ORIGINAL RESEARCH

Fraxin Ameliorates Ulcerative Colitis by Modulating Oxidative Stress, Inflammation and TLR4/NF-κB and MAPK Signaling Pathways

Wenkai Sun, MM; Li Hang, MM; Liping Chen, MM; Yun Qu, MM; Yingchun Zhang, MM; Zhiwei Miao, MD; Xiaoyu Wang, MD

ABSTRACT

Objective • UC is a chronic gastrointestinal disorder of uncertain etiology. However, effective therapeutic drug options for UC are relatively limited. Fraxin represents a principal active constituent within the traditional Chinese medicinal herb known as Cortex Fraxini or Qinpi. Nevertheless, the impact of Fraxin on UC remains uncharted. This study aims to explore the potential of Fraxin, a key component of Cortex Fraxini, in inhibiting DSS-induced intestinal inflammation in mice and to unravel the underlying mechanisms.

Methods • In vitro experiment,the RAW264. 7 cells were induced by LPS as the model.In vivo experiment,the mice were induced by DSS as the animal model for a ten day experiment.The ELISA, western blots, measurement of oxidative stress markers and other relevant methods were used to discuss the effect of Fraxin on LPS-induced RAW264.7 cells and the inhibitory effect of Fraxin on intestinal inflammation induced by DSS in mice and underlying mechanisms.

Results • Our findings indicated that Fraxin significantly reduced symptoms of UC, such as body weight loss, colonic length shortening, and histological damage. At the molecular level, it inhibited ROS generation, reduced pro-inflammatory cytokines, and regulated key pathways including TLR4/NF-κB and MAPK.The findings indicated that Fraxin diminished the expression of p-NF-κB and p-IκB, downregulated iNOS and COX-2 expression, and lessened p38, JNK and ERK phosphorylation.

Conclusion • Taken together, Fraxin ameliorates UC by regulating oxidative stress, inflammation, and TLR4/NF-κB and MAPK pathways, and Fraxin may be a new treatment for UC. Our findings suggest that Fraxin could offer a novel therapeutic approach for UC, targeting oxidative stress and key inflammatory pathways. (*Altern Ther Health Med.* 2024;30(12):117-125).

Wenkai Sun, MM, Resident doctor; **Li Hang,** MM, Resident doctor; **Liping Chen,** MM, Resident doctor; **Yingchun Zhang,** MM, Resident doctor; **Zhiwei Miao,** MD, Associate chief physician; **Xiaoyu Wang,** MD, Chief physician; Department of Gastroenterology, Zhangjiagang TCM Hospital Affiliated to Nanjing University of Chinese Medicine, Suzhou, China. **Yun Qu,** MM, Resident doctor; Department of Neonatology, Zhangjiagang TCM Hospital Affiliated to Nanjing University of Chinese Medicine, Suzhou, China.

Corresponding author: Xiaoyu Wang, MD
E-mail: wsy0082023@163.com
Corresponding author: Zhiwei Miao, MD
E-mail: hmy09262023@163.com

INTRODUCTION

Ulcerative colitis(UC) is classified as a form of inflammatory bowel disease (IBD). The disease presents a chronic inflammatory reaction state. The lesions are continuous and can involve different parts of the rectum and colon. The specific cause is not clear. The clinical features are alternated with onset, remission, and recurrence. It is a common intractable digestive system disease. Because it is difficult to cure, easy to relapse, and evolve into a tumor, The World Health Organization categorizes it as an intractable condition. Per pertinent research, ulcerative colitis incidence and prevalence have risen in recent years.[1]

Noxious oxidative processes and deleterious inflammatory cascades are purported to fulfill pivotal pathogenetic functions in ulcerative colitis. The products of oxidative stress have pro-inflammatory properties, which can destroy cell structure through lipid peroxidation, and ultimately lead to cell apoptosis and necrosis.[2] Aberrant overproduction of proinflammatory cytokines, exemplified by tumor necrosis factor-α(TNF-α) and interleukin-6(IL-6), represents a signature manifestation of enteritis.[3] toll-like receptors(TLRs) are transmembrane protein receptors that play crucial roles in nonspecific innate immune defense.[4] Within the TLRs family, TLR4 is critical, binding and signaling downstream to MAPK and NF-κB via receptor dimerization.[5] The constituent

elements of triggered MAPK and NF-κB signaling cascades modulate inflammation via upregulating interleukin-1β(IL-1β), IL-6, TNF-α, and other proinflammatory cytokines.[6,7] Moreover, research indicates targeting the TLR4/NF-κB and MAPK signaling pathways may provide an alternative approach to treating inflammatory diseases.[8]

5-aminosalicylic acid, Immunosuppressive drugs, antibiotics, and steroids are the primary pharmacological UC.[9-11] However, the long-term use of these drugs can induce serious toxicities,including infection, gastrointestinal adverse reactions, and bone marrow suppression. Thus, selecting novel therapeutics with low toxicity and robust efficacy is especially critical. Traditional Chinese medicine is increasingly involved in the comprehensive treatment of UC, and its effectiveness has been continuously proven. The use of effective Chinese medicine, Chinese medicine prescriptions and their active ingredients to treat UC is also becoming a hot spot in clinical research.

Fraxin is an important active ingredient in Cortex Fraxini. We reviewed the literature and found that Qinpi glycoside possesses a multitude of biological properties, encompassing anti-inflammatory, antio-xidant, analgesic, antibacterial, antiviral, immunomodulatory, anti-hyperuricemia and diuretic activitie.[12] Among these biological properties, anti-inflammatory and antio-xidant effects are particularly important in improving UC related inflammatory responses.

Prior studies of Fraxin's anti-inflammatory activity primarily concentrated on lung injury[13], liver injury[14], kidney injury[15,] and endotoxic shock.[16] However, Fraxin's therapeutic effects on UC have not yet been reported. Baitouweng decoction is a classical Chinese prescription,derived from Treatise on Febrile Diseases,consisting of Pulsatilla chinensis (Bunge) Regel (Baitouweng), Coptis chinensis Franch (Huanglian), Phellodendron chinense C. K. Schneid (Huangbai), and Cortex Fraxini (Qinpi) has been proven to protect the intestinal mucosal barrier and reduce the release of pro-inflammatory cytokines.[17] In addition, the Fraxetin in Cortex Fraxini can alleviate TNBS-induced experimental colitis.[18] These findings lay the groundwork for the hypothesis that Fraxin could improve UC. Accordingly, the objective of our investigation is to elucidate the putative mechanisms and actions of Fraxin on ulcerative colitis utilizing integrated in vivo and in vitro methodologies. Our results demonstrate that Fraxin can ameliorate UC by regulating oxidative stress, inflammation, and TLR4/NF-κB and MAPK pathways.

MATERIALS AND METHODS

Drugs and Reagents

2', 7'-dichloro-fluorescein (DCFH-DA) was procured from Beyotime. DSS (MW36000–50000) was procured from MP Biomedicals (Santa Ana, CA, USA). The Cell Counting Kit-8 (CCK-8) was obtained from MedChemExpress LLC (New Jersey, USA). FBS was procured from Biological Industries Co., Ltd (Beijing, China). Sulfasalazine (SASP) was acquired from Shanghai Sine Tianping Pharmaceutical. Dimethylsulfoxide (DMSO) was obtained from Thermo Fisher Scientific (Scoresby, Australia). SOD and MDA evaluation kits were procured from Yifeixue Bio-Tech (Nanjing, China). Mouse TNF-α, IL-6, IL-10, and IL-1β ELISA kits were obtained from Yifeixue Bio-Tech (Nanjing, China). Mouse monoclonal [AC-15] to beta-actin (β-actin), anti-Cyclooxygenase 2 (COX2) antibody, anti-iNOS antibody, anti-TLR4 antibody, anti-NF-κB p65 antibody, anti-NF-κB p65 (phospho T254) antibody, horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were obtained from Abcam (Cambridge, UK). The anti-JNK,anti-phosphorylate JNK, anti-IκBα, anti-phosphorylate IκBα, anti-ERK, anti-phosphorylate ERK, anti-p38 and anti-phosphorylate p38 were obtained from Cell Signaling Technology Inc (Danvers, MA, USA).

Cell culture and Cell Viability Assay

The RAW264.7 cells were propagated in DMEM enriched with 10% FBS at 37°C in a 5% CO_2 atmosphere. Cells underwent treatment with varying Fraxin concentrations (0.1-400 μM) for 24 hours. Subsequently, a CCK-8 assay was executed to assess cellular viability. Absorbance values were ascertained at 450 nm employing a microplate spectrophotometer.

Detection of intracellular ROS

The DCFH-DA fluorescence assay was utilized to detect intracellular ROS. RAW264.7 cells were seeded in a 6-well plate at 1.0×10^5 cells/well for 24 hours.The cells were pretreated with different concentrations of Fraxin (10 μM, 20 μM, 50 μM) for 1 hour, and then LPS (1 μg/ml) was added and incubated for 4 hours. Then, after removing all the cell culture medium, 1 mL of DCFH-DA diluted with a serum-free medium at 1:1000 was added. After incubating at 37°C in the dark for 20 minutes, wash with PBS 3 times to fully remove the DCFH-DA that has not been internalized by the cells. An inverted fluorescence microscope observed the cells, and the DCF fluorescence distribution at the excitation wavelength was assessed utilizing a flow cytometer. The excitation and emission wavelengths are 488 and 525 nm respectively.

Animal and DSS-induced experimental colitis in mice

The experimental protocol was approved by the Animal Ethics Committee of Zhangjiagang Hospital of Traditional Chinese Medicine Affiliated to Nanjing University of Traditional Chinese Medicine on ethical issues.

Male C57BL/6J mice (18-22 grams, n=60) were obtained from Suzhou University (Suzhou, China) (certificate no. SCXK (Su) 2018-0006). The mice were placed in an environment with temperature (25 ± 2°C), humidity (55% ± 15%) and a 12-hour light-dark cycle, and were given sterile food and water.Subsequent to one week of acclimatization, they were randomly partitioned into 6 cohorts comprising 10 creatures per group: 1) control set; 2) DSS set; 3) DSS+SASP set; 4) Low-dose Fraxin treatment set; 5) Medium-dose Fraxin treatment set; 6) High-dose Fraxin treatment set. The murine subjects were provided 2.5% DSS in their potable water for a period of 7 days, except for the control group. Subsequently, the water was replaced with normal drinking water. The low, medium, and high dose Fraxin treatment

groups were given oral 10 mg/kg, 20 mg/kg, and 50 mg/kg Fraxin, respectively. Each dosage was diluted using distilled water, ensuring that every mouse received a consistent amount of the administered compound. The control group was given the same amount of distilled water orally. Colitis development was monitored, and on the 10th day, the mice were euthanized for subsequent analysis.

Morphological and histopathological examination

Throughout colitic pathogenesis, 3 independent observers surveilled murine weight decrement, stool morphology, and fecal blood quotidianly, constituting the DAI, as antecedently documented. On the 10th day, the length of the colon from the anus to the appendix was recorded. Colonic segments from each murine subject per group were maintained in 4% PBS-buffered formaldehyde, embedded in paraffin, and consecutively sectioned into 5μm slices. The histopathological scores came from three researchers who were unaware of the experimental conditions. The criteria were delineated as follows: 0: no inflammatory indices; 1: low-grade inflammation with dispersed monocytic infiltration; 2: multifocal moderate inflammation with monocytic predominance; 3: high-grade inflammation with augmented vascular density, significant mural thickening; 4: maximal inflammation, transmural leukocytic infiltration, and goblet cell demise.

Histological Analysis

The colon tissue was fixed with 4%(wt/vol) paraformaldehyde.The paraffin-embedded samples were processed to obtain 5 μm thick sections.Paraffin sections were deparaffinized with xylene, dehydrated with ethanol, and then stained with hematoxylin and eosin (H&E) to assess colon damage.Sections were observed under an inverted microscope (Olympus CX43).

ELISA

Centrifuge the mouse plasma at 4°C at 3000 r/min for 10 min, aspirate the supernatant, and store it in aliquots at -80°C. The concentration of IL-1β, IL-6, IL-10, and TNF-α in mouse plasma was determined using an ELISA kit, and the lower limit of the quantitative concentration of each cytokine was 7.8 μg/ml.

Take the logarithmic growth phase RAW264.7 cell suspension, adjust the cell density to 10×10^4 cells/mL, inoculate it in a 6-well plate, 2 mL per well, group and administer cells, and collect the supernatant after culturing for 24 h. The concentration of IL-1β, IL-6, IL-10 and TNF-α in the supernatant was determined with an ELISA kit.

Western Blot Analysis

Protein was extracted from colon tissue and RAW264.7 cells using RIPA buffer (Beyotime) and protease inhibitor cocktail (200 m MAEBSF, 30 μM instatin, 13mM instatin, 1.4m ME64 and 1mM DMSO, 1:100). The protein was extracted after centrifugation. A BCA kit (Beyotime) was taken to determine protein concentration. Subsequently, polyacrylamide gel electrophoresis (Bio-Rad) was performed and the protein samples were transferred to 0.45 μm PVDF membranes (Millipore, MA, USA). After blocking with 5% BSA for 1 h,the membranes were incubated overnight at 4°C with the primary antibodies: TLR4, COX-2, NF-κB, p-NF-κB, β-actin, iNOS, p-JNK1/2, p-IκBα, p-ERK, p-p38MAPK, JNK1/2, IκBα, ERK, p38, and then incubated with secondary antibodies at room temperature for 1 h.The secondary antibody was then washed away, and chemiluminescence was measured (ECL Plus; Beyotime).

Immunofluorescence staining

Immunofluorescence was employed to assess colon tissue inflammation and intestinal barrier integrity. Tissues were firstly deparaffinized and cleansed. After that, 0. 01 M citrate-buffered antigen and sodium solution (pH 6) were supplemented. The colonic sections were barricaded with 5% BSA and incubated overnight under refrigerated 4°C conditions with diverse primary antibodies. Sections were incubated for 1 hour at ambient temperature with Alexa Fluor 488-conjugated donkey anti-rabbit IgG antibody. The sections were subsequently stained with DAPI. Tissues were visualized by confocal microscopy.

Measurement of Oxidative Stress Markers

MDA and SOD levels were measured in colonic tissue. In brief, 100 mg of colon tissue was weighed and homogenized in PBS. The homogenized colonic sample was centrifuged. The consequent supernatant was subsequently assayed per the manufacturer's directives.

Take the logarithmic growth phase RAW264. 7-cell suspension, adjust the cell density to 10×10^4 cells/mL, inoculate it in a 6-well plate, 2 mL per well, group and administer cells, and collect the supernatant after culturing for 24 h. Likewise, the resulting supernatant was analyzed.

RNA Isolation and Quantitative Real-Time PCR and Statistical Analysis

Total RNA was extracted from a total RNA Extraction Reagent (Vazyme Biotech Co. Ltd., China) and then reverse transcribed by Hifair™ II 1st Strand cDNA Synthesis SuperMix for qPCR (Yeasen, Shanghai, China).The primers were purchased from Sangon Biotech (Shanghai) Co. Ltd., and the primer sequences (designed and checked by Primer Premier 6.0) were shown in Table 1. Real-time PCR was conducted using a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA).The relative amount of target mRNA was normalized to the level of β-actin, and the result was calculated using the $2^{-\Delta\Delta Ct}$ method.

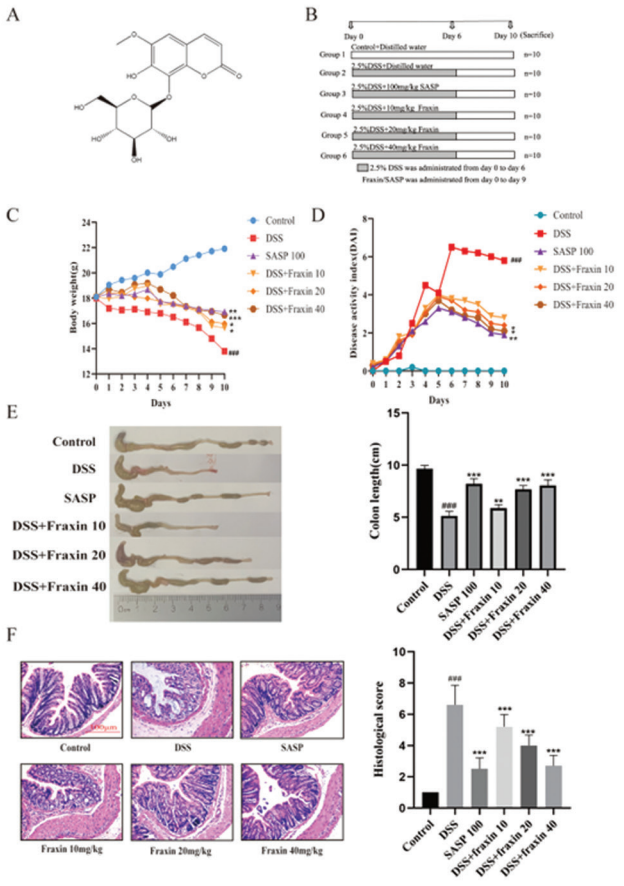
Statistical Analysis

For statistical analysis, GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA) was used to analyze and calculate all data values and expressed as the average of three determinations ± the standard error of the average (SEM). One-way analysis of variance and Dunnett's test were used for the data, and $P < .05$ was considered statistically significant.

Table 1. Specifific primers used in real-time PCR analysis.

Genes	Primer	Sequence(5'->3')
IL-6	FW	TGGAAATGAGAAAAGAGTTGTGC
	RV	CCAGTTTGGTAGCATCCATCA
IL-1β	FW	TTCATCTTTGAAGAAGAGCCCAT
	RV	TCGGAGCCTGTAGTGCAGTT
TNF-α	FW	GTGATCGGTCCCCAAAGG
	RV	GGTGGTTTGCTACGACGTG
β-Actin	FW	CGCCACCAGTTCGCCATGGA
	RV	TACAGCCCGGGGAGCATCGT

Figure 1. Fraxin alleviates DSS-induced UC. (A) Chemical structure of Fraxin. (B) Schematic diagram of the animal experimental design. (C) Body weight loss. (D) DAI score. (E) Intestine images and Statistics of colon length in each group. (F) The pathological characters were reduced after Fraxin treatment.



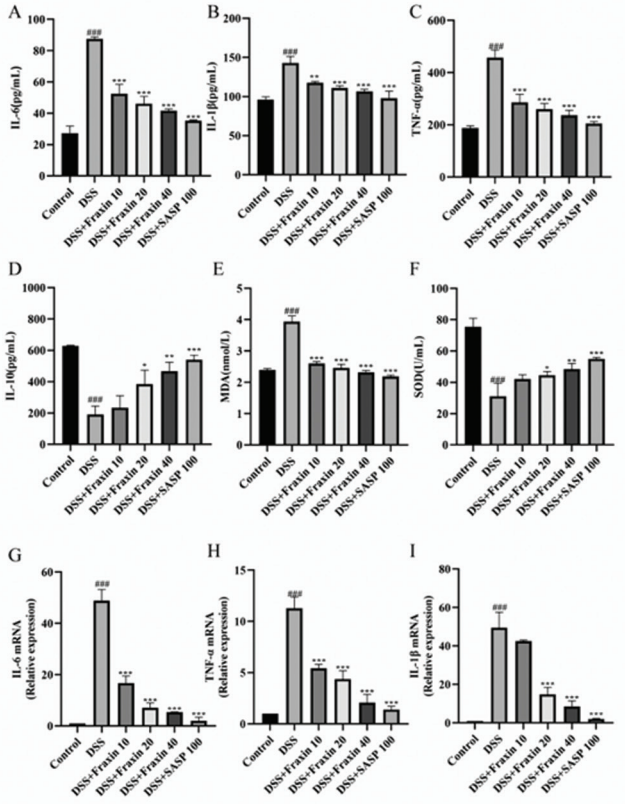
Note: H&E staining and histological colitis score. Data are expressed as mean ± SEM (n=10) $^{###}P < .001$ compared with the control group and $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ compared with the vehicle treated UC model group.

RESULTS

Fraxin alleviated the colonic damage.

It was broadly accepted that DSS elicits grave illness in murine subjects, hallmarked by substantial weight diminution, conspicuous diarrhea/loose excrement, and discernible fecal blood, as adjudged by the DAI.[19] With SASP as the positive control, the animal experiment design diagram is shown in Figure 1B. The results exhibited that disease advancement was attenuated in the SASP cohort and Fraxin group relative to the DSS group, concurrent with reduced weight diminution,

Figure 2. Effects of Fraxin on colon cytokines in DSS-treated mice. Expression levels of (A) IL-6, (B) IL-1β (C) TNF-α and (D) IL-10 in the colon of acute colitis model mice were determined by commercial ELISA kits. (E–F) The activity of SOD and the amount of MDA were determined in colon tissues of mice using commercial ELISA kits.



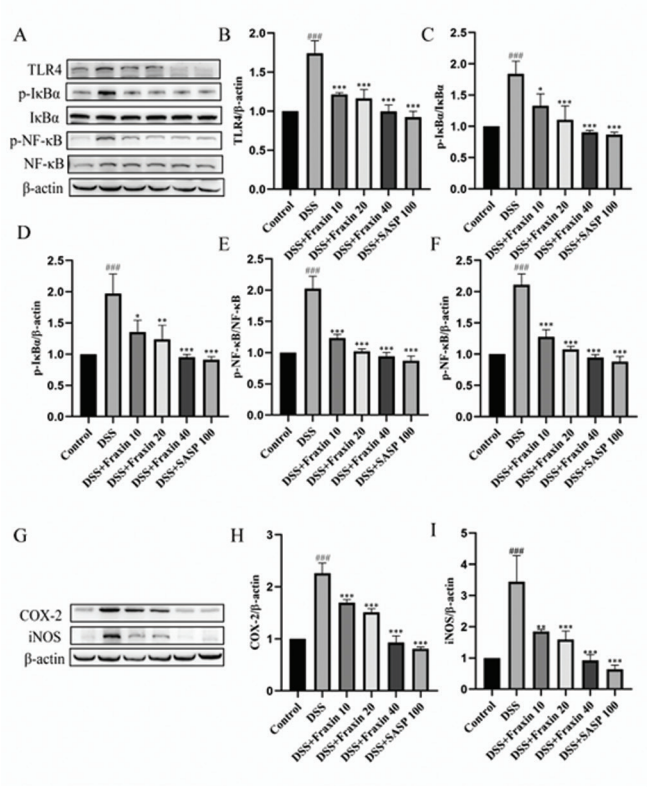
Note: Data are expressed as mean ± SEM (n=10), $^{###}P < .001$ compared with the control group and $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ compared with the vehicle treated UC model group. Expression levels of (G) IL-6, (H) TNF-α and (I) IL-1β in the colon of acute colitis model mice determined by RT-PCR. Data are expressed as mean ± SEM (n=10), $^{###}P < .001$ compared with the control group and $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ compared with the vehicle treated UC model group.

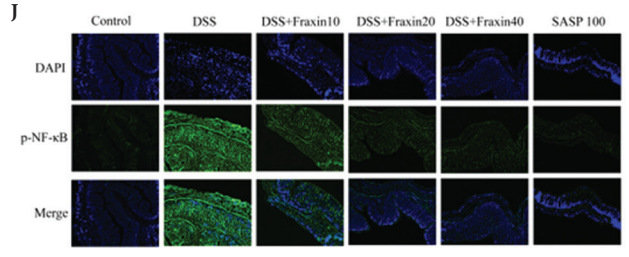
decreased DAI, and ameliorated colon length (Figure 1C-E). H&E stained histological analysis was employed to confirm the protective effect of Fraxin on colitis. The histological features of DSS-treated mice included partial defect of mucosal epithelium, disordered or disappeared gland structure, a great number of inflammatory cells, cell infiltration and cryptitis, goblet cell reduction, and ulcer formation. Fraxin treatment significantly alleviated these pathological characteristics (Figure 1F). All of these parameters were attenuated in a dose-dependent fashion. These findings indicate that Fraxin can ameliorate DSS-induced ulcerative colitis in mice.

Fraxin negated the secretion of pro-inflammatory cytokines and antioxidant suppression elicited by DSS.

While DSS treatment markedly elevated IL-6, IL-1β, and TNF-α and reduced IL-10 levels in colon tissue, Fraxin treatment significantly showed the opposite changes (Figure 2A-D). Notably, Fraxin (40 mg/kg) nearly restored the

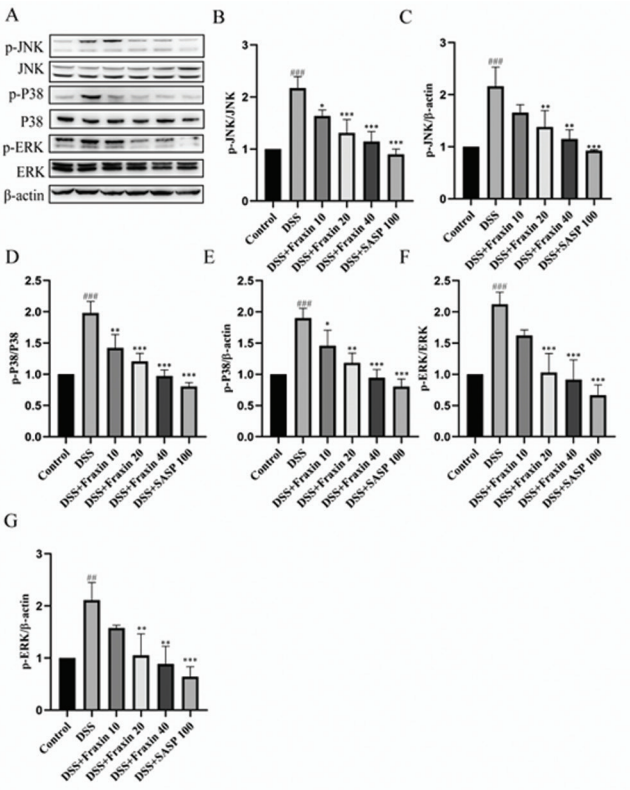
Figure 3. Fraxin inhibits the activation of TLR4/NF-κB signaling pathway in DSS-induced UC mice. (A) Western blots and related quantifification of (B) TLR4, (C-D) p-IκBα and (E-F) p-NF-κB expressed as percent of control. (G) Western blots and related quantifification of (H) COX-2 and (I) iNOS expressed as percent of control. (J) Colon sections were stained with DAPI (blue), p-NF-κB (green) and observed under a flfluorescence microscope.





Note: All data are presented as mean values ± SEM for n = 3. $^{###}P < .001$ compared with the control group and $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ compared with the vehicle-treated UC model group.

Figure 4. Fraxin inhibits the activation of MAPK signaling pathway in DSS-induced UC mice. (A) Western blots and related quantifification of (B-C) p-JNK, (D-E) p-P38 and (F-G) p-ERK expressed as percent of control.



Note: All data are presented as mean values ± SEM for n = 3. $^{##}P < .01$ and $^{###}P < .001$ compared with the control group and $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ compared with the vehicle-treated UC model group.

secretion of these three proinflammatory cytokines to normal levels. In addition, some representative oxidative stress products and antioxidant factors, such as MDA and SOD respectively, were also detected in this section. Relative to the control group, colonic tissue MDA content was markedly elevated, and SOD levels were substantially decreased in DSS-treated mice. However, Fraxin treatment significantly reversed this trend (Figure 2E-F). There was a similar trend in mRNA expression in colon tissue (Figure 2G-I). These findings indicate that Fraxin can suppress pro-inflammatory cytokine release and boost antioxidant defense against colitis.

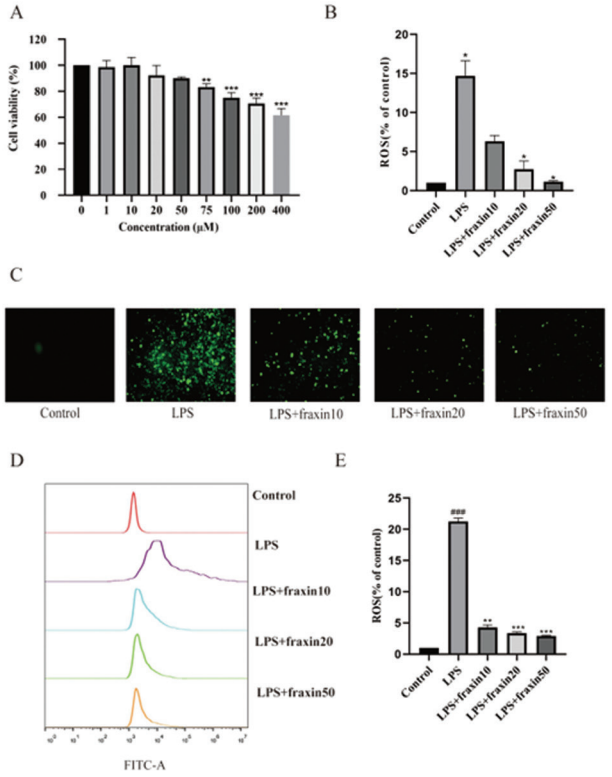
Fraxin inhibited inflammation in mice

Inflammation drives the pathogenesis of ulcerative colitis. TLR4 functions as an activator of signaling cascades, triggering NF-κB and MAPK activation and pro-inflammatory responses.[20] Immunoblotting experiments were conducted to gain a deeper understanding of the mechanisms behind Fraxin's anti-inflammatory effects in mice. As exhibited, DSS administration markedly upregulated the expression of p-NF-κB, TLR4 and p-IκB in the NF-κB signaling cascade and p-P38, p-ERK and p-JNK in the MAPK signaling pathway (Figure 3A-F and Figure 4). The expression profile of inflammatory effectors mirrored the stimulation of the NF-κB signaling cascade (Figure 3G-I). Compared with the DSS group, SASP and Fraxin treatment markedly decreased the phosphorylation levels of these target proteins and inhibited TLR4/NF-κB and MAPK signaling pathway activation (Figure 3-4). In addition, immunofluorescence staining revealed that the fluorescence intensity of p-NF-κB in DSS-induced mouse colon tissue was more pronounced, whereas the fluorescence intensity in both the Fraxin and SASP groups was diminished (Figure 3J).

Toxicity of Fraxin on RAW264. 7 cells

To determine the appropriate Fraxin concentration to use *in vitro*, we first examined the cytotoxicity of Fraxin on

Figure 5. Effects of Fraxin on cell viability and ROS production in LPS-stimulated RAW264. 7 cells. (A) Effect of Fraxin on RAW264. 7 cell viability. (B-C) Intracellular ROS were detected by DCFH-DA fluorescence method. (D-E) ROS production was detected using flow cytometry.



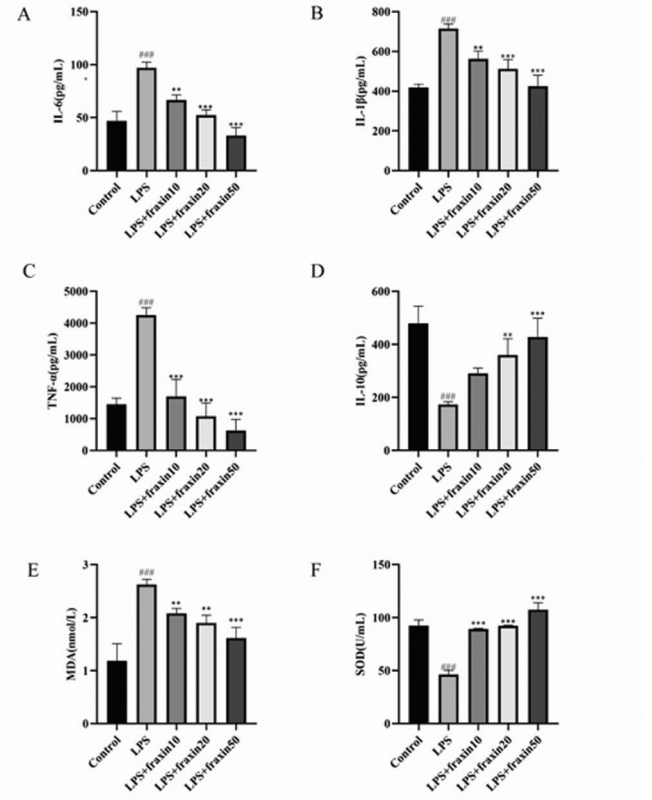
Note: All data are presented as mean ± SD standard deviation of three replicate experiments. $^{\#}P < .05$, $^{\#\#\#}P < .001$ compared with the control group and $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ compared with LPS group.

RAW264. 7 cells. When Fraxin concentrations ranged from 1 to 50 μM, Pretreatment with Fraxin showed no notable effect on cell viability relative to the control group. At concentrations exceeding 50 μM, Fraxin markedly suppressed cell survival ($P < .01$). The cell viability findings delineate the safe concentration range. Therefore, in subsequent experiments, selected concentrations of 10, 20 and 50 μM were used to examine Fraxin on LPS-stimulated RAW264. 7 cells (Figure 5A).

Fraxin efficaciously diminished ROS generation in LPS-induced RAW264. 7 cells.

Oxidative stress, marked by increased intracellular reactive oxygen species levels, is considered a key mediator of inflammation.[21] Fraxin significantly reduced the fluorescence intensity compared with the LPS group (Figure 5B-C). LPS (1 μg/ml, 24 h) provoked excessive ROS generation, which was suppressed by Fraxin treatment. (Figure 5D-E). These findings reaffirmed that Fraxin could counteract the LPS-induced elevation in reactive oxygen species.

Figure 6. Effects of Fraxin on colon cytokines in LPS-stimulated RAW 264. 7 cells. RAW 264. 7 cells were pretreated with the indicated concentrations of Fraxin for 1 h and then stimulated with LPS (1 μg/mL) for 24 h. Expression levels of (A) IL-6, (B) IL-1β, (C) TNF-α and (D) IL-10 in cell culture supernatants were determined by commercial ELISA kits. (E–F) The activity of SOD and the amount of MDA were determined in cell culture supernatants using commercial ELISA kits.



Note: Data are expressed as mean ± SEM (n = 3 independent experiments), $^{\#\#\#}P < .001$ compared with the control group and $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ compared with LPS group.

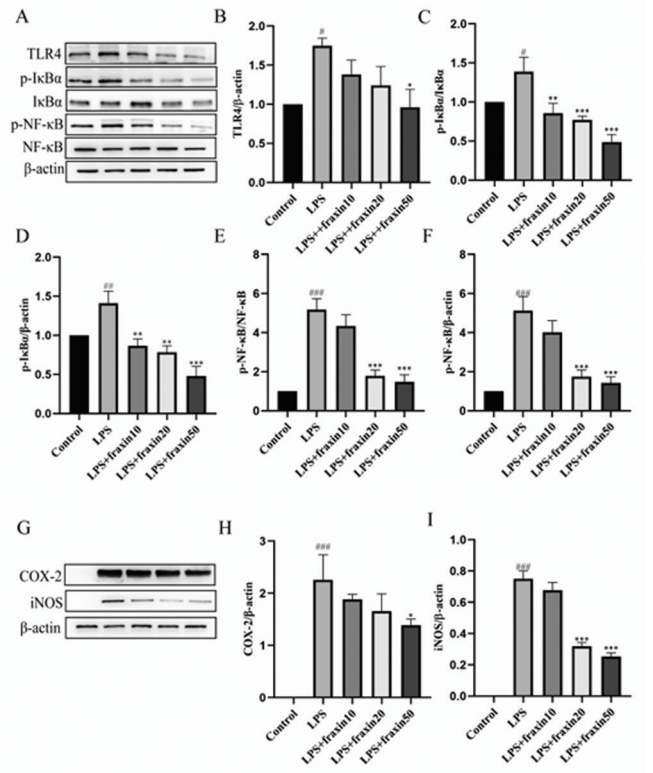
Fraxin attenuated the generation of pro-inflammatory cytokines and potentiated antioxidant activity in LPS-induced RAW264. 7 cells.

As hypothesized, ELISA outcomes displayed a dose-dependent decrease in IL-1β, IL-6, and TNF-α concentrations concurrent with an elevation in IL-10 ensuing Fraxin treatment in the supernatants of RAW264. 7 cells (Figure 6A-D). We also detected the oxidative stress products and antioxidant factors, such as MDA and SOD. MDA decreased, and SOD increased after Fraxin treatment (Figure 6E-F).

Fraxin inhibits LPS-induced activation of TLR4/NF-κB and MAPK signaling pathways in RAW264. 7 cells

To delve deeper into the modulation of these pathways by Fraxin, LPS-stimulated RAW264. 7 cells were examined. We performed immunoblotting experiments similar to the findings. (Figure 7). In addition, Fraxin markedly suppressed the activation of the MAPK signaling cascade and the phosphorylation of JNK, p38, and ERK (Figure 8).

Figure 7. Fraxin inhibits the activation of TLR4/NF-κB signaling pathway in LPS-stimulated RAW 264. 7 cells. RAW 264. 7 cells were pretreated with the indicated concentrations of Fraxin for 1 h and then stimulated with LPS (1 μg/mL) for 24 h. (A) Western blots and related quantifification of (B) TLR4, (C-D) p-IκBα and (E-F) p-NF-κB expressed as percent of control. (G) Western blots and related quantifification of (H) COX-2 and (I) iNOS expressed as percent of control.

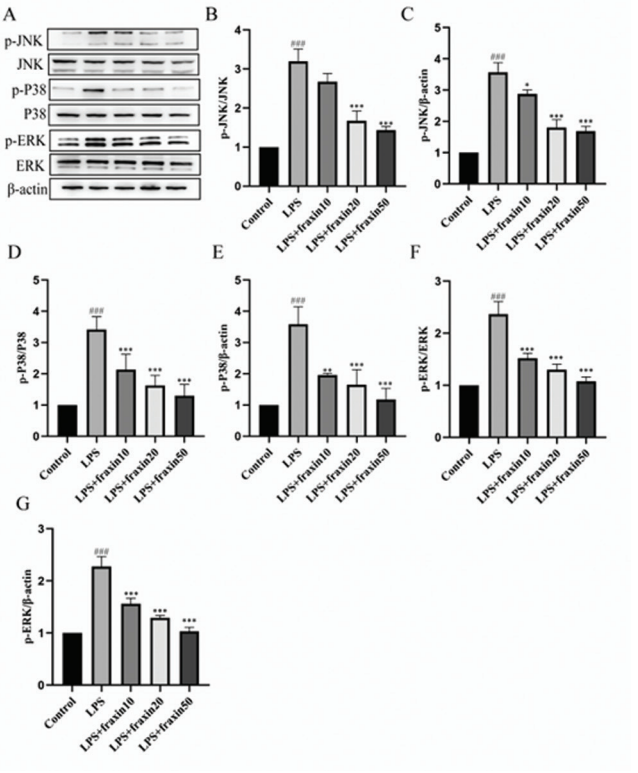


Note: All data are presented as mean values ± SEM for n = 3. $^{\#}P < .05$, $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ compared with the control group and $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ compared with LPS group.

DISCUSSION

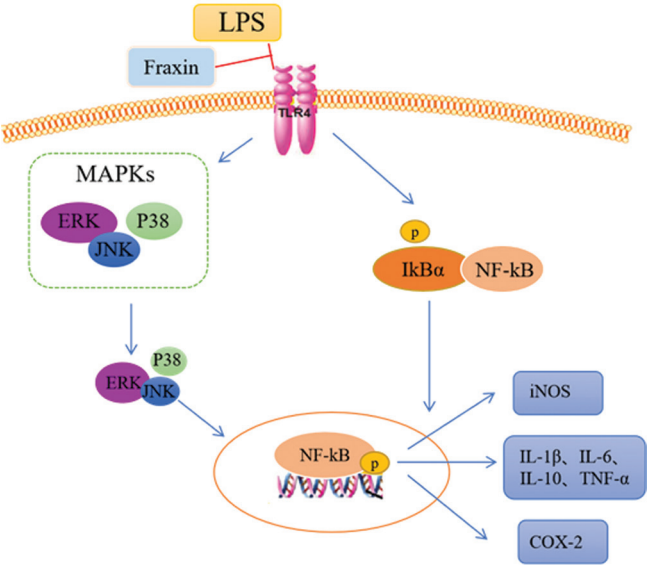
UC is known for its high prevalence around the world. At present, the treatment drugs for UC mainly focus on aminosalicylic acid preparations, glucocorticoids, immunosuppressants, etc. Some patients may experience allergies, gastrointestinal adverse reactions, etc. after taking such drugs. For such patients, finding drugs with high safety and minimal side effects has become a future trend. Therefore,finding natural anti-inflammatory products in Chinese herbal medicine is an attractive and safe alternative to traditional methods of regulating inflammatory diseases. Fraxin is the active ingredient in Qinpi, which is used to treat infections and inflammations.[22] The UC model is established by DSS, a classical method widely used to simulate the pathophysiological process of UC with high operability. In this experiment, We assessed Fraxin's therapeutic efficacy against DSS-induced murine experimental colitis. Experimental results showed that Fraxin could alleviate colon damage and reduce intestinal inflammation, such as weight loss, shortening of colon length, mucosal damage, and

Figure 8. Fraxin inhibits the activation of MAPK signaling pathway in LPS-stimulated RAW 264. 7 cells. Western blots and related quantifification of (B-C) p-JNK, (D-E) p-P38 and (F-G) p-ERK expressed as percent of control.



Note: All data are presented as mean values ± SEM for n = 3. $^{\#\#}P < .01$ and $^{\#\#\#}P < .001$ compared with the control group and $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ compared with LPS group.

Figure 9. The mechanism of action of Fraxin in a mouse model of colitis. Fraxin can simultaneously inhibit the activation of NF-κB and MAPK, thereby reducing the protein and mRNA levels of pro-inflammatory cytokines and relieving colitis.



ulceration. At the same time, Fraxin effectively mitigated the release of pro-inflammatory mediators while bolstering antioxidant defenses. *In vitro*, we found that Fraxin reduced ROS production, decreased MDA, and increased SOD,and decreased proinflammatory cytokine production in LPS-induced RAW264.7 cells. Furthermore, Fraxin suppressed the expression of p-IκBα, TLR4, p-NF-κB and pMAPKs in mice and macrophages, suggesting that Fraxin may inhibit DSS-induced ulcerative colon in mice eand LPS-induced macrophage inflammation by downregulating the phosphorylation of essential cytokines in the TLR4/NF-κB and MAPK signaling pathways. (Figure 9).

Current research shows that colitis and inflammation are closely related.[23] The proinflammatory cytokines modulate signaling cascades during inflammation.[24] TNF-α is a notable proinflammatory cytokine involved in numerous inflammatory conditions and diseases.[25] IL-6 serves as a key marker for gauging the severity of inflammatory conditions.[26] IL-1β levels are substantially elevated following exogenous antigen stimulation, which can consequently elicit the secretion of pro-inflammatory cytokines.[27] As expected, serum concentrations of the proinflammatory cytokines increased in the colitis set; nevertheless, Fraxin treatment effectively lowered these elevated levels, indicating that Fraxin possesses anti-inflammatory properties. In addition, in LPS-induced RAW264. 7 experiments ELISA assays exhibited that the concentrations of TNF-α, IL-6, and IL-1β declined in a dose-dependent fashion subsequent to Fraxin administration. The IL-10 family of anti-inflammatory factors can promote tissue healing after injury caused by infection or inflammation.[28] In this study, we discovered that Fraxin markedly stimulated the release of IL-10 in the murine serum. Similar results were obtained in LPS-induced macrophages. Therefore, we can deduce that Fraxin ameliorates colitis by modulating the equilibrium between pro- and anti-inflammatory cytokines.

Oxidative stress is another important factor leading to tissue destruction in UC.[29] Previous studies report that Fraxin can alleviate LPS-induced ARDS by downregulating oxidative damage.[13] Fraxin protects against chemically induced liver toxicity by attenuating oxidative stress.[14] We deduce that Fraxin may reduce UC tissue damage by reducing oxidative stress. We first examined the impact of Fraxin on LPS-induced RAW264. 7 cells and found that Fraxin significantly alleviated the LPS-induced intracellular ROS overaccumulation. In addition, we also detected SOD and MDA in the supernatant of colon tissue of DSS-induced UC mice and found that SOD decreased and MDA increased. Fraxin treatment improved SOD activity and decreased MDA levels in a concentration-dependent manner. Similar results were obtained *in vitro*. These provide evidence that Fraxin improves UC through antioxidant effects.

NF-κB is widely recognized for its critical role in regulating inflammatory responses, regulating the expression of various cytokines involved in inflammation.[30] Additionally, TLR4 is crucial for upstream signaling proteins involved in NF-κB activation.[31] In the inactive state, the translocation of NF-κB is usually hidden in the cytoplasm by the inhibitory protein IκBα. Upon activation, NF-κB undergoes translocation to the nucleus, facilitated by the phosphorylation and rapid degradation of its inhibitory protein, IκBα, activating downstream gene transcription, such as inflammatory genes (expression of iNOS and COX-2).[32,33] Therefore, we focus on the regulation of Fraxin on TLR4/NF-κB signaling pathway. DSS markedly increased the expression of TLR4, p-NF-κB, and p-IκB, which in turn increased the levels of iNOS and COX-2. Fraxin treatment markedly decreased TLR4 expression, p-NF-κB, and p-IκB, as well as iNOS and COX-2. Paralleling the in vivo findings, Fraxin also suppressed TLR4 expression in vitro, p-NF-κB, and p-IκB in LPS-stimulated RAW264. 7 cells. Consequently, it can be deduced that the suppression of TLR4/NF-κB signaling pathway activation may contribute to some of the therapeutic effects of Fraxin in UC.

Likewise, MAPK activation is important in inflammatory responses.[34] Activation of TLR4 stimulates a family of MAPKs and the MAPK signaling pathway appears to play a considerable regulatory role in inflammatory responses through its three major proteins, ERK, p38, and JNK.[35] Furthermore, MAPKs modulate the synthesis of proinflammatory cytokines TNF-α, IL-1β, and IL-6, in addition to the anti-inflammatory cytokine IL-10.[36] In our investigation, Fraxin effectively hindered the phosphorylation of p38, JNK, and ERK both in vivo and in vitro. These findings suggest that Fraxin's anti-inflammatory properties could be mediated, in part, by the inactivation of MAPK signaling pathways.

CONCLUSION

In summation, our study shows that Fraxin can ameliorate LPS-induced inflammation in macrophages and in experimental animal models of UC. Surprisingly, Fraxin treatment downregulates phosphorylation of key cytokines in the TLR4/NF-κB and MAPK signaling pathways, suppressing further inflammation. Meanwhile, Fraxin treatment can improve oxidative stress. Our study may provide a new insight into Fraxin as a prospective therapeutic for UC treatment. There is currently no clear explanation regarding the safety and toxicity, biological mechanism, and use of Fraxin. However, Fraxin's potential has broad prospects and further research is needed.

DATA AVAILABILITY

The data used to support the findings of this study are available from the corresponding author upon request.

ETHICAL COMPLIANCE

This study was approved by the Animal Ethics Committee of Zhangjiagang TCM Hospital, Affiliated to Nanjing University of Chinese Medicine Animal Center.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

WS, LH, ZM, and XW designed the study and performed the experiments, LC, YQ and YZ collected the data and analyzed the data, WS, LH, ZM and XW prepared the manuscript. All authors read and approved the final manuscript. WS and LH contributed equally to this work.

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