Anti-inflammatory effects of kudingcha methanol extract (*Ilex kudingcha* C.J. Tseng) in dextran sulfate sodium-induced ulcerative colitis

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Abstract. The present study aimed to investigate the anti-inflammatory effects of *Ilex kudingcha* C.J. Tseng methanol extracts (KME) on 3% dextran sulfate sodium (DSS)-induced ulcerative colitis (UC) in mice (C57BL/6J strain). Body weight, disease activity index (DAI), colon length, colon weight to length ratio, colonic myeloperoxidase (MPO), glutathione (GSH) and malondialdehyde (MDA) levels were measured. Histological changes were observed by hematoxylin and eosin staining. Colonic levels of tumor necrosis factor-α (TNF-α), interleukin(IL)-1β and IL-6 were measured with an enzyme-linked immunosorbent assay. The mRNA expression of TNF-α, IL-1β, -6, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the colon tissue, was quantified by RT-PCR. KME significantly suppressed DSS-induced body weight loss, colon length shortening and decreased the colon weight to length ratio. It also resulted in increased GSH and reduced MPO and MDA levels in the colon tissue. Histological observation suggested that KME prevented edema, mucosal damage and loss of crypts, which are induced by DSS. In addition, KME decreased the levels of TNF-α, IL-1β and -6 in the colon tissues, while inhibiting the mRNA expression of these cytokines, as well as iNOS and COX-2. The results of this study suggested that KME has anti-inflammatory effects on DSS-induced UC in mice (C57BL/6J strain) by reducing the colonic levels and inhibiting the mRNA expression of pro-inflammatory cytokines.

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Introduction

Human inflammatory bowel disease (IBD), a type of serious gastrointestinal tract disease that includes ulcerative colitis (UC) and Crohn's disease (CD), is characterized by recurrent chronic inflammation and mucosal tissue damage of the gastrointestinal tract (1). However, the etiology of IBD remains unknown. Generally, IBDs are the result of various factors including environmental, genetic and lifestyle effects, as well as immunological disorders (1,2). In particular, immunological disorders have resulted in an imbalance between pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-1β, -6 and -12, and anti-inflammatory cytokines such as IL-4 and -10. Therefore, cytokines may be important in the pathological process of IBD (3). The inflammatory response begins with an infiltration of neutrophils and macrophages. Activated macrophages produce a potent mixture of broadly active inflammatory cytokines, such as TNF-α, IL-1β and -6 which subsequently causes damage to the colonic tissues (4,5). Downregulation of the aberrant immune response and inhibition of the pro-inflammatory cytokines that induce inflammatory cascades, has been recognized as a major therapeutic target in IBD treatment (6).

Traditional therapeutic agents, including 5-aminosalicylaes (5-ASA) and corticosteroids, which are used in clinical IBD treatment, are also associated with serious complications and undesirable side effects (7). 5-ASA is well tolerated but diarrhea, cramps and abdominal pain are occasional side effects and these may be accompanied by fever, rash or kidney problems. Corticosteroids also result in systemic immunosuppression and have several well-known side effects, such as rounding of the face, acne, increased body hair, diabetes, weight gain and high blood pressure (8). For these reasons, the development of effective and safe therapeutic agents to treat IBD, has become increasingly important.

Kudingcha is a bitter tea that is prepared from *Ilex kudingcha* C.J. Tseng. It has been consumed traditionally as a herbal tea in China, Vietnam and various regions of Southwest Asia (9). Kudingcha has been demonstrated to exhibit numerous beneficial functions, including antioxidant, -obesity, -diabetic, -inflammatory, cardiovascular, hepatoprotective and neuroprotective activities (10-18). Previous studies have identified

that kudingcha is rich in polyphenolic compounds, such as caffeoylquinic acid (CQA) and its derivatives (11,12,19). In the present study, we investigated the anti-inflammatory effects of *Ilex kudingcha* C.J. Tseng methanol extract using an *in vivo* animal model of IBD, induced by DSS.

Materials and methods

Chemicals and reagents. DSS (molecular weight: 36,00-50,000) was obtained from MP Biomedicals (Solon, OH, USA). TRIzol reagent, OligodT_{18} primer, murine maloney leukemia virus (MMLV) reverse transcriptase, RNase inhibitor, ethidium bromide (EtBr) and agarose, were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). All other reagents were of analytical grade.

Plant extract preparation. Fresh kudingcha (*Ilex kudingcha* C.J. Tseng.) leaves were purchased from a local market in Chongqing, China in October, 2012. The fresh kudingcha leaves were freeze-dried and then ground into a fine powder. A 12-fold volume of methanol (80%, vol/vol) was added to the powdered samples and extracted three times by stirring overnight. Kudingcha methanol extracts (KME) were concentrated by heat evaporation, cryodessication and stored at 4˚C until further study.

Animal studies. Male mice (C57BL/6J strain; age, 6 weeks) were purchased from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). The mice were housed in a standard 12-h light/dark cycle at room temperature, and had *ad libitum* access to food and water. Colitis was induced in mice by administration of 3% (wt/vol) DSS in the drinking water for 7 days. Mice were randomly divided into four groups with 6 mice per group: Group 1, the normal controls were treated with 0.9% normal saline; group 2, DSS-treated mice and groups 3 and 4 received DSS and were administered with KME (50 and 200 mg/kg) daily via an intragastric route (0.2 ml/mouse) for 7 days, until sacrifice. The animal protocol used in this study was reviewed by the Animal Ethics Committee of Chongqing Medical University.

Evaluation of disease activity index (DAI). The DAI was used to evaluate the grade and extent of intestinal inflammation. Body weight, stool consistency and blood in the stools were monitored daily for determination of DAI. Each score was provided as follows: Body weight loss (0, none; 1, 1-5%; 2, 5-10%; 3, 10-20%; 4, >20%), diarrhea (0, normal; 2, loose stools; 4, watery diarrhea) and blood (0, normal; 2, slight bleeding; 4, gross bleeding). The DAI score ranged from 0 to 12 (total score) (20). The mice were sacrificed on day 7, and the weight and length of the colon were measured.

Histological observations. The distal colons from each animal were subjected to histological examination. The colon tissues were fixed in 10% (vol/vol) neutral-buffered formalin, dehydrated in ethanol and embedded in paraffin. Colon tissue sections (4 μm) were then cut and stained with hematoxylin and eosin (H&E).

Myeloperoxidase (MPO) activity. MPO activity was assessed as described previously (21), but with modifications. Colon tissues (50 mg) were washed, homogenized in cooled phosphate-buffered saline (PBS, 80 mM, pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) and centrifuged at 12,000 x g, for 20 min at 4˚C. The supernatant was added to a mixture of 150 μl 3,3`,5,5`-tetramethylbenzidine (2 mM), 50 μl H_2O_2 (300 mM), 250 μl PBS (pH 5.4, 80 mM) and incubated for 30 min at 25˚C. The reaction was quenched by 2.5 ml H_2SO_4 (200 mM) and the absorbance of the resulting mixture was measured at 450 nm with a UV-2401PC spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Lipid peroxidation levels. Lipid peroxidation was measured by the thiobarbituric acid (TBA)-reactive substance (TBARS) assays for malondialdehyde (MDA) following a previously described method (22), but with modifications. Colon tissue (100 mg) was washed and homogenized in cooled PBS. Total protein was determined with a bicinchoninic acid (BCA) assay. The suspension was mixed with 1 ml TBA (0.67%, w/v) and 1 ml trichloroacetic acid (TCA; 25%, w/v), heated for 45 min at 95˚C and centrifuged at 12,000 x g for 20 min at 4˚C. TBA reacted with the oxidative degradation products of lipids, yielding red complexes that are absorbed at 535 nm. The volume of MDA was determined using a spectrophotometer (UV-2401PC).

Glutathione (GSH) levels. GSH levels were assessed as described previously by Ellman (23). Colon tissue (100 mg) was washed and homogenized in cooled PBS. The homogenate (0.5 ml) was well mixed with 10% TCA (0.5 ml) and centrifuged at 3,000 x g for 5 min. An aliquot of supernatant (0.1 ml) was mixed with 1.7 ml of potassium phosphate buffer (0.1 M, pH 8.0) and 0.1 ml of Ellman's reagent. After 5 min, the optical density was measured at 412 nm against a blank using a spectrophotometer (UV-2401PC).

Measurement of colonic pro-inflammation cytokine levels. Colon samples were washed and homogenized for 5 min in 3 ml PBS (0.1 M, pH 7.4) at 4˚C. The tissue homogenates were centrifuged at 12,000 x g for 5 min at 4˚C. Colonic levels of TNF-α, IL-1β and -6 were measured with a commercial enzyme-linked immunosorbent assay kit (ELISA MAX, BioLegend Inc., San Diego, CA, USA) according to the manufacturer's instructions.

RT-PCR assay. mRNA expression of TNF-α, IL-1β, -6, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the colon tissue, was measured with RT-PCR. Total RNA was isolated with TRIzol reagent and centrifuged at 12,000 x g, for 15 min at 25˚C, following the addition of chloroform. Isopropanol was added to the supernatant at a 1:1 ratio and the RNA was pelleted by centrifugation at 12,000 x g for 15 min. After washing with ethanol, the RNA was solubilized in diethyl pyrocarbonate-treated RNase-free water and quantified by measuring the absorbance at 260 nm using a spectrophotometer (UV-2401PC). Equal amounts of RNA (1 μg) were reverse transcribed in a master mix containing 1X reverse transcriptase buffer, 1 mM dNTPs, 500 ng of oligodT_{18} primers, 140 units of MMLV reverse transcriptase and 40 units of RNase inhibitor for 45 min at 42˚C. PCR was then carried out in an automatic thermocycler

Table I. Effects of kudingcha methanol extract on the levels of malondialdehyde and glutathione in the colon tissue of dextran sulfate sodium-treated mice.

Groups	GSH (μmol/mg protein)	MDA (nmol/mg protein)
Normal control	8.70±0.40[a]	0.47±0.02[d]
DSS	2.62±0.34[d]	0.98±0.10[a]
DSS + KME (50 mg/kg)	4.58±0.44[c]	0.79±0.07[b]
DSS + KME (200 mg/kg)	5.84±0.49[b]	0.63±0.07[c]

Data are represented as the mean ± standard deviation. [a-d]Different letters are significantly different (P<0.05) by Duncan's multiple range tests. MDA, malondialdehyde; GSH, glutathione; DSS, dextran sulfate sodium, KME, kudingcha methanol extract.

(Bioneer, Daejeon, South Korea) for 25 cycles (94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 40 sec) followed by an 8 min extension at 72˚C. The PCR products were separated in 2% agarose gels and visualized by EtBr staining. β-actin was used for normalization.

Statistical analysis. Data were presented as the mean ± standard deviation. Differences between the mean values for individual groups were assessed by a one-way analysis of variance with Duncan's multiple range tests. P<0.05 was considered to indicate a statistically significant difference. The SAS v9.1 statistical software package (SAS Institute Inc., Cary, NC, USA) was used for analysis.

Results

KME attenuated DSS-induced colitis symptoms. No animals died during the experimental period. As shown in Fig. 1A, the body weight gain of mice in the DSS group was significantly lower than that in the normal control group. KME-treated groups prevented the DSS-induced body weight loss. The symptoms of DSS-induced colitis in mice were similar to those observed in humans, such as body weight loss, diarrhea and gross bleeding (24). We quantitatively scored these symptoms according to the DAI. The DAI score indicated that KME alleviated the severity of DSS-induced colitis (Fig. 1C). DSS-induced colitis is associated with a marked decrease in colon length (25). As shown in Fig. 1B, DSS significantly decreased the colon length in the DSS treatment group (5.7±0.5 cm), compared with that in the normal control group (9.0±0.4 cm). However, KME was able to reduce the DSS-induced colon shortening in mice with colitis. In addition, colon weight to length ratio was used as an indicator of disease-associated intestinal wall thickening and intensity of inflammation. KME significantly inhibited DSS-induced intestinal wall thickening compared with that of the normal control group (Fig. 1D).

Effects of KME on histological changes and MPO activity in DSS-induced colitis mice. The H&E staining assay was used to evaluate the therapeutic effects of KME in DSS-induced colonic inflammation and mucosal injury in colitis mice. As is evident in Fig. 2A, the tissue sections from normal mice showed intact surface epithelium, cryptal gland, stroma and submucosa, while the tissue sections from the DSS-induced colitis mice showed distorted crypt epithelium and extensive mucosal damage with a large number of inflammatory cells (Fig. 2B). However, tissue sections from KME-treated DSS-colitis mice had more intact surface epithelium, crypt glands and less inflammatory reactions than those in the DSS-colitis mice (Fig. 2C and D).

MPO activity, which is an indicator of acute inflammation, reflects the volume of neutrophil infiltration (26). DSS significantly increased the colonic MPO activity in colitis mice. However, following a treatment of 50 and 200 mg/kg KME significantly suppressed MPO accumulation in the colonic tissues of DSS-induced colitis mice (Fig. 2E).

Effects of KME on GSH and MDA levels in DSS-induced colitis mice. DSS significantly increased the colonic MDA levels (to 0.98±0.10 nmol/mg protein) compared with that of the normal control group (0.47±0.02 nmol/mg protein) (Table I). KME significantly reduced the colonic MDA levels of 0.79±0.07 and 0.63±0.07 nmol/mg protein at 50 and 200 mg/kg KME, respectively. In addition, KME also attenuated the DSS-induced reduction in colonic GSH levels in the colitis mice. The colonic GSH levels of the DSS-colitis mice significantly increased following treatment with KME, with the increased levels ranging from 4.58±0.44 to 5.84±0.49 μmol/mg protein at 50 and 200 mg/kg KME, respectively.

Effects of KME on the colonic and mRNA levels of pro-inflammatory cytokines in DSS-induced colitis mice. Increased pro-inflammatory cytokine levels are associated with the UC pathological process. As shown in Fig. 3A, DSS significantly increased the colonic levels of TNF-α, IL-1β and -6. KME significantly reduced the levels of TNF-α, IL-1β and -6 compared with that in the DSS-induced mice with colitis. To investigate the anti-inflammatory effects of KME on DSS-induced colitis in mice, mRNA expression of TNF-α, IL-1β and -6 in colonic tissue was analyzed by RT-PCR. As shown in Fig. 3B, colonic inflammation induced by DSS. resulted in an elevated expression of all pro-inflammatory cytokines. Our findings demonstrate that administration of KME effectively reduced the mRNA expression of TNF-α, IL-1β and -6 in the colon tissue of mice with DSS-induced colitis.

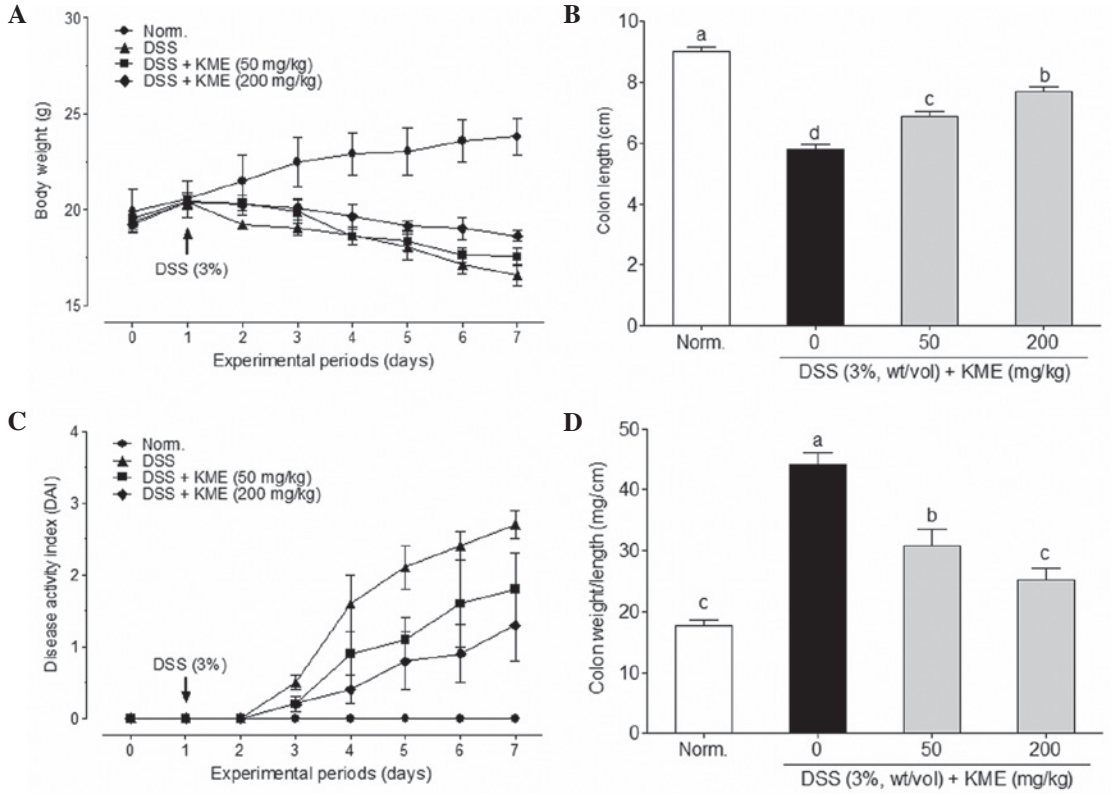

Figure 1. Effects of kudingcha methanol extract (KME) on (A) body weight changes (B) colon length; (C) disease activity index (DAI) and (D) colon weight to length ratio in dextran sulfate sodium (DSS)-treated mice. Data are presented as the mean ± standard deviation. [a-d]Different letters are significantly different (P<0.05) by Duncan's multiple range tests.

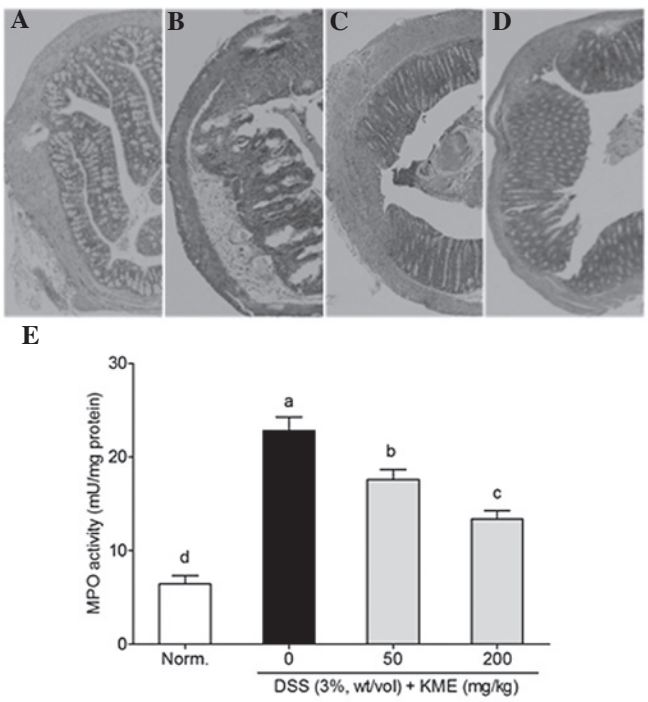

Figure 2. Effects of kudingcha methanol extract (KME) on colonic histological observations and myeloperoxidase (MPO) activity in dextran sulfate sodium (DSS)-treated mice. (A) Normal control group received water; (B) mice exposed to 3% DSS in vehicle; (C and D) co-treatment with 50 and 200 mg/kg KME, respectively, for 7 days together with DSS administration (magnification for A-D, x40); (E) MPO activity was assayed according to criteria defined in Materials and methods. Data are presented as the mean ± standard deviation. [a-d]Different letters are significantly different (P<0.05) by Duncan's multiple range tests.

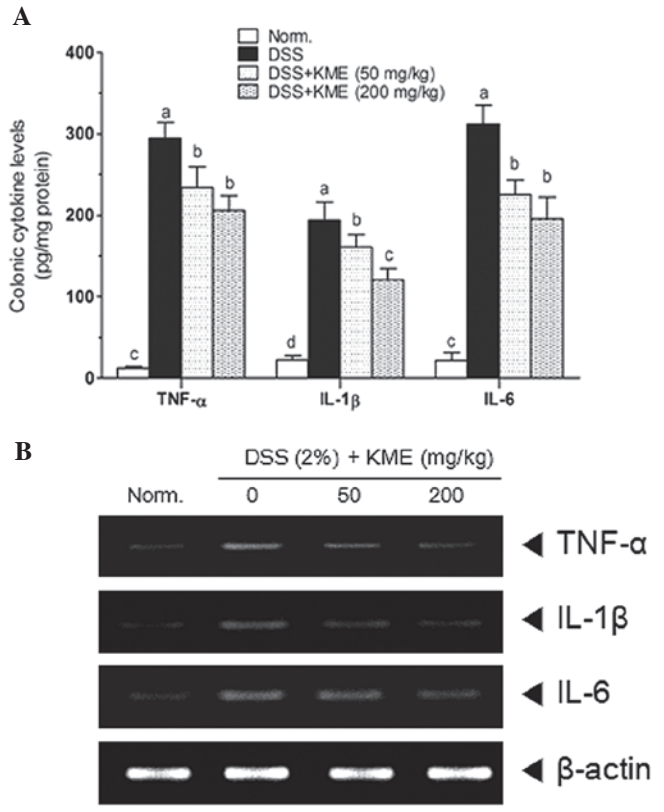

Figure 3. Effects of kudingcha methanol extract (KME) on colonic (A) and mRNA (B) levels of TNF-α, IL-1β and -6 in dextran sulfate sodium (DSS)-treated mice. Data are presented as the mean ± standard deviation. [a-d]Different letters are significantly different (P<0.05) by Duncan's multiple range tests.

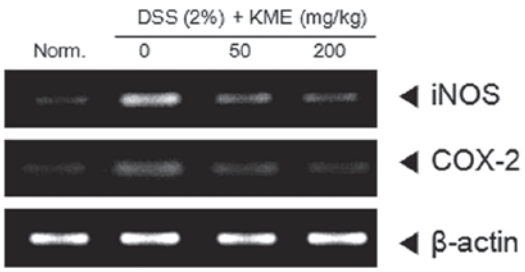

Figure 4. Effects of kudingcha methanol extract (KME) on the mRNA levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the colon tissue of dextran sulfate sodium (DSS)-treated mice.

KME inhibited the iNOS and COX-2 gene expression in DSS-induced colitis mice. iNOS and COX-2 are two types of inflammation-related enzymes and are important in the pathological process of UC. Therefore, we evaluated the effects of KME on iNOS and COX-2 mRNA expression in the colonic tissue of DSS-colitis mice compared with that of the untreated DSS-colitis mice. As shown in Fig. 4, DSS significantly increased the mRNA levels of iNOS and COX-2 in the colonic tissue of DSS-induced colitis mice. In addition, KME significantly and dose-dependently reduced the iNOS and COX-2 mRNA levels.

Discussion

In western countries, the incidence and prevalence of IBD has increased in the past 50 years from 8-14/100,000 to 120-200/100,000 in individuals with UC (27). Recently, the prevalence of UC has been on the increase in the Asian-Pacific region (28). In general, therapeutic treatment for UC includes anti-inflammation and immunosuppression. However, these treatments have also been associated with undesirable side effects. Therefore, natural medicine has become an alternative therapy in addition to the conventional therapies that are used to treat UC (29). In the present study, we investigated the anti-inflammatory activity of kudingcha methanol extracts (KME) using DSS-induced mice colitis model. KME (50 and 200 mg/kg) and DSS (3%) in mice were orally coadministered and then clinical colitis was assessed by examining body weight loss, shortening of the colon length, increasing colon weight to length ratio and the DAI.

KME administration attenuated body weight loss, colonic shortening and intestinal wall thickening that was induced by DSS (Fig. 1). DSS significantly induced inflammatory cell infiltration, mucosal erosion, distortion and loss of crypts (Fig. 2B), as well as elevated MPO accumulation in the colon tissue of DSS-colitis mice (Fig. 2E). MPO, a member of the hemeperoxidase-cyclooxygenase superfamily, is abundantly expressed in neutrophils, and to a lesser extent in monocytes and certain types of macrophages (31). It is a specific marker that may be used to determine neutrophil influx into the colon tissue. The decrease in MPO activity may be explained through the reduction of neutrophil accumulation in inflamed tissues (32). In the present study, KME markedly reduced leukocyte (neutrophil and macrophage) infiltration and also decreased the colonic MPO levels to ameliorate the inflammatory conditions in the colonic tissues of DSS-induced colitis mice.

A typical development of DSS-induced colitis is leukocyte infiltration into the colonic tissues. Leukocyte infiltration is an important source of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which act as cytotoxic agents by cross-linking proteins, lipids and nucleic acids, thus causing cell damage (33). Excess ROS markedly disrupts the oxidant/antioxidant balance as shown by the increased lipid peroxidation and reduction in colonic GSH content (34,35). Oxidative stress or cellular damage with its dual of free radicals, generates profound lipid peroxidation and are the hallmarks of UC (36). As a good *in vitro* free radical scavenger (12,19), KME markedly increased the colonic GSH levels and reduced the generation of MDA to attenuate the DSS-induced colitis in mice.

It is well known that the increased pro-inflammatory cytokines (TNF-α, IL-1β and -6) amplify the inflammatory cascade and result in intestinal tissue damage in patients with UC, as well as in animal models of DSS-induced colitis (5,37,38). The downregulation and/or blockade of pro-inflammatory cytokine activity was useful in the treatment of IBD (39). For example, anti-TNF-α antibody (Infilximab, a mouse monoclonal antibody) had effective therapeutic effects on UC in a clinical case (40,41). In addition, IL-1β and -6 are two key mediators of the progression of UC. IL-1β is known to stimulate diarrhea and the reduction of its function subsequently suppressed the infiltration of inflammatory cells into the intestinal tissue and inhibited intestinal necrosis in animals with UC (42-44). Furthermore, anti-IL-1β antibody attenuated not only the symptoms of DSS-induced colitis, but also IL-6 gene expression (45). In the present study, we observed that the colonic levels of TNF-α, IL-1β and -6 in the DSS-induced colitis mice were markedly decreased by KME administration, as identified by ELISA (Fig. 3A). The RT-PCR assay also confirmed that the mRNA levels of these pro-inflammatory cytokines were reduced by KME in the colonic tissue of DSS-induced colitis mice (Fig. 3B). These results indicate that KME may exert anti-inflammatory effects on DSS-induced colitis by reducing the activity of TNF-α, IL-1β and -6.

Previous studies have indicated that iNOS and COX-2 are considered to be vital in the inflammatory process of UC (5,46,47). Pro-inflammatory cytokines induced the mRNA expression of iNOS and increased the generation of nitric oxide (NO), which is an inflammatory mediator associated with the pathological process of UC. Suppression of iNOS attenuated UC in human and animal cases (47,48). Moreover, the inhibition of excessive COX-2 activity induced the generation of prostaglandins E2 (PGE2), which may result in the suppression of DSS-induced colitis in mice (49,50). In the present study, we demonstrated that KME decreased the mRNA expression of iNOS and COX-2 in the colonic tissue of DSS-induced colitis mice (Fig. 4).

In conclusion, results of the present study have demonstrated the potential anti-inflammatory effects of KME in DSS-induced colitis mice. These results suggest that KME administration prevents DSS-induced body weight loss, colonic shortening and modulates MPO activity, as well as reducing intestinal wall thickening. In addition, KME administration increased the colonic GSH levels, decreased colonic lipid peroxidation, and reduced the production and mRNA levels of TNF-α, IL-1β and -6. Results of the present study suggest

that the potential mechanism of KME involves suppressing the production of TNF-α, IL-1β, -6, iNOS and COX-2, and may be considered an important anti-inflammatory treatment agent against colonic inflammation.

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