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Research article

Rubia cordifolia L. ameliorates DSS-induced ulcerative colitis in mice through dual inhibition of NLRP3 inflammasome and IL-6/JAK2/STAT3 pathways

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ABSTRACT

The aerial part of *Rubia cordifolia* L. has been used as an herbal medicine for a long time with various pharmacological activities, including anti-inflammatory, anticancer, and antibacterial activities. The most notable usage of these was that this herbal medicine had good therapeutic effects on diarrhea caused by various factors. However, the mechanism for the ethanolic extract of *R. cordifolia* L. (RCEE) to treat Ulcerative colitis (UC) effectively is still unclear. In this study, DSS successfully induced UC mice and then intervene using different polar parts of RCEE. The results indicated that RCEE-treatment inhibited colonic combination NLRP3 inflammasome formation and IL-6/JAK2/STAT3 activation in vivo, significantly ameliorating the clinical symptoms, including alleviating colonic mucosal damage and infiltration of macrophages, suppressing the release of inflammatory cytokines, and reducing mortality. Taken together, this study suggests that dual inhibition of NLRP3 inflammasome and IL-6/JAK2/STAT3 pathways activation using RCEE may be a promising therapeutic strategy for preventing the progression of UC.

1. Introduction

Ulcerative colitis (UC) is a chronic non-specific intestinal disease, which mainly affects the colonic and rectal mucosa and submucosa. It is difficult to treat, with a high recurrence rate and high cancer rate. There is an urgent need for the development of drugs aiming at new targets for the therapy of UC. The cause of the disease is still unclear, but it is thought to be related to genetic, environmental, intestinal microecological, and immune imbalance factors [1]. Acute and chronic inflammatory responses contribute to the pathogenesis of UC. The Janus-activated kinase 2 signal transducer and activator of transcription 3 (JAK2/STAT3) pathway is an extremely important intracellular signal transduction pathway that is widely accepted as a critical modulator of various biological processes including innate and adaptive immunity and inflammation. Available evidence has shown that IL-6 is closely related to the severity of the clinical features of UC and plays a significant role in T cell proliferation and differentiation [2]. IL-6 is responsible for regulating inflammation and the immune response and activates downstream JAK2 and promotes the phosphorylation and nuclear localization of STAT3. It has been reported that blocking the JAK2/STAT3 pathway modulates innate and acquired immune responses and attenuates chronic inflammation in the intestine [3]. Another critical player in inflammation and immunity is interleukin-1β (IL-1β). IL-1β has been shown to play a pivotal role in the progression of UC [4,5]. IL-1β maturation and release are controlled by the inflammasome, a large multimeric complex that recruits and activates caspase-1 to process inactive pro-IL-1β and pro-IL-18 into active pro-inflammatory cytokines, IL-1β and IL-18 [6,7]. NLRP3 inflammasome is the most characterized member that can be activated by multiple endogenous stress-related signals, mainly containing the receptor NLRP3 protein, the adaptor protein ASC, and pro-caspase-1 protein. Thus, activation of NLRP3 inflammasome may be one of the most

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critical steps in the progression of UC. UC's current treatment modalities are still based on anti-inflammatory and immunomodulatory agents, mainly including salicylic acid agents, corticosteroids, immunosuppressive agents, and biological agents. Traditional drugs for the disease have more adverse effects and are prone to drug resistance [8,9], and newer biologics are less toxic but more costly and difficult to popularize. Therefore, it is important to find new UC treatment drugs that are efficient, safe, and inexpensive. Traditional Chinese medicine has received increasing attention due to its long clinical practice and reliable efficacy, one of which is *R. cordifolia* L. researched in this manuscript. *R. cordifolia* is a perennial climbing vine that belongs to the Rubiaceae family, which has a long history of application in China and India [10–13]. The aerial part of it has various pharmacological activities, such as anti-inflammatory, anticancer, antioxidant, hemostatic, and antibacterial activities [14–18]. In Shanxi Province, water decoction of the aerial part of *R. cordifolia* has been used to treat acute diarrhea in children for over a century, with remarkable efficacy and nontoxic side effects. The herb itself is also one of the main components of anti-diarrheal medicine as "Er-Xie-Ting". Moreover, the antidiarrheal effect of this herb has also been included in the Chinese Materia Medica Mongolia Volume [19]. Previously, YY Sun [20] discovered that *R. cordifolia* aerial extract could inhibit rotavirus multiplication by promoting virus-induced apoptosis in MA-104 cells, thus effectively treating acute diarrhea caused by rotavirus. XP Gong [21] also found that its extracts could reduce the evacuation index with senna-induced diarrhea, delay the formation of semisolid feces and inhibit propulsive movement in castor oil-induced intestinal transit. All the above-mentioned evidence indicates that the aerial extract of *R. cordifolia* has anti-diarrheal and anti-inflammatory activities and confirms the traditional use of this plant as a valuable natural herb for the treatment of diarrhea.Considering its clinical application and biological activity, we hypothesized that the ethanolic extract of *R. cordifolia* L. (RCEE) ameliorates DSS-induced UC in mice through dual inhibition of NLRP3 inflammasome and IL-6/JAK2/STAT3 signaling pathways, thereby reducing the release and expression of proinflammatory cytokines. To test this hypothesis, we investigated the protective effects and potential mechanisms of RCEE in DSS-induced UC mice.

2. Materials and methods

2.1. Experimental animals

Male BALB/c mice (6–8 weeks old, 27 ± 3 g) were obtained from Changzhou Cavens Laboratory Animal Limited Liability Company (Changzhou, China). All animals were acclimatized and fed for one week prior to the experiment. Mice were kept in specific pathogen-free conditions where they received standard food and sterilized water ad libitum under constant humidity (65–70%) and temperature (22 ± 2 °C) with a cycle of 12 h light/dark. All animals were fed ad lib and kept in a pathogen-free environment. The procedures for the care and use of animals were approved by the Ethics Committee of Kunming Institute of Botany, Chinese Academy of Sciences, and all applicable institutional and governmental regulations concerning the ethical use of animals were followed.

2.2. Plant material

R. cordifolia L. was harvested from Shushan National Forest Park in Hefei, Anhui Province, and authenticated by Professor Shoujin Liu (College of Pharmacy, Anhui University of Chinese Medicine, China). The plant specimen was stored at the same college, and the voucher number was NP2020081503.

2.3. Preparation of plant extract

The plant was air-dried at room temperature (43 kg) and cut with an electric shredder, and the herbs were extracted with 75% and 95% ethanol by infiltration, with each concentration repeated 2–3 times and the extracts combined. The extracts were concentrated in a rotary evaporator at 50 °C, and finally, we obtained 8.8 kg crude extract with a 20.47% yield. The RCEE was extracted using petroleum ether, ethyl acetate, and n-butanol in turn, and the four different polar segments of RCPEE (Petroleum ether extract from *R. cordifolia* L.), RCEAE (Ethyl acetate extract from *R. cordifolia* L.), RCNBAE (n-Butyl alcohol extract from *R. cordifolia* L.) and RCAPE (Aqueous phase extract from *R. cordifolia* L.) were finally obtained.

2.4. Drugs and reagents

Mesalazine enteric-coated tablets (Sunflower Pharmaceutical Group Co., Ltd.); Dextran sodium sulfate (DSS, MW: 36000–50000, MP Biomedicals, USA); The myeloperoxidase (MPO) assay kit, total superoxide dismutase (T-SOD) assay kit (hydroxylamine method), malondialdehyde (MDA) assay kit (TBA method) and nitric oxide (NO) assay kit (nitrate reductase method) were purchased from Nanjing Jiancheng Bioengineering Institute; LXR-MultiDTM (Shanghai Universal Biotech Co., Ltd.); Occult blood test kit (Beijing Leagene Biotechnology Co., Ltd.); Primary antibodies against NLRP3, ASC, Caspase-1, JAK2, phospho-JAK2, STAT3, phospho-STAT3, IL-6, GAPDH and secondary antibody (goat anti-rabbit IgG, HRP-linked antibody) supplied by Cell Signaling Technology (Danvers, MA, USA);

2.5. Analysis of RCEE by high-performance liquid chromatography (HPLC)

INERTSIL ODS-SP column (4.6 × 250 mm; 5 μm) was used. The mobile phase consisted of 0.1% H_3PO_4 solution (A) and acetonitrile (B). The gradient elution process was as follows: 0–5 min, 5%–5% B; 5–10 min, 5%–10% B; 10–15 min, 10%–12% B; 15–25 min, 12%–12% B; 25–37 min, 12%–25% B; 37–50 min, 25%–25% B; 50–52 min, 25%–40% B; 52–58 min, 40%–80% B; 58–75 min, 80% B; with equilibration of the gradient elution for 5 min. The column temperature was 30°C, and the injection volume was 2 μL. The detection wavelength was 254 nm.

2.6. The biosafety of RCEE

The biosafety of RCEE was tested by continuous gavage administration (RCEE, 500 mg/kg) to experimental mice for one month. In comparison with normal mice, 500 mg/kg RCEE (i.g.) did not affect the growth of mouse weight (Figure 1A). The number of red blood cells and platelets and the concentration of hemoglobin in RCEE-treated mice were also consistent with those in normal mice. Multiple important physiological indicators, such as albumin, alanine aminotransferase, creatinine, urea, uric acid, and aspartate transaminase, did not show significant differences between RCEE-treated mice and normal mice (Figure 1B). Moreover, immunohistochemical analysis of the heart, liver, spleen, lung, and kidney revealed neither inflammatory cell infiltration nor organ tissue necrosis in RCEE-treated mice (Figure 1C). All these results indicated that RCEE possesses satisfactory biosafety.

2.7. Determination of the dose of RCEE administered in animal experiments

To determine the optimal dose of RCEE to be administered in animal experiments, we first calculated the human equivalent of RCEE in clinical administration based on its extraction rate and then converted it into the applicable dosage for mice based on the principle of drug dosage conversion between humans and mice. On this basis, the safety and efficacy of these RCEE dosages were fully evaluated through preliminary experiments. Finally, the dosages of RCEE in UC mice were determined to be 250 and 500 mg/kg (i.g.).

2.8. Induction of acute UC model and treatment methods

UC was induced by a 3% DSS (dissolved in drinking water) supply to mice to drink freely for 1 week. Eighty-eight mice were randomly divided

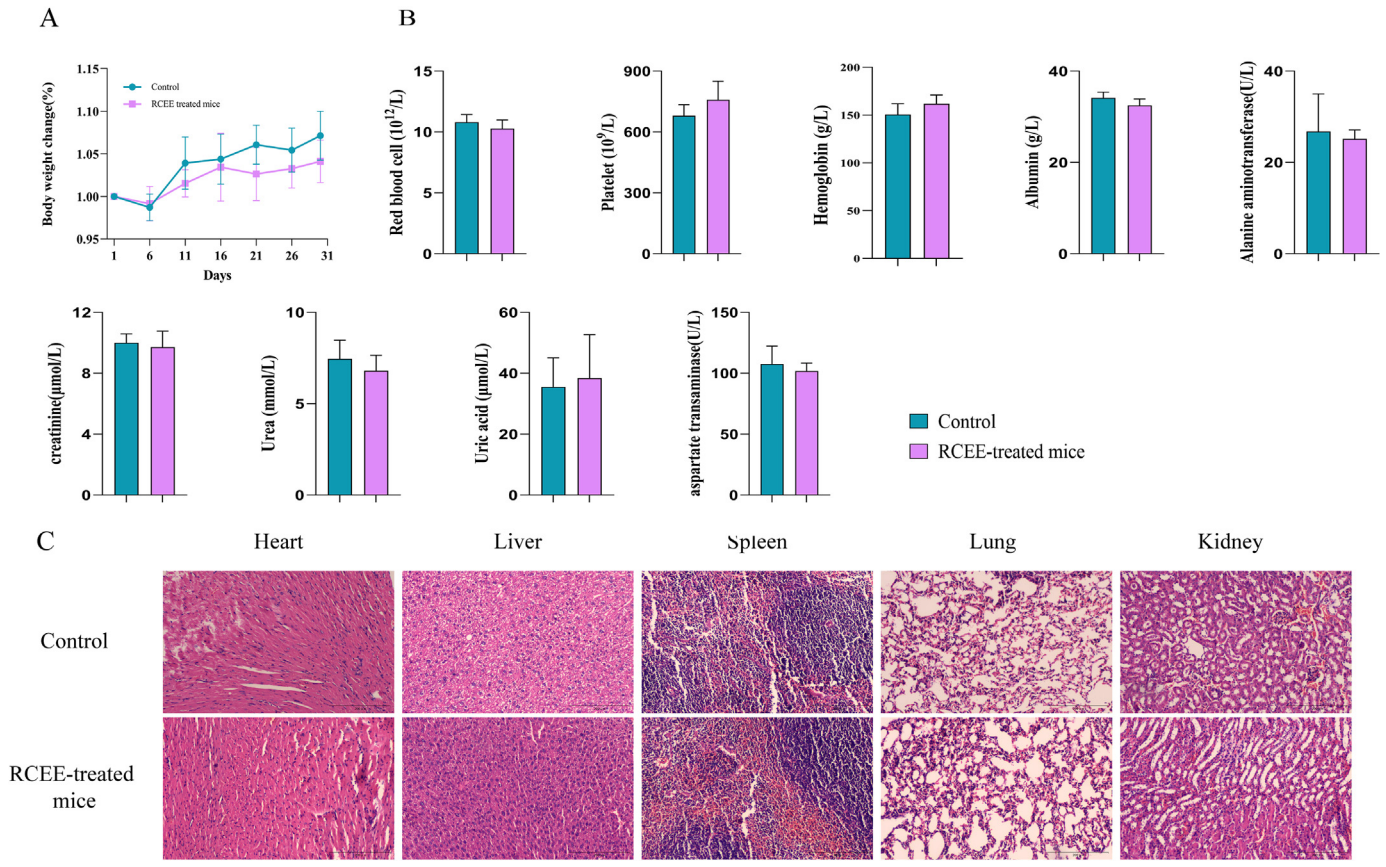

Figure 1. The biosafety of RCEE. A. The weight of mice in the control group (normal mice) and RCEE-treated group (500 mg/kg RCEE). B. The number of red blood cells and platelets and the concentrations of hemoglobin, albumin, alanine aminotransferase, creatinine, urea, uric acid, and aspartate transaminase in mouse serum samples from the control group (normal mice) and RCEE-treated group (500 mg/kg RCEE). C. Immunohistochemical images (H&E staining) of the heart, liver, spleen, lung, and kidney from the control group (normal mice) and the RCEE-treated group (500 mg/kg RCEE). Scale bar = 200 μm.

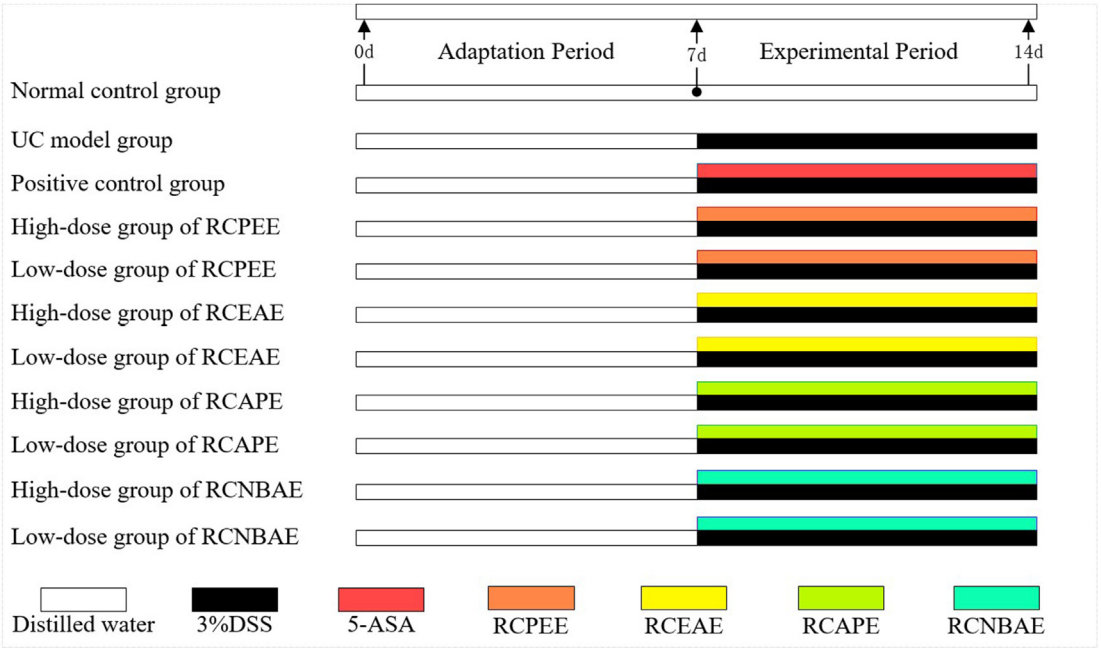

Figure 2. Schematic diagram of the treatment schedule in ulcerative colitis.

into eleven groups with 8 mice in each group, named the normal group, positive control group, model group, RCPEE group, RCEAE group, RCNBAE group, and RCAPE group (the latter four drug groups were set in separate high and low doses: 250 and 500 mg/kg, i.g.) (Figure 2). Except for the normal group, all remaining groups of mice could only drink 3% DSS freely throughout all experimental periods. The eight drug

intervention groups were given high and low doses of four different polar segment extracts, and the positive control group was given mesalazine (5-ASA) solution (200 mg/kg, i.g.). All drugs were dissolved in 2% Tween-80 solution, and the normal group and model group were simultaneously gavaged with 2% Tween 80 as a control. (40 ml/kg, i.g.) once daily, respectively.

2.9. Specimen preparation and HE staining

(1) After the mice were sacrificed by the cervical dislocation method, the colon was quickly dissected and flushed with PBS, and the length of the colon was compared among the groups and recorded. From the anus upwards, three tissue specimens (5 mm × 10 mm) were taken from each animal's distal colon, frozen in liquid nitrogen, and stored in a -80 °C freezer for nucleic acid, protein, and cytokine detection.
(2) One colon tissue specimen with obvious inflammation or ulceration was taken, rinsed, and placed in 4% paraformaldehyde in 10 times the volume of the tissue specimen for fixation, after which pathological sections (tissue paraffin embedding, sectioning, H&E staining) were made by conventional methods. Histopathological inflammation of the colonic mucosa was observed microscopically.

2.10. General sign observation and disease activity index (DAI)

General conditions, such as mental status, diet, activity, and hair, of the mice in each group were observed. Body weight, fecal traits, and occult blood were recorded, and the disease activity index (DAI) was calculated for each group of mice according to the Cooper HS scale [22]. (Table 1).

2.11. Evaluation of colonic pathological changes and colon mucosa damage index

Colon fragments were fixed with 10% formalin, embedded in paraffin, and sectioned into 5–6 μm slices. Sections were hydrated and stained with HE followed by microscopic observations. We used a blinded fashion to assess the colon mucosa damage index (CMDI), as described in Table 2 [23].

2.12. Evaluation of splenomegaly and spleen coefficient

The cervical dislocation method was adopted to execute the experimental mice. The spleen and thymus of the mice were separated and photographed, and the weight was recorded. The organ coefficients were calculated by formula (1).

$$\text{Organ coefficient } (\%) = W_S/W_B \quad (1)$$

W_S and W_B were the organ weight and body weight, respectively, on the 8th day.

Table 1. Criteria for scoring the disease activity index.

Weight loss (%)	Stool consistency	Occult	Score
<1	Normal stool	Normal (-)	0
1–5	Loose	Positive Fecal Occult Blood (+)	1
5–10	Loose	Slight blood in the stool (++)	2
10–15	Diarrhea (++)	Blood in the stool (+++)	3
>15	Diarrhea (+++)	Bloody stools visible to the naked eye	4

DAI = (Weight loss + Stool consistency + Occult)/3.
*Normal stool: well-formed pellets; Loose: pasty stool that does not stick to the anus; Diarrhea: liquid stool that sticks to the anus.
Note: Fecal occult blood was detected using the Occult blood test kit and following its steps.

Table 2. Evaluation of CMDI.

Colon mucosa damage	Score
Inflammatory cells	
Presence of occasional inflammatory cells in the lamina propria	0
Increased numbers of inflammatory cells in the lamina propria	1
The confluence of inflammatory cells, extending into the submucosa	2
Transmural extension of the infiltrate	3
Tissue damage	
No mucosal damage	0
Discrete lymphoepithelial lesions	1
Surface mucosal erosion or focal ulceration	2
Extensive mucosal damage and extension into deeper structures of the bowel wall	3

2.13. MPO activity assay

Neutrophil infiltration is an important feature of chronic colitis and associated colorectal cancer, where external stimuli can lead to massive accumulation of neutrophils and consequent release of myeloperoxidase. As its characteristic enzyme, MPO induces the production of proinflammatory factors that trigger inflammation and kill microorganisms in phagocytes by catalyzing and oxidizing chloride ions to produce hypochlorous acid and destroying a variety of target substances. MPO levels and activity are positively correlated with the severity of inflammation [24], so its activity can be measured to evaluate the degree of neutrophil infiltration.

2.14. Evaluation of oxidative stress levels

T-SOD, MDA, and NO were measured by the assay kit, and all operations were carried out in strict accordance with the procedure in the instructions.

2.15. Measurement of cytokines

The levels of inflammatory cytokines in the colon tissues, including IL-1β, IL-6, and TNF-α, were measured by Luminex testing technology. Data were analysed using MILLIPLEXTM Analyst software and quantified by standard curves. All procedures were performed according to the manufacturer's guidelines.

2.16. Immunofluorescence staining

Immunofluorescence staining was carried out according to Wu [25] et al.

2.17. Immunohistochemical (IHC)

Immunohistochemistry (IHC) was carried out according to Akanda MR [26] et al.

2.18. Western blot analysis

Protein was extracted from colon tissue by a whole-cell lysis assay, and the protein concentration was determined by a detergent compatible Bradford protein assay kit. Proteins were separated by SDS–PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were sealed [5% Nonfat-Dried Milk in Tris-buffered saline tween 20 (TBST)] for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies against IL-6, p-JAK2, JAK2, p-STAT3, STAT3, NLRP3, ASC, and Caspase-1. The membranes were then washed 3 times and incubated with horseradish peroxidase (HRP)-conjugated

secondary antibodies for 1 h at room temperature. Bands were semi-quantified using ImageJ software.

3. Statistical analysis

Statistical analysis was implemented using SPSS version 18.0 (Chicago, IL, USA). All results were expressed as the mean ± SD. Group comparisons were performed by one-way analysis of variance (ANOVA) followed by Welch's ANOVA when appropriate. P values less than 0.05 were considered statistically significant.

4. Results and discussion

4.1. Identification of RCEE by HPLC

As shown in Figure 3, HPLC was used to analyze the RCEE (A), RCPEE (B), RCEAE (C), RCNBAE (D), and RCAPE (E). The following standard substance, Rutin (F), was used for the analysis. The existence of RCEE was confirmed by comparison to the peak retention times of the standard substance.

4.2. General sign observation and disease activity index (DAI)

The colon absorbs water and electrolytes and forms, stores, and excretes feces. When colon function is disturbed, absorption is affected, and symptoms such as diarrhea, constipation, and bloating can occur. Weight loss and DAI are therefore often used to assess clinical conditions (Figure 4).

On day 3, the mice in the UC model group had bloody stools and loose stools; on day 5, they started to lose weight and had obvious signs of bloody and watery stools. The mice were huddled in piles, depressed, with dull coat color and a significant reduction in the amount of food and water consumed. All three groups, H/L-RCPEE and H-RCEAE, significantly alleviated weight loss and significantly reduced DAI scores compared to the model group ($p < 0.01$).

4.3. Histopathological observation and colon mucosa damage index (CMDI)

Studies have shown that during the pathogenesis of UC, the colon is significantly shortened, and the feces change to loose or watery [27].

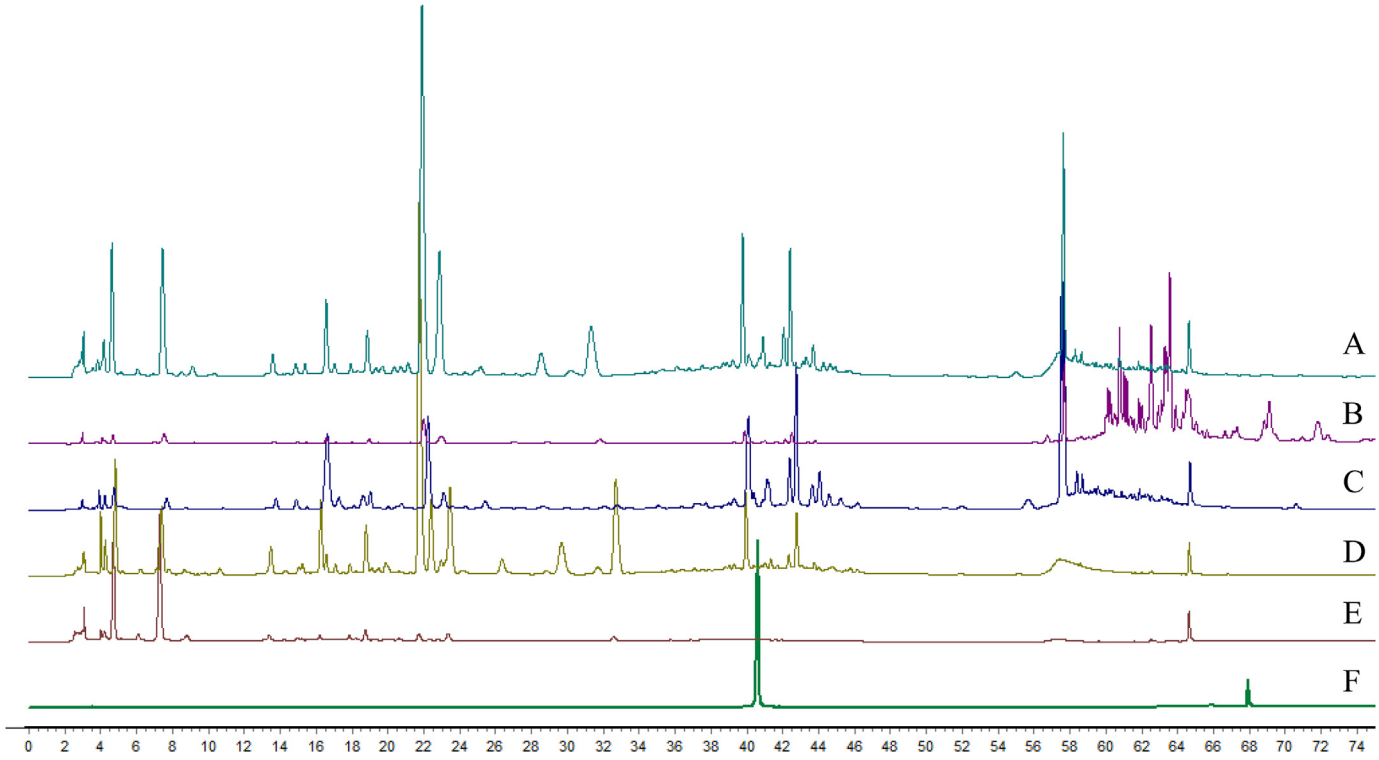

Figure 3. HPLC chromatogram of different polar parts of RCEE and standard solution Rutin.(A) RCEE. (B) RCPEE. (C) RCEAE. (D) RCNBAE. (E) RCAPE. (F) Rutin.

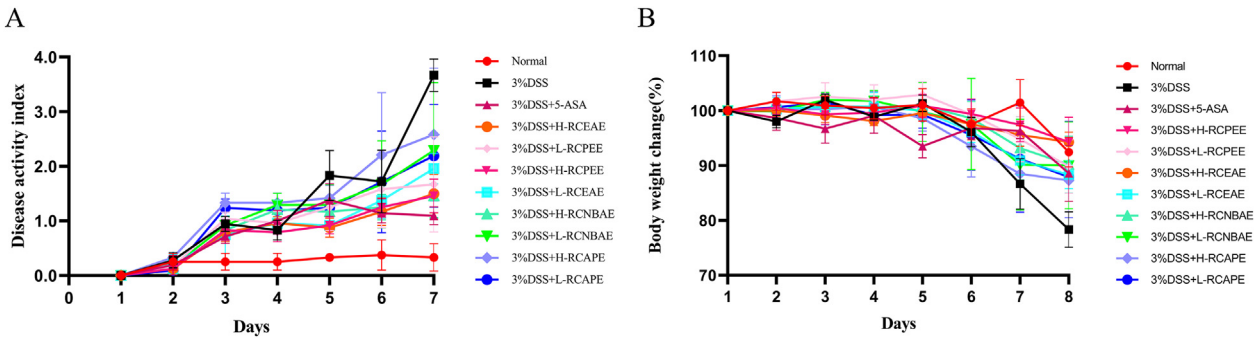

Figure 4. (A) Disease active index. (B) Bodyweight changes.

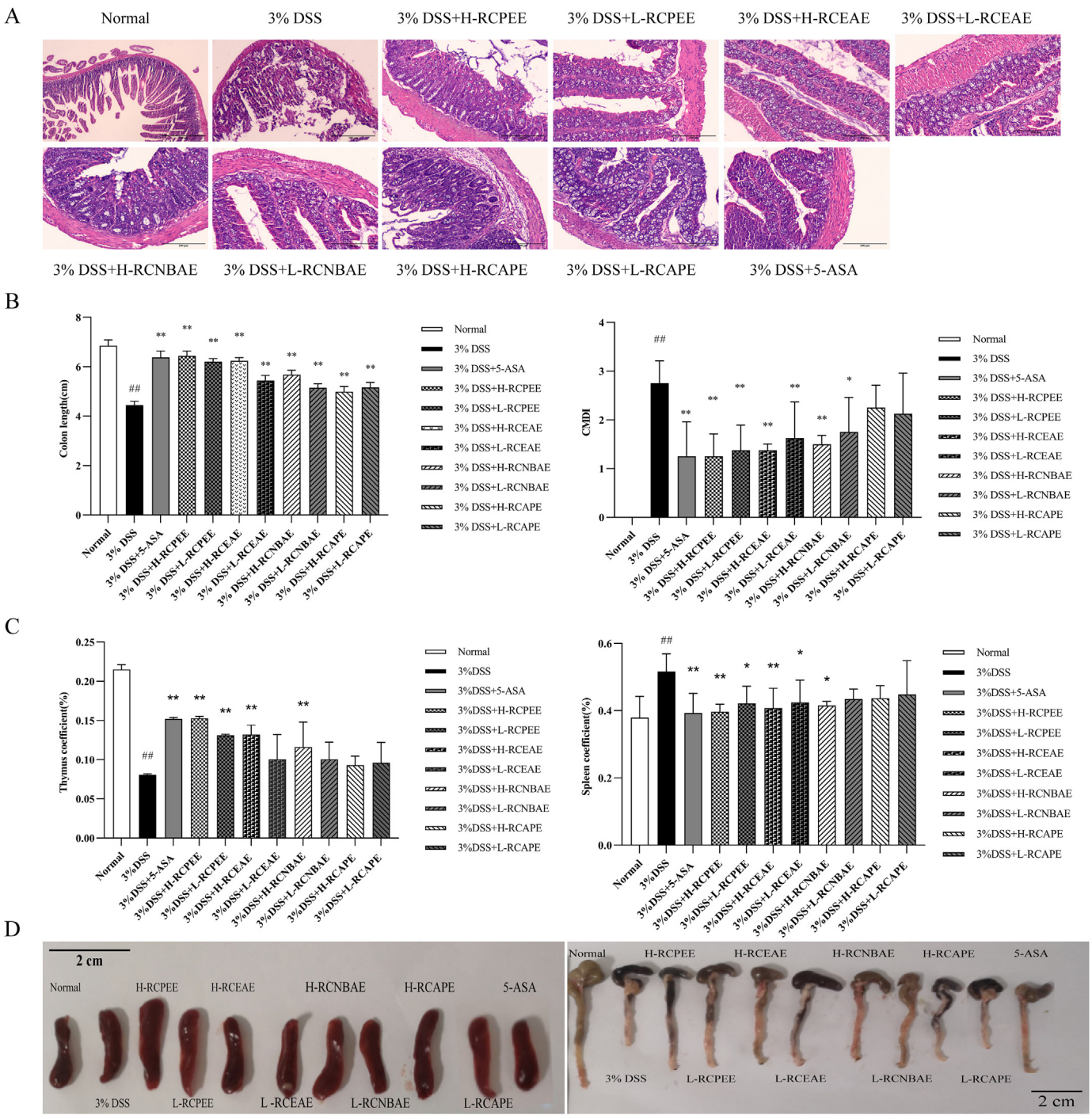

Figure 5. Effects of RCEE on colonic pathological changes and spleen in DSS-induced ulcerative colitis mice. (A) Representative images of hematoxylin and eosin (H&E) staining in colon tissues (magnification × 100). (B) Colon length and CMDI. (C) Thymus coefficient and Spleen coefficient. (D) Representative pictures of the spleen and colon in each group. All of the data are expressed as the mean ± SD (n = 8), *P < 0.05, **P < 0.01 compared with the UC group, and #P < 0.05, ##P < 0.01 compared with the control group. Scale bar = 200 μm.

The experimental results showed that the colon of the mice in the model group was significantly shortened, the intestinal wall was thickened and stiffened, the intestinal mucosa was swollen and congested, and obvious ulcerated surfaces were visible. The lesions were mostly concentrated in the cecum and distal colon, irregular ulcers and erosions in the distal colon were mostly found within 3–4 cm from the anus, with the degree of lesions gradually decreasing from the bottom to upwards; the colon length of mice in both the drug and mesalazine groups increased significantly compared to the model group ($p < 0.01$) (Figure 5 B, D).

The histopathological score is an important indicator to evaluate the success of modeling. Histopathological sections of the colon of the experimental mice showed that there was basically no ulcer formation or inflammatory cell infiltration in the normal group, the crypt structure was intact, and the glands were closely arranged, regularly and clearly structured. In contrast, it is clear that a large number of inflammatory

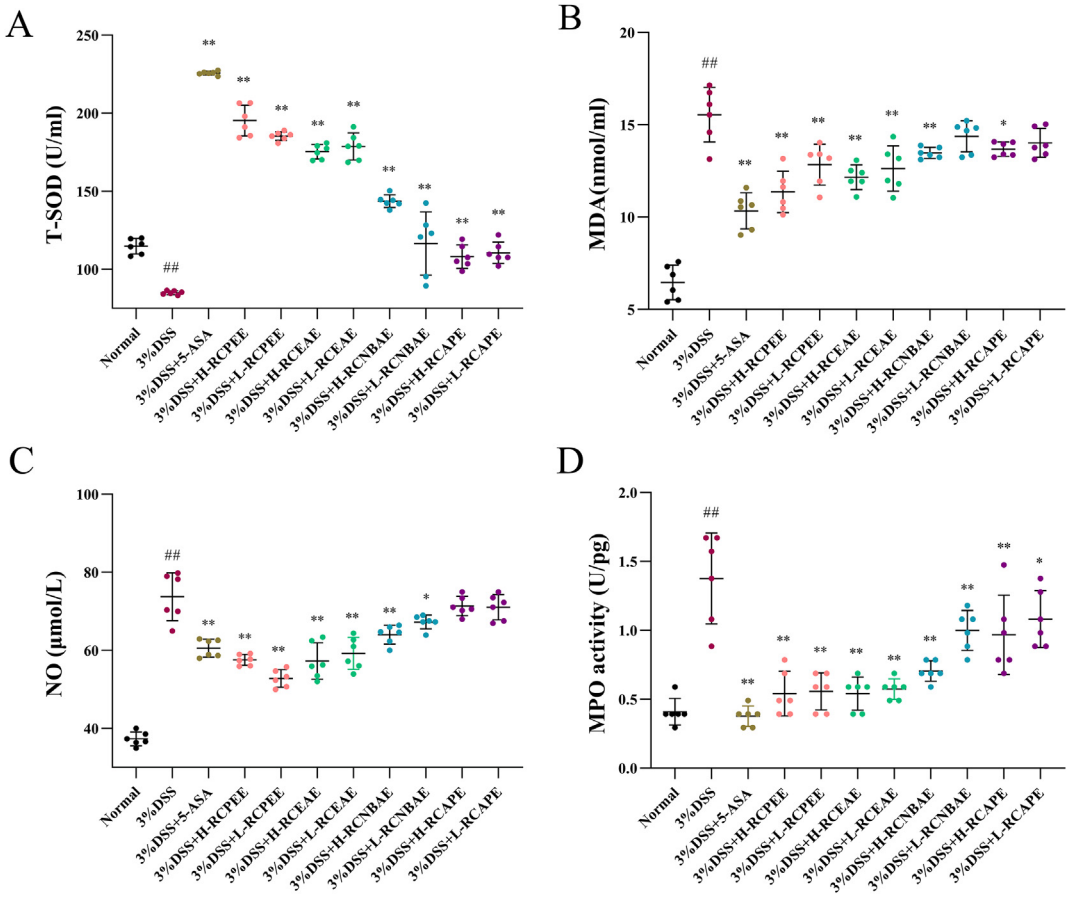

Figure 6. (A) T-SOD. (B) MDA. (C) NO. (D) MPO. Data were based on the serum. *P < 0.05, **P < 0.01 compared with the UC group, and #P < 0.05, ##P < 0.01 compared with the control group.

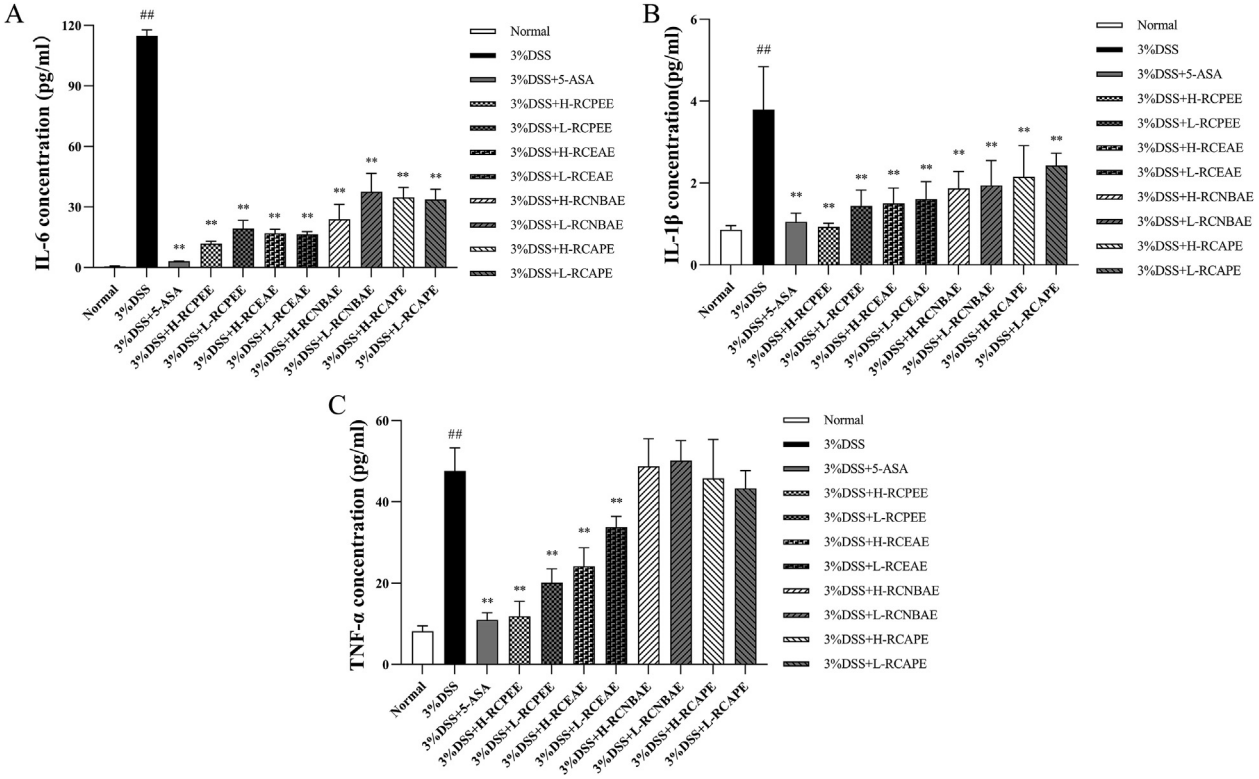

Figure 7. Effects of RCEE on the secretion levels of proinflammatory cytokines in DSS-induced ulcerative colitis mice. The levels of proinflammatory cytokines for IL-6 (A), IL-1β (B), and TNF-α (C). Data are shown as the mean ± SD of 6–7 mice in each group. *P < 0.05, **P < 0.01 compared with the UC group, and #P < 0.05, ##P < 0.01 compared with the control group.

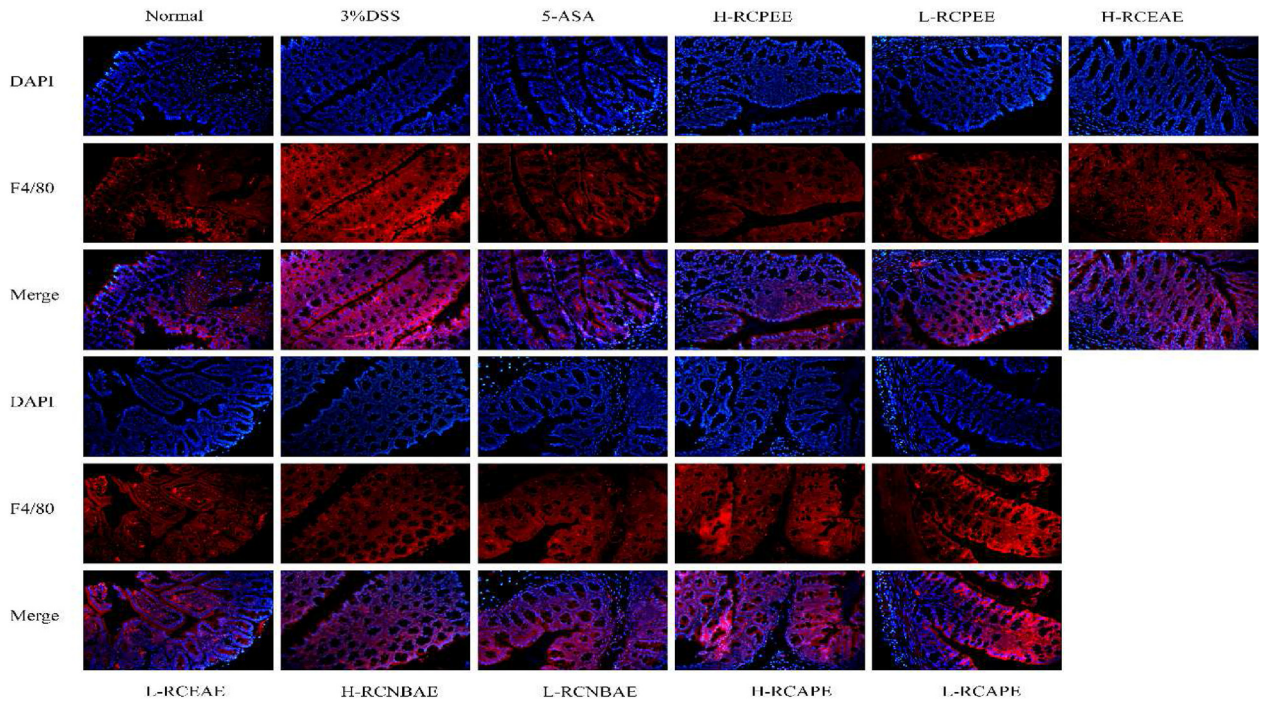

Figure 8. Immunofluorescence analysis of F4/80 (×200).

cells infiltrated in the model group, mainly including neutrophils and lymphocytes, without cupped cells, and the crypt and glands were severely deformed, which was significantly different from the blank group (Figure 5 A).

In contrast, the crypt structure was clearly visible in the Mesalazine group, but there was still inflammatory cell infiltration, and the treatment effect on tissue damage was average, while the improvement of the H/L-RCPEE and H-RCEAE groups was more pronounced, the crypt structure was clearly visible, the glands were neatly arranged, inflammatory cell infiltration and the degree of inflammation was significantly reduced. The experimental results showed that the H/L-RCPEE, H/L-RCEAE, and H-RCNBAE groups all improved the tissue damage in the colonic site of DSS-induced ulcerated mice to some extent, while the L-RCNBAE and H/L-RCAPE groups were less effective in treatment.

4.4. The effects of RCEE on immune organs in DSS-induced colitis mice

Mice with DSS-induced acute ulcerative colitis develop splenomegaly, which leads to immune dysfunction and elevated levels of inflammation [28]. The present study found that both the H-RCPEE and H-PCEAE groups of drugs significantly reduced spleen coefficients compared to the model group. In addition, thymic coefficients were also significantly increased after administration of H/L-RCPEE, H-RCEAE and H-RCNBAE compared to the model group (Figure 5 C). The results suggest that RCEE can restore the immune system of mice with DSS-induced colitis by suppressing splenomegaly and thymus atrophy (Figure 5 D).

4.5. Effects of RCEE on T-SOD, MDA, NO, and MPO activity in the serum of DSS-induced colitis mice

The data showed that the NO (73.71 ± 6.11 μmol/L) level in the serum of mice in the model group was significantly increased, which triggered oxidative stress in the body and caused oxidative damage by free radicals entering the tissues [29,30], resulting in a significant increase in the content of lipid peroxides such as MDA (15.55 ± 1.48 nmol/mL) and further aggravating the damage in the body. Compared with the model group, the NO and MDA contents of the H/L-RCPEE and H/L-RCEAE groups decreased significantly, with the NO values in the H/L-RCPEE group decreasing by 21.9% and 28.3%, and the MDA values by 26.9% and 17.4%, respectively, and the NO values in the H/L-RCEAE groups decreasing by 22.3% and 19.6%, and the MDA values by 21.8% and 18.8% (Figure 6 B, C). T-SOD protects cells from damage by scavenging excess $\cdot O^{2-}$ and thus inhibiting lipid peroxidation. Compared with the normal group (114.72 ± 4.91 U/mL), the serum T-SOD activity was significantly lower in the model group (84.95 ± 1.24 U/mL), indicating that the oxidative damage was more serious. The T-SOD activity of the four groups, H/L-RCPEE and H/L-RCEAE, increased by 130.0%, 118.2%, 106.4%, and 110.3%, respectively, all of which were significantly different compared to the model group, indicating that the oxidative damage of the organism was repaired to a certain extent and the inflammatory degree of ulcerative colitis formed by oxidative damage was further alleviated (Figure 6 A). During the inflammation process, the influx of a large number of neutrophils at the lesion site causes oxidative damage, which further leads to mucosal and tissue damage at the inflammation site. The body produces MPO for self-repair through self-feedback regulation, increasing MPO levels, and its level and activity are positively correlated with the severity of inflammation. Compared with the normal group, the MPO activity in the serum of mice in the model group was significantly higher (P <0.01); after the administration of the drug, MPO secretion gradually decreased, the inflammatory symptoms of the organism were relieved, and the mucous membrane and tissue damage were repaired. This suggests that RCEE may alleviate the inflammatory symptoms of ulcerative colitis by inhibiting MPO activity (Figure 6 D).

4.6. RCEE suppresses the release of inflammatory cytokines in DSS-induced colitis mice

IL-1β, IL-6, and TNF-α are all important cytokines in the development of UC disease. Neutrophils secrete large amounts of IL-1β and participate in the recruitment and retention of leukocytes in inflammatory tissues

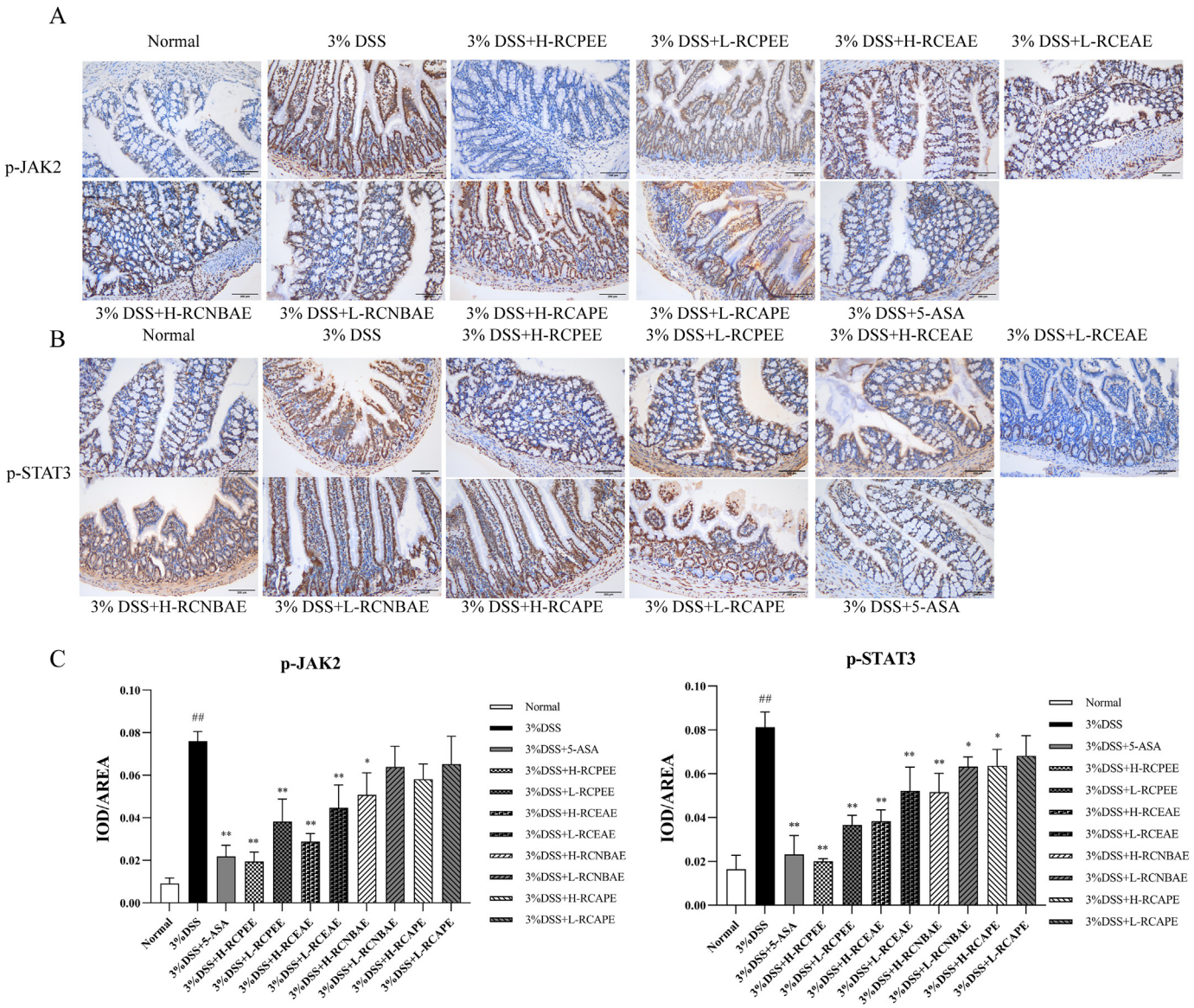

Figure 9. RCEE inhibited the activity of the IL-6/JAK2/STAT3 pathway in DSS-induced colitis mice. Immunohistochemical staining showed p-JAK2 (A) expression and p-STAT3 expression (B) in the diseased colons. Statistical analysis of the positive area expression of p-JAK2 (C) and p-STAT3 (D). *P < 0.05, **P < 0.01 compared with the model group, and #P < 0.05, ##P < 0.01 compared with the control group.

through the upregulation of their secretion and stimulate the production of inflammatory transmitters and cytokines [31] while blocking the activation and secretion of IL-1β significantly reduces the extent of mucosal damage and inhibits the formation of tumor cells [32]. IL-6 is a lymphokine produced by T cells and macrophages [33] that is involved in the development and progression of many diseases, and its blood levels are closely associated with inflammation, viral infections, and autoimmune diseases. In the inflammatory response, elevated levels of IL-6 precede other cytokines, and procalcitonin (PCT) and C-reactive protein (CRP) are long-lasting and can be used to aid in the early diagnosis of acute infections. In sepsis, IL-6 is regarded as an early warning index and used in combination with PCT and CRP to monitor the high-risk population of sepsis continuously and dynamically. TNF-α is mainly produced by NK cells, macrophages, and T cells and has immunomodulatory functions. During the development of UC, serum levels of TNF-α far exceed normal levels, and excessive TNF-α triggers chemotaxis of neutrophils and monocytes, stimulating the release of other cytokines, such as IL-6 and IL-1β, creating a cascade amplification effect of inflammation and further aggravating the level of UC inflammation [34,35]. As shown in Figure 7, the levels of TNF-α (47.62 ± 5.64 vs. 8.19 ± 1.27 pg/mg), IL-6 (114.89 ± 2.85 vs. 0.54 ± 0.20 pg/mg), and IL-1β (3.79 ± 1.05 vs. 0.86 ± 0.10 pg/mg) in the serum of mice in the model group were significantly higher than those in the normal group, and the levels of each administered. The levels of cytokines in all groups were decreased to different degrees compared with the model group. Among them, both H/L-RCPEE and H/L-RCEAE demonstrated good efficacy. In contrast, data analysis of H/L-RCNBAE and H/L-RCAPE in this index of TNF-α showed $P > 0.05$, which was not different from the DSS-induced model group. The experimental results showed that both H/L-RCPEE and H/L-RCEAE could effectively inhibit the secretion of TNF-α, IL-6, and IL-1β during the development of UC, reducing the level of inflammatory factors in the intestine and decreasing the damage of intestinal mucosal irritation, which has a certain improvement effect on UC.

4.7. Immunofluorescence staining

Macrophages are a group of leukocytes located in the peripheral blood and inflammatory tissues. In animals, macrophages participate in

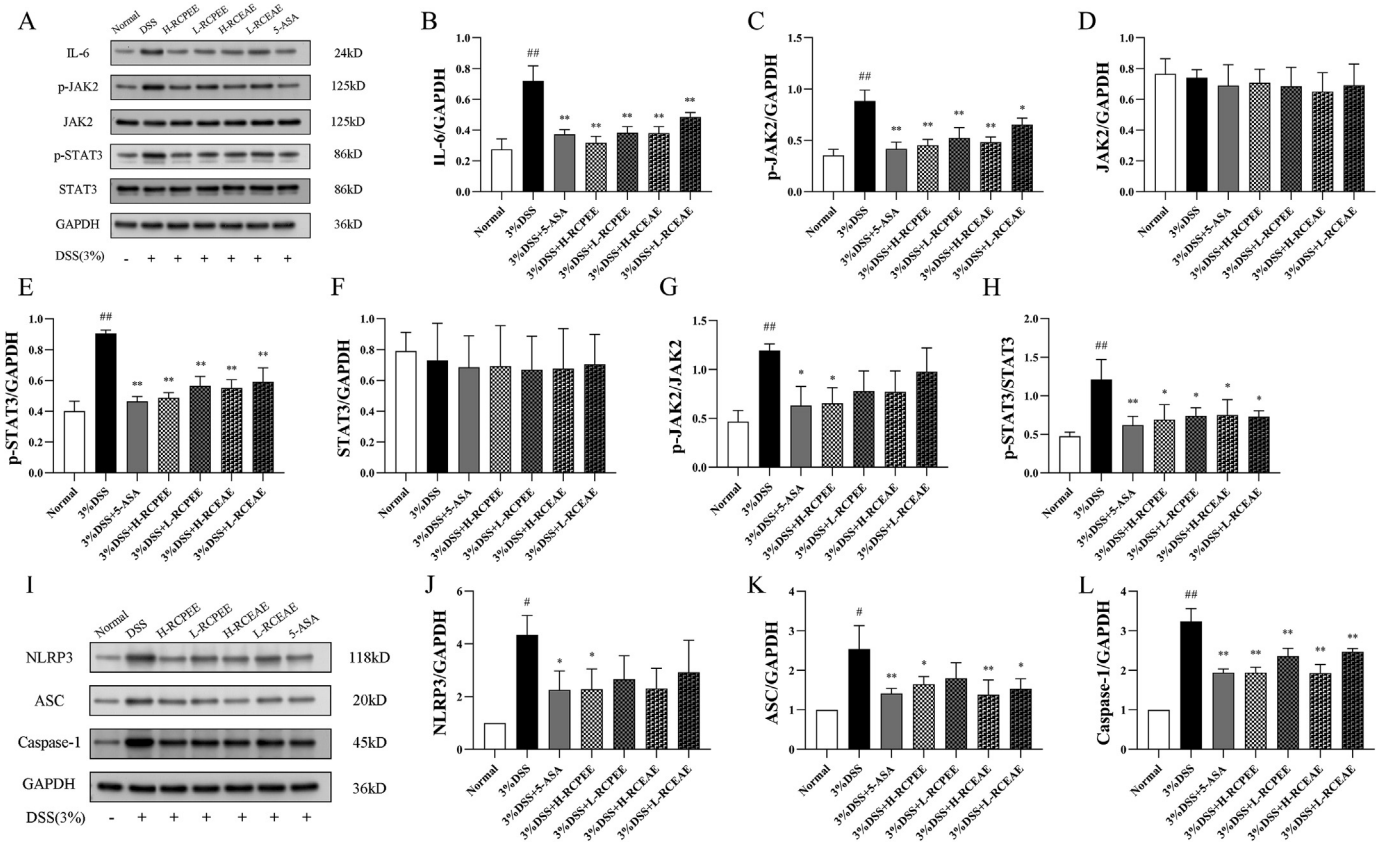

Figure 10. Effects of RCEE on IL-6/JAK2/STAT3 pathways and NLRP3 inflammasome expression in DSS-induced colitis mice. The protein expression levels of IL-6, p-JAK2, JAK2, p-STAT3, STAT3 (A), NLRP3, ASC, and Caspase-1 (I) were detected by Western blot in colonic tissue. The protein expression levels of IL-6 (B), p-JAK2 (C), JAK2 (D), p-STAT3 (E), STAT3 (F), NLRP3 (J), ASC (K), and Caspase-1 (L) were quantitated by ImageJ software. The data are representative of at least 3 independent experiments. All the values are presented as the mean ± SD. *P < 0.05, **P < 0.01 compared with the UC group, and #P < 0.05, ##P < 0.01 compared with the control group.

nonspecific immune regulation mainly by phagocytosing bacteria, dead cells, and cellular debris and subsequently digest phagocytosed material and present its characteristics to subsequent lymphocytes and other immune cells for specific immune regulation. Macrophage infiltration was significantly increased in the DSS model group compared to the normal group, while macrophage infiltration was reduced to varying degrees in all dosing groups (Figure 8).

4.8. RCEE suppressed the IL-6/JAK2/STAT3 pathways and NLRP3 inflammasome activation in DSS-induced colitis mice

H/L-RCPEE and H/L-RCEAE inhibited JAK2 and STAT3 activation and blocked the development and persistence of colonic inflammation by reducing IL-6 expression. We also found that the levels of p-JAK2 (Figure 9 A, C) and p-STAT3 (Figure 9 B, D) in the model group were significantly higher than those in the normal group by immunohistochemical staining analysis, while H/L-RCPEE and H-RCEAE significantly reduced the expression of the two phosphorylated proteins. ASC expression was increased in neutrophils at severely symptomatic inflammatory sites, whereas ASC expression levels were significantly reduced after treatment with H-RCPEE and H/L-RCEAE, with a corresponding reduction in the activation levels of Caspase-1 involved in their induction. We speculate that RCEE may also alleviate UC by suppressing ASC expression, reducing Caspase-1 activation, and thereby inhibiting NLRP3 inflammasome. Taken together, RCPEE and RCEAE inhibited activation of IL-6/JAK2/STAT3 pathways and the formation of NLRP3 inflammasome.

5. Discussion

The Janus-activated kinase signal transducer and activator of transcription (JAK-STAT) pathway is an extremely important intracellular signal transduction pathway involved in a variety of cell proliferation, differentiation, apoptosis, and immune regulation.Janus kinase (JAK) is a nonreceptor-type tyrosine protein kinase. The highly conserved mammalian JAK family has four members, JAK1, JAK2, TYK2, and JAK3. The first three members are widely present in various tissues and cells, while JAK3 expression is only present in the bone marrow and lymphoid systems [36,37]. STATs are substrates of JAK, a family of DNA-binding proteins that can be coupled with tyrosine phosphorylation signaling pathways to play a role in transcriptional regulation and mediate a variety of biological effects. Activation of the JAK2/STAT3 signaling pathway is achieved through IL-6, a multifunctional cytokine with both pro-inflammatory and anti-inflammatory properties that are involved in a variety of physiological and pathological processes. Its receptor can exist either in transmembrane form (IL-6R) or insoluble form (SIL-6R, soluble interleukin-6 receptor). IL-6 binds to both forms of receptors separately to form a complex, which in turn interacts with glycoprotein 130 (gp130) to trigger downstream signaling and gene expression, thus completing the classical pathway and trans-signaling. Most of the proinflammatory effects of IL-6 are attributed to trans signaling pathways, whereas anti-inflammatory and regenerative signals are mediated by classical signaling. During UC development, IL-6 binds to SIL-6R to form a complex, which further activates gp130 on the cell membrane surface, thereby activating JAK

associated with gp130, which activates JAK and binds to signal transducers and activators of transcription 3 (STAT3) protein to promote STAT3 phosphorylation, activating the nuclear transcription factor NF-κB, which enters the nucleus and regulates the expression of inflammatory cytokines. Planell N [38] and Arijs I [39] found that transcripts of JAK1, JAK2, TYK2, and JAK3 were significantly upregulated during the active phase of UC and significantly decreased during the remission phase by transcriptional analysis of intestinal mucosa from UC patients.We found that RCPEE and RCEAE inhibited JAK2 and STAT3 activation and blocked the development and persistence of colonic inflammation by reducing IL-6 expression (Figure 10 A-H). Related studies have also confirmed that blocking IL-6/STAT3 pathway transduction can be effective in treating IBD, such as blocking SIL-6R with IL-6R monoclonal antibodies and synthetic reagents targeting downstream gp130 [40–42]. Suzuki A [43] found that in mice with IL-6 gene deletion, STAT3 activation was reduced, and the process of DSS-induced UC pathogenesis was slowed. Becker C [44] also found that TGF could inhibit colon tumor growth by inhibiting IL-6 production and interfering with the IL-6/STAT3 pathways, demonstrating the feasibility of interfering with the IL-6/STAT3 pathway in the treatment of colorectal cancer (CRC). NLRP3 inflammasome is a group of protein complexes that can be activated by multiple endogenous stress-related signals, mainly containing the receptor NLRP3 protein, the adaptor protein ASC, and pro-caspase-1 protein. NOD-like receptor protein 3 (NLRP3) in NLRP3 inflammasome is activated by agonists and binds to the pyrin domain (PYD) of ASC through its PYD, which then binds to the caspase recruitment domain (CARD) of pro-caspase-1 through its CARD to trigger the self-cleavage and maturation of caspase-1. On the one hand, activated caspase-1 promotes the maturation and secretion of several pro-inflammatory factors, including IL-1β and IL-18; on the other hand, it triggers pyroptosis of damaged cells and clears pathogens [45, 46]. We hypothesize that RCPEE and RCEAE may alleviate UC by suppressing ASC expression, reducing Caspase-1 activation, and thereby inhibiting NLRP3 inflammasome (Figure 10 I-L). Loher F [47] and Bauer C [48] also found that the Caspase-1 inhibitor pralnacasan significantly reduced the degree of inflammation in DSS-induced ulcerative colitis by inhibiting the cleavage maturation of IL-1β and the IL-18 precursor, thereby effectively reducing the number of proinflammatory cytokines and impairing IFN-γ-induced synthesis by IL-18.The anti-ulcerative colitis active fractions of RCEE were concentrated in petroleum ether- and ethyl acetate-extracted fractions. The petroleum ether fraction was mostly volatile oils and other small polar components, most of which had broad-spectrum antibacterial activity, which may be one of the reasons for its good biological activity. The ethyl acetate-extracted fraction mainly contains anthraquinones, phenolic acids, tannins, and amino acids, which have anti-inflammatory and antibacterial pharmacological effects, especially anthraquinones. A large number of anthraquinones and their derivatives have been obtained from RCEE by identification and isolation. These two anti-ulcerative colitis active fractions inhibited activation of IL-6/JAK2/STAT3 pathways and formation of NLRP3 inflammasome, preventing the persistence and progression of ulcerative colitis. Based on these results, RCEE holds promise as a candidate for the treatment of acute UC and other inflammatory diseases.

6. Conclusion

RCEE administration could ameliorate the experimentally DSS-induced colitis in mice. The protective effect of RCEE appears to be associated with mitigating the inflammation and oxidative stress through attenuation of pro-inflammatory cytokines mediated by NLRP3 inflammasome and IL-6/JAK2/STAT3 signaling pathways. These findings suggest that RCEE could be a potent phytomedicine and have advantages for prospective clinical applications in the future for the treatment of IBD or correlated disorders.

Declarations

Author contribution statement

Wei Qin; Han Luo: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Liu Yang; Di Hu; Su-Ping Jiang; Dai-Yin PENG; Jiang-Miao Hu; Shou-Jin Liu: Conceived and designed the experiments.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

[1] K. Nanki, M. Fujii, M. Shimokawa, et al., Somatic inflammatory gene mutations in human ulcerative colitis epithelium[J], Nature 577 (7789) (2020) 254.
[2] S. Grivennikov, E. Karin, J. Terzic, et al., IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer, Cancer Cell 15 (2) (2009) 103–113.
[3] A. Fernández-Clotet, J. Castro-Poceiro, J. Panés, Tofacitinib for the treatment of ulcerative colitis, Expet Rev. Clin. Immunol 14 (11) (2018) 881–892.
[4] H. Okuno, H. Ogino, E. Ihara, et al., Interleukin-1β as a Predictor of Glucocorticoid Response in Ulcerative Colitis. Digestion 102 (3) (2021) 357–367.
[5] S. Okada, K. Hata, T. Shinagawa, et al., A Polymorphism in Interleukin-1β Gene Is Associated with the Development of Pouchitis in Japanese Patients with Ulcerative Colitis, Digestion 102 (3) (2021) 489–498.
[6] K. Schroder, J. Tschopp, The inflammasomes, Cell 140 (6) (2010) 821–832.
[7] BK. Davis, H. Wen, JP. Ting, The inflammasome NLRs in immunity, inflammation, and associated diseases, Annu Rev Immunol 29 (2011) 707–735.
[8] L. Peyrin-Biroulet, W. Sandborn, B.E. Sands, et al., Selecting therapeutic targets in inflammatory bowel disease (STRIDE): determining therapeutic goals for treat-to-target, Am. J. Gastroenterol. 110 (9) (2015) 1324–1338.
[9] S.V. Kane, Systematic review: adherence issues in the treatment of ulcerative colitis, Aliment. Pharmacol. Ther. 23 (5) (2006) 577–585.
[10] D. Gupta, S. Kumari, M. Gulrajani, Dyeing studies with hydroxyanthraquinones extracted from Indian madder. Part 1: dyeing of nylon with purpurin, Color. Technol. 117 (2001) 328–332.
[11] P.S. Vankar, R. Shanker, D. Mahanta, et al., Ecofriendly sonicator dyeing of cotton with *Rubia cordifolia* L. using biomordant, Dyes Pigments 76 (2008) 207–212.
[12] M. Yusuf, M. Shahid, S.A. Khan, et al., Eco-dyeing of wool using aqueous extract of the roots of Indian Madder (*Rubia cordifolia* L.) as natural dye, J. Nat. Fibers 10 (2013) 14–28.
[13] N.D. Namsa, M. Mandal, S. Tangjang, et al., Ethnobotany of the Monpa ethnic group at Arunachal Pradesh, India, J. Ethnobiol. Ethnomed. 7 (2011) 31.
[14] S. Chakrabortee, Y. Liu, L. Zhang, et al., Macromolecular and small-molecule modulation of intracellular Aβ42 aggregation and associated toxicity, Biochem. J. 442 (2012) 507–515.
[15] S. Lodi, V. Sharma, L. Kansal, The protective effect of *Rubia cordifolia* against lead nitrate-induced immune response impairment and kidney oxidative damage, Indian J. Pharmacol. 43 (2011) 441–444.
[16] I. López-Expósito, A. Castillo, N. Yang, et al., Chinese herbal extracts of *Rubia cordifolia* and Dianthus superbus suppress IgE production and prevent peanut-induced anaphylaxis, Chin. Med. 6 (2011) 35.
[17] P.N. Shilpa, S. Venkatabalasubramanian, S.N. Devaraj, Induction of apoptosis by methanolic extract of *Rubia cordifolia* Linn. in HEp-2 cell line is mediated by reactive oxygen species, Asian Pac. J. Cancer Prev. APJCP 13 (2012) 2753–2758.
[18] P.N. Shilpa, S. Venkatabalasubramanian, S.N. Devaraj, Ameliorative effect of methanol extract of *Rubia cordifolia* in N-nitrosodiethylamine-induced hepatocellular carcinoma, Pharm. Biol. 50 (2012) 376–383.
[19] The editorial board of Chinese Materia Medica, Chinese Materia Medica Mongolia Volume, Shanghai Scientific & Technical Publishers, Shanghai, 2004.

[20] Y.Y. Sun, X.P. Gong, J.Y. Tan, et al., *In vitro* antiviral activity of *Rubia cordifolia* aerial Part Extract against rotavirus, Front. Pharmacol. 7 (2016) 308.
[21] X.P. Gong, Y.Y. Sun, W. Chen, et al., Anti-diarrheal and anti-inflammatory activities of aqueous extract of the aerial part of *Rubia cordifolia*, BMC Compl. Alternative Med. 17 (1) (2017) 20.
[22] H. Cooper, Clinicopathologic study of dextran sulfate sodium experimental murine colitis. [J], Lab. Invest. 69 (2) (1993) 238–249.
[23] B. Siegmund, G. Fantuzz, F. Rieder, et al., Neutralization of Interleukin-18 Reduces Severity in Murine Colitis and Intestinal IFN-Gamma and TNF-Alpha Production, Am J Physiol Regul Integr Comp Physiol, 2001, pp. R1264–R1273.
[24] S.J. Klebanoff, Myeloperoxidase: friend and foe[J], J. Leukoc. Biol. 77 (5) (2005) 598–625.
[25] X.F. Wu, Z.J. Ouyang, L.L. Feng, et al., Suppression of NF-κB signaling and NLRP3 inflammasome activation in macrophages is responsible for the amelioration of experimental murine colitis by the natural compound fraxinellone, Toxicol. Appl. Pharmacol. 281 (1) (2014) 146–156.
[26] M.R. Akanda, H.H. Nam, W. Tian, et al., Regulation of JAK2/STAT3 and NF-κB signal transduction pathways; Veronica polita alleviates dextran sulfate sodium-induced murine colitis, Biomed. Pharmacother. 100 (2018) 296–303.
[27] Mohammad Yousef, Rath Pichyangkura, Sunhapas Soodvilai, et al., Chitosan oligosaccharide as potential therapy of inflammatory bowel disease: therapeutic efficacy and possible mechanisms of action[J], Pharmacol. Res. 66 (1) (2012).
[28] J. Liang, J. Liang, H. Hao, et al., The extracts of Morinda officinalis and its hairy roots attenuate dextran sodium sulfate-induced chronic ulcerative colitis in mice by regulating inflammation and lymphocyte apoptosis, Front. Immunol. 8 (2017) 905.
[29] H. Zhu, Y.R. Li, Oxidative stress and redox signaling mechanisms of inflammatory bowel disease: updated experimental and clinical evidence, Exp. Biol. Med. 237 (5) (2012) 474–480.
[30] Y. Ren, Y. Geng, Y. Du, et al., Polysaccharide of Hericium Erinaceus attenuates colitis in C57BL/6 mice via regulation of oxidative stress, inflammation-related signaling pathways, and modulating the composition of the gut microbiota, J. Nutr. Biochem. 57 (2018) 67–76.
[31] J. Puleston, M. Cooper, S. Murch, et al., A distinct subset of chemokines dominates the mucosal chemokine response in inflammatory bowel disease[J], Aliment. Pharmacol. Ther. 21 (2) (2005) 109–120.
[32] Y. Wang, K. Wang, G.C. Han, et al., Neutrophil infiltration favors colitis-associated tumorigenesis by activating the interleukin-1 (IL-1)/IL-6 axis, Mucosal Immunol 7 (5) (2014) 1106–1115.
[33] J. Mudter, M.F. Neurath, IL-6 signaling in inflammatory bowel disease: pathophysiological role and clinical relevance[J], Inflamm. Bowel Dis. 13 (8) (2007) 1016–1023.
[34] K. Zhang, Y. Gao, M. Zhong, et al., Hepatoprotective effects of Dicliptera Chinensis polysaccharides on dimethylnitrosamine-induced hepatic fibrosis rats and its underlying mechanism, J. Ethnopharmacol. 179 (2016) 38–44.
[35] Haruhiko Ogata, Toshifumi Hibi, Cytokine and anti-cytokine therapies for inflammatory bowel disease[J], Curr. Pharmaceut. Des. 9 (14) (2003) 1107–1113.
[36] R. Yan, S. Small, C. Desplan, et al., Identification of a Stat gene that functions in Drosophila development, Cell 84 (3) (1996) 421–430.
[37] C. Liongue, T. Taznin, A.C. Ward, Signaling via the CytoR/JAK/STAT/SOCS pathway: emergence during evolution, Mol. Immunol. 71 (2016) 166–175.
[38] N. Planell, J.J. Lozano, R. Mora-Buch, et al., Transcriptional analysis of the intestinal mucosa of patients with ulcerative colitis in remission reveals lasting epithelial cell alterations, Gut 62 (7) (2013) 967–976.
[39] I. Arijs, G. De Hertogh, K. Lemaire, et al., Mucosal gene expression of antimicrobial peptides in inflammatory bowel disease before and after first infliximab treatment, PLoS One 4 (11) (2009), e7984.
[40] R. Carey, I. Jurickova, E. Ballard, et al., Activation of an IL-6: STAT3-dependent transcriptome in pediatric-onset inflammatory bowel disease, Inflamm. Bowel Dis. 14 (4) (2008) 446–457.
[41] K. Mitsuyama, S. Matsumoto, S. Rose-John, et al., STAT3 activation via interleukin 6 trans-signaling contributes to ileitis in SAMP1/Yit mice, Gut 55 (9) (2006) 1263–1269.
[42] H. Ito, Treatment of Crohn's disease with anti-IL-6 receptor antibody, J. Gastroenterol. 40 (2005) 32–34.
[43] A. Suzuki, T. Hanada, K. Mitsuyama, et al., CIS3/SOCS3/SSI3 plays a negative regulatory role in STAT3 activation and intestinal inflammation, J. Exp. Med. 193 (4) (2001) 471–481.
[44] C. Becker, M.C. Fantini, C. Schramm, et al., TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling, Immunity 21 (4) (2004) 491–501.
[45] B.Z. Shao, Z.Q. Xu, B.Z. Han, et al., NLRP3 inflammasome, and its inhibitors: a review, Front. Pharmacol. 6 (2015) 262.
[46] A. Baroja-Mazo, F. Martín-Sánchez, A.I. Gomez, et al., The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response, Nat. Immunol. 15 (8) (2014) 738–748.
[47] F. Loher, C. Bauer, N. Landauer, et al., The interleukin-1 beta-converting enzyme inhibitor pralnacasan reduces dextran sulfate sodium-induced murine colitis and T helper 1 T-cell activation, J. Pharmacol. Exp. Therapeut. 308 (2) (2004) 583–590.
[48] C. Bauer, F. Loher, M. Dauer, et al., The ICE inhibitor pralnacasan prevents DSS-induced colitis in C57BL/6 mice and suppresses IP-10 mRNA but not TNF-alpha mRNA expression, Dig. Dis. Sci. 52 (7) (2007) 1642–1652.