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Original Article

Immune enhancing activity of garlic polysaccharide, selenizing *Codonopsis pilosula* compounds in chickens

Li Liang-liang[1,2], Liu Kuan-hui[3], Xie Shi-hui[3], Li Ting[3], Zhu Guang-huang[3], Mikhlid H. Almutairi[4], Muhammad Zahid[5], Mushtaq Ahmad Gondal[6], Qudratullah[6], Khalid Mehmood[5*], Yi Wu[1*]

[1]College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, China
[2]College of Veterinary Medicine, Henan Agricultural University, Zhengzhou, China
[3]Wuhu Institute of Technology, Wuhu, China
[4]Zoology Department, College of Science, King Saud University, Riyadh, Saudi Arabia
[5]Faculty of Veterinary and Animal Sciences, The Islamia University of Bahawalpur, Pakistan
[6]Cholistan University of Veterinary and Animal Sciences Bahawalpur, Pakistan

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**Corresponding author email:*
wuyi2001cn@163.com
khalid.mehmood@iub.edu.pk

Abstract

Codonopsis pilosula polysaccharide is a main ingredient of Codonopsis. The key component in Condoposis is identified as *Codonopsis pilosula*. This *Codonopsis* has multiple functions including stimulation of spleen, lungs and tonifying the middle Jiao and Qi. *Allium sativum* has functions of regulating vital energy, appetizing, detoxification, and disinfection. Polysaccharide of the bulbous is key component to perform the functions of this bulbous. In current study, the immune enhancing activity of GP, sCPP and GP-sCPP through in-vivo and in-vitro trials was evaluated. For in-vivo trials, a total of 150 one day old white roman chicks were randomly divided into five groups, namely immune control (VC), blank control (BC), GP-sCPP, sCPP and GP groups. Chicks in group GP-sCPP, sCPP and GP was intramuscularly treated with 0.5 mg, 0.5 mg and 4.5 mg of polysaccharide, respectively, in all experimental days, and chicks in VC and BC were treated with equal volume of normal saline injections daily. All chicks were bred according to the standards for the rearing of laying hens. For in-vitro trials, lymphocyte cells were cultured in a 12-well plate along with different concentrations of GP-sCPP, sCPP and GP were supplemented in the cells and incubated at 37.5℃. After that, MTT was employed to measure the cytotoxicity of those polysaccharides. The results showed that GP, sCPP and GP-sCPP could increase immune-enhancing activities by releasing cytokines of IL-2 and IFN-γ. In-vivo experiments in chicks showed significantly higher serum antibody titer, T lymphocyte proliferation, and IFN-γ, IL-2 and IL-6 levels in GP-sCPP group. These results demonstrated that GP-sCPP can significantly promote immune enhancing activity, which could be recognized as a novel candidate for new type of immune modulator.

Keywords: Lymphocyte proliferation, *Codonopsis Pilosula*, Chickens, mRNA expression, Antibody titer, Cytokines

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Introduction

Codonopsis pilosula is the dried root of Campanulaceae plants. In the Traditional Chinese Medicine (TCM), *Codonopsis* has several functions like; invigoration of the spleen and lungs, tonifying the middle Jiao and Qi (Morshdy et al., 2021; Nuriyasa et al., 2022). This *bulbous* contains polysaccharides, glycosides, steroids, etc. (Gao et al., 2019; Li et al., 2022). The estimated molecular weight of *Codonopsis pilosula* polysaccharide (CPP) is 1.1×10^4 Da. It has net carbohydrate content of 95.6%. Gas chromatography showed that D-galactose, D-rhamnose, and D-arabinose in the ratio of 1.12:1.00:1.12 constituted CPP. Further, galactopyranosyl are the backbone chains of CPP. Most galactosyl residues possessed arabinofuranosyl in the branches (Yongxu and Jicheng, 2008; Alshamiri et al., 2021). In several studies, it is found that CPP has hepatoprotective, antioxidant, immunity-enhancing and anticancer activities (Zou et al., 2019; Luan et al., 2021).

Allium sativum is scientific name of garlic, is a bulb crop belonging to the family Liliaceae. The regulation of vital energy, detoxification, appetite and disinfection are reported functions of garlic (Adaki et al., 2014; Bila and Tyasi, 2022). In addition, A. *sativum* contains polysaccharides, amino acids, lipids, steroids and glycosides. Garlic polysaccharide (GP) is a homogeneous polysaccharide and the main active constituent of garlic. The molecular weight of GP is 4.54×10^3 Da, and GP is composed of fructose and glucose in a 14:1 ratio. The GP chain is primarily composed of a (2→1)-β-D-fructopyranose linked at the non-reducing end to the terminal (2→1)-α-D-glucopyranose and a (2→6)-β-D-fructopyranose branches on its backbone (Chen et al., 2013). In addition, GP has immune-enhancing and antioxidant activities (Jiang et al., 2022; Kumari et al., 2022).

Selenium is a micro mineral and an essential element for several biological functions (Lin et al., 2023). Key functions of the selenium include the amplification of immune activity, antioxidant, anticancer and prevents accumulation of heavy metals in a body (Khan et al., 2022; Liu et al., 2021; Men et al., 2022). Inorganic form of selenium is found naturally, but cannot be easily absorbed by the cells with highly toxic effect and induce mutation in cells. The characteristic of organic selenium differs with inorganic selenium (Khan et al., 2022; Liu et al., 2021). It is reported that polysaccharides can increase the bioavailability of selenium so, selenizing polysaccharide played dual role for trace selenium and polysaccharide (Hou et al., 2016; Imran and Alsayeqh, 2022).

In traditional Chinese medicine, polysaccharides act as good immune modulators and can significantly trigger immunity (Zeng et al., 2019; Liu et al., 2021; Orinetha et al., 2022). Several studies have shown that single polysaccharide can strengthen immunity. However, limited information is available on polysaccharide compounds that possess immune enhancing activity. Several combinations of polysaccharides were used to develop a mixture compound that could enhance immunity compared to a single polysaccharide (Wang et al., 2019). The aim of this study is to check immune enhancing activities of GP, sCPP and GP-sCPP through in-vivo and in-vitro trials, which may provide the theoretical/ Practical basis for developing a novel immune enhancer.

Material and Methods

Ethical approval

All the experiments were performed under the instructions and approval of Laboratory Animals Research Centre of Wuhu Institute of Technology Anhui, China.

Traditional Chinese medicines and reagents

CPP was purchased from Nanjing Jinling Pharmacy of the Xunzhou Pengzu Chinese Herbal Drugs Factory (Nanjing, China). Garlic was procured from the Nanjing farmer's market. Sodium selenite was purchased from Xiya Chemical Industry Co., Ltd (Linyi, China). Hydrochloric acid, Nitric acid, Ascorbic acid and Dimethylsulfoxide were procured from Nanjing Chemical Reagent Co., Ltd (Nanjing, China). Fetal bovine serum was purchased from Zhejiang-Tianhang Biotech Co., Ltd (Huzhou, China). Roswell Park Memorial Institute 1640 medium was purchased from Gibco (MT, USA). Newcastle disease vaccine (La Sota strain, No. 119087) was bought from Nanjing Tianbang Biotechnology Co., Ltd (Nanjing, China). ELISA kits for interleukin-2, interleukin-6 and interferon-γ were purchased from Suzhou Kaerwen Biotechnology Inc (Suzhou, China).

Preparation of GP and sCPP

The extraction and purification of CPP and GP were performed as per previous study (Qiu et al., 2014).

Briefly, *Codonopsis pilosula*/garlic was crushed, dried, and incubated in 95% ethanol, and then incubated in water bath at 80℃ to remove fat. Water-extraction and ethanol-precipitation of CPP and GP were performed and the total polysaccharide contents were measured as per previous study (He et al., 2023).

Selenization of CPP was performed based on nitric acid-sodium selenite method as previous researches described (Qiu et al., 2014). Briefly, CPP and sodium selenite (5:3) were mixed in 5% nitric acid at 70 ℃ for 8 h, and confirmed by infrared spectroscopy results showed that sCPP was successfully modified. Then the polysaccharide and selenium contents were examined by employing phenol-sulfuric acid method and hydride generation-atomic fluorescence spectrometry, respectively.

The immune enhancing activities of GP-sCPP, sCPP and GP in-vivo trials

A total of 150 one day old white roman chicks were purchased from Tangquan poultry farm (Nanjing, China) and were randomly divided into five groups, namely immune control (VC), blank control (BC), GP-sCPP, sCPP and GP groups. Chicks in group BC, GP-sCPP, sCPP and GP were vaccinated with ND virus on the 14th and 28th day. The birds were reared in cages in semi-controlled shed, and we did not notice any disease in the birds during the entire treatment period. Each chick in GP-sCPP, sCPP and GP was intramuscularly treated with 0.5 mg, 0.5 mg and 4.5 mg of polysaccharide (Yongxu and Jicheng, 2008; Qin et al., 2016; Zou et al., 2019) respectively in all experimental days, and chicks in VC and BC were treated with equal volume of normal saline injections daily. All chicks were bred according to the standards for the rearing of laying hens. On the 7th (D7), 14th (D14), 21st (D21), and 28th (D28) days, blood samples were collected from four chicks in each group to examine the proliferation rate of lymphocytes. The hemagglutination inhibition antibody titer was measured of six chicks in each group through β-micro method. Serums were separated from blood samples for detection of IFN-γ, IL-2, and IL-6 levels by employing commercial ELISA kits.

The cytotoxicity of GP-sCPP, sCPP, and GP on lymphocytes

Firstly, blood samples (5 mL/per chick) from the heart of white Roman chickens (60 days) were collected in the Tangquan poultry farm, and then immediately transferred to Wuhu Institute of Technology. Samples were diluted with Hanks' solution and mixed with RPMI 1640 medium (4mL), followed by centrifugation (x 2000 rpm) for 15 min to separate Lymphocytes. The Lymphocytes layer was washed twice with hanks' solution, and then centrifuged at 1500 rpm for 10 min. Secondly, lymphocytes were re-suspended with RPMI 1640 medium containing 10% FBS and seeded in a 96-well culture plate. Different concentrations of GP-sCPP (1:9), sCPP and GP were added to the cells and incubated at 37.5 ℃ with 5% CO_2 for 44hrs. Then MTT was employed to measure the cytotoxicity of those polysaccharides.

The immune enhancing activities of GP-sCPP, sCPP and GP in-vitro trials

Lymphocyte cells were cultured similarly in a 12-well plate containing GP-sCPP, sCPP and GP and incubated at 37.5℃ for 12h with 5% CO_2. Three independent repetitions were performed for each test.

Relative expressions of IFN-γ and IL-2 in chicken lymphocytes

RNA extraction from lymphocytes was preformed through Super Easy RNA pure Total RNA Kit. Primers (Table 1) generated by SANGON Biotech (Shanghai, China) and PrimeScript^{TM} RT Master Mix were utilized for real-time quantitative PCR. The RT-PCR was performed through ABI-StepOne system (Applied Biosystems, CA, USA) and gene relative expression analysis was performed by method of $2^{-\Delta\Delta Ct}$.

Table-1. Primer pairs used in the present study.

Genes	Primers
GAPDH	5'-TGGAGAAACCAGCCAAGTAT-3'
	5'-CGCATCAAAGGTGGAAGAAT-3'
IL-2	5'-ATGGAAAACTCTTCAAACA-3'
	5'-ACTTCTCCCAGGTAACAC-3'
IFN-γ	5'-GCTGACGGTGGACCTATT-3'
	5'-TCCTCTGAGACTGGCTCCTT-3'

Statistical analysis

Data was depicted as mean ± SD. One-way ANOVA test of multiple comparison among chick groups was performed vis SPSS (26.0), and statistical significance was recognized when $P<0.05$.

Results

The cytotoxicity of GP-sCPP, sCPP, and GP on lymphocytes.

The polysaccharide concentration of CPP and GP was 98.19% and 94.5%, respectively. In sCPP, the polysaccharide content of CPP was 56.2% and the selenium content was 11.86 mg/g. The results showed that there was no cytotoxicity of GP-sCPP, sCPP, and GP on lymphocytes with higher A_{570} values found in all polysaccharide wells when compared with control wells (Table 2), which indicated that those polysaccharides can promote the proliferation of lymphocytes.

Comparison of the immune enhancing activities of GP-sCPP, sCPP, and GP in-vivo trials

The A_{570} value of lymphocytes in chicks in the GP-sCPP group between D_7~D_{28} and the sCPP group on D_{14} was remarkably higher than that in both VC and BC ($p < 0.05$) (Table 3). The serum antibody titer in the GP-sCPP group between D_7~D_{28}, the sCPP group on D_{14} and D_{21}, and the GP group on D_{28} were significantly higher than that in VC and BC ($p < 0.05$) (Table 4). The serum levels of IL-2 in chicks in GP-sCPP group between D_7~D_{28}, the sCPP group on D_{14} and D_{21}, and the GP group on D_{21} and D_{28}) were significantly elevated than that in VC and BC ($p < 0.05$) (Table 5). The serum levels of IL-6 in chicks in the GP-sCPP group between D_7~D_{28} were found to be significantly higher than that of group VC and BC ($p < 0.05$) (Table 6). The serum levels of IFN-γ in the GP-sCPP group between D_7~D_{28}, the sCPP group on D_{14} and D_{28}, and the GP group on D_{14} point were remarkably higher than that in group VC and BC ($p < 0.05$) (Table 7).

Table-2. A_{570} value of lymphocytes from all the groups. Data was depicted as mean ± SD. (n=6). Different letter means significant difference.

sCPP-GP concentration (μg/mL)	A_{570} value	sCPP concentration (μg/mL)	A_{570} value	GP concentration (μg/mL)	A_{570} value
20	0.415±0.013^{a}	2	0.383±0.009^{a}	18	0.380±0.009^{a}
10	0.424±0.011^{a}	1	0.364±0.015^{ab}	9	0.370±0.012^{a}
5	0.406±0.014^{ab}	0.5	0.360±0.010^{ab}	4.5	0.358±0.015^{ab}
2.5	0.367±0.016^{b}	0.25	0.350±0.019^{ab}	2.25	0.351±0.007^{ab}
1.25	0.352±0.013^{b}	0.125	0.327±0.009^{bc}	1.125	0.329±0.012^{bc}
0	0.312±0.009^{c}	0	0.312±0.009^{c}	0	0.314±0.008^{c}

Table-3. Changes in the proliferation of lymphocytes in all groups. Data was depicted as mean ± SD. (n=6). Different letter means significant difference.

Groups	D_7 (A_{570})	D_{14} (A_{570})	D_{21} (A_{570})	D_{28} (A_{570})
GP-SCPP	0.190±0.007^{a}	0.290 ±0.006^{a}	0.274±0.027^{a}	0.287±0.013^{a}
SCPP	0.185±0.007^{ab}	0.252 ±0.006^{b}	0.235±0.015^{ab}	0.252±0.019^{ab}
GP	0.174±0.003^{b}	0.218 ±0.002^{c}	0.207±0.035^{b}	0.230±0.015^{bc}
VC	0.172±0.003^{b}	0.202 ±0.009^{c}	0.204±0.011^{b}	0.190±0.017^{c}
BC	0.155±0.007^{c}	0.165±0.006^{d}	0.145±0.005^{c}	0.141±0.012^{d}

Table-4. Comparison of serum antibody titer in different chick groups. Data was depicted as mean ± SD. (n=6). Different letter means significant difference.

Groups	D_7 (log2)	D_{14} (log2)	D_{21} (log2)	D_{28} (log2)
GP-sCPP	6.750±0.250^{a}	8.000±0.408^{a}	7.250±0.479^{a}	6.750±0.479^{a}
sCPP	4.750±0.250^{b}	6.500±0.500^{b}	6.000±0.408^{ab}	6.500±0.500^{a}
GP	5.000±0.408^{b}	6.000±0.408^{bc}	5.750±0.250^{ab}	6.250±0.250^{a}
VC	4.250±0.250^{b}	5.000±0.408^{c}	4.500±0.289^{b}	4.250±0.479^{b}
BC	2.750±0.479^{c}	3.250±0.250^{d}	2.500±0.289^{d}	2.250±0.250^{c}

Table-5. Comparison of serum IL-2 levels in different chick groups. Data was depicted as mean ± SD. (n=6). Different letter means significant difference.

Groups	D_7 (ng/L)	D_{14} (ng/L)	D_{21} (ng/L)	D_{28} (ng/L)
GP-sCPP	202.521±0.894^{a}	303.704±9.143^{a}	313.529±10.244^{a}	267.978±6.589^{a}
sCPP	184.414±7.811^{b}	233.256±27.753^{bc}	239.455±18.529^{b}	203.935±12.181^{bc}
GP	163.838±3.067^{c}	217.284±13.518^{c}	235.340±16.693^{b}	225.000±18.666^{b}
VC	161.883±8.916^{c}	180.941±3.567^{c}	195.010±7.134^{c}	176.235±4.440^{c}
BC	120.010±3.151^{d}	124.614±8.920^{d}	153.858±1.098^{d}	131.327±5.642^{d}

Table-6. Comparison of serum IL-6 levels in different chick groups. Data was depicted as mean ± SD. (n=6). Different letter means significant difference.

Groups	D_7 (ng/L)	D_{14} (ng/L)	D_{21} (ng/L)	D_{28} (ng/L)
GP-sCPP	34.254±0.594^{a}	38.183±3.53^{0a}	30.961±0.457^{a}	33.584±1.663^{a}
sCPP	29.001±0.995^{b}	30.885±1.653^{b}	22.621±0.810^{b}	23.060±1.738^{bc}
GP	29.674±0.815^{b}	29.886±1.958^{b}	22.240±1.119^{b}	23.071±1.632^{bc}
VC	27.186±0.948^{b}	26.934±0.657^{b}	21.728±2.043^{b}	22.676±2.267^{c}
BC	18.992±0.410^{c}	19.361±0.744^{c}	17.309±0.251^{c}	20.053±2.157^{d}

Table-7. Comparison of serum IFN-γ levels in different chick groups. Data was depicted as mean ± SD. (n=6). Different letter means significant difference.

Groups	D_7 (ng/L)	D_{14} (ng/L)	D_{21} (ng/L)	D_{28} (ng/L)
GP-sCPP	55.191±0.142^{a}	73.995±5.718^{a}	71.396±1.40^{1a}	70.654±3.187^{a}
sCPP	42.947±2.166^{b}	61.520±6.064^{ab}	56.479±3.520^{b}	63.549±6.320^{ab}
GP	40.637±1.667^{bc}	69.094±4.994^{a}	57.997±2.606^{b}	55.629±5.510^{bc}
VC	37.304±1.727^{c}	49.985±1.324^{b}	56.479±1.062^{b}	47.188±4.222^{c}
BC	29.614±0.650^{d}	30.777±0.935^{c}	34.861±0.951^{c}	33.352±1.782^{d}

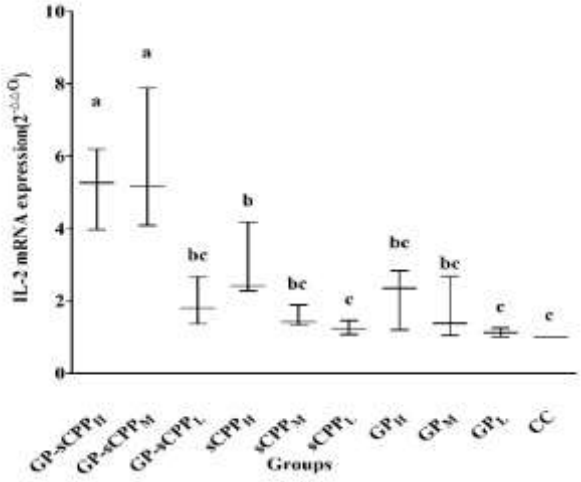

Figure-1. Fold changes of the relative expression of IL-2 in lymphocytes. Data was depicted as mean ± SD. (n=3). Different letter means significant difference.

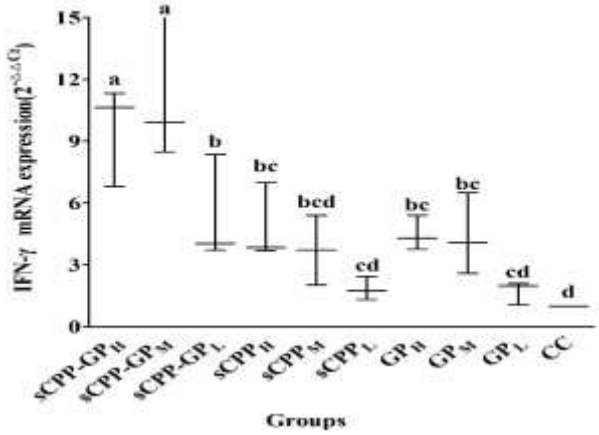

Figure-2. Fold changes of the relative expression of IFN-γ in lymphocytes. Data was depicted as mean ± SD. (n=3). Different letter means significant difference.

The immune enhancing activities of GP-sCPP, sCPP, and GP in-vitro trials

The expression of IL-2 expression in the GP-$sCPP_H$, GP-$sCPP_M$, and $sCPP_H$ groups were obviously higher compared to control group ($p < 0.05$) (Fig 1). The expression level of IFN-γ in the GP-$sCPP_H$, GP-$sCPP_M$, GP-$sCPP_L$, $sCPP_H$, GP_H, and GP_M groups were statistical significantly than control group ($p < 0.05$) (Fig 2).

Discussion

The MTT assay is widely used to determine the functions of lymphocytes in poultry and has several advantages, like fast, high accuracy, and sensitivity (Qiu et al., 2014; Raheel et al., 2021; Radwan et al., 2022). Hence, in this study, MTT assay was employed to determine the immune-enhancing activity of GP-sCPP in vitro. Phytohaemagglutinin (PHA) is an oligosaccharides-protein complex that can enhance lymphocyte proliferation and differentiation (Hou et al., 2016). Similar results were described by Qiu et al. (2014) that sGPSs can encourage lymphocytes proliferation, while sGPS3, sGPS5 and sGPS6 existing strong efficiency. The A_{570} values of lymphocytes are positively correlated with lymphocyte proliferation. We observed that the A_{570} values of lymphocytes treated with the GP-sCPP compound were higher compared to lymphocytes in the cell control group at five concentrations. These results indicated that GP-sCPP at varying concentrations alone and on stimulation with PHA could significantly promote the proliferation of lymphocytes. Results of the present study are consistent with previous study in which research *Sulfated epimedium* polysaccharide along with an *Astragalus* polysaccharide exert a synergistic effect in reducing immunosuppression (Guo et al., 2012).

The A_{570} values of T lymphocyte treated with GP-sCPP on D_7, D_{14}, D_{21}, and D_{28} were significantly higher as compared to immune control and blank control groups. The average proliferation rates of lymphocytes were higher at four different time points. These results suggest that GP-sCPP is involved in inducing the proliferation of T lymphocytes significantly. Studies have shown that T lymphocytes primarily mediate cell-mediated immune responses. Results of this study indicate that GP-sCPP could significantly promote immune-enhancing activity in vivo. Previous study also demonstrated similar results that lymphocytes were significantly increased but they did mention further details about T lymphocyte or B lymphocytes (Qiu et al., 2014). Actually, Antigen stimulation induces the proliferation of B lymphocytes and the differentiation of plasma cells to mediate humoral immunity. Therefore, serum-specific antibody titer directly reflects the strength of the humoral immune response (Throngnumchai et al., 2021). According to our results, the serum antibody titers of chickens from all the groups treated with GP-sCPP were significantly higher at four different time points compared to the immune control and blank control groups. Thus, it could be inferred from the results that the GP-sCPP compound could significantly promote humoral immune responses in-vivo study. Furthermore, Qin et al. (2016) also reported the similar results but their study that was on Macrophage Modulatory Activities rather than on lymphocytes.

Prior studies have demonstrated that polysaccharides play an immunomodulatory role by inducing cell proliferation and cytokine differentiation (Lee et al., 2015; Sun et al., 2015; Li et al., 2022). Helper T-cells not only induce the proliferation and differentiation of IFN-γ and IL-2 but also stimulate T-lymphocyte proliferation and differentiation (Azamor et al., 2021; Hernandez et al., 2022). Various cells secrete IL-6, which is a significant mediator of immune response and anti-infection (Yang et al., 2021). Hence, the serum IFN-γ, IL-2, and IL-6 levels indicate immune functions. Results of this study showed significantly high levels of IFN-γ, IL-2, and IL-6 in the serum of chickens treated with GP-sCPP compared to the immune control and blank control groups at four different time points. These results indicate that GP-sCPP plays an immunomodulatory role by a significant increase in the secretion of cytokines.

Immune-enhancing drugs mediate its effect via cytokines (Qiu et al., 2014; Gao et al., 2016). Cytokines regulate immune responses; specifically, IFN-γ and IL-2, produced primarily by helper T cells, function significantly in immune response and are natural immune enhancers (Gao et al., 2019). Con A promotes T lymphocyte differentiation and secretes several cytokines, like IFN-γ and IL-2. Therefore, we determined the expression of cytokines like IFN-γ and IL-2 in lymphocytes present in the peripheral blood. The IFN-γ expression level in the lymphocytes of chickens treated with varying GP-sCPP concentrations was significantly higher in comparison to the cell control group. This is congruent to some previous studies in which

expression of cytokines was increased (Qiu et al., 2014; Gao et al., 2019).

Additionally, IL-2 expression level in the lymphocytes of chickens treated with 10～20μg·mL^{-1} GP-sCPP was found to be significantly higher than the cell control group. Gao et al. (2019) reported the expression of IL-2 to check the garlic polysaccharide-based effect on immune-enhancing activity and got matching results to our study. On the basis of our findings, it may be proposed that GP-sCPP causes a significant increase in the expression levels of cytokines such as IFN-γ and IL-2 mRNA, by elevating the immune-enhancing activity.

Conclusion

In conclusion, it was found that GP-sCPP could significantly promote immune-enhancing activity, which may be recognized as a novel candidate for new-type of immuno potentiator.

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Contribution of Authors

Liang-liang L, Kuan-hui L & Almutairi MH: Conceived idea, conducted the experiment, wrote and edited manuscript, supervised study and secured funding for research

Shi-hui X: Conducted the experiment and wrote first draft of manuscript

Ting L & Guang-shuang Z: Conducted the experiment and collected data

Zahid M: Reviewed literature, wrote and edited manuscript

Gondal MA, Qudratullah & Mehmood K: Analyzed and interpreted data

Wu Y: Supervised study and secured funding for research