

The Healing Effects of Hydroalcoholic Extract of *Carum Copticum L.* on Experimental Colitis in Rats

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What's Known

- Ulcerative colitis is a disease with indefinite treatment. Common medical treatments for ulcerative colitis can cause serious adverse effects.
- *Carum copticum L.* has positive effects on the treatment of colic, dyspepsia, indigestion, and diarrhea. It has antioxidant, anti-inflammatory, and anti-ulcer effects.

What's New

- The effect of *Carum copticum L.* on the treatment of colon inflammation and ulceration is demonstrated.
- *Carum copticum L.* is a natural and effective supplement for the treatment of Ulcerative colitis without serious adverse effects.

Abstract

Background: Ulcerative colitis is an inflammatory disease with indefinite treatment. The present study aimed to assess the anti-inflammatory and antioxidant effects of *Carum copticum L.* (CC) extract on induced colitis in rats.

Methods: Sixty male rats were randomly divided into six groups (n=10 per group). Acetic acid-induced colitis rats were orally administered with doses of 100, 200, and 400 mg/kg CC extract, and 100 mg/kg sulfasalazine for seven consecutive days, respectively. Colonic biopsies were taken to measure histopathological parameters as well as the tumor necrosis factor (TNF- α), interleukin-6 (IL-6), myeloperoxidase (MPO), malondialdehyde (MDA), and glutathione (GSH). Data analysis was performed using the one-way ANOVA and Tukey's test for normally distributed data. Kruskal-Wallis test followed by Dunn's test was used for non-normally distributed data. The analysis was performed at $P \leq 0.05$ using SigmaStat software (version 10.0).

Results: The control colitis group had a significantly higher total colitis index ($P=0.01$), TNF- α ($P=0.01$), IL-6 ($P=0.01$), MPO ($P=0.01$), and MDA ($P=0.01$); and lower GSH ($P=0.01$) than those of the sham group. The colitis group receiving a dose of 200 mg/kg/day CC extract had a significantly lower total colitis index ($P=0.01$), TNF- α ($P=0.01$), IL-6 ($P=0.01$), MPO ($P=0.01$), and MDA ($P=0.01$); and higher GSH ($P=0.01$) than those of the control colitis group. The colitis group receiving a dose of 200 mg/kg/day CC extract had a significantly lower total colitis index ($P=0.04$), TNF- α ($P=0.03$), IL-6 ($P=0.04$), MPO ($P=0.03$), and MDA ($P=0.03$); and higher GSH ($P=0.01$) than those of the colitis group receiving sulfasalazine.

Conclusion: The present study revealed that CC extract had healing effects on colitis, possibly due to its antioxidant and anti-inflammatory properties.

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Keywords • Colitis • Ulcerative • Rats • Cytokines • Antioxidants • Anti-Inflammatory agents

Introduction

Ulcerative colitis (UC) is a set of chronic and recurrent diseases typically known as a type of inflammatory bowel disease (IBD).¹ The annual incidence of the disease is reported at 6.3, 19.2, and 24.3 per 100,000 person-years in Asia and the Middle East,

Europe, and North America, respectively.² UC affects the mucosa in the large intestine and has acute symptoms with chronic inflammation, which may result in ulceration of the colon, bloody diarrhea, rectal bleeding, colic, and weight loss.³ It is also associated with a higher incidence of bowel cancer.⁴ Although the etiology of UC is not known, factors such as gastrointestinal infections, urban lifestyle, use of some medications, reactive oxygen species (ROS), and genetics might be involved.⁵ Oxidative stress⁶ and cytokines (e.g. TNF- α , IL-6, and IL-1 β)⁷ are also known to play a vital role in the pathophysiology of UC. However, the complete treatment of this disease is still unrealized. The most common medical treatments for UC are glucocorticoids, salicylates, immune modulators, and antibiotics; however, these drugs only provide temporary relief and are associated with serious adverse effects.³

Recently, there has been a growing interest in the use of herbal medicine to treat UC.⁸ Medicinal plants are an important source of new chemical materials with antioxidant and anti-inflammatory properties which might be beneficial in UC treatment.⁹ A number of herbs including *Tormentil*,¹⁰ *Carum carvi*,¹¹ and *Hypericum perforatum*¹² have been reported to have palliative effects in human and/or animal models of UC. The benefits of such herbs have been attributed to the antioxidant and anti-inflammatory effects.¹⁰⁻¹²

Carum copticum L. is a grassy annual plant of the Apiaceous family, which grows in India, Iran, and Egypt. It has white flowers and small brownish fruits.¹³ This plant is commonly used as either a spice, food additive, or food preservative.¹⁴ The chemical compounds of CC are steroptin, cumene, thymene, tannin, and dietary fiber. CC essential oil also contains phenolic compounds such as thymol (35-63%), terpinene, p-cymene, pinene, and carvacrol as the main compounds.¹³ This plant is known to have positive effects on the treatment of colic, dyspepsia, indigestion, and diarrhea. It has also been reported to have antioxidant, anti-inflammatory, and anti-ulcer effects.¹⁵ Several studies have demonstrated the effects of CC in treating different diseases such as acute and sub-acute inflammation in rats,¹³ liver injury in mice, and peptic ulcer in rats.¹⁶

In previous studies, the effects of CC in treating some gastrointestinal diseases and ulcer have been demonstrated. However, there is no report on the possible effect of CC on colitis. Consequently, the present study was designed to evaluate the effects of CC extract on acetic acid-induced colitis model in rats.

Materials and Methods

Plant Collection and Extract Preparation

Dried fruits of CC were locally purchased and its exact species was determined in the Herbarium of Pharmacology Department, Shiraz University of Medical Sciences, Shiraz, Iran (herbarium number: PM950). The hydroalcoholic extract of the dried fruit was prepared by the Pharmacology Department of Shiraz University of Medical Sciences in accordance with the percolation method. Briefly, the dried fruit was ground into powder and soaked in an adequate volume of ethanol:water (80:20) solution for 72 hours. The extract was subsequently filtered and the solvent was evaporated in a rotary evaporator under reduced pressure, and then dried at 50 °C for 72 hours.¹⁷

Determination of Total Phenolic Content (TPC)

The total phenol concentration was measured using the Folin-Ciocalteu method. This method involves the reduction of the Folin-Ciocalteu reagent by phenolic compounds, whereby the concentration of the total phenolic is expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GA/g dE).¹⁸

Animals

Sixty male Sprague-Dawley rats, weighing 180-220 g, were obtained from the Laboratory of Animal Breeding Center, Shiraz University of Medical Sciences, Shiraz, Iran. They were kept individually in single cages under standard conditions (12:12 light:dark cycle, humidity of 50 \pm 10%, and temperature of 22 \pm 2 °C) with standard rat chow and drinking water *ad libitum*. All animal experiments were approved by the Animal Experimentation Ethics Committee of Shiraz University of Medical Sciences (number: 93-7406) prior to the experiments. In addition, the "Guide for the Care and Use of Laboratory Animals" published by the United States National Institutes of Health (NIH)¹⁹ was taken into account.

Experimental Protocol and Design

Induction of Colitis

The animals were anesthetized with ketamine 10% (100 mg/kg) and xylazine 2% (10 mg/kg) (Alfasan, Woerden, The Netherlands). A cannula (2 mm diameter) was inserted into the animal's rectum and pushed 8 cm forward such that its tip laid in the colon. The animals were then injected through the cannula with either 2 ml saline solution or 2 ml 3% acetic acid (Merck, Darmstadt, Germany) to induce UC. The rats were maintained in head down position for 30

seconds to prevent leakage.²⁰

Experimental Groups

The animals were randomly divided into six groups (10 rats per group) as follows:

- Group I (sham group): Received 2 ml/kg distilled water orally (p.o.) as a vehicle without colitis induction.
- Group II (control colitis group): Received 2 ml/kg distilled water orally (p.o.) as a vehicle following induction of colitis.
- Group III: Received a dose of 100 mg/kg CC extract orally following induction of colitis.
- Group IV: Received a dose of 200 mg/kg CC extract orally following induction of colitis.
- Group V: Received a dose of 400 mg/kg CC extract orally following induction of colitis.
- Group VI (reference group): Received a dose of 100 mg/kg sulfasalazine orally following induction of colitis.

All treatments started the day after the onset of bloody diarrhea in the animals and continued daily for seven consecutive days. On the eighth day, the rats were sacrificed by cervical dislocation and tissue samples were obtained from their colon for histopathological investigation and biochemical markers (TNF- α , IL-6, MPO, MDA, and GSH). For the measurement of biochemical markers, tissue samples were immediately kept frozen at -80 °C in a nitrogen tank until analysis.

Assessment of Colon Histological Damage

Sections of 4 μ m thickness from the colonic tissues were prepared and stained with hematoxylin and eosin (H&E). The sections were then examined for inflammation severity, and their extent and crypt damage were measured using a modified version of a validated scoring system.²¹ The total colitis index was measured by summing three sub-scores (inflammation severity, inflammation extent, and crypt damage). This measurement was conducted by a pathologist who was blind to the treatment protocols.

Biochemical Analysis

Tissue samples were homogenized and the homogenate was used for the measurement of biochemical markers (TNF- α , IL-6, MPO, MDA, and GSH). MDA, MPO, and GSH were measured using the methods described by Ohkawa, Krawisz, and Moran, respectively.²⁰ Their activities and contents were expressed per gram wet tissue weight. All materials were purchased from Sigma, Germany. TNF- α and IL-6 levels were analyzed using a commercial enzyme-linked immunosorbent assay (ELISA)

kit (Sigma, Germany). Analyses of all samples, standards, and controls were performed in duplicate. Cytokine activities were expressed as pg/mg protein.

Statistical Analysis

The data were examined for normality of distribution and the obtained data were analyzed using the one-way analysis of variance (ANOVA). Where a significant difference was obtained with ANOVA, the source of the difference was located using the Tukey-Kramer test. Normally distributed data were analyzed by the Kruskal-Wallis test followed by Dunn's test. The analysis was performed at $P \leq 0.05$ using SigmaStat software (version 10).

Results

Analysis of the Extract

The TPC in the 80% ethanol extract, calculated from the gallic acid standard calibration curve, was 192.2 ± 2.96 mg GAE/g dE.

Histopathological Study

Except for the inflammation extent, the values of crypt damage, inflammatory severity, and total colitis index were normally distributed. No indication of histological damage of inflammation was observed in colonic tissues of the rats in the sham group. Colonic tissues of the rats with acetic acid-induced colitis showed extensive necrotic destruction of the epithelium and submucosal edema as well as acute inflammation with neutrophil infiltration in mucosa and submucosa. The acetic acid-induced colitis rats also showed ulceration with adjacent glandular architecture disruption, crypt dilatation, and goblet cell depletion (figure 1). As shown in table 1, the crypt damage, inflammation extent, inflammation severity, and the total colitis index of the sham group were zero. Moreover, the crypt damage, inflammation extent, inflammation severity, and total colitis index of the control colitis group were significantly ($P=0.01$) higher than those of the sham group. The crypt damage, inflammation extent, inflammation severity, and the total colitis index of groups receiving the CC extract at doses of 100, 200, 400 mg/kg/day and sulfasalazine were significantly ($P=0.03$, $P=0.01$, $P=0.02$, and $P=0.02$, respectively) lower than those of the control colitis group (table 1). As shown, there was no significant difference in the crypt damage, inflammation extent, inflammation severity, and total colitis index of the sulfasalazine-treated colitis group and those of the groups treated with a dose of 100 or 400 mg/kg/day CC extract. However, the crypt

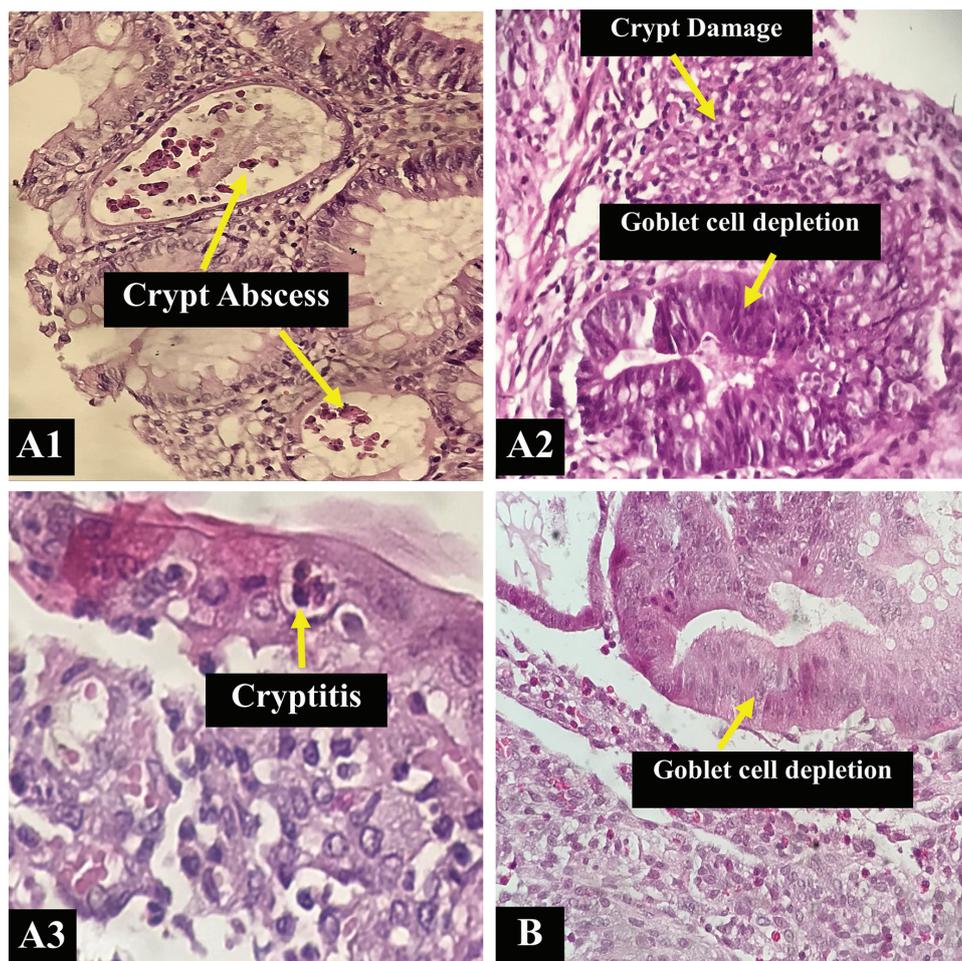


Figure 1: Microscopic images (H&E, ×400) of colonic tissues from colitis groups receiving vehicle. It shows extensive damage of (A1) crypt abscess, (A2) crypt damage and goblet cell depletion, and (A3) cryptitis. (B): Hydroalcoholic extract of CC (100 mg/kg) shows mild regeneration of colon with necrosis, ulceration, and inflammation.

Groups	Crypt damage	Inflammation extent	Inflammation severity	Total colitis index
Sham	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.0
Control colitis	3.6±0.5 [*]	3.3±0.5 [*]	3.6±0.5 [*]	10.6±1.3 [*]
Colitis-E1	2.8±0.8	3.0±0.9 [#]	2.1±0.9 [#]	8.0±2.5 [#]
Colitis-E2	0.6±0.7 ^{*,+}	1.5±0.7 ^{*,+}	1.2±0.7 ^{*,+}	3.3±1.6 ^{*,+}
Colitis-E3	2.0±0.7 [#]	2.5±0.7 [#]	2.3±0.6 [#]	6.8±1.9 [#]
Colitis-SUL	1.3±0.5 [#]	2.4±0.5 [#]	2.4±1.5 [#]	6.1±3.01 [#]

Data (n=10 each) are presented as mean±SEM. E1: 100 mg/kg extract, E2: 200 mg/kg extract, E3: 400 mg/kg extract, SUL: Sulfasalazine; ^{*}Significance difference in the control colitis group; [#]Significance difference from the control colitis group; ⁺Significance difference from the colitis-SUL group

damage, inflammation extent, inflammation severity, and total colitis scores of the group treated with a dose of 200 mg/kg/day CC extract were significantly (P=0.04) lower than those of the sulfasalazine-treated colitis group (table 1).

Biochemical Markers

Except for the MPO and GSH, the TNF- α , IL-6, and MDA parameters were normally-distributed and homogeneous. Tissue TNF- α concentrations in the control colitis group

were significantly (P=0.01) higher than those of the sham group (figure 2A). Tissue TNF- α concentrations in the colitis group receiving the CC extract at doses of 100, 200, and 400 mg/kg, and sulfasalazine were significantly (P=0.04, P=0.01, P=0.03, and P=0.03, respectively) lower than those of the colitis control group. There was no significant difference between tissue TNF- α concentrations in the colitis groups treated with CC extract at a dose of 100 or 400 mg/kg/day and sulfasalazine (figure 2A). However, tissue

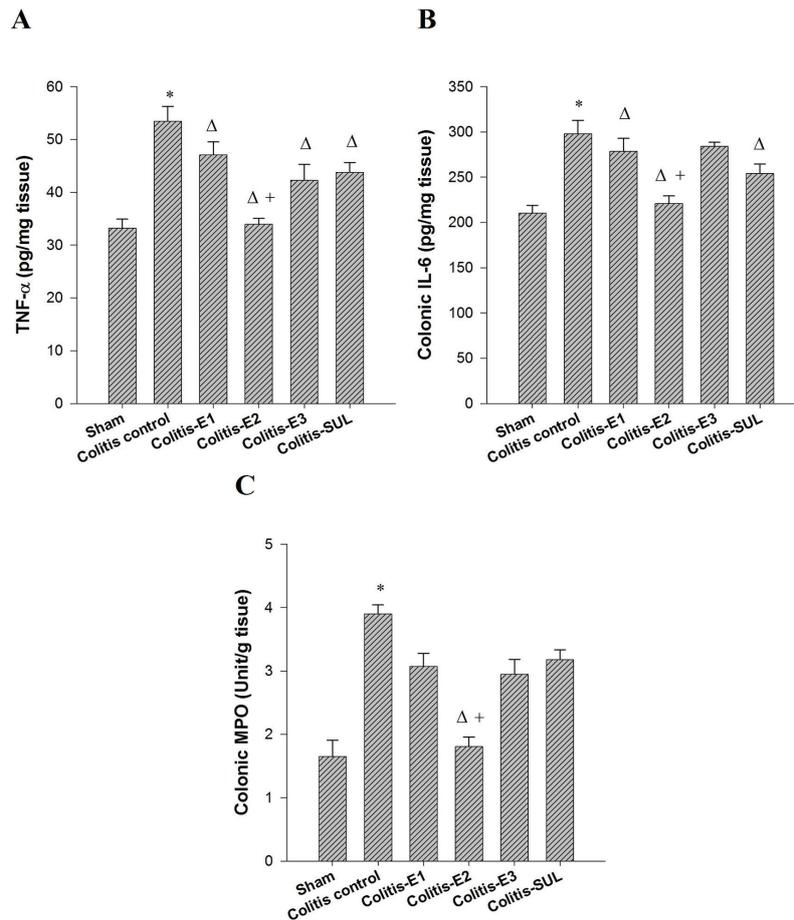


Figure 2: Shows the colonic tissue levels (mean±SEM, n=10 each) of TNF-α, IL-6 and MPO. Sham: Group treated with vehicle, Colitis control: Groups treated with vehicle, Colitis-E1: 100 mg/kg/day CC hydroalcoholic extract, Colitis-E2: 200 mg/kg/day CC hydroalcoholic extract, Colitis-E3: 400 mg/kg/day CC hydroalcoholic extract, Colitis-SUL: 100 mg/kg/day sulfasalazine; *Significant different from the sham group; ^ΔSignificant difference from the control colitis group; ^{Δ+}Significant difference from the sulfasalazine-treated colitis group

TNF-α concentrations in the colitis groups treated with CC extract at a dose of 200 mg/kg/day were significantly ($P=0.03$) lower than those of the sulfasalazine-treated group (figure 2A).

Tissue IL-6 concentrations in the control colitis group were significantly ($P=0.01$) higher than those of the sham group (figure 2B). Tissue IL-6 concentrations in the colitis group receiving the CC extract at doses of 100 and 200 mg/kg/day, and sulfasalazine were significantly ($P=0.04$, $P=0.01$, and $P=0.03$, respectively) lower than those of the colitis control group. There was no significant difference between tissue IL-6 concentrations in the colitis groups treated with CC extract at a dose of 100 or 400 mg/kg/day and sulfasalazine (figure 2B). However, tissue IL-6 concentrations in the colitis groups treated with a dose of 200 mg/kg/day CC extract were significantly ($P=0.04$) lower than those of the sulfasalazine-treated group (figure 2B).

Tissue MPO concentrations in the control colitis group were significantly higher ($P=0.01$)

than those of the sham group (figure 2C). Tissue MPO concentrations in the colitis group receiving the CC extract at doses of 100, 200, and 400 mg/kg, and sulfasalazine were lower than those of the colitis control group; only the extract at a dose of 200 mg/kg was significant ($P=0.01$). There was no significant difference between tissue MPO concentrations in the colitis groups treated with CC extract at a dose of 100 or 400 mg/kg/day and sulfasalazine (figure 2C). However, tissue MPO concentrations in the colitis groups treated with a dose of 200 mg/kg/day CC extract were significantly ($P=0.03$) lower than those of the sulfasalazine-treated group (figure 2C).

Tissue MDA concentrations in the control colitis group were significantly ($P=0.01$) higher than those of the sham group (figure 3A). Tissue MDA concentrations in the colitis group receiving the CC extract at doses of 100, 200, and 400 mg/kg, and sulfasalazine were significantly ($P=0.03$, $P=0.01$, $P=0.02$, and $P=0.03$, respectively) lower

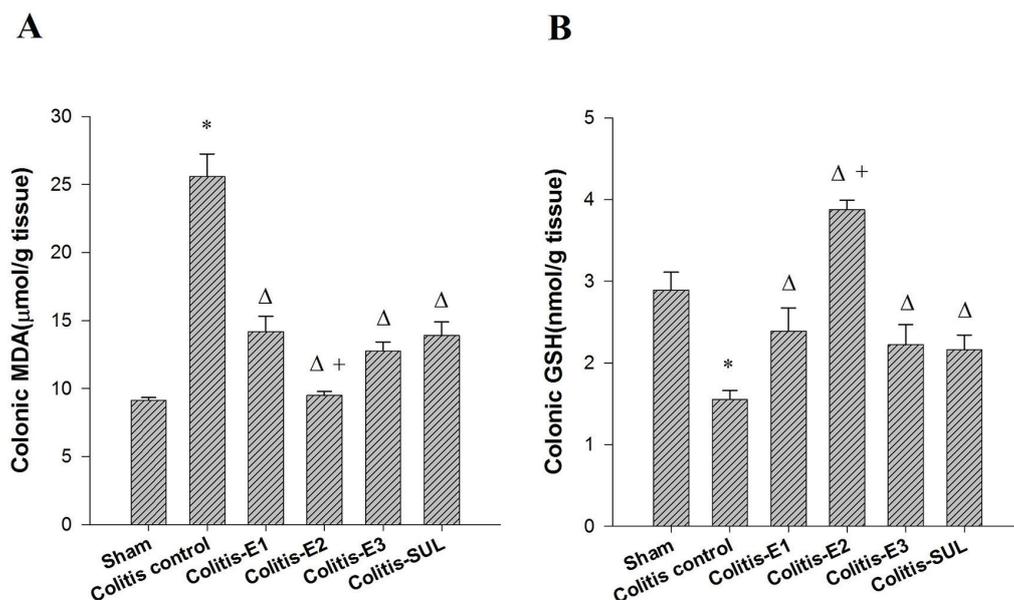


Figure 3: Shows colonic tissue levels (mean±SEM, n=8-10 each) of MDA (A), and GSH peroxidase (B). Sham: Group treated with vehicle, Colitis control: Groups treated with vehicle, Colitis-E1: 100 mg/kg/day CC hydroalcoholic extract, Colitis-E2: 200 mg/kg/day CC hydroalcoholic extract, Colitis-E3: 400 mg/kg/day CC hydroalcoholic extract, Colitis-SUL: 100 mg/kg/day sulfasalazine; *Significant different from the sham group; ^ΔSignificant difference from control colitis group; ⁺Significant difference from sulfasalazine-treated colitis group

than those of the colitis control group. There was no significant difference between tissue MDA concentrations in the colitis groups treated with CC extract at a dose of 100 or 400 mg/kg/day and sulfasalazine (figure 3A). However, tissue MDA concentrations in the colitis groups treated with CC extract at a dose of 200 mg/kg/day were significantly ($P=0.03$) lower than those of the sulfasalazine-treated group (figure 3A).

Tissue GSH concentrations in the control colitis group were significantly ($P=0.01$) higher than those of the sham group (figure 3B). Tissue GSH concentrations in the colitis group receiving the CC extract at doses of 100, 200, and 400 mg/kg, and sulfasalazine were significantly ($P=0.03$, $P=0.01$, $P=0.02$, and $P=0.02$, respectively) lower than those of the colitis control group. There was no significant difference between tissue GSH concentrations in the colitis groups treated with CC extract at a dose of 100 or 400 mg/kg/day and sulfasalazine (figure 3B). However, tissue GSH concentrations in the colitis groups treated with CC extract at a dose of 200 mg/kg/day were significantly ($P=0.01$) lower than those of the sulfasalazine-treated group (figure 3B).

Discussion

The findings of the present study indicated that intrarectal administration of acetic acid resulted in colitis characterized by extensive necrotic destruction of the epithelium, submucosal edema, neutrophil infiltration in mucosa and

submucosa; ulceration with adjacent glandular architecture disruption, crypt dilatation, and goblet cell depletion. In addition, the present model of colitis was associated with an increase in MDA, MPO, TNF- α , and IL-6 concentrations, and decreased GSH concentrations in colonic tissues. It was also shown that treatment with either CC hydroalcoholic extract or sulfasalazine not only prevented the development of colitis but also reduced the colonic tissue levels of MDA, MPO, TNF- α , and IL-6, and increased the level of GSH.

Previous studies revealed that pro-inflammatory cytokines are involved in the pathogenesis of UC.⁷ Certain pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 β are known to play a crucial role in modulating the mucosal immune system in UC patients.⁷ Therefore, we measured TNF- α and IL-6 level in colonic tissues and found that the present model of colitis is associated with an increase in the pro-inflammatory cytokines (TNF- α and IL-6). These findings are in line with previous studies that showed acetic acid induction was associated with increased levels of pro-inflammatory cytokines.^{22,23} Also, the anti-inflammatory effects of CC have been supported in several studies.^{13,14} These effects are suggested to be based on several mechanisms such as the effect on the synthesis of kinin, prostaglandin, bradykinin,¹⁴ and inhibitory effect on infiltrating inflammatory cells.²⁴ Although thymol and carvacrol (the main components of CC) suppress pro-inflammatory

cytokines (TNF- α , IL-1 β , and IL-6) and NF- κ B expressions,²⁵ our results showed that CC extract reduced the levels of TNF- α and IL-6 in colonic tissue.

In UC, the inflammation caused by infiltration of neutrophils into the colon plays a role in both the initiation and development of the disease. Thus, inhibition of neutrophils secretion is an important step in the prevention and treatment of the disease.¹ The level of colonic MPO is a valid marker to assess neutrophils influx and subsequent acute intestinal inflammation.²⁶ MPO is a peroxidase enzyme that exists in neutrophils and is released in response to tissue damage and, as a result, causes inflammation.²⁷ The results of the present study showed that acetic acid caused an increase in the MPO level. This result is also supported by previous animal studies that showed an increase of MPO level in UC.^{22, 23} CC hydroalcoholic extract improved infiltration of neutrophils as evidenced by the alleviated colonic level of MPO and histological improvement.

Oxidative stress is another factor playing a major role in the etiopathogenesis of UC.²⁸ Infiltrating leukocytes in the mucosa of a patient with UC produces a large amount of highly reactive metabolite of oxygen that causes tissue destruction.²⁹ In the present study, the extent of oxidative stress was evaluated by measuring MDA and GSH levels. Reactive oxygen species (ROS) have an aggressive effect on the lipid membrane of cells and cause lipid peroxidation.³⁰ MDA, frequently used for the measurement of lipid peroxide levels, exhibits a good correlation with the degree of lipid peroxidation.¹² GSH is one of the primary non-enzymatic peptides in the oxidative defense system. The reduction of this antioxidant causes both cellular and colonic injury.³¹ Studies have shown a significant decrease of GSH level in acute and chronic experimental colitis models.³² In the present study, acetic acid induced oxidative stress by increasing the MDA level and decreasing GSH level. These findings are consistent with previous studies that showed acetic acid induction was associated with increased levels of oxidant markers and reduced level of antioxidant marker.^{22, 23} Previous studies have attributed the beneficial effects of herbs to the reduction of oxidative stress and an increase of antioxidant defense in UC.³³ Furthermore, CC is known as a plant with high antioxidant properties. This plant acts as antioxidant by scavenging free radicals, restoration of GSH, superoxide dismutase activity, and catalase.³⁴ Our results showed that CC hydroalcoholic extract reduced the MDA level and increased GSH level in colonic tissue.

These findings are similar to those of Sancheti and colleagues³⁵ and Kim and colleagues³⁶ who demonstrated that thymol, as the main component of CC, improved oxidative stress in several animal models by decreasing the MDA level and increasing GSH level.

The beneficial effects of herbs in UC have been attributed to phenols and polyphenols.³⁷ We measured the phenol constituent of CC hydroalcoholic extract because CC contains different important phenolic compounds such as carvacrol, thymol, terpinene, p-cymene, and β -pinene.¹⁶ Since our results showed that CC hydroalcoholic extract is rich in phenolic compounds, it is suggested that the beneficial effects of the extract can be partly attributed to such compounds.

The present study also showed that the effects of hydroalcoholic extract were biphasic; the first two doses increased the beneficial effects, but the third dose had reserve effects. Although the reason for such a biphasic behavior is not clear, it could probably be due to the fact that unlike one-molecule synthetic chemicals, multiple molecules are involved in the extract. It is tempting to speculate that by increasing the dose of the extract, the balance between the beneficial and non-beneficial components of the extract could tilt in favor of the non-beneficial effects. It must be noted that such behavior has also been reported on other herbs, including a combination of *thyme* with *oregano* and curcumin from *Turmeric*.^{25, 38}

The main limitation of the present study was the lack of toxicity investigation. However, it has been shown³⁹ that the administration of 125 mg/kg, twice daily for two weeks (total of 3 g), is safe and not associated with significant changes in liver enzymes (ALT and AST). Consequently, doses used in the present study were tailored such that the total amount of the highest dose (2.8 g) was even lower than the recommended safe amount.

Conclusion

It is demonstrated that CC causes biphasic dose effects and the medium dose is more effective on inflammatory cytokines, oxidative markers, and healing ulcer than that of a higher dose or even sulfasalazine. While our findings encourage future clinical usage of CC, as a natural and effective supplement in UC, further investigation is required to evaluate its effective therapeutic dose.

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Conflict of Interest: None declared.

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