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RESEARCH ARTICLE

Effect of Ethanol Extract and Fractions of Physalis Calyx Seu Fructus on Inflammation and *Mycoplasma Gallisepticum*

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A B S T R A C T

The aim of the study was to investigate whether ethanol extract and fractions from D-101 macroporous resin of Physalis Calyx Seu Fructus has the anti-inflammatory and anti-Mycoplasma gallisepticum (*MG*) activity in vitro. Extract (P) was isolated from the dried calyces of Physalis Calyx Seu Fructus with 80% ethanol under reflux. The five major fractions (P1, P2, P3, P4 and P5) were obtained with ethanol gradient elution from D-101 macroporous resin column loaded with P. P and Fraction P2 was enriched with luteoloside content, with 1.39% and 2.548%, respectively. P3 had significantly inhibited the expression of IL-6, TNF-α and NO both at protein and gene level in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages by the qRT-PCR analysis and ELISA assay. Moreover, P2, P3, and P4 at its minimum inhibitory concentration inhibited the growth of *MG*. Conclusively, the present study suggested that P3 is a potential agent against *MG* and inflammation.

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INTRODUCTION

Physalis Calyx Seu Fructus is the dry calyxes and fruits of *Physalis alkekengi L. var. franchetii (Mast.) Makino*, one of an edible plant of Solanaceae family. It is usually used to treat cough, excessive phlegm, sore throat, and pharyngitis in traditional Chinese medicines (Li *et al.*, 2018). Various biologically active compounds of Physalis Calyx Seu Fructus have been isolated and identified, including steroids, flavonoids, alkaloids, phenylpropanoids, polysaccharides etc. (Chen *et al.*, 2014; Shu *et al.*, 2016; Yang *et al.*, 2016; Zhang *et al.*, 2016; Kranjc *et al.*, 2017). These extracts showed several pharmacological properties, such as anti-inflammatory, anti-microbial, anti-tumor (Zhu *et al.*, 2016; Yang *et al.*, 2017; Wang *et al.*, 2018; Shin *et al.*, 2019). Steroids and flavonoids have been considered the main component of Physalis Calyx Seu Fructus on anti-inflammatory and antimicrobial (Helvacı *et al.*, 2010; Shu *et al.*, 2016; Wang *et al.*, 2017; Li *et al.*, 2018). These compounds may exert their antimicrobial action via inhibiting the proliferation of bacteria or the immunosuppressive activity. However, the effect of Physalis Calyx Seu Fructus against *MG* is still unknown.

MG is an extracellular pathogen that induces chronic respiratory disease in chickens and sinusitis in turkeys characteristics with nasal discharge, tracheal rales, coughing, dyspnea and sneezing (Matyushkina *et al.*, 2016). The decreased flocks' performance, reduced egg production and broiler weight, and high infection rate make *MG* infection become a most cost disease. Eradicating positive flocks or vaccination and medicine is the main way to control the infection of the disease. Furthermore, antibiotic was always the primary choice of farmers to against *MG*, such as macrolide antibiotics. The longtime or unreasonable use of that has caused mutations in mycoplasma and resulted more difficulty on controlling its infection. Recently, it has reported that *MG* mediated severe inflammatory response in vitro (Majumder *et al.*, 2014; Majumder *et al.*, 2015; Yu *et al.*, 2018). Inflammation, an immune defense response of the body to harmful stimuli, is involved in the pathogenesis of many diseases, including atherosclerosis, cancer, asthma, cardiovascular and other deadly disease (Bishayee, 2014; Wu *et al.*, 2014; Taleb, 2016; Mishra *et al.*, 2018). The tumor necrosis factor (TNF-α), interleukin-6 (IL-6) and nitric oxide (NO) play a major role in inflammatory response.

In recent years, it has reported that compounds extracted from herbal plants having less toxicity and biodegradability, play a critical role in the prevention or treatment of various diseases. In our previous researches, many herbal compounds have anti-inflammatory, anti-viral, and anti-cancer activities in vitro (Sun *et al*., 2016; Fan *et al*., 2018; Sun *et al.,* 2019). Therefore, the present study was aimed to investigate the anti-inflammatory effect of Physalis Calyx Seu Fructus ethanol extract and its fractions with an effect on the growth of *MG* and provide a base line study for clinical application of Physalis Calyx Seu Fructus. (Write correctly and mechanism of activity against bacteria).

MATERIALS AND METHODS

Reagents: Dulbecco's modified Eagle's medium (DMEM) was obtained from HyClone, USA; fetal bovine serum (FBS) were purchased from Biological Industries, Israel; RAW264.7 cells were obtained from Shanghai Institutes for Biological Sciences; KM2 mycoplasma hyopneumoniae broth medium was obtained from Jiangsu Academy of Agricultural Sciences, Nanjing, China; ELISA kits were obtained from Cusabio, Wuhan, China.

Extract P from physalis calyx seu fructus: 1 kg of dried Physalis Calyx Seu Fructus powder was soaked overnight in 20 volumes of 80% ethanol, extracted under reflux for 2 h for two times, and collected the filtrates. The two filtrates were combined, concentrated under reduced pressure on a rotary evaporator. The extract was dried under vacuum, named P.

Fractionization of P: P was dissolved in distilled water, added to a neutral D-101 microporous resin for overnight adsorption and eluted with water and 30, 50, 75 and 95% (v/v) ethanol gradient, to obtain five fractions P1, P2, P3, P4 and P5, respectively. All factions were concentrated and dried under vacuum. The luteoloside contents of extract and fractions were determined by HPLC.

Cell culture and treatment: RAW264.7 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin, incubated at 37°C with 5% CO_2. Cells were treated with P or its fractions for 48 h to determine the cells viability using MTT assay. Cells were co-treated with P or its fractions and LPS for 24 h to determine inflammatory cytokines. The cells were co-treated with LPS and P3 for 24 h (Model 1), with LPS for 12 h and then treated with P3 for 12 h (Model 2), with P3 for 12 h and then treated with LPS for 12 h (Model 3).

Determination of TNF-α, IL-6 and Nitric Oxide (NO): The supernatants were collected and the pro-inflammatory cytokines (IL-6 and TNF-α) were measured using ELISA. The production of NO was detect using Griess (reference) (Guevara *et al.,* 1998).

qRT-PCR analysis: Gene expression levels of TNF-α, IL-6 and iNOS were measured using the $2^{-\triangle\triangle CT}$ method (REFERENCE)(Sun *et al.,* 2019). β-actin was used as invariant internal control. The following primers were used: TNF-α, forward: 5′-GATCGGTCCCCAAAGGGA TG-3′ and reverse: 3′-GTTTGCTACGACGTGGGCT-5′; IL-6, forward: 5′-GTCCTTCCTACCCCAATTTCCA-3′ and reverse: 3′-TAACGCACTAGGTTTGCCGA-5′; iNOS, forward: 5′-GAGTTCAGCCAGTTGTGC-3′ and reverse: 3′-CTCCAGGATGTTGTAGCG-5′; β-actin, forward: 5′-CTGAGCTGCGTTTTACACCC-3′ and reverse: 3′-CGCCTTCACCGTTCCAGTTT-5′.

Determination of Mycoplasma Color Changing Unit (CCU): The *MG* (gift of Jiangsu Academy of Agricultural Science) was cultured in KM2 mycoplasma hyopneumoniae broth medium and incubated at 37ºC. 10-fold serial dilutions of *MG* were seeded in 96-well plates at 37ºC, monitored the medium color until it not changed. The mycoplasma CCU was calculate according to the reference (Calus *et al.,* 2010).

Determination of Minimal inhibitory concentration (MIC) and Minimum mycoplasmacidal concentration (MMC): 180 µL P or its fractions were added to the 96-well plates followed by 20 µL/well of *MG* with the final concentrations of 10^4 CCU/mL. The color change was observed every 6 h until positive control was changed from red to yellow (phenol red indicator). The lowest drugs concentration at which medium color did not change to yellow was MIC. 200 µL medium from MIC well, were transferred into a small ampoule containing 1.8 mL KM2 mycoplasma hyopneumoniae broth medium incubated at 37ºC and monitored the medium color. The lowest drugs concentration at which the color did not change to yellow was MMC.

The inhibitory effect of extract (P) and its fractions on *MG* growth: 10-fold serial dilutions of 10^8 CCU/mL *MG* were seeded in 96-well plates. P and its fractions at MIC were added to each well, monitored for the color change at different time interval until the positive control change to yellow.

Statistical analysis: Data were expressed as mean ± SD. One-way analysis of variance was used to assess differences between multiple treatments using GraphPad Prism 5. $P<0.05$ was considered significant.

RESULTS

Physalis Calyx Seu Fructus extract P and its fractions: In this study, 295.1 g dried ethanol extract P from 1 kg Physalis Calyx Seu Fructus powder was obtained and purified by D-101 macroporous resin column. Five fractions as P1 (88.2 g), P2 (42.1 g), P3 (17.3 g), P4 (13.0 g) and P5 (22.5 g) were obtained and evaluated by determining the content of luteoloside. The contents of luteoloside in Physalis Calyx Seu Fructus_was 0.1735%, and P, P1, P2, P3, P4 was 1.39, 0.022, 2.548, 0.72 and 0.018%, respectively with no luteoloside was detected in P5 (Fig. 1, Table 1).

The cytotoxicity of extract P and its fractions in RAW264.7 macrophages: Before the study of the biological activity of these fractions, cytotoxicity of P, P1, P2, P3, P4 and P5 in RAW264.7 macrophages were performed. The results were shown in Table 1, and the maximum non-toxic concentration (MNTC) was used in the follow-up study.

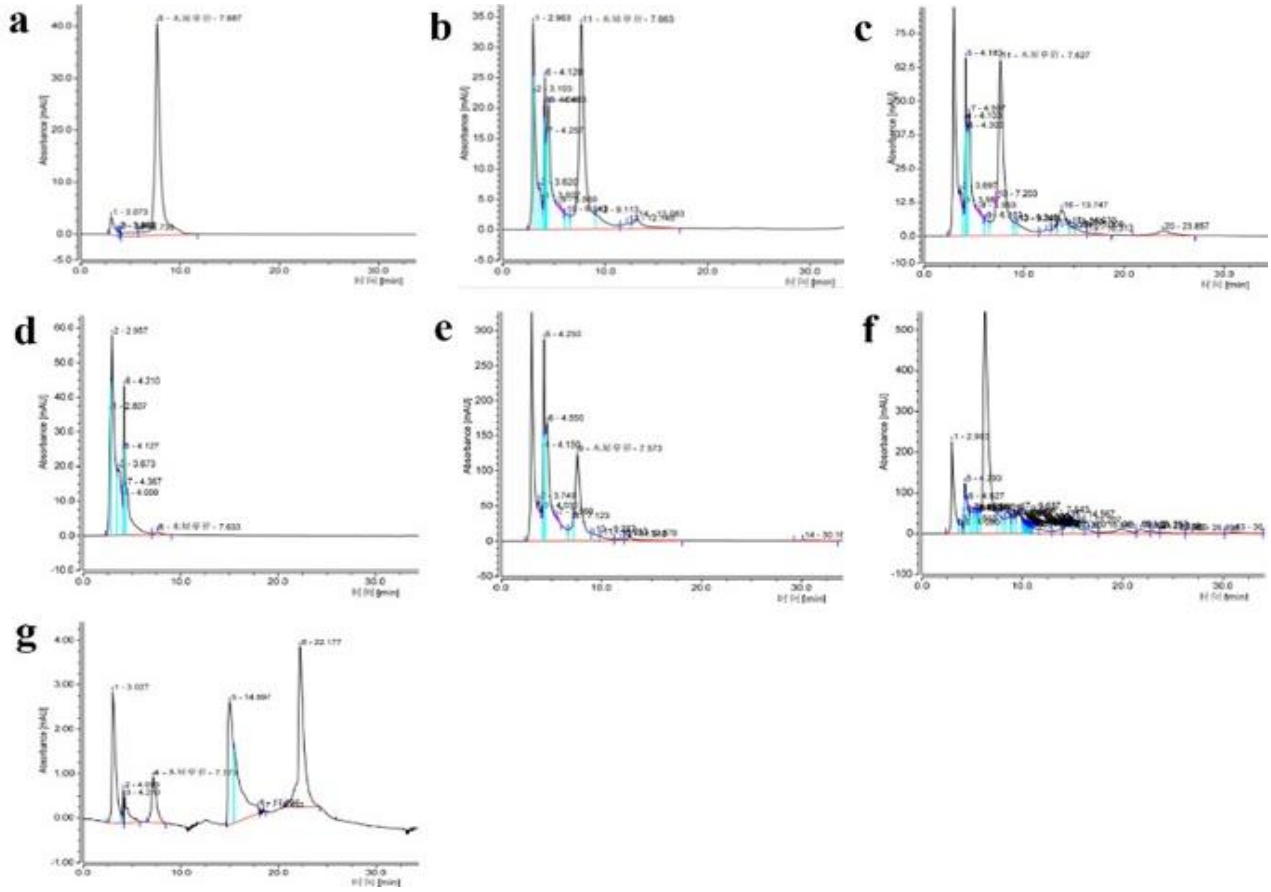

Fig. 1: The content of Luteoloside determined by HPLC. (a) standard of Luteoloside. (b) Physalis Calyx Seu Fructu. (c) Ethanol extracts P from Physalis Calyx Seu Fructus. (d) P1. (e) P2. (f) P3. (g) P4.

Table 1: Characteristics of ethanol extract (P) and its fractions (P1, P2, P3, P4 and P5)

Sample	Physalis Calyx Seu Fructu	P	P1	P2	P3	P4	P5
Luteoloside	0.1735%	1.39%	0.022%	2.548%	0.72%	0.018%	-
MNTC (μg/mL)		8	0.03125	64	64	8	32
CC_{50} (μg/mL)		1430±0.17	0.14±0.08	206860±8.27	78.91±2.93	40.74±1.21	86.01±3.57
Inhibition rates of No		60.37%	-8.34%	20.44%	99.38%	40.19%	19.66%

Effects of extract P and its fractions on LPS-stimulated inflammation in RAW264.7 macrophages: P, P1, P2, P3, P4 and P5 were co-treated with LPS in RAW264.7 macrophages and the results (Fig. 2A) showed that comparing to LPS alone, the protein level of IL-6 was significantly decreased by P3, that of TNF-α was significantly reduced when treated with P1, P2 and P3, and that of NO were significantly reduced by P2, P3, P4 and P5. The NO inhibition rate of P3 reached 99.38% which was similar to that of the control group (Table 1). Similarly, as shown in Fig. 2B, comparing with LPS, mRNA expression levels of IL-6 were significantly decreased by P3, that of TNF-α was significantly decreased by P1, P2, P3 and P5, and that of iNOS was markedly reduced by P3 and P5.

The role of P3 in LPS-stimulated inflammation on RAW264.7 macrophages: To investigate the effects of P3 on LPS-stimulated inflammation in RAW264.7 macrophages, cells were treated with P3 and LPS in three different treatment ways. The ELISA and qRT-PCR data (Fig. 3) showed that the protein and mRNA expression levels of IL-6, TNF-α and NO (iNOS) was significantly reduced by P3. Comparing to the other two models, P3 co-treated with LPS, had the strongest inhibitory effect on the production of NO by LPS with the inhibition rate was 99.38% as shown in Table 2.

MIC and MMC of extract P and its Fractions: The MIC and MMC of extract P and its fractions against *MG* were examined as shown in Fig. 4 and Table 3. The MIC values of P, P2, and P3 were 0.625 mg/mL and that of P4 and P5 were 1.25 mg/mL, 2.5 mg/mL, respectively while that of tiamulin was 0.0003125 mg/mL. P1 (20 mg/mL) had no inhibition effect on the *MG* growth. DMSO, as the solvent control, has no effect on the growth of *MG* at a concentrations less than 20 μL/mL. The MMC values of P, P2, P3, P4 and P5 were 10 mg/mL, 5 mg/mL, 5 mg/mL, 10 mg/mL and 20 mg/mL, respectively while that of tiamulin was 0.000625 mg/mL.

Effect of the extract P and its fractions on the growth of *MG:* As showed in Fig.5 and Table 3. P4 at a concentration of 1.25 mg/mL inhibited the growth of $\leq 10^6$ CCU/mL *MG*. P2, P3 and P5 at a concentration of 0.625 mg/mL, 0.625 mg/mL and 2.5 mg/mL, respectively decreased the growth of $\leq 10^5$ CCU/mL *MG*. However, the inhibition effect of P5 was lower than that of P2 and P3.

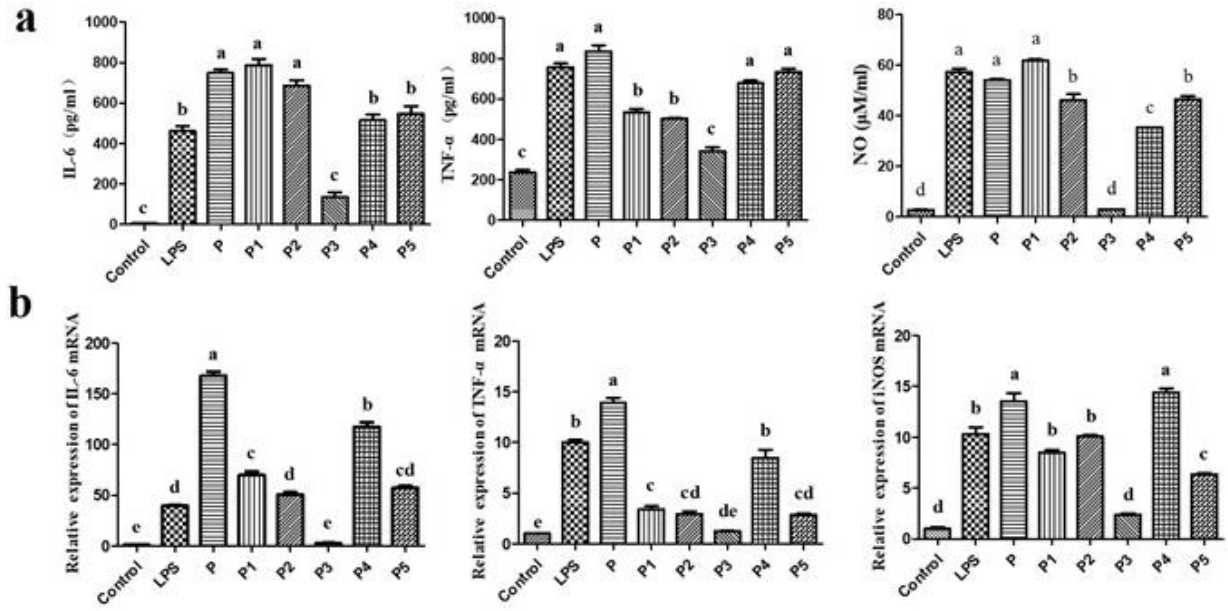

Fig. 2: Expression levels of IL-6, TNF-α and NO in RAW264.7 macrophages treated with ethanol extract and its fractions. cells were co-treated with P, P1, P2, P3, P4, P5 and LPS for 24 h. (a) The protein level of IL-6, TNF-α and NO. (b) The gene level of IL-6, TNF-α and NO. Dates are presented as means ± SD, different letters indicate significant differences between groups (P<0.05).

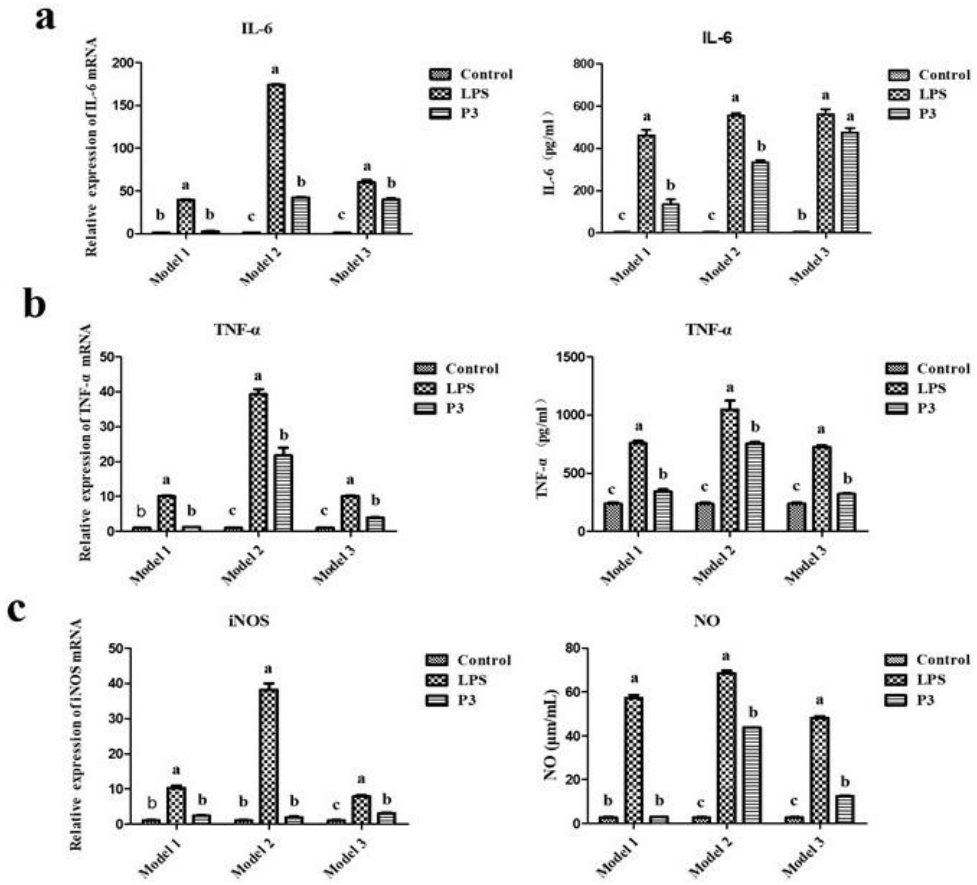

Fig. 3: The protein and mRNA expression levels of IL-6、TNF-α and NO treated with P3. (a) The expression of IL-6 in all models. (b) The expression of TNF-α in all models. (c) The expression of NO in all models. Results are presented as means ± SD, data with different letters indicate significant differences between groups (P<0.05).

DISCUSSION

Physalis Calyx Seu Fructus is involved in various biological activities including anti-inflammatory, anti-tumor, anti-bacterial, ect. (Helvacı *et al.*, 2010; Li *et al.*, 2014; Shu *et al.*, 2016). In the present study, we used 80% ethanol under reflux to get the maximum active contents and the yield of the ethanol extract P was 29.52%. The content of luteoloside in the control plant we used was 0.1735% which was higher than that mentioned in the pharmacopeia. The contents of luteoloside in the ethanol extract P was 1.39%, enriched up 7-folds. After being further separated by D-101 macroporous resin, the content of luteoloside in P2 was the highest, reaching 2.548%, which was 13.7-folds higher compared with the original material. These data suggested that high content of luteoloside was obtained by eluting D-101 macroporous resin with 30% ethanol, which could be used for the extraction of luteoloside in the future.

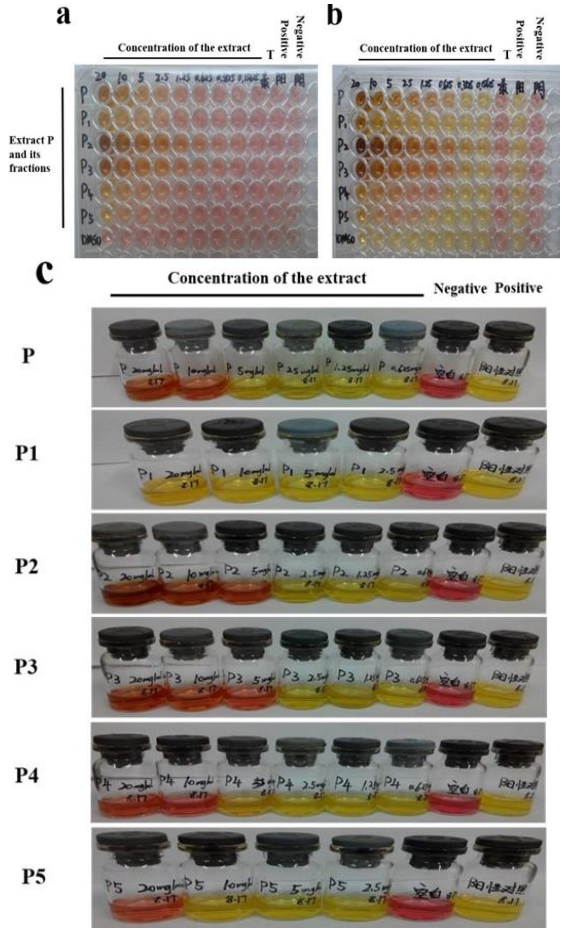

Fig. 4: The MIC and MMC of extract P and its fractions. (a) Cultured *MG* for 0 h. (b) The color of positive control turned to yellow after *MG* was cultured for 5 days. (c) *MG* treated with extract P and its fractions. Notes: T, tiamulin; Positive, only *MG*; Negative, only KM2 medium.

Acute inflammatory response is a rapid response for the body to infectious microbes or tissue damage. LPS is an activator of macrophages by promoting the release of inflammatory cytokines and chemotactic factors (Abekura *et al.*, 2019; Li *et al.*, 2019). Most studies have shown plant extracts have a strong anti-inflammatory activity. Wang *et al*. (Wang *et al*., 2015) reported that a neutral polysaccharide from North American ginseng possessed a strong anti-inflammatory by decreasing the pro-inflammatory cytokine ratios and reduced the release of NO in LPS activated RAW264.7 macrophages. The phenolics extracted from Jujube peel markedly inhibited LPS-stimulated inflammation in murine macrophages (Wang *et al*., 2019). In the present study, the results showed that P1, P2, P3, P4 and P5 inhibit the expression of TNF-α, IL-6 and NO in LPS-stimulated inflammatory response on RAW264.7 macrophages both at the gene and protein level. P3 possessed the strongest anti-inflammatory effect among all extracts. To further evaluate the potential of P3, different experimental models were designed in which the expression of inflammatory cytokines was decreased. However, NO production was strongly inhibited by the P3 in the model simultaneously treat with LPS. Previous research reported that the extract of polysaccharides from Sargassum horneri had a stronger inhibition of NO production in their preventive and repair experimental model (Wen *et al*., 2016). To certain degree, the anti-inflammatory effect of the drug is related to the application of the drug under the specific experiment conditions.

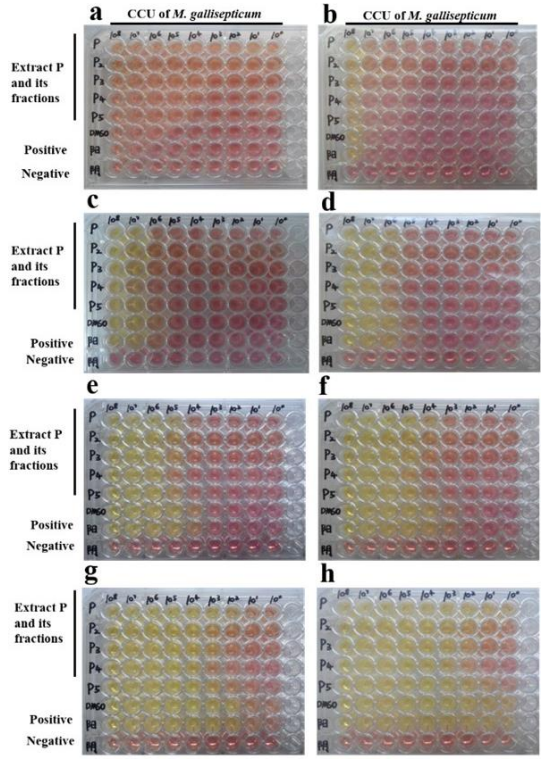

Fig. 5: Inhibitory effect of extracts and its fraction on the growth of *MG* at MIC. a, b, c, d, e, f, g, h represented cultured 0 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, 168 h; DMSO: vehicle control; Positive, only *MG*; Negative, only KM2 medium.

Table 2: The inhibition rates of P3 on NO production in different models

Treatment	Inhibition rates
Model 1	99.38%
Model 2	37.65%
Model 3	78.48%

Table 3: Effect of methanol extract (P) and its fractions (P1, P2, P3, P4 and P5) on *MG*

	P	P1	P2	P3	P4	P5	Tiamulin
MIC (mg/mL)	0.625	-	0.625	0.625	1.25	2.5	0.0003125
MMC (mg/mL)	10	-	5	5	10	20	0.000625
Inhibited *MG* concentration (CCU)	$<10^4$	-	$\leq 10^5$	$\leq 10^5$	$\leq 10^6$	$\leq 10^5$	

MG mainly causes chronic respiratory disease in chicken or sinusitis in turkey, which has made a huge economic loss in modern poultry farming industries. It also has been reported that *MG* could induced the up-regulation of proinflammatory genes. Yu found that the *MG* derived lipid-associated membrane proteins increased the production of IL-1β (Yu *et al*., 2018). Majumder reported that *MG* in chicken tracheal epithelial cells promoted the macrophage chemotaxis and activation by up-regulating the expression of IL-1β, IL-6, and IL-8 (Majumder *et al*., 2014; Majumder *et al*., 2015). The dichloromethane extract from the calyces of Physalis alkekengi L. (Solanaceae) and physalin D possessed a strong antibacterial activity, especially the Gram-positive bacterial (Helvacı *et al*., 2010). Shu reported that the physalins and flavones extracted from Physalis Alkekengi var. franchetii could significantly against the bacterial activity (including Gram-negative bacteria and Gram-positive bacteria) and reduce the inflammation induced by LPS both in vivo and vitro (Shu *et al*., 2016). Therefore we investigated the effect of extract on the growth of *MG*. Consistent with the previous studies, our results showed that the ethanol extract and its fractions form Physalis

Calyx Seu Fructus against *MG*. P2 and P3 had the lowest MIC and MMC values among all fractions, which indicated that P2 and P3 had the stronger inhibitory effect on the *MG* growth. P1 was the fraction eluted with water, the main component was glucose, which is essential for *MG* growth. That may be the reason why P1 had no inhibitory function against *MG* even with a high concentration (Snell, 1981).

To further investigate the inhibition of extract P and its fractions on the growth of *MG*, it was treated with P, P1, P2, P3, P4 and P5 at their MIC. The results showed that P4 has the strongly inhibited the growth of $\leq 10^6$ CCU/mL *MG*, while P2 and P3 inhibited that of $\leq 10^5$ CCU/mL *MG*. The anti-mycoplasmal effect of P3 may be related to its strongest anti-inflammatory activity, which will be further explored.

Conclusions: Using 80% ethanol under reflux, the extract of Physalis Calyx Seu Fructus was enriched with the luteoloside. P3 possessed the strongest anti-inflammatory activity in LPS-stimulated RAW264.7 macrophages by significantly reducing the release level of proinflammatory cytokines (IL-6, TNF-α, NO) among the ethanol extract and different fractions of Physalis Calyx Seu Fructus. P2, P3 and P4 inhibited the growth of 10^4 CCU/mL *MG*. Taken together, P3 could to be developed as an effective anti-mycoplasma and anti-inflammatory agent in the future. Furthermore, the identification of active compounds components and structures of P3 and the anti-mycoplasma mechanism will be further studied.

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Authors contribution: YY, HL and NS conceived and designed the study. YY, MY and PS executed the experiment and analyzed the data. JC guided the RT-qPCR. YY, AK and JG provided the manuscript editing. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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