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Original Article

Study of the protective effect of carvacrol on acetic acid-induced colitis in rats; its oxidative stress and inflammation-modulating role

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Abstract

Background and aims: Inflammatory bowel disease is a chronic inflammatory disease whose prevalence is rising worldwide. The treatment-related challenges of this disease have expanded the research on other compounds with suitable therapeutic properties. Carvacrol monoterpene phenolic compound, with a wide range of therapeutic properties, can be an appropriate choice. This study aims to investigate the protective effect of carvacrol on acetic acid-induced colitis in mice, further emphasizing the modulating role of oxidative stress and inflammation.

Methods: This experimental study was conducted on 60 mice divided into six groups. Colitis was induced by intrarectal injection of acetic acid. Five groups of mice received carvacrol at doses of 12.5, 25, 50, and 100 mg/kg and normal saline (1 ml/kg). One group was considered normal (without colitis) and received normal saline (1 ml/kg). The severity of colitis complications was assessed through histopathological examination of colon tissue samples. Furthermore, the malondialdehyde (MDA) level, total antioxidant capacity (TAC), and gene expression of inflammatory markers were investigated in the colon samples. Data analysis was done by PRISM version 8 using one-way ANOVA and Tukey's test.

Results: The results showed that the induction of colitis caused significant damage to the intestinal mucosal layers, and the administration of carvacrol reduced the severity of this damage. Interestingly, the TAC of all groups that received carvacrol was higher than that of the group that received normal saline ($P<0.05$). The administration of carvacrol decreased the MDA level ($P<0.05$). In addition, the gene expression of interleukin-1beta (IL-1β), Toll-like receptor 4 (TLR4), and tumor necrosis factor-alpha (TNF-α) reduced after carvacrol administration ($P<0.05$).

Conclusion: Carvacrol exerted a protective effect on the acetic acid-induced colitis in mice, probably via inhibiting the inflammatory cascade and modulating oxidative stress.

Keywords: Inflammatory bowel disease, Oxidative stress, Carvacrol, Inflammation

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Introduction

An inflammatory bowel disease is a group of diseases that cause inflammation of the colon and small intestine wall. Ulcerative colitis and Crohn's disease are among the most common diseases of this type (1). The incidence of this disease has increased significantly in the last few decades due to its chronic nature and progressive course, which impose a considerable cost on the countries' health systems. Although this disease was more prevalent in western countries in the past, statistics show that its incidence is also increasing substantially in developing countries (2). According to one study conducted in Iran, the incidence of this disease is rising, which can be an alarm for the increase of its risk factors (3). Various studies on monozygotic twins and the incompatibility of disease occurrence in them have shown that environmental risk factors play a critical role in the pathogenesis of inflammatory bowel disease, and researchers have implicated pharmaceutical, infectious, nutritional, stress, and socio-economic factors in increasing the incidence of such diseases (4). Regarding the nutritional factors, the consumption of monounsaturated fats, polyunsaturated fatty acids, and vitamin B6 are among the most critical factors for the increased incidence of these diseases (5).

Epidemiological studies have shown that consuming fruits and vegetables reduces the risk of inflammatory bowel diseases, which can be due to the role of oxidative stress in the etiology of these diseases. The consumption of antioxidants significantly reduces the incidence of this disease (6). Monoterpenes are a group of terpenes that consist of two isoprene units and have a special status in the pharmaceutical, cosmetic, agricultural, and food industries. The potential anti-inflammatory properties of these compounds indicate the therapeutic potential of this chemical group for the treatment of inflammatory diseases (7). Carvacrol is a phenolic compound belonging to monoterpenes that have significant anti-inflammatory and antioxidant effects

along with antimicrobial, antitumor, neuroprotective, and cardioprotective properties (8). The efficacy of this compound against diseases with inflammatory etiology, including asthma, shows its role in modulating the immune system (9). Given the numerous side effects of standard therapies for inflammatory bowel diseases and the high economic burden of these compounds, it is necessary to use natural compounds with comparably fewer side effects (10); Meanwhile, carvacrol, which is a natural monoterpene with multiple therapeutic effects, can play a noticeable therapeutic role in inflammatory diseases such as inflammatory bowel disease (11). In this study, the protective role of carvacrol in the acetic acid-induced colitis model in mice was assessed considering histopathological evaluations and assessment of its effects on oxidative stress and inflammatory responses in the colon tissue.

Materials and Methods

This study used 60 male NMRI mice according to the protocol and principles of working with laboratory animals. We kept the mice under controlled laboratory conditions, including 22 ± 1°C temperature, a 12-hour light/dark cycle, and free access to food and water. Animals were divided into six groups (n = 10). After induction of colitis, treatment was started and continued for seven days. Then mice were euthanized, and colon samples were collected for histopathological, molecular, and biochemical investigations.

Colitis induction

First, the mice were fasting for 24 hours with free access to water. Then they were anesthetized with the combination (1:1 ml/kg; v/v 1) of xylazine 2% (10 mg/kg) and ketamine 10% (50 mg/kg). Finally, a medical-grade polyurethane cannula (external diameter of 2 mm) was inserted into the anus of the mice (7 mm). Through it, 1 ml of 4% acetic acid was injected into the colon (12). Then the mice were divided into the following groups: Group 1: received normal saline at a dose of 1 ml/kg; Groups 2-5: received carvacrol (Sigma Aldrich) at doses of 12.5, 25, 50, and 100 mg/kg; Group 6 as control normal group (colitis did not induce) received normal saline at a dose of 1ml/kg. All administrations continued for 7 days. The dose and time of drug administrations were chosen based on the pilot study as well as previous studies (13).

Examining the histopathological changes of colon tissue

After the treatment, the mice were subjected to deep anesthesia, the intestinal tissue was removed, and the pathological changes were examined using H&E staining in the colon tissue. For this purpose, the removed colon was first washed with phosphate buffer and then fixed in 10% formalin. Then samples were sliced into small pieces with a thickness of 5 µm using a microtome, and finally stained with H&E and checked for mucosal damage, hyperemia, necrosis, submucosal edema, cellular infiltration, and cellular hyperplasia (14).

Measurement of total antioxidant capacity in the colon samples

At the end of the study, the colon was extracted on the ice-cold surface and directly placed into liquid nitrogen, and stored at -70-C freezer until the molecular and biochemical assessments. The antioxidant capacity of the colon tissues was determined by measuring the ability to reduce Fe^{3+} to Fe^{2+} using the ferric-reducing ability of plasma (FRAP) assay. FRAP working solution contains 25 mL of acetate buffer, 2.5 mL of TPTZ solution, and 2.5 mL of $FeCl_3$. For this purpose, the colon was removed, homogenized, and centrifuged at 10 000 rpm, and the supernatant was used to evaluate the antioxidant capacity. Fifty microliters of this solution were added to 1.5 mL of freshly prepared working solution at 37°C. After 10 minutes, a complex of Fe^{2+} and TPTZ was formed, which created a blue color. Its absorption was calculated at a wavelength of 593 nm, and $FeSO_4$ was used as a standard solution in this method (15).

Measurement of malondialdehyde level in the colon tissue

One ml of homogenized colon tissue was placed in a 20 mL glass tube and incubated at 37 ± 1 °C in a metabolic shaker for 60 minutes. Afterward, 1 mL of 5% tetrachloroacetic acid along with 1 mL of 67% thiobarbituric acid was added to it and mixed well after each step. The content of each vial was transferred to a centrifuge tube and centrifuged at 2000 rpm for 15 minutes. Next, the supernatant solution was transferred to another tube and left in a boiling water bath. After 10 minutes, the test tubes cooled down, and the absorbance of each part was measured at 535 nm (16).

Expression of inflammatory genes in the colon tissue

After the experiment, the mice were killed, the colon was removed, and the gene expression of inflammatory genes, including interleukin-1 beta (IL-1β), tumor necrosis factor (TNF-α), and Toll-like receptor 4 (TLR4), were analyzed using real-time reverse transcription polymerase chain reaction (RT-PCR). The reaction was done in triplicate for each gene and was repeated twice. The required specific primers were designed using Primer 3 software version 0.4.0 (http://frodo.wi.mit.edu). The genes and their primers are listed in Table 1. B2m was used as a housekeeping gene (normalizer), and alterations in the expression of each target mRNA in comparison with B2m were measured based on the $2^{-\Delta\Delta Ct}$ relative expression

Table 1. Primer sequences

Primers	Reverse	Forward
B2m	AGGGGTGATACGCTTTACCTTTA	TCATCGACACCTGAAATCTAGGA
TNF-α	GGCTTGTCACTCGAATTTTGAGA	CTGAACTTCGGGGTGATCGG
Il-1β	TGGATGCTCTCATCAGGACAG	GAAATGCCACCTTTTGACAGTG
TLR4	GAGGCCAATTTTGTCTCCACA	ATGGCATGGCTTACACCACC

formula, as described in our previous publication.

Data analysis

PRISM statistical software (version 7) was used to collect and analyze the data. One-way ANOVA and Tukey's post hoc tests were performed. Statistically significant results were considered when $P < 0.05$ was used.

Results

Intestinal tissue antioxidant capacity

As illustrated in Figure 1, the antioxidant capacity of the saline-treated colitis group is lower than that of the saline-treated normal group ($P < 0.001$). We showed that carvacrol at doses of 12.5 ($P < 0.01$), 25 ($P < 0.001$), 50 ($P < 0.001$), and 100 mg/kg ($P < 0.001$) significantly increased the antioxidant capacity of colitis groups compared with the saline-treated colitis group.

Malondialdehyde level in the colon tissue

As shown in Figure 2, the induction of colitis by acetic acid increased the malondialdehyde (MDA) level in the colon tissue compared to the normal group ($P < 0.001$). Meanwhile, the administration of carvacrol at doses of 12.5 ($P < 0.05$), 25 ($P < 0.01$), 50 ($P < 0.01$), and 100 mg/kg ($P < 0.001$) significantly decreased MDA levels in colitis groups in compared with the saline-treated colitis group.

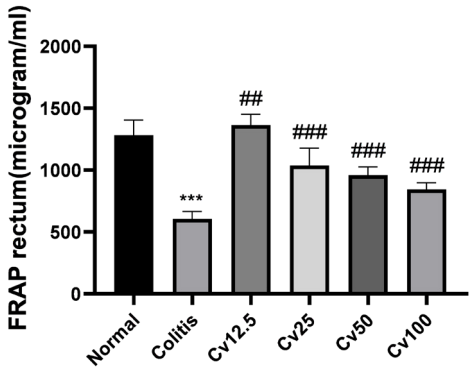

Figure 1. The total antioxidant capacity in the colon samples. The data are expressed as mean ± standard deviation. ***Significant difference compared to the normal group ($P < 0.001$). ##Significant difference with the saline-treated colitis group ($P < 0.01$) and ###Significant difference with the saline-treated colitis group ($P < 0.001$). CV; Carvacrol.

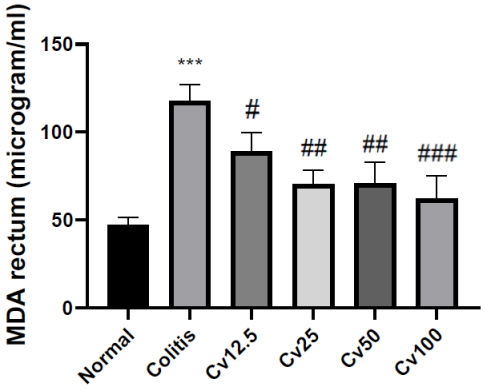

Figure 2. Malondialdehyde levels in the colon of the studied groups. The data are expressed as mean ± standard deviation. ***Significant difference compared to the normal group ($P < 0.001$). #Significant difference with the saline-treated colitis group ($P < 0.05$), ##Significant difference with the saline-treated colitis group ($P < 0.01$) and ###Significant difference with the saline-treated colitis group ($P < 0.001$). Abbreviations: MDA, malondialdehyde; CV; carvacrol.

Histopathological changes of colon tissue

Figure 3 shows the histopathological findings of the colon tissue in the studied groups. As observed, colon tissue remained healthy in the normal group and showed no signs of damage to the crypts and infiltration of inflammatory cells. Meanwhile, in the saline-treated colitis group, in addition to the destruction of the epithelium and bleeding, inflammation and infiltration of inflammatory cells are evident. We showed that following treatment with carvacrol, the intensity of involvement is lower than that of the saline-treated group.

IL-1β gene expression

Figure 4 shows the gene expression of the inflammatory cytokine IL-1β in the colon of the studied groups. As shown, the gene expression of IL-1β in the saline-treated colitis group is significantly higher than in the normal group ($P < 0.01$). We showed that carvacrol at doses of 12.5 and 25 mg/kg significantly decreased the gene expression of IL-1β in the colon of colitis groups ($P < 0.01$).

TLR4 gene expression

Figure 5 shows the gene expression of the inflammatory cytokine TLR4 in the colon of the studied groups. As shown, the gene expression of TLR4 in the saline-treated colitis group is significantly higher than in the normal group ($P < 0.01$). We showed that carvacrol at the dose of 25 mg/kg significantly decreased the gene expression of TLR4 in the colon of colitis groups ($P < 0.05$).

TNFα gene expression

Figure 6 shows the gene expression of the inflammatory cytokine TNFα in the colon of the studied groups. As shown, the gene expression of TNFα in the saline-treated colitis group is significantly higher than in the normal group ($P < 0.001$). We showed that carvacrol at doses of 25 ($P < 0.01$) and 50 mg/kg ($P < 0.001$) significantly decreased the gene expression of TNFα in the colon of colitis groups.

Discussion

The present study aimed to determine the protective effect of carvacrol on acetic acid-induced colitis in mice. Carvacrol could reduce tissue damage caused by acetic acid by modulating oxidative stress and inflammation. Examining the antioxidant capacity of the intestinal tissue of the studied groups showed that the administration of carvacrol increased the antioxidant capacity in a dose-dependent manner compared to the group that received saline instead of carvacrol, which can show the improving effect of carvacrol against the destructive effects of oxidative stress. In this study, the MDA level

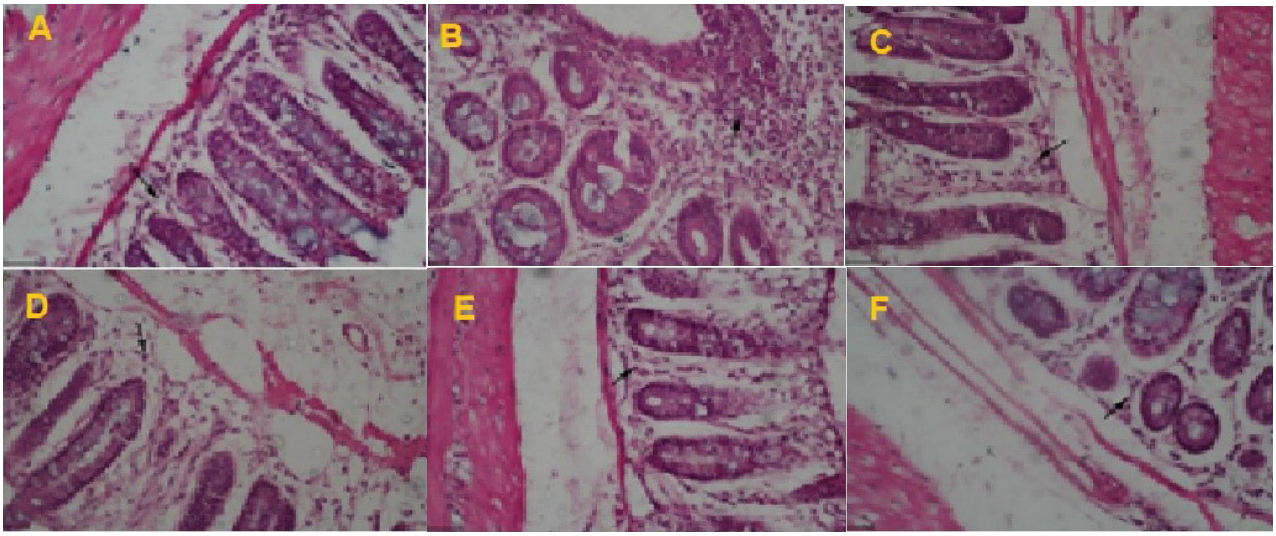

Figure 3. Histopathological findings colon tissue stained with hematoxylin & eosin (optical microscope; 40×magnification). (A) In the colon of the normal group, the number of inflammatory cells is low, and the mucous layer and crypts are normal. (B) In the colon tissue of the saline-treated colitis group, there is mucosal and submucosal inflammation, as well as damage to the epithelium and infiltration of inflammatory leukocytes. (C) The colitis group was treated with carvacrol at 12.5 mg/kg. (D) The colitis group was treated with carvacrol at a 25 mg/kg dose. (E) The colitis group was treated with carvacrol at 50 mg/kg. (F) The colitis group was treated with carvacrol at a 100 mg/kg dose. As observed, the number of inflammatory cells decreased in the groups that received carvacrol. Arrowhead points to inflammatory cells. Scale bar=25 µm.

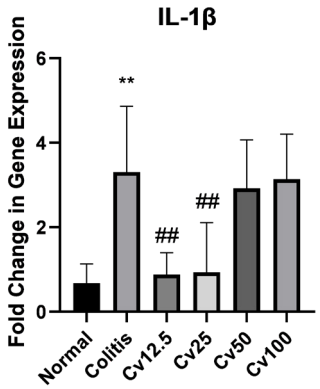

Figure 4. The gene expression of IL-1β in the colon of studied groups. The data are expressed as mean±standard deviation and were analyzed using a one-way analysis of variance followed by Tukey's test. **Significant difference compared to the normal group ($P<0.01$) and ##Significant difference compared to the saline-treated colitis group ($P<0.01$). CV; Carvacrol.

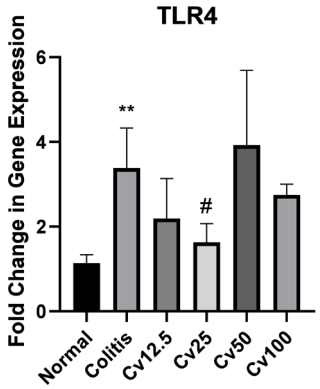

Figure 5. The gene expression of TLR4 in the colon of studied groups. The data are expressed as mean±standard deviation and were analyzed using a one-way analysis of variance followed by Tukey's test. **Significant difference compared to the normal group ($P<0.01$) and #Significant difference compared to the saline-treated colitis group ($P<0.05$). CV; Carvacrol.

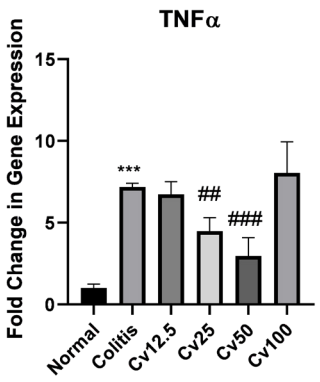

Figure 6. The gene expression of TNFα in the colon of studied groups. The gene expression of TNFα in the colon of studied groups. The data are expressed as mean±standard deviation and were analyzed using a one-way analysis of variance followed by Tukey's test. ***Significant difference compared to the normal group ($P<0.001$), ##Significant difference compared to the saline-treated colitis group ($P<0.01$) and ###Significant difference compared to the saline-treated colitis group ($P<0.001$). CV; Carvacrol.

was higher in the group in which colitis was induced by giving acetic acid, and the administration of carvacrol could increase the MDA level in the studied groups in a dose-dependent manner. The histopathological findings of the study and other cited findings indicate the healing properties of carvacrol against the destructive effects of acetic acid. Previous studies have shown that oxidative stress and intestinal tissue inflammation play an important role in the development of colitis (17). In colitis, damage to the epithelium increases the intestinal epithelium's absorption of bacterial endotoxins such as lipopolysaccharide. TLR4 in the intestinal wall detects these lipopolysaccharides (18). The interaction between the TLR4 receptor and antigens such as lipopolysaccharide can lead to the activation of the TLR4/NF-κB pathway

and the production and secretion of pro-inflammatory cytokines such as TNF-α and IL-1, which in turn can lead to the recruitment of other inflammatory cells and their penetration into intestinal tissue (19). Studies have shown that following colitis, the expression of inflammatory cytokines increases in colon tissue (20). In this regard, clinical and preclinical studies have shown that excessive production of oxidative stress indicators is involved in the pathophysiology of colitis (21). The chronic inflammation in the intestinal mucosa due to the mentioned mechanism leads to the peroxidation of lipids, which increases the level of MDA and other indicators of oxidative stress (22). Total antioxidant capacity (TAC) in the colon has been shown to decrease following colitis (23). In agreement with the above studies, we observed that the level of MDA was significantly increased, and FRAP was reduced considerably in the colitis group. Furthermore, we found that colitis was associated with a significant increase in inflammatory cytokine gene expression, including TLR4, TNF-α, and IL-1β.

Various studies have confirmed that some natural compounds, including caffeic acid (24), nerolidol (25), tricine (26) as well as *α-ketoglutarate* (27), are effective in reducing the inflammatory response in the colon following colitis. Carvacrol is a natural phenolic monoterpenoid present in the essential oils of some plants. Many studies have shown this compound's anti-inflammatory, antioxidant, immune system-modulating, and antimicrobial effects, which indicates its high potential for improving diseases such as asthma, allergies, rheumatoid arthritis, and other inflammatory and autoimmune diseases (8,28,29). Studies have attributed the toxicity of this phenolic compound to animal cells to its very high doses (median lethal dose: 810 mg/kg) (30), which is much higher than the effective dose of this compound in previous studies for its anti-inflammatory properties (31,32). The present study showed that carvacrol significantly decreased the level of MDA, increased FRAP, and decreased gene expression of inflammatory cytokines such as TLR4, TNF-α, and IL-1β in the intestinal tissue of the colitis group. Several studies have shown that the intrarectal injection of acetic acid causes experimental colitis in rodents (33). Colitis is associated with edema, damage to the colon epithelium, and infiltration of inflammatory cells, such as macrophages and neutrophils, into the epithelium (34). In the present study, infiltration of inflammatory cells and edema were observed along with epithelial lesions in the colon tissue. The findings showed that carvacrol reduced edema, epithelial damage, and the infiltration of inflammatory cells into the colon tissue.

Few studies have addressed the effect of essential oils containing carvacrol on inflammatory bowel disease to date. Yet, the possible ways of producing the effect of this compound in this disease have not been investigated (35). The present study, for the first time and at least partially, showed that carvacrol inhibits the destructive effects of colitis by reducing oxidative stress (as a decrease in MDA levels and an increase in FRAP) along with inducing an anti-inflammatory response (reduction of inflammatory cytokine gene expression). In other words, it was shown that carvacrol has beneficial effects in reducing colitis through its antioxidant and anti-inflammatory effects in the experimental model. However, further studies are needed to seek out other underlying mechanisms for the impact of carvacrol on colitis.

Conclusion

Taken together, the findings of the present study aimed at investigating the healing properties of carvacrol on acetic acid-induced colitis in mice with a further emphasis on the modulating role of oxidative stress and inflammation, showed that carvacrol potentially reduces the histopathological damage in the colon as well as decreased oxidative stress and the inflammatory response in colon tissue.

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Writing—Review and Editing: Narges Kargar-Dastgerdi, Hossein Amini-Khoei, Mohammad Rahimi-Madiseh.

Conflict of Interest Disclosures

The authors have no conflicts of interest to disclose.

Ethical Approval

The ethics approval was obtained from Shahrekord University of Medical Sciences with the ethical code of IR.SKUMS.REC.1399.036.

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