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Original Article

Effect of *Cleistocalyx operculatus* and *Morus alba* extracts on DSS-induced acute colitis in mice

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ABSTRACT

The inflammatory bowel disease (IBD) is a multifactorial disorder and complex. Previous studies have shown that *Cleistocalyx operculatus* Roxb. Merr. et Perry and *Morus alba* L. have anti-inflammatory properties. This study aims to evaluate the effects of combined *Cleistocalyx operculatus* Roxb. Merr. et Perry and *Morus alba* L. (**CM**) extracts on dextran sulfate sodium (DSS)-induced acute colitis in mice. The combined *Cleistocalyx operculatus* Roxb. Merr. et Perry and *Morus alba* L. leaves were extracted with 50% ethanol and then suspended in water and partitioned successively with ether and ethyl acetate. The ethyl acetate extract was combined and evaporated to obtain **CM** extract. Mice were induced colitis, by using DSS for seven days. Mice were treated with combined **CM** extracts at doses of 150 mg/kg/b.w. and 300 mg/kg/b.w. Throughout the experiment, the disease activity index (DAI) was evaluated. Following the last day of treatment, blood samples were collected for analysis of IL-1β, INF-γ and TNF-α by using a sandwich enzyme-linked immunosorbent assay (ELISA) method. The **CM** extract significantly decreased the disease activity index and myeloperoxidase activity in the **CM** extract-treated mice as compared with DSS group mice. The combined **CM** extracts also suppressed the levels of pro-inflammatory IL-1β, INF-γ and TNF-α in colon tissues of the **CM** extract-treated mice as compared with DSS group ($p < 0.05$). Our results showed that the combined **CM** extract has strong beneficial effects on colitis and could be used for the treatment of IBD.

KEYWORDS: *Cleistocalyx operculatus* Roxb. Merr. et Perry, *Morus alba* L., dextran sulfate sodium, colitis, inflammatory bowel disease.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a group of inflammatory conditions of the colon and small intestine [1]. Crohn's disease and ulcerative colitis are the principal types of inflammatory bowel disease [2]. IBD can be acute or chronic. IBD symptoms include abdominal pain, mild to serve pain (depending on the stage of the disease), diarrhea, blood in the stool, loss of bowel movements, flatulence, fatigue, anorexia and unexplained weight loss [2]. In mild cases, painful inflammatory lesions appear. In severe cases, ulcers, hemorrhage, or possibly abscesses in the colon may appear. Persistent colitis is difficult to treat completely and greatly affects the physical health and functional activities of patients. The etiology and pathophysiology of IBD are still poorly understood, but they can include a number of causes such as intestinal infection, persistent constipation, some intestinal diseases such as colon ischemia, enteritis, side effects of drugs including antibiotics, arsenic poisoning, lead, mercury, herbicides, psychological stress, Crohn's disease and tuberculosis. Molecular pharmacology study has shown that in an IBD patient, there is an increase in infiltrating neutrophils.

Moreover, IBD patients have also experienced increased expression of pro-inflammatory cytokines such as interleukin (IL)-1β, interferon (INF)-γ and tumor necrosis factor (TNF)-α [1]. Reducing the occurrence of neutrophils and these pro-inflammatory factors contributes to the reduction of colon disease. Medicinal plants have some advantages in the treatment of diseases, such as health safety, no side effects, and relieving the symptoms of IBD [3]. Medicinal plants are used for acute and chronic gastrointestinal diseases, including IBD and colitis. Many medicinal plants with antioxidant, anti-inflammatory and antibacterial activities for the prevention and treatment of IBD are increasingly used, providing long-term effects in the treatment of the disease [4]. In particular, the use of combinations of medicinal plants will increase their effects in individuals.

Cleistocalyx operculatus belongs to the Myrtaceae family. *C. operculatus* is a well-known perennial tree, and is widely distributed throughout China, Vietnam and other tropical countries. The phytochemical composition of the plant contains tannins, vitamins and some minerals, essential oils, active substances polyphenol and flavonoids [5]. The leaves and buds of *C. operculatus* have been used as an herbal tea for gastrointestinal disorders [6]. The leaves of *C. operculatus* are effective in reducing gas, bloating, indigestion, chronic colitis, dull abdominal pain, diarrhea, dermatitis, itching, lowering blood fat, cholesterol, diabetes and hepatitis.

The mulberry tree, *Morus alba* L. (Mulberry), is a plant of the family Moraceae and the genus *Morus* [7]. Phytochemical reports on *M. alba* L. showed that the plant contains flavonoids, tannins, triterpenes, anthocyanins, anthraquinones, phytosterols, sitosterols, benzofuran derivatives, morusimic acid, oleanolic acid, alkaloids, steroids, saponins, and phenolic compounds [8]. *Morus alba* L. leaves have various pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, cytotoxic, antihyperglycemic, hypolipidemic, anti-diabetic, α-glucosidase inhibition, anti-atherosclerotic, anti-obesity and cardioprotective [7]. However, until now there has been no study to show the anti-colitis effect of the combination of these two plants. This study aimed to evaluate the effects of combined *Cleistocalyx operculatus* and *Morus alba* (**CM**) extract on dextran sulfate sodium (DSS)-induced acute colitis in mice. The disease activity index (DAI) and myeloperoxidase activity (MPO) is measured to assess the inflammatory response as an index of neutrophil infiltration in the mucosa. Cytokines including pro-inflammatory IL-1β, INF-γ and TNF-α were also determined.

MATERIALS AND METHODS

Preparation of CM extract

The dried leaves of *Cleistocalyx operculatus* Roxb. Merr. et Perry and *Morus alba* L. were collected in July 2020 from Bac Ninh province, Vietnam. The raw powder of the leaves of *Cleistocalyx operculatus* and *Morus alba* leaves were mixed in the ratio of 1:1 to obtain 500 g. This combined material was extracted by using ethanol 50% by heating for 1 hour (5 L × 3 times). The ethanol extract was combined, filtrated, and evaporated to dryness *in vacuum* at 50 °C, giving 45 g of the total ethanol extract. The total ethanol extract was suspended in water and partitioned successively with ether and ethyl acetate. The ethyl acetate extract was combined and evaporated to dryness *in vacuum* at 50 °C to obtain 35 g (**CM** extract). The yield was about 7%.

Animals and feeding regimens

Studies were carried out using adult *Swiss* albino mice of both sexes; the average weight was 25 ± 2 g. The mice were obtained from the National Institute of Hygiene and Epidemiology. The animals were grouped and housed in cages with not more than ten animals per cage and maintained under standard laboratory conditions of humidity (50 ± 5%), temperature (25 ± 2 °C) with dark and light cycle (12/12 h). The animals were fed with a standard pellet diet supplied by the National Institute of Hygiene and Epidemiology and fresh water *ad libitum*. All of the mice were acclimatized to laboratory conditions for a week before the commencement of the experiment.

Mice were randomly divided into four groups each with ten individuals per group. The animals were orally administrated for seven successive days as follows:

Just before starting, animals were randomly divided into four groups: Control, DSS, CM150 and CM300. The control group received water *ad libitum* as

vehicle and standard diet administration; DSS group received the solution of 3% (w/v) dextran sodium sulfate (DSS; molecular weight 36-50 kDa, MP Biomedicals, Vietnam) as vehicle and the standard diet administration; CM150 and CM300 groups received the solution of 3% (w/v) DSS and were treated with **CM** extract at doses of 150 and 300 mg/kg/b.w., respectively.

Water consumption was measured daily to ensure that equivalent amounts of DSS were consumed. Throughout the experiment, the Disease Activity Index (DAI) was evaluated. The animals were sacrificed using an overdose of anesthetic. Colon tissues of mice were removed, weighed and homogenized in the buffer system Nonidet-P40 (NP40) including 150 mM sodium chloride, 1.0% NP-40 and 50 mM Tris-HCl, pH 8.0. Then, samples were centrifuged at 12,000 g, for 15 minutes at 4 ^{o}C and the supernatant was collected for analysis. Protein concentration was analyzed by Bradford's method.:

Disease activity index (DAI)

Bodyweight, stool consistency and presence or absence of blood in stool were recorded every day. The disease activity index was measured daily using the protocol described in our previous study [9]. DAI is the average of weight loss, stool consistency and stool blood scores. Each score is determined as follows: change in body weight (0: < 1%; 1: 1–5%; 2: 5–10%; 3: 10–15%, 4: >15%), stool consistency (0: normal, 2: soft, 4: diarrhea) and presence or absence of fecal blood (scored as: 0, negative hemoccult test; 2, positive hemoccult test; 4, gross bleeding). Bodyweight loss was calculated as the percentage difference between the original body weight (day 0) and the bodyweight on any particular day.

Myeloperoxidase activity

Myeloperoxidase (MPO) is an enzyme found in neutrophils. The MPO activity, as a marker of inflammatory cell infiltration, was used as a convenient and valuable tool in evaluating the anti-inflammatory activity of CM extract. MPO activity, which is an indicator of polymorphonuclear leukocyte accumulation, was determined as previously described [9]. Colon tissues were suspended in potassium phosphate buffer (pH 6.0) containing 0.3% hexadecyltrimenthyl ammonium bromide and a cocktail of protease inhibitors (Sigma). Samples were homogenized on ice and sonicated. Following sonication, suspensions were centrifuged at 15000 x g for 15 minutes at 4 °C, and the supernatant was collected. Potassium phosphate buffer (pH 6.0; 625 µL) containing 0.5 mM O-dianisidine dihydrochloride (D3252 Sigma-Aldrich, Vietnam) was added to 125 µL of supernatant and 0.05% hydrogen peroxide. Changes in absorbance at 450 nm were recorded with a spectrophotometer every 30 seconds over 3 minutes. MPO was expressed in units per milligram of tissue, where 1 unit corresponds to the activity required to degrade 1 µmol H_2O_2/min/mL at 37 °C.

Measurement of cytokines

Single-use aliquots of the homogenate colon were used to measure cytokine levels. Colon's IL-1β, INF-γ and TNF-α were determined with a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturers' instructions. Analysis of cytokine IL-1β, INF-γ and TNF-α was performed using a sandwich ELISA method. Briefly, 96-well plates were coated overnight with 100 µl of monoclonal antibody against IL-1β (2,0 µg/ml) or IFN-γ (3,0 µg/ml) or TNF-α (1,0 µg/ml) or in PBS 1x buffer at 4 ^{o}C (pH 7.2). The plate was then washed four times with wash buffer (PBS 1x + 0.05% Tween-20), blotted dry, and then incubated with blocking solution (PBS 1x + 1% bovine serum albumin) for 1 h. The plate was then washed and 100 µl of each homogenate sample or standard was added. Then the plate was incubated at room temperature for 2 h, followed by washing, and 100 µl of detection antibody IL-1β (0.5 µg/ml) or IFN-γ (0.3 µg/ml) or TNF-α (0.25 µg/ml) was added. The antibody was incubated at room temperature for 2 h. Following additional washing, 100 µl of avidin-HRP (horseradish peroxidase) conjugated (1:2000) was added to each well, followed by a 30 min incubation. After thorough washing, plate development was performed using ABTS (2,2'- Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]- diammonium salt) liquid substrate solution. Then the plate was incubated at room temperature for color development and the color was monitored using a microplate reader at 405 nm with wavelength correction set at 650 nm. The standard curve for the ELISA was established by

using murine standard IL-1β, IFN-γ or TNF-α diluted in PBS 1x buffer. All standard curves obtained an r2 value between 0.98 and 1. Results were normalized to total protein content in the colon samples. Total protein content was determined by Bradford's method. Data are reported as cytokines per milligram protein.

Statistical analysis

All results were expressed as mean ± SEM (n = 10 animals/ group). Serial measurements were analyzed by using Student's t-test or two-way ANOVA with Bonferroni's *post hoc* test using SigmaStat 3.5 program (Systat Software Inc). The critical significance level α was established at 0.05, then, statistical significance was defined as $p < 0.05$.

RESULTS

Effects of the CM extract in DSS-induced acute colitis

The DAI was used to analyze the therapeutic benefit of **CM** extract. In the group of mice treated with DSS, we observed body weight loss and diarrhea with bloody stools, which resulted in an increase in the DAI from day 2 onwards. The mice treated with DSS had significantly increased DAI index as compared with control healthy mice. However, the DAI index value in mice treated with **CM** extract at the doses of 150 and 300 mg/kg/b.w was decreased significantly as compared to the DSS group mice (Figure 1). Thus, the **CM** extract has the effect of reducing the DAI index induced by DSS-induced acute colitis in mice.

Effects of the CM extract on MPO activity in colon homogenates

Neutrophils are the first cells to participate at the site of inflammation. They have a critical role in the innate immune response and associate with lymphocytes to raise epithelial dysfunction and injury associated with IBD. MPO activity, a marker of inflammatory cell infiltration, was used as a convenient and valuable tool in evaluating the anti-inflammatory activity of **CM** extract. We observed that the MPO activity was significantly higher in the DSS group as compared with the control group mice. Interestingly, the MPO activity in mice treated with **CM** extract at both the doses of 150 and 300 mg/kg/b.w was decreased significantly as compared to the DSS group mice (Figure 2). Our study showed that **CM** extract demonstrates a protective role in mouse models of IBD by reduced mucosal neutrophil infiltration.

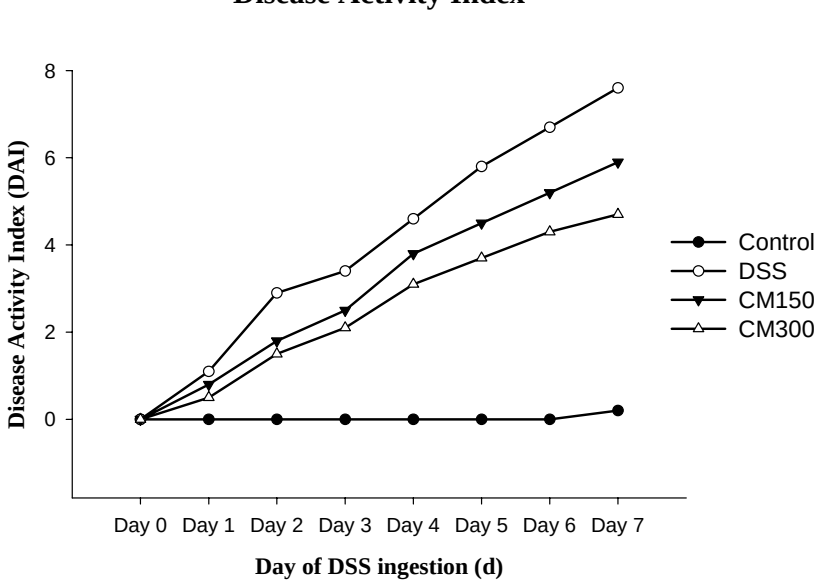

Figure 1. Disease activity index (DAI) of colitis in four experimental groups of mice. The DAI value was calculated as described in the 'Materials and methods' section (n = 10 per group).

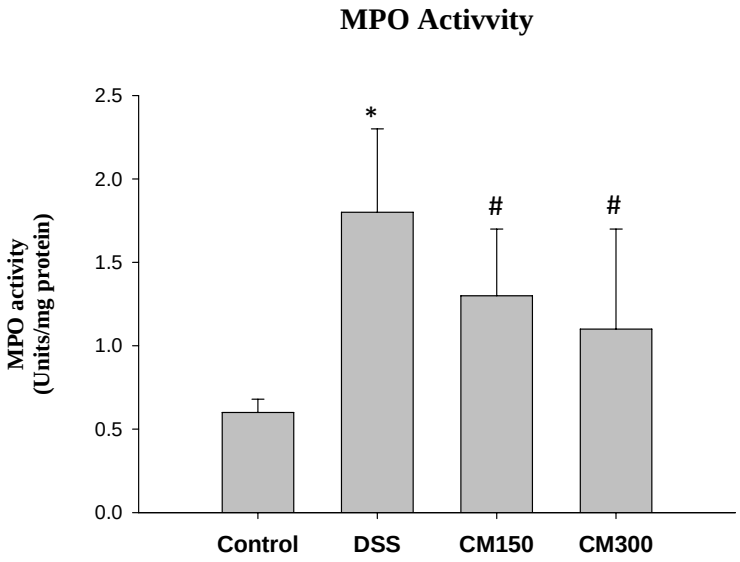

Figure 2. MPO activity in colon homogenates**.** The MPO activity was determined as indicated in the 'Materials and methods' section. *Significantly different between DSS group and control group ($p < 0.05$). #Significantly different between DSS group and the CM150 and CM300 groups ($p < 0.05$).

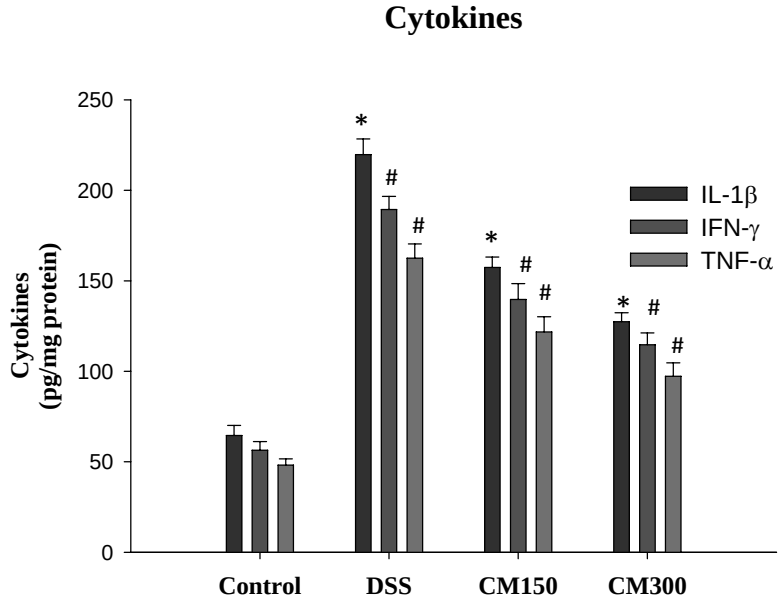

Figure 3. Cytokine expression in colons from four groups. Cytokines were measured by ELISA. *Significantly different between DSS group and control group ($p < 0.05$). #Significantly different between DSS group and the CM150 and CM300 groups ($p < 0.05$).

CM extract inhibits cytokine release of DSS-induced acute colitis

Colonic levels of the pro-inflammatory cytokines IL-1β, INF-γ and TNF-α were determined (Figure 3). The levels of IL-1β, INF-γ and TNF-α were significantly increased after 7 days in DSS group mice as compared with the control group. In group CM150 and CM300, the levels of IL-1β, INF-γ and TNF-α were significantly decreased as compared with the DSS group. Therefore, our

study showed that **CM** extract demonstrates a protective role in mouse models of IBD by reduced pro-inflammatory cytokines.

DISCUSSION

Cleistocalyx operculatus Roxb. Merr. et Perry and *Morus alba* L. have been widely used in Vietnamese traditional medicine. Many beneficial pharmacological effects of *Cleistocalyx operculatus* Roxb. Merr. et Perry and *Morus alba* L. have been described. They include antioxidant, anti-inflammatory, antihyperglycemic, hypolipidemic activities, etc [10, 11]. It is an attractive approach for the treatment of various inflammatory disorders, such as ulcerative colitis [12]. Inflammatory bowel disease (IBD) encompasses a range of intestinal pathologies, the most common of which are ulcerative colitis (UC) and Crohn's Disease (CD). Both UC and CD, when present in the colon, generate a similar symptom profile which can include diarrhea, rectal bleeding, abdominal pain, and weight loss [13]. DDS-induced colitis [14], an experimental model that mimics human IBD, has been widely studied and has shown high amounts of cytokines (TNF-α, IL-1β, IFN-γ) [15]. This model is also associated with an important inflammatory infiltration of neutrophils into the colon [16]. Our study has demonstrated that DSS-induced colitis caused a critical degree of inflammation with an increase in a series of cytokines such as TNF-α, IL-1β, IFN-γ and infiltration of neutrophils. These changes were significantly reduced in mice treated with CM extract. Our study showed that the anti-inflammatory effects of CM extract involve a reduction in MPO activity and cytokines. Our results are consistent with previous data using different colonic models' and dosages which demonstrated that ingredients in Mulberry and *C. Operculatus* are capable of decreasing the degree of inflammation associated with experimental colitis [17]. Yang Wang *et al*. showed the beneficial effects of mulberry against ulcerative colitis in DSS-induced colitis in mice. These authors have demonstrated that the DAI, histological colitis score and the MPO activity were all significantly higher in DSS-treated mice than in DSS plus mulberry-treated mice. Moreover, Zhengjiang Qian *et al.* elucidated that mulberry fruit attenuated LPS-induced inflammatory responses by blocking the activation of both NF-κB/p65 and pERK/ MAPK pathways [12]. Neutrophils are the first cells to participate at the site of inflammation. They have a critical role in the innate immune response and associate with lymphocytes to raise epithelial dysfunction and injury associated with IBD. Our data have shown that the CM extract decreased the level of neutrophil infiltration by reducing MPO activity [18]. Proinflammatory cytokines such as IL-1β, IFN-γ and TNF-α are induced by the immune cells after activation of NF-κB in colitis tissue. These proinflammatory cytokines play a critical role in the pathogenesis of IBD. They increase the inflammatory response of inflammatory mediators, destructive enzymes and increase the free radicals that cause tissue damage [19]. In our study, we have shown that the level of TNF-α, IL-1β, and INF-γ was also reduced in **CM** extract-treated mice colitis. A previous study showed that chronic colitis in mice fed mulberry extract could be attenuated by decreasing the mRNA expression of IL-1β and NLRP3 inflammasomes, suggesting that mulberry fruit acts as a powerful antioxidant and cancels the ROS influence in the colonic microenvironment [17]. Another study showed that the essential oil of the *C. operculatus* buds significantly inhibited lipopolysaccharide (LPS)-induced secretion of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), in RAW 264.7 cells, a mouse macrophage-like cell line. Also the mRNA expression of TNF-α and IL-1β was suppressed by treatment with the essential oil of the C. operculatus buds in LPS-stimulated RAW 264.7 cells. Moreover, reporter gene analysis revealed that the essential oil of the C. *operculatus* buds significantly blocked LPS-induced transcriptional activation of NF-kappaB in RAW 264.7 cells [20].

CONCLUSION

In conclusion, our study has demonstrated that the CM extract could reduce DSS-induced colitis in mice. Our results suggest that the CM extract would be a promising therapeutic for the treatment of IBD because of its ability to modulate pathophysiological activity during colonic inflammation.

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AUTHORS' CONTRIBUTIONS

Nguyen, T. T., Bui, T. T. conceived and designed the study. Nguyen, T. T., Bui, T. T. and Phan, H. M. participated in the writing process. Bui, T. T., Bui, T. M., Duong, T. H. L. and Phan, H. M. performed general experiments. Nguyen, T. T., Phan, H. M. and Bui, T. T. read and approved the final manuscript.

ETHICAL CONSIDERATIONS

All animal handling and procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 85–23, revised 1996). The current protocol was confirmed by the Ethical Committee of VNU University of Medicine and Pharmacy, Vietnam National University Hanoi and followed the international rules for animal research.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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