



Limonin ameliorates ulcerative colitis by regulating STAT3/miR-214 signaling pathway

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ABSTRACT

Ulcerative colitis (UC) is a major inflammatory bowel disease (IBD) which has become a global public health problem. Limonin is a triterpenoid extracted from citrus which possesses the capacities to against inflammations and cell apoptosis. However, the efficacy and the underlying mechanisms of limonin in the treatment of UC remain unclear. In this study, we first investigated the therapeutic effects of limonin on dextran sodiumsulfate (DSS)-induced UC *in vivo* by examining the changes of disease activity index (DAI), the colon length, the colon histology, and cyto/chemokine levels. We found that limonin markedly reduced DAI, intestinal damages, and the levels of pro-inflammatory cytokines, such as TNF- α and IL-6. *In vitro*, limonin significantly repressed the productions of pro-inflammatory cytokines in cultured normal colonic epithelial cells. Mechanistically, we demonstrated that limonin improved the prognosis of UC mainly through downregulating p-STAT3/miR-214 levels. Collectively, our results suggested that limonin was a novel therapeutic agent and it was expected to be translated into the clinic to improve the prognosis of UC.

1. Introduction

Ulcerative colitis (UC) is a type of chronic inflammatory disease that affects the innermost lining of large intestine (colon) and rectum. The incidence of UC is steadily rising worldwide [1]. However, the pathogenesis of UC remains ambiguous by thus far. Geography, age, sex, genetic factors, and environmental factors are potential risk factors involved in the development of UC [2]. In the clinic, UC not only impairs the life qualities but also augments the risk to be developed into colon cancer in patients. Currently, 3-Aminosalicylate is the primary medication for mild to moderate UC treatment, besides the application of topical and systemic steroids. In comparison, in moderate to severe UC, immunosuppressants and biological drugs are generally to be considered in the clinical setting [3, 4]. However, it is worth of pointing out that these current medications for UC actually have unexpected side effects if they are used over long periods of time, conventional remedies developed to treat UC are unfortunately lacking [5]. Although multiple supplements or phytonutrients, such as Yuzu (*Citrus junos* Tanaka) [6],

Chinese Herbal Formula IBS-20 [7], and liriodendra [8] have been sporadically reported as sort of options for UC treatment, it is still an urgent need to design more effective and safe therapeutic strategies in UC management.

Limonin is a triterpenoid compound that exists in plants of the Rutaceae and Meliaceae families (chemical structure shown in Fig. 1A) [9,10]. It is an important bioactive component that possesses the capacities in anti-inflammation [11], anti-cancer [12], anti-bacteria [13], and anti-oxidant stress [14]. However, the therapeutic effects and the underlying mechanisms of limonin on UC treatment are largely unknown. In the present study, we hypothesized that, as an effective candidate, limonin is able to attenuate DSS-induced UC in mice.

MicroRNAs (miRNAs) are small noncoding RNAs, approximately 18 to 25 nucleotides in length, which regulate genes expression through binding to the 3'-untranslated region of target mRNAs [15]. It is well known that miRNAs can regulate inflammatory signaling pathways [16] and is capable of dictating the prognosis of multiple inflammatory diseases [17], including inflammatory bowel disease (IBD) [18,19].

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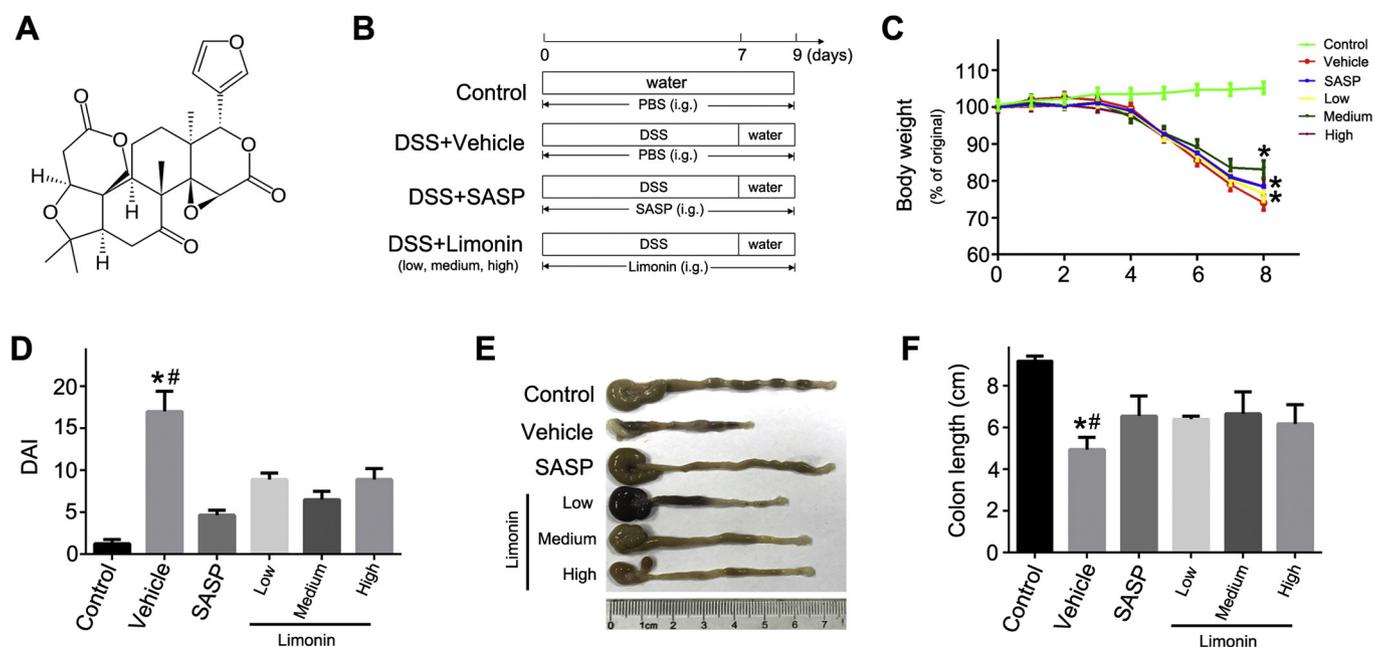


Fig. 1. Limonin ameliorated DSS-induced UC in mice. (A) The chemical structure of limonin. (B) Animal model of DSS-induced colitis in mice. (C) Body weight changes. * $P < 0.05$ vs vehicle group, $n = 5$. (D) The DAI score was calculated as the average of weight loss score, diarrhea score, and fecal blood score at day 9th. * $P < 0.05$ vs control, # $P < 0.05$ vs SASP and limonin groups, $n = 5$. (E) The colons collected in the UC model. (F) The difference of colon length among six groups. * $P < 0.05$ vs control, # $P < 0.05$ vs SASP and limonin groups, $n = 5$.

Previous studies have shown that miR-214 is an inflammatory effector molecule which is dysregulated in patients with colitis [20]. The signal transduction and activator of transcription 3 (STAT3) binds to the miR-214 promoter by which to manipulate the activity of the signaling pathway. In presence to Interleukin 6 (IL-6), IL-6 directly leads to IL-6/STAT3-dependent miR-214 upregulation and subsequent activation of both the PDZ and LIM domain protein 2 (PDLIM2)/nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) and Phosphatase and tensin homolog (PTEN)/Akt signaling pathways [20]. Meanwhile, up-regulated miR-214 also activates the NF- κ B/IL-6 signaling pathway, which suggesting the existence of a miR-214/STAT3/IL-6 feedback loop [16]. In the current study, we found that limonin did prevent UC progression by suppressing the STAT3/miR-214 signaling pathway.

2. Materials and methods

2.1. Chemicals and reagents

Limonin (purity > 96.8%) was obtained from the Zhejiang Nanyang Pharmaceutical Group (China). Sulfasalazine (SASP) was purchased from Xinyi Pharmaceutical Group (China) and it was used as a positive control in the present study. Dextran sodiumsulfate (DSS, molecular weight 36,000–50,000 Da, Cat No: 160110) was purchased from MP Biomedicals (California, USA). Dulbecco's Modified Eagle's Medium (DMEM/ high glucose) and fetal bovine serum (FBS) were purchased from Biological Industries. Mouse IL-6 and IL-10 ELISA kits were purchased from MultiSciences (Lianke) Biotech Co., Ltd. Cell counting kit-8 (CCK8) was purchased from EnoGene (Nanjing, China). Antibodies against the following proteins were used: p-STAT3, STAT3, and PDLIM2 (Cell Signaling Technology, Inc., MA, USA); PTEN and NF- κ B p65 (Proteintech, Nanjing, China); and GAPDH and p-NF- κ B (Affinity, USA). The BCA Protein Assay Reagent Kit was supplied by Thermo Scientific (Waltham, MA, USA). The transfection reagent jet PRIME was supplied by Polyplus-transfection company (FRANCE). Digoxigenin HRP-conjugated antibody was purchased from Jackson ImmunoResearch.

2.2. Animals

C57BL/6J male mice (18–22 g) were purchased from Qinglong Mountain Animal Breeding Farm, Jiangning District, Nanjing, China [License No: SYXK (Su) 2016-0011]. The mice were housed under standard temperature (20 ± 2 °C) and humidity ($50 \pm 10\%$) conditions on a 12 h light/dark cycle. Mice were acclimated to these conditions for at least 7 days before starting experiments. All animal treatments were performed in strict accordance with the National Institutes of Health Guidelines for the Care and use of laboratory animals. The experiments were approved by the Animal Ethics Committee of the China Pharmaceutical University.

2.3. Cell culture

Normal colonic epithelial cells (NCM460) were obtained from ATCC (Manassas, VA, USA) and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin in an incubator with 5% CO₂. The culture medium was replaced every other day. Cells at 80% confluence were used for all of the assays. For the *in vitro* experiments, NCM460 cells were treated with IL-6 (5, 10, 20 ng/mL) and limonin at different concentrations for 24 h.

2.4. Cell viability assay

Cell viability was assayed by CCK-8 assays. Cells were seeded in 96-well plates at a density of 8000 cells per well in 0.2 mL of DMEM. After 24 h, cells were divided into 8 groups, including the control group and groups treated with different concentrations of limonin (2.5, 5, 10, 20, 40, 80 and 160 μ g/mL), and incubated for 24 h. Then, CCK-8 was added to the cells, which were incubated for another 4 h. Finally, the optical density at 450 nm was measured with a multifunction microplate reader.

2.5. Cell transfection

At 24 h before transfection, cells were inoculated into 6-well plates

at the rate of 5×10^5 /well. Cells transfection followed the procedure: (1) when the fusion degree of the cells reached 60%–80% in the 6-well plate, the fresh culture medium was replaced. (2) add 4 μ L miRNA mimics into the sterile 1.5 mL EP tube and mix with 200 μ L jet PRIME buffer, vortex for 10 s. (3) add 4 μ L jet PRIME reagent, vortex for 10 s, and rest at room temperature for 10 min. (4) add the mixed solution to the 6-well plate, shake the culture plate gently and mix it, and then put it in the cell incubator for another 48 h culturing.

2.6. Establishment of the DSS-induced acute colitis model in mice

C57BL/6J mice were divided into 6 groups: control group, DSS + vehicle group, DSS + sulfasalazine (SASP)-treated group (200 mg/kg), and DSS + limonin groups (40, 80, and 160 mg/kg). Acute colitis in mice was induced by the administration of DSS in drinking water (3%, w/v). The mice in DSS + limonin group were given limonin *via* gavage from the day of DSS treatment. The control group and DSS + vehicle group were given the same amount of PBS (Fig. 1B). The mice were sacrificed after DSS administration at 9 days. The entire colons were dissected and the lengths were measured. Colons were rinsed quickly with cold PBS to remove feces and blood. The samples were collected for multiple future purposes.

2.7. Assessment of the DAI score

During the test period, body weight, stool consistency, and gross blood in the feces were recorded daily and scored as the disease activity index (DAI). Briefly, the DAI was obtained based on the following parameters [21]: a) body weight loss (0, no loss; 1, 1–5% loss; 2, 6–10% loss; 3, 10–20% loss; or 4, over 20% loss); b) diarrhea (0, normal; 2, loose stools; or 4, watery diarrhea); and c) hematochezia (0, no bleeding; 2, slight bleeding; or 4, gross bleeding).

2.8. Histologic analysis of the colon

Mice were sacrificed after DSS administration at 9 days. The colon was removed. Then, the colons were washed, and small segments of the colon were fixed in 10% neutral-buffered formalin solution for 24 h, embedded in paraffin and sectioned into 3 μ m slices that were subsequently subjected to hematoxylin–eosin (H&E) staining and immunohistochemical staining. The severity of inflammation was evaluated using H&E-stained sections as described previously [22].

2.9. Immunohistochemical staining

The paraffin sections of colon tissue were dried in a constant temperature oven at 60 °C for 1 h and washed three times in xylene for 5 min each, twice with 100% ethanol for 10 min, twice with 95% ethanol for 10 min each, and twice with double distilled water for 5 min each. Then, the paraffin sections were placed in a microwave oven at medium-medium-strength at for 8 min for antigen retrieval. Hydrogen peroxide (3%) was used to inhibit endogenous peroxidase activity, and blocking solution was used to block nonspecific antigens. Then, the sections were incubated with anti-p-STAT3 antibody and anti-IL-6 antibody at 4 °C overnight and then incubated with the secondary antibodies, followed by DAB staining. Finally, the sections were dehydrated and mounted, and observed under microscopes.

2.10. Cytokine analysis by ELISA

Colon tissues were homogenized and sonicated in normal saline to obtain 2.5% homogenate, which was centrifuged at 3000 \times g for 10 min at 4 °C, and the supernatant was collected to examine the levels of cytokines. The concentration of inflammatory cytokines (IL-6 and IL-10) was determined using ELISA kits according to the manufacturer's protocols (MultiSciences, China).

2.11. Western blot analysis

Colon and cellular protein lysates were prepared using RIPA lysis buffer. A BCA protein assay was used to determine protein concentration, and 20 μ g of protein was separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, Massachusetts), as described previously [23]. After the membranes were blocked with 5% fat-free milk in Tris-buffered saline + Tween (TBST), proteins were detected by incubating with primary antibodies at 4 °C overnight. Next, the membrane was incubated with the secondary antibodies according to the appropriate protocols. Specific signals were detected using Chemstar High-sig ECL Western Blotting Substrate (Thermo Fisher, USA). Protein levels were quantified by density analysis using Quantity One software (Bio Rad).

2.12. RNA extraction, cDNA synthesis, and quantitative analysis

Total RNA was extracted by TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. qRT-PCR (Quantitative real-time polymerase chain) reaction was performed on triplicate samples in a reaction mix of SYBR Green (Vazyme, China) with ABI Step One Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). mRNA and miRNA levels were normalized to GAPDH or U6 RNA, respectively. The primers were as follows: GAPDH: Forward: AAGGTCGGAGTCACC GGATT, Reverse: CTGGAAGATGGTGATGGGATT; STAT3: Forward: GAGGAGCCATTCGGAAAAGTATT, Reverse: CAGGTCGTTGGTGTCCAC ACA; PTEN: Forward: ACCAGGACCAGAGG-AAACCT, Reverse: GCTA GCCTCTGGATTTGACG; IL-6: Forward: CGGAGAGGAGACTTCACA GAG, Reverse: CATTTCCACGATTTCCAGA; PDLIM2: Forward: ATGG CCACGATTATGTCTCC, Reverse: GCCCATCATGGTGACTAAGG; miR-214-3p: Forward: CGCTTTACAGCAGGCACAGA, Reverse: TAAGGTTC ATCACGACAGGTTTCAC; U6: Forward: CAGCACATATACTAAAATTGG AAGG, Reverse: ACGAATTTGCGTGTCCATCC.

2.13. Fluorescence *in situ* hybridization (FISH) of miR-214

Fluorescence *in situ* hybridization for miR-214 was performed on the frozen slice. The frozen slice was rehydrated in citrate buffer. Proteinase K digestion was used to treat fixed tissues at 37 °C for 20 min. After digestion, the slice was hydrated through a graded series of alcohol to tap water after immersing in RNase-free water for 3 min and then air dried. Hybridization was carried out overnight at 37 °C using miR-214-3p probes with the sequence of 5'-DIG-ACTGCCTGTCT GTGCCTGTGT-DIG-3'. After washing, the sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) for 20 min and observed with the confocal microscopy.

2.14. Statistical analysis

All data were expressed as mean \pm SEM. Data analysis was performed using GraphPad Prism 5 software. Comparison between groups was made using one-way ANOVA test. *P*-values of < 0.05 was considered significant (*, # *p* < 0.05).

3. Results

3.1. Limonin ameliorated DSS-induced UC in mice

To explore the protective roles of limonin (Fig. 1A) on UC, as shown in Fig. 1B, the mouse model of DSS-induced acute colitis was established by administrating mice with 3% DSS for 7 days. We then tested the efficacy of limonin which the dosages ranging from 40 to 160 mg/kg *in vivo*. SASP was served as a positive control in this study. At 9 days after the model construction, the body weight changes, DAI score, and colon length in mice were evaluated. Compared with the control group, the vehicle group showed a significant decrease in body weight on day

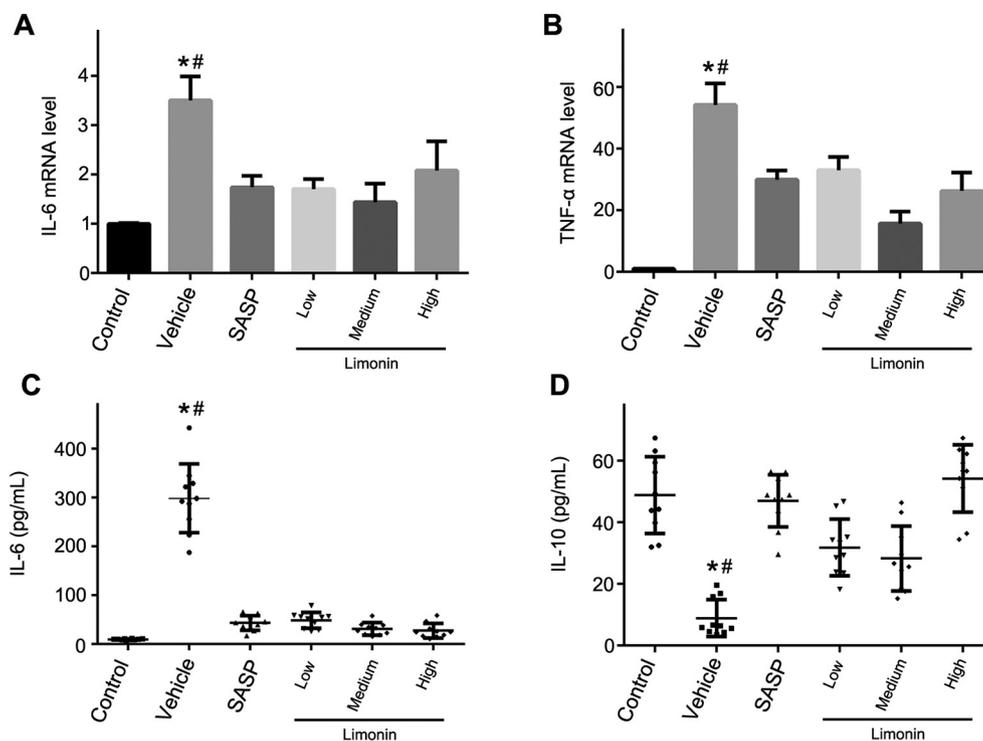


Fig. 2. Limonin alleviated inflammation in DSS-induced colitis *in vivo*. Limonin repressed mRNA expression of pro-inflammatory cytokines IL-6 (A) and TNF- α (B) (n = 5). IL-6 (C) and IL-10 (D) protein levels were analyzed by ELISA (n = 10). *P < 0.05 vs control, # P < 0.05 vs SASP and limonin groups.

4, with a continuous daily decrease thereafter. Administration of limonin markedly rescued the body weight loss induced by DSS (Fig. 1C). Meanwhile, the DAI of DSS-treated mice were higher than the value in the control group, while it was significantly improved by limonin (Fig. 1D). Interestingly, limonin partially reversed the shortening in colon length due to DSS administration (Fig. 1E and F). Of note, in a separated experiment, we found that limonin did not cause obvious DAI changes or pathologic lesions on colon tissues after administration at 9 days in the healthy mice, as presented in supplementary Fig. 1A through E. Collectively, these results provide the first impression on the protective effects of limonin in UC treatment in mice.

3.2. Limonin alleviated inflammation in DSS-stimulated colitis *in vivo*

Because UC is a chronic inflammatory disease, we first examined the mRNA levels of major proinflammatory cytokines by using qRT-PCR analyses. As shown in Fig. 2A–B, the mRNA levels of IL-6 and Tumor necrosis factor- α (TNF- α) were induced in diseased colon after DSS administration, while limonin markedly abolished the productions of both cytokines. We then confirmed these findings by detected the levels of IL-6 and IL-10 in the diseased colon with enzyme-linked immunosorbent assay (ELISA), as presented in Fig. 2C and D. Consistently, HE staining showed that there is no histopathologic damage was observed in the control group, whereas DSS-induced colon damage was characterized by a marked decrease of the number of crypts, loss of the surface epithelium, and inflammatory cells infiltration in the mucosa and submucosa. These pathologic damages were dramatically improved by limonin in mice, as illustrated in Fig. 3A and B. Furthermore, the immunohistochemical staining also indicated that DSS caused a pronounced increase of IL-6 expression in mice, while a high dosage of limonin clearly eliminated IL-6 expression in the diseased colons (Fig. 3B). These data suggested that limonin could improve the pathology of the diseased colons in UC.

3.3. Limonin blocked the activity of STAT3/miR-214 signaling pathway *in vivo*

Since previous research has shown that STAT3/miR-214 signaling is activated in IBD mice [16], we speculated that limonin may exert its protective role in UC mainly by blocking the activity of STAT3/miR-214 axis. As shown in Fig. 4A and E, expression of miR-214 was dramatically induced by DSS, while it was repressed by limonin, particular in the group using medium dosage. However, limonin was not able to change miR-214 levels in the healthy mice (Supplementary Fig. 1F). Consistent with miR-214 levels in the disease colon in UC mice, expression of phosphorylated STAT3 was also detected by western blot and immunohistochemical staining, as illustrated in Fig. 4B to 4D. The results suggested that limonin suppressed the activity of STAT3/miR-214 signaling pathway in UC mice.

3.4. Limonin restored the expression of PTEN and PDLIM2 *in vivo*

To further verify the biologic functions of limonin, we further evaluated whether limonin could increase expression of the miR-214 downstream targets, such as PTEN and PDLIM2. In the healthy mice, limonin was demonstrated that it has little effects on PTEN and PDLIM2 expression in colon tissue (Supplementary Fig. 1G and H). Impressively, as shown in Fig. 5A and B, qRT-PCR analyses revealed that PTEN and PDLIM2 mRNA expression were diminished by DSS, while their levels were significantly improved after limonin administration. Western blot analyses indicated the similar results in the diseased colon in UC mice (Fig. 5C–E). Of note, the medium dosage of limonin displayed the best pharmacologic efficacy, at least in the current study.

3.5. Limonin suppressed the STAT3/miR-214 signaling pathway *in vitro*

In vitro, we sought to further confirm the therapeutic effects of limonin on blocking the activity of STAT3/miR-214 signaling pathway. First, we examined the cytotoxicity of limonin on NCM460 cells. The

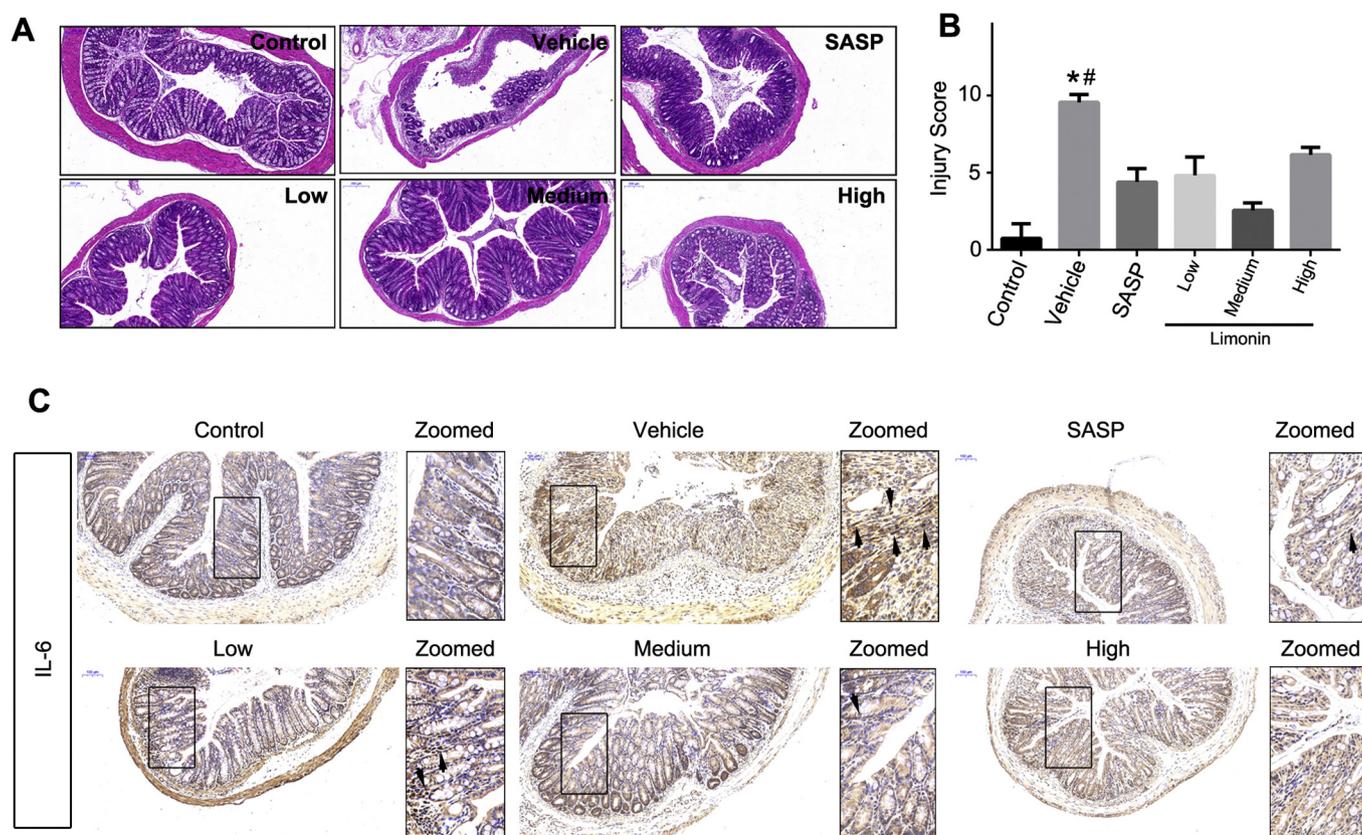


Fig. 3. Limonin improved colon histology in DSS-induced colitis *in vivo*. (A) Representative H&E-staining micrographs and histology changes were scored (B). Scale bar: 100 μ m. *P < 0.05 vs control, # P < 0.05 vs SASP and limonin groups, n = 5. (B) Representative micrographs showed IL-6 expression in the diseased colons. Arrows denote positive staining.

results showed that limonin had little cytotoxicity in the concentration ranges of 0–160 μ g/mL (Fig. 6A). As shown in Fig. 6B and C, after incubation with different concentrations of IL-6 and limonin at 24 h, the secretion of IL-17 and TNF- α were markedly increased in IL-6-stimulated NCM460 cells, this effect was then suppressed by limonin. Furthermore, we found that limonin restored IL-6-induced dysregulations of p-STAT3, miR-214, PTEN, and PDLIM2 in cultured NCM460 cells, as presented in Fig. 6D to G.

3.6. Limonin attenuated IL-6-induced inflammation via miR-214 *in vitro*

Finally, we need to make sure whether limonin attenuated IL-6-induced inflammation was associated with the activity of miR-214 *in vitro*. As shown in Fig. 7A to C, limonin indeed blocked IL-6-induced secretion of IL-17 and TNF- α in cultured NCM460 cells. However, this effect was impaired by miR-214 overexpression. Furthermore, miR-214 overexpression also largely weakened the capacities of limonin in inhibiting IL-6-induced downregulation of PTEN and PDLIM2 (Fig. 7D–F). Taken together, these results suggested that limonin suppressed IL-6 induced inflammation mainly through inhibiting STAT3/miR-214 signaling *in vitro*.

4. Discussion

At present, UC is a chronic inflammatory disease with an increasing incidence and frequent recurrence that not only affects the quality of life of patients but also significantly increases their risk to colorectal cancer. Current therapeutic strategies mainly involve the use of 5-aminosalicylic acid, hormones, immunosuppressive agents, biological agents, other medical treatments, and surgical procedures. These methods can alleviate inflammation to some extent, but considerable

limitations in their safety and efficacy are existed [24–26]. Therefore, there is an urgent need to develop more safe and effective medications for UC [27]. Over past years, considering cost-effectiveness in developing biological agents as well as emerging drug-resistant seriously affects the efficacy and safety in UC treatment, international pharmaceutical companies start to pay more attentions to the research and development of natural products. It has now become an important direction in designing UC management strategy by finding the main active ingredients from traditional herbal medicine. Previous studies have shown that limonin has significant effects in promoting cancer cell apoptosis, regulating low-density lipoprotein, treating colon cancer, and combating bacterial infections [28]. Expanding the application of limonin to treat UC is believed to possess great medical value and broad market prospects in the field.

Diarrhea and blood in stool are the main symptoms of UC [29]. In this study, mice were given free access to 3% DSS showed weight loss, diarrhea, and blood in the stool. The overall scores of these three indicators directly reflect the DAI or the disease severity in mice. As a novel extract, our data indicated that limonin is a strong candidate in improving UC clinical symptoms. We demonstrated that limonin is able to alleviate colon shortening and its pathologic damages, although the pathogenesis for colon shortening remains unclear in the field [30]. Meanwhile, we confirmed that limonin improved UC prognosis mainly through blocked inflammation in diseased animals. Limonin reduced IL-6 level and increased IL-10 level in the DSS-induced UC model. In particular, our results indicated that medium to high dosages of limonin could significantly improve the prognosis of UC. Intriguingly, the medium dose of limonin showed stronger protective ability. A possible reason may be the threshold of limonin saturation was relatively low. Once the blood concentration of limonin reaches the peak, its therapeutic effects will be limited no matter how to increase the dosage of

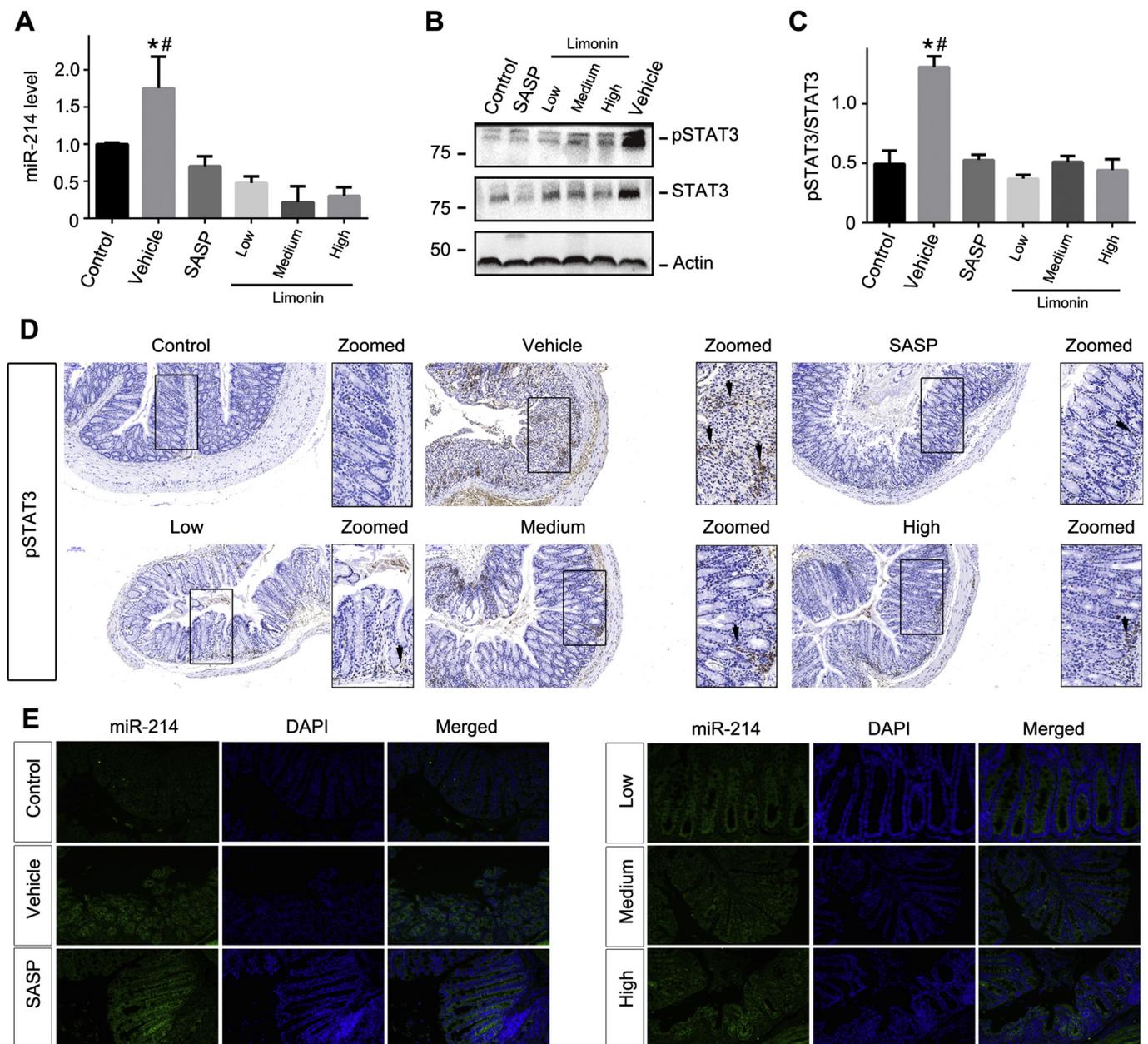


Fig. 4. Limonin blocked the activity of STAT3/miR-214 signaling pathway *in vivo*. (A) qRT-PCR analyses revealed reductions of miR-214 after administration of limonin in UC model. * $P < 0.05$ vs control, # $P < 0.05$ vs SASP and limonin groups, $n = 5$. (B–C) Western Blot analyses indicated limonin repressed STAT3 phosphorylation in the diseased colons and quantitative data was presented (C). * $P < 0.05$ vs control, # $P < 0.05$ vs SASP and limonin groups, $n = 5$. (D) Representative micrographs showed p-STAT3 expression in diseased colons. Arrows indicated positive cells. Scale bar: 100 μm . (E) *In situ* hybridization was used to reveal miR-214 (green) distribution in diseased colons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

limonin. In many cases, it does have low efficacy on a higher dose because the binding with targeted proteins or the mechanisms of action has been changed.

The incidence of UC is associated with inflammation, immune abnormalities, infection, heredity, and environmental factors [31]. Earlier studies have shown that the occurrence of UC is closely related to the abnormal expression of miRNAs, such as miR-146 and miR-206 [32], as well as aberrantly secreted cytokines, such as IL-6 and IL-17 [33]. Therefore, uncontrolled expression of cytokine in transcription levels may be the main biological mechanisms of UC development [34]. STAT3, one of the seven STAT family members, is a bifunctional protein which involved in signal transduction *via* tyrosine phosphorylation in cytoplasm [35]. Over activation of STAT3 leads to abnormal cell

proliferation and apoptotic disorders, which promotes inflammation and tumor formation and development. STAT3 is activated through phosphorylation and then translocated into the nucleus to turns on the downstream target genes expression. Overactivation of STAT3 causes an uncontrolled inflammatory response [16]. Disorders in the IL-6/STAT3 signaling pathway are causally related to a variety of gastrointestinal and rheumatic diseases [36], including UC [37].

The miR-214 expression is associated with UC severity and disease duration [38]. It is well known that miR-214 is an inflammatory effector molecule that is dysregulated in patients with colitis-associated colon cancer. STAT3 is able to bind to the promoter region of miR-214. Once present to IL-6, IL-6 directly upregulated miR-214, thereby modulates the downstream PDLIM2/NF- κB and PTEN/Akt signaling

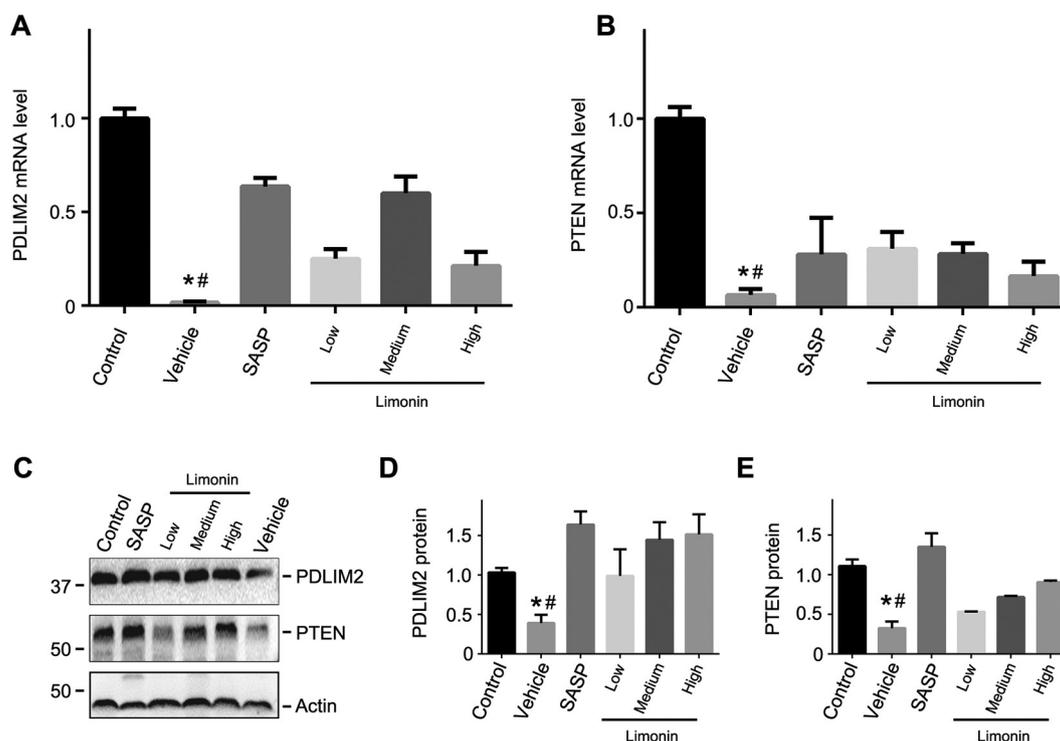


Fig. 5. Limonin restored PTEN and PDLIM2 expression *in vivo*. (A–B) qRT-PCR analyses revealed PTEN and PDLIM2 levels in the diseased colons among multiple groups. *P < 0.05 vs control, # P < 0.05 vs SASP and limonin groups, n = 5. (C–E) Representative Western Blot analyses indicated PTEN and PDLIM2 levels in the diseased colons and quantitative data was presented (E–D). *P < 0.05 vs control, # P < 0.05 vs SASP and limonin groups, n = 5.

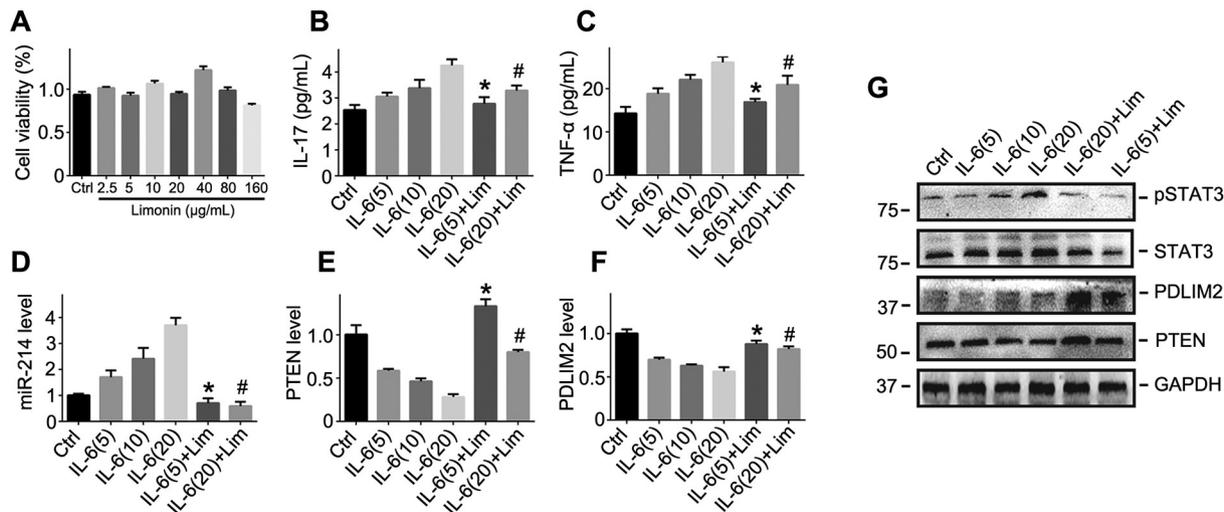


Fig. 6. Limonin suppressed the activity of STAT3/miR-214 signaling pathway *in vitro*. (A) The cytotoxicity of limonin at different concentrations of 0–160 µg/mL on NCM460 cells was determined by CCK-8 assay. (B–C) ELISA revealed that limonin reduced TNF-α and IL-17 level in NCM460 cells incubated with IL-6 (ng/mL) *P < 0.05 vs IL-6(5), # P < 0.05 vs IL-6(20), n = 3. (D–F) qRT-PCR analyses indicated limonin reduced miR-214 level (D) and increase the PTEN (E) and PDLIM2 (F) levels in NCM460 cells after stimulation by IL-6. (G) Western blot analyses showed p-STAT3, PDLIM2, and PTEN expression in NCM460 cells incubated with IL-6 and limonin.

pathways. In addition, upregulation of miR-214 activates the NF-κB/IL-6 pathway, indicating the presence of a miR-214 involved feedback loop [16]. Our data further indicated that miR-214 level was significantly increased in DSS-treated mice. After the administration of different concentrations of limonin, miR-214 level was significantly decreased. Meanwhile, mRNA and protein levels of the downstream factors, PTEN and PDLIM2, have increased accordingly. Overall, we expanded the application of limonin to UC treatment for the first time which indeed exerted therapeutic effects both *in vivo* and *in vitro*. One shortcoming of this study is that more detailed mechanisms of miR-214

in the treatment of UC need to be further explored. It is worth of pursuing whether limonin could regulate other targets through miR-214 as well as whether PTEN and PDLIM2 are also regulated by other miRNAs in near future.

5. Conclusion

Administration of limonin improved the prognosis of DSS-induced UC in mice. The protective effects of limonin were tightly associated with its capacities in alleviating the expression of pro-inflammatory

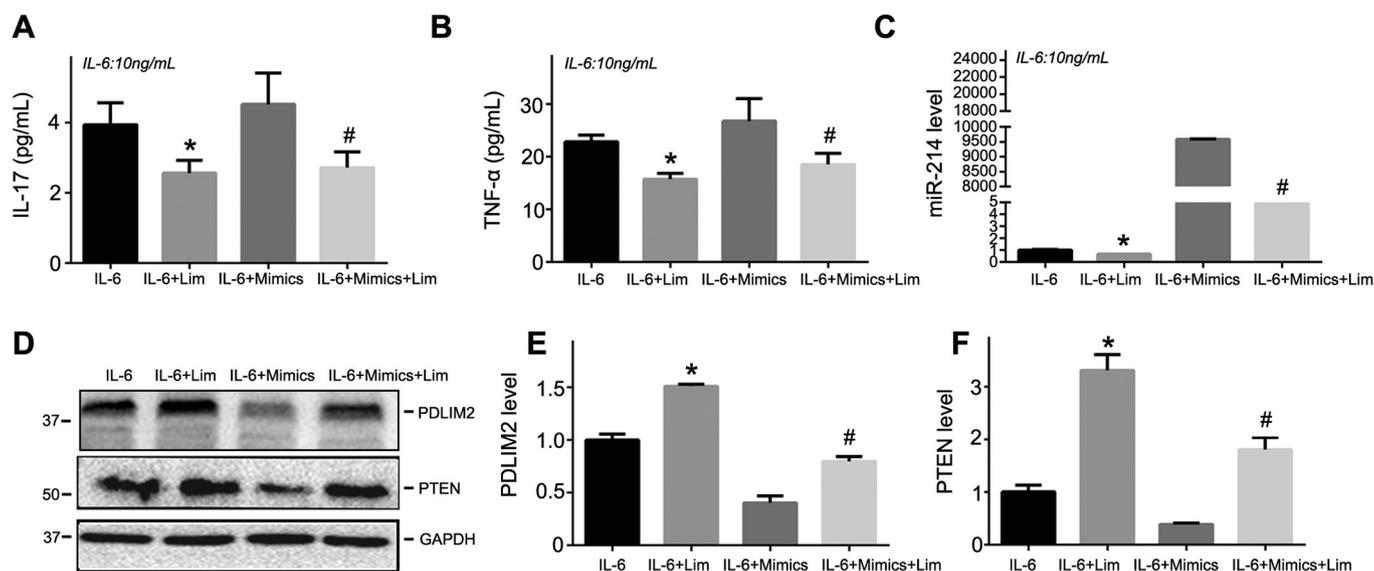


Fig. 7. Limonin attenuated IL-6-induced inflammation depending on miR-214 activity *in vitro*. (A–B) ELISA showed limonin reduced IL-17 and TNF- α in NCM460 cells incubated with IL-6 or miR-214 mimics. (C) miR-214 levels changes. (D–F) Western blot analyses showed PDLIM2 and PTEN expression in NCM460 cells incubated with IL-6, miR-214 mimics, or limonin. *P < 0.05 vs IL-6, # P < 0.05 vs IL-6 + mimics, n = 3.

cytokines and related proteins in p-STAT3/miR-214 signaling pathway. These results suggested that limonin is an effective botanical medication and have advantages in the future for prospective clinical applications in designing the strategies for UC.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2019.105768>.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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