

## Original Article

# The Suppression of Adjuvant-induced Inflammation and the Inhibition of the Serum and Tissue IL-17, TNF- $\alpha$ and IL-1 $\beta$ levels by Thymol and Carvacrol

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## Abstract

**Background and Aim:** Thymol and carvacrol are two important components of thyme that have multiple medicinal uses. This study investigates the *in vivo* effects of these natural products on adjuvant-induced inflammation and secretion of interleukin (IL)-17 and key inflammatory cytokines in rats.

**Materials and Methods:** We injected complete Freund's adjuvant (CFA) into the hind paws of rats in order to induce inflammation. Each of the CFA-treated rat groups received gavages of thymol, carvacrol, or vehicle (CFA-only group). Rats' paws and ankle edema were measured and then we were able to determine an inflammatory score based on the results. After 72 h of inflammation induction, sera were collected and subsequently inflamed tissue extracts were prepared for cytokine assay by ELISA.

**Results:** Both components significantly decreased paw edema in rats ( $p < 0.01$ ). Thymol decreased ankle edema to 61.6% of edema in CFA-only rats ( $p < 0.001$ ). We observed a decreased inflammatory score in the thymol and carvacrol-treated rats. The evaluation of the tissue and serum inflammatory cytokine levels showed that both components decreased tumor necrosis factor (TNF)- $\alpha$  levels ( $p < 0.05$ ). Thymol and carvacrol reduced interleukin (IL)-1 $\beta$  serum and tissue levels, respectively. These components reduced tissue levels of IL-17 from  $148.4 \pm 13.4$  pg/ml in CFA-only rats to  $90.1 \pm 18.9$  pg/ml (thymol) and  $82.3 \pm 9.2$  pg/ml (carvacrol). Both components decreased serum IL-17 levels in rats ( $p < 0.05$ ). In comparison, the anti-inflammatory drug, indomethacin, reduced the inflammatory score and decreased tissue TNF- $\alpha$  and IL-1 $\beta$  levels but did not affect IL-17 production.

**Conclusion:** Carvacrol and thymol could relieve inflammation symptoms possibly by downregulating serum and tissue IL-17 expression in addition to key pro-inflammatory cytokines, TNF $\alpha$  and IL-1 $\beta$ .

**Keywords:** Thymol, carvacrol, anti-inflammatory, adjuvant-induced inflammation

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## Introduction

Uncontrolled, persistent inflammation damages various organs of the body and appears to play a role in many chronic diseases. Inflammation is characterized by an interaction between anti-inflammatory and pro-inflammatory cytokines (1). The anti-inflammatory cytokines, interleukin (IL)-10 and transforming growth factor (TGF)- $\beta$ , are immunoregulatory molecules that control pro-inflammatory cytokine responses and reduce the severity of inflammatory reactions (2). Pro-inflammatory cytokines IL-1 $\beta$  and tumor necrosis factor (TNF)- $\alpha$  are believed to contribute to the pathogenesis of many inflammatory diseases (3,4). IL-17, a T-helper 17 (T<sub>H</sub>17) cytokine, also induces the expression of many mediators of inflammation, including those involved in the maturation, proliferation, and chemotaxis of neutrophils. Dysregulated IL-17 production has been associated with tissue damage and several inflammatory diseases. Experimental targeting of this molecule in animal models is an approach to assess the anti-inflammatory effects of drugs (5,6).

In recent decades, the therapeutic potential assessment of plants essential oils and their extracts has been the goal of scientific researches in the discovery of new drugs of plant origin, in particular those that display anti-inflammatory properties (7).

Thyme essential oil is a mixture of the monoterpenes thymol and carvacrol, both of which possess anti-inflammatory activities. A recent study has shown the *in vitro* inhibitory effects of these components on the production of inflammatory cytokines (IL-1 $\beta$  and TNF- $\alpha$ ) by macrophages via the modulation of signaling molecules and transcription factors of inflammatory processes (8). These components have suppressive activities on dendritic cell maturation and function (9). In addition, they have induced production of cytokines such as IL-2 and IFN- $\gamma$  in Jurkat T cells (10). The majority of these studies have investigated the *in vitro* anti-inflammatory effects of thymol and carvacrol. However, there is less information about their *in vivo* anti-inflammatory effects, particularly their effects on local inflammatory cytokine production. We have used adjuvant-induced inflammation in rats that served as

animal model of inflammation to assess the effects of thymol and carvacrol on the production of inflammatory mediators in the periphery and at the site of inflammation.

## Materials and Methods

Complete Freund's adjuvant (CFA), Phenylmethylsulfonyl fluoride (PMSF), thymol and carvacrol were purchased from Sigma-Aldrich Co. (St. Louis, MO). Indomethacin was obtained from Iran Hormone Company (Tehran, Iran). IL-1 $\beta$ , TNF- $\alpha$  and IL-17A enzyme-linked immunosorbent assay (ELISA) kits were purchased from eBioscience (San Diego, CA). Other chemicals and solvents were of reagent grade and available.

### Animals and Experimental Design

Male Wistar rats that weighed 120–160g were obtained from the Center of Comparative and Experimental Medicine at Shiraz University of Medical Sciences, Shiraz, Iran. Rats were kept under standard conditions and the relevant Ethics Committee approved all protocols for animal care and treatments. We divided 30 rats into five groups, with six animals per group. In four of the groups, we injected 0.1 ml CFA, that contained 1mg heat killed *Mycobacterium tuberculosis* bacilli, into the animals' left hind paws. The fifth group (negative control) received normal saline injections. CFA-treated rat groups received either olive oil as the vehicle (CFA-only), thymol (100 mg/kg), carvacrol (100 mg/kg), or indomethacin (10 mg/kg) for three consecutive days by gavage. These concentrations of the compounds were based on previous *in vivo* studies with thymol and carvacrol and on the anti-inflammatory effective doses of indomethacin (11-14). The negative control group also received the vehicle in parallel with the test groups.

### Inflammation Scoring and Sample Collection

Rats were firstly weighed, and then their ankle as well as paw edema, and their redness were measured daily with a digital caliper. The degree of inflammation (edema and redness) was estimated on a score from 0 to 5 as follows: 0(none); 1(mild); 2(mild/moderate); 3(moderate); 4(moderate/severe); and 5(severe). After three days, the animals were sacrificed and their inflamed tissues were removed for tissue extract preparation. Blood samples were also collected to measure a series of hematologic parameters using a

Sysmex analyzer (Japan). Rats' sera were prepared and kept in  $-80^{\circ}\text{C}$  for cytokine assay.

### The Preparation of the Homogenates of Inflamed Paw Tissue

The collected inflamed tissues were weighed and immediately placed in 1mL of ice-cold homogenization buffer (Tris, 10mol/L;  $\text{MgCl}_2$ , 2.5 mmol/L; NaCl, 50 mmol/L, pH 7.4) containing PMSF (1mM) as protease inhibitor. The tissues were then homogenized using a TissueLyser device (Qiagen, Hilden, Germany). The tissue homogenates were spun at  $20000 \times g$  for 30 min at  $4^{\circ}\text{C}$ , and then the supernatant was collected to store at  $-80^{\circ}\text{C}$  for cytokine assay.

### ELISA Assay

The IL-1 $\beta$ , TNF- $\alpha$  and IL-17A cytokine levels in the supernatant of the homogenates of inflamed paw tissue as well as rat sera were measured using related ELISA kits with the sensitivity of 8 pg/ml (for IL-1 $\beta$  and TNF- $\alpha$ ) and 4 pg/ml (for IL-17A) according to the manufacturer protocols. Briefly, 96-well microplates were coated with 100 $\mu\text{l}$  capture antibody for an overnight at  $4^{\circ}\text{C}$ . The plates were blocked and then 100 $\mu\text{l}$ /well of samples and standards were added for 2 h at room temperature (RT). 100 $\mu\text{l}$  of detection antibody (kit-provided) was added to each well and incubated at RT for 1 h. After washing, avidin-horseradish peroxidase (HRP) and then tetramethylbenzidine (TMB) substrate and stop solution were added, respectively. The absorbance of reaction was measured at 450 nm with a background subtraction at 570 nm using a microplate reader (Biotek, Carson City, NV).

### Statistical Analysis

Data were expressed as mean  $\pm$  standard deviation (SD). The significant differences between groups were evaluated by GraphPad prism software (San Diego, CA) and appropriate statistical tests, e.g., one- and two-way ANOVA with suitable post hoc test.  $P < 0.05$  was considered statistically significant.

## Results and Discussion

### The Effects of Treatments on Paw and Ankle Edema and Inflammatory Score

The effects of thymol and carvacrol on inflammation induced by CFA are depicted in Figures 1 and 2. At 72 h after induction of CFA inflammation, the paw

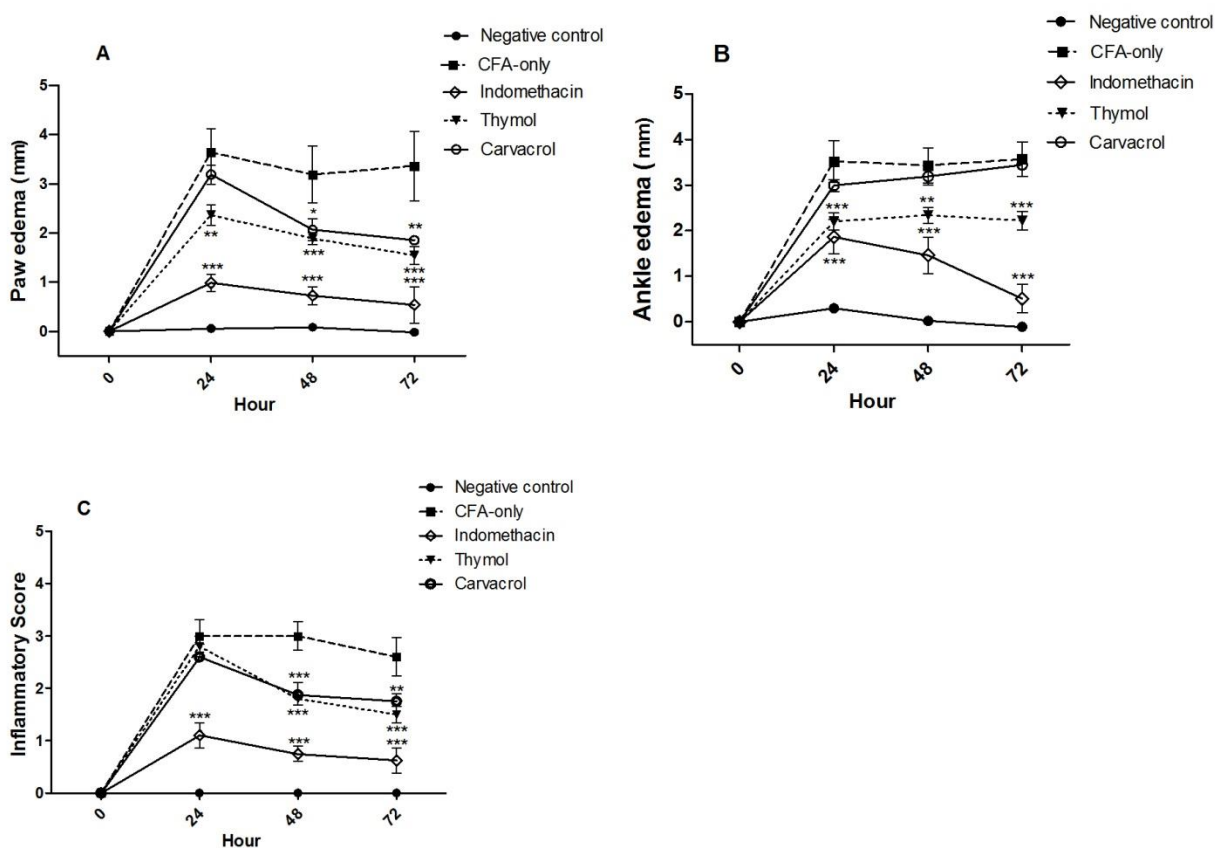
edema (Figure 1A) increased to  $3.36 \pm 1.6$  mm ( $p < 0.001$ ) and ankle edema (Figure 1B) increased to  $3.57 \pm 0.83$  mm in the CFA-only group ( $p < 0.001$ ). The paw and ankle edema had been reduced in thymol treated rats to  $1.5 \pm 0.4$  mm;  $p < 0.001$  and  $2.2 \pm 0.46$  mm;  $p < 0.001$  respectively. Although the paw edema had also decreased to  $1.85 \pm 0.1$  mm ( $p < 0.01$ ) in carvacrol-treated rats, ankle edema had not been affected. Treatment of rats with indomethacin as an anti-inflammatory drug reduced paw edema to  $0.54 \pm 0.5$  mm (Figure 1A;  $p < 0.001$ ) and ankle edema to  $0.51 \pm 0.44$  mm (Figure 1B;  $p < 0.001$ ). As shown in Figure 1C, CFA injection increased the inflammatory score from zero in the negative control group to  $2.6 \pm 0.8$  in the CFA-only group after 72 h. Treatment of rats with thymol, carvacrol, or indomethacin decreased this score to  $1.5 \pm 0.4$  (thymol,  $p < 0.001$ ),  $1.8 \pm 0.3$  (carvacrol,  $p < 0.01$ ), and  $0.6 \pm 0.5$  (indomethacin,  $p < 0.001$ ). Examples of the effects of the compounds on CFA-induced inflammation in rats are presented in Figure 2.

### The Effects of Treatments on Cytokines in Tissue Extracts

In order to find the effects of these components on local inflammatory cytokine secretions, the homogenates of inflamed Paw tissue were prepared and cytokine assay was performed by enzyme-linked immunosorbent assay (ELISA). As shown in Figure 3, the levels of cytokines in the extracted tissues from the CFA-only group were:  $214.4 \pm 20.3$  pg/ml (IL-1 $\beta$ ),  $519.1 \pm 45.2$  pg/ml (TNF- $\alpha$ ), and  $148.4 \pm 13.4$  pg/ml (IL-17). Treatment of rats with carvacrol reduced levels of IL-1 $\beta$  ( $136.4 \pm 15.2$  pg/ml;  $p < 0.05$ ), TNF- $\alpha$  ( $282.2 \pm 63.9$  pg/ml;  $p < 0.05$ ), and IL-17 ( $82.2 \pm 9.2$  pg/ml;  $p < 0.05$ ). Thymol had no significant effect on IL-1 $\beta$  levels but it decreased TNF- $\alpha$  ( $289.3 \pm 68.1$  pg/ml;  $p < 0.05$ ) and IL-17 ( $90.1 \pm 18.9$  pg/ml;  $p < 0.05$ ) levels. Indomethacin had no significant effect on IL-17 levels but reduced IL-1 $\beta$  to  $117.8 \pm 5.8$  pg/ml ( $p < 0.05$ ) and TNF- $\alpha$  to  $296 \pm 13.9$  pg/ml ( $p < 0.05$ ).

### The Effect of Treatments on Serum Cytokine Levels

Rat sera were evaluated for cytokine levels by ELISA (Figure 4). The serum levels were: IL-1 $\beta$  ( $53.8 \pm 2.1$  pg/ml), TNF- $\alpha$  ( $67 \pm 3.2$  pg/ml), and IL-17 ( $57.3 \pm 8.9$  pg/ml) in the CFA-only group. Thymol reduced IL-1 $\beta$  serum levels to  $34.3 \pm 7.4$  pg/ml but carvacrol had no



**Figure 1. Effects of thymol and carvacrol on paw and ankle edema and inflammatory score.** Inflammation was induced in rats by injections of complete Freund's adjuvant (CFA) in the left hind paws of the rats. Rats received either olive oil as the vehicle (CFA-only), thymol (100 mg/kg), carvacrol (100 mg/kg), or indomethacin (10 mg/kg) for three consecutive days by gavage. The negative control consisted of a group injected with normal saline that received vehicle in the same manner as the test groups. We obtained daily measurements of the paw edema (A) and ankle edema (B) with a digital caliper. The degree of inflammation (edema and redness) was estimated by a score from 0 to 5 (C). The values are presented as mean  $\pm$  SD in each group (n=6). \* $p$ <0.05, \*\* $p$ <0.01 and \*\*\* $p$ <0.001 show significant differences with the CFA-only group.

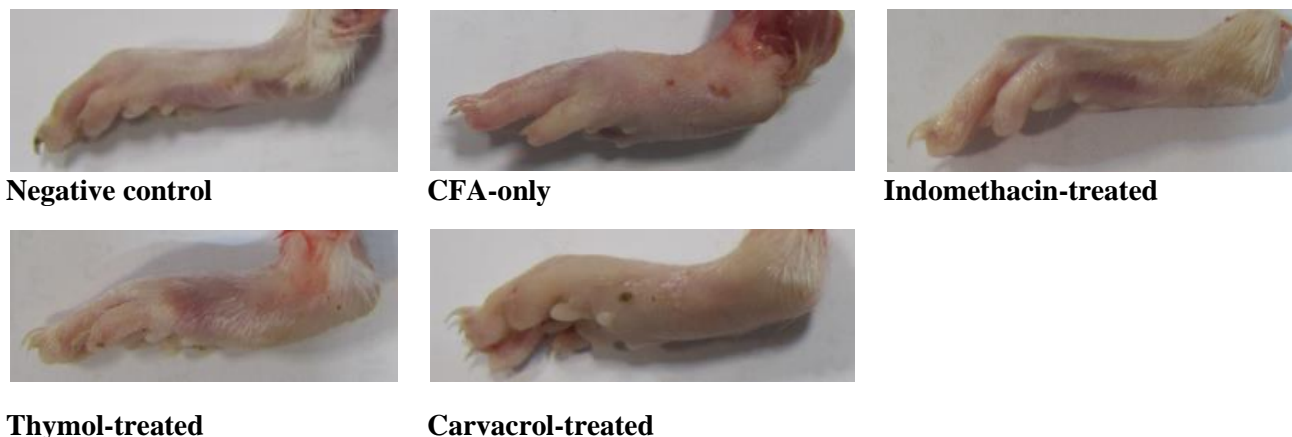
significant effect (Figure 4A). Both thymol and carvacrol reduced TNF- $\alpha$  levels to  $43.1 \pm 7.4$  pg/ml (thymol,  $p$ <0.05) and  $45.4 \pm 3.2$  pg/ml (carvacrol,  $p$ <0.05; Figure 4B). IL-17 levels also were reduced by thymol ( $21.2 \pm 0.4$  pg/ml,  $p$ <0.05) and carvacrol ( $18.9 \pm 5.2$  pg/ml,  $p$ <0.05) as seen in Figure 4C. IL-1 $\beta$  serum levels had decreased in rats treated with indomethacin ( $22 \pm 0.8$  pg/ml,  $p$ <0.01; Figure 4A).

#### The Effect of Thymol and Carvacrol on Weight and Hematological Parameters

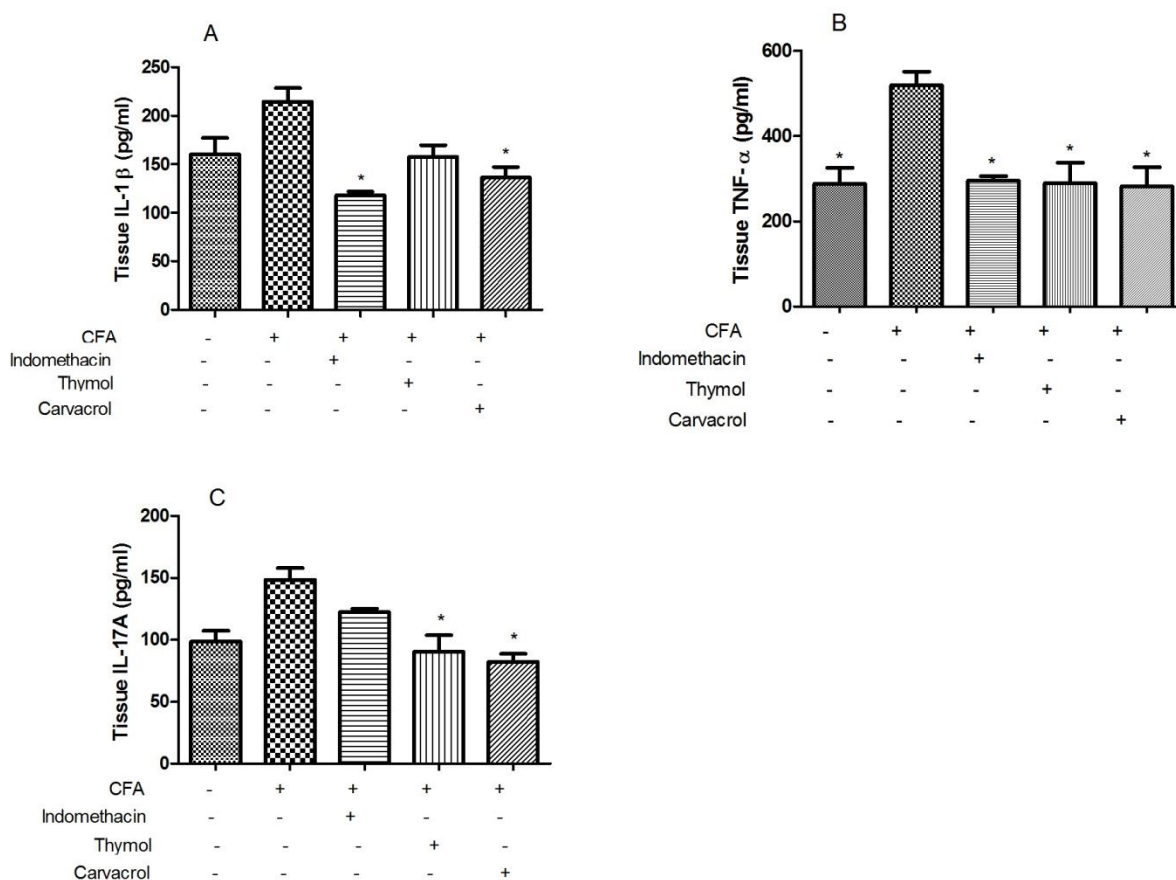
Table 1 lists the changes in animals' weight and hematological parameters after three consecutive days of treatment with thymol and carvacrol. Inflammation induction triggered weight loss from  $(4.25 \pm 2)$  g in the normal control group to  $-1.2 \pm 1$  g ( $p$ <0.05). As seen in Table 1, thymol and carvacrol did not significantly affect weight and the

hematological parameters of white blood cells (WBCs), red blood cells (RBCs), platelet numbers, and hemoglobin (Hb). These components did not affect the percentages of lymphocytes, monocytes, and granulocytes in peripheral blood.

Thymol and carvacrol are the main components of thyme essential oil. *In vitro* anti-inflammatory effects of these components have been reported in various studies (15,16). Carvacrol has been shown to have the ability to decrease cyclooxygenase (Cox)2 and prostaglandin E<sub>2</sub> production whereas thymol decreased TNF- $\alpha$ , IL-6, and IL-1 $\beta$  production in the supernatants of lipopolysaccharide (LPS)-stimulated mouse mammary epithelial cells (15,16). These components reduced IL-1 $\beta$  and TNF- $\alpha$  inflammatory cytokines through the modulation of several transcription factors involved in the inflammatory processes such as c-Jun

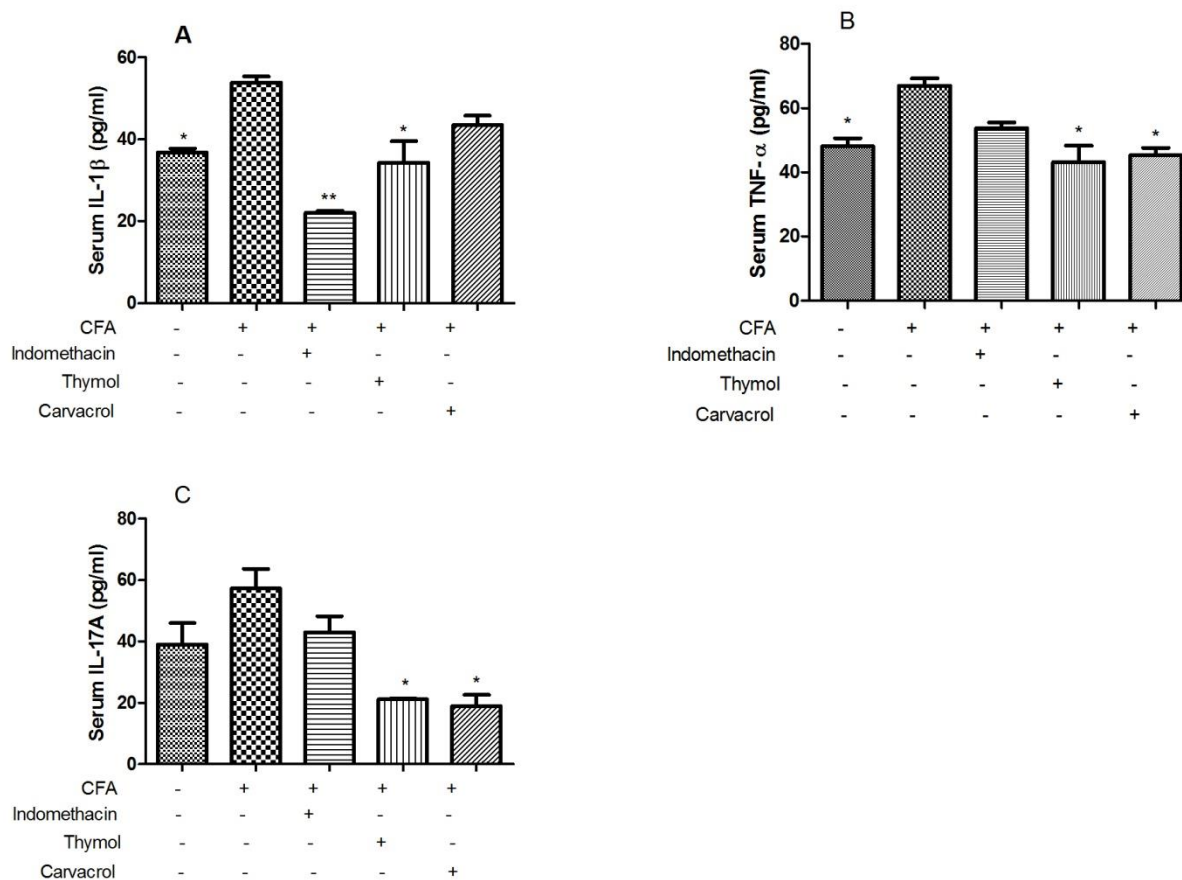


**Figure 2. Effects of thymol and carvacrol on inflammation reduction.** Inflammation was induced in rats by injections of complete Freund’s adjuvant (CFA) in the left hind paws of the rats. Rats received either olive oil as the vehicle (CFA-only), thymol, carvacrol, or indomethacin for three consecutive days by gavage. The negative control consisted of a group injected with normal saline that received vehicle in the same manner as the test groups. Paw and ankle edema in samples of rats from different groups after 72 h are shown.



**Figure 3. Effects of thymol and carvacrol on cytokine release in tissue extracts.** Inflammation was induced in rats by injections of complete Freund’s adjuvant (CFA) in the left hind paws of the rats. Rats received either olive oil as the vehicle (CFA-only), thymol, carvacrol, or indomethacin for three consecutive days by gavage. After three days, animals were sacrificed and their inflamed paw tissues removed for homogenate preparation and cytokine assay by ELISA. The values are presented as mean ± SD in each group (n=6). \**p* < 0.05 shows significant differences with CFA-only group.

N-terminal kinases (JNK), signal transducer and activator of transcription 3 (STAT3), activator protein-



**Figure 4. Effect of thymol and carvacrol on serum cytokine levels.** Inflammation was induced in rats by injections of complete Freund's adjuvant (CFA) in the left hind paws of the rats. Rats received either olive oil as the vehicle (CFA-only), thymol, carvacrol, or indomethacin for three consecutive days by gavage. After three days, animals were sacrificed and their blood was collected for cytokine assay by ELISA. The values are presented as mean  $\pm$  SD in each group (n=6). \*p < 0.05 and \*\*p < 0.01 show significant differences with CFA-only group.

1 (AP-1), and nuclear factor of activated T-cells (NFATs) (8). Our previous study showed that thymol and carvacrol suppressed T-cell responses as well as dendritic cell maturation and function (9). Both components significantly reduced IFN- $\gamma$  and IL-4 production by T cells and downregulated the expressions of co-stimulatory molecules, CD86 and CD40 on dendritic cells (9). These two components with the reduction of IL-2 and IFN- $\gamma$  production modulated T cell activity in part by the downregulation of NFAT-2 and AP-1 transcription factors (10). According to our previous study, these components also reduced IL-17 and ROR $\gamma$ c gene expression in splenocytes and IL-17 production in sera of ovalbumin-immunized mice (11).

In the current study, we evaluated the *in vivo* anti-

inflammatory effects of thymol and carvacrol in an adjuvant-induced inflammation model. After the induction of inflammation by CFA, the rats received thymol, carvacrol, and indomethacin. We chose indomethacin, a potent immunosuppressive drug, as the control. The results showed that thymol had the capacity to reduce the extent of paw and ankle edema to 45.8% and 61.6% of CFA-only rats, respectively. The effect of carvacrol was mainly on paw edema (55% of the CFA-only group). A decreased inflammatory score in treated rats was obtained by both thymol and carvacrol which indicated their strong *in vivo* anti-inflammatory effects. In various studies, the effects of thymol and carvacrol on a number of inflammatory models have been investigated. Zhou *et al.*, showed that administration of thymol could

**Table 1:** Effects of thymol and carvacrol on weight and hematological parameters.

	Negative control rats	CFA-only rats	Indomethacin-treated rats	Thymol-treated rats	Carvacrol-treated rats
<b>Weight gain (+) or loss (-) (gram)</b>	+ (4.25±2)	- (1.2±1) *	- (15.5±2) ***	+ (1±2.8)	+ (1.75±2.3)
<b>RBC number (×10<sup>6</sup>/mm<sup>3</sup>)</b>	6.7±0.7	6.8±0.2	4.6±1.2 **	6.9±0.3	7.1±0.5
<b>Hemoglobin (g/dl)</b>	13±1.4	13.3±0.2	8.8±2.9 **	13.3±0.7	13.4±0.7
<b>Hematocrit (%)</b>	40.5±3	38.3±1.1	24.5±8.7 **	40.7±3.5	38.9±2.6
<b>WBC number (×10<sup>3</sup>/mm<sup>3</sup>)</b>	11.3±2.5	10.5±2.5	9.9±1.1	9±2.5	10.1±2.2
<b>Lymphocytes%</b>	76±4.8	71.8±15.5	62.6±5.4	73.4±6.1	61±8.5
<b>Monocytes%</b>	3.4±2.3	1.8±1.3	0.8±0.4	1.2±0.8	1.2±0.4
<b>Granulocytes%</b>	20.6±7.1	26±15.5	36.6±5.2	25.4±5.6	37.8±8.4
<b>Platelet number (×10<sup>3</sup>/mm<sup>3</sup>)</b>	566±75	540±73	396±69 *	556±55	626±156

Inflammation was induced in rats by injection of CFA in the left hind paw of rats. Rats were treated with vehicle (CFA-only), thymol, carvacrol and indomethacin for three consecutive days. After three days, animals were sacrificed and blood samples were collected for measuring a series of hematologic parameters. The values are presented as mean ± SD (except weight; mean ± SE) in each group (n=6). \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001 show significant differences with negative control group. RBC (red blood cell), WBC (white blood cell)

attenuate the allergic airway inflammation in ovalbumin-induced mouse asthma (17). The anti-inflammatory and wound healing activities of thymol in carrageenan-induced hind paw edema and peritonitis models of rodents have been shown by the reduction in edema and diminished influx of leukocytes to the injured area (18). Arigesavan and Sudhandiran suggested a role for carvacrol in a 1,2-dimethyl hydrazine plus dextran sodium sulfate induced inflammation rat model. In this study, carvacrol served as an excellent dietary antioxidant. Moreover, it reduced nitric oxide (NO), inducible-nitric oxide synthase (iNOS), and IL-1β (19). The protective effects of carvacrol on acute pancreatitis-induced liver injury via antioxidant response (20) and on acute lung injury induced by LPS in mice (21) have also been reported. These data, in addition to the results of the current study, confirmed the benefits of thymol and carvacrol as alternatives in the development of new anti-inflammatory therapeutic strategies.

In order to evaluate the effects of these components on the formation of inflammatory cytokines, we measured IL-1β and TNF-α levels both in inflamed tissues and sera. We have observed decreased secretion levels of both cytokines in samples of thymol- and carvacrol-treated rats. IL-1β and TNF-α are key mediators of inflammatory responses, and their over expressions can lead to severe pro-inflammatory reactions. A previous study has reported that pre-treatment of macrophages with thymol and carvacrol significantly reduced *in vitro*

IL-1β and TNF-α protein and/or gene expressions (22). Carvacryl acetate, a derivative of carvacrol, reduced the inflammatory response in a mouse peritonitis model by decreasing the levels of IL-1β in the peritoneal exudates and increasing IL-10, an anti-inflammatory cytokine (23). Thymol has also attenuated inflammation in isoproterenol-induced myocardial infarcted rats by inhibiting the release of lysosomal enzymes and downregulating the expressions of TNF-α, IL-6, and IL-1β genes (24). This finding provided further evidence for the beneficial use of these components in attenuating inflammatory responses.

IL-17 is an important pro-inflammatory cytokine, produced by CD4+ T cells, that acts as a potent mediator of inflammation. Secretion of this cytokine can recruit monocytes/macrophages and neutrophils to the inflammation site. IL-17 in combination with IL-1β and TNF-α has been shown to promote inflammation (25,26). Production of this cytokine is induced by IL-23. According to a research, the IL-17/IL-23 axis can result in immune activation and, ultimately, chronic inflammation. Targeting IL-17 can be a new therapeutic to treat chronic inflammatory diseases (27,28). In the present study, we have observed that treatment with thymol and carvacrol, but not indomethacin, led to the reduction of IL-17 formation in inflamed tissues as well as sera from the treated rats which suggested a possible new benefit for these components in the treatment of IL-17-based pathologies.

An evaluation of the thymol and carvacrol adverse

effects indicated that neither of these components had significant effects on hematological parameters of WBCs, RBCs and platelet numbers, as well as Hb and differential count. In comparison, indomethacin at the administered concentration, induced anemia and diminished platelet numbers in the rats' peripheral blood. Indomethacin caused significant weight loss in the animals. Although thymol and carvacrol significantly reduced the inflammatory score to a lesser extent than indomethacin, they had no significant adverse effects on the animals.

## Conclusion

Apart from reducing the tissue inflammatory reactions, thymol and carvacrol decreased local and circulating levels of IL-17, IL-1 $\beta$  and TNF- $\alpha$  inflammatory cytokines. They also showed no serious side effects during their administration to the rats. These features make these compounds promising supplementary agents in the suppression of inflammatory diseases.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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