SPANDIDOS PUBLICATIONS

Dracocephalum moldavica L. extract alleviates experimental colitis in rats by modulating gut microbiome and inflammatory pathways

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Received April 4, 2023; Accepted September 26, 2023

DOI: 10.3892/mmr.2023.13115

Abstract. Several studies have revealed that an imbalance of the intestinal microbiota is involved in intestinal inflammation associated with ulcerative colitis (UC). Therefore, regulating the homeostasis of gut microbiota is critical for treating UC. *Dracocephalum moldavica L.* (DML) extract, a common traditional Chinese medicine, has been demonstrated to possess numerous pharmacological effects, such as antioxidative, anti-inflammatory, and antibacterial properties. The aim of the present study was to evaluate the beneficial effects of DML extract and the probable mechanism of action in a dextran sulfate sodium-induced chronic colitis model. It was found that DML extract ameliorated UC by improving disease activity index, weight loss, colon length, and histological scoring. DML extract administration also enhanced the count of *Lactobacillus* and reduced the count of *Romboutsia*. Furthermore, the results of network pharmacology analysis revealed that the active ingredients (including luteolin, rosmarinic acid, oleanolic acid, ursolic acid, apigenin, acacetin, kaempferol, and isorhamnetin) in the DML extract were closely associated with anti-inflammatory activity via various signaling pathways, including the NF-κB, IL-17, TNF, and Toll-like receptor (TLR) signaling pathways. Western blot analysis further indicated that DML extract downregulated the expression of members of the TLR4/NF-κB signaling pathway, which was associated with colitis. Thus, it was hypothesized that DML extract exerted its anti-colitis effects by modulating the gut microbiota and inflammatory pathways.

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Key words: Dracocephalum moldavica L., ulcerative colitis, gut microbiota, inflammation

Introduction

Ulcerative colitis (UC) is a chronic inflammatory bowel disease, which causes numerous sporadic symptoms, including abdominal pain, diarrhea, and bloody mucopurulent stool (1). The pathogenesis of UC, which is generally considered to be influenced by multiple factors, is not clearly understood.

The human microbiota comprises a wide array of microorganisms (bacteria, viruses, protozoa, archaea, and fungi), which are integral in multiple physiological processes of the host (2). Microbial dysbiosis is defined as a disturbance in the composition of the microbiome. A state of imbalance in the human microbiota is often caused by infection, inflammation, or immunity (3). Dysbiosis of the gut microbiome has been reported in various inflammatory and immune-related diseases. To date, several studies have focused on the role of the gut microbiota in UC development (4-6). Therefore, targeting the gut microbiota may be a potential treatment for UC.

Recent developments in sequencing technologies and bioinformatics have enabled researchers to explore the mechanisms underlying the gut microbiota-mediated impact on the progress of diseases, leading to developments in novel therapeutics, such as prebiotics, probiotics, fecal transplantation, and drugs (7). Previous studies have focused on the role of traditional Chinese medicines (TCMs) in modulating gut microbiota. Various studies have indicated that TCM can modulate the composition and metabolism of the gut microbiota via direct and indirect actions (8,9). *Dracocephalum moldavica L.* (DML), a traditional ethnic medicine, possesses a wide range of pharmacological properties such as antioxidative, cardioprotective, anti-inflammatory, and antimicrobial activities (10-12). As DML contains a range of biologically active compounds that belong to different chemical classes such as flavonoids, steroids, glycosides, saponins, tannins, phenols, and essential oils (13), it was speculated that it may regulate the composition and metabolism of gut microbiota to achieve its effects. Thus, the present study was designed to explore the anti-colitis effect of DML in a dextran sulfate sodium (DSS)-induced colitis model. Moreover, the mechanisms by

which DML exerted its beneficial effects on this disease were also investigated by focusing on gut microbiota and inflammatory pathways.

Materials and methods

Animals. A total of 24 male Sprague-Dawley rats (5-6 weeks old, weighing 140-180 g) [Beijing Vital River Laboratory Animal Technology Co., Ltd.; animal certificate no. SCXK (Jing) 2016-0006] were housed individually in a ventilated cage, with free access to food and water, at 23.0±2.0˚C and a 12/12-h light/dark cycle. Animals were acclimated to the laboratory environment for 1 week before the experiments. The animal experiments were approved by the Baotou Medical College Research and Ethics Review Committee (approval no. 2021040).

Drug. The aerial parts of DML were obtained in the city of Tongliao (Inner Mongolia Autonomous Region, China). A voucher specimen (IMKLDRB-2022-01) was deposited in Inner Mongolia Key Laboratory of Disease-Related Biomarkers, Baotou Medical College (Baotou, Inner Mongolia, China). The samples were air-dried before being ground into a fine powder. Next, DML extracts were prepared as previously described (11). Briefly, the plant powder was subjected to extraction twice with 65% ethanol for 120 min at 60˚C. After removing the ethanol by vacuum distillation, the obtained extracts were separated with ethyl acetate using separating funnels.

Experimental design. After a 1-week of acclimation, animals were randomly allocated to one of three groups (n=8): Control, model, and treatment. The control and model groups received normal drinking water, while the treatment group received DML extract (400 mg/kg) by oral gavage for 5 days. On day 6, 5% DSS dissolved in drinking water was administered to the rats in the model and treatment groups to induce UC. From days 11-15, the control and model groups were administered normal drinking water only, while the treatment group received DML extract at a dose of 400 mg/kg by oral gavage. At the end of the experiment, the rats were euthanized with an overdose of pentobarbital sodium (200 mg/kg) by intraperitoneal injection. Fresh feces were collected and stored at -80˚C for future use. The length of the colon was determined, and then the colon was removed and washed with ice-cold PBS. One part of colon tissue was rapidly frozen in liquid nitrogen and stored at -80˚C until required, and the remaining parts were fixed in 10% formalin for 24 h at room temperature for histological examination.

Evaluation of the disease activity index. The body weight, gross rectal bleeding, and stool consistency of rats were monitored daily. A disease activity index (DAI) score, an indicator of disease activity, was calculated by grading on a scale of 0-4 using the following parameters: weight loss (0, normal; 1, 0-5%; 2, 5-10%; 3, 10-20%; 4, >20%), stool consistency (0, normal; 2, loose stools; 4, watery diarrhea) and the occurrence of gross blood in the stool (0, negative; 4, positive). DAI was determined by averaging numerical scores of weight loss, stool consistency and bleeding.

Histological analysis. The rat colon was fixed in 10% buffered formalin for 24 h at room temperature, embedded in paraffin, and stained with hematoxylin and eosin (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Each sample was assessed under light microscopy at x200 magnification (Nikon Corporation).

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted from frozen tissue using an RNAprep Pure Tissue Kit according to the manufacturer's protocol (Tiangen Biotech Co., Ltd.). RNA concentration and quality were evaluated using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). First-strand cDNA was performed on total RNA using a Prime Script RT MasterMix according to the manufacturer's protocol (Takara Bio, Inc.). The mRNA levels of IL-17 and TNF-α were measured by qPCR analysis using TB Green Premix Ex Taq II (Takara Bio, Inc.). Each sample was assayed in triplicate on an ABI PRISM 7500 System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermal cycler parameters were as follows: Denaturation at 95˚C for 30 sec; followed by 40 cycles of denaturation at 95˚C for 5 sec and combined annealing/extension at 60˚C for 30 sec. The relative quantification of genes was performed using the $2^{-\Delta\Delta Cq}$ method, with β-actin as an internal reference for normalization. The sequences of primers are listed in Table SI (14).

ELISA. Colon tissues were homogenized in ice-cold PBS. The homogenates were centrifuged at 3,000 x g for 10 min at 4˚C and the supernatants were then assayed to evaluate the levels of IL-17 (cat. no. M17F0) and TNF-α (cat. no. RTA00) using ELISA kits according to the manufacturer's protocols (R&D Systems, Inc.).

Fecal DNA extraction, metagenomic sequencing, and analysis. MoBio Power Fecal DNA Isolation kit (MO BIO Laboratories, Inc.) was used for DNA extraction according to the manufacturer's instructions. The quality of the extracted DNA was measured using 1% agarose gel electrophoresis (Beijing Solarbio Science & Technology Co., Ltd.). DNA libraries were constructed using TruSeq DNA LT Sample Prep Kit v2 according to the manufacturer's protocol (Illumina, Inc.). Metagenomic sequencing was performed on a HiSeq 3000 platform (Illumina, Inc.). Bioinformatics analysis was performed as previously described (15).

Network pharmacology analysis. Network pharmacology analysis was performed as previously described (16). Briefly, active ingredients of DML were collected from the PubChem database (pubchem.ncbi.nlm.nih.gov). The potential UC-related targets were searched in the Human Gene Database (GeneCards, https://www.genecards.org/) using the search term 'ulcerative colitis'. The potential protein targets of active ingredients and UC-related targets were imported into a Venn diagram web tool (https://bioinfogp.cnb.csic.es/tools/venny/), and common targets were identified as UC-related targets of the active ingredients (Table SII) for further investigation. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (17) was performed using the Database for

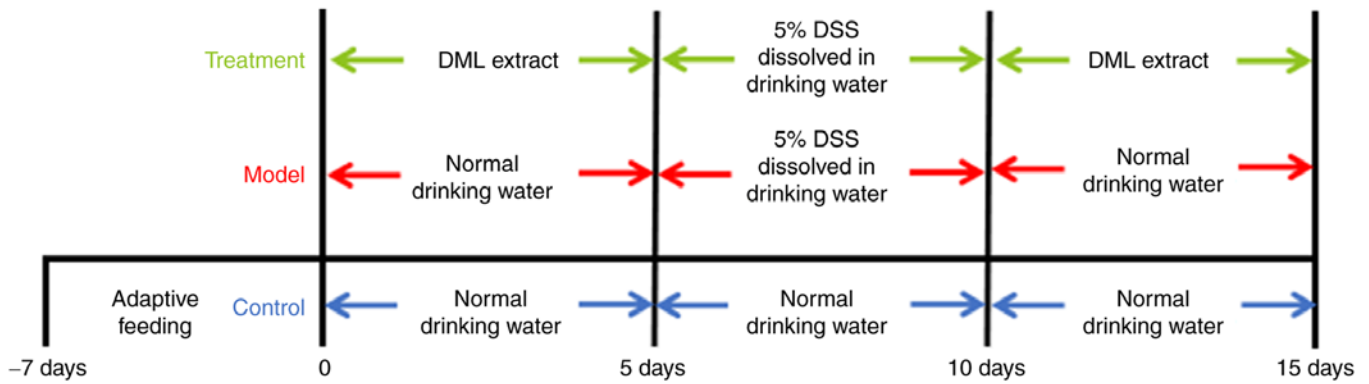

Figure 1. Experimental design to evaluate the effects of *Dracocephalum moldavica L.* extract on dextran sulfate sodium-induced rats.

Figure 2. *Dracocephalum moldavica L.* extract attenuates dextran sulfate sodium-induced colitis in rats. (A) Body weight of rats. (B) Disease activity index of rats. (C) Representative images of colons (ruler numbers represent cm). Data are presented as the mean ± SD (n=8). *P<0.05 vs. control; #P<0.05 vs. model.

Annotation, Visualization and Integrated Discovery database (https://david.ncifcrf.gov/) (18,19).

Western blotting. Colon tissue protein was extracted using Pierce™ RIPA Buffer (Thermo Fisher Scientific, Inc.). The protein concentration was measured using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). Total protein (60 μg) was separated by 10% SDS-PAGE. After transferring the proteins onto PVDF membranes (MilliporeSigma), the membranes were blocked with 5% non-fat milk and incubated overnight at 4˚C with antibodies against Toll-like receptor (TLR)4 (cat. no. ab217274, 1:1,000, Abcam), MyD88 (cat. no. ab219413, 1:1,000, Abcam), NF-κB p65 (cat. no. ab16502, 1:1,000, Abcam), phospho-NF-κB p65 (cat. no. ab76302, 1:1,000, Abcam), and GAPDH (cat. no. ab313650, 1:2,500, Abcam). After washing 5 times in TBS containing Tween 20, each membrane was subsequently incubated with HPR-labeled IgG (cat. no. ab6721, 1:10,000, Abcam) at room temperature

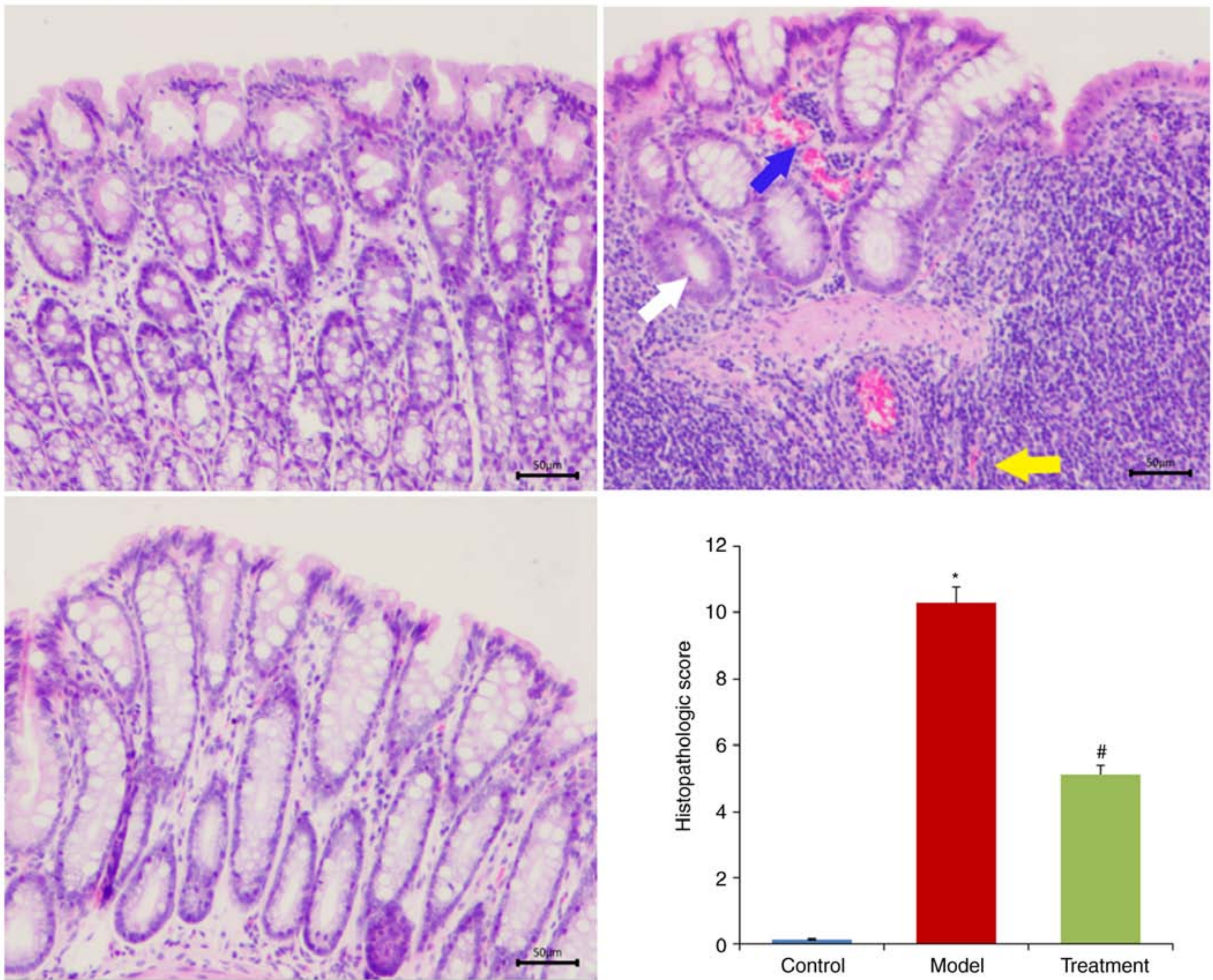

Figure 3. Representative images of hematoxylin and eosin-stained colonic tissue (scale bar, 50 μm) and histological scores. The white arrow indicates destruction of the crypt structure; the yellow arrow indicates inflammatory cell infiltration; the blue arrow indicates loss of goblet cells. Data are presented as the mean ± SD (n=8). *P<0.05 vs. control; $^{\#}$P<0.05 vs. model.

for 2 h. Signals were visualized using an enhanced chemiluminescence reagent (Bio-Rad Laboratories, Inc.). The ratio of the gray value of TLR4 and MyD88 to GAPDH, or the ratio of the gray value of phospho-NF-κB p65 to NF-κB p65, was analyzed using Image Lab software version 6.1.0 (Bio-Rad Laboratories, Inc.).

Statistical analysis. All data were analyzed using SPSS 22.0 software (IBM Corp.). After performing a normality test, statistical significance was evaluated using an unpaired Student's t-test or one-way ANOVA followed by a Student-Newman-Keul's test. Data are presented as the mean ± SD. P<0.05 was considered to indicate a statistically significant difference.

Results

DML extract administration attenuates DSS-induced rat colitis. The experimental design to assess the effects of DML extract on DSS-induced rats is shown in Fig. 1. A steady increase in the body weight of all three groups was observed on the first 5 days of the experiment (Fig. 2A). Subsequently, the rats were administered water containing 5% DSS. The model group showed a significant reduction in body weight and an increase in DAI score compared with the control group (P<0.05). The maximum body weight loss was 12.7% at the end of 5 days after DSS administration.

DML extract administration alleviated body weight loss and reduced the DAI score (both P<0.05; Fig. 2A and B). Additionally, DML extract administration significantly attenuated DSS-induced colonic shortening (Fig. 2C). Histological analysis showed that DSS treatment destroyed crypt structure and goblet cells and induced inflammatory cell infiltration. However, DML extract administration attenuated the DSS-induced tissue morphological changes (Fig. 3).

DML extract administration inhibits the expression of TNF-α and IL-17. The relative expression levels of TNF-α and IL-17 were significantly increased in the model group compared with the control group (P<0.05; Fig. 4). By contrast, the increase in

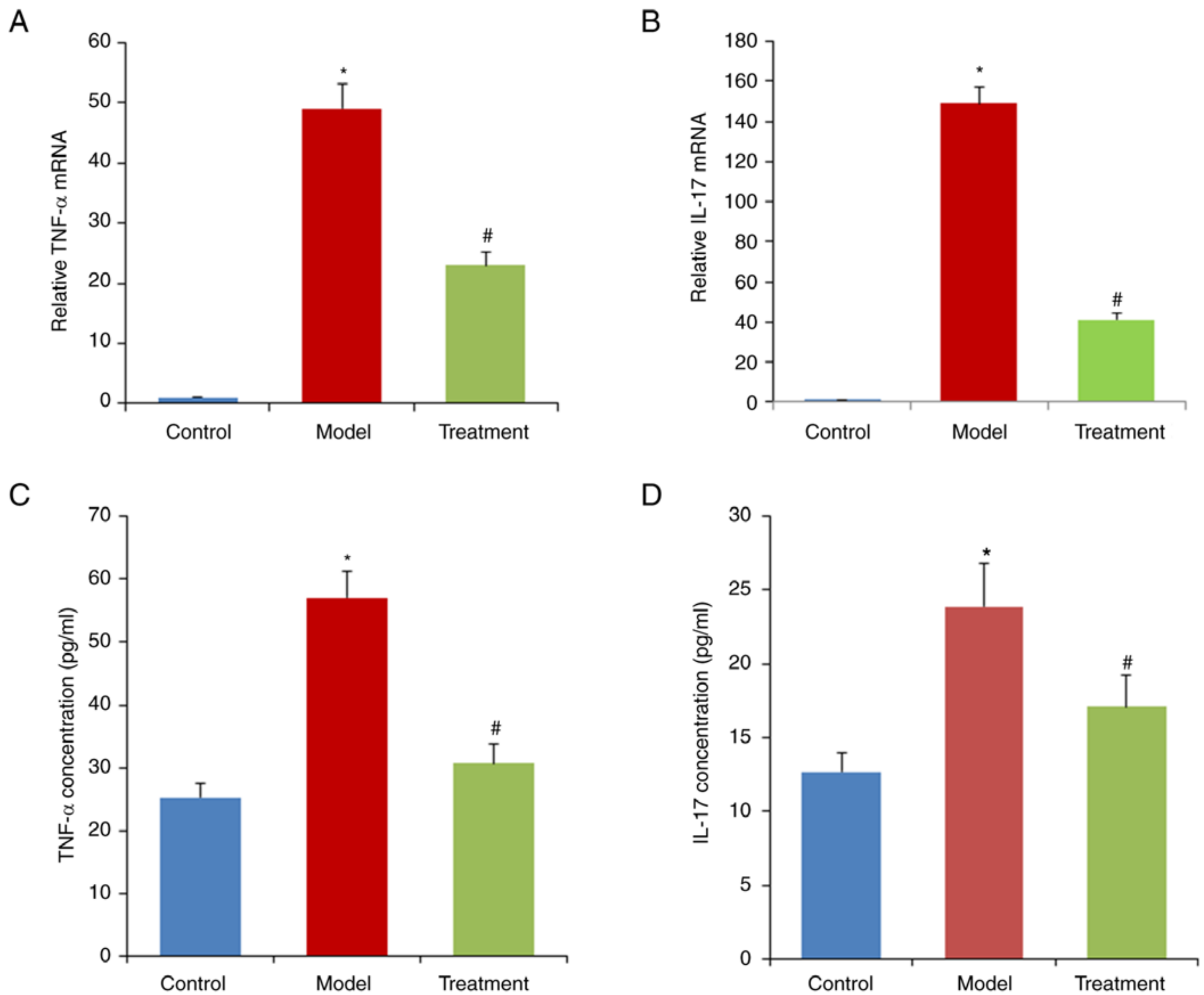

Figure 4. Relative mRNA expression of (A) TNF-α and (B) IL-17 in the colon, as evaluated by reverse transcription-quantitative PCR. The protein levels of (C) TNF-α and (D) IL-17 in the colon, as evaluated by ELISA. Data are presented as the mean ± SD (n=8). *P<0.01 vs. control; #P<0.01 vs. model.

TNF-α and IL-17 induced by DSS was alleviated in the treatment group (P<0.01; Fig. 4).

DML extract administration alters the gut microbiota profile in rats with DSS-induced UC. The results from the species accumulation curve and rank abundance curves indicated that the sequencing depth was sufficient to provide coverage for the majority of microbial species in each sample (Figs. S1 and S2). As shown in Fig. 5A, the dominant phyla were *Firmicutes* and *Actinobacteria*. At the genus level (Fig. 5B), the relative abundance of 4 genera, namely *Romboutsia*, *Lactobacillus*, *Clostridium sensu stricto*, and *Allobaculum*, was enriched in the three groups. Compared with the control group, the model group had an over-representation of *Romboutsia* and a lower abundance of *Lactobacillus* (P<0.05). DML extract administration prevented the decrease in *Lactobacillus* as well as the increase in *Romboutsia* (Fig. 5B).

The linear discriminant analysis (LDA) effect size (LEfSe) analysis showed that 15 taxa were enriched in the model and control groups (Fig. 6A). The control rats primarily showed higher enrichment of *Coriobacteriaceae*, *Olsenella*, *Erysipelotrichia*, *Allobaculum*, *Ruminococcaceae*, *Clostridium_XlVa*, *Turicibacter*, *Erysipelotrichaceae*, *Peptococcaceae*, *Lachnospiraceae*, *Enterorhabdus*, *Erysipelotrichales*, *Adlercreutzia*, *Coriobacteriales*, and *Peptococcus*. Based on the results of LEfSe analysis, taxa with significantly different abundances between the model group and the treatment group were also observed (Fig. 6B). The model group had higher scores for *Salmonella*. However, the proportion of *Erysipelotrichia*, *Clostridium_XlVa*, *Turicibacter*, *Erysipelotrichaceae*, *Lachnospiraceae*, *Erysipelotrichales*, and *Adlercreutzia* in the treatment group was similar to that of the control rats, indicating that the DML extract administration improves the imbalance of intestine microbiota composition in DSS-induced UC rat to a normal-like level.

DML extract administration regulates the inflammatory pathway in rats with DSS-induced UC. Upon analyzing PubChem database, 31 active ingredients of DML were identified. Among them, 16 active ingredients were selected for

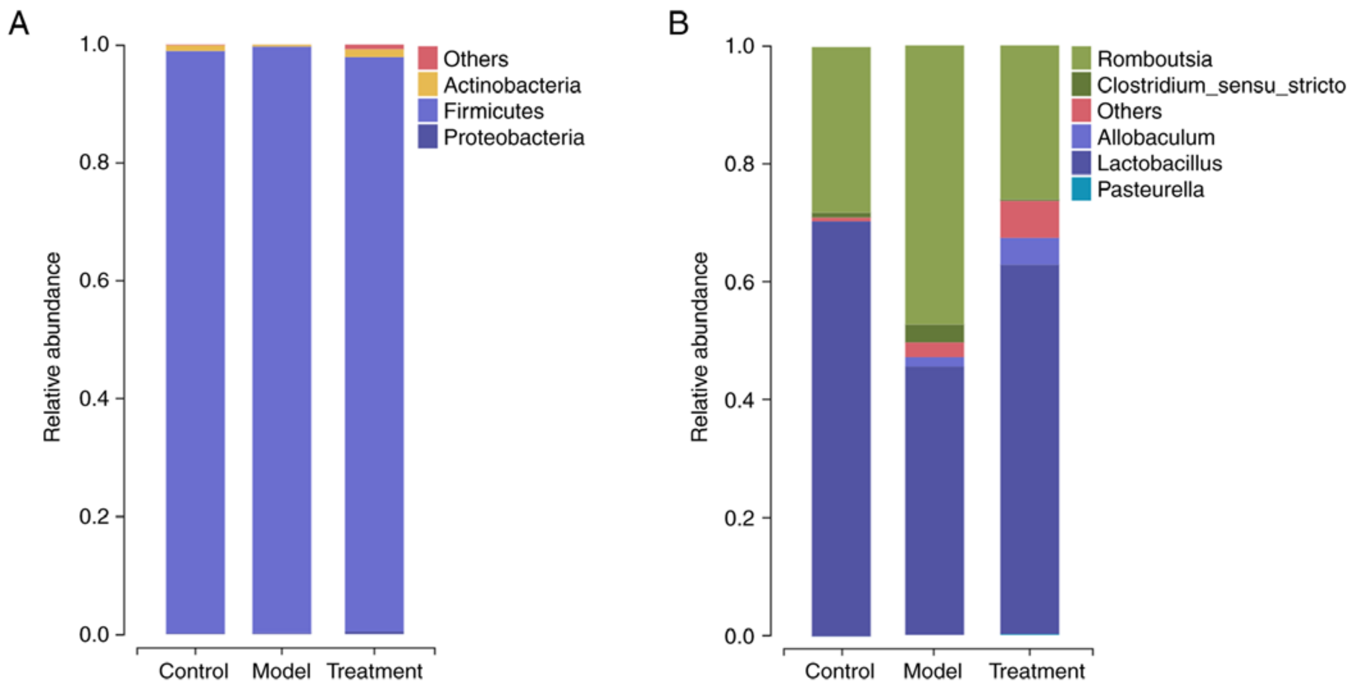

Figure 5. *Dracocephalum moldavica L.* extract restructures the gut microbiota in rats with dextran sulfate sodium-induced ulcerative colitis. Community bar plot analysis at the (A) phylum and (B) genus levels.

identifying potential targets (Table SII). Candidate targets of active ingredients in DML and UC disease were searched. A total of 194 targets related to active components in DML, 7,855 targets related to UC, and 170 targets overlapping between active components in DML and UC were found (Figs. S3 and 4). KEGG annotation showed that the common targets of active components in DML and UC were primarily involved in the inflammatory response and immune regulation, such as the NF-κB, IL-17, TNF, and TLR signaling pathways (Fig. 7). These results suggested that DML extract alleviated UC by controlling pro-inflammatory pathways in response to gut microbiota imbalances.

Furthermore, western blotting was performed to verify the effect of DML extract on the TLR and NF-κB signaling pathways. The results demonstrated that the protein expression levels of TLR4, MyD88, and phosphorylated NF-κB p65 in colon tissues were higher in the model group than in the control group. However, these changes were reversed in the treated group (Fig. 8).

Discussion

The pathogenesis of UC is complex and involves imbalances in the gut microbiota and disorders of immune regulation. DSS-induced animal models of experimental colitis have been widely used for investigating the pathological mechanisms of UC (20-22). Thus, the current study investigated the effect of DML extract on colitis in a rat model. Microbial imbalances were evaluated in the absence and presence of DML extract treatment. Next, network pharmacology analysis was performed to determine the mechanism by which DML extract was involved in UC regulation.

To study the alleviating effect of DML extract on colitis, an experimental colitis model was established in rats by feeding them with 5% DSS. Oral DML extract intervention alleviated DSS-induced colitis, as evidenced by the marked increase in the area of goblet cells, relieved infiltration of inflammatory cells, and decreased the presence of colonic mucosal ulcers. These experimental results indicate that DML extract has a therapeutic effect against DSS-induced UC in rats.

DSS can induce a severe immune response in the intestinal mucosa and can induce the release of a large number of inflammatory mediators such as IL-17 and TNF-α (23). It has been reported that the induction of inflammatory mediators promotes the occurrence and development of UC (24). Therefore, the present study investigated the effects of DML extract on inflammatory factors in the colon of rats with UC induced by DSS. Decreased expression of IL-17 and TNF-α after DML extract treatment was observed. These data indicate that DML extract suppresses inflammatory responses and thereby exerts a protective effect against DSS-induced UC in rats.

The interplay between the gut microbiota and the host immune system plays a crucial role in modulating intestinal function. Multiple studies have shown that natural active products lead to alterations in the intestinal microbiota and improve DSS-induced colitis symptoms (25,26). The current study aimed to determine whether DML extract could affect the gut microbiota in UC rats. Analysis of the gut microbiota indicated that administration of DML extract significantly increased the number of *Lactobacillus*. This is in agreement with another study, which reported that *Lactobacillus* could alleviate DSS-induced UC (27). The current study also showed that the relative abundance of *Romboutsia* was markedly increased after chronic DSS induction. *Romboutsia* has been identified as a potential pathobiont in UC (28). However, DML extract treatment led to higher counts of *Lactobacillus* and lower counts for *Romboutsia*, indicating that DML extract

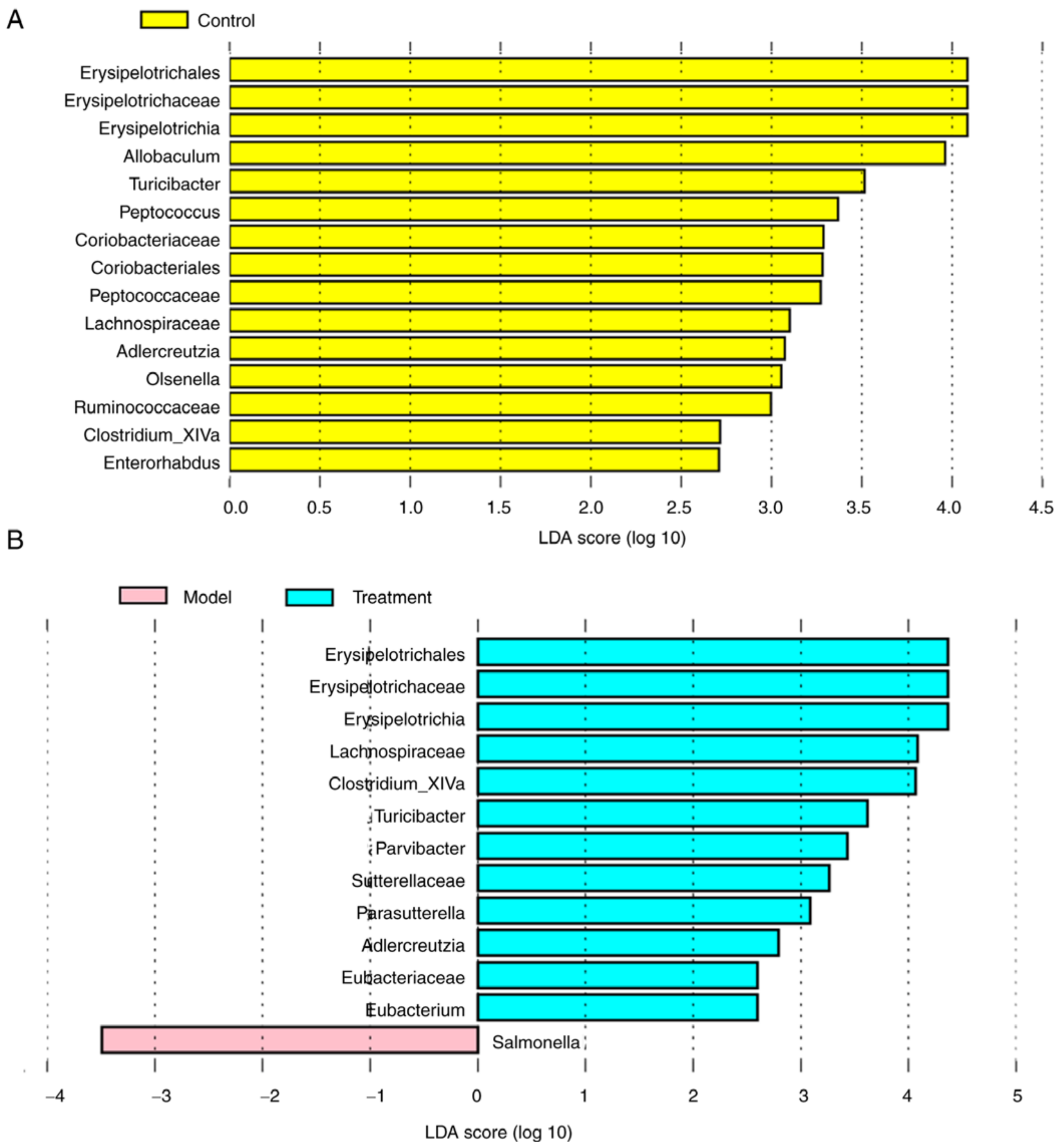

Figure 6. LEfSe analysis of the abundant taxa among the control, model, and treatment groups. (A) LEfSe analysis of gut microbiota in the control and model groups. (B) LEfSe analysis of gut microbiota in the model and treatment groups. (LDA score >2.0; P<0.05). LDA, linear discriminant analysis; LEfSe, LDA effect size.

administration restored the imbalance in the gut microbiota in rats with UC.

The LEfSe algorithm has been widely used to analyze bacteria in different groups. The present study showed that *Salmonella* played an important role in DSS-treated rats. It has been reported that *Salmonella* infection promotes the recurrence of UC (29). It is well established that a variety of *Lactobacilli* can effectively alleviate UC, which is possibly related to the upregulation of short-chain fatty acids (SCFAs) and balancing of profiles of intestinal microbiota (30,31). A previous study showed that *Lactobacillus* reduced the release of inflammatory factors (TNF-α and IL-17) in mice with DSS-induced colitis (32). *Lactobacilli* suppressed the production of inflammatory factors through the TLR4/NF-κB signaling pathway in the colon tissues of mice with UC (33). The current findings showed that DML extract could decrease the levels of TNF-α and IL-17 in rats with colitis. Thus, the involvement of the TLR4/NF-κB pathway in the anti-colitis effects of DML extract was next investigated.

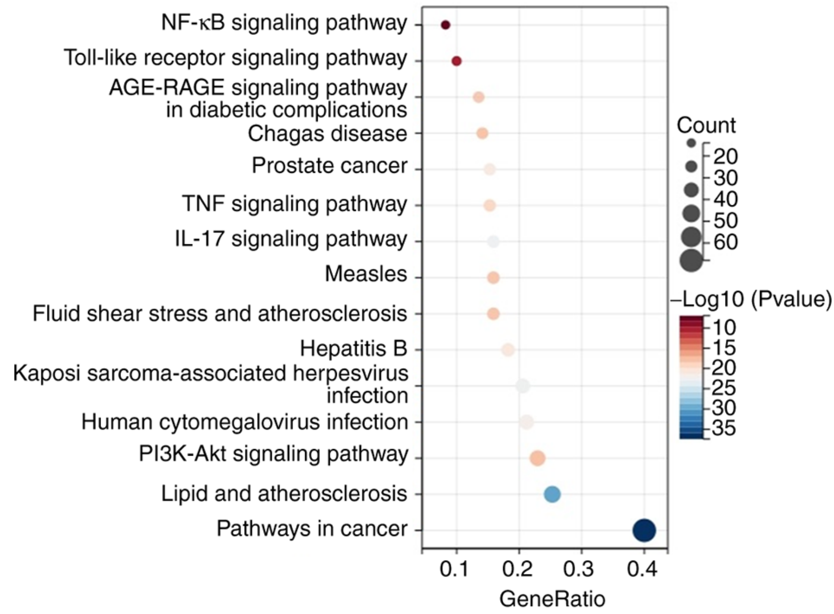

Figure 7. KEGG pathway analysis of potential targets of *Dracocephalum moldavica L*. among ulcerative colitis-related proteins. The KEGG term is indicated as a node, and the size of the node indicates its importance. KEGG, Kyoto Encyclopedia of Genes and Genomes.

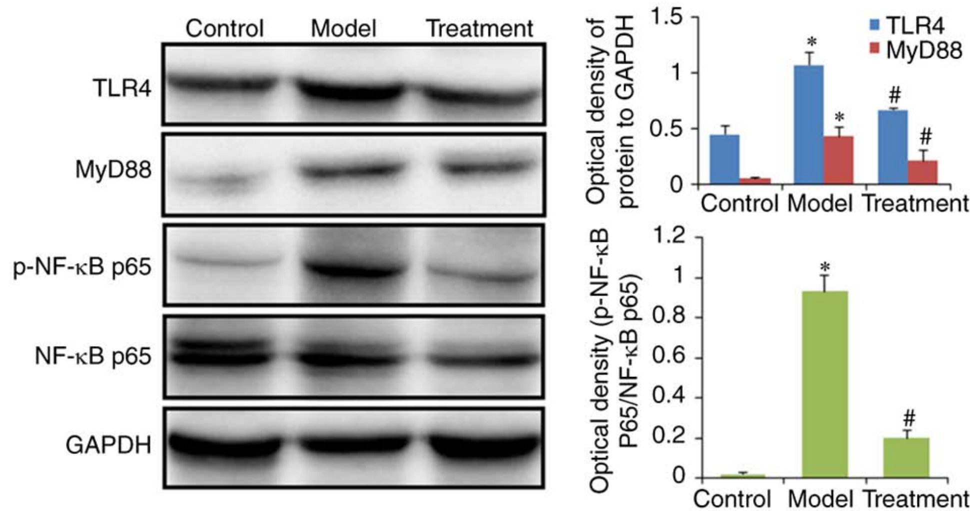

Figure 8. Expression of Toll-like receptor 4, MyD88 and NF-κB in rat colonic tissues. Data are presented as the mean ± SD (n=8). *P<0.05 vs. control; #P<0.05 vs. model.

In the present study, network pharmacology approaches were used to explore the potential mechanism by which DML extract relieves colitis in rats. Ingredients-targets network analysis revealed that luteolin, rosmarinic acid, oleanolic acid, ursolic acid, apigenin, acacetin, kaempferol, isorhamnetin, and other active components could regulate multiple targets. Previous studies have found that these components have potential therapeutic effects on UC (34-40). The findings suggested that these active ingredients may be related to the anti-colitis effects of DML in UC. To explore the mechanism by which DML extract relieves colitis in rats, KEGG pathway enrichment analysis was carried out for the 170 targets of DML with activity in UC. The results revealed pathways associated with UC included the NF-κB, IL-17, TNF, and TLR signaling pathways. TLR4 is a specific receptor for lipopolysaccharides (LPS) derived from Gram-negative bacteria and is a key regulatory factor in colon immune inflammation regulation (41). It has been reported that microbiota and their metabolites can inhibit the TLR4 signaling pathway. Downregulating TLR4 decreased the expression of NF-κB, and reduced the expression and release of inflammatory factors, which led to the alleviation of UC (42). To further verify whether DML extract can affect the expression of TLR4/NF-κB, the expression of TLR4, MyD88, and NF-κB in the colon was examined. The results confirmed that TLR4/NF-κB signaling was downregulated by DML extract. These results agree with a previous report, which demonstrated that DML extract could exert anti-inflammatory effects via downregulation of the NF-κB signaling pathway (43).

Taken together, intestinal microbiota dysbiosis in rats with colitis may result in increased LPS production,

causing intestinal inflammation by activating TLR4 and consequently upregulating cytokine secretion. DML extract reshaped the profile of the gut microbiota and its metabolites (such as SCFAs), thereby suppressing LPS-induced TLR4/NF-κB signaling, as well as the production of the cytokines, TNF-α and IL-17, to alleviate DSS-induced UC damage. In the present study, there was no direct evidence of gut microbiota regulating TLR4/NF-κB signaling. However, the present study showed that gut microbiota can regulate TLR4/NF-κB signaling and the release of inflammatory mediators (44).

In summary, the current results suggest that DML extract may mediate inflammatory regulation through the intestinal flora and TLR4-related signaling pathways to exert its anti-colitis effects. However, the exact mechanism of this effect requires further investigation. The present findings offer alternative treatment strategies for UC by using natural medicines.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant nos. 82060084, 82260092, and 82170296), and the Inner Mongolia Autonomous Region Science and Technology Innovation Guidance Project (grant no. CXYD2021BT02).

Authors' contributions

SG, WB and HY designed the experiments. SG and WB performed the experiments. WB and ZW participated in data curation. HY, ZW, and GA participated in data interpretation and discussion, and writing of the manuscript. All authors reviewed the manuscript. WB and ZW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Availability of data and materials

The data that support the findings of the present study have been deposited into CNGB Sequence Archive of China National GeneBank database with accession no. CNP0003841 (http://db.cngb.org/cnsa/project/CNP0003841_c66117f4/reviewlink/).

Ethics approval and consent to participate

Ethical approval was obtained from Baotou Medical College Research and Ethical Review Committee (approval no. 2021040).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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