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Original Article

Anti-inflammatory effect of *Perilla frutescens* seed oil rich in omega-3 fatty acid on dextran sodium sulfate-induced colitis in mice

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Abstract

Background and purpose: Ulcerative colitis is a chronic inflammatory bowel disease that involves diffused inflammation of the large intestine. Omega-3 fatty acid (FA) has been known to regulate the inflammatory response associated with ulcerative colitis pathogenesis. *Perilla frutescens* is a valuable source of omega-3 FA and α-linolenic acid (ALA) contained in its seed oil. Therefore, the aim of this study was to evaluate the anti-inflammatory effect of *Perilla* seed oil (PSO) on colitis induced by dextran sulfate sodium (DSS) in a mouse model.

Experimental approach: PSO was extracted using a cold-pressed extractor and FA composition of PSO was analyzed by GC-MS. Acute colitis in mice was induced with 3% DSS in drinking water for 7 days. Some mice were treated with PSO (20, 100, 200 mg/kg BW) for 3 weeks before the DSS administration. Sulfasalazine was used as a positive control. The clinical features, histopathologic, serum, and gene expression of proinflammatory cytokines in the colon were assessed.

Finding/Results: PSO contained the highest proportion of ALA (61.51%). Furthermore, PSO pretreatment evidently reduced body weight loss, diminished diarrhea, gross bleeding, and DSS-induced colon shortening. PSO pretreatment attenuated histopathological changes in response to DSS-induced colitis. PSO pretreatment also markedly decreased inflammatory response in serum and the colon tissue of DSS-induced mice.

Conclusion and implication: ALA in PSO is suggested to be mainly responsible for the reduction of DSS-induced colitis through suppressing inflammatory markers. PSO could be further developed as a functional health supplement, which would be beneficial for anti-inflammation in the colonic mucosa.

Keywords: Anti-inflammation; Inflammatory bowel disease; Inflammatory cytokines; Omega-3 fatty acid; *Perilla* seed oil.

INTRODUCTION

Inflammatory bowel disease (IBD) is a long-term inflammatory disorder that directly affects the gastrointestinal tract with relapse and remission of course. IBD consists of two main clinical forms, including Crohn's disease which involves transmural ulceration of any portion of the gastrointestinal tract, and ulcerative colitis (UC) which involves diffused inflammation of the colonic mucosa (1). The incidence of IBD is increasing worldwide, and long-term chronic inflammation of the colon is also associated with colorectal cancer development (2). The pathogenesis of IBD currently remains unknown; however, evidence of IBD pathogenesis suggests that it occurs from multifactorial factors interactions between genetic, environmental, microbial, and immunological factors (3).

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Conventional therapy with aminosalicylates, immunosuppressants, and monoclonal antibodies is presently insufficient and inefficient; moreover, it may cause several adverse side effects, including opportunistic infections (4), acute kidney injury (5), and liver toxicity (6). Therefore, alternative therapeutic approaches for IBD are needed to regulate the mucosal immune response in the area of inflammation to diminish the side effects.

Dextran sulfate sodium (DSS)-induced colitis in an animal model has been shown to closely resemble human UC in several facets of the pathogenesis of UC, involving abnormal immune response and clinical manifestations (7). In this model, the innate immune cells, including macrophages and epithelial cells in mucosa and submucosa, responded to stimuli and secreted excessive inflammatory cytokines which resulted from the trigger of the adaptive immune system response and led to intestinal damage (7). Thus, the induction of UC by DSS in mice has been useful to investigate the effects of functional foods. Several studies have reported that functional foods and ingredients, including omega-3 fatty acid (FA), phenolic, and flavonoids possess antioxidant and anti-inflammatory activities which contribute to their regulation of the innate immune cells for the productions of several cytokines in IBD (8-11).

Perilla frutescens, a plant grown in northern Thailand, is used as a medicinal herb and functional food in Asia. *Perilla* seed oil (PSO) contains large quantities of omega-3 FA, especially α-linolenic acid (ALA) at 54-79% of total FAs (12,13). Recent investigations have shown that PSO exhibits several biological activities, such as having antioxidant, anti-inflammatory, anti-asthmatic, anticancer, and antihypercholesterolemic effects as well as improves memory and cognitive function (14-19). It has been reported that PSO reduces the production of the inflammatory cytokines in lipopolysaccharide-induced macrophage cell lines, decreases the levels of prostaglandin E2, and leukotriene B in colitis induced by DSS (18). In animal models, the PSO supplementation was found to decrease the level of arachidonic acid yet increase the level of ALA, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) in the colon. The combination of PSO with lipoxygenase inhibitor suppresses the increase of chloride ion secretion-related diarrhea in mice's colitis (20). Furthermore, the advantage of PSO is that it is inexpensive, free of heavy metal contamination, and safe (21). However, the protective effect of PSO and its anti-inflammatory effects on DSS-induced colitis in mouse models have never been investigated and reported. Therefore, this study determined whether there are protective effects of PSO on colitis induced by DSS in a mouse model and how they affect colitis. The effects are evaluated by the macroscopic and histology parameters along with the inflammatory factors.

MATERIALS AND METHODS

Preparation of PSO and FA composition analysis

The *Perilla* seeds were collected from cultivated areas in the Maehongson province of Thailand. The seeds were washed, dried, and crushed. PSO was then extracted using a cold-pressed extractor. These oils were centrifuged at 4000 *g* for 10 min, filtered for clarity, and stored at 4 ℃. The FA composition of PSO was subsequently measured using a gas chromatography-mass spectrometer (GC-MS) analysis (Agilent 6890N, DE, USA) by the Central Lab (Thailand) Co., Ltd. in accordance with the in-house protocol based on AOAC method 996.06. The FA content in PSO was presented as a percentage of total FAs.

Animals and experimental design

Four-week-old male C57BL/6 mice (National Laboratory Animal Center, Bangkok, Thailand) were housed in plastic cases with the maintained temperature (25 ± 1 ℃), humidity (50 ± 10%), and a 12/12-h light/dark cycle. Mice were acclimatized for 7 days before the experiment. All animal experiments procedures were approved by the Animal Ethics Committee of the Faculty of Medicine, Chiang Mai University, Thailand (Ethics ID. 40/2558). All mice were randomly separated into six groups (n = 6). The mice of the normal group (N) and the DSS group were fed with

soybean oil. In the pretreatment groups, mice were orally administered the PSO at the dose of 20 (DSS + PSO20), 100 (DSS + PSO100), and 200 (DSS + PSO200) mg/kg BW daily for 4 weeks. At the fourth week of PSO pretreatment, acute colitis was induced by DSS (MP Biomedicals, Solon, OH, USA) at a concentration of 3% (w/v) in drinking water for 7 days (22). In the positive control group, mice were orally administered the sulfasalazine (SSZ) at 50 mg/kg BW (DSS + SSZ group) during DSS administration. The SSZ was dissolved in 0.5% carboxymethylcellulose, while the PSO was dissolved in a vehicle of soybean oil because it lacked ALA. Mice were euthanized under CO_2 asphyxiation at the end of the study for sample collection. The experimental protocol of this study is shown in Fig. 1.

Assessment of disease activity index

The disease activity index (DAI) was calculated by summing the scores for body weight change, stool consistency, and gross bleeding, divided by 3. The scoring was graded as follows: body weight change (0: none, 1: 1-5% weight loss, 2: 5-10% weight loss, 3: 10-15% weight loss, and 4: > 15% weight loss), stool consistency (0: formed pellets, 2: loose stool, and 4: diarrhea), and gross bleeding (0: no rectal bleeding, 2: hemoccult-positive, and 4: visible gross bleeding) (23). The body weight in each mouse was recorded during DSS administration. On the sacrifice day, the stool consistency was observed and graded depending on the severity of diarrhea. The gross bleeding was determined using the Hema-Screen® Lab Pack for fecal occult blood (EKF Diagnostics, TX, USA).

Colon collection

After the mice were sacrificed, the colon was harvested, cut longitudinally, and gently cleaned with ice-cold phosphate-buffered saline. Then, the lengths of the colons were measured. The isolated colon tissues were prepared for further analysis, including histological examination and RNA isolation.

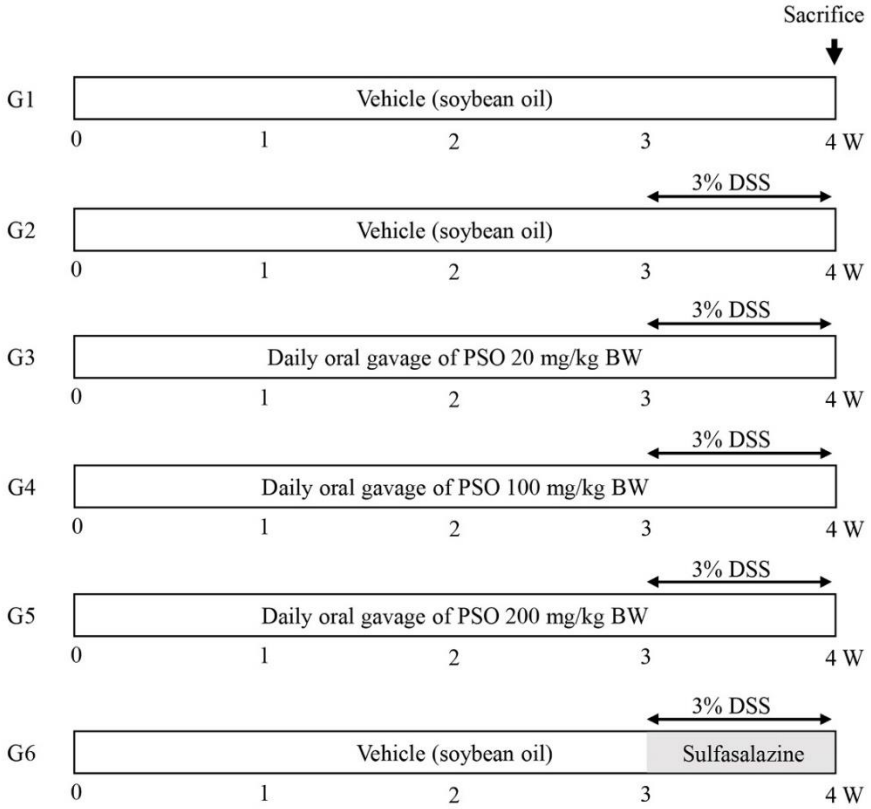

Fig. 1. Experimental design for evaluating the protective effects of PSO on DSS-induced colitis in a mouse model. Mice were orally administered PSO (20, 100, and 200 mg/kg BW) daily for 4 weeks. At the fourth week of PSO pretreatment, colitis was induced with 3% DSS in drinking water for 7 days. In the positive group, mice were orally administered the sulfasalazine (50 mg/kg BW) before sacrificed for 7 days. PSO, *Perilla* seed oil; DSS, dextran sulfate sodium.

Table 1. Primer sequences were used in this study.

Genes	Forward sequences	Reverse sequences
COX-2	5'-GCCCACCAACTTACAATGTGC-3'	5'-CATGGGAGTTGGGCAGTCAT-3'
TNF-α	5'-AAATGGGCTCCCTCTCATCAGTCC-3'	5'-TCTGCTTGGTGGTTTGCTACGAC-3'
IL-1β	5'-CACCTCTCAAGCAGAGCACAG-3'	5'-GGGTTCCATGGTGAAGTCAAC-3'
IL-6	5'-TCCTACCCCAACTTCAATGCTC-3'	5'-TTGGATGGTCTTGGTCCTTAGCC-3'
GAPDH	5'-GACATGCCGCCTGGAGAAAC-3'	5'-AGCCCAGGATGCCCTTTAGT-3'

Histopathologic evaluation

Tissues from the distal colon were fixed immediately in 10% buffered formalin overnight for paraffin processing. The paraffin tissue sections were stained with hematoxylin and eosin (H&E). The histopathological index was evaluated on the basis of the severity of inflammation (0 to 4 depending on the percentage of area affected by inflammation), erosion or ulceration (0-3 considering the areas of ulceration with lamina propria, submucosa, or transmural involvement), crypt loss (0 to 3 considering the percentage of damaged crypts), the extent of follicle aggregates, edema and infiltration of mononuclear and polymorphonuclear cells (scored 0 to 3 for the absent, weak, moderate, and severe degree, respectively) (22). The histopathological index was scored by two independent specialists.

Reverse transcription-quantitative polymerase chain reaction

The total RNA was isolated from the colon tissue using TRIzol reagent (Invitrogen; Carlsbad, CA) in accordance with the recommendations of the manufacturer. The cDNA was produced using a ReverTraAce® reverse-transcription-quantitative polymerase chain reaction (RT-qPCR) kit (TOYOBO, Tokyo, Japan). The RT-qPCR was subsequently performed using the SensiFAST SYBR® Lo-ROX kit (Bioline, Singapore). The fold change of the genes of interest including cyclooxygenase-2 (COX-2), tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and IL-6 was calculated by the method of $2^{-\Delta\Delta Ct}$. The PCR primers in this study are shown in Table 1. GAPDH was used as the housekeeping gene.

Measurement of pro-inflammatory cytokines

Blood samples were collected. Samples were centrifuged at 3,000 rpm for 15 min at 4 °C, and supernatants were stored at -20 °C until use. The serum levels of IL-1β, IL-6, and TNF-α were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (Biolegend, San Diego, CA, USA) according to the manufacturer's instructions.

Statistical analysis

All data are expressed as the means ± SEM. Body weight changes were analyzed with two-way ANOVA. Other data were analyzed by one-way ANOVA followed by Fisher's LSD *post hoc* test for comparative analysis. Differences were considered significant at $P < 0.05$.

RESULTS

The composition of FAs in PSO

The composition and content of total FAs in PSO were analyzed by GC-MS. The major constituents of PSO were mainly ALA (61.51 ± 0.09%), followed by linoleic acid (LA, 16.55 ± 0.06%), oleic acid (11.62 ± 1.14%), palmitic acid (6.87 ± 0.53%), and stearic acid (2.96 ± 0.09%), respectively.

Effects of PSO pretreatment on the severity of colitis induced by DSS

The pathologic symptoms including the body weight change, diarrhea, and bloody stool were found to be aggravated in the DSS-induced colitis. In this study, the body weight of all mice was monitored during DSS administration. As shown in Fig. 2A, we found that body weight gradually increased in the normal group. However, the body weight of the DSS group significantly decreased after days 5-7 of DSS administration compared to the normal group, while the PSO pretreatment groups, especially at 100 and 200 mg/kg BW, and the SSZ group attenuated the body weight change compared to the DSS group. The DAI

score is one of the clinical parameters for the representation of the severity of colitis (7). The DAI score was markedly augmented in the DSS group. However, the PSO pretreatment groups for all doses and the SSZ group marked a significantly diminished DAI score when compared to the DSS group (Fig. 2B). The length of the colon was significantly shortened after induction of colitis by DSS (Fig. 2C-D), while PSO pretreatment (100 and 200 mg/kg BW) led to restoring the length of the colon, which affected the SSZ group similarly.

Effects of PSO pretreatment on DSS-induced colitis histopathological changes

Protective effects of PSO on DSS-induced colonic inflammation were also confirmed by histopathological assessment. As shown in Fig. 3A, architectural changes of colonic damage including crypt loss, inflammatory cell infiltrations, ulceration, and submucosal edema were markedly increased in the DSS group. In contrast, PSO pretreatment ameliorated the colon architectural damage and inflammatory cell infiltrations. Histopathological scores were thus significantly lower in the PSO pretreatment (100 mg/kg BW) and the SSZ group compared to the DSS group (Fig. 3B).

PSO pretreatment suppressed DSS-induced colitis by decreasing the inflammatory cytokine markers.

To determine whether PSO had a protective effect on DSS-induced proinflammatory cytokines' production, the level of IL-1β, IL-6, and TNF-α in the serum were determined by ELISA assay and mRNA expressions of inflammatory cytokine markers in the colon were determined using RT-qPCR analysis. All these pro-inflammatory cytokine markers in the serum level were significantly increased after DSS administration (Fig. 4). However, PSO pretreatment and the SSZ group markedly reduced the serum level of IL-1β (Fig. 4A), IL-6 (Fig. 4B), and TNF-α (Fig. 4C). Similarly, mRNA expression of IL-1β, especially at 200 mg/kg BW (Fig. 5A), IL-6 (Fig. 5B), and TNF-α (Fig. 5C) were also significantly decreased in comparison with those pro-inflammatory cytokines in the DSS group. In addition, the COX-2 produced excessive inflammatory mediators, which damaged the mucosal integrity and contributed to the expansion of intestinal injury (24). Our result showed that the COX-2 expression was markedly decreased by PSO pretreatment at 100 and 200 mg/kg BW (Fig. 5D) compared with that in the DSS group.

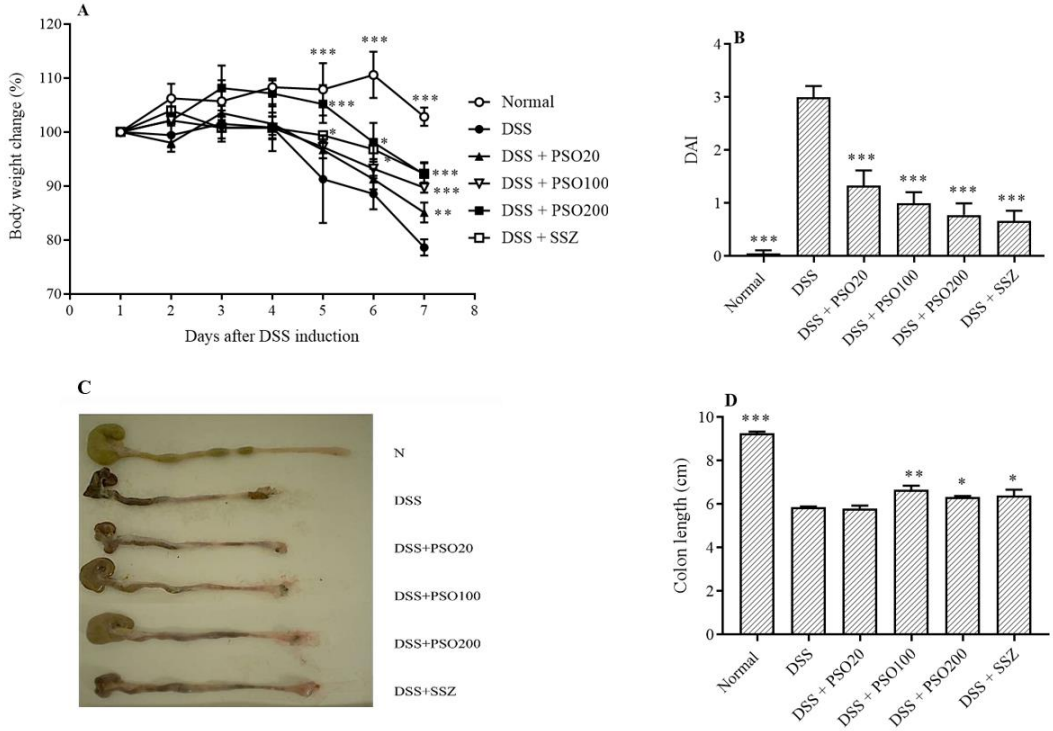

Fig. 2. Effects of PSO pretreatment on the severity of DSS-induced colitis in mice. (A) The body weight change; (B) DAI; (C) gross appearance of the colon tissue; and (D) the length of the colon. Data are expressed as mean ± SEM, n = 6. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate significant differences versus DSS group. PSO, *Perilla* seed oil; DSS, dextran sulfate sodium; DAI, disease activity index; SSZ, sulfasalazine.

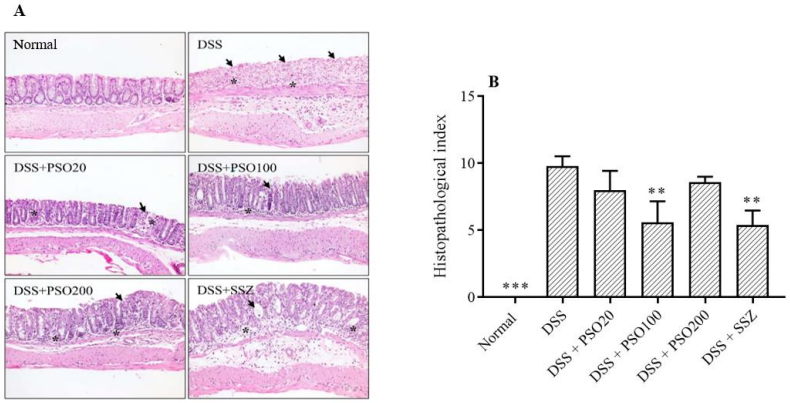

Fig. 3. Effect of PSO on histopathological changes of colonic inflammation induced by DSS. (A) Microscopic of the colon tissue damage induced by DSS (H&E staining; magnification ×200), the black arrow indicates crypt damaged area and the asterisk indicates the inflammatory cell infiltration. (B) The histopathological index was scored from the crypt loss, the intensity of area affected by inflammation, the extent of follicle aggregates, edema, ulceration/erosion in the mucosa, and the inflammatory cell infiltration. Data are expressed as mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences in comparison with DSS group. PSO, *Perilla* seed oil; DSS, dextran sulfate sodium; SSZ, sulfasalazine.

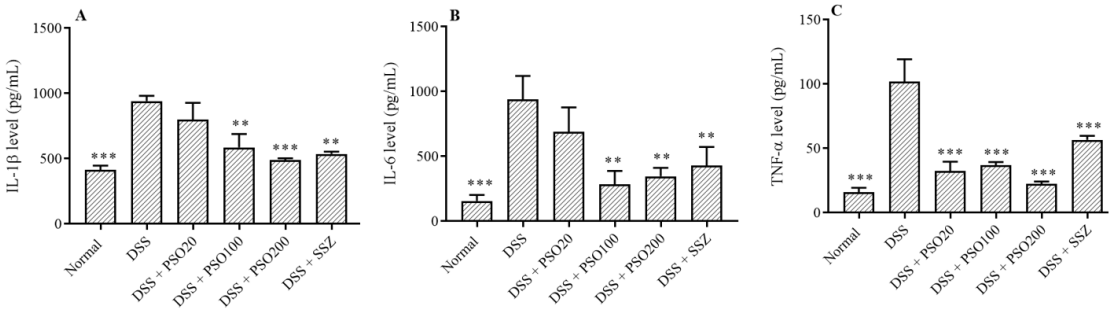

Fig. 4. Effect of PSO pretreatment on the level of pro-inflammatory cytokines in the serum. (A) IL-1β; (B) IL-6; (C) and TNF-α were determined by ELISA assay. Data are expressed as mean ± SEM, n = 3. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences in comparison with DSS group. PSO, *Perilla* seed oil; DSS, dextran sulfate sodium; SSZ, sulfasalazine; IL, interleukin; TNF, tumor necrosis factor.

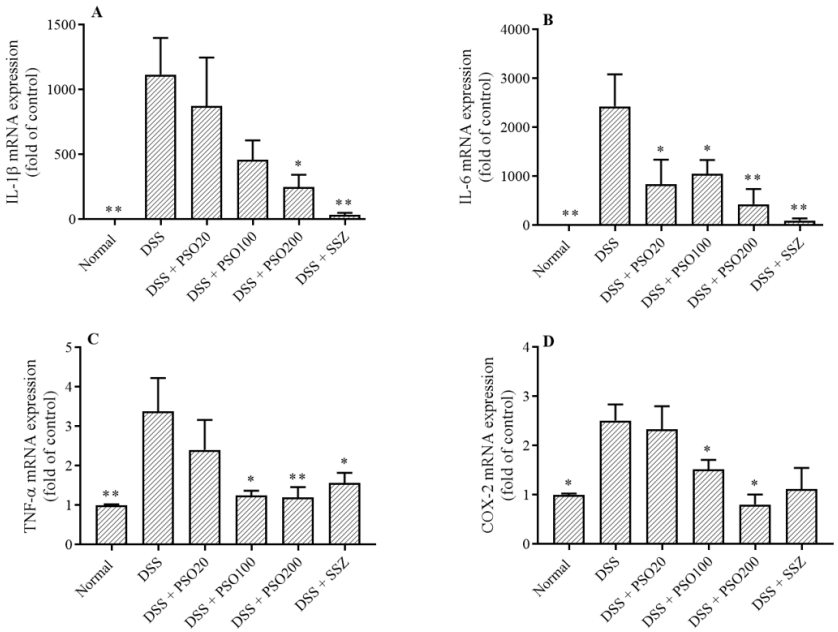

Fig. 5. The mRNA expression levels of inflammatory markers in the colon including (A-D) IL-1β, IL-6, TNF-α, and COX-2, respectively were determined using RT-qPCR analysis. Data are expressed as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate significant differences in comparison with DSS group. PSO, *Perilla* seed oil; DSS, dextran sulfate sodium; SSZ, sulfasalazine; IL, interleukin; TNF, tumor necrosis factor; COX, cyclooxygenase.

DISCUSSION

The present study clearly showed that the pretreatment of PSO had a strong protective effect on DSS-induced colitis in mice evidently by the reduction of the severity of clinical symptoms, histopathological changes, the expression, and the production of inflammatory cytokines. ALA, predominantly found in PSO, was mainly recommended to use in lowering the effect on colonic mucosal damage by anti-inflammatory mechanisms. Our results confirmed that pretreatment with PSO for a period of 4 weeks exerted anti-inflammatory effects. This treatment period was suitable for gastrointestinal absorption and availability for the conversion of ALA into EPA and DHA which have more potent anti-inflammatory properties (25). It was shown that by decreasing the dietary intake of LA/ALA ratio for 4 weeks, ALA could be increasingly converted into long-chain omega-3 FAs in human plasma and erythrocytes phospholipids (26). Accordingly, the administration of ALA extracted from fermented black radish (30 and 60 mg/kg/day) for 7 days alleviated DSS-induced UC by suppressing colon injury and inflammation (27). Therefore, the period of dietary omega-3 FA intake for 4 weeks would be sufficient and efficient for the management of IBD.

Wen *et al.* found that the protective effects of given ALA (150 mg/kg) dose on mouse colitis induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS) and suggested its possible underlying mechanisms of the interactions *via* the Th1/Th2/Th17 pathway (28). ALA from the diet could be incorporated into tissue membrane phospholipids or enzymatic conversion to EPA and DHA (29). The anti-inflammatory effects of EPA and DHA could be explained that suppressed the conversion of arachidonic acid to pro-inflammatory eicosanoids such as prostaglandins, leukotrienes, and lipoxins (30). These suggested that ALA was changed to EPA and DHA which subsequently interfered with the enzymatic synthesis of pro-inflammatory eicosanoids.

PSO is indeed fully rich in polyunsaturated FA (PUFAs) such as omega-3 FA (ALA) and omega-6 FA (LA). PUFAs were reported as health benefits in several ways such as suppressing inflammation, reducing serum cholesterol and triglyceride, and preventing colon cancer. The efficacies of PUFAs are dependent on the optimal ratio of omega-6 FA to omega-3 FA (LA:ALA) as evidently found in the animal studies using *Perilla* and other plant oils (12,16,17). A low ratio (about 1-5:1) of LA:ALA in the diet has particularly been recommended for health beneficial effects by several investigators (25,31). Therefore, PSO is one of the most beneficial plant oils due to its lowest ratio of LA:ALA, approximately 1:4 (16.55%:61.51%).

The present study demonstrated that pretreatment with ALA-rich PSO for 4 weeks clearly decreased the loss of body weight and restored the shortening of the colon. In addition, the severity of colitis was determined using the DAI score after DSS administration for a week. The DAI score was remarkably increased in the mice that received only DSS. However, PSO pretreatment showed a significantly lower DAI score than the untreated group. Several studies have reported that the DSS-induced colitis model could present histopathological changes, including colonic ulceration/erosion, loss of intestinal crypt, and infiltration of inflammatory cells (7). Our data similarly showed that PSO pretreatment (100 mg/kg BW) evidently attenuates histopathological change as well (Fig. 3). PSO pretreatment had a markedly preventive effect against DSS-induced colonic inflammation with PSO at the higher dose was more effective than the lower ones. Similarly, krill oil, which is rich in EPA and DHA, potentially protected colitis by DSS through the restoration of colon length and reduction of oxidative markers and cytokines as well as the increase of prostaglandins E_3 levels and anti-inflammatory transcription factors (32).

IBD patients and the DSS-induced colitis model have been associated with excessive pro-inflammatory cytokines and enzymes regulated by the activation of NF-κB and signal transducer and activator of transcription 3 pathway (23). Evidently, omega-3 FAs could be used in the management of IBD since it could attenuate the clinical symptoms and recovery of the mucosal damage by anti-inflammatory effect. It has been shown that Fat-1 transgenic

mice which rich in endogenous omega-3 FA protected DSS-induced colitis by reducing the expression of inflammatory cytokines (IL-1β, IL-6, and TNF-α) and inactivating NF-κB while enhancing the mucoprotective effect (33). Our results indicated that PSO strongly reduced the levels of IL-1β, IL-6, and TNF-α in the serum (Fig. 4A-C) and mRNA expression (Fig. 5A-C). Furthermore, COX-2 was induced in the area of inflammation in order to respond to the pro-inflammatory cytokines (24). This study found that PSO had an anti-inflammatory activity which was further confirmed by the significant decrease of the COX-2 expression (Fig. 5D), thereby indicating that PSO provided high efficacy in ameliorating acute colitis. The results obtained in our study were consistent with the previous data demonstrated that omega-3 FAs from other sources such as fish, krill, and flaxseed oils were capable of decreasing the degree of inflammation associated with experimental colitis. The dietary omega-3 FAs inhibited TNBS-induced colitis by suppressing the inflammatory markers including IL-1β, IL-6, TNF-α, COX-2, and leukotriene B4 (25). Similarly, the supplementation with sage oil, which rich in ALA, inhibited DSS or TNBS-induced inflammatory response *via* decreasing the expression of the pro-inflammatory markers including COX-2, IL-8, and inducible nitric oxide synthase (34). In the senescence-accelerated mouse model, diets rich in omega-3 FA from *Perilla* oil and fish oil significantly attenuated ileitis by down-regulating mRNA levels of IL-6, monocyte chemoattractant protein 1, and interferon-gamma (35). This study evidently confirmed that PSO which rich in ALA could reduce and prevent *in vivo* colitis by decreasing the inflammatory cytokines.

CONCLUSION

PSO has significant protective effects against experimental colitis induced by DSS in mice. ALA enriched in PSO is suggested to be mainly responsible for the prevention of DSS-induced colitis through suppressing inflammatory markers. For this reason, PSO could be further developed as a potential functional supplement to be beneficial for ameliorating colonic inflammation in alternative and complementary medicine.

Acknowledgments

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Author's contribution

N. Kangwan and M. Suttajit contributed to the concept, design experiments, manuscript preparation, and editing. N. Kangwan, C. Khanaree, K. Pintha, S. Kongkankha, and T. Chewonarin performed the experimental studies. N. Kangwan performed the data acquisition and statistical analysis. All authors had been read and approved manuscript to be published.

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