

Salvia Miltiorrhiza Ameliorates Disease Progression in Dextran-Sodium-Sulfate Induced Colitis in Mice

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1. Abstract

Salvia miltiorrhiza (SM, or Danshen) extract has been approved by China FDA for the treatment of cardiovascular and cerebrovascular diseases owing to its potent anti-inflammatory effects. Whether SM may be used to treat Inflammatory Bowel Disease (IBD) remains elusive. In the current study, Dextran-Sodium-Sulfate (DSS) induced colitis in mice was used as a model of IBD, and SM was given orally for 7 days. SM administration has significantly reduced the disease activity index (DAI) score and weight lost and colon shortening in the DSS-induced colitis mice. The macrophage infiltration was significantly reduced in the SM treatment group. To explore the mechanisms, macrophage processor cell line Raw 264.7 was used to verify the anti-inflammatory effect of SM. SM treatment inhibited lipopolysaccharide (LPS)-induced macrophage activation in RAW264.7 cells and significantly reduced the production of pro-inflammatory factors. The current study provided evidence that oral administration of

SM ameliorates pathological deterioration of IBD in mice, and warrants future clinical application of SM for the management of IBD.

2. Introduction

Inflammatory Bowel Diseases (IBD), including ulcerative colitis, and Crohn's disease, is malfunction of gastrointestinal system with multiple causes. It is characterized with abnormal immune-mediated intestinal chronic and recurrent inflammation [1] and is significantly associated with colorectal cancer risks [2]. In recent decades, the global prevalence of IBD keeps increasing [3]. The pathogenesis of IBD remains elusive, environmental factors that act on genetic susceptibility may be the main cause. With the participation of intestinal flora, IBD initiates natural intestine immunity and active immune response. Immune hyper activation results in pathological changes such as intestinal mucosal barrier damage, ulcer healing and inflammatory hyperplasia. Various immune cells and cytokines are involved in the pathogenesis and treatment of IBD [4-6]. DSS damag-

es intestinal epithelial cells and tight junction barriers and ultimately increases intestinal permeability to induce acute colitis. In our study, the DSS-induced mouse acute colitis model was used to mimic the clinical symptom of IBD.

Indeed, anti-inflammatory agents and immunomodulators have been employed to treat IBD. In the long-term, both two types of drugs have limited effectiveness with side effects, such as gastrointestinal reactions, osteoporosis, and myelosuppression. Recently, two anti-tumor necrosis factor (anti-TNF) drugs, infliximab and adalimumab, have been proved their clinical efficacy [7, 8]. However, 50% of patients lose the effective treatment response within 1 year [9]. The IBD is a chronic process, and most patients have recurrent episodes, so a supplemental and alternative treatment is needed.

Recently herbal therapy as a potential alternative medical therapy have drawn attention. Many herbs have been shown to treat mice colitis effectively [10-14]. *Salvia miltiorrhiza* (SM, or Danshen), a traditional Chinese herb, has been widely used in Asian countries for cerebral ischemia-reperfusion injury, blood rheology, platelet function, anti-hypertensive, anti-inflammatory and even protecting the cardiovascular system [15]. Studies indicate SM have anti-tumor, antioxidant, anti-diabetic and anti-fibrotic effects, which plays a positive role in promoting butyric acid metabolism and regulating intestinal flora [16, 17]. In addition, a study has been reported that salvianolic acid B, which was an effective component of SM, could restore impaired barrier function in rat colitis model [18]. Other studies have shown that salvianolic acid A does not affect normal coagulation [19]. Compared with heparin, SM may be safer as it has not been found to increase the risk of blood thinning, no report on hemorrhage in the animal studies. Despite anticoagulant treatment in IBD management is still debatable [20, 21], using heparin for treatment of thrombotic UC achieved good results and it is generally accepted [22-24]. It has been found that IBD patients have an increase in peripheral platelet count and abnormal activation which in turns to. release a variety of inflammatory mediators, leading to aggregation of inflammatory cells and worsening the inflammatory responses of IBD [25-27]. Hence platelets activation is related to the severity of IBD, whereas SM has anti-platelet aggregation effects, therefore SM was selected to test their roles in DSS-induced colitis in mice.

3. Materials and Methods

3.1 Chemicals and Reagents

Medical grade SM extract was purchased from Chiatai Qingchunbao Pharmaceutical Co., Ltd (National Medicine Standard Z33020177, Hangzhou, Zhejiang, China). Dextran Sulfate Sodium (DSS, molecular weight 36–50 kDa) was purchased from MP Biologicals (US). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (US). Fetal bovine serum (FBS), penicillin, and alpha-MEM medium were purchased from Life Technologies. Griess reagent (modified) from Sigma was used. Rat anti-mouse F4/80 was purchased from BIORAD (US). Goat Anti-Rat IgG H&L was purchased from Abcam (US).

3.2. Animals

Male C57BL/6 mice, 6–10-weeks-old, were provided by the Laboratory Animal Research Centre of the Chinese University of Hong Kong with animal experimental ethical approvals. Animal experiment was carried out under the animal license issued by the Hong Kong SAR government and the approval of the institutional animal care and use committee (AECC: 20-191-MIS). The efforts were made to minimize the suffering of the animals.

3.3. Ulcerative Colitis (UC) Mouse Model and Treatment of SM

Acute colitis was induced in C57BL/6 mice by oral administration of 3.0% DSS in drinking water for 7 days. They were randomly assigned to 3 groups: (a) Control group (n=4), mice received a drinking water only. (b) DSS group (n=4), mice were given 3.0% DSS in drinking water for 7 days. (c) SM group (n=4), mice were given 3.0% DSS in water and SM (5ml/kg) gavage feeding once daily for 7 days. Mice were terminated at the end of the experiment, the colons (from cecum to anus) and spleens were harvested. The length of the colons was measured using a ruler, and the samples were fixed in 4% formaldehyde for 24 hours, and prepared for paraffin embedding, sectioning and histological examinations.

3.4. Evaluation of Disease Activity Index (DAI)

DAI was used for evaluation of the severity of colitis [28]. The scores were determined by combining scores of bleeding, diarrhea, and body weight loss every two days.

3.5. Histological Assessments

The distal end of colons was fixed in 4% formaldehyde for 24 h and stained with according to standard protocols[29]. Then the histological score was calculated according to (a) the severity of inflammation: 0 = none; 1 = slight; 2 = moderate; 3 = severe; 4 = very severe; (b) depth of lesion: 0 = none; 1 = mucosal layer; 2 = submucosal layer; 3 = muscle layer; 4 = transmural; (c) crypt damage: 0 = none; 1 = basal 1/3 damaged; 2 = basal 2/3 damaged; 3 = only surface epithelium intact; 4 = entire crypt and epithelium lost; resulting in a score from 0(normal) to 12 (severe colitis) [12].

3.6. Immunofluorescent Analysis

To assess the extent of infiltration of mucosal macrophages by immunofluorescence, the paraffin-embedded colon tissue sections were labeled with F4/80 antibody. First, the sections were deparaffinized. After exposing the antigens, the colon sections were incubated with 1:50 primary antibodies overnight at 4°C, then co-incubated with 1:1000 secondary antibodies for 1 hour at room temperature. Finally, sections were sealed with DAPI and observed under a microscope at magnification, x20, x40.

3.7. RAW264.7 Cell Culture and Nitric Oxidase (NO) Production Assay

RAW264.7 cells (Beyotime, China) were grown in alpha-MEM containing 10% FBS and 100 U/ml penicillin. Cells were incubated at 37°C in a humidified incubator with 5% CO₂. These RAW264.7 cells

are often used as an in vitro model of macrophage activation. Raw 264.7 cells (4×10^4 /well) were cultured in 96-well plates. Cells were pretreated in the presence or absence of SM (0.2%) in for 1h and then stimulated with LPS ($10\mu\text{g}/\text{ml}$) for 24 h. The NO levels in the culture media were evaluated by using modified Griess reagent to measure the nitrite accumulation. The culture supernatant and Griess reagent were mixed with equal volume and incubated for 10 min. The absorbance at 540 nm was measured with a microplate reader. Sodium nitrite (NaNO_2) was used to generate a standard curve for quantification.

3.8. RNA Extraction and Quantitative Real-Time PCR

Total RNA of cells was extracted by using Trizol (Takara, USA), and RNA purity and concentration were determined by measuring the absorbance at 260 and 280 nm. Then total RNA was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific USA). Real-time PCR was performed with Step one plus real-time PCR system (Applied Biosystems). The reaction conditions consisted of 10ul reaction volumes with diluted cDNA template 1, 5ul SYBR-Green master mix (2x), 1.6ul PCR-grade water, and 0.4ul of each primer. Amplification conditions were followed: first at 95°C for 5 min, and then 50 cycles of 95°C for 15 s and 60°C for 60 s.

Primer sequences were as follows: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward: $5\text{'-ggcatggactgtggtcatgag-3'}$, reverse: $5\text{'-tgcaccaccaactgttagc-3'}$;

IL-1 β forward: $5\text{'-ttcaggcaggcagatcactc-3'}$, reverse: $5\text{'-gaaggtccacgg-gaaagacac-3'}$;

IL-6 forward: $5\text{'-tagtcttctaccaccaatttc-3'}$, reverse: $5\text{'-ttggctcttagc-cactcttc-3'}$;

IL-10 forward: $5\text{'-gctcttactgactggcatgag-3'}$, reverse: $5\text{'-cgcagctctag-gagcatgtg-3'}$. The relative quantification of gene expression was analyzed with $2^{-\Delta\Delta\text{CT}}$ method, normalized with GAPDH expression level.

4. Statistical Analysis

All values were presented as means \pm SD. Statistical significance was assessed by Student's t-test or one-way ANOVA using Prism Graph-Pad 5.0 (San Diego, CA, USA). Probabilities (p) < 0.05 were considered significant.

5. Results

5.1. SM Improves the Symptoms of DSS-Induced Colitis in Mice

Acute colitis is commonly induced by administration of 2-5% DSS for 4-9 days [30, 31]. Our mouse colitis model was induced by 3.0% DSS in drinking water for 7 days, and the treatment group received daily SM via gavage (Figure 1A). Bodyweight presented as the percentage of original body weight, diarrhea and bleeding were assessed every two days. The results showed that SM treatment group attenuated the body weight loss and significantly reduced the Disease Activity Index (DAI), compared to the DSS control group (Figure 1B-C). In the DSS only group, the colonic length of mice was markedly shortened than that of the SM treatment group (Figure 1D-E). The control group (normal) mice had an average colon length of 8.38 ± 0.76 cm, while the DSS treatment led to a significant reduction of colon length to 4.9 ± 0.84 cm. SM treatment reduced the extent of colon shortening to 6.35 ± 0.39 cm). It has been reported that DSS induced splenomegaly was associated with increased splenic macrophages infiltration [32]. The DSS only group had significantly increased the size and weight of the spleen comparing to other groups (Figure 1 F-G).

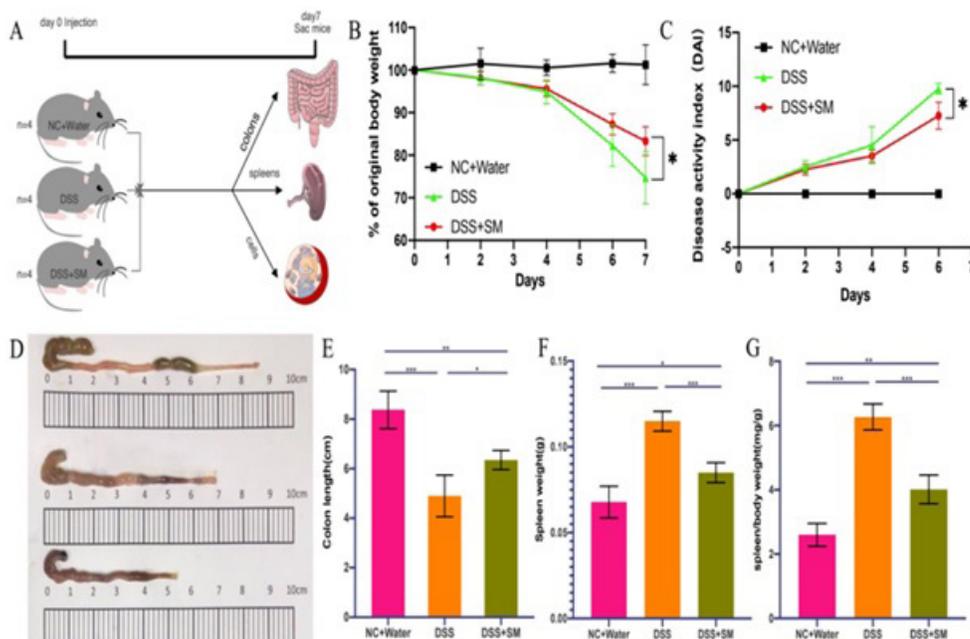


Figure 1: SM treatment ameliorates the symptoms of DSS induced colitis. (A) Experimental schematic. Body weights (B), disease activity indexes on diarrhea, bleeding, and body weight loss (C) of all mice in groups were assessed on days 0, 2, 4, 6. All mice were terminated on day 7, colons and spleens were collected to measure the colon length and spleen weight (D-G). The values were presented as the means \pm SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 4$.

5.2. SM Treatment Attenuated the Histological Changes of DSS-Induced Colitis in Mice

We next evaluated the severity of colonic inflammation by histopathological analysis using H&E staining. The higher histological scores indicate severer damages. Damage of crypts, loss of goblet

cells, infiltration of mononuclear cells, and formation of serious ulcers in the colons were examined. Comparing to the SM treatment group, the DSS group had significantly increased the infiltration of inflammatory cells, signs of epithelial loss and crypt destruction (Figure 2A) with higher histological scores (Figure 2B).

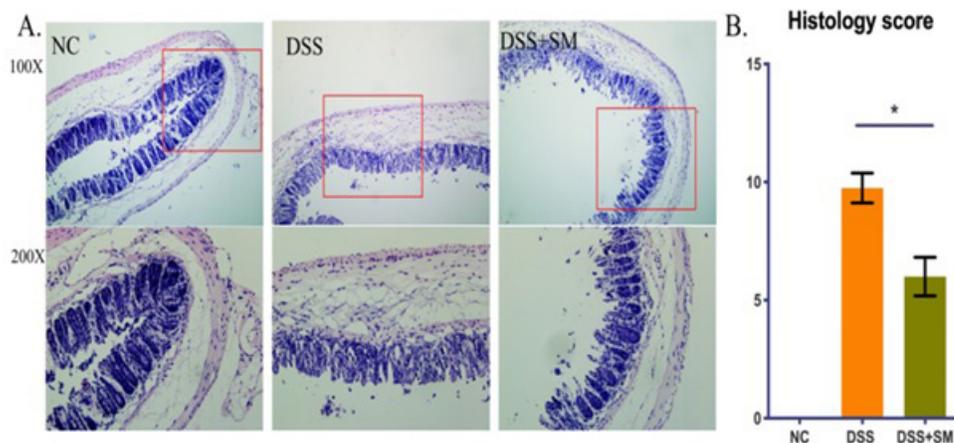


Figure 2: Clinical assessment of DSS-induced colitis in SM injection. (A) Representative hematoxylin and eosin stained colon sections (up 10X, down 20X magnification). (B) The histological damage scores. The results are presented as the means \pm SD. T-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3. SM Treatment Suppressed Infiltration of Macrophages in DSS-Induced Colitis in Mice

Immunofluorescence analysis of F4/80, a marker of macrophages, was employed to evaluate the inflammatory phenotype of SM treat-

ment in DSS-induced colitis mice. An increased number of F4/80 inflammatory cells was observed in the DSS only mice whereas SM treatment significantly reduced the number of macrophages in mucosa tissues (Figure 3A-B).

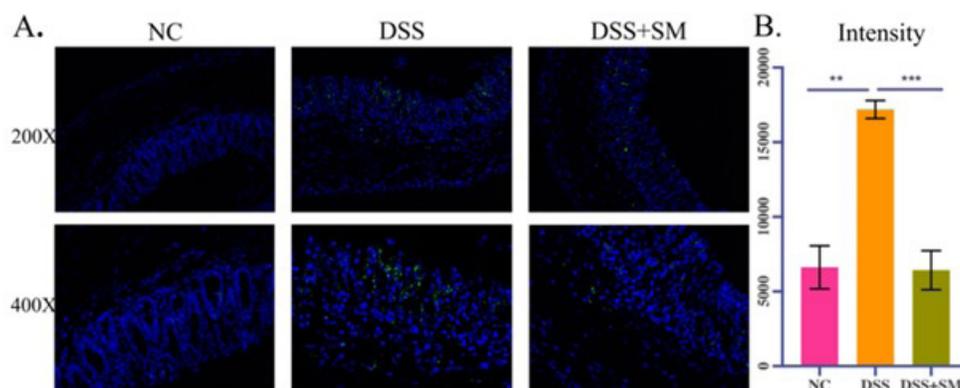


Figure 3: SM treatment reduced the macrophage infiltration in the colons of DSS-induced colitis mice. (A) Cross sections of colon were stained for the macrophage marker F4/80. (B) Quantitative analysis of F4/80 expression intensity. The results were presented as the means \pm SE, Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.4. SM Inhibited NO Production and Downregulated Proinflammatory Cytokine Expression in LPS-Stimulated RAW 264.7 Cells

A hallmark of macrophage activation is the production of NO in response to LPS [33]. Therefore, we investigate the effect of SM on the production of NO in LPS-stimulated RAW 264.7 cells. Data showed

that LPS induced a significant increase in NO production while SM treatment suppressed NO production in LPS-activated RAW 264.7 cells (Figure 4A). We further tested the effects of SM treatment on some proinflammatory factors expression (IL-1 β , IL-6 and IL-10) by q-PCR. Following SM treatment, the mRNA expressions of IL-1 β , IL-6 and IL-10 in LPS-stimulated RAW267.4 cells were significantly reduced comparing to the control groups (Figures 4B-D).

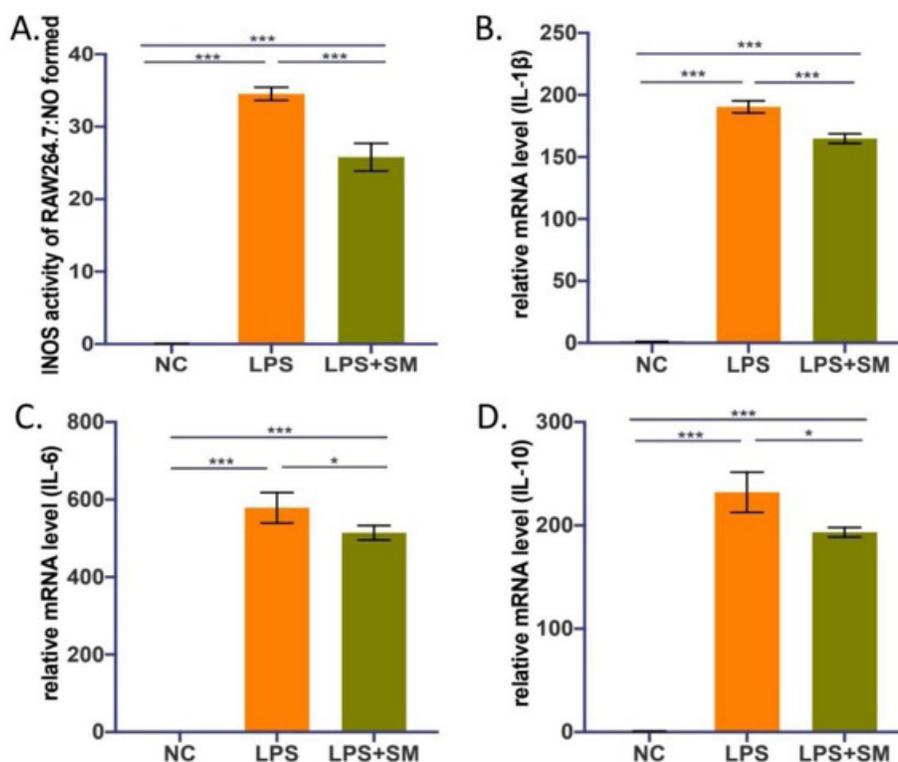


Figure 4: SM inhibited NO production and down regulated the expression of proinflammatory cytokines in LPS-stimulated RAW264.7 cells. (A) NO production. The mRNA levels of IL-1 β (B), IL-6 (C) and IL-10 (D). The results were presented as the means \pm SE, * p < 0.05, ** p <0.01, *** p < 0.001.

6. Discussion

This study evaluated the effects of SM on DSS-induced colitis model in mice. This model has been used to mimic many manifestations of human UC, including mucosal ulcers and systemic symptoms such as colon shortening, diarrhea, bloody stools, splenomegaly [34, 35]. DSS damages intestinal epithelial cells and tight junction barriers and ultimately increases intestinal permeability to induce acute colitis. The current study found that SM treatment alleviated clinical symptoms of IBD in mice, including maintaining colon length, body weight, and reduction of inflammatory factors production.

Increased platelets, oxygen free radicals and inflammatory cells are associated with occurrence of UC or IBD [36-38]. The effect of SM in promoting microcirculation is widely recognized in the cardiovascular field, but its anti-inflammatory effect is underestimated. Its role in gastrointestinal inflammation is largely unexplored, especially for the treatment of UC or IBD [15]. Cao et al. found that Cryptotanshinone (CTN), one of the main constituents of SM, has anti-inflammatory effects. CTN inhibits prostaglandin E2 production and COX-2 expression via suppression of TLR4/NF- κ B signaling pathway in LPS-stimulated Caco-2 cells [39]. Jin et al. also proved that Rosmarinic acid (RA), a phenolic ester also found in SM, resulted in the reduction of the inflammatory-related cytokines, such as IL-6, IL-1 β , and IL-22, and protein levels of COX-2 and iNOS in mice with DSS-induced colitis [42]. Furthermore, RA effectively and pleiotropically inhibits the activation of NF- κ B and STAT3, and

subsequently reduces the activity of pro-survival genes that depend on these transcription factors [40]. These previous published results support the current findings that SM does possess anti-inflammation potential in IBD conditions.

The gut microbiota of IBD patients is imbalanced [41]. In recent years, probiotics are found to be one important therapeutic target for maintaining the normal immune response and the integrity of the intestinal mucosal [42, 43]. SM has been reported to increase the number of intestinal probiotics and reverse the intestinal disordered microbiota. SM extracts significantly regulated *Peptococcus*, *Peptostreptococcaceae* and *Ruminococcus* and reduced inflammation in guts [44]. Dihydroshinone I (DHTS), a liposoluble extract of SM, efficiently restored disordered fecal microbiota community and increased the abundance of *Akkermansia* in chemotherapy-induced intestinal mucositis mice. *Akkermansia*, a genus in the phylum *Verrucomicrobia*, a potential probiotic, was reported to use their mucous to produce acetate and propionate. DHTS also enriched bacterial species which promote butyric acid metabolism or negatively correlated with inflammatory factors [45]. Whether SM treatment could stabilize gut microbiota via inhibiting intestinal inflammation, is the subject for future investigation, to explore the impact of SM treatment on gut microbiota diversity and severity of intestinal inflammation.

SM extract (SME) has been reported to halt the disease progression in DSS-induced colitis mice, in that 25 and 50 mg/kg SME intraperitoneal injection inhibited colonic shortening in a dose-related man-

ner [46]. In the current study, we gave SM orally which is in medical grade injection from at a dose of 5ml/kg, and we have obtained comparable results in slowing down disease progression as previous studies [46] with much low dose SM. We also analyzed the spleen/body weight ratio and inflammatory factors level in plasma. When tanhinone *IIA*, a main component of SM, was given by intraperitoneal injection at a dose 20 mg/kg [47], there was no statistical difference in body weight, colon length and plasma IL-6 levels in the DSS-induced colitis mice [28], while the above indices were significantly reduced in our study. The level of iNOS and IL-1 β in the SM treatment group was also lower than that of tanhinone *IIA* treatment group [28] or cryptotanshinone treatment group (50 mg/kg, oral administration) [48], indicating that SM is a more potent anti-inflammation modulator. Histological results further confirmed that SM treatment protects the intestinal mucosa and structure as well as reduced inflammatory cells infiltration. Our study also suggests that oral administration of SM may be more effective than intraperitoneal injection for the management of UC/IBD. These results taken together explain partially the underlying mechanisms why SM could alleviate symptoms of UC (IBD).

The current study reported a potential new application of a widely used herbal drug SM for the management of UC/IBD through oral administration. SM may be used clinically as a supplementary drug for the treatment of Crohn's disease or colorectal cancer. In future studies, different doses of SM combined with glucocorticoids or other biological reagents for the management of IBD shall be investigated through animal studies and clinical trials.

7. Funding

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