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PREPARATION PROCESS OF *Passiflora edulis* LEAVES EXTRACT AND ITS POTENTIAL PHARMACOLOGICAL MECHANISMS IN MICE: IMMUNOMODULATORY AND ANTI-INFLAMMATORY ACTIVITIES

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ABSTRACT

Passiflora edulis (*P. edulis*) is a medicinal and food plant with several pharmacological activities, but its leaves are not well exploited and utilized. The preparation procedure of Passiflora edulis leaf extract (PELE) was optimized using the orthogonal design on L9(4^3) parameters based on the transfer rate of orientin from raw material and the quality control methods were established to a method for determining the total flavonoid content by UV-vis and the orientin content by HPLC. The evaluation of immunomodulatory and anti-inflammatory activities of the PELE were used by establishing a mouse model of immunosuppression and paw edema. The results showed that 80% ethanol, 60 min extraction at 90°C and 1:8 ratio was the best extraction process for PELE with a transfer rate of 11.15 ± 0.05 %. The total flavonoids and orientin content in PELE were measured to be about 67.79 ± 5.51 and 3.89 ± 0.03 mg/g, and the transfer rate of orientin was 56.49 ± 0.41%. In the immunosuppression model, PELE pretreatment delayed the atrophy of immune organs and reversed the down-regulation of immunoglobulin IgA, IgG, IgM and pro-inflammatory factors TNF-α, IFN-γ, IL-2, IL-10 levels in mice. In a mouse paw edema model, PELE significantly reduced paw swelling and also showed down-regulation of pro-inflammatory factors TNF-α, IL-1β, IL-6 and up-regulation of anti-inflammatory factors IFN-γ, IL-2, IL-4, IL-10. In conclusion, PELE has good immunomodulatory and anti-inflammatory activities and is a good candidate for the development of drugs for the treatment of related diseases.

Keywords: anti-inflammatory, immunomodulatory, quality control, *Passiflora edulis* leaves extract, *Passiflora edulis*

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INTRODUCTION

Passiflora edulis (*P. edulis*) is a member of the family Passifloraceae originated in Southern Brazil, Paraguay and northern Argentina (Fotsing *et al*., 2023). Favoring its sweet and nutrient-rich fruits, *P. edulis* has been commercially cultivated in tropical and subtropical areas (He X, *et al*., 2020). The *P. edulis* leaves (PEL) and their extract prepared by ethanol or water extraction have been widely used in folk medicines for the treatment of alcoholism, anxiety, migraine, nervousness and insomnia in South America (Fotsing *et al*., 2023; LOPEZ *et al*., 2016). *P. edulis* is used as a Zhuang medicine, and it is included in the "*Chinese Zhuang Yao Tuan Jian*". Its fruit has the functions of clearing lungs and moisturizing, soothing the nerves, and relieve pain as medicine. It is clearly stated in the "*List of Yunnan Traditional Chinese Medicine Resources*" that the roots, stems, leaves, flowers, and fruits of *P. edulis* can be used as medicine, and have the effects of removing wind and dampness, promoting blood circulation and relieving pain, clearing heat, relieving cough, and reducing phlegm. It is recorded in the first volume of the Second Edition of the "*Traditional Chinese Medicine Dictionary* " that *P. edulis* is the whole grass of the family Passifloraceae, and it is a perennial grass sapwood, which has been introduced and cultivated in Guangxi, Guangdong, Yunnan and other places in China (Nanjing University of Traditional Chinese Medicine, 2014). It has the functions of expelling wind, dehumidifying, promoting blood circulation and relieving pain. It is mainly used for colds and headaches, exogenous wind heat cough, hernia, dysmenorrhea, etc. Additionally, modern pharmacology studies showed that PEL and their extract have significant anxiolytic (Li *et al*., 2011), anti-inflammatory (Urrego *et al*., 2021), antidepressant-like (Wang *et al*., 2013), anti-Giardia (Neiva *et al*., 2014), liver protection (Zhang *et al*., 2016) and antioxidative (Malvezzi *et al*., 2018; He X, *et al*., 2020) activities in vivo. A recent systematic review indicates that phenolic compounds are the most investigated constituents in *P. edulis* and flavonoid is the most frequent phenolic compounds with 35 flavonoids out of total 52 phenolic compounds, such as vitexin, isovitexin, isoorientin, orientin, and their derivatives (Lucena *et al*., 2018). These flavonoids, presenting strong in vitro and in vivo anti-inflammatory, antiplatelet and antithrombotic effects, might be the effective constituent of PEL (Wonhwa and Jong-Sup, 2015; He X, *et al*., 2020). However, to our knowledge, there still lack a comprehensive and effective standard for the quality control of *P. edulis* leaves extract and a systemically study on its immunomodulatory and anti-inflammatory activities.

Immunosuppression is a state of temporary or long-lasting immunity dysfunction causing by stress, virus infection, chemotherapy, radiotherapy, unbalanced diet, pollution, or immunosuppressive agents (Malvezzi *et al*., 2018; Shevtsov *et al*., 2019). While in the breeding industry, inflammation and immunosuppression are widespread in animals due to expansion of breeding density, the decrease of animal excise and the increasing frequency of infectious diseases (Jackwood, 2011; Liu *et al*., 2019). Immunosuppression and inflammation lowers the body's resistance against endogenous and exogenous immunogen, leading to the worsening of diseases, secondary infection and vaccination failure, causing huge economic lose in both human life and animal breeding (Chen *et al*., 2012; Fu *et al*., 2017). Great efforts have focused on the development of exogenous agents modulating immunity and inflammatory response to improve the conditions of patients or cultivated animals (Fu *et al*., 2017; Vareille *et al*., 2019; Makni *et al*., 2019).

Guangxi belongs to the subtropical region and its climate is particularly suitable for the growth Passifloraceae. The planted area of *P. edulis* in Guangxi is more than 100,000 acres and these plants produce more than 50,000 kg passion fruits for the industry of flavor, juice and food every year. Currently, there is no exploration on the medical applications of the leaves, resulting in a waste of 10,000 tons PEL per year by directly burning which also cause environmental pollution. This study is carried out to explore the medical applications of PEL, a byproduct of *P. edulis* industry. Firstly, the extraction method for the total flavonoid from PEL was optimized by the orthogonal design. A comprehensive and effective system for the quality control of the extraction of PEL (PELE) was established by ultraviolet spectrophotometry (UV), thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) according to the total flavonoid content, presence of orientin and isoorientin, and the content of orientin. The immunomodulatory and anti-inflammatory activities of PELE were then studied using BALB/c mice administrated with cyclophosphamide (CTX) or carrageenan, respectively.

MATERIALS AND METHODS

Chemicals and drugs: Vitexin (purity of 94.9%), orientin (purity of 97.9%) and isoorientin (purity of 94.0%) were purchased from National Institutes for Food and Drug Control (Beijing, China). Astragalus polysaccharides (purity of 75.0%) was purchased from the Sanxingdui Plant Chemical Co., Ltd. (Guanghan, China). Cyclophosphamide (CTX, purity >98%) was from the Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Dexamethasone (purity of 99.7%), carrageenan (purity of 99.0%) was purchased from the Solarbio Science & Technology Co., Ltd. (Beijing, China). ELISA kits for immunoglobulin A (IgA), IgG, IgM, interleukin 1β (IL-1β), IL-2, IL-4, IL-6, IL-10, tumor necrosis factor α (TNF-α), and interferon-γ (IFN-γ) were purchased from MLBIO Biotechnology (Shanghai, China). All other chemical reagents were at least analytical grade and used as received.

Collection, identification and processing of the plant material: The leaves of *P. edulis* were collected in Lingshan, Guangxi, China, in April 2018. The plant botanical identification was realized by Professor Songji Wei in Guangxi University of Chinese Medicine. A voucher specimen (GXCM 2018063) was deposited at the Specimen Room, Guangxi University of Chinese Medicine. Leaves were dried in a circulating air oven at 37 ± 2 °C, milled and sieved to pass through the ASTM # 60 mess. Subsequently, the sample was packaged, labeled and stored at room temperature.

Preparation of PELE: Ethanol extraction method was applied to prepare PELE from dried and milled *P. edulis* leaves. The extraction conditions was optimized using the orthogonal design on $L9(4^3)$ parameters based on the transfer rate of orientin from raw material to PELE (Liu *et al*., 2018). The parameters included ethanol concentrations of 60 %, 70 % and 80 %, extraction temperature of 70 ℃, 80 ℃ and 90 ℃, extraction time of 30 min, 60 min and 90 min, as well as material-liquid ratio of 1:8 g/mL, 1:10 g/mL and 1:12 g/mL. The orthogonal optimization process was repeated triplicate. Specifically, dried and milled *P. edulis* leaves were extracted by ethanol solution at specific material-liquid ratio under predefined temperature for certain extraction time using a reflux condenser. The extraction procedure was repeated once for the residues. Then the extraction was combined and filtered through a 60-mesh press cloth. The filtrate was concentrated by a rotary evaporator and dried to constant weight at 60 °C to obtain the final product of PELE.

Standard establishment for the quality control of PELE

Test solution preparation: 0.5 g of PELE was dissolved in 20 mL water under sonication for 15 min, followed by filtrating through a 70-µm filter paper. The filtrate was further extracted with 20 mL petroleum ether for twice. The lower layer was further extracted with ethyl acetate for twice. Obtained ethyl acetate extract was combined and dried using a rotary evaporator. The residue was then dissolved in 2 mL methanol and filtered with 0.45 µm filter paper (CHROMAFIL® Xtra). The obtained solution was used as stock solution for TLC, UV and HPLC detection.

Reference solution preparation: Reference solution of vitexin, orientin and isoorientin were prepared at 1 mg/mL in methanol and filtered with 0.45 µm filter papers after completely dissolved.

Thin-layer chromatography: To identify the chemical constituents of PELE, thin-layer chromatography was carried out on a commercial polyamide sheet (10*20 cm) using methanol: water (80:20 V/V) system as mobile phase. Specifically, the reference solution of orientin and isoorientin, as well as the test solution prepared from three batches of PELE, was dropped at one end of the polyamide sheet in a volume of 2 µL. After they developed over a path of 15 cm, the polyamide sheet was dried at 100-105°C. The crystalline aluminum chloride solution (0.5 g/L in 5 % methanol) was sprayed on top of the polyamide sheet and allowed to dry in air for 30 min. Results were observed and imaged under UV exposure.

UV-vis determination of total flavonoid content: 0.5 g PELE were refluxed in 25 mL methanol at 80 °C for 30 min and then filtered with neutral filter paper. The filtrate was diluted with a dilution ratio defined in preliminary experiments and mixed with 0.5 mL $NaNO_2$ (5%, m/v). After standing for 6 min at room temperature, 0.5 mL $AlCl_3$ solution (10%, m/v) and 5 mL of NaOH (1 M) were added into the mixture. The volume of obtained mixture was adjusted to 10 mL with methanol and kept for 15 min. The absorbance (A) at 493 nm was measured and total flavonoid content was calculated according to the standard curve prepared by measuring the absorbance of series concentrations of orientin following the same method. All determinations were performed in triplicate.

HPLC quantification of orient: The HPLC method was performed on a Shimadzu LC-20A liquid chromatograph with a UV diode array detector equipped with Intertsil ODS-3 C18 column (250 mm×4.6 mm, 5 µm). The gradient elution was: solvent A - methyl cyanide (100%), solvent B – 0.1% Phosphoric acid in water (0~25 min, 19% A; 25~30 min, 19~50% A; 30~40 min, 50% A; 40~45 min, 50% to 19% A; 45~60 min, 19% A). Injection volume for both extract and standard solutions (orientin and vitexin) was 10 µL. Flow rate was 1.0 mL/min and detection wavelength was set as 348 nm. The column temperature was 25°C. Identification of the compounds was achieved by the comparison of retention times with those of standard solutions. The content of orientin in individual extracts was calculated according to the calibration curve prepared from series concentrations of orientin.

Evaluation of immunomodulatory and anti-inflammatory effects of PELE

Animals: Male and female BALB/c mice (SPF grade, 4-week-old, 18-20 g) were purchased from the Hunan SJA Laboratory Animal Co., Ltd (Changsha, China). Animals were kept in micro-isolator cages and acclimated at 22 ± 2°C with a 12-light and dark cycle for 7 days. During this period, mice had free access to standard commercial diet and water. Experiments were carried out following the protocol approved by the Ethical Committee on Animal Research of Guangxi University (Protocol number GXU2015-008) and animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Drug preparation for intraperitoneal injection: Powdery drugs of astragalus polysaccharides (AP), cyclophosphamide (CTX), dexamethasone (Dex) and PELE were dissolved in saline for injection to required concentrations. Drug solutions were filtered with 0.22 µm filter papers before intraperitoneal injection. The PELE under this study is endotoxin free.

Experimental design for immunomodulatory effects evaluation: The immunomodulatory activity of PELE was evaluated using CTX-induced immunosuppressed mouse models as described by Di Wang with slight modification (Wang *et al.*, 2018). Astragalus polysaccharides, a widely used immunopotentiator, was used as positive control drugs in this study. After one-week acclimatization, mice were randomly divided into 6 groups with 10 mice (male and female in half) per group. Day 1 to 7, mice in control, CTX, AP and PELE groups were intraperitoneally injected with saline, AP (400 mg/kg) or PELE (250, 500 and 1000 mg/kg.bw) in a volume of 0.1 mL/10 g (body weight.bw). 4 h after the last drug administration, all mice expect those in control group were intraperitoneally injected with cyclophosphamide in a dose of 250 mg/kg.bw. 24 hr later, all mice were weighted and sacrificed. Blood, thymus and spleen were collected before or immediately after mice sacrificed. The spleen and thymus were weighted and organ indexes were calculated as the ratio of spleen or thymus weight (mg) to body weight (g).

Experimental design for anti-inflammatory effects assessment: The anti-inflammatory activity of PELE was evaluated using carrageenan induced acute inflammation in the right mouse paw as described by Makni with modifications (Makni *et al*., 2019). Dexamethasone, a common steroidal anti-inflammatory drug, was used as positive control. Mice were divided into six groups with ten mice per group. Mice in different groups were intraperitoneally injected with 0.2 mL of saline, dexamethasone (2 mg/kg.bw) or PELE (250, 500, and 1000 mg/kg.bw), respectively. 1 hr after the last drug treatment, the right hind paw of all mice except those in control group were subcutaneously injected with 50 μL of carrageenan (1% m/v in saline). The paw thickness was measured before and 30 min, 60 min, 90 min and 120 min after the carrageenan injection using a vernier caliper. 2 hr after the carrageenan administration, blood samples were collected and all mice were sacrificed. The right hind paw was dissected immediately after sacrifice and fixed in 10% buffered formalin for histological analysis. The swelling rate were calculated as percentage of $(X_t - X_0)/X_0$ where Xt represents the paw thickness after carrageenan injection at 't' min and X_0 denotes the paw thickness before carrageenan injection. The edema inhibition rate of drug treatment was calculated percentage of (Swelling rate of carrageenan group − Swelling rate of treated group)/Swelling rate of carrageenan group.

Enzyme Linked Immunosorbent Assay (ELISA): The blood samples were centrifuged at 3000 rpm, 4 °C for 15 min to separate serum. Contents of IgA, IgG, IgM, IL-2, IL-10, TNF-α and IFN-γ in the serum collected in the experiment of immunomodulatory effects, and contents of IL-1β, IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ in the serum collected in the study of anti-inflammatory effects were analyzed using commercial kits according to the manufacturers' instructions.

Histological analysis: Spleen, thymus and the right hind paw sections were fixed in 10% buffered formalin (phosphate buffer 10 mM, pH 7.4) for 1 week. Subsequently, tissues were embedded in paraffin, sectioned at 5-µm thickness and stained with hematoxylin and eosin. The pathological changes of these tissues were observed and imaged under light microscopy equipped with a camera (Nikon, Japan).

Statistical analysis: Data were expressed as mean values ± standard deviation (Mean ± SD). The statistical analysis of the data was carried out by one-way ANOVA, followed by Tukey's multiple comparisons test using GraphPad Prism 8.0.1 software. Difference was considered statistical different in a significance level of $p < 0.05$.

RESULTS

Preparation and quality control of PELE: Table 1 and table 2 shows the orientin transfer rate under different conditions and it was found that extraction temperature played the most important role on the extraction efficiency, followed by ethanol concentration, material-liquid ration and then extraction time. Significant effect on the orientin transfer rate from raw material to PELE was significantly affecting by different extraction temperature, showing a F value of $F_{0.05}(2,2) = 19 < F_B = 83.780 < F_{0.01}(2,2) = 99$, where B denotes extraction temperature. No significant effect on the extraction efficiency was observed from ethanol concentration, extraction time and material-liquid ratio, as their F values were smaller than 19. From the results obtained orthogonal design, the optimized extraction conditions was determined as 80 % ethanol, extraction temperature of 90℃ , extraction time of 60 min and a material-liquid ration of 1:8. The optimal conditions was repeated triplicate to evaluate the repeatability and stability of the extraction process. The dark brown power obtained was denoted as *P. edulis* leaves extract (PELE) and the extraction yield was determined as 11.15 ± 0.05 % from three different batches of PELE.

Constituent determination via thin-layer chromatography: From the TLC results, clear background, good separation between different components and negligible interference were observed. Similar TLC spectra was observed from three different batches of PELE, indicating the stability on PEPE preparations. The dots corresponding to the standard chemicals of orientin and isoorientin were clearly present in the lanes of PELE samples, suggesting that orientin and isoorientin might be the main constituents of PELE (Fig.1).

Total flavonoids content in PELE determined via UV-vis: Standard curve for orientin determination were prepared by measuring the absorbance of series concentrations of orientin 493 nm using UV-vis method. A good linear relation was obtained with a R^2 of 0.9993, and the linear range was determined as 0.22 to 1.10 mg/mL. According to the standard curve, the content of total flavonoids in PELE was calculated as 67.79 ± 5.51 mg/g.

Identification of orientin and vitexin and quantification of orientin in PELE by HPLC: Besides TLC, orientin and vitexin in PELE were further analyzed by HPLC (Fig.2). Under the optimized HPLC conditions, the retention time for orientin and vitexin were 10.2 min and 17.6 min, respectively. The target peak of orientin or vitexin was clearly separated from the neighboring peaks of other constituents in PELE. The calibration plot for orientin was in good linear relation with a R^2 of

0.999987. Based on the calibration plot, the content of orientin in PELE was calculated as 3.89 ± 0.03 mg/g and its transport rate from raw material to PELE was determined to be 56.49 ± 0.41 %.

Table 1 The results of orthogonal experiment (Mean ± SD, n = 3)

Group	Ethanol concentration (A, %)	Extraction temperature (B, ℃)	Extraction time (C, min)	Material-liquid ratio (D, g/mL)	Orientin transfer rate (%)
1	1(60)	1(70)	1(30)	1(1:8)	17.99±1.82
2	1(60)	2(80)	2(60)	2(1:10)	22.37±1.26
3	1(60)	3(90)	3(90)	3(1:12)	42.28±0.47
4	2(70)	1(70)	2(60)	3(1:12)	21.33±2.23
5	2(70)	2(80)	3(90)	1(1:8)	24.81±1.30
6	2(70)	3(90)	1(30)	2(1:10)	52.71±0.42
7	3(80)	1(70)	3(90)	2(1:10)	24.48±1.45
8	3(80)	2(80)	1(30)	3(1:12)	23.30±1.79
9	3(80)	3(90)	2(60)	1(1:8)	56.20±0.75
Average 1	26.88	21.27	31.33	33.00	
Average 2	32.95	23.50	33.30	33.19	
Average 3	34.66	49.73	29.86	28.30	
Range	7.78	28.46	3.45	4.88	
Optimal level	A_3	B_3	C_2	D_1	

Optimum assembly $A_3 B_3 C_2 D_1$
Primary and secondary order B>A>D>C

Table 2 The results of variance analysis

Factors	Deviation sum of squares	Degrees of freedom	Variance	F value	Sig
A. Ethanol concentration (%)	100.337	2	50.169	5.593	
B. Extraction temperature (℃)	1503.118	2	751.559	83.780	*
D. Material-liquid ratio (g/mL)	45.950	2	22.975	2.561	
Error	17.941	2	8.971		

Note: Because there is no blank column in the orthogonal design, thus the extraction time with the lowest effect on extraction efficiency was chosen as the source of error analysis. $F_{0.05}(2, 2) = 19$; $F_{0.01}(2, 2) = 99$

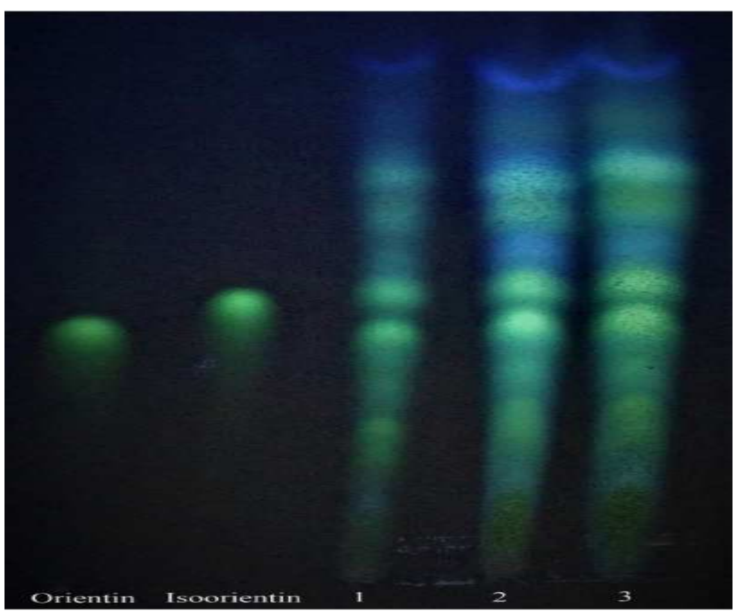

Fig.1 Thin-layer chromatography profile of orientin standard, isoorientin and three batches of PELE (1-3).

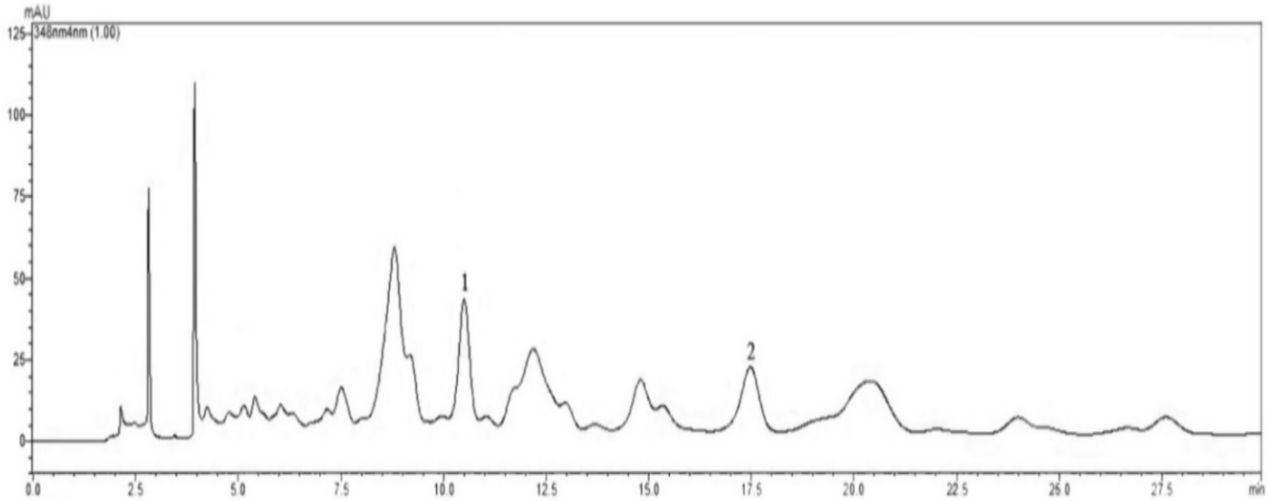

Fig.2 HPLC chromatography of PELE under the UV detection wavelength of 348 nm. 1. Orientin, 10.2 min; 2. Vitexin, 17.6 min.

Immunomodulatory activity evaluation of PELE: During the seven-day PELE administration, no obvious difference in food and water intake, movement, hair appearance, behavior, and control of urination were observed between mice in different groups before CTX administration. However, spirit dispirited, lethargy and anorexia were observed in mice after CTX injection. PELE pretreatment alleviated these kinds of symptoms in immunosuppressed mice.

Protective effect of PELE on CTX induced damage in thymus and spleen: Upon the mice sacrifice, obvious atrophy of the immune organs including thymus and spleen were observed from mice in model group, while astragalus polysaccharides (AP) and PELE pretreatment prevented the atrophy of immune organs. Fig.5A and 5B shows the thymus and spleen indexes of mice after PELE and CTX administration. Significantly lower thymus and spleen indexes were observed in CTX treated mice ($p < 0.0001$), comparing to those of normal mice. Pretreatment of mice with AP at doses of 400 mg/kg and PELE at 250, 500 and 1000 mg/kg strongly inhibited CTX induced the decrease of thymus index in immunosuppressed mice ($p < 0.05$). Regarding to the spleen index, 400 mg/kg.bw AP and all tested doses of PELE significantly improved the spleen index in immunosuppressed mice after 7-day pretreatment in a significant level of $p < 0.0001$. The preventative effect of PELE on CTX induced immune organ atrophy increased as the dose increased, exhibiting a dose-dependent manner.

Morphological changes in the thymus and spleen were further examined via H&E staining (Fig.3 and 4). Compared with thymus of normal mice, the thymus of mice injected with CTX but without drug pretreatment was significantly atrophied, their cortex became thinner, the structure of cortex and medulla was destroyed, the number of lymphocytes decreased, and vacuolar degeneration, nuclear concentration and nuclear fragmentation were observed, in consistent with the spleen index mentioned above. The thymus of mice pretreated with AP or PELE showed completed thymic lobules, clear boundaries between cortex and medulla, significantly increased cortical thickness and upregulated number of lymphocytes. Interestingly, mice in the low-dose PELE group (250 mg /kg.bw) exhibited the best preventive effect on CTX induced thymus injury, showing more integrated thymoid lobules, clearer cortex and medulla, less vacuolar degeneration, smaller nuclear concentration and less nuclear fragmentation.

Similar effects were observed on the pathological changes in the spleen of CTX treated mice with or without drug pretreatment (Fig.4). The spleen parenchyma was composed of white pulp, red pulp and marginal areas with clear boundaries between white pulp and red pulp in the spleen of normal mice. CTX administrated caused obviously tissue atrophy, disorder of tissue structure, destroy of the boundary between red pulp and white pulp, and shrinkage of the spleen corpuscle. AP and PELE pretreatment alleviated CTX induced pathological damage in the spleen, showing clear boundary between red pulp and white pulp, obvious enlargement of spleen corpuscle and increased lymphocyte density in the spleen of treated mice.

Regulatory Effect of PELE on immunoglobulins and lymphokines production in CTX treated mice: Lymphokine contents in the serum were examined using commercial ELISA and results were showed in Fig.5C~5F. CTX injection significantly downregulated the secretion of IL-2, IL-10, TNF-α and IFN-γ, consistent with the atrophy and pathological damage of immune organs. However, AP and PELE pretreatment strongly inhibited the CTX induced downregulation of these lympokines ($p < 0.05$). AP at a dose of 400 mg/kg.bw and PELE at doses of 500 and 1000 mg/kg.bw showed the strongest inhibitory effects and the difference from model group were statically different at $P < 0.0001$ for all tested lymphokines.

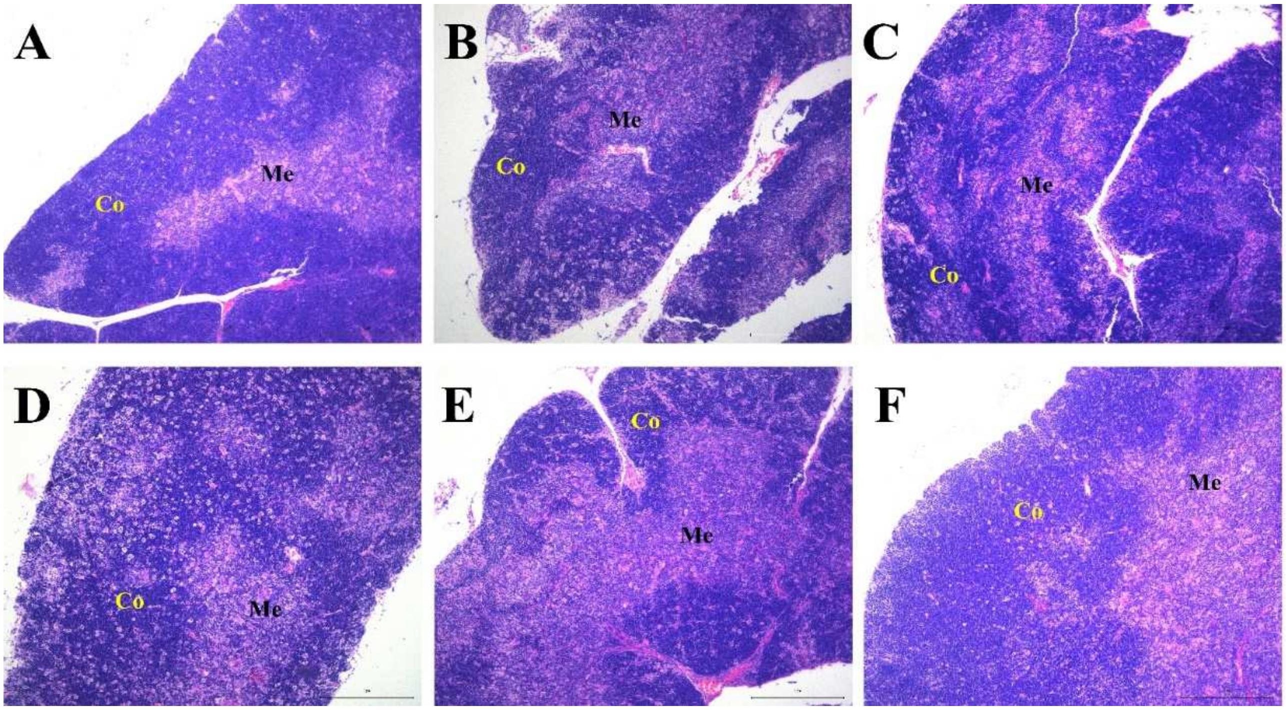

Fig.3 Pathological sections of thymus in CTX-induced immunosuppressed BALB/c mice with or without PELE pretreatment. (A) Normal mice, (B) Model mice, (C) mice with 400 mg/kg.bw AP pretreatment, (D-F) mice with 250, 500 and 1000 mg/kg.bw PELE pretreatment. Me, medulla; Co, cortex. (H&E staining, magnification × 10).

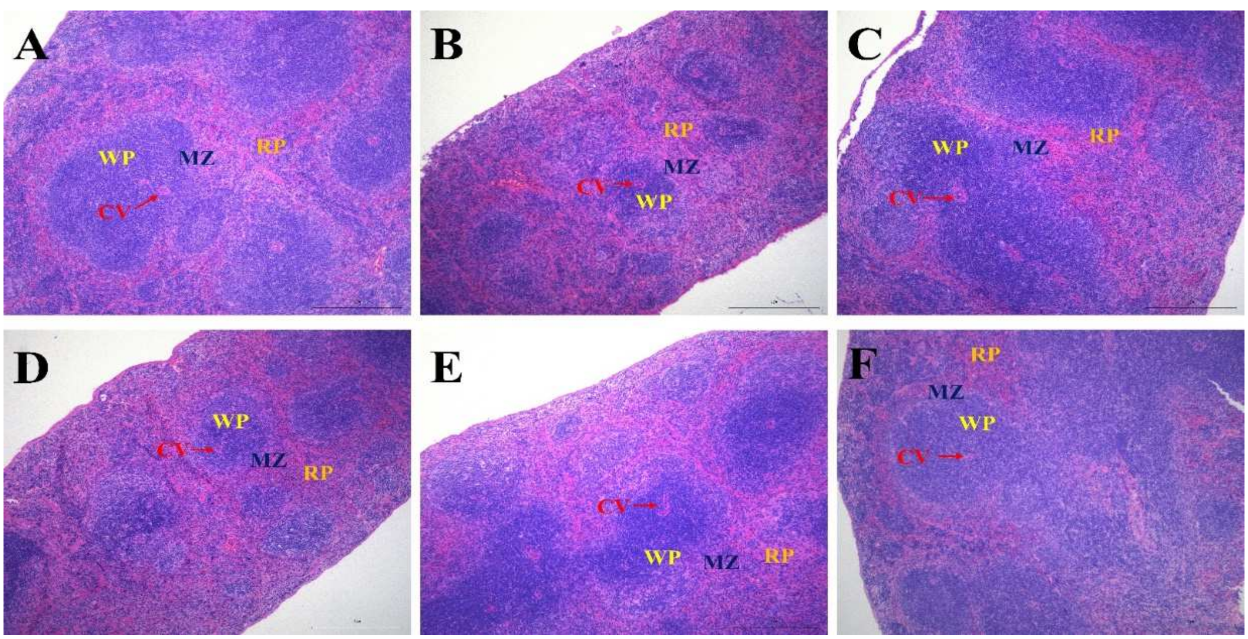

Fig.4 Pathological changes of spleen in CTX-induced immunosuppressed BALB/c mice with or without PELE pretreatment. (A) Normal mice, (B) Model mice, (C) mice with 400 mg/kg.bw AP pretreatment, (D-F) mice with 250, 500 and 1000 mg/kg.bw PELE pretreatment. CV, central vein; MZ, marginal zone; RP: red pulp; WP: white pulp. (H&E staining, magnification × 10).

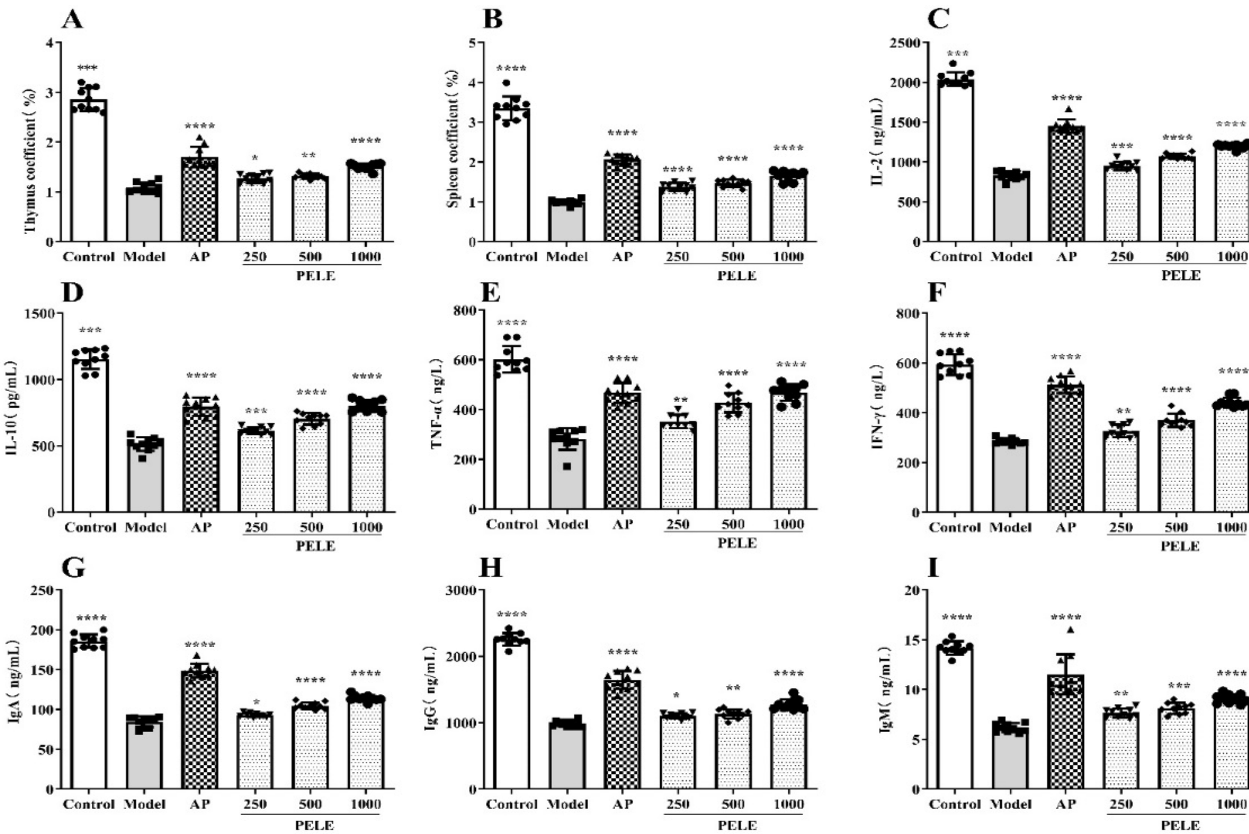

Fig.5 Effects of PELE on organ index and serum level of lymphokines and immunoglobulins in CTX-induced immunosuppressed BALB/c mice. (A) thymus index, (B) spleen index, (C) IL-2, (D) IL-10, (E) TNF-α, (F) IFN-γ, (G) IgA, (H) IgG and (I) IgM. *, **, * and **** indicated statistical difference from that of model group in significant level of $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$. (n = 10).**

CTX administration also resulted in significantly decrease in the serum content of immunoglobulins including IgA, IgG and IgM ($p < 0.0001$). AP (400 mg/kg.bw) and PELE in a dose of 1000 mg/kg.bw was able to inhibit the CTX induced immunoglobulin production reduction in a significant level of $p < 0.0001$. IgA, IgG and IgM levels in mice pretreated with PELE at doses of 250 and 500 mg/kg.bw were significantly higher than those in model mice ($p < 0.05$, 0.01, 0.001 or 0.0001) (Fig.5G~5I.). The inhibitory effect on CTX induced immunoglobulin production increased as the doses of PELE increased, showing a dose-dependent manner.

Anti-inflammatory activity of PELE

Effect of PELE on carrageenan induced paw edema: The anti-inflammatory activity of PELE was evaluated using carrageenan induced paw edema models in mice. After the subcutaneous injection of carrageenan, paw edema was observed in 30 min and the paw thickness reached the peak in 60 min (Fig.6). The paw thickness was slightly decreased after 60 min but the paw was still much thicker than that of normal mice during the 2 hr observation. The paw swelling rate of mice pretreated with 250 and 500 mg/kg.bw PELE lower than that of model mice, showing similar trends as model group. It was worth to note that pretreatment of mice with 2 mg/kg.bw DEX (Dexamethasone) or 1000 mg/kg.bw PELE strongly inhibited the paw edema upon carrageenan administration, and the paw swelling rate exhibited a sharp decreased trend during the 2 hr observation period. Inhibition rates of 15.44%, 17.72%, 45.38% and 48.73% were observed at the time point of 30 min, 60 min, 90 min and 120 min, respectively, in mice pretreated with 1000 mg/kg.bw PELE, showing a similar effect as DEX (15.21%, 30.70%, 47.08% and 51.74% at 30 min, 60 min, 90 min and 120 min, respectively).

Effect of PELE on pro-inflammatory and anti-inflammatory factors secretion: Pro-inflammatory and anti-inflammatory factors play important roles in controlling the inflammatory response. Upon carrageenan administration, the production of pro-inflammatory factors, including TNF-α, IL-1β and IL-6 were significantly elevated ($p < 0.0001$), suggesting the formation of acute inflammation (Fig.7A~C). DEX (2 mg/kg.bw) and PELE (500 and 1000 mg/kg.bw) pretreatment strongly inhibited the production of these pro-inflammatory factors in mice injected with carrageenan ($p < 0.0001$), showing a dose dependent manner.

Opposite from pro-inflammatory factors, the serum levels of anti-inflammatory factors, including IL-2, IL-4, IL-10 and IFN-γ, were dramatically decreased in mice injected with carrageenan ($p < 0.0001$) (Fig.7D~G). A dose of 250 mg/kg.bw PELE significantly upregulated the serum content of IL-4 ($p < 0.05$), and a dose of 500 mg/kg.bw was able to inhibit carrageenan included reduction of both IL-2 and IL-4 ($p < 0.0001$). The contents of all tested anti-inflammatory cytokines in the serum of DEX (2 mg/kg.bw) and PELE (1000 mg/kg.bw) pretreated mice were significantly higher than their contents in the serum of mice in the model group ($p < 0.01$ or $p < 0.0001$).

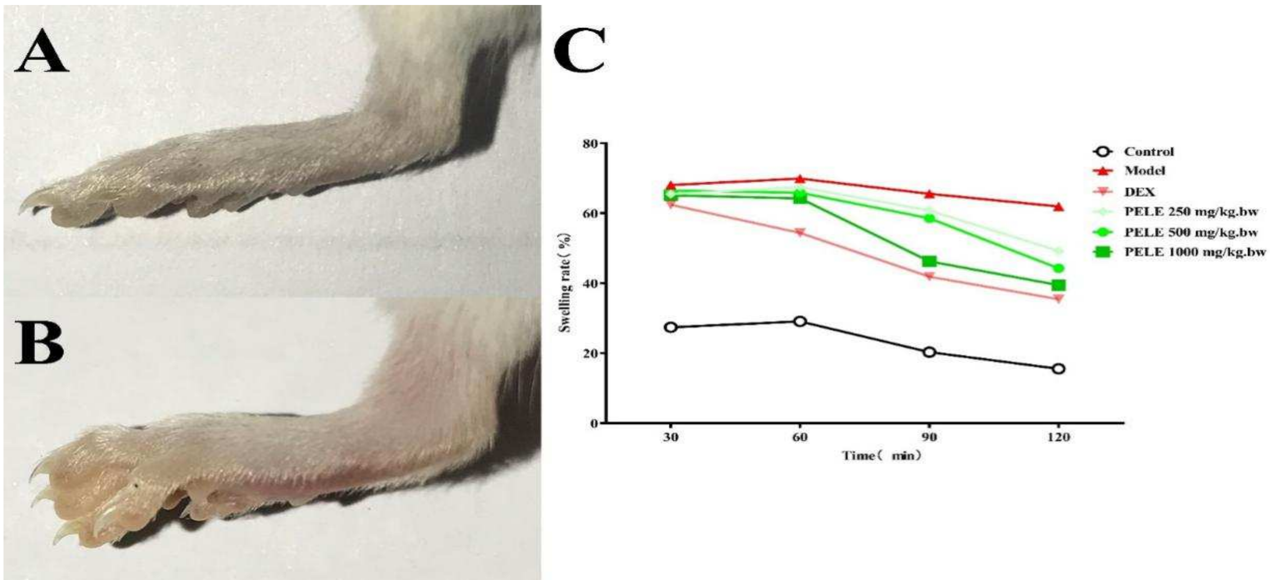

Fig.6 Carrageenan-induced swelling of hind paw in BALB/c mice. (A) paw of normal mice; (B) paw of mice 30 min after *carrageenan* injection; (C) Paw swelling time curve.

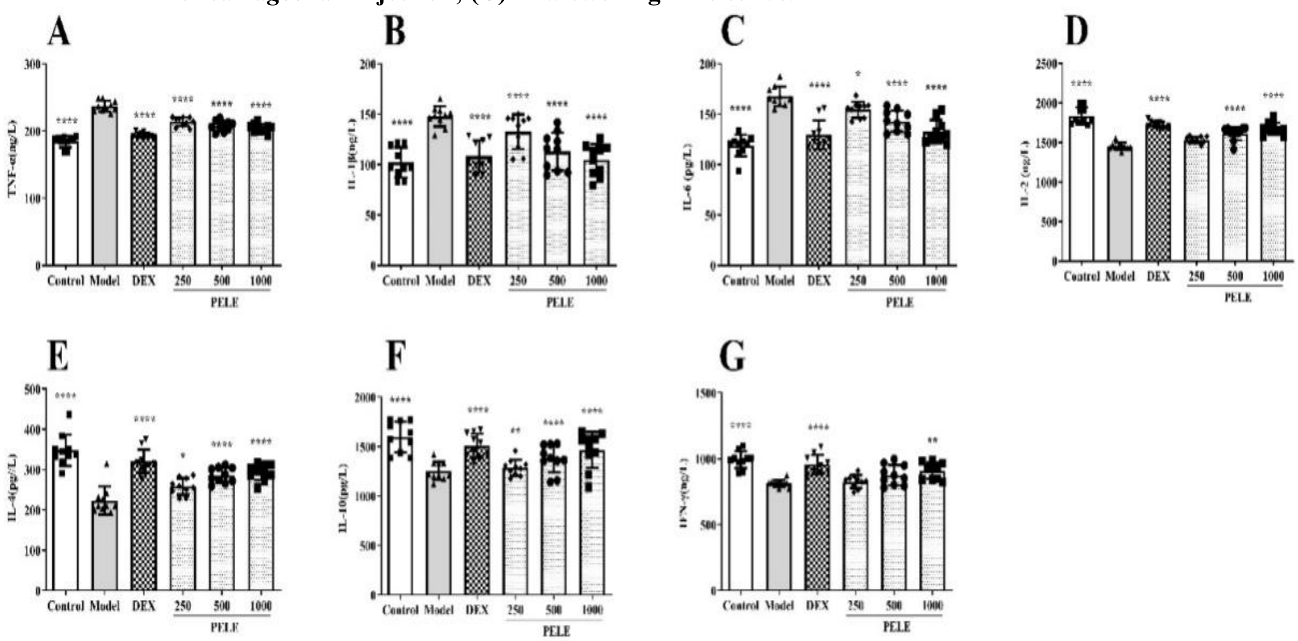

Fig.7 Effects of PELE on the swelling rate and inhibition rate of carrageenan-induced paw edema and serum levels of inflammation associated factors in BALB/c mice. (A) TNF-α, (B) IL-1β, (C) IL-6, (D) IL-2, (E) IL-4, (F) IL-10 and (G) IFN-γ. *, ** and ** indicated statistical difference from that of model group in significant level of $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$. (n = 10).**

Histological analysis of hind paw: The pathological change in the hind paw were studied by histological analysis and showed in Fig.8. Carrageenan injection resulted in tissue edema, destruction of connective tissues and polymorphonuclear cell cloning. PELE and DEX pretreatment obviously alleviated these deteriorations and showed a significant reduction of edematous condition and the inflammatory cell infiltration.

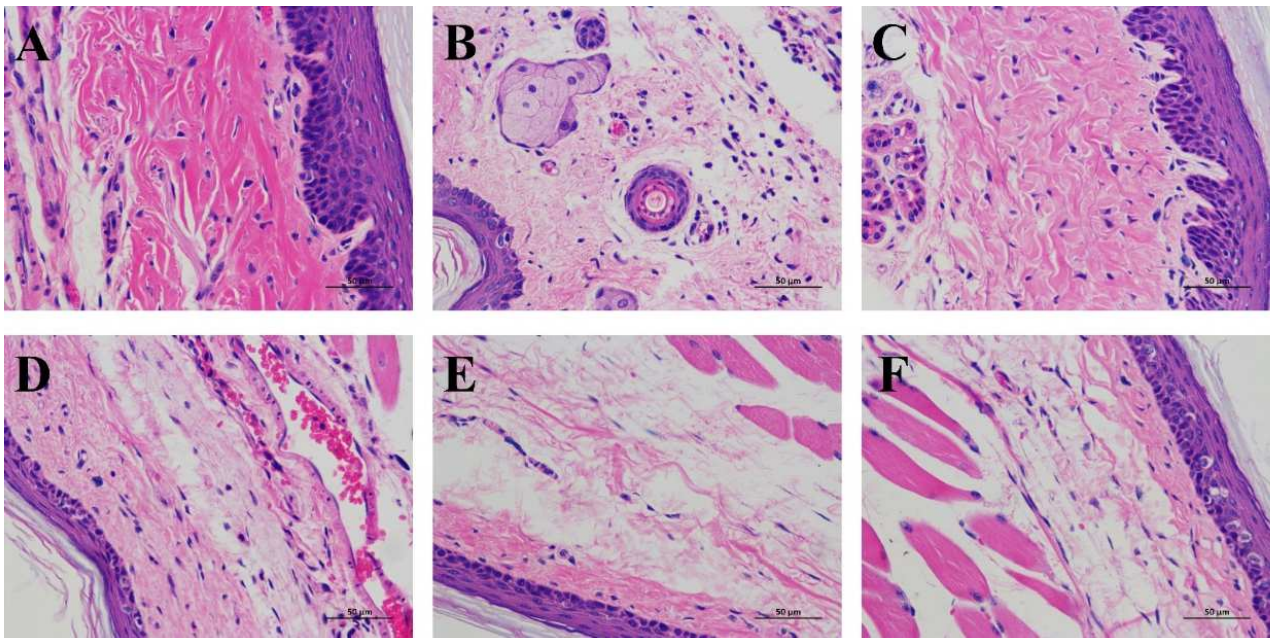

Fig.8 Histological examination of hind paw of carrageenan administrated BALB/c mice. (A) normal mice, (B) Model mice, (C) mice with 400 mg/kg.bw AP pretreatment, (D-F) mice with 250, 500 and 1000 mg/kg.bw PELE pretreatment. (H&E staining, magnification ×400).

DISCUSSION

Traditional Chinese medicine extract has been classified as a new kind of raw material characterized by the enrichment of active ingredients. It was reported that polysaccharide, flavonoids, alkaloids, phenols, triterpene and volatile oil are the main effective constituents of many Chinese herbs(Xiang *et al*., 2022). Due to their ability in improving the quality and nutrition value of plants, and their well-documented pharmocological activities of anti-cancer, anti-oxidative, anti-inflammatory and anti-mutagenic, flavonoid have attracted increasing attentions in the modern pharmacology (Chen *et al*., 2019; Ibrahim *et al*., 2017). *P. edulis* is a plant widely cultivated in Guangxi for the supply of passion fruits for the industry of flavor, juice and food, leaving 10,000 tons of their leaves directly burned. Great effort has been carried out to explore the medical applications of *P. edulis* leaves in other countries and activities of anxiolytic, anti-inflammatory, liver protection and antioxidative have been observed from preparations and extracts of *P. edulis* leaves. However, there still lacks a comprehensive and effective standard for the quality control of the preparation and extract of *P. edulis* leaves, limiting their widely medical applications.

In the 9th edition of British pharmacopoeia, hyperoside and rutin are the designated ingredients for the identification of *P. edulis* flowers dry extract via TLC method (Commission., 2008). The content of total flavonoids in the flower of *P. edulis* has also been previously determined by UV-vis using hypericin, rutin or vitexin as reference (Muller *et al*., 2005). However, these constituents cannot be detected in PELE prepared in this study and raw PEL corrected in Guangxi using reported methods, including UV, TLC and HPLC. In other literatures, orientin, isoorientin, vitexin, isovietin, chrysin and luteolin were detected in the stem and leaf of *P. edulis* (Lucena *et al*., 2018). In this study, the content of total flavonoids in PELE was determined to be 67.79 ± 5.51 mg/g by UV-vis analysis using orientin equivalents. Two identification methods, including TLC and HPLC were established for the identification of orientin and isoorientin and the identification of orientin and vitexin in PELE, respectively. The content of orientin in PELE was quantitative determined using HPLC method under optimized conditions. The results of TLC and HPLC in this study showed the characteristics of strong specificity, good reproducibility and negligible interference. The combination of UV-vis, TLC and HPLC under the conditions optimized in this study could be an ideal method for the quality control of PELE and PEL in planted Guangxi province.

Cyclophosphamide is a widely used chemotherapy agents for malignant tumor. Severe side effects, such as leukopenia, immunosuppression and myelosuppression, are frequently observed on patients during the chemotherapy treatment period (Turk and Parker, 1982). CTX induced immunosuppressed animal models have been frequently used for the evaluation of immunomodulatory activities of Chinese herbs, plant extract and other immunomodulator. Atrophy of immune organs (spleen and thymus), decrease of immunoglobulin production (IgA, IgG and IgM) and reduction of lymphokine secretion (TNF-α, IFN-γ, IL-1β, IL-4, and IL-6) were the main characteristics of immunosuppression in CTX administrated mice (Chen *et al*., 2019b; Wang *et al*., 2018). TNF-α plays an important role in the cellular immune process by activating macrophages and T lymphocytes. IFN-γ, an essential cytokine for host defense against pathogens, activate neutrophil, NK cells and macrophages to eliminate bacterial and tumor cell. IL-2 promotes T cell response by regulating homeostasis and the differentiation of T cells, while IL-10 is critical in controlling the balance of immune response. In this study, intraperitoneal injection of mice with CTX (250 mg/kg) significantly reduced the thymus and spleen index, decreased serum levels of IgA, IgG and IgM, lowered the levels of IL-2, IL-10, TNF-α and IFN-γ in 24 hr, in agreement with previous reports (Wang *et al*., 2018; Yang *et al*., 2018). However, such changes in tissue index, immunoglobulin production and lymphokine secretion were inhibited by PELE pretreatment, suggesting that PELE could be a potential candidate of immunoenhancer and can be used in the treatment of immunosuppression-associated disease.

Carrageenan, a polysaccharide derived from seaweeds of the genus Eucheuma, Gigartina, Iridaea and Chondrus, is a strong chemical that can causes reproducible inflammatory reactions (Morris, 2003). It has been widely used to establish inflammatory animal models for the screening of anti-inflammatory candidates (Catelan *et al*., 2018; Gardner, 1960). Different from pathogen induced systemic inflammation, carrageenan induced edema does not accompany with the release of histamine which has been speculated from the fact that no preventive activity on carrageenan induced edema were observed when large doses of potent antagonist against serotonin and histamine were used (Winter *et al*., 1962) Blood vessels dilate and their permeability increase during the first phase (0~120 min) of inflammatory response to the carrageenan injection, leading to obvious edema in the hind paw (Li *et al*., 2019). Mediators such as pro-inflammatory cytokines play critical role in the development of inflammatory reactions (Monika *et al*., 2010). The cytokines include interleukins (IL-1β and IL-6) and tumor necrosis factors (TNF-α). Besides functioning as immunomodulator, IL-2 and IL-10 are also important anti-inflammatory cytokines to prevent excessive inflammation (Ip *et al*., 2017; Klatzmann *et al*., 2015). In this study, accompanying the obvious paw edema, carrageenan injection also induced increase of TNF-α, IL-1β and IL-6 production and decrease of IL-2, IL-4, IL-10 and IFN-γ secretion. PELE pretreatment inhibited the pro-inflammatory factor production and the anti-inflammatory factor consumption, showing excellent inhibitory effect on carrageenan induced inflammation in BALB/c mice.

Vitexin, orientin and isoorientin are considered as the main ingredients of PELE. In previous studies, isoorientin alleviated LPS induced inflammatory responses in BV-2 microglia (Yuan *et al*., 2014), vitexin down-regulated the level of pro-inflammatory mediators and inhibited the neutrophil migration to inflammatory focus (Rosa *et al*., 2016), and orientin exhibited antidepressant-like effects on the behavior of CUMS-induced depressed mice (Liu *et al*., 2015). Besides, anti-inflammatory activity of vitexin and isoorientin has been previously confirmed by Kupeli using the method of carrageenan induced edema in rats and male Swiss albino mice (Kupeli *et al*., 2004). The similar anti-inflammatory results obtained in this study and previous reports suggested that flavonoids in PELE might account for the major pharmacology activity of PELE, and vitexin, orientin and isoorientin could be the main effective constituents. However, we did not evaluate the immunomodulatory and anti-inflammatory effect of vitexin, orientin and isoorientin in this research, but will be carried out in our further studies to find out the exact functional mechanism of PELE on immunosuppression and anti-inflammation. Since both inflammation and immunosuppression can be observed under the conditions of radiation (Shevtsov *et al*., 2019), cancer (Xiang *et al*., 2020), virus infection (Kandasamy *et al*., 2020; Xiao *et al*., 2020), stress, PELE exhibiting both immunomodulatory and anti-inflammatory effect could be promising candidates for this kind of disease.

Overall Assessment and Recommendations: This study quantitatively analyzed the active ingredients in the PELE and investigated its preliminary anti-inflammatory and immunomodulatory effects, providing a research basis for future research on the anti-inflammatory and immunomodulatory mechanisms of the PELE. Here, we suggest using the PELE and its corresponding active ingredients for re oxidation, antibacterial, and anti-inflammatory experiments to determine the specific effects of various active ingredients in the extract to justify the findings of these results.

Conclusion: In conclusion, PELE was prepared using ethanol extraction. A quality control method combining UV-Vis, thin layer chromatography and high performance liquid chromatography was developed. The total flavonoid content of PELE was estimated to be 67.79 ± 5.51 mg/g and the content of orientin in PELE was determined to be 3.89 ± 0.03 mg/g. The ability of PELE pretreatment to attenuate CTX-induced immune damage and inhibit acute inflammation induced by carob gum makes it a potential candidate for the treatment of immunosuppression and inflammation-related diseases.

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Conflicts of Interest: The authors declare no conflict of interest.

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