

Effect of carvacrol on various cytokines genes expression in splenocytes of asthmatic mice

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ABSTRACT

Objective(s): With regard to pharmacological effects of carvacrol on the respiratory system, its effect on cytokines genes expression in splenocytes of asthmatic mice was examined in this study.

Materials and Methods: Splenocytes were isolated from non-sensitized (control group), sensitized mice to ovalbumin (OVA) (group S), and S animals treated with dexamethasone, and three concentrations of carvacrol. IL-4, IFN- γ , TGF- β , FOXP3, and IL-17 genes expression were carried out in cultured splenocytes using the real-time PCR method.

Results: Compared to the control group, IFN- γ and FOXP3 genes expression were significantly decreased ($P < 0.001$ for both cases), but IL-4 and IL-17 genes expression were significantly increased in the S group ($P < 0.001$ and $P < 0.05$, respectively). IL-4 gene expression due to treatment of all concentrations of carvacrol, TGF- β gene expression due to its two higher concentrations, and IL-17 gene expression due to its high concentration were significantly decreased compared to group S ($P < 0.01$ to $P < 0.001$). IFN- γ gene expression was significantly increased due to last carvacrol concentration (300 $\mu\text{g/ml}$, $P < 0.01$), and FOXP3 due to its two last concentrations (150 and 300 $\mu\text{g/ml}$, $P < 0.05$ and $P < 0.001$, respectively) in treated S splenocytes. Dexamethasone treatment of sensitized splenocytes only showed significant inhibitory effect on IL-4 and TGF- β genes expression ($P < 0.001$ for both cases).

Conclusion: These results showed the immunomodulatory effect of carvacrol indicating increased IFN- γ and FOXP3 but decreased IL-4, TGF- β , and IL-17 genes expression, which was more selective than the effect of dexamethasone in sensitized mice splenocytes, which indicates its possible therapeutic value in allergy, autoimmunity, and infectious diseases.

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Introduction

The pathologic manifestations of asthma include airway inflammation, airway remodeling and mucus hypersecretion (1). In asthmatics, CD4⁺ T cells productions such as IL-4, IL-5, and IL-13 have been identified in bronchoalveolar lavage (BAL) and airway biopsies. These cytokines are secreted in the airways of patients with mild or asymptomatic asthma (2, 3). Th2 cells have been identified in the airways of asthmatics, and because Th2 cytokines are required for the development of airway eosinophilia and IgE, it has been proposed that Th2 cells stimulate an inflammatory response that results in asthma (1). Th1 has proved to inhibit Th2 responses, and thus one goal of asthma therapy should be focusing on increasing the activity of Th1 (4). The forkhead/winged helix transcription factor FOXP3 serves as a master regulator for Treg (regulatory T cell) development and is currently

found to be the most specific Treg marker (5-7). It has been shown, in both animals and humans that the absence of FOXP3 is associated with the development of immune abnormalities such as severe allergic inflammation and high immunoglobulin E (IgE) levels (8). IL-17 has been proposed to have an important role in the development of autoimmune disorders and in the induction and maintenance of chronic inflammation (9). IL-17 also appears to play an upstream role in T cell-triggered inflammation and hematopoiesis by stimulating stromal cells to secrete other cytokines and growth factors (10).

Carvacrol or cymophenol, C₆H₃CH₃(OH)(C₃H₇) is one of the main constituents of several medicinal plants including *Zataria multiflora* (11) that have various therapeutic effects on respiratory diseases (11, 12).

All plants containing carvacrol have shown relaxant effects on different smooth muscles including trachea

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(13-19) and have also shown anti-inflammatory effects (20-27). The relaxant effect of essential oil from *Carum copticum* on tracheal chains has been shown to be mainly due to its fraction 2, which is suggested to be carvacrol (28). The relaxant effect of carvacrol on tracheal smooth muscle has also been observed in another study (29). Various effects of carvacrol on sensitized guinea pigs including effect on tracheal responsiveness, pathological changes in the lung, inflammatory cells, and inflammatory mediators were also demonstrated in previous studies (29-34). These studies indicate that carvacrol or plant containing this chemical may affect asthma therapy.

Therefore, in the present study, the effect of carvacrol on genes expression of some cytokines in asthmatic mice splenocytes was studied.

Materials and Methods

Experimental animals and their sensitization

Pathogen-free, 6 to 8 week-old, male BALB/c mice, weighing 18 to 20 g, were purchased from Razi Institute (Mashhad, Iran) and maintained in a pathogen-free animal room in School of Medicine. They were kept in hygienic cages and in air-conditioned rooms 12 hr light/12 hr dark cycle with food and water available *ad libitum*.

The mice were sensitized by two intraperitoneal injections, on days 0 and 14 of the experiment, with 10 µg/0.1 ml chicken egg albumin (Ovalbumin, grade V, 98% pure; Sigma, St. Louis, MO, USA) together with Al (OH)₃ as an adjuvant. The animals were then exposed to an aerosolized 2.5% ovalbumin (OVA) concentration for 30 min/day, three days/week for eight weeks beginning from the 21st day of the study. The mice in the control group received IP and inhaled normal saline instead of OVA using the same procedure as OVA administration in sensitized animals (35, 36). Exposures were carried out in a whole body inhalation exposure chamber. A solution of 2.5% OVA in normal saline was aerosolized by delivery of compressed air to a jet nebulizer (37).

Preparation of splenocytes culture and experimental groups

Splenocytes were removed and suspended in complete RPMI 1640 with 15% FBS at a density of 5×10⁶/ml.

The study was carried out in the following groups (n=5 for groups 1 to 3 and n=6 for treated groups with carvacrol):

- (1) Non-sensitized splenocytes (control group).
- (2) Sensitized splenocytes (group S).
- (3) Sensitized splenocytes treated with dexamethasone (0.1 mM/L, Sigma Chemicals, LTD.), (group D).
- (4) Sensitized splenocytes treated with carvacrol at 3 concentrations of 75, 150, and 300 µg/ml (groups C1, C2, and C3).

Splenocytes in all groups were cultured as follows; 5×10⁶ cells were suspended in culture environment (15%) and dexamethasone (0.1 mM/L), carvacrol (75, 150, and 300 µg/ml) were added and were incubated for 18h (T:37°, PCO₂:5%). Then, cells were separated and 1 ml Tripure per every microtube was added.

RNA extraction and real-time PCR analysis

Total RNA was isolated from splenocytes with Tripure Isolation Reagents (Roche Applied Science, Germany) according to the manufacturer's instructions.

Samples were incubated for 10 min at room temperature. Then 200 µl chloroform solution was added to the microtubes, and the vortexing intensity was 15 sec. It was incubated for 15 min at 4°C in dim light. Samples were centrifuged at 4°C with 12000 rpm for 15 min. Clear supernatant was removed carefully and transferred to the other microtubes. Cold isopropanol 500 µl was added and incubated for 10 min at 4°C in dim light, then centrifuged for 10 min at 12000 rpm and 4°C. At the end of this stage, sediment RNA was visible as a tiny white pellet. The supernatant was removed and washed, the RNA precipitated by alcohol 75%, and then centrifuged at 4°C and 7500 rpm for 5 min. Supernatant was carefully and completely emptied and the remaining ethanol in the microtubes was removed by air flow. DEPS water was added to microtubes containing RNA, and for the duration of 10 min at 56°C was placed on a dry-block device; eventually moved to -20°C and held until cDNA synthesis.

Synthesis of cDNA

Due to the low half-life of extracted mRNA, it must be transcribed to cDNA, which was performed by the enzyme reverse transcriptase. This DNA sequence was then used as a template in PCR and second strand DNA synthesis by gene-specific primers.

A fixed volume of input RNA (1 µl) was used for each cDNA reaction. Reverse transcription reaction was carried out with Revert Aid™H Minus M-MulV First Strand (Fermentase, Germany) according to manufacturer's instructions.

Quantitative real-time PCR (qPCR)

The real-time PCR primers and quantitation probes were designed with mRNA sequences for β2M (β2-Microglobulin), IFN-γ (Interferon gamma), IL-4, IL-17, and TGF-β (Transforming growth factor beta), and FOXP3 was obtained from the National Center for Biological Information website (<http://www.ncbi.nlm.nih.gov>), using the Beacon Designer software version 7.9. The criteria for primers and probes design included a melting temperature (T_m) for the primers of 58–60°C, a T_m for the probe of 10°C higher than that of the primers and a maximum amplicon length of less than 150 base pairs (bp). The primers and probes set crossed the intron-exon

boundaries, ensuring amplification of mRNA only. The primers and probe were as follows:

1. **β 2-Microglobulin**; 5-GCCGAACATACTGAACTGCTAC-3(forward)
5-CTTGCTGAAGGACATATCTGACATC-3(reverse)
5-AACACAGTTCCACCCGCTCACATTGA-3 (probe).
2. **IFN- γ** ; 5- GTATTGCCAAGTTTGAGGTCAAC-3(forward)
5- GCTTCCTGAGGCTGGATTC-3(reverse)
5- CCACAGTCCAGCGCCAAGCATTCAA-3 (probe)
3. **IL-4**; 5-TCTCACAGCAACGAAGAAC-3 (forward)
5-CAAGCATGGAGTTTTCCCATG-3 (reverse)
5-AGCACCTTGAAGCCCTACAGACGAGC-3(probe)
4. **IL-17**; 5-CAGACTACCTCAACCGTTC-3 (forward)
5-TTCCCTCCGCATTGACAC-3 (reverse)
5-ACTCTCCACCGCAATGAAGACCCTGA-3 (probe).
5. **TGF- β** ; 5-CCTGGATACCAACTATTGCTTCAG-3 (forward)
5-CAGACAGAAGTTGGCATGGTAG-3 (reverse)
5-TCCACTTCCAACCCAGGTCCTTCCT-3 (probe)
6. **FOXP3**; 5-GGTACACCCAGGAAAGACAG-3 (forward)
5-GCTTGGCAGTGCCTTGAGA-3 (reverse)
5-TGGCTCCTCGAAGACCTTCTCACAACC-3 (probe)

Beta 2 microglobulin (β 2M) was used as reference gene to normalize the mRNA expression levels and control the errors between samples.

The real-time PCR reactions were performed in glass capillaries (Qiagen, Germany) in a final volume of 10 μ l containing 2 μ l cDNA template, 0.4 μ l of primer pairs (200 nM, β 2M, IFN- γ , IL-4, IL-17, TGF- β , and FOXP3), and 5 μ l of the probe Master Mix. Two standard curve methods were used for target and reference genes quantification by a Rotor-Gene Q Real-Time PCR machine (Corbett Research, Australia). The Rotor-Gene 6000 software (Corbett Research, Australia) was used to analyze the standards and the unknown RNA copy numbers. The relative quantity of each mRNA was normalized to the relative quantity of β 2M mRNA. Then the relative gene of interest expression level for each sample was calculated by an equation:

Normalized index^{1/4} copy number of gene of interest (IFN γ , IL-4, ...)/copy number of reference gene (β 2M). Then the fold change in expression of gene of interest for each group was calculated by the following equation:

Mean of gene of interest Normalized Index of test group/mean of gene of interest Normalized Index of healthy controls.

Statistical analysis

All data were expressed as mean \pm SEM. SPSS 16 software was used to analyze the data. The statistical analyses were carried out using one-way ANOVA with Tukey-Kramer *post hoc* tests. The results were

considered statistically significant if the *P* value was less than 0.05.

Results

Comparison of gene expression between sensitized and control splenocytes

In sensitized splenocytes, IFN- γ and FOXP3 genes expression significantly decreased ($P < 0.001$), but IL-4 ($P < 0.001$) and IL-17 genes expression increased ($P < 0.05$), compared to the control group. In addition, IFN- γ /IL-4 ratio was significantly decreased in group S compared to the control group ($P < 0.001$). However, there was no significant difference in TGF- β gene expression between the two groups (Figures 1–6).

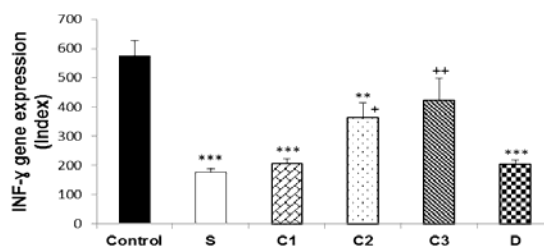


Figure 1. Values (mean \pm SEM) of IFN- γ gene expression in splenocytes from control mice (control) and sensitized animals (S), and S groups treated with dexamethasone (D) and carvacrol at 3 concentrations of 75, 150, and 300 μ g/ml (C1, C2, and C3, respectively), (n=5 for groups C, S, and D, and n=6 for groups treated with carvacrol). The levels of gene expression were gene fold/mL but presented as Normalized Index, as explained in the methods section. Significant difference between control and other groups: ** $P < 0.01$, *** $P < 0.001$. Significant difference between dexamethasone and carvacrol vs sensitized group: + $P < 0.05$, ++ $P < 0.01$. The statistical comparisons were made using ANOVA with Tukey-Kramer multiple *post hoc* test

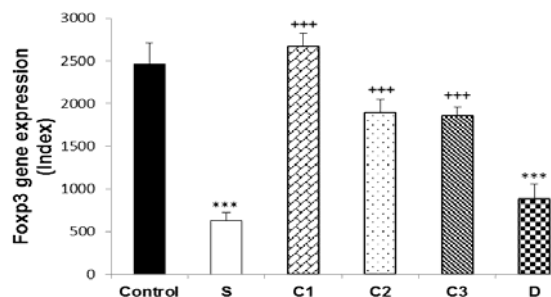


Figure 2. Values (mean \pm SEM) of FOXP3 gene expression in splenocytes from control mice (control) and sensitized animals (S), and S groups treated with dexamethasone (D) and carvacrol at 3 concentrations of 75, 150, and 300 μ g/mL (C1, C2, and C3, respectively), (n=5 for groups C, S, and D, and n=6 for groups treated with carvacrol). The levels of gene expression were gene fold/mL but presented as Normalized Index as explained in the methods section. Significant difference between control and other groups: *** $P < 0.001$. Significant difference between dexamethasone and carvacrol vs sensitized group: +++ $P < 0.001$. The statistical comparisons were made using ANOVA with Tukey-Kramer multiple *post hoc* test

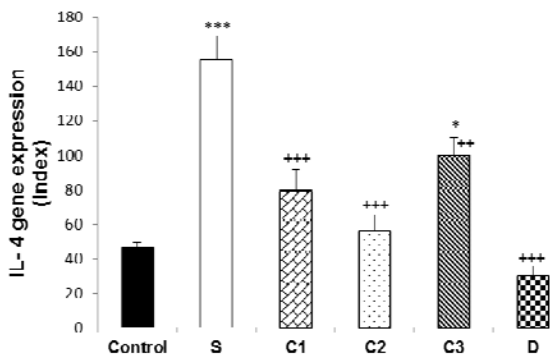


Figure 3. Values (mean±SEM) of IL-4 gene expression in splenocytes from control mice (control) and sensitized animals (S), and S groups treated with dexamethasone (D) and carvacrol at 3 concentrations of 75, 150, and 300 µg/ml (C1, C2, and C3, respectively), (n=5 for groups C, S, and D, and n=6 for groups treated with carvacrol). The levels of gene expression were gene fold/ml but presented as Normalized Index as explained in the methods section. Significant difference between control and other groups: * $P < 0.05$, *** $P < 0.001$. Significant difference between dexamethasone and carvacrol vs sensitized group: ++ $P < 0.01$, +++ $P < 0.001$. The statistical comparisons were made using ANOVA with Tukey-Kramer multiple *post hoc* test

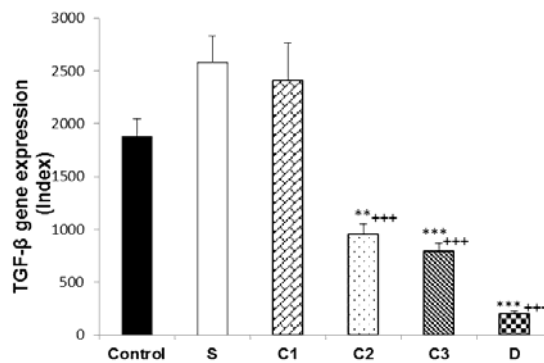


Figure 4. Values (mean±SEM) of TGF-β gene expression in splenocytes from control mice (control) and sensitized animals (S), and S groups treated with dexamethasone (D) and carvacrol at 3 concentrations of 75, 150, and 300 µg/ml (C1, C2, and C3, respectively), (n=5 for groups C, S, and D, and n=6 for groups treated with carvacrol). The levels of gene expression were gene fold/ml but presented as Normalized Index as explained in the methods section. Significant difference between control and other groups: ** $P < 0.01$, *** $P < 0.001$. Significant difference between dexamethasone and carvacrol vs sensitized group: +++ $P < 0.001$. The statistical comparisons were made using ANOVA with Tukey-Kramer multiple *post hoc* test

Cytokine gene expression in splenocytes of sensitized mice treated with carvacrol

IFN-γ gene expression was significantly increased due to two higher carvacrol concentrations (150 and 300 µg/ml), ($P < 0.05$ and $P < 0.01$, respectively), but FOXP3 gene expression was increased by all concentrations ($P < 0.001$ for all cases) in treated compared to non-treated sensitized splenocytes. IL-4 gene expression was significantly decreased due to all carvacrol concentrations ($P < 0.001$ for 75 µg/ml and 150 µg/ml, $P < 0.01$ for 300 µg/ml); TGF-β gene due to its two higher concentrations (150 and 300 µg/ml, $P < 0.001$ for both cases), and IL-17 gene expression

due to its last concentration (300 µg/ml, $P < 0.001$) in treated compared to non-treated sensitized splenocytes. IFN-γ/IL-4 ratio was significantly increased in the treated groups with two higher carvacrol concentrations ($P < 0.001$ for 150 and $P < 0.05$ for 300 µg/ml) compared to group S (Figures 1–6). However, the IFN-γ genes expression in groups treated with C1 and C2, IL-4 gene expression in group treated with C3, TGF-β in groups treated with C2 and C3 and IFN-γ/IL-4 ratio in groups treated with all carvacrol concentrations were significantly different from those of the control group (Figures 1, 3, 4 and 6).

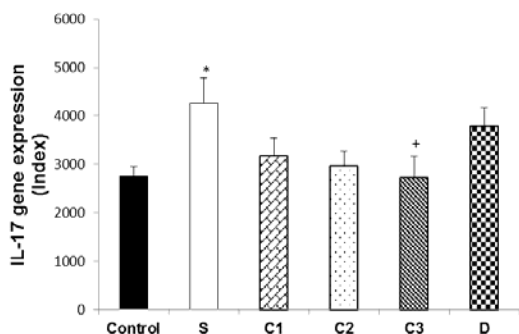


Figure 5. Values (mean±SEM) of IL-17 gene expression in splenocytes from control mice (control) and sensitized animals (S), and S groups treated with dexamethasone (D) and carvacrol at 3 concentrations of 75, 150, and 300 µg/ml (C1, C2, and C3, respectively), (n=5 for groups C, S, and D, and n=6 for treated groups with carvacrol). The levels of gene expression were gene fold/ml but presented as Normalized Index as explained in the methods section. Significant difference between control and sensitized group: * $P < 0.05$. Significant difference between dexamethasone, and carvacrol vs sensitized group: + $P < 0.05$. The statistical comparisons were made using ANOVA with Tukey-Kramer multiple *post hoc* test

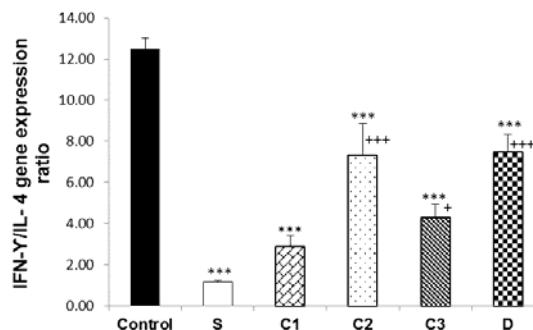


Figure 6. Values (mean±SEM) of IFN-γ / IL-4 gene expression ratio in splenocyte control mice (control), sensitized animals (S), and S groups treated with dexamethasone (D) and carvacrol at 3 concentrations of 75, 150 and 300 µg/ml (C1, C2, and C3 respectively), (n=5 for groups C, S and D and n=6 for treated groups with carvacrol). Significant difference between control and other groups: *** $P < 0.001$. Significant difference between dexamethasone, and carvacrol vs sensitized group: + $P < 0.05$, +++ $P < 0.001$. The statistical comparisons were made using ANOVA with Tukey-Kramer multiple *post hoc* test

Table 1. The values of IFN- γ , IL-4, TGF- β , FOXP3 and IL-17 genes expression as well as IFN- γ /IL-4 ratio in control (C), non-treated sensitized (S), sensitized treated with dexamethasone (0.1 mM/L) and carvacrol at concentrations of 75, 150, and 300 μ g/ml (C1, C2, and C3, respectively) mice

Variable	IFN- γ	FOXP3	IL-4	TGF- β	IL-17	IFN- γ /IL-4 ratio
S+C1	205.33 \pm 17.80	2671.82 \pm 148.10 ***	79.33 \pm 12.29 *	2412.16 \pm 351.47 ***	3176.42 \pm 377.06	2.88 \pm 0.52 ***
S+C2	363.6 \pm 49.52	1898.93 \pm 150.80 ***#	56.00 \pm 9.06	950.16 \pm 100.30 *###	2968.46 \pm 309.14	7.33 \pm 1.52 ###
S+C3	422.16 \pm 74.88 ***#	1857.75 \pm 100.90 ***	99.66 \pm 10.61 ***	793.16 \pm 74.25 ###	2728.16 \pm 432.77	4.30 \pm 0.63 *
S+D	203.00 \pm 16.86	915.20 \pm 172.92	29.80 \pm 5.76	200.40 \pm 21.99	3789.71 \pm 381.44	7.46 \pm 0.90

Values are presented as mean \pm SEM. The level of each gene expression (IFN- γ , IL-4, IL-17, TGF- β , and FOXP3) was gene fold/ml but presented as Normalized Index which was explained in the methods section.

Significant difference between treated groups with carvacrol vs dexamethasone (D): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Significant difference between S+C2 and S+C3 vs S+C1 treatment groups: # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$.

Statistical comparisons were made using ANOVA with Tukey-Kramer multiple *post hoc* test.

Cytokine gene expression in splenocytes of sensitized mice treated with dexamethasone

Dexamethasone treatment of sensitized splenocytes showed a significant inhibitory effect on IL-4 and TGF- β genes expression ($P < 0.001$ for both cases). However, dexamethasone treatment of sensitized splenocytes did not significantly affect IFN- γ , FOXP3, and IL-17 genes expression. In addition, IFN- γ /IL-4 ratio was significantly increased in the group treated with dexamethasone ($P < 0.001$) compared to group S (Figures 1–6). IFN- γ , FOXP3, and TGF- β genes expression and IFN- γ /IL-4 ratio were significantly different in animals treated with dexamethasone than those of the control group (Figures 1, 2, 4, and 6).

Comparison of the effects of carvacrol and dexamethasone treatments on cytokine gene expression in splenocytes of sensitized mice

The effects of high carvacrol concentration treatment on IFN- γ gene expression in sensitized splenocytes were significantly higher than the effect of dexamethasone treatment ($P < 0.01$, Table 1). The effects of all carvacrol concentrations treatments on FOXP3 gene expression in sensitized splenocytes were significantly higher than the effect of dexamethasone treatment ($P < 0.01$ to $P < 0.001$, Table 1). The effects of higher and lower carvacrol concentrations treatments on IL-4 gene expression in sensitized splenocytes were significantly lower than the effect of dexamethasone treatment ($P < 0.001$ and $P < 0.05$ for high and low carvacrol concentrations, respectively, Table 1). The effect of low carvacrol concentration treatment on TGF- β gene expression in sensitized splenocytes was significantly lower than the effect of dexamethasone treatment ($P < 0.001$, Table 1). However, the effects of

low and high carvacrol concentrations on IFN- γ /IL-4 ratio were significantly lower than the effect of dexamethasone ($P < 0.001$ and $P < 0.05$ for low and high concentrations, respectively, Table 1).

Comparison of the effects of three concentrations of carvacrol on cytokine gene expression in splenocytes of sensitized mice

There was no significant difference in IL-4 and IL-17 genes expression of sensitized splenocytes among three carvacrol concentrations (Table 1). However, the effect of only high carvacrol concentration (300 μ g/ml) treatment on IFN- γ gene expression in sensitized splenocytes was significantly higher than the effect of its low concentration (75 μ g/ml), ($P < 0.01$, Table 1). The effect of lower carvacrol concentration (75 μ g/ml) on FOXP3 expression potentiation was also significantly higher compared to its other two concentrations ($P < 0.05$ for both cases, Table 1). In addition, the effects of the last two carvacrol concentrations (150 and 300 μ g/ml) on reduction of TGF- β gene expression in sensitized splenocytes were significantly higher compared to its lower concentration ($P < 0.001$ for both cases, Table 1). The effect of medium carvacrol concentration (150 μ g/ml) treatment on IFN- γ /IL-4 ratio was significantly higher than its low concentration ($P < 0.001$, Table 1).

Discussion

The results of the present study showed decreased IFN- γ and FOXP3 genes expression but increased IL-4 and IL-17 genes expression in sensitized animals. The IFN- γ /IL-4 ratio was also decreased in sensitized animals compared to the controls.

The immunologic features of asthma is a balance shift from Th1 to Th2 (31). Th2 productions such as IL-4, IL-5, and IL-13 have been identified in BAL and airway biopsies of patients with mild or asymptomatic asthma (2, 3). Th2 cytokines are required for the development of airway eosinophilia and IgE, stimulating an inflammatory response that results in asthma (1). IL-17 has also been demonstrated to have an important role in the development of autoimmune disorders and chronic inflammation (9). This cytokine also plays an upstream role in T cell-triggered inflammation and hematopoiesis by secretion of other cytokines and growth factors from stromal cells (10). IL-17 might be a potential player in the cytokine network involved in asthma, and a possible indirect effect of IL-17 on airway remodeling is suggested (38). IL-17 is up-regulated in asthma, which in turn increases synthesis of IL-6 and IL-11 by bronchial fibroblasts derived from bronchial biopsies of asthmatic subjects (38). Molet *et al* reported that Th2-type cytokines, including IL-4 and IL-13, enhance IL-17 induced release of IL-6 from fibroblasts, suggesting that IL-17 might be involved in asthma, which is a Th2-mediated disease (38). IL-17 has been reported to be involved in inflammatory cell recruitment into airways, particularly neutrophils (38). Th1 has proved to inhibit Th2 response by secretion of IFN- γ , IL-2, and TNF β and thus one goal of asthma therapy should be increasing the balance of Th1/Th2 (39). In addition, it was shown that the absence of FOXP3 is associated with the development of immune abnormalities, such as severe allergic inflammation and high immunoglobulin E (IgE) levels (8). The reduction of FOXP3 protein expression is reported in patients with stable asthma (40). Therefore, reduction of IFN- γ and FOXP3 genes expression and IFN- γ /IL-4 ratio but increased IL-4 and IL-17 genes expression in sensitized animals confirmed their sensitization and indicates the induction of an animal model of asthma in mice.

In splenocytes of sensitized animals treated with carvacrol, IFN- γ gene expression was significantly increased due to its last concentration and FOXP3 due to its last two concentrations. In addition, IL-4 gene expression due to all carvacrol concentrations, TGF- β due to its two higher concentrations and IL-17 due to its high concentration were significantly decreased compared to group S. In addition, IFN- γ /IL-4 ratio was significantly increased in the groups treated with two higher carvacrol concentrations. Therefore, carvacrol treatment showed potentiating of anti-inflammatory and reduction of pro-inflammatory cytokines genes expression in splenocytes of sensitized mice.

These results suggest a preventive therapeutic effect of carvacrol on inflammatory disorders associated with immunological dis-regulation such as asthma. In fact, a previous study showed

enhancement effect of carvacrol on serum level of IFN- γ and its reduction effect on IL-4 of sensitized guinea pigs as well as increased IFN- γ /IL4 cytokine (Th1/Th2) balance (31). The results of this study support the findings of the present study. In addition, the effect of carvacrol on tracheal responsiveness (41), pathological changes (30), and inflammatory mediators (31, 41) in sensitized guinea pigs were also shown in previous studies, which also support the possible preventive therapeutic effect of carvacrol on asthma.

The effects of high carvacrol treatment on IL-4, FOXP3 genes expression, and IFN- γ /IL-4 ratio were lower than its other two concentrations. These results may show that the maximum effect of carvacrol is occurring at its medium concentration.

The excitatory effect of carvacrol on FOXP3 gene expression in sensitized animals was also demonstrated in the present study. Peripheral blood CD⁴⁺CD²⁵⁺ Tregs were found to suppress Th2 cytokine production from both atopic and non-atopic donors (42). Therefore, carvacrol may suppress Th2 cytokine production in this manner, which will help in the treatment of asthma.

Treatment of sensitized animals with carvacrol also caused significant reduction in IL-17 gene expression. The direct role of IL-17 in contributing to the severity of asthma was demonstrated in mouse studies. Mice deficient in IL-17RA or IL-17A have markedly diminished recruitment of neutrophils into the lung in response to allergens or gram-negative bacteria (43, 44). In animal models of asthma, transfer or antigen-specific IL-17 producing Th2 cells triggered influx of heterogeneous leukocytes, including neutrophils, eosinophils, macrophages, and lymphocytes, resulting in distinct pathophysiological features of severe asthma (45). IL-17 producing Th2 cell cytokines may represent the key pathogenic Th2 on promoting exacerbations of allergic asthma (46). The inflammatory response may be propagated in the airway and regulate the maintenance of Th2 and Th17 cell subsets during the chronic phase of the disease (46). Therefore, carvacrol may also have therapeutic effects on asthma by suppression of IL-17 gene expression.

The results of the present study also indicated that carvacrol decreased TGF- β gene expression in splenocytes of sensitized mice. TGF- β has an effective role in origins of airway remodeling in asthma (47) and pro-inflammatory effects on inflammatory cells (47). In addition, TGF- β is synthesized during the late phase of lung inflammation (48). It was shown that TGF- β gene expression increases in asthmatic airways after repeated exposure to low doses of cat allergen, accompanied by asthma symptoms (49). Therefore, these results showed that carvacrol may affect asthma therapy by reducing TGF- β gene expression.

Previous studies have also shown the preventive effect of carvacrol on systemic inflammation, tracheal responsiveness, and lung pathology in guinea pigs model of COPD (22, 50, 51). The anti-inflammatory effects of carvacrol in various inflammatory disorders were also shown previously (20-27). The results of the above studies may also support the findings of the present study, indicating anti-inflammatory effect of carvacrol.

The results also showed that dexamethasone treatment of sensitized splenocytes only inhibited the pro-inflammatory cytokines including IL-4 and TGF- β genes expression in splenocytes of sensitized mice, but dexamethasone had no significant effect on IFN- γ and FOXP3 genes expression. Although dexamethasone significantly increased IFN- γ /IL-4 ratio, this effect was due to suppression of pro-inflammatory rather than the effect on anti-inflammatory cytokines. However, treatment of splenocytes of sensitized mice with carvacrol affects both anti-inflammatory and pro-inflammatory cytokine genes expression. This impact totally differed from dexamethasone's effect because dexamethasone inhibited the activities of only Th2 cell subpopulations, but carvacrol potentiated Th1 and inhibit Th2 activities. These results showed that carvacrol has more selective therapeutic effects in disorders associated with pro and anti-inflammatory cytokine imbalances such as asthma than dexamethasone. In fact, similar results were observed for carvacrol on serum IFN- γ /IL4 cytokine (Th1/Th2) ratio of sensitized guinea pigs (36), which supports the results of this study.

Conclusion

The results of this study showed that carvacrol potentiated anti-inflammatory cytokines, and IFN- γ and FOXP3 genes expression, but decreased pro-inflammatory, IL-4, TGF- β , and IL-17 genes expression in splenocytes of sensitized mice. However, dexamethasone treatment only reduced pro-inflammatory IL-4 and TGF- β genes expression. Therefore, the results may indicate the more selective therapeutic value in allergy, autoimmunity, and infectious diseases by both potentiating anti-inflammatory and suppressing pro-inflammatory cytokines.

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Conflict of interest

The authors declare that they have no conflict of interest.

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