

Original Research Article

Thymol exerts anti-inflammatory effect in dextran sulfate sodium-induced experimental murine colitis

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Abstract

Purpose: To investigate the effect of thymol on dextran sulfate sodium (DSS)-induced experimental colitis in mice.

Methods: Colitis in mice was induced by drinking water with 2.5 % (w/v) DSS. The levels of myeloperoxidase (MPO), malondialdehyde (MDA), glutathione (GSH) and superoxide (SOD) in colonic tissues were determined using commercial kits. Histological changes were evaluated by hematoxylin and eosin staining. The production of nitric oxide (NO) was measured by Griess assay. The levels of pro-inflammatory cytokines including TNF- α , IL-1 β and IL-6 were assessed by quantitative PCR and ELISA kits. Nuclear factor-kappaB (NF- κ B) pathway activation was determined by Western blot.

Results: Thymol markedly reduced disease activity index (DAI) scores, and recovered the colon length. Histological damage and MPO levels in the colonic tissues were markedly inhibited ($p < 0.05$) by thymol, which also reduced mRNA expressions of TNF- α , IL-1 β and IL-6 in the colon. In addition, it down-regulated MDA level but elevated GSH and SOD levels. Moreover, *in vitro* data showed that thymol significantly inhibited ($p < 0.05$) lipopolysaccharide-induced secretion of NO, TNF- α , IL-1 β and IL-6 in macrophages, and suppressed ($p < 0.05$) the activation of NF- κ B pathway.

Conclusion: Thymol attenuates experimental colitis by down-regulating the activation of NF- κ B pathway. Therefore, thymol is a potential candidate drug for the management of ulcerative colitis.

Keywords: Thymol, Ulcerative colitis, Dextran sulfate sodium, Macrophages, Nuclear factor-kappaB

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory disorder in the colon, which not only harms the life quality of patients, but elevates the probability of colorectal cancer [1,2]. The precise cause of UC is poorly understood. However, bowel inflammation could be elicited by dysregulated immune response to commensal gut flora in

individuals with genetic susceptibility [3]. Dextran sulfate sodium (DSS)-induced colitis in mice has been extensively applied for UC study, which is characterized by many symptoms mimic some clinical features in human UC such as body weight loss, diarrhea, blood in stool and mucosal ulceration [4].

Traditional therapies of UC are 5-

aminosalicylates (5-ASA) and corticosteroids, which mainly focus on the reduction of the inflammatory response and abnormal immune responses. However, adverse effects such as diarrhea, cramps, fever, rash and kidney problems are associated with 5-ASA administration. In addition, some immune modulators like azathioprine are also used in the treatment of UC patients, but accompanied by severe adverse events or poor therapeutic effects [5,6]. Thus, it is necessary to develop more effective therapeutic modalities that attenuate the colonic inflammation.

Thymol, a natural monoterpene compound, is one of the active components of Chinese herb *Thymus vulgaris*, and the constituent of other plants such as *Monarda punctate* and *Origanum vulgare* spp [7]. Previous studies showed that thymol had various bioactivities, such as anti-inflammatory [8,9], anti-bacterial [10], anticancer and antioxidant properties [11]. Although thymol had wide-ranging pharmacological actions, the anti-inflammatory ability of thymol on experimental colitis has not been studied. The present study was to investigate the effect of thymol on DSS-induced colitis in mice. The mechanisms of how the thymol inhibits oxidative stress, pro-inflammatory mediator's production and NF- κ B signaling pathway were explored.

EXPERIMENTAL

Chemicals and reagents

Thymol (> 98 % purity) and lipopolysaccharide (LPS, B5) were purchased from Sigma (St. Louis, USA). Dextran sulfate sodium (DSS, 36000-50000 Da) was obtained from MP Bio (Aurora, USA). HiScript Q RT SuperMix and AceQ qPCR SYBR Green Master Mix were from Vazyme Biotech (Nanjing, China); Antibodies with phosphorylation of I κ B α , NF- κ B-p65 and GAPDH were obtained from Bioworld Biotech (Nanjing, China). ELISA kits for mouse TNF- α , IL-6 and IL-1 β were obtained from Dakewe Biotech (Shenzhen, China). Malondialdehyde (MDA), glutathione (GSH), superoxide (SOD) and myeloperoxidase (MPO) assay kits were products of Nanjing JianCheng Bioengineering Institute (Nanjing, China). MTT and TRIzol were obtained from Invitrogen (Carlsbad, USA).

Animals

Male C57BL/6 mice, 6-8 weeks old, were used (Beijing Vital River Laboratory Animal Tech, Beijing, China). The study protocol was approved by the Ethics Committee of Chengdu University of Traditional Chinese Medicine (no. 2014 DL-

023) in accordance to "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [12].

Colitis induction

DSS-induced colitis was performed as described previously [13]. Mice were randomly assigned into five groups: normal, model, thymol (30 mg/kg), thymol (60 mg/kg) and 5-ASA (100 mg/kg). In addition to mice in normal group, other mice were provided with drinking water with 2.5 % (w/v) DSS for seven days *ad libitum*, from day 0 to day 6, and then with water from day 7 to day 9. Thymol (30, 60 mg/kg) and 5-ASA (100 mg/kg) were orally administered for 10 consecutive days, from day 0 to day 9.

The mice were observed and recorded daily for body weight, diarrhea and gross bleeding in feces. Disease activity index (DAI) score = (body weight + diarrhea + gross bleeding) / 3 [14]. Mice were euthanized by CO₂ inhalation at day 9 after DSS induction. The colon was harvested and the length was determined.

Histological assessment

The colon tissues were fixed in paraformaldehyde and further embedded in paraffin. Then, the slides of colon tissues stained with hematoxylin and eosin. Histological score was carried out by analyzing the inflammation severity, inflammation extent and crypt damage as previously described [15].

Evaluation of MDA, GSH, SOD and MPO

Levels of MDA, GSH, SOD and MPO in colon homogenate were determined according to the manufacturer's protocol. Total protein was measured by a bicinchoninic acid protein kit (Beyotime Biotech, Shanghai, China).

Real-time PCR analysis

Total RNA was extracted from colon tissue using TRIzol reagent and reverse transcribed to cDNA. The mRNA expression were performed on a CFX96 Real-time PCR Detection System (Bio-rad) according to the instructions of HiScript Q RT SuperMix and AceQ qPCR SYBR Green Master Mix. The primer sequences are listed in Table 1. The expression of GAPDH was used for the normalization.

Cell culture

RAW 264.7 cells were obtained from Shanghai Institute of Cell Biology (Shanghai, China). RAW

Table 1: Prime sequences of target genes

Target gene	Forward	Reverse
TNF- α	ACC CTC ACA CTC ACA AAC CA	ATA GCA AAT CGG CTG ACG GT
IL-6	CCC AAT TTC CAA TGC TCT CCT	AAC GCA CTA GGT TTG CCG A
IL-1 β	TGC CAC CTT TTG ACA GTG ATG	AAG GTC CAC GGG AAA GAC AC
GAPDH	TCA GGA GAG TGT TTC CTC GT	TTC CCA TTC TCG GCC TTG AC

264.7 cells were cultured in DMEM medium (Gibco, Carlsbad, USA) with fetal bovine serum (10 %), penicillin (100 U/mL), and streptomycin (100 μ g/mL) under a normal (37 °C, 5 % CO₂) humidified air.

RAW 264.7 cell growth was measured by MTT assay. RAW 264.7 cells were plated in 96-well plate at a density of 2×10^5 cells per well, and stimulated with various concentrations of thymol (0, 25, 50, 100, 200 and 400 μ M), with or without 1 μ g/mL LPS for 20 h [16]. Cell viability was measured absorbance at 570 nm using a microplate reader.

Measurement of nitric oxide (NO) and cytokines

RAW 264.7 cells were plated in 96-well plate at a density of 2×10^5 cells per well, and stimulated with 1 μ g/mL LPS in the presence of thymol (50, 100 and 200 μ M) for 24 h. NO level in supernatant was evaluated as described before [17]. The absorbance at 550 nm was measured using a microplate reader (Thermo). According to the manufacturer's protocol, the levels of TNF- α , IL-6 and IL-1 β in cell supernatant were determined using specific ELISA kits.

Western blot assay

Cells (1×10^6 cells/mL) were pretreated with thymol (50, 100 and 200 μ M) for 20 h, and further cultured with 1 μ g/mL LPS for another 30 min. Total proteins were extracted using RIPA lysis buffer and loaded onto a 10 % SDS-PAGE. Blots transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, USA). The primary antibodies used in this study including p-I κ B α (1:1000), p-NF- κ B-p65 (1:1000), and GAPDH (1:5000). The signals were measured using the Western HRP substrate (Bio-rad).

Statistical analysis

Data are shown as mean \pm SEM. One-way ANOVA followed by Dunnett's test were used to determine the level of significance, and $p < 0.05$ was considered statistically significant. The data were analyzed using GraphPad Prism 5 (La Jolla, USA).

RESULTS

Effect of thymol on colitis

To determine the effect of thymol on experimental colitis, colitis was induced by adding DSS into drinking water in mice. DSS markedly induced DAI elevation in mice. Thymol (60 mg/kg) as well as 5-ASA decreased the DAI score when compared to the DSS group (Figure 1A). Figure 1B shows that thymol (60 mg/kg) significantly recovered the colon length when compared with the DSS group, so as 5-ASA did. Figure 1C shows that the activities of MPO in colons were also decreased in thymol (60 mg/kg) and 5-ASA.

Effect of thymol on colonic pathological changes

Histological analysis of colons from DSS-induced mice demonstrated severe damage to the epithelium, goblet cells lost, inflammatory cells infiltration, and severe damages in mucosal and submucosal. However, these pathological changes were much attenuated by thymol (60 mg/kg) and 5-ASA (Figure 2A). The histological scores of each group were shown in Figure 2B.

Effect of thymol on the production of pro-inflammatory cytokines in colons

To analyze the effect of thymol on the pro-inflammatory cytokines involved in the induction of UC, the mRNA expressions of TNF- α , IL-6 and IL-1 β in colon tissues were determined by real-time PCR. In DSS group, the mRNA expressions of TNF- α , IL-6 and IL-1 β were markedly increased when compared with normal group. Thymol (60 mg/kg) and 5-ASA treatments significantly reduced the level of TNF- α , IL-6 and IL-1 β in colon tissues (Figure 3A-C).

Effect of thymol on oxidative stress in colon tissues

To determine the effect of thymol on oxidative stress involving in the inflammatory response and intestinal damage, the levels of MDA, GSH and SOD in colon tissues were measured. The DSS administration increased MDA level, decreased levels of GSH and SOD in colon tissues. However, thymol (60 mg/kg) treatment

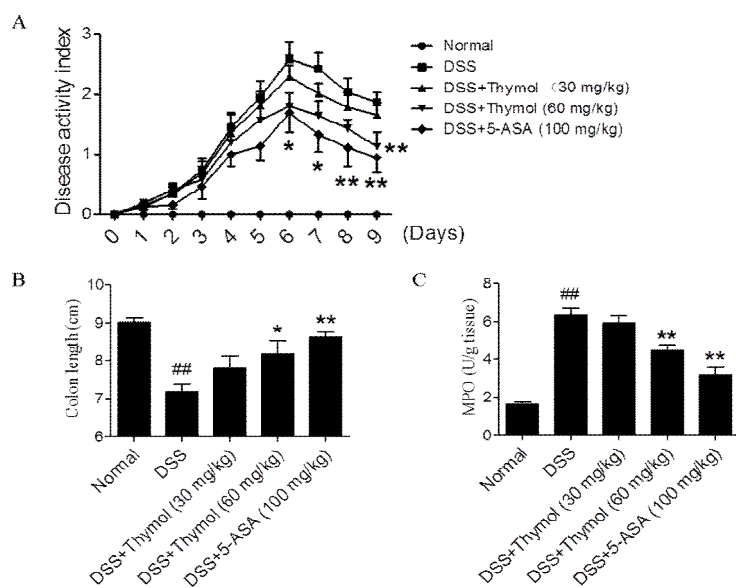


Figure 1: Effect of thymol on murine colitis. (A) DAI scores; (B) colon length; (C) MPO activity in colon tissues ($n = 6$). $^{##}p < 0.01$ vs normal, $^{*}p < 0.05$, $^{**}p < 0.01$ vs DSS

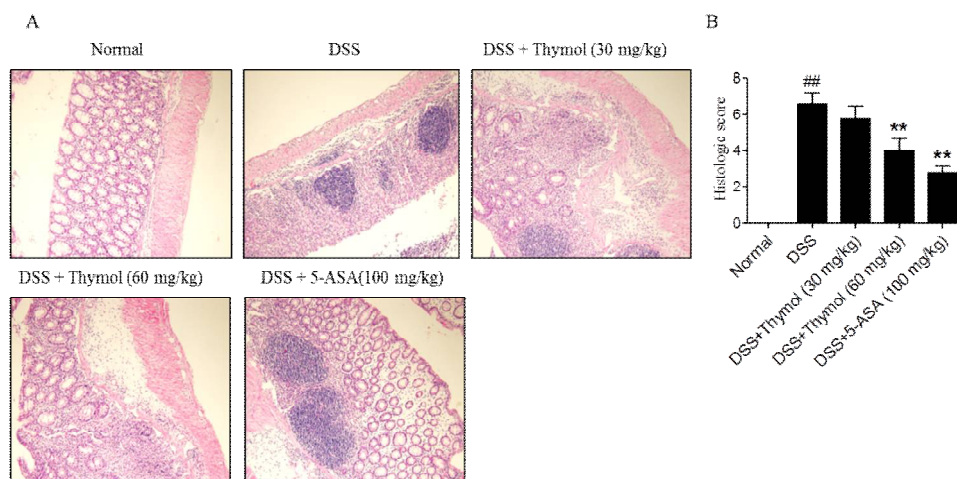


Figure 2: Effect of thymol on the histological changes. (A) Histological changes in colon tissues. The original scale was 200 micrometer. (B) Histologic scores from each group ($n = 6$). $^{##}p < 0.01$ vs normal, $^{*}p < 0.05$, $^{**}p < 0.01$ vs DSS

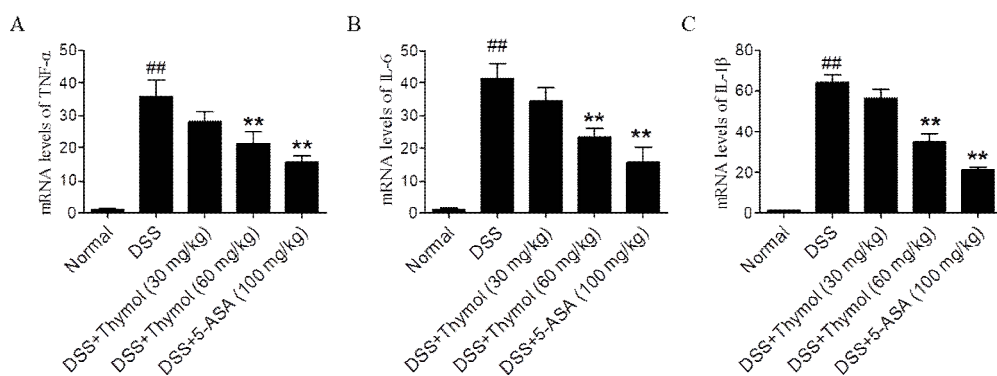


Figure 3: Effect of thymol on the production of pro-inflammation cytokines in colon tissues. The mRNA levels of (A) TNF- α , (B) IL-6 and (C) IL-1 β in colon tissues were assessed by real-time PCR ($n = 6$). $^{##}p < 0.01$ vs normal, $^{*}p < 0.05$, $^{**}p < 0.01$ vs DSS

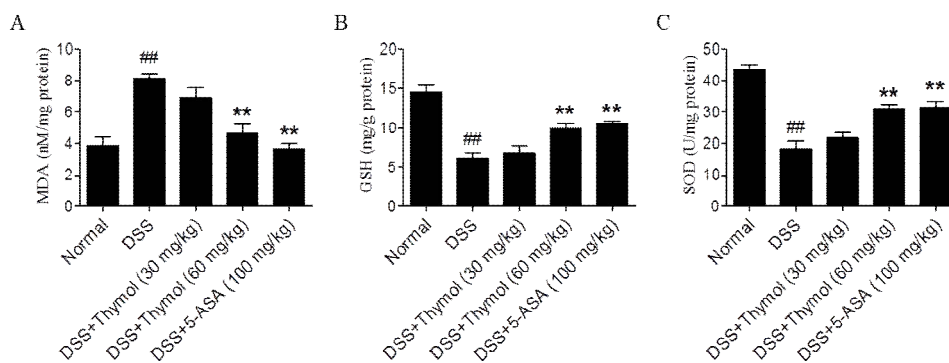


Figure 4: Effect of thymol on oxidative stress in colon tissues. The levels of (A) MDA, (B) GSH and (C) SOD in the colon tissues were measured by commercial kits ($n = 6$). ^{##} $P < 0.01$ vs normal, $p < 0.05$, ^{**} $p < 0.01$ vs DSS

attenuated MDA level, improved levels of GSH and SOD in colon tissues (Figure 4A-C).

Effect of thymol on the cell viability in macrophages

Macrophages play key roles in the secretion of pro-inflammatory cytokines. Firstly, the effect of thymol on the cell viability in the presence or absence of LPS in RAW264.7 cells was investigated. Figure 5 A and B showed thymol had no significant cytotoxicity on RAW 264.7 cells at concentrations below 400 μM .

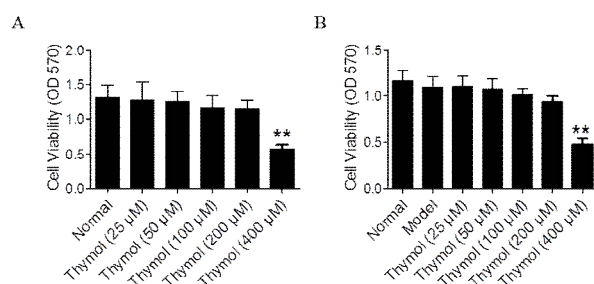


Figure 5: Effect of thymol on the cell viability in macrophages. Cell viability was determined by using MTT assay. RAW264.7 cells were treated with various concentrations of thymol (25, 50, 100, 200 and 400 μM) (A) in the absence or (B) presence of LPS (1 $\mu\text{g}/\text{mL}$) for 24 h. Data are representative of three independent experiments; ^{**} $p < 0.01$ vs normal

Effect of thymol on levels of inflammatory mediators in LPS-induced macrophages

The effect of thymol on the secretion of inflammatory mediators, such as NO, TNF- α , IL-6 and IL-1 β in LPS-induced macrophages was evaluated. LPS stimulation markedly increased the production of NO, TNF- α , IL-6 and IL-1 β in RAW264 cells. However, thymol (100 and 200 μM) inhibited the LPS-induced increases in levels of NO, TNF- α , IL-6 and IL-1 β in a concentration-dependent manner (Figure 6 A-D).

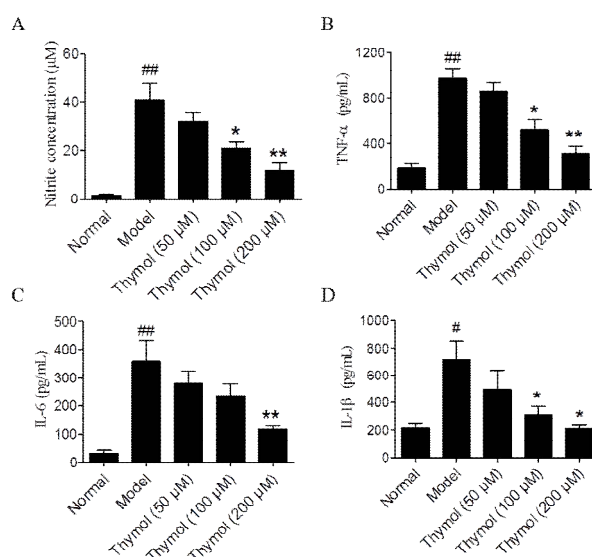


Figure 6: Effects of thymol on levels of inflammatory mediators in LPS-induced macrophages. RAW264.7 cells were cultured with thymol (50, 100 and 200 μM) in the presence or absence of 1 $\mu\text{g}/\text{mL}$ LPS. After treatment with 24 h, the culture supernatants were collected. (A) NO level was measured by using Griess assay. The levels of (B) TNF- α , (C) IL-6 and (D) IL-1 β were determined by using ELISA kits. Data are representative of three independent experiments; ^{##} $p < 0.01$ vs normal, $*p < 0.05$, ^{**} $p < 0.01$ vs model

Effect of thymol on activation of NF- κB pathway in LPS-induced macrophages

In general, LPS binds to toll-like receptors and activates many downstream signaling pathways, such as NF- κB pathway [18]. To address the mechanism for thymol inhibiting the levels of pro-inflammatory mediators, effect of thymol on the activation of NF- κB signaling by using Western blot was determined. Figure 7 showed that LPS treatment markedly increased the level of phosphorylation of I $\kappa\text{B}\alpha$ and NF- κB -p65 in RAW264.7 cells. Thymol treatment significantly down-regulated the activation of NF- κB signaling pathway.

DISCUSSION

It is indispensable to optimize the therapeutic options for UC treatment. The present study aimed to find a potent drug candidate for the treatment of UC. The study report that thymol attenuates colitis induced by DSS in mice by inhibiting pro-inflammatory mediator production from macrophages via down-regulation of activation of NF- κ B pathways.

In the clinic, UC is often associated with the goblet cell depletion, destruction of crypt architecture, inflammatory cells infiltration, and crypt inflammation [19]. In the results, thymol treatment inhibited inflammatory damage such as loss of goblet cell, mononuclear cell infiltration. The level of MPO indicated that infiltration of neutrophils in the colon tissues, and thymol showed a markedly reduction in MPO levels in the colon.

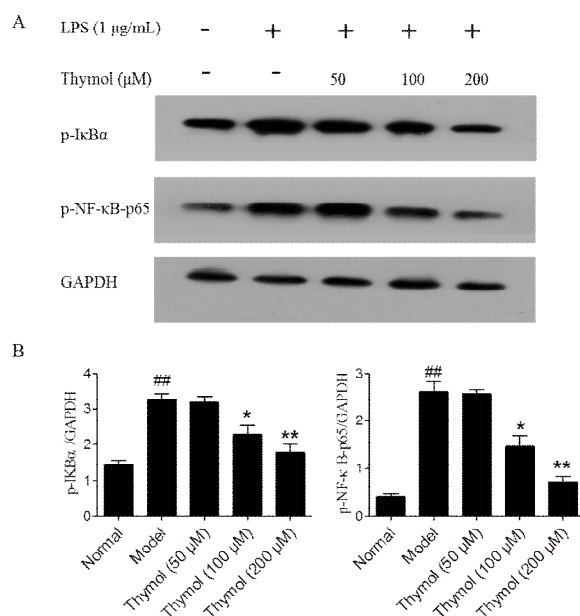


Figure 7: Effect of thymol on activation of NF- κ B pathway in LPS-induced macrophages. RAW264.7 cells were pre-treated with thymol (50, 100 and 200 μ M) for 20 h, then stimulated with LPS (1 μ g/mL) for 30 min. Total protein were extracted. (A) Levels of p-I κ B α , p-NF- κ B-p65 and GAPDH were analyzed by using Western blot. (B) Band densitometry were analyzed, and the quantitative data were shown. Data were representative of three independent experiments; ^{##} $p < 0.01$ vs normal, ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs model

Previous studies showed that overproduction of inflammatory mediators from macrophages, such as pro-inflammatory cytokines and reactive oxygen metabolites (e.g., NO), is mostly involved in the inflammation cascade reaction and subsequent colon damage in UC [20]. Macrophages secrete TNF- α , IL-6 and IL-1 β ,

which are considered as crucial inflammatory mediators. In the colon mucosa of patients with UC, their levels are markedly increased [21]. The results demonstrated that thymol inhibited production of pro-inflammatory mediators in the colons of colitis mice, as well as in LPS-stimulated macrophages.

Previous studies showed that oxidative stress kept the inflammation response active in the colon mucosa. The lipid peroxidation product MDA cause tissue damage. In parallel, reduction of GSH and SOD, the main intracellular antioxidant, have been detected in the colonic mucosa of UC patients [22]. The results showed that treatment with DSS markedly reduced the levels of GSH and SOD, and enhanced MDA level in the colonic tissues, which is in consistent with the previous finding [23]. The results shows that thymol significantly suppressed oxidative stress induced by DSS.

LPS are recognized by mucosal macrophages via TLR4. Then macrophages have an elevated ability to produce TNF- α , IL-6 and IL-1 β , causing the activation of NF- κ B [24]. Hence, it is speculated that the anti-inflammatory effect of thymol was due to the suppression of NF- κ B pathway, regulating the expression of numerous inflammatory factors. The inducible form of NF- κ B is typically a heterodimer composed of a p50 and p65 subunits, present in cytoplasm as an inactive complex associated with inhibitory protein I κ B. The activation of this complex by various stimuli lead to the phosphorylation, ubiquitination and degradation of I κ B proteins. Then, NF- κ B heterodimer (p65/p50) will rapidly translocate into the nucleus, regulate the transcriptions of a large number of target genes, such as pro-inflammatory cytokines (TNF- α , IL-6 and IL-1 β) [25,26]. Although thymol inhibited NF- κ B-p65 phosphorylation in mouse epithelial cells [27], the results demonstrated it can inhibit LPS-induced NF- κ B activation in macrophages.

CONCLUSION

Thymol shows protective effects against DSS-induced colitis, and reduces the production of pro-inflammatory mediators and oxidative damage in colon tissues. The mechanism involves the inhibition of the NF- κ B activation. Thus, thymol is a potential candidate drug for UC treatment.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. De-Ming Liu and Wen Li conceived and designed the study; Ping Wang and Chun-Yan Zhou collected and analyzed the data; De-Ming Liu and Xian-Li Meng wrote the manuscript. All authors have read the manuscript and approved for publication.

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