Codonopsis thalictrifolia Wall. var. mollis (Chipp.) L. T. Shen exhibits anti-inflammatory activity against LPS-induced RAW264.7 cells and peritonitis in mice

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Abstract. Codonopsis thalictrifolia Wall. var. mollis (Chipp.) L. T. Shen (C. thalictrifolia), a traditional medicinal plant used in Tibetan medicine, has been previously reported to exhibit a variety of pharmacological characteristics. In the present work, we assessed the anti-inflammatory action of 70% ethanolic extract of C. thalictrifolia (ECW) on RAW264.7 cells induced by lipopolysaccharide (LPS) and mice with peritonitis. High-performance liquid chromatography analysis of ECW identified two major compounds, caffeic acid and luteolin. Our results demonstrate that ECW remarkably repressed the generation of pro-inflammatory mediators, which include interleukin-6 (IL-6), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), nitric oxide (NO), tumor necrosis factor α (TNF-α), and interleukin-1β (IL-1β), in the RAW264.7 cells stimulated with LPS. Moreover, ECW suppressed LPS-induced degradation and phosphorylation of IκBα and the nuclear translocation of nuclear factor kappa B (NF-κB) p65 in RAW264.7 cells, indicating its modulation of pathway of NF-κB. In peritonitis mice induced by LPS, ECW treatment ameliorated disorders of immune cells and decreased the pro-inflammatory mediator levels. Our findings suggest that C. thalictrifolia possesses potent anti-inflammatory activity, probably via modulating the NF-κB signaling pathway.

Keywords: Codonopsis thalictrifolia; RAW264.7; peritonitis; mice; NF-κB.

1. Introduction

As a defense against the external environment, the immune system can identify pathogens, such as viruses and bacteria, and prevent serious infections and diseases [1]. However, in immune system disorders, damaged tissues or cells can be wrongly identified as foreign invaders. Specifically, the antibodies or immune cells produced may attack normal tissues or cells, inducing inflammation and immune responses, which can result in a range of immune-related diseases [2, 3].

NF-κB is extensively viewed as one of the most significant regulators of inflammation in controlling a variety of inflammatory mediators and the activation of inflammatory cells [4-6]. NF-κB is normally restricted to the cytoplasm and coupled to its inhibitory factor, IκB. This complex is disrupted when cells are exposed to diverse stimuli, leading to the translation of free NF-κB to the nucleus, in which it interacts with specific sites of DNA recognition. Eventually, such interaction leads to raised expression of several inflammatory genes, inclusive of COX-2, iNOS together with other pro-inflammatory cytokines [7-9]. Recent research have shown that attenuating the transcriptional activity of NF-κB in macrophages can effectively prevent the overproduction of proinflammatory mediators [10, 11].

C. thalictrifolia, a member of the Campanulaceae family, is primarily found in the Tibet Autonomous Region [12]. Recent chemical studies have revealed that C. thalictrifolia extract is abundant in flavonoids, triterpenes, and phenolic acids, such as caffeic acid and luteolin, among other chemical compounds [13]. Besides, C. thalictrifolia has been broadly adopted in conventional Tibetan medicine, especially for treating swelling and inflammation, nourishing impotence, strengthening the spleen and stomach, and other medicinal purposes [14]. Numerous modern

pharmacological researches have claimed that C. thalictrifolia exhibits the following pharmacological activities: promotion of immunity, antioxidant activity, antitumor activity, increase of blood gastrin and motilin concentrations after severe burns, and regulation of burns and scalds leading to gastrointestinal dysfunction [15, 16]. Despite these findings, the overall impact and underlying mechanisms of C. thalictrifolia on inflammation remain unstudied.

Therefore, in order to clarify the bioactivity of C. thalictrifolia, this study was conducted for assessing the anti-inflammatory characteristics and action mechanisms of the ethanol extract of C. thalictrifolia in LPS-induced peritonitis mice and murine macrophages.

2. Materials and methods

2.1 Chemicals and reagents

Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM) as well as other tissue culture reagents were provided by GIBCO BRL Co. (Grand Island, NY, USA). Primary antibodies like anti-COX-2, mouse/goat/rabbit anti-iNOS, anti-phospho-IκBα, anti-IκBα, anti-p65, together with the secondary antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for IL-6, IL-1β and TNF-α were derived from Boster Biological Technology Co. Ltd (Wuhan, China). IgG secondary antibody labeled with Fluorescein isothiocyanate (FITC) and the 4′,6-diamidino-2-phenylindole (DAPI) dye solution were provided bySanta Cruz Biotechnology Company (Beijing, China). FITC anti-mouse F4/80 antibody, FITC anti-mouse Ly6G antibody, 2',7'-dichlorofluorescein diacetate (DCFH-DA), Cell Staining Buffer, APC-anti-mouse CD11b antibody, and purified anti-mouse CD16/32 antibody were supplied by Elabscience Biological Technology Co. Ltd (Wuhan, China).

2.2 Plant material and preparation of ECW

The sun-dried and crushed aeial parts of C. thalictrifolia were extracted by 70% EtOH two times under reflux for 1.5 hours. The extract was concentrated to obtain ethanolic extract of C. thalictrifolia (ECW), which was stored at -20°C. ECW was solubilized with DMSO and distilled water in cellular and animal experiments, respectively. CBTM-E107, a voucher specimen, was stored at the Component Bank of Tibetan Medicine (CBTM) in Lhasa, Tibet, China.

2.3 Experimental animals

Kunming mice (male, with 8-9 weeks of age) were supplied by the Qingdao Institute for Food and Drug Control (with the approval number of SYXK (Lu) 2020 0001). All of the animals had free water and food during the experiment in a 22 ± 2°C room with 12-hour light/dark cycle. The associated facilities together with experimental procedures were implemented as per the Technical Standards of the Animal Ethics Committee of Qingdao University of Science and Technology (approval number: ACQUST-2020-053).

2.4 Characterization of ECW

The HPLC conditions were: stationary phase: Hypersil C18 ODS-10 μm-4.6 mm×250 mm (Thermo, USA), with a mobile phase of 0.5% aqueous phosphoric acid (A)/acetonitrile (B), at 25°C column temperature, with the UV detection wavelength and flow rate of 330 nm and 0.5 mL/min. Samples were detected using a gradient elution procedure, system: Agilent 1220 Infinity LC system (Agilent Technologies, USA), mobile phase: first 10 min, A/B=85/15 (v, v), second 10-30 min, 85-63% (A), 15-37% (B).

2.5 Cell culture and viability assay

Macrophage cell line (RAW264.7) was provided by the American Type Culture Collection. They were grown in DMEM complemented with penicillin (100 U/mL) and 10% heat-inactivated FBS at 37°C and 5% CO_2.

MTT assay was employed for identifying the ECW cytotoxicity. Concentrations of 12.5, 25, 50, 100, 200, 400 and 800 μg/ml of ECW were added to 96-well plates inoculated with RAW264.7 macrophages (100 μL, 1 × 105 cells/mL per well). After 24 h, MTT (which interacts with live cells to produce Formazan crystals) at a 2.5 mg/mL concentration was added to each well, which was cultivated for 4 hours. The absorbance of the solution of crystals produced by dissolving 150 μL of DMSO was detected at 490 nm utilizing an enzyme standard.

2.6 Measurement of Inflammatory Factors

RAW264.7 cells (1×105 cells/mL) were initially cultivated with ECW for 12 h, followed by the addition of LPS for 24 h. The supernatant was derived by centrifugation and kept at -80°C for later experiments. The amount of nitrite generated by Griess reaction reflects the amount of nitric oxide (NO) in the cells. Briefly, the 100 μL supernatant was mixed with Griess reagent at a volume ratio of 1:1 and the absorbance of the mixed solution at 525 nm was determined with an enzyme marker. The IL-6, IL-1β along with TNF-α levels were identified via ELISA kits.

2.7 Extraction of Nuclear and Cytosolic Fractions

The extraction of cytoplasm and nuclei was implemented as per the instructions of manufacturer. In brief, cells were collected and then refloated in cytosolic extraction reagent to facilitate cell lysis. Cytoplasmic extracts were obtained by incubating the mixture at a temperature of 4°C for 10 minutes followed by centrifugation. The nuclear precipitate was added to the nuclear extraction reagent, vortexed at 4 °C for 15 min to promote nuclear lysis, and the supernatant obtained by centrifugation was the nuclear extract. The protein content of each component was identified through applying Bradford's method.

2.8 Western Blot Analysis

For electrophoresis, equal amounts of cell homogenate protein were placed on a 10% SDS-polyacrylamide gel and later transferred to a nitrocellulose membrane. Incubate for 2 hours with TBST blocking solution containing 5% skimmed dry milk. After washing, incubating with the proper concentration of primary antibody for 1.5 hr. After rinsing, the membrane was incubated with the goat anti-rabbit/mouse antibody that was conjoined with horseradish peroxidase for an hour, later rinsed thrice with TBST and the bands were observed with Amersham Bioscience's ECL Western Blotting substrate. A ChemiDoc image analyzer was used to measure protein intensity.

2.9 DNA-Binding Activity of NF-κB

DNA binding activity of NF-κB was detected in nuclear extracts of RAW264.7 macrophages with chosen concentrations of ECW treatment for 12 hours, and subsequently treated with LPS (1 μg/ml) for an hour via the TransAM kit. In brief, complete binding buffer (30 μL, DTT, herring sperm DNA and binding buffer AM3) was added to nuclear extracts (20 μg) from the cells after treatment diluted with complete lysis (20 μL) buffer, the samples were cultivated at room temperature for 1 hour at 100 rpm. NF-κB antibody and HRP-conjugated antibody were diluted at 1:1000 with 1× antibody binding buffer and 1× antibody binding buffer, respectively. Each well was washed with washing buffer and NF-κB antibody (100 μL) was added and cultivated at 100 rpm for an hour. After that, antibody conjugated with HRP was added in accordance with the same operation, and eventually the developing solution was added for the reaction for 5 min. At 450 nm, the absorbance of cleaned supernatant was determined by a spectrophotometer within five minutes.

2.10 Immunofluorescence

To localize p65, RAW264.7 macrophages were added to tissue-cultured glass coverslips and paraformaldehyde was added to fix them for 30 minutes. Subsequently, cells were permeabilized (to promote the entry of p65 antibody into the cells) and labeled with Triton X-100 and FITC-labeled secondary antibody successively. Finally, the nuclei were viewed under a fluorescence microscope (CKX53, Olympus, Japan) in cells localized after incubation with DAPI solution for 30 min.

2.11 LPS-induced peritonitis

The experiments were divided into six groups, each containing 10 male Kunming mice. The ECW groups were divided into three groups, which were intragastriced (i.g.) with low (L-ECW), medium (M-ECW) and high (M-ECW) doses of ECW at 0.0757, 0.1510 and 0.3030 g/kg, respectively. The remaining three groups (model group, blank and positive control group) were were intragastriced (i.g.) with the same amount of 0.9% saline, all performed continuously for 14 days. The blank control group and the remaining 5 groups were injected i.p. with sterile saline and LPS (1 mg/kg, serotype 055:B5), where the positive control group was injected intraperitoneally with dexamethasone (0.5 mg/kg) 60 min before LPS injection. After 4 hours, the peritoneal cavity of the opened mice was cleaned with sterile phosphate-buffered saline (1 mL) with heparin (25 UI/mL).

2.12 Measurement of TNF-α, IL-6, and IL-1β in mice

The IL-6, IL-1β as well as TNF-α levels in peritoneal lavage fluid of mice were determined according to the method in "2.6". Briefly, the peritoneal lavage fluid gathered was centrifuged at 3000 rpm for 15 minutes, and the supernatant was gathered (placed at -80°C) for analysis.

2.13 Flow cytometry

The cellular sediment from the centrifuged mouse peritoneal lavage solution was resuspended with cell staining buffer and washed 2 times. Erythrocyte lysis solution was incorporated into the cells and subsequently lysed for 5 minutes at 4°C. The cells were cleaned twice after centrifugation, later resuspended in the cell staining buffer, and adapted to a 1×107/mL cell density. Cells were cultivated via purified anti-mouse CD16/32 antibody [2.4G2] at ambient temperature for 5 minutes, and next incubated with the APC anti-mouse CD11b antibody [M1/70], flow cytometry antibody, FITC anti-mouse F4/80 antibody [CI:A3-1] and FITC anti-mouse Ly6G antibody [1A8] at 4°C for a further 30 minutes in darkness. Cells were continuously washed with cell staining buffer (5 mL, centrifuged for 5 minutes) after incubation and resuspended in cell staining buffer (0.5 mL) and subjected to analysis utilizing flow cytometry.

2.14 Statistical analysis

All data are expressed as mean ± SD. Student's t-test was applied to detect significant differences between both groups. GraphPad Prism 8 was adopted for the statistical analysis. At $p < 0.05$, between groups, the differences were regarded as significant.

3. Results

3.1 HPLC analysis of ECW

The HPLC analysis of ECW was performed and its chromatogram was recorded at 330 nm. the retention times of the components of ECW were compared with those of the two known bioactive components of C. thalictrifolia (caffeic acid and lignocaine) (Fig.1). The retention times of caffeic acid and lignocaine in the chromatograms of the reference components were 8.557 min and 21.917 min. The retention times of the ECW sample peaks were essentially the same as those of the standards, and the presence of both components in ECW was determined.

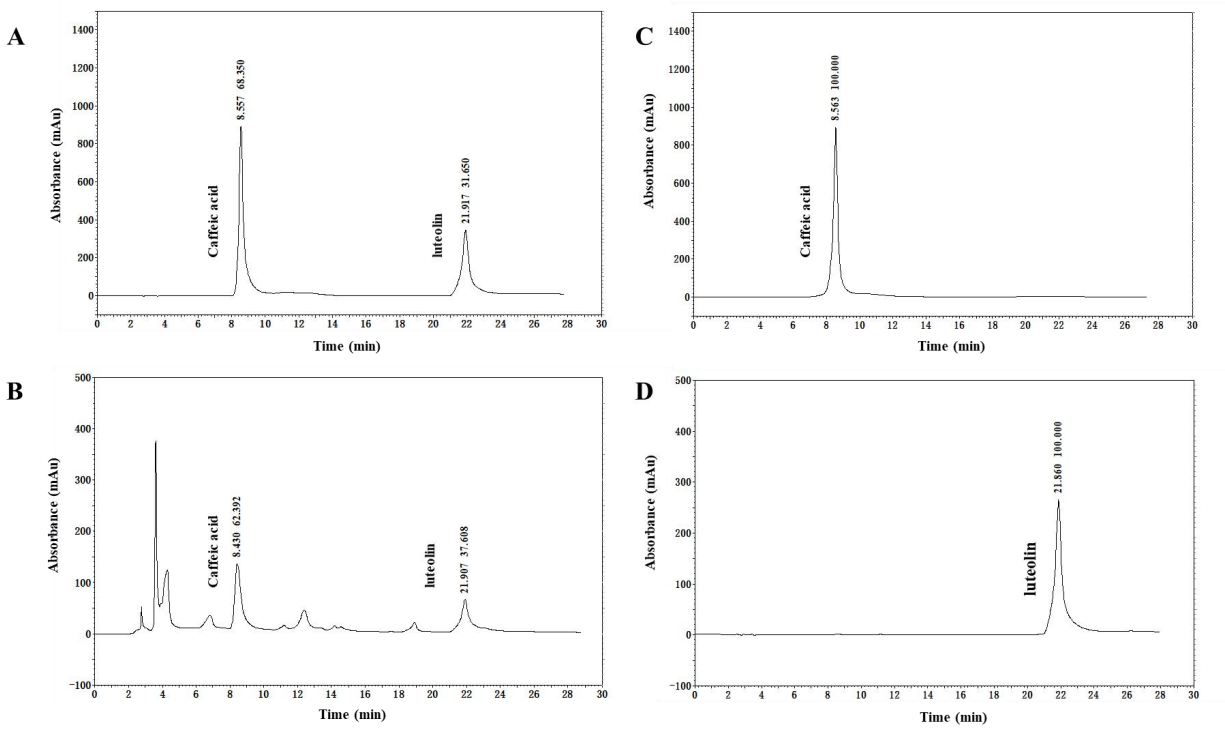

Fig. 1 Results of HPLC analysis. Mixture of reference samples (A); ECW (B); caffeic acid standard (C); luteolin standard (D).

3.2 Effects of ECW on cell viability

For establishing the ECW cytotoxic potential, MTT assay was applied for detecting the cell viability. As illustrated in Fig. 2, ECW demonstrated no cytotoxicity in 12.5-200 μg/mL concentration. Therefore, the ECW concentrations of treated cells in subsequent experiments ranged from 12.5-200 μg/mL.

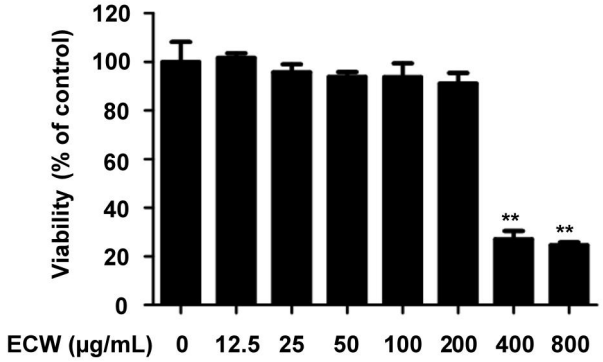

Fig. 2 Impact of ECW on the RAW264.7 macrophage viability. Cell viability of cells that was interacted with the specific concentrations of ECW (12.5, 25, 50, 100, 200, 400, 800 μg/mL) for 24 hours was identified via MTT, here, the control group was set to be 100%. Data denote the mean ± SD of three separate experiments. **$p < 0.01$, in comparison with the control.

3.3 Impacts of ECW on the generation of proinflammatory cytokines in RAW264.7 cells

To examine the impact of ECW on the pro-inflammatory mediator generation, the NO (Fig. 3 A), TNF-α (Fig. 3 B), IL-1β (Fig. 3 C) as well as IL-6 (Fig. 3 D) expression levels were determined in RAW264.7 macrophages with the specific concentrations of ECW treatment applying ELISA kits. In contrast to the LPS group, ECW inhibited the production of the above four cellular inflammatory factors and showed a dose-dependent effect. In addition, Fig. 4 exhibits that ECW also suppressed the iNOS (A) and COX-2 (B) expression versus the LPS group.

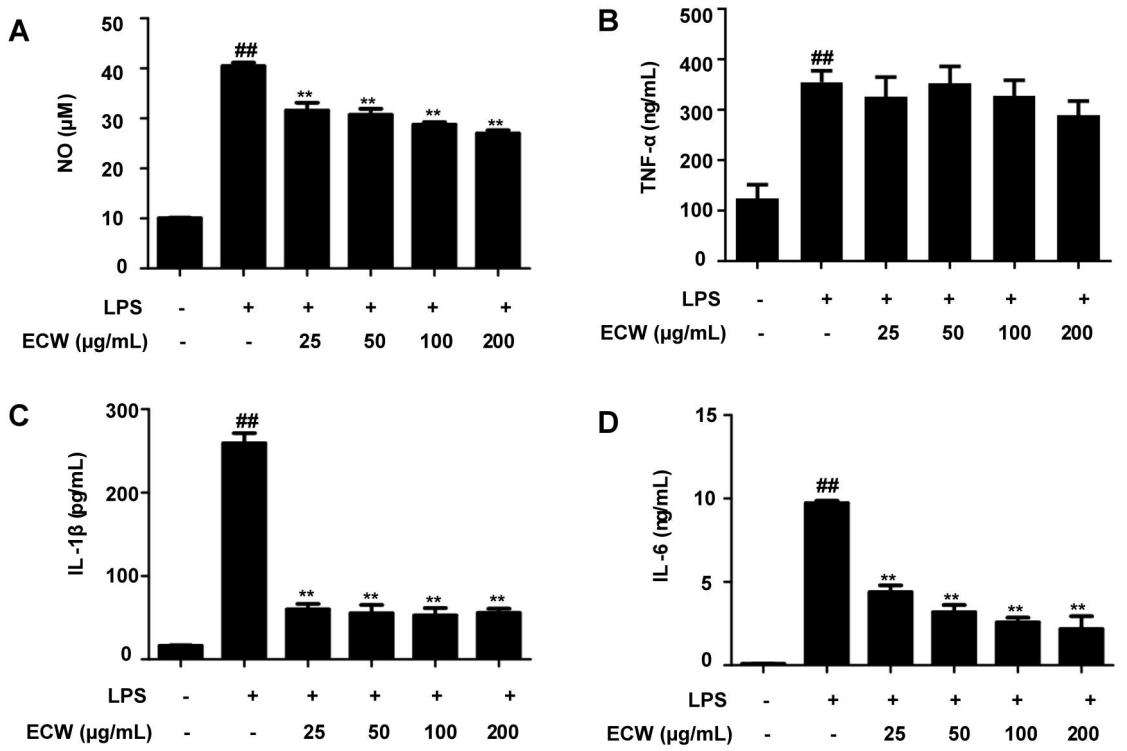

Fig. 3 Effect of ECW on the proinflammatory cytokines in RAW264.7 cells. The NO (A), TNF-α (B), IL-1β (C) together with IL-6 (D) expression levels in the RAW264.7 macrophages that were first treated by the specific concentrations of ECW (25, 50, 100, 200 μg/mL) for 12 hours and later continued to be cultivated for 24 hours after adding LPS (1 μg/mL) were assayed through ELISA kits. Data denote the mean ± SD of three separate experiments. ##$p < 0.01$, versus the control; *$p < 0.05$, **$p < 0.01$, versus the LPS.

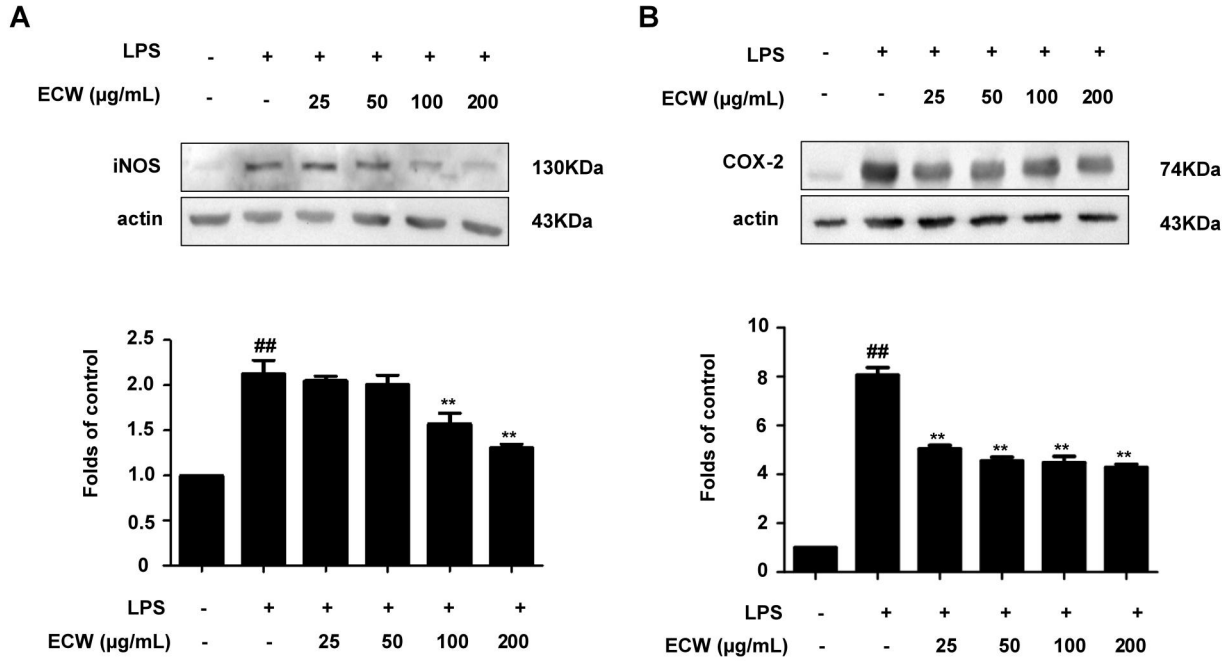

Fig. 4 Impact of ECW on the levels of COX-2 and iNOS in RAW264.7 cells. The iNOS (A) and COX-2 (B) expression levels in the RAW264.7 macrophages that were first treated by the specific concentrations of ECW (25, 50, 100, 200 μg/mL) for 12 hours and later continued to be cultivated for 24 hours after adding LPS (1 μg/mL) were assayed through western blot. Data denote the mean ± SD of three independent experiments. ##$p < 0.01$, in contrast to the control, **$p < 0.01$, in contrast to the LPS.

3.4 Effects of ECW on the NF-κB pathway activity in RAW264.7 cells

To examine the impact of ECW on the component expression of the NF-κB (p65) signaling pathway, which modulates the pro-inflammatory mediator expression primarily through activation and transcription. As shown in Fig. 5 A, pretreatment with ECW (25, 50, 100, 200 μg/mL) for 12 hours significantly inhibited the IκBα phosphorylation in RAW264.7 macrophages resulted from the treatment of LP. Fluorescence microscopy analysis also displayed that ECW impaired the p65 nuclear translocation (Fig. 5 C). Fig. 5 B exhibits that ECW evidently decreased NF-κB DNA binding activity in RAW264.7 cells stimulated by LPS dose-dependently.

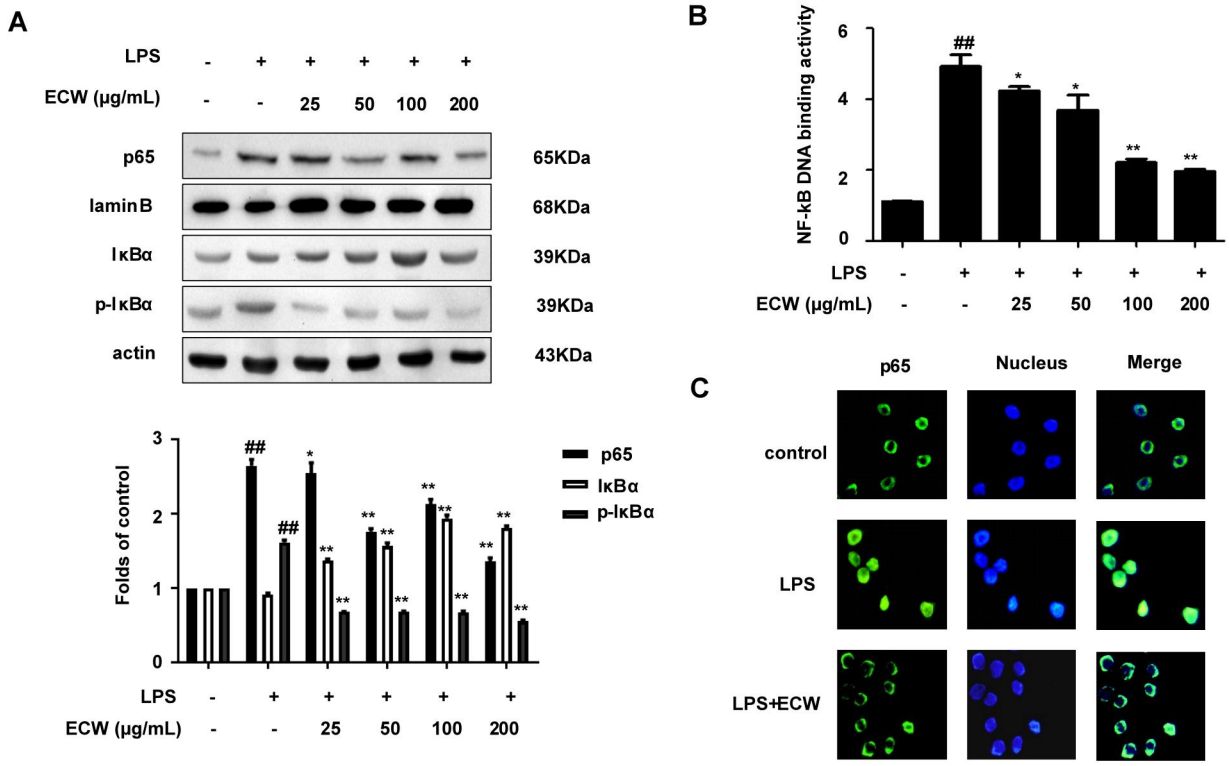

Fig. 5 Impact of ECW on the pathway of NF-κB in RAW264.7 cells. The p-IκB A, IκBα and p65 protein expression levels in the RAW264.7 macrophages that were processed by the specific concentrations of ECW (25, 50, 100, 200 μg/mL) for 12 hours and later continued to be cultivated for 24 hours after adding LPS (1 μg/mL) were assayed through western blot. (A). NF-κB ELISA (Active Motif) was applied for examining the nuclear extracts to identify the NF-κB binding extent (B). The p65 nuclear translocation with ECW treatment for 12 hours and subsequently the LPS (1 μg/mL) addition for continued incubation for 1.5 h was observed by immunofluorescence. The nuclei of cells were stained by DAPI (blue) and viewed under a fluorescence microscope at 400× (C) magnification. Data denote the mean ± SD of three separate experiments. ##$p < 0.01$, vs. the control, *$p < 0.05$, **$p < 0.01$, vs. the LPS.

3.5 Impact of ECW on TNF-α, IL-6, and IL-1β levels in mice

To confirm the in vivo anti-inflammatory influence of ECW, the TNF-α (Fig. 6 A), IL-6 (Fig. 6 B) and IL-1β (Fig. 6 C) levels were measured in mice treated with ECW for LPS-induced peritonitis using ELISA kits. As shown in Fig. 6, treatment with ECW suppressed the raise in the expression levels of the inflammatory factors resulted from the treatment of LPS dose-dependently, indicating that ECW can be effectively applied to treat peritonitis induced by LPS in mice.

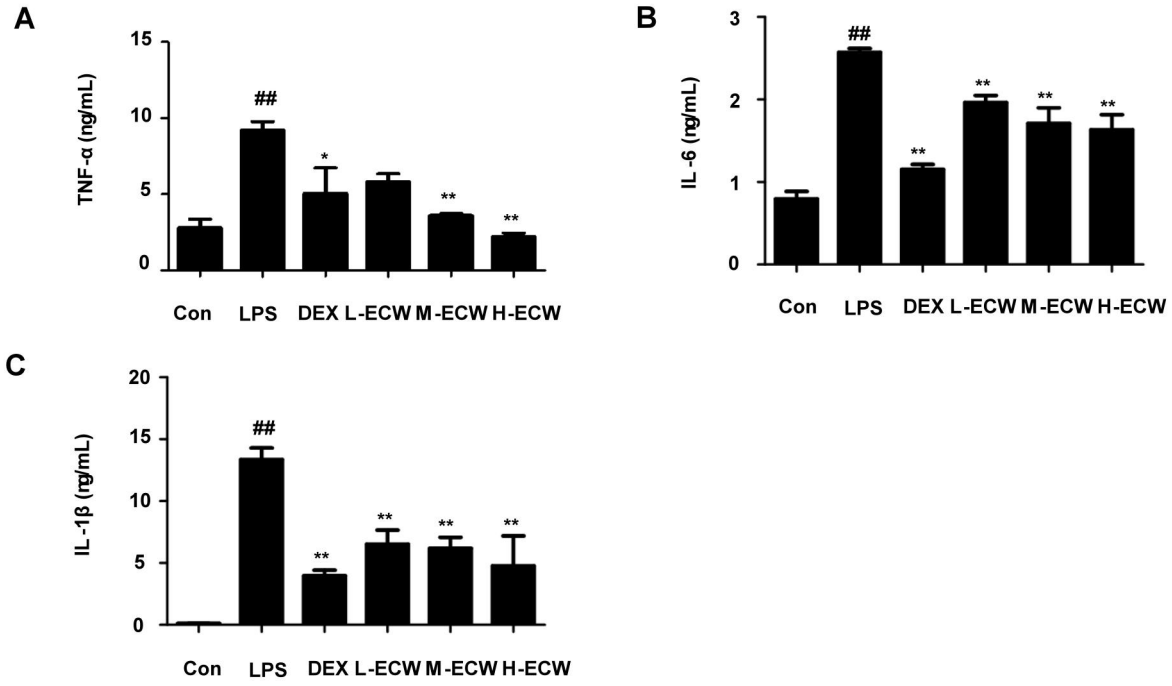

Fig. 6. Impact of ECW on IL-1β, IL-6, along with TNF-α in mice. The TNF-α (A), IL-6 (B) and IL-1β (C) expression levels in supernatants acquired by centrifugation of the peritoneal lavage of mice that was treated by various doses of ECW for 14 consecutive days and later molded with LPS (1 mg/kg) were detected with ELISA kits. Data denote the mean ± SD of three separate experiments, ##$p<0.01$, in contrast to the control; *$p<0.05$, **$p<0.01$, in contrast to the LPS.

3.6 Effects of ECW on changes of immune cells in peritoneal lavage solution in mice

Inflammation is an excessive immune response. The quantity and composition of immune cells change when inflammation onset and progression. Flow cytometry was applied for investigating the peritoneal lavage fluid of peritonitis mice. In contrast to control group, the number of macrophages in the peritoneal lavage fluid was remarkably higher in peritonitis mice. After administration of ECW, the number of macrophages in the low-, middle-, and high-dose groups of ECW was significantly reduced. In particular, the proportion of macrophages in the H-ECW group was reduced to 40.32% (Fig. 7A).

In contrast to the control group, the percentage of neutrophils in peritonitis mice was significantly higher at 7.22%. After administration of ECW, the number of neutrophils significantly decreased, and the proportion of neutrophils in the high-dose group was attenuated to 2.06%. Hence, we found that ECW can improve inflammation by reducing the accumulation of neutrophils in the abdominal cavity of mice (Fig. 7B).

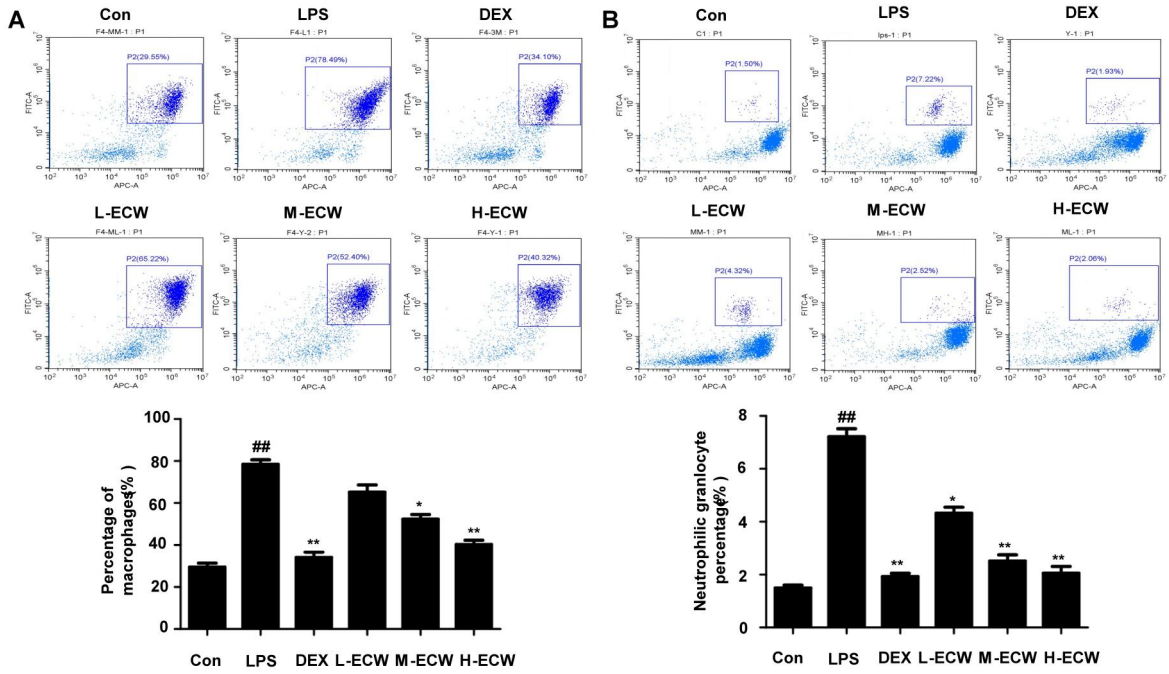

Fig. 7 Impact of ECW on immune cells in peritoneal lavage fluid of mice. The numbers of macrophages (A) and neutrophils (B) in the peritoneal lavage fluid of mice molded with LPS (1 mg/kg) after 14 days of administration with different doses of ECW were determined by flow cytometry. Data denote the mean ± SD of three separate experiments, ##$p < 0.01$, vs. the control, *$p < 0.05$, **$p < 0.01$, vs. the LPS.

4. Discussion

In most cases, moderate and managed inflammation is beneficial because it helps to localize infections and eliminates pathogens. However, prolonged or dysregulated inflammatory reactions can cause local tissue dysfunction, which may evolve into chronic inflammation, sepsis, and even death [17]. Macrophages serve a pivotal role in immune response, inflammation and allergies; via phagocytosis, they protect the human body from external invasion [18, 19]. In aseptic inflammation, tissue damage can also cause excessive neutrophil activation, leading to inappropriate neutrophil migration, adhesion, and increased cell numbers, thereby exacerbating the inflammatory response and leading to a vicious circle [20, 21]. LPS is a lysing component of Gram-negative bacteria's cell wall, which upregulates the expression of various proinflammatory factors by binding to the TLR4 receptor on the surface of immune cells, and activates intracellular inflammatory signaling pathways for instance NF-κB, MAPK, and PI3K-Akt to mediate the inflammatory response of the organism [22-25]. In this work, we adopted LPS-induced RAW264.7 macrophage cells and peritonitis mice to examine whether ECW can regulate the expression of proinflammatory factor-related proteins to exert anti-inflammatory effects. Our findings presented that the inflammatory factor expression levels in LPS-induced RAW264.7 cells and peritonitis mice were evidently raised. However, ECW suppressed the COX-2 and iNOS expression levels together with the generation of inflammatory factors in RAW264.7 cells induced via LPS, and it alleviated peritonitis in mice. Moreover, immune cell abnormalities such as macrophages and neutrophils were also substantially improved.

According to research, macrophage activation increases the inflammatory process with the raising signaling ofNF-κB and regulating the coding of immunological processes with inflammatory response-related genes [26, 27]. Our findings showed that ECW treatment greatly reduced the IκBα degradation to prevent NF-κB activation and nuclear translocation, and considerably inhibiting

NF-κB binding activity. These data imply that ECW can decrease NF-κB activation and thus suppress the inflammatory response.

As a traditional Tibetan medicine, C. thalictrifolia contains active ingredients such as caffeic acid and luteolin. Previous research have shown that caffeic acid can decrease numerous inflammatory mediators for instance IL-6, iNOS, COX-2, PGE2 and IL-1β via regulating the signaling pathways of AGEs, Nrf2, and NF-κB, hence reducing the generation and progression of many inflammatory illnesses (neuroprotective, intestinal-protective, liver-protective, antirheumatic, and anti-atherothrombotic effects) [28, 29]. In vitro and in vivo research have presented that luteolin regulates the synthesis of numerous inflammatory mediators by targeting transcription factors like SOCS3 in the STAT3 pathway and MAPK in the activator protein AP-1 pathway. Besides, a clinical trial with a luteolin formulation revealed a significant reduction in serum TNF-α together with IL-6 levels in children with autism spectrum disorders (ASDs) [30, 31]. In our follow-up research, we will continue to explore other anti-inflammatory ingredients of this plant in addition to the above two compounds to provide more supporting materials in the subsequent development and utilization.

5. Conclusion

In conclusion, our results display that ECW can suppress the NF-κB signaling pathway, thereby suppressing the generation of proinflammatory enzymes and cytokines and improving inflammation in RAW264.7 cells induced by LPS and peritonitis mice. These results give a pharmacological underpinning and a potential mechanism for ECW's anti-inflammatory activity, and underscore its potential as an anti-inflammatory therapeutic agent. In future studies, it will be necessary to perform pharmacokinetic and quality standard evaluations of the active components to give a more comprehensive theoretical fundament for the application of C. thalictrifolia-related products.

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