

Original Research Article

***Curcuma longa* extract suppresses inflammation in mice with DSS-induced acute colitis**

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Abstract

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The etiology and pathophysiology of inflammatory bowel disease (IBD), which is a multifactorial disorder, remain poorly understood. *Curcuma longa* extract has been demonstrated to exhibit anti-inflammatory activity in the literature. Here, we examined the effect of *C. longa* extract on dextran sulfate sodium (DSS)-induced colitis over a period of 7 days. The extract (150 mg/kg/b.w.) was mixed with the diet of the mice. *C. longa* significantly reduced the Disease Activity Index and myeloperoxidase activity. The extract also suppressed the levels of pro-inflammatory IL-1 β , IL-6, IL-17, INF- γ , TNF- α and increased the level of IL-10. Additionally, the protein expression levels of COX-2 and iNOS were reduced by *C. longa* extract. Our results suggest that *C. longa* extract exerts beneficial effects on experimental colitis; therefore, we propose that this extract may have therapeutic implications for ulcerative colitis.

Keywords: *Curcuma longa* extract, Colitis, DSS, Inflammation, Mice

INTRODUCTION

Inflammatory bowel diseases (IBDs) are chronic inflammatory disorders of the gastrointestinal tract. IBDs cause abdominal pain, fever, diarrhea with blood and mucus and increase risk of colon cancer. The etiology and pathophysiology of IBD is still poorly understood. There are two main subtypes of IBD: Crohn's disease and ulcerative colitis (Podolsky 2002). Ulcerative colitis causes mucosal layer inflammation of the colon while Crohn's disease originates transmural inflammation and appears anywhere in the gastrointestinal tract (Wallace, McKnight et al. 1998). In IBD, neutrophils play important role in contributing to tissue injury. It has been reported an increase of infiltrating neutrophils in IBD (Bian, Guo et al. 2012). Moreover, the IBD is also characterized by an activation of the pro-inflammatory transcription factor nuclear factor- κ B (NF- κ B) and increased expression of pro-inflammatory cytokines such as interleukine (IL)-1 β , IL-6, IL-10, IL-17, interferon (INF)- γ , tumor necrosis factor (TNF)- α and enzyme cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) in immune cells (Brown and Mayer 2007).

Curcuma longa (Zingiberaceae), turmeric, is a plant belonging to the ginger family found in south and southeast tropical Asia. It is widely used for centuries as food and medicine. A great number of compounds have been identified as constituents in turmeric especially in rhizomes. *C. longa* rhizome contains phenolic compounds, terpenoids, polysaccharide and fatty acids. Curcumin and relative compounds as curcuminoids are the major components of *C. longa* rhizomes. They are responsible for the biology activity of *C. longa* (Ammon and Wahl 1991). Some studies showed that curcumin decreased degree of inflammation associated with experimental colitis (Sugimoto, Hanai et al. 2002, Salh, Assi et al. 2003, Ukil, Maity et al. 2003, Jian, Mai et al. 2005). However, the activity mechanism still remains and needs to be answered. Taking all into account, the aim of this study is to get a better understanding of effects and mechanism of *C. longa* 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. Cytokine such as IL-1 β , IL-6, IL-10, IL-17, INF- γ , TNF- α and the expression of iNOS, COX-2 were also determined.

MATERIAL AND METHODS

Preparation of *Curcuma longa* extract

The dried rhizomes of *Curcuma longa* (8 kg) were collected in July 2012 from Bac Ninh province, Vietnam and identified by Prof. Hai Nguyen Thanh (School of Medicine and Pharmacy, Vietnam National University, Hanoi). A voucher specimen (No. SMP-2013-0013) was deposited at the Herbarium of SMP, VNU. The dried rhizomes of *Curcuma longa* (8 kg) were extracted with ethanol (20 L \times 3 times) at room temperature for a week. After filtration, the combined ethanol extract was then concentrated to yield a dry residue (840 g) and the extract was stored at -20°C .

Animals and feeding regimens

A total of 36 eight-week-old male C57BL/6J mice were used in our study. Animals were housed into enriched environmental conditions in groups of 10 animals per polycarbonate cage in a colony room under a 12 h light/dark cycle (12:00 AM – 12:00 PM) under controlled temperature ($22 \pm 3^{\circ}\text{C}$) and humidity. All animals were maintained accordingly to a protocol approved by the Ethical Committee of the Vietnam National University and following the international rules for animal research.

Just before starting, animals were randomly divided in three groups: Control, DSS and (DSS+Ex). Control group received water *ad libitum* as vehicle and standard diet administration (AIN-93M); DSS group received the solution of 3% (w/v) dextran sodium sulfate (DSS; molecular weight 36-50kDa, MP Biomedicals, Vietnam) as vehicle and the standard diet administration; and (DSS+Ex) group received the solution of 3% (w/v) dextran sodium sulfate and the diet mixed with *C. longa* extract (150 mg/kg/b.w.).

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 anaesthetic.

Disease Activity Index

Body weight, stool consistency and stool blood were recorded every day. The disease activity index was measured daily using the protocol previously described by Cooper *et al.* (Cooper, Murthy *et al.* 1993). DAI is 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 hemocult test; 2, positive hemocult test; 4, gross bleeding). Body weight loss was calculated as the percent difference between the original body weight (day 0) and the body weight on any particular day.

Tissue homogenization

For determination of the cytokine and Western Blotting procedures, frozen colonic tissues were weighed and homogenized in ice-cold buffer (50 mM Tris-HCl, pH 7.5, 8 mM MgCl₂, 5 mM ethylene glycol bis (2-aminoethyl ether)-N,N,N',N'- tetraacetic acid (EGTA), 0.5 mM EDTA, 0.01 mg/ml leupeptin, 0.01 mg/ml pepstatin, 0.01 mg/ml aprotinin, 1 mM phenyl- methylsulfonyl fluoride (PMSF) and 250 mM NaCl). Homogenates were centrifuged (12,000 g, 15 min, 4°C) and the supernatants were collected and stored at -80°C . Protein concentration was determined by Bradford's method (Bradford 1976).

Myeloperoxidase Activity

Myeloperoxidase (MPO) is an enzyme found in neutrophils and, much less in monocytes and macrophages. The use of MPO activity as a marker of inflammatory cell infiltration was used as a convenient and valuable tool in evaluating the anti-inflammatory activity of extract. MPO activity, an indicator of polymorphonuclear leukocyte accumulation, was determined as previously described (Mullane, Kraemer *et al.* 1985). Colon tissues were suspended in potassium phosphate buffer (pH 6.0) containing 0.3% hexadecyltrimethyl ammonium bromide and a cocktail of protease inhibitors (Sigma). Samples were homogenized on ice and sonicated. Following sonication, suspensions were centrifuged at $15000 \times g$ for 15 minutes at 4°C , and 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 $\mu\text{mol H}_2\text{O}_2/\text{min/mL}$ at 37°C .

Measurement of cytokines

Single-use aliquots of the homogenates colon were used to study. Colon's IL-1 β , IL-6, IL-10, IL-17, INF- γ and TNF- α were determined with commercially available Enzyme-

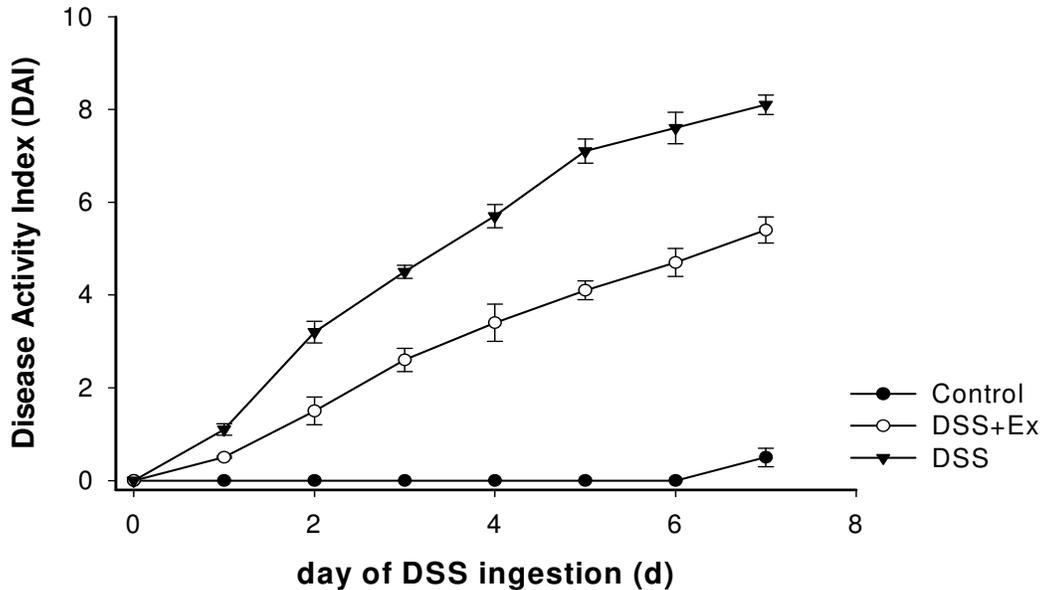


Figure 1. Disease activity index (DAI) of colitis in three experimental groups of mice. The DAI value was calculated as described in Materials and Methods (n=12 per group).

Linked Immuno Sorbent Assay (ELISA) (Thermo Fisher Scientific, Pierce, USA) kits according to the manufacturers' instructions. Analysis of cytokine IL-1 β , IL-6, IL-10, IL-17, IFN- γ and TNF- α were performed using a sandwich ELISA method. Briefly, 96-well plates were coated overnight at 4°C with 100 μ l of monoclonal antibody against IL-1 β (2,0 μ g/ml) or IL-6 (2,0 μ g/ml) or IL-10 (2,0 μ g/ml) or IL-17 (1,0 μ g/ml) or TNF- α (1,0 μ g/ml) or IFN- γ (3,0 μ g/ml) in PBS 1x buffer (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 μ l of each homogenate sample or standard was added. Then the plate was incubated at room temperature for 2 h, followed by washing, and addition of 100 μ l of detection antibody IL-1 β (0,5 μ g/ml) or IL-6 (0,5 μ g/ml) or IL-10 (0,5 μ g/ml) or IL-17 (0,25 μ g/ml) or TNF- α (0,25 μ g/ml) IFN- γ (0,3 μ g/ml). The antibody was incubated at room temperature for 2 h. Following additional washing, 100 μ l of avidin-HRP 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-1 β , IL-6, IL-10, IL-17 or TNF- α diluted in PBS 1x buffer. All standard curves obtained an r^2 value between 0.98 and 1. Results were normalized to total protein content in the liver

samples, determined by Bradford's method. Data are reported as cytokine per milligram protein. Cytokine standard curves were prepared in ELISA buffer, and samples from the tissue homogenates were calculated from these standard curves.

Western blot analysis

Equal amounts of protein homogenates (50 μ g) were separated on a 10% acrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Then, membranes were blocked with 5% skim milk dissolved in 0.5 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween-20 for 1 h at room temperature. The membranes were subsequently incubated with the primary antibodies anti-COX-2, anti-iNOS (Cell signaling, USA). After three washes with Tris-buffered saline with 0.1% Tween-20 (TBST), blots were incubated with secondary horseradish peroxidase conjugated goat anti-rabbit antibody (Calbiochem, Germany) in TBST with 5% skim milk at a 1:10,000 dilution for 1 h at room temperature. Membranes were then washed three times in TBST and developed using an enhanced chemiluminescence detection substrate ImmobilonTM Western Chemiluminescent HRP Substrate (Merk Millipore, Germany). Protein levels were visualized by the ChemiDocTM XRS+ System and compiled with Image LabTM 4.0.1 Software (Bio-Rad Laboratories, USA) for quantification. To control equal loading, the membranes were analysed for β -actin expression using an anti- β -actin antibody (Santa Cruz Biotechnology, CA, California, USA).

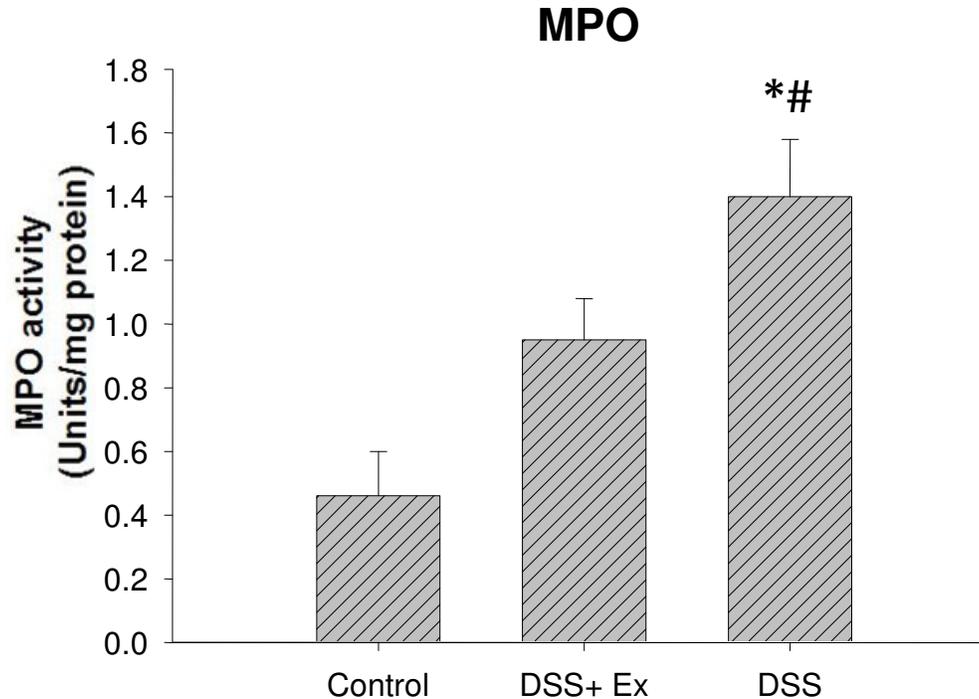


Figure 2. MPO activity in colon homogenates. The MPO activity was determined as indicated in Methods section. *Significantly different between DSS group and control group ($p < 0.05$). #Significantly different between DSS group and (DSS+Ex) group ($p < 0.05$). DSS, dextran sulphate sodium; MPO, myeloperoxidase.

Statistical analysis

All results were expressed as mean \pm SEM (n=12 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 and figureures were performed by using SigmaPlot, 10.0 programs (Systat Software Inc). The critical significance level α was established at 0.05 then, statistical significance was defined as $p < 0.05$.

RESULTS

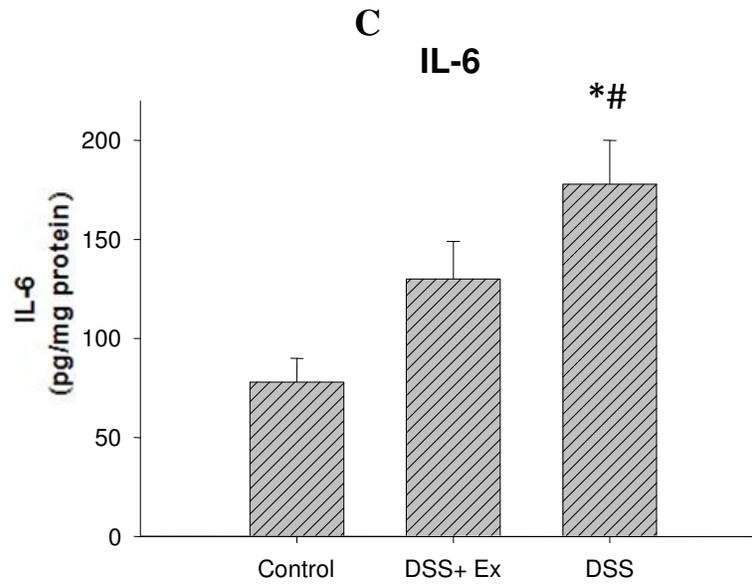
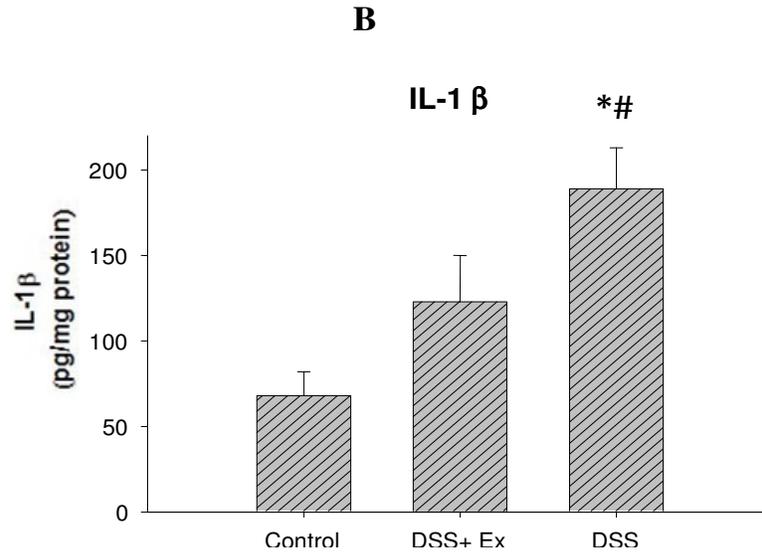
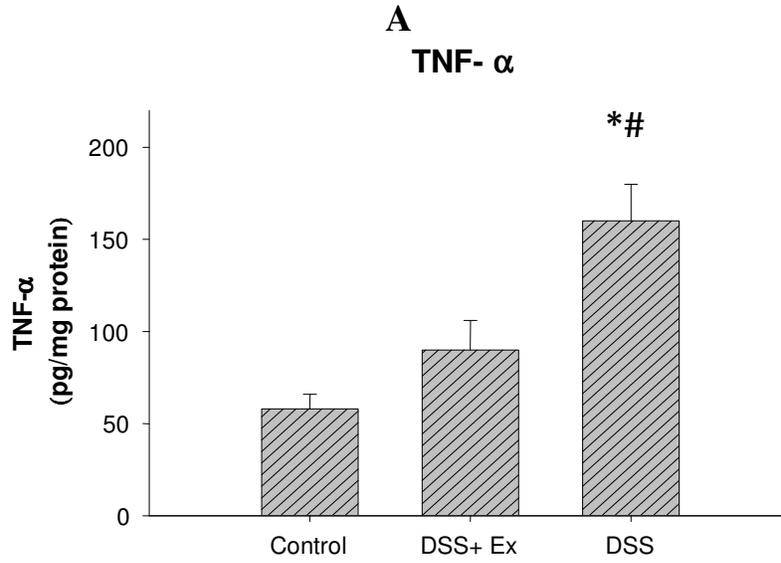
Effects of *C. longa* extract in DSS-induced acute colitis

A combination of DAI (body weight loss, stool consistency and stool blood) was used to analyze the therapeutic benefit of *C. longa* extract. In mice with DSS-induced acute colitis, we observed the body weight loss and marked significantly diarrhea with bloody stools, which resulted in an increase of the disease activity index (DAI) from day 2 onwards, compared with control healthy mice. However, we have observed that the DAI score was significantly decreased in (DSS + Ex) group as compared to DSS group by effect of *C. longa* (Figure. 1).

Polymorphonuclear leukocytes, mainly neutrophils are major contributors of tissue injury in colitis (Wallace, McKnight et al. 1998). Here we evaluated if *C. longa* extract reduced the neutrophil population in the intestinal mucosa. After seven days of the initiation of DSS treatment, mucosal neutrophils infiltration into the colon was determined indirectly by measuring MPO activity. Colonic samples taken from group DSS treated mice showed significantly increased MPO levels relative to group control healthy mice. Notably, *C. longa* extract has significantly reduced MPO levels in (DSS + Ex) group as compared to DSS treated group (Figure. 2). All indicate that *C. longa* extract attenuates the severity of DSS-induced acute colitis.

Curcuma longa extract inhibits cytokine release of DSS-induced acute colitis

Colonic levels of the pro-inflammatory cytokines TNF- α , IL-1 β , IL-6, IL-17, INF- γ and IL10 were determined. The level of TNF- α , IL-1 β , IL-6, IL-17 and INF- γ were significantly increased after 7 days of DSS-induced acute colitis (Figure. 3). In case of IL-10, the levels were decreased significantly after the treatment by DSS (Figure. 3F). In the (DSS + Ex) group, *C. longa* extract treatment significantly reduced the levels of TNF- α by



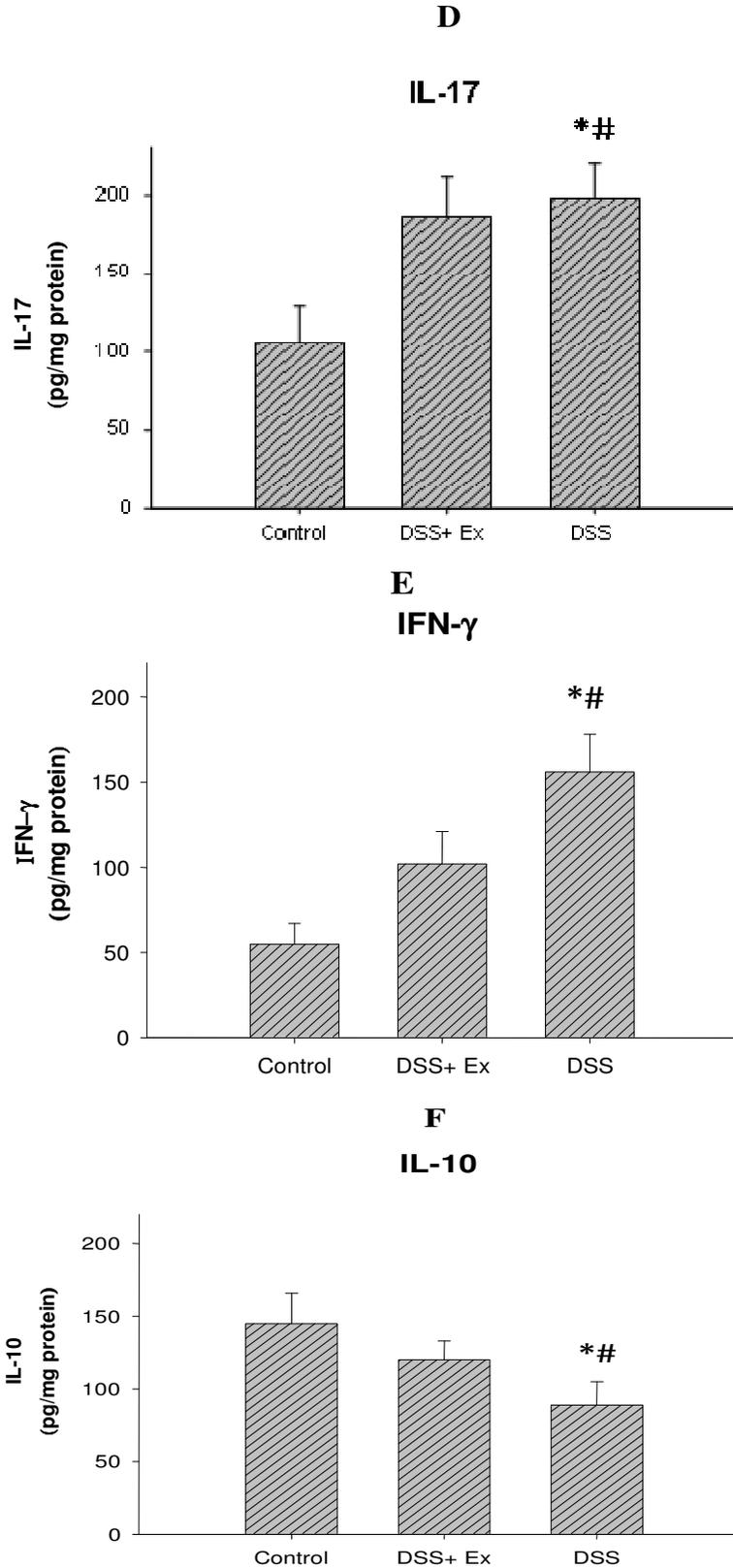


Figure 3. Cytokine expression in colons from three groups. Cytokines were measured by ELISA of colon homogenates as indicated in Method section. n=12 per group. *Significantly different between DSS group and control group ($p < 0.05$). #Significantly different between DSS group and (DSS+Ex) group ($p < 0.05$).

44%, IL-1 β by 35%, IL-6 by 27%, INF- γ by 36% and incremented the levels of IL-10 by 35% ($P < 0.05$) as compared to DSS treated group in colon tissue (Figure. 3A, B, C, E, F). In the case of IL-17, the levels tended to decrease 6% by *C. longa* extract treatment in (DSS + Ex) group as compare as DSS treated group (Figure. 3D).

***C. longa* extract treatment also inhibits COX-2 and iNOS levels of DSS-induced acute colitis**

We also determined the expression of other factors in inflammation such as COX-2 and iNOS levels. As in the case of cytokines, colonic levels of COX-2 and iNOS in mice increased by DSS induction (Figure. 4A and B). And the treatment of *C. longa* extract in (DSS + Ex) group significantly reduced significantly the levels of COX-2 and iNOS as compared to DSS treated group (Figure. 4A & B).

DISCUSSION

Ulcerative colitis and Crohn's disease are main type of inflammatory bowel disease that affects millions of people worldwide. It is characterized by chronic uncontrolled inflammation of intestinal mucosa. However, the pathogenesis of IBD is still not completely understood. Researchers suggest that the imbalance between pro-inflammatory cytokines, such as TNF- α , IL-1 β , IL-6, IL-17, INF- γ and anti-inflammatory cytokines, such as IL-10, plays a pivotal role in colitis inflammation (Ardizzone and Bianchi Porro 2005). Previous study showed that in IBD the inflammatory responses began with an infiltration of neutrophils and macrophages (Hanauer 2006). Then, the activated macrophages produce a potent combination of broadly active inflammatory cytokines, including TNF- α , IL-1 β , IL-6 (Podolsky 2002).

C. longa rhizome has been widely used in Vietnamese traditional medicine. Curcumin and relative compounds are the major components of *C. longa* rhizome. Many beneficial pharmacological effects of curcumin have been described. They include antioxidant, antiinflammatory, anticarcinogenic, antibacterial, antitumor, hepatoprotective activities, etc. It is an attractive approach for the treatment of various inflammatory disorders, such as ulcerative colitis (Ammon and Wahl 1991).

DSS-induced colitis, an experimental model that mimics human IBD, has been widely studied and showing high amounts of cytokines (TNF- α , IL-1 β , INF- γ , IL-16, IL-17) but low levels of IL-10. This model is also associated with an important inflammatory infiltration of neutrophils into the colon (Stack and Hawkey 1997).

Our study has demonstrated that DSS-induced colitis caused a critical degree of inflammation with an increase of series cytokine and tissue injury in the mouse colon,

which was characterized by infiltration of inflammatory cells, neutrophils, by increased the MPO activity. These changes were significantly reduced in mice treated with *C. longa* extract. The study showed that the anti-inflammatory effects of *C. longa* extract involve a reduction in MPO activity, cytokine and enzyme COX-2, iNOS expression. Our results are consistent with previous data using different models of colonic models and dosages which demonstrated that curcumin is capable of decreasing the degree of inflammation associated with experimental colitis (Sugimoto, Hanai et al. 2002, Salh, Assi et al. 2003, Ukil, Maity et al. 2003, Jian, Mai et al. 2005). Deguchi *et al.* showed that curcumin compounds reduced significantly the development of DSS-induced colitis. The 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 curcumin-treated mice (Deguchi, Andoh et al. 2007). Also in DSS plus curcumin-treated mice, the microscopically, mucosal edema, cellular infiltration, and epithelial disruption were much less severe than in DSS-treated mice. Similar, Zeng Z *et al.* have showed that curcumin improves colonic inflammation in a rat colitis model through inhibition of the TLR4/NF- κ B signaling pathway and IL-27 expression (Zeng, Zhan et al. 2013).

Neutrophils are the first cells to enroll at the site of inflammation. They have critical role in the innate immune response and associating with lymphocytes to raise epithelial dysfunction and injury associated with IBD. Our data have showed the *C. longa* extract decreased the MPO activity, which is indirectly index expression of level neutrophil infiltration. In previous study showed that curcumin demonstrates a protective role in mouse models of IBD and in human ulcerative colitis by a reduced mucosal neutrophil infiltration. Curcumin attenuated the expression and secretion of IL-1 β , keratinocyte chemoattractant and macrophage inflammatory protein 1 α in colonic epithelial cells and in macrophages. Curcumin also inhibited the neutrophil migration (Larmonier, Midura-Kiela et al. 2011).

Pro-inflammatory cytokines such as IL-1 β , IL-6, IL-17, INF- γ and TNF- α are induced by the immune cells after activation of NF-B in colitis tissue. These pro-inflammatory cytokines play critical role in the pathogenesis of IBD. They increase inflammatory response of inflammatory mediators, destructive enzymes and free radicals that cause tissue damage (Podolsky 2002). Cyclooxygenase-2 (COX-2), a key enzyme of prostanoid biosynthesis, is an inducible cyclooxygenase that its production is stimulated by IL-1 β , TNF- α , and many other mediators (Sakamoto 1998). It has been described the overexpression protein and mRNA level of COX-2 in colitis. Also the enzyme inducible nitric oxide synthase (iNOS) is an enzyme dominantly expressed during inflammatory reactions in IBD. Overproduction of nitric oxide by the inducible form

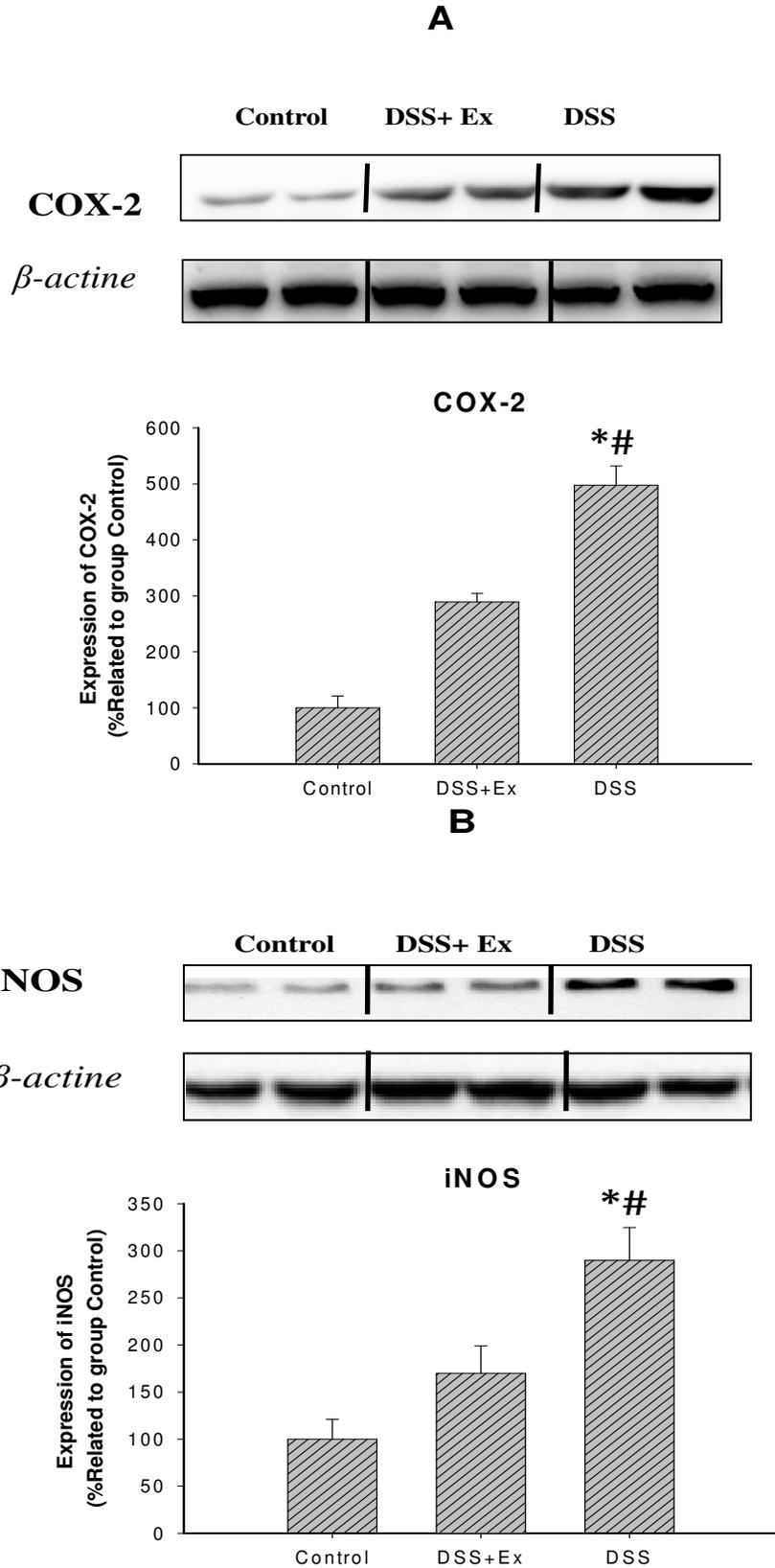


Figure 4. Protein expression in colons from three groups by WB. Protein levels of COX-2 and iNOS were determined by WB as indicated in Method section
^{*}Significantly different between DSS group and control group ($p < 0.05$).
[#]Significantly different between DSS group and (DSS+Ex) group ($p < 0.05$).

of iNOS has been implicated in colitis (Ogawa, Yamamoto et al. 2001). These end points iNOS, COX-2 are key mediators of colitis (El-Medany, Mahgoub et al. 2005). We have showed that the protein expression of both iNOS and COX-2 was also reduced in mice DSS-induced acute colitis consuming *C. longa* extract. Curcumin has been shown to attenuate chronic colitis by decreasing the expression of COX-2 and iNOS and increasing prostaglandin E2 (Camacho-Barquero, Villegas et al. 2007).

There are many factors have been involved in the IBD. Among these factors, the major transcription factors involve in the proinflammatory gene regulation is NF- κ B. NF- κ B is generally considered to be present in the cytoplasm as a heterodimer complex of p65/p50 subunits combined with an inhibitory protein, I κ B. Inflammatory stimuli induce rapid degradation of I κ B, and subsequently the free NF- κ B molecule translocates into the nucleus, binds to target DNA elements and activates transcription of genes encoding proteins involved with inflammation responses (Baldwin 1996). The previous report from author Deguchi has showed that NF- κ B activation, which transcriptionally regulates iNOS and COX-2, was inhibited in the mucosa by curcumin in DSS plus curcumin-treated mice (Deguchi, Andoh et al. 2007).

In conclusion, our study has demonstrated that anti-inflammatory effects of *C. longa* extract in DSS-induced experimental colitis. The mechanism may be mediated by the inhibition of cytokine, COX-2 and iNOS activity. Our results suggest that the *C. longa* extract would be a promising therapeutic for the treatment of IBD by the ability of modulation of pathophysiological activity during colonic inflammation.

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Conflict of interest

None

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