having many pharmacological effects, including hepatoprotection (Gao, Piao, Guo, Sui, & Ren, 1996), immunomodulation (Bhavan, 1992; Khajuria et al., 2007; Smit et al., 2000), anti-aging (Sun, Lv, Yu, Lv, & Wang, 2001), antitumor (Yu, Li, & Wang, 2010), antioxidative (Huang, Chen, & Zhang, 2005; Xie, Zhan, Zhang, & Yang, 2002), antidiabetic (Cai & Han, 2010), and anti-atherosclerosis (Gao, Meng, & Li, 2008; Wang, Hou, Ye, Liu, & Sun, 2009) effects. However, few studies have focused on *K. marmorata* and the *Kadsura* polysaccharide (KPS).

The chicken is the most numerous livestock species in the world, with approximately 50 billion birds produced every year as a source of food, for both their meat and their eggs. In order to meet food security concerns, poultry production will increase dramatically in the next few decades. The current and increasing intensity of poultry production is only possible through the use of vaccination, with multiple vaccines being given during the short, five-week or less, life of a commercial broiler chicken (Sharma, 1999). To help the chicken establish strong and effective immune responses to vaccines in a shortest time frame, we are interested in investigation on the immune modulatory, enhancer or vaccine adjuvant for chicken.

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We have reported for the first time the isolation and structural description of nine compounds derived from the fruit of *K. marmorata* (Wang, Wu, Zhou, Deng, & Wang, 2012). In this study, we successfully isolated and purified a polysaccharide named *Kadsura* polysaccharide III (KPSIII) from the fruit of *K. marmorata*. The immunomodulatory effects of the KPSIII on chicken lymphocytes were also investigated.

2. Materials and methods

2.1. Isolation and purification of KPSIII

Fruiting bodies of *K. marmorata* were obtained from the Guizhou Traditional Chinese Medicine Hospital (Guizhou, China). They were identified by Dr. Qishi Sun, Professor of Traditional Chinese Medicine Identification Section at the Shenyang Pharmaceutical University, Shenyang, China, as coming from Schisandraceae *K. marmorata*. The KPS was extracted and isolated from *K. marmorata* as previously described (Wang et al., 2013) and subjected to an additional purification with Sephadex G-25 (2.5 cm × 160 cm), resulting in the yield of a single compound, designated as KPSIII. This compound was subjected to chromatography and acetate membrane electrophoresis to analyze its composition.

2.2. Determination of the molecular weight for KPSIII

To determine the molecular weight of KPSIII, six molecular weight reference glucans (T-10, T-20, T-40, T-70, T-110 and T-500) (Sigma, MO, USA), were used as standards. All of the standards and samples were loaded onto TSK-Gel G2000PW columns (TOSOH Company, Japan) and run on a HITACHI HPLC Pump system (HITACHI, Japan) equipped with a Refractive index detector (Shodex RI SE-51, Japan) in 0.2 mol/L phosphate buffer (pH 6.0) at a flow rate of 0.5 mL/min, and monitored at 206 nm. Air flow volume (V_o), which was determined by the retention time of blue glucan-2000, column bed volume (V_t), which was measured by the retention time of glucose, and elution volume (V_e), which was assessed by the retention time of either the reference glucan or the sample, were recorded to calculate the distribution coefficient (Kav) and logarithmic molecular weight (lgM) according to the two formulas listed below. This allowed for the generation of a standard curve. The molecular weight of KPSIII was then calculated based on the standard curve.

$$\mathrm{Kav} = \frac{V_e - V_o}{V_t - V_o} \quad (1)$$

$$\mathrm{Kav} = K_1 - K_2 lg\mathrm{M} \quad (2)$$

2.3. Isolations of T-lymphocytes, B-lymphocytes and peritoneal macrophages from chickens

Specific-pathogen-free Hy-line variety brown chickens (male, 7–14 days old, weight in 100–150 g) were obtained from the Experimental Animal Center of Liaoning Medical University (Jinzhou, Liaoning province, China) and housed in standard conditions of temperature, humidity and light. Animal experiments were approved by the Experimental Animal Center of Liaoning Medical University and performed in accordance with the guidelines for the care and use of laboratory animals published by the US National Institutes of Health.

Chickens were sacrificed, and a single splenocyte suspension was prepared as previously described (Liu et al., 2008, 2012) with few modifications. Briefly, spleens were aseptically removed and gently smashed on a sterile plastic strainer. Red blood cells were lysed with an RBC lysis buffer, and the splenocytes were washed twice with PBS, followed by resuspension in complete DMEM medium (DMEM media supplemented with 10% fetal bovine serum, 2.5 mM L-glutamine, 0.031 mg/mL pyridoxine hydrochloride, 100 IU/mL penicillin, 100 IU/mL streptomycin and 15 mM HEPES). Approximately 5×10^6 splenocytes were seeded in each well of a 6-well plate and incubated in 5% CO_2 at 37 °C for 2 h. The supernatant, together with the non-adherent cells, was collected by centrifugation at 1000 rpm for 10 min. T-lymphocytes and B-lymphocytes were isolated from the splenocyte suspensions by a nylon fiber column (Kisker GbR, Germany) (Trizio & Cudkowicz, 1974) and spun down. The cell pellets were resuspended in complete DMEM medium and adjusted to 5×10^5 cells/mL.

2.4. Proliferation of T-lymphocytes, B-lymphocytes and peritoneal macrophages

The 96-well flat-bottom microplates were rinsed three times with PBS prior to seeding with 1×10^5 cells into each well. Different concentrations of KPSIII (0 μg/mL, 50 μg/mL, 100 μg/mL, and 150 μg/mL) were added to the medium either alone or together with 0.5 mg/mL cyclophosphamide (CTX, Sigma, MO, USA) and 5 μg/mL concanavalin A (Con A, Sigma, MO, USA), 10 μg/mL phytohaemagglutinin (PHA, Sigma, MO, USA) or 20 μg/mL lipopolysaccharide (LPS, Sigma, MO, USA) and cultured for 68 h. The MTT assay was used to evaluate cell proliferation. Briefly, 20 μL of MTT solution (5 μg/mL) was added to each well after removing the medium. After incubating for 4 h, the purple formazan crystals were solubilized by adding 100 μL of DMSO. The optical density was measured at 599 nm using a microplate reader (Tecan, Austria). Peritoneal macrophages suspensions were prepared as previously described (Cheng, Wan, Wang, Jin, & Xu, 2008; Lee, Ryu, & Byun, 2005). The proliferation of peritoneal macrophages was processed and measured as described above. Each treatment was performed in quadruplicate.

2.5. Detection of cytokines

The 96-well flat-bottom microplates were rinsed three times with PBS prior to seeding 1×10^5 of either T-lymphocytes or peritoneal macrophages in 200 μL culture medium to each well. Different concentrations of KPSIII plus either 5 μg/mL Con A or 10 μg/mL LPS were added to each well and then incubated in a 5% CO_2 incubator at 37 °C for 48 h. The supernatants from the T-lymphocyte cultures were collected to measure IL-2 and IFN-γ expression levels, whereas the macrophage culture supernatants were collected to detect TNF. An ELISA max kit (Biolegend, USA) was used to measure cytokine expression levels according to the manufacturer's instructions. Briefly, each well of the ELISA microplate was coated with chicken IL-2, IFN-γ, and TNF-α ELISA capture antibodies and incubated overnight at 4 °C. After blocking with 5% nonfat milk in PBS, the culture supernatants and the standards were added to the wells and incubated at room temperature (RT) for 2 h. Biotinylated chicken IL-2, IFN-γ, and TNF-α detection antibodies were added to each well after washes and incubated at RT for 30 min. An HRP–avidin solution was added to the plates and incubated for 30 min at RT, followed by the addition of 100 μL of TMB substrate solution after several washes to develop for 10 min. A 50 μL stop solution (2 N H_2SO_4) was added to halt the reaction, and the absorbance was measured at 450 nm. The cytokine expression levels were calculated based on the linear portion of the standard curve (Byun, Ryu, & Lee, 2006; Cheng et al., 2008; Lee et al., 2005).

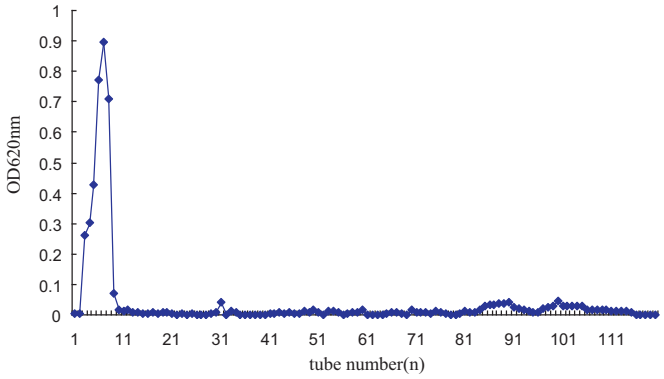

Fig. 1. Purification and composition analysis of KPSIII. Approximately 200 mg of KPSIII was dissolved in 2 mL distilled H_2O and loaded onto a Sephadex G-25 column, which was gradient-eluted and monitored at an OD of 206 nm. The eluent was comprised of distilled H_2O, 0.01 mol/L $NaHCO_3$, and 0.05 mol/L $NaHCO_3$ respectively. For each tube, 4.0 mL of eluent was collected into at a flow rate of l.0 mL/min.

2.6. Statistical analysis

All data were analyzed by the SPSS 11.5 statistical software and reported as the mean ± SD. Significant differences were examined by independent one-way variance analysis (one-way ANOVA) and the Pearson correlation test. A *P*-value of less than 0.05 was considered to be statistically significant. A *P*-value of less than 0.01 was considered to have a high degree of statistical significance.

3. Results

3.1. Isolation and purification of KPSIII

A total of 688 grams of KPS was isolated from 20 kg of *K. marmorata* fruit powder after extraction and precipitation processing. The KPS isolated was precipitated with 20%, 40% and 60% ethanol consequently. Around 52.31% KPS was precipitated under 60% ethanol. This portion of KPS was subjected a further purification with Sephadex G-25 (2.5 cm × 160 cm), resulting in the yield of single compound and named as KPSIII. Liquid chromatography analysis showed only one elution peak (Fig. 1), suggesting that this KPSIII was a single compound (Qin, YU, Ning, & Lin, 2005). Additionally, there was a single positive signal observed on the membrane after electrophoresis (Fig. 2), suggesting that the polysaccharide KPSIII obtained in this study was a single compound with high purity (Song, 2007; Wang et al., 2013).

3.2. Molecular weight of KPSIII

Our results showed that the retention time of blue glucan-2000 (V_o) was 20.65 min and that the retention time of glucose (V_t) was 40.12 min. All of the other parameters of the reference glucans V_e, lgM, and Kav are shown in Table 1. A standard curve (Fig. 3) was generated using the statistic software SPSS version 17.0 to analyze the data acquired. The linear regression equation Kav = 1.3409–0.1798 lgM, R^2 = 0.9994, was also obtained. For the samples of KPSIII, the average retention time was 28.85 min; the molecular weight was thus calculated using the two known equations to be 130,406 Da.

Table 1
V_e, lgM, Kav of reference glucans.

Reference glucan	T-10	T-20	T-40	T-70	T-110	T-500
Mw	10,400	21,600	44,400	68,500	110,000	450,000
lgM	4.017	4.334	4.647	4.836	5.041	5.653
V_e/min	32.82	31.73	30.65	29.66	28.43	27.38
Kav	0.625	0.569	0.514	0.463	0.400	0.346

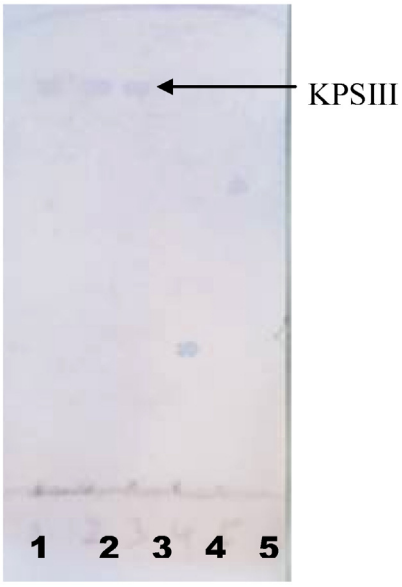

Fig. 2. Acetate membrane electrophoresis of KPSIII. The purity of KPSIII was analyzed by acetate membrane electrophoresis in 0.05 mol/L borax and 0.2 mol/L sodium hydroxide solution (pH 10.0). The acetate membrane was stained with 0.5% water-soluble aniline blue for 10 min and rinsed with water for 5 min after electrophoresis. Lanes 1–5 represent different KPS fractions that were isolated by ethanol precipitation. The arrow designates KPSIII.

3.3. KPSIII enhanced the proliferation of chicken T-lymphocytes, B-lymphocytes, and peritoneal macrophages

To test whether KPSIII promotes the proliferation of immune cells, chicken T-lymphocytes, B-lymphocytes, and peritoneal macrophages were isolated and cultured with either KPSIII alone or KPSIII plus CTX and ConA, PHA or LPS. The proliferation of B-lymphocytes cultured with 50 μg/mL KPSIII was significantly increased (Table 2), whereas the cell proliferations of T-lymphocytes and peritoneal macrophages cultured with either 100 μg/mL or 150 μg/mL KPSIII were significantly increased (Tables 3 and 4). Interestingly, low doses of KPSIII could enhance the ability of PHA to stimulate B-lymphocyte proliferation (Table 2). These results suggested that KPSIII could enhance the cell proliferation of B-lymphocytes, which implies that KPSIII might be beneficial for regulating humoral immune functions. Additionally, these results suggested that KPSIII could antagonize the inhibitory

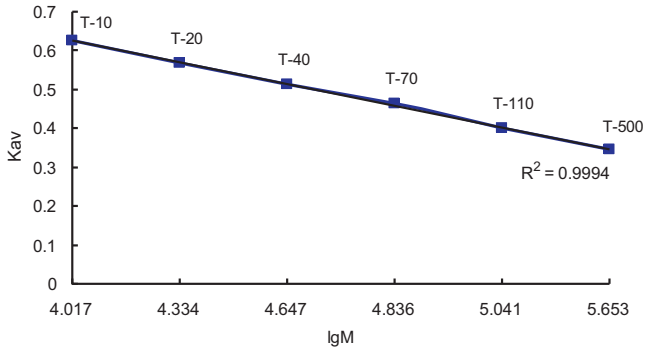

Fig. 3. Linear curve of lgM and Kav. Six glucans (T-10, T-20, T-40, T-70, T-110 and T-500) were used as standards and run on HPLC to generate a standard curve using SPSS11.5 statistical software.

Table 2
The effects of KPSIII on the proliferation of chicken B-lymphocyte.

Group	KPSIII doses (mg/L)			
	0	50	100	150
Mock	0.435 ± 0.019	1.508 ± 0.05^{AbC}	1.030 ± 0.03^{AbC}	0.833 ± 0.02^{aC}
PHA	0.788 ± 0.021	2.288 ± 0.05^{ABC}	1.810 ± 0.03^{ABC}	1.613 ± 0.02^{ABC}
CTX	0.049 ± 0.016	0.290 ± 0.01	0.415 ± 0.02^{C}	0.095 ± 0.01

a represents significant difference to compare with mock ($P < 0.05$); A represents very significant different to compare with mock ($P < 0.01$); b represents significant difference to compare with PHA ($P < 0.05$), B represents very significant difference compare with PHA ($P < 0.01$); C represents very significant difference compare with CTX ($P < 0.01$).

Table 3
The effects of KPSIII on the proliferation of chicken T-lymphocyte.

Group	KPSIII doses (mg/L)			
	0	50	100	150
Mock	0.156 ± 0.02	0.218 ± 0.02^{aC}	0.227 ± 0.02^{aC}	0.275 ± 0.02^{abC}
ConA	0.206 ± 0.01	0.323 ± 0.02^{ABC}	0.332 ± 0.02^{ABC}	0.380 ± 0.02^{ABC}
CTX	0.061 ± 0.01	0.195 ± 0.01	0.212 ± 0.01^{C}	0.288 ± 0.02^{aC}

a represents significant difference to compare with mock ($P < 0.05$); A represents very significant different to compare with mock ($P < 0.01$); b represents significant difference compare with ConA ($P < 0.05$); B represents very significant difference compare with ConA ($P < 0.01$); C represents very significant difference compare with CTX ($P < 0.01$).

effect of CTX on immunocyte proliferation and could serve as an antagonist for immunosuppression.

3.4. KPSIII increased the expression levels of IL-2, IFN-γ, and TNF-α

The IL-2, IFN-γ, and TNF-α expression levels were measured from cell-free supernatants derived from either T-lymphocyte or peritoneal macrophage treated with KPSIII plus Con A or LPS. The results showed that KPSIII treatment elevated the expression of IL-2, IFN-γ, and TNF-α after sensitization with either Con A or LPS (Fig. 4). Additionally, KPSIII increased the expression levels of IL-2, IFN-γ, and TNF-α in a dose-dependent manner (Fig. 4), and the expression levels of IFN-γ and TNF-α were significantly increased after treatment with 100 μg/mL KPSIII (Fig. 4B and C). Taken together, KPSIII elevated IL-2, IFN-γ, and TNF-α expression for both T-lymphocytes and peritoneal macrophages *in vitro*.

4. Discussion

In this study, the immunomodulatory effects of KPSIII were investigated *in vitro*, and the results showed that KPSIII stimulated the proliferation of T-lymphocytes, B-lymphocytes, and peritoneal macrophages. KPSIII also antagonized the inhibition of CTX in the proliferation of T-lymphocytes, B-lymphocytes, and peritoneal macrophages. Furthermore, the expression levels of IL-2, TNF-α, and IFN-γ were significantly increased after KPSIII treatment. These results demonstrated that KPSIII might have potential effects on the regulation of the immune system.

A number of research groups have reported that some compounds potentiate the proliferative ability of Con A, PHA, and LPS on immunocytes, suggesting that they have important effects in immune function (Caballero-Hernándeza et al., 2005; Gupta, K, & S, 2006; Manosroi, Saraphanchotiwitthaya, & Manosroi, 2005). The assessment of substances that either promote or inhibit immunocyte proliferation was crucial to the studies of immunomodulatory drug discovery. KPSIII significantly increased the proliferation of T-lymphocytes, B-lymphocytes, and peritoneal macrophages at concentrations of 50–150 μg/mL, which were markedly enhanced by the presence of Con A, PHA or LPS. T cells are involved in cell-mediated immunity, whereas B cells are primarily responsible for humoral immunity. Our data showed the KPSIII could enhance both cellular immunity and humoral immunity.

Aside from enhancing the proliferation of immune cells, KPSIII can also stimulate the secretion of IL-2, IFN-γ, and TNF-α, which are secreted by Th1-type cells after activation. The Th1 response supports protective immunity against intracellular infections such as viruses, bacteria, and protozoa as well as against cancer cells (Malik et al., 2007). The ratio of Th1 to Th2 cytokines is critical for the orientation of the inflammatory response toward either cell-mediated or humoral-mediated responses. The communication network between Th1 and Th2 cytokines may act either in synergy or in opposition to promote lymphocyte proliferation and differentiation according to the interaction between the timing of their secretion, their relative concentrations and the experimental system used (Vander & Schellekens, 1996). The results from this study showed that KPSIII has a significant stimulatory effect on inducing the release of Th1 cytokines (IL-2, IFN-γ, and TNF-α) and exerts an effect on immunological regulation by upregulating B-lymphocyte production in the immune system. These results suggested that B-lymphocytes might be one of the target cells of KPSIII. Additionally, our data confirmed that KPSIII has a general effect on the cell-mediated immune responses and suggested that it could be used as a vaccine adjuvant.

In conclusion, the polysaccharide compound KPSIII isolated from *K. marmorata* enhances both innate and adaptive immunity

Table 4
The effects of KPSIII on the proliferation of chicken peritoneal macrophages.

Group	KPSIII doses (mg/L)			
	0	50	100	150
Mock	0.286 ± 0.016	0.338 ± 0.02^{AC}	0.402 ± 0.02^{AbC}	0.395 ± 0.02^{AbC}
LPS	0.322 ± 0.018	0.388 ± 0.05^{AC}	0.512 ± 0.03^{ABC}	0.413 ± 0.02^{AbC}
CTX	0.125 ± 0.011	0.253 ± 0.01^{c}	0.317 ± 0.02^{aC}	0.310 ± 0.03^{aC}

a represents significant difference to compare with mock ($P < 0.05$); A represents very significant different to compare with mock ($P < 0.01$); b represents significant difference to compare with LPS ($P < 0.05$); B represents very significant difference compare with LPS ($P < 0.01$); c represents significant difference compare with CTX ($P < 0.05$); C represents very significant difference compare with CTX ($P < 0.01$).

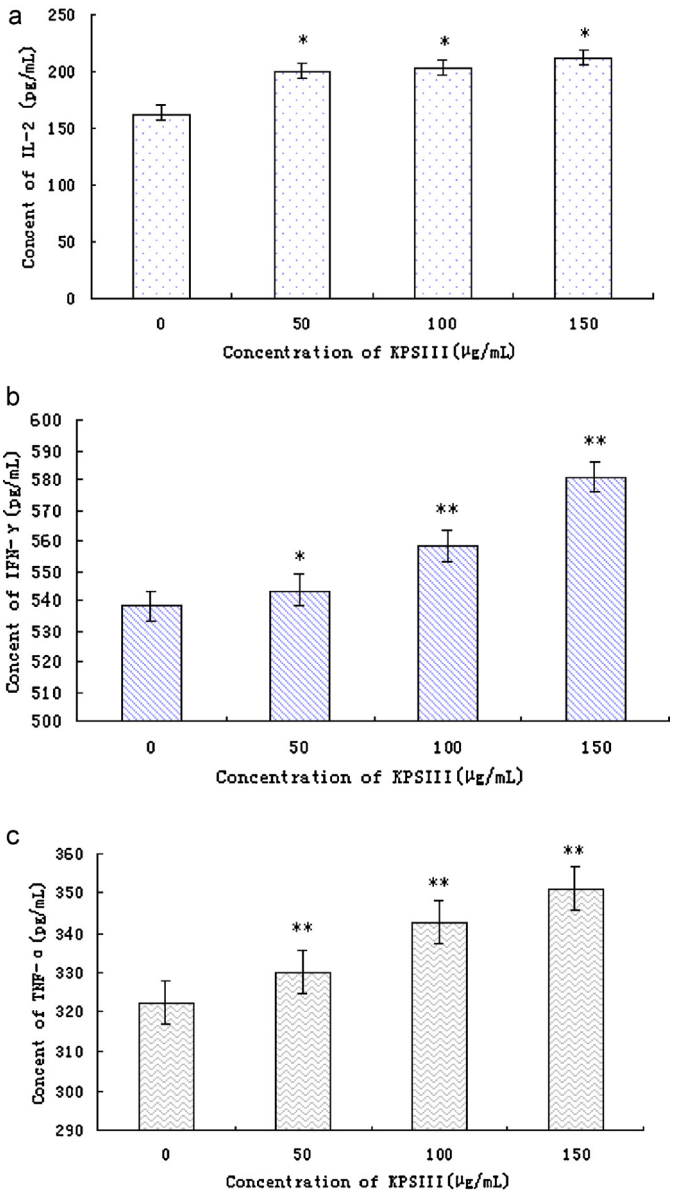

Fig. 4. KPSIII increased the expressions of IL-2, IFN-γ, and TNF-α. T-lymphocytes and peritoneal macrophages were cultured with different concentrations of KPSIII alone or in combination with either Con A (final concentration: 5 mg/L) or LPS (20 mg/L) for 48 h. The expression levels of IL-2 (A) and IFN-γ (B) from the T-lymphocyte culture and TNF-α (C) from macrophage culture were assessed by ELISA assay. The results were from three independent experiments and presented as the mean ± SD. * represents $P < 0.05$; ** represents $P < 0.01$.

through the stimulation of proliferation in chicken T-lymphocytes, B-lymphocytes, and peritoneal macrophages as well as cytokine production *in vitro*. Further studies are necessary to address the molecular target of KPSIII and its potential immunomodulatory effects *in vivo*.

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