



Immunomodulatory effect of characterized extract of *Zataria multiflora* on Th₁, Th₂ and Th₁₇ in normal and Th₂ polarization state



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ABSTRACT

The effect of the extract of *Zataria multiflora* (*Z. multiflora*) on IFN- γ , FOXP3, IL-4, TGF- β , and IL-17 gene expression was evaluated in cultured splenocytes obtained from control, nontreated asthma or sensitized mice (group S). Sensitized animals treated with dexamethasone or three concentrations of *Z. multiflora* extract (200, 400 and 800 μ g/ml) ($n = 6$, for each group). IFN- γ and FOXP3 gene expressions were significantly decreased ($P < 0.001$ for both cases) but IL-4 ($P < 0.001$) and IL-17 ($P < 0.05$) were increased in group S compared to control group. *Z. Multiflora* extract 800 μ g/ml, significantly upregulated IFN- γ gene expression ($P < 0.01$) and its 400 and 800 μ g/ml concentrations increased FOXP3 gene expression ($P < 0.05$ and $P < 0.001$, respectively) compared to group S. *Z. multiflora* extract at all concentrations (200, 400 and 800 μ g/ml) decreased TGF- β gene expression and its lowest concentration significantly reduced IL-17 gene expression compared to group S ($P < 0.001$ for all cases). Only IL-4 and TGF- β gene expression was significantly decreased following treatment with dexamethasone ($P < 0.001$ for both cases). The results indicated an increase in IFN- γ and FOXP3 but decrease in TGF- β and IL-17 gene expression profile in sensitized splenocytes treated with the extract, which might be partially due to the presence of one of its constituent, carvacrol.

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1. Introduction

T cells as a part of adaptive immune system, are divided to effectors and regulator subdivisions. Type 2 T helper (Th₂) cells are the ones which produce IL-4, IL-5 and IL-13 and they are also known as anti-inflammatory cells that are increased in the airway of asthmatic patients (Walker et al., 1992; Robinson et al., 1992). The role of Th₂ cells and their cytokines in the development of airway eosinophilia and Immunoglobulin E (IgE) production as well as induction of an inflammatory response that results in asthma has

been shown (Ray and Cohn, 1999). Type 1 T helper (Th₁) cells, also known as inflammatory cells, can antagonize Th₂ activity, and enhancement of their activity could be considered as a therapeutic target in asthma therapy (Boskabady et al., 2013). The forkhead/winged helix transcription factor FOXP3 is a regulatory T cell (T_{reg}) developmental regulator and is the most specific T_{reg} marker (Hori et al., 2003; Fontenot et al., 2003; Khattri et al., 2003). The absence of FOXP3 is associated with the development of immune abnormalities such as severe allergic inflammation and high immunoglobulin E levels in both animals and humans (Chatila, 2005). In addition, IL-17 has also an important role in the development of autoimmune disorders and maintenance of chronic inflammation (Annunziato et al., 2008). This cytokine plays an upstream role in T cell-triggered inflammation by stimulating stromal cells to secrete other cytokines and growth factors (Wong et al., 2001). In addition, Th₂ dominant condition in cancers may prevent alleviation of

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treatment by cytokine (e.g. IL-4 and IL-5) production (Ivanova and Orekhov, 2015).

Zataria multiflora (*Z. multiflora*) is a perennial plant which grows in Iran, Pakistan, and Afghanistan (Scop, 1997; Ali et al., 2000) and contains various compounds particularly terpenes such as thymol and carvacrol which might be responsible for its therapeutic effects (Scop, 1997). In Iran, *Z. multiflora* (in Persian called “Avishan shir-azi”) has been used in traditional medicine for its antiseptic, analgesic, and carminative properties (Ali et al., 2000). Antioxidant (Sharaf-Kandi, 1985), immune-regulatory (Shokri et al., 2006) and anti-inflammatory effects (Hosseinzadeh et al., 2000; Boskabady et al., 2011b; Nakhai et al., 2007), as well as an effect on gastrointestinal disorders (Nakhai et al., 2007; Alireza Ghannadi et al., 2004), have also been reported for this plant. The antispasmodic effect of the plant on smooth muscle (Meister et al., 1999; A, 2006; Reiter and Brandt, 1985; Gharib Naseri et al., 2010) and the relaxant effect of another plant of this family (*Thymus vulgaris*) on tracheal smooth muscle (Boskabady et al., 2006) were demonstrated. The inhibitory effect of the extract of *Z. multiflora* and carvacrol on histamine (H_1) (Boskabady and Tabanfar, 2011) and muscarinic receptors (Boskabady et al., 2011a, 2012; Zahra Jafari et al., 2011) and their stimulatory effect on β -adrenoceptors (Boskabady et al., 2011b) were also documented. The effect of the extract of *Z. multiflora* on IFN- γ , IL-4 and IFN- γ /IL-4 ratio (Th_1/Th_2 balance) (Boskabady et al., 2013), tracheal responsiveness (Boskabady et al., 2014a,b), pathological changes of the lung (Mohammad Hossein Boskabady et al., 2014a,b) and inflammatory mediators (Boskabady et al., 2013) in sensitized guinea pigs were shown.

To clarify the cellular and molecular basis of the effect of the plant on Th_2 polarized inflammatory disorders such as asthma, the effect of *Z. multiflora* extract on IFN- γ , FOXP3, IL-4, TGF- β , and IL-17 gene expression in splenocytes obtained from sensitized mice was examined in the present study.

2. Material and methods

2.1. Plant and extracts

Z. multiflora was collected from a mountain area in a fluorine mine, central region of Iran between Tabas and Yazd. The plant was identified by Mr. Joharchi, and a sample was kept in the Herbarium of the Faculty of Sciences, Ferdowsi University of Mashhad (herbarium number: 35314). The hydro-ethanolic extract of *Z. multiflora* was prepared by mixing 100 g of dried shoots and powdered plant with 875 mL of 50% ethanol. The mixture was shaken for 72 h at room temperature. The extract was then passed through the filter paper, and the solvent was removed under reduced pressure. Then, it was freeze-dried and dissolved in DMSO (200 mg of extract in 1 mL of analytical grade of DMSO) which has anti-bacterial effect. Subsequently, 2 mL of complete RPMI-1640 containing 1% penicillin/streptomycin was added to the mixture. Finally, the extract at the concentration of 20 mg/mL was filtered using 0.2- μ m-pore-size anti-microbial filter. Considering the anti-bacterial effect of DMSO and also by using 0.2 μ m anti-microbial filter, it was concluded that the extract was sterile. The dried extract was collected and kept in the refrigerator. The yield of the extract was 33.2 g. The concentration of the final extract was adjusted to 10 mg/mL by adding distilled water to the dried extract. Three concentrations of 200, 400 and 800 μ g/mL of the extract were then used in our study.

2.2. Characterization of the extract of *Zataria multiflora* by HPLC

The extract of *Z. multiflora* was characterized in our previous

study (Boskabady et al., 2011b; Boskabady et al., 2014b) by HPLC (Waters 474, Waters Corporation, Milford, MA, USA) finger print. Fig. 1b illustrates the chromatographic profile of pure carvacrol (5/1000) with retention time of about 9 min. All solvents used on this study were HPLC grade and supplied by Caledon Laboratories Ltd, Georgetown, ON, Canada.

2.3. Animal sensitization

Male BALB/c mice (6–8 week old; weighing 18–20 g), were purchased from Razi Institute (Mashhad, Iran). They were kept in hygienic macrolene cages in air-conditioned rooms with 12 h light/12 h dark cycle and they had free access to food and water *ad libitum* during experimental period.

Mice were sensitized by two intraperitoneal injections, on days 0 and 14 of the experiment, with 10 μ g/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 aerosolized ovalbumin (OVA) for 30 min/day, three days/week for eight weeks beginning from the 21st day of the study (Temelkovski et al., 1998; Babayigit et al., 2009). 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 (Hocaoglu et al., 2012). Non-sensitized animals (control group) received aerosol saline instead of OVA during experimental period.

2.4. Preparation and culture of splenocytes and groups

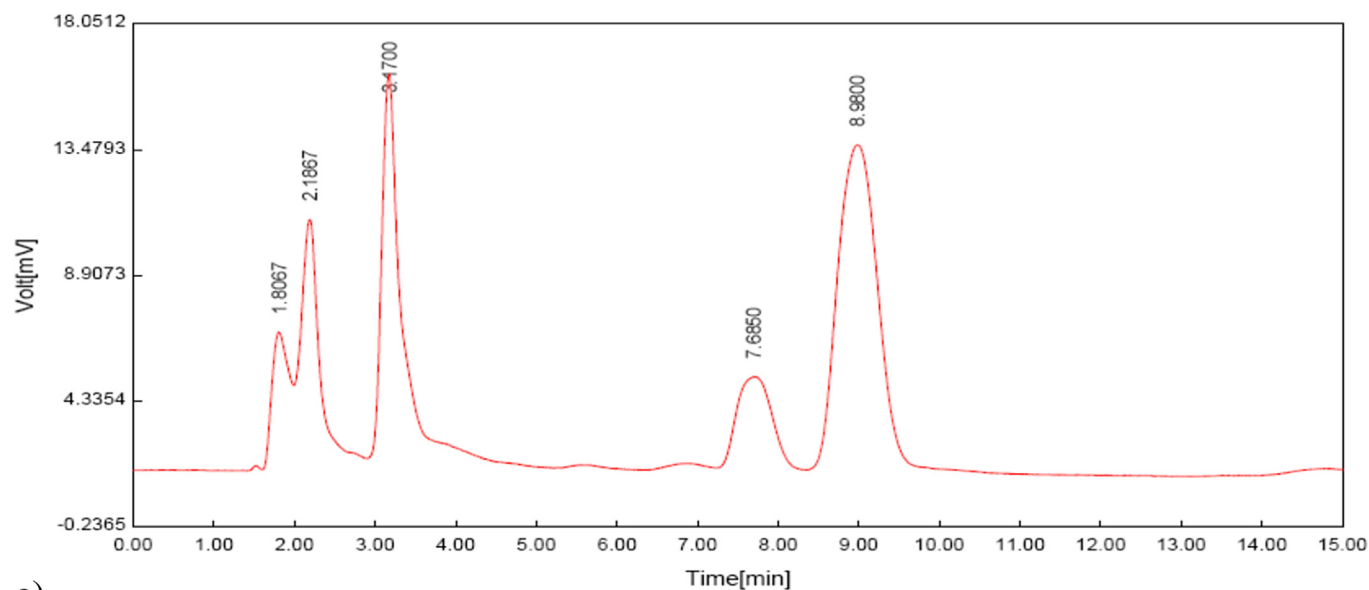
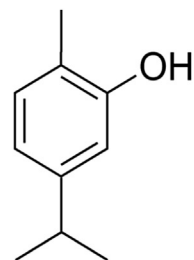
Both groups of animals were sacrificed on day 78 and splenocytes were removed and suspended in complete RPMI 1640 with 10% FBS at a density of 5×10^6 /mL. Splenocytes isolation was carried out using Ficoll gradient. The study was carried out in the following groups:

- (1) Non-sensitized splenocytes (control group)
- (2) Non-treated sensitized splenocytes (group S).
- (3) Sensitized splenocytes treated with dexamethasone (0.1 mM, Sigma Chemicals, LTD.) (group D).
- (4) Sensitized splenocytes treated with *Z. multiflora* extract 200,400 and 800 μ g/mL (groups Z1, Z2 and Z3) based on our previous study (Boskabady et al., 2012).

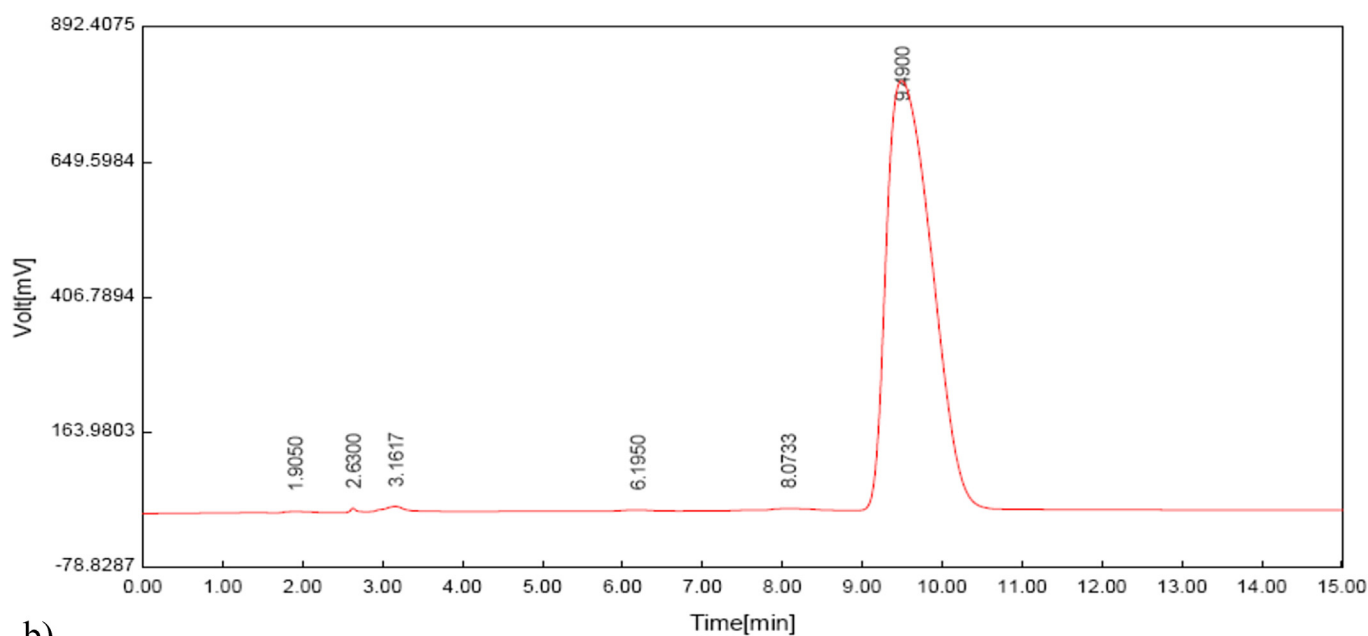
Splenocytes (5×10^6) obtained from all groups were suspended in culture media. Dexamethasone (0.1 mM) and three concentrations of the extract (200,400 and 800 μ g/mL) were added and incubated for 18 h (at 37 °C with P_{CO_2} 5%). Then, the cells were separated and 1 mL Tripure was added to each microtube. The procedure of sensitization, splenocyte culture and their treatment was illustrated in Fig. 2.

2.5. RNA extraction

Using Tripure Isolation Reagents (Roche Applied Science, Germany), total RNA was isolated from splenocytes as described by the manufacturer's instructions. Samples were incubated for 10 min at room temperature. Then, 200 μ l of chloroform solution was added to the microtubes and the mixture was vortexed for 15 s. Then, the mixture was incubated for 15 min at 4 °C in dim light. Samples were centrifuged at 12,000 rpm at 4 °C for 15 min. A clear supernatant was carefully removed and transferred to other microtubes. Afterwards, 500 μ l of cold isopropanol was added and incubated for 10 min at 4 °C in dim light and centrifuged at 12,000 rpm at 4 °C for



a)



b)

Fig. 1. HPLC fingerprint of (a) the extract of *Z. multiflora* (50 $\mu\text{g/mL}$) and the (b) chromatographic profile of pure carvacrol ($\text{C}_{10}\text{H}_{14}\text{O}$, 5/1000) with retention time of about 9 min. MW = 150.217 (Boskabady et al., 2011b). The peak appears on 8.98 min has the same retention time (RT) of pure carvacrol (9.49 min).

10 min. At the end of this stage, sediment RNA was visible as a tiny white pellet. The supernatant was removed and the precipitated RNA was washed by alcohol 75% and then centrifuged at

7500 rpm at 4 °C for 5 min. Supernatant was emptied and the remaining ethanol was removed by the air flow. Diethylpyrocarbonate (DEPC) water was added to RNA containing microtubes and

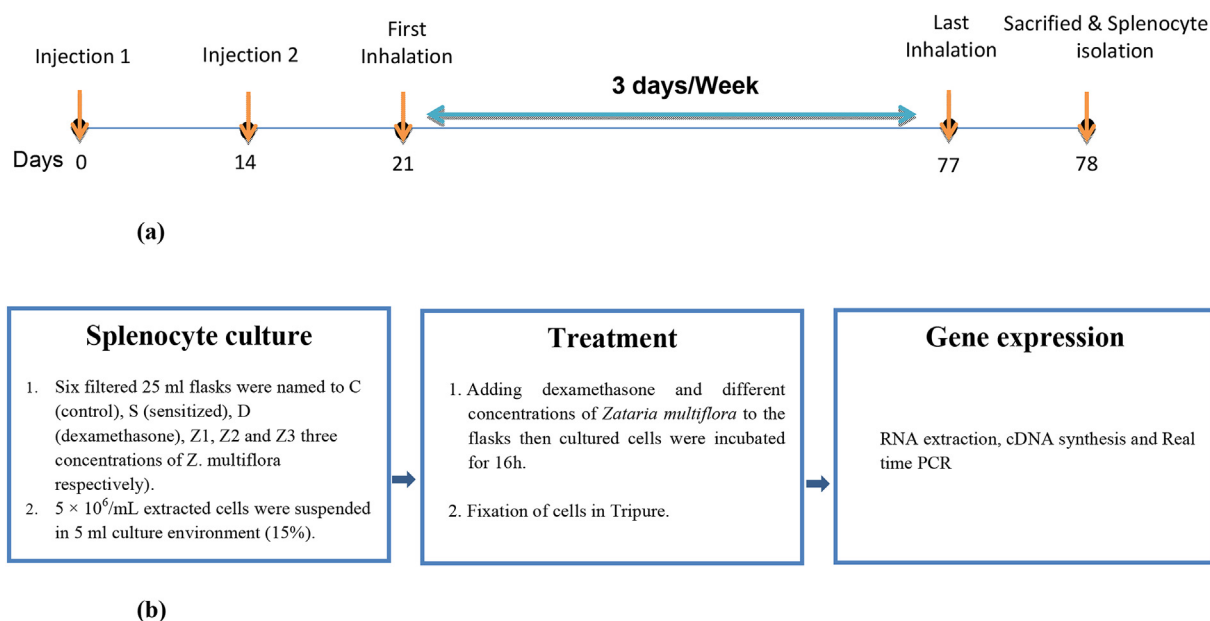


Fig. 2. The schematic time-table of animal sensitization, splenocyte isolation, culture and treatment.

A Induction of sensitization in mice.

In sensitized animals, OVA was used for injection and inhalation but in control animals saline was administrated instead of OVA.

B Culture and treatment of splenocytes with dexamethasone and three concentrations of *Z. multiflora* extract.

- In groups C and S: cells were treated with saline
- In group D: cells were treated with dexamethasone (0.1 mmol)
- In groups Z1, Z2 and Z3: cells were treated with *Z. multiflora* extract in concentrations of 200, 400 and 800 µg/mL respectively.

the microtubes were placed on a dry-block device for 10 min at 56 °C and then kept –20 °C until cDNA synthesis.

2.6. Synthesis of cDNA

Transcription of extracted mRNA to cDNA was performed by the enzyme reverse transcriptase. This DNA sequence was then used as a template in PCR and synthesis of the second strand DNA 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 the manufacturer's instructions.

2.7. Quantitative real-time PCR (qPCR)

For β2M (β2-Microglobulin), IFN-γ (Interferon gamma), IL-4, IL-17, TGF-β (Transforming growth factor beta) and FOXP3, Real-Time PCR primers and quantitation probes were designed using mRNA sequences, and 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 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.

2.7.1. β₂-microglobulin

(forward5-GCCGAACATACTGAAGTCTAC-3) (reverse5CTTGCT-GAAGGACATATCTGACATC-3)
(probe5-AACACAGTTCCACCCGCCTCACATTGA-3).

2.7.2. IFN-γ

(forward5-GTATTGCCAAGTTTGAGGTCAAC-3)
(reverse5-GCTTCTGAGGCTGGATTTC-3) (probe5CCA-CAGGTCCAGCGCCAAGCATTCAA-3).

2.7.3. IL-4

(forward5-TCCTCACAGCAACGAAGAAC-3)
(reverse5-CAAGCATGGAGTTTCCCATG-3)
(probe5-AGCACCTTGAAGCCCTACAGACGAGC-3).

2.7.4. IL-17

(forward 5-CAGACTACCTCAACCGTTCC-3)
(reverse5-TTCCCTCCGCATTGACAC-3)
(probe 5-ACTCTCCACCGCAATGAAGACCTGA-3).

2.7.5. TGF-β

(forward 5-CCTGGATACCAACTATTGCTTCAG-3)
(reverse5-CAGACAGAAGTTGGCATGGTAG-3)
(probe5-TCCACTTCCAACCCAGGTCCTTCTCT-3).

2.7.6. FOXP3

(forward5-GGTACACCCAGGAAAGACAG-3)
(reverse5-GCTTGGCAGTGCTTGAGA-3)
(probe 5-TGGCTCCTCGAAGACCTTCTCACAACC-3).

Beta 2 microglobulin (β2M) was used as the reference gene to normalize RNA expression levels and control the errors between samples. The real time PCR reactions were performed in glass capillaries (Qiagene, Germany) at a final volume of 10 µL containing 2 µL cDNA template, 0.4 µL of primer pairs (200 nM, β2M, IFN-γ, IL-4, IL-17, TGF-β, and FOXP3), and 5 µL of the probe Master Mix. Two standard curves 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 $\beta 2M$ mRNA. Then, the relative expression levels of each gene for each sample was calculated by the following equation: normalized index/copy number of gene of interest (IFN each cytokine)/copy number of reference gene ($\beta 2M$). Then, the fold change in expression of each gene for each group was calculated by the following equation: mean gene of interest normalized index of test group/mean gene of interest normalized index of healthy controls.

2.8. Statistical analysis

The results were expressed as mean $\pm$ SEM. The statistical analyses were carried out using one way ANOVA with Tukey–Kramer *post hoc* tests. The results were considered statistically significant if $p < 0.05$. SPSS software, version 16 was used for data analysis.

3. Results

3.1. Zataria multiflora extracts characterization

The extract of *Z. multiflora* was identified using HPLC finger print which showed five major peak from 1 to 10 min. Carvacrol is one of the main constituent of the extract which appeared approximately at 9.5 min (Fig. 1). Using the calibration curve, the quantification of carvacrol in a sample of the extract of *Z. Multiflora* showed that this compound constituted about 0.18% (w/w, Fig. 1) of the extract.

3.2. Cytokines gene expression in splenocytes of sensitized as compared to control group

IFN- γ and FOXP3 genes expression and IFN- γ /IL-4 ratio were significantly decreased ($P < 0.001$ for all cases) but IL-4 ($P < 0.001$) and IL-17 ($P < 0.05$) genes expression were increased in sensitized splenocytes as compared to the control group. However, there was no significant difference in TGF- β gene expression between the two groups (Figs. 3–8).

3.3. Effect of treatment of sensitized splenocytes with the extract of Zataria multiflora on cytokines gene expression

Treatment of sensitized splenocyte with the highest concentration of the extract (800 $\mu\text{g/mL}$) caused significant increase in IFN- γ ($P < 0.01$) and treatment with 400 and 800 $\mu\text{g/mL}$ increased

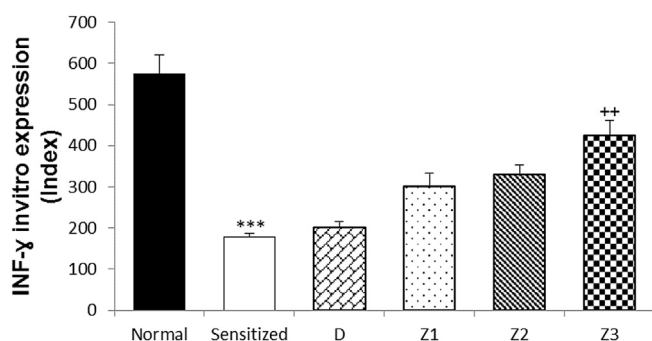


Fig. 3. Values (mean $\pm$ SEM) of IFN- γ gene expression in splenocyte from control mice, sensitized animals, and S treated with dexamethasone (D) and the extract of *Z. multiflora* at concentrations 200,400 and 800 $\mu\text{g/mL}$ (groups Z1, Z2 and Z3 respectively), ($n = 6$ for each group). The levels of gene expression was gene fold/mL but presented as Normalized Index as explained in method section. *** $P < 0.001$, compared to control group. ++ $P < 0.01$, compared to sensitized group. The statistical comparisons were made using ANOVA with Tukey–Kramer multiple post hoc test.

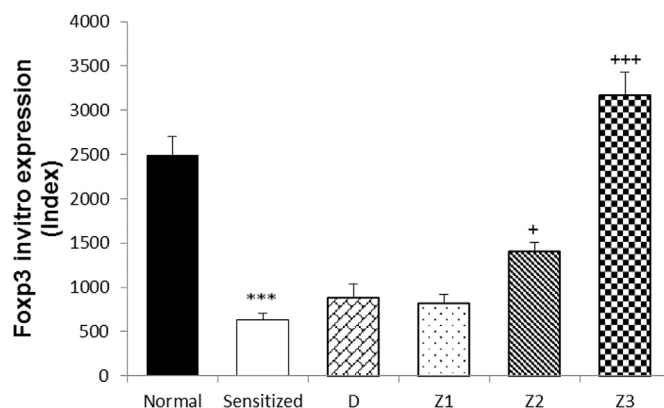


Fig. 4. Values (mean $\pm$ SEM) of FOXP3 gene expression in splenocyte from control mice, sensitized animals, and S treated with dexamethasone (D) and the extract of *Z. multiflora* at concentrations 200,400 and 800 $\mu\text{g/mL}$ (groups Z1, Z2 and Z3 respectively), ($n = 6$ for each group). The levels of gene expression was gene fold/mL but presented as Normalized Index as explained in method section. *** $P < 0.001$, compared to control group. + $P < 0.05$, +++ $P < 0.001$, compared to sensitized group. The statistical comparisons were made using ANOVA with Tukey–Kramer multiple post hoc test.

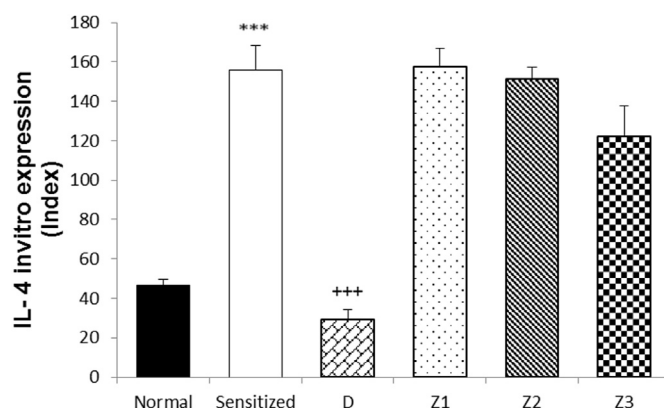


Fig. 5. Values (mean $\pm$ SEM) of IL-4 gene expression in splenocyte from control mice, sensitized animals, and S treated with dexamethasone (D) and the extract of *Z. multiflora* at concentrations 200,400 and 800 $\mu\text{g/mL}$ (groups Z1, Z2 and Z3 respectively), ($n = 6$ for each group). The levels of gene expression was gene fold/mL but presented as Normalized Index as explained in method section. *** $P < 0.001$, compared to control group. +++ $P < 0.001$, compared to sensitized group. The statistical comparisons were made using ANOVA with Tukey–Kramer multiple post hoc test.

FOXP3 gene expression as well as IFN- γ /IL-4 ratio ($P < 0.05$ and $P < 0.001$, respectively). However, IL-17 gene expression was significantly decreased by the highest concentration of the extract (800 $\mu\text{g/mL}$), ($P < 0.001$) and TGF- β gene expression was increased by all extract concentrations ($P < 0.001$ for all cases) in treated sensitized splenocytes (Figs. 3–8).

3.4. Effect of treatment of sensitized splenocytes with dexamethasone on cytokines gene expression

Treatment of sensitized splenocytes with dexamethasone showed significant inhibitory effect on IL-4 and TGF- β genes expression ($P < 0.001$ for both cases) but it increased IFN- γ /IL-4 ratio. However, dexamethasone treatment did not significantly affect IFN- γ , FOXP3 and IL-17 genes expression in sensitized splenocytes (Figs. 3–8).

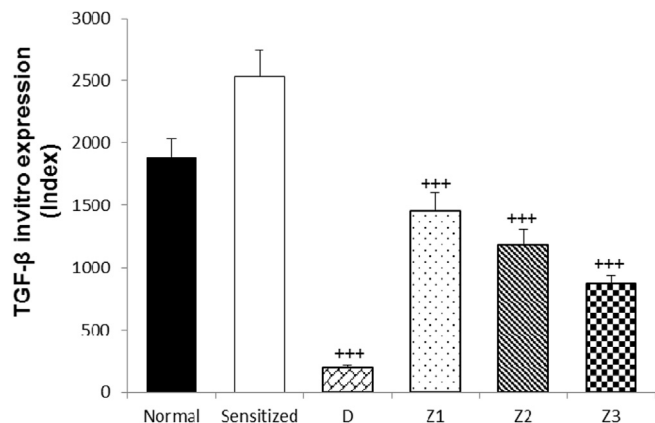


Fig. 6. Values (mean $\pm$ SEM) of TGF- β gene expression in splenocyte from control mice, sensitized animals, and S treated with dexamethasone (D) and the extract of *Z. multiflora* at concentrations 200, 400 and 800 μ g/mL (groups Z1, Z2 and Z3 respectively), ($n = 6$ for each group). The levels of gene expression were presented as Normalized Index as explained in method section. Compared to control group. +++ $P < 0.001$, compared to sensitized group. The statistical comparisons were made using ANOVA with Tukey–Kramer multiple post hoc test.

3.5. Cytokines gene expression in splenocytes of sensitized mice treated with the extract of *Zataria multiflora* as compared to dexamethasone treatment

The effect of the treatment of sensitized splenocytes with high concentration of the extract on IFN- γ and FOXP3 genes expression was significantly more pronounced than that of dexamethasone treatment ($P < 0.01$ and $P < 0.001$ respectively, Table 1). The inhibitory effects of all extract concentrations on IL-4 and TGF- β genes expression in sensitized splenocytes were significantly lower than the effect of dexamethasone ($P < 0.05$ to $P < 0.001$, Table 1). However, the effect of high concentration of the extract on IL-17 gene expression in sensitized splenocytes was significantly higher than the effect of dexamethasone ($P < 0.001$, Table 1). In addition, the increase in IFN- γ /IL-4 ratio following treatment with all extract concentrations were significantly lower than the effect of dexamethasone ($P < 0.01$ to $P < 0.001$, Table 1).

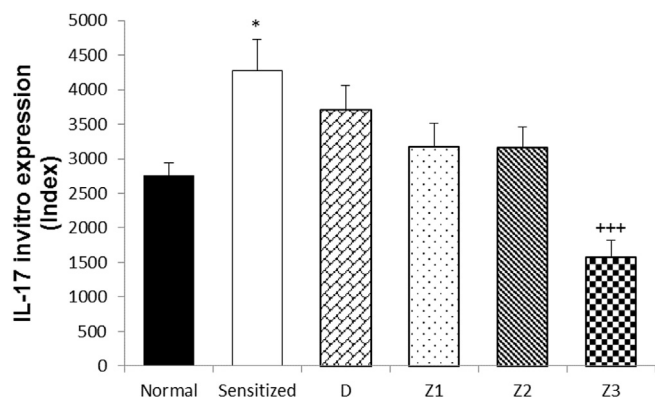


Fig. 7. Values (mean $\pm$ SEM) of IL-17 gene expression in splenocyte from control mice, sensitized animals, and S treated with dexamethasone (D) and the extract of *Z. multiflora* at concentrations 200, 400 and 800 μ g/mL (groups Z1, Z2 and Z3 respectively), ($n = 6$ for each group). The levels of gene expression were presented as Normalized Index as explained in method section. * $P < 0.05$, compared to control group. +++ $P < 0.001$, compared to sensitized group. The statistical comparisons were made using ANOVA with Tukey–Kramer multiple post hoc test.

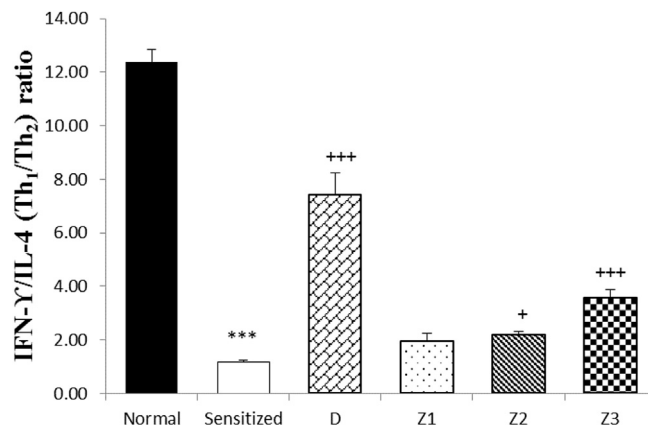


Fig. 8. Values (mean $\pm$ SEM) of IFN- γ /IL-4 gene expression ratio in splenocyte from control mice, sensitized animals, and S treated with dexamethasone (D) and the extract of *Z. multiflora* at concentrations 200, 400 and 800 μ g/mL (groups Z1, Z2 and Z3 respectively), ($n = 6$ for each group). The levels of gene expression were presented as Normalized Index as explained in method section. *** $P < 0.001$, compared to control group. + $P < 0.05$, +++ $P < 0.001$, compared to sensitized group. The statistical comparisons were made using ANOVA with Tukey–Kramer multiple post hoc test.

3.6. The difference of cytokines gene expression in sensitized splenocytes treated with three concentrations of *Zataria multiflora* extract

Z. multiflora extract 800 μ g/mL potentiated FOXP3 and inhibited IL-17 genes expression in sensitized splenocytes and this effect was significantly higher than the other two concentrations (for both concentrations, $P < 0.001$ for FOXP3 and $P < 0.05$ for IL-17, Table 1). However, there was no significant difference among the effects of the three extract concentrations on IFN- γ , IL-4 and TGF- β genes expression in sensitized splenocytes (Table 1). There was a concentration-dependent increase in IFN- γ /IL-4 ratio following treatment with the extract, but the differences among three extract concentrations in this regard were not statistically significant (Table 1).

Decreased IFN- γ and FOXP3 but increased IL-4 and IL-17 genes expression in sensitized were shown compared to control animals. Decreased IFN- γ /IL-4 ratio was also seen in sensitized animals which indicated a decrease in Th1/Th2 balance.

4. Discussion

A shift in Th1/Th2 balance toward Th2 dominance is well documented in asthmatic animals (Jalali et al., 2013). Th2 cytokines such as IL-4, IL-5 and IL-13 are required for the development of airway inflammatory response in asthma (Ray and Cohn, 1999) and the presence of these cytokines in BAL and airway biopsies of asthmatic patients has been shown (Walker et al., 1992; Robinson et al., 1992). Moreover, Th2 cytokines can induce anti-inflammatory effect by suppression of Th1 activity. IL-17 also plays an upstream role in T cell-triggered inflammation and hematopoiesis by secretion of other cytokines and growth factors from stromal cells (Wong et al., 2001) suggesting an important role in the development of autoimmune disorders and chronic inflammation (Annunziato et al., 2008; Molet et al., 2001). It was shown that IL-17 is up-regulated in asthma, and in turn increases the synthesis of IL-6 and IL-11 by Th2 cytokines such as IL-4 and IL-13. IL-17 induced release of IL-6 from fibroblasts and inflammatory cell recruitment into airways, particularly neutrophils suggesting that IL-17 might be involved in asthma (Molet et al., 2001). Increasing

Table 1

The values of IFN- γ , IL-4, TGF- β , FOXP3 and IL-17 gene expressions as well as IFN- γ /IL-4 ratio in control (C), non-treated sensitized (S) sensitized treated with dexamethasone (0.1 mM), the extract of *Z.mutiflora* at concentrations 200,400 and 800 μ g/mL (groups Z1, Z2 and Z3 respectively) splenocyte.

Variable	IFN- γ	FOXP3	IL-4	TGF- β	IL-17	IFN- γ /IL-4 ratio
S + Z1	301.50 $\pm$ 31.04	819.43 $\pm$ 101.80	157.66 $\pm$ 9.20 ***	1454 $\pm$ 147.40 ***	3173.50 $\pm$ 343.80	1.97 $\pm$ 0.28 ***
S + Z2	330.16 $\pm$ 22.86	1411.33 $\pm$ 98.52	151.33 $\pm$ 5.84 ***	1180 $\pm$ 130.99 **	3162.55 $\pm$ 301.26	2.18 $\pm$ 0.13 ***
S + Z3	424.66 $\pm$ 37.74 **	3164.08 $\pm$ 266.42 ***## $\dagger\dagger$	122.33 $\pm$ 15.26 ***	872.50 $\pm$ 61.93 *	1574.25 $\pm$ 250.19 ***# $\dagger$	3.59 $\pm$ 0.28 **
S + D	200.83 $\pm$ 15.24	884.16 $\pm$ 157.74	29.16 $\pm$ 5.20	200.00 $\pm$ 19.67	3709.56 $\pm$ 350.46	7.43 $\pm$ 0.80

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

* P < 0.05, ** P < 0.01, *** P < 0.001, compared to dexamethasone (D) treated group.

P < 0.05, ## P < 0.001, compared to Z1 groups group.

$\dagger$ P < 0.05, $\dagger\dagger$ P < 0.001, compared to Z2 groups group.

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

Th₁/Th₂ balance could be considered as a therapeutic target in asthma therapy because Th₁ has been proved to inhibit Th₂ response (Randolph et al., 1999). The absence of FOXP3 is associated with the development of immune abnormalities, such as severe allergic inflammation and high immunoglobulin E levels (Chatila, 2005) and the reduction of FOXP3 protein expression is reported in patients with stable asthma (Provoost et al., 2009). Therefore, increased IL-4 and IL-17 genes expression but reduction of IFN- γ and FOXP3 genes expression and IFN- γ /IL-4 ratio in sensitized animals confirmed their sensitization and induction of a mice model of asthma in the present study. Regarding the use of splenocyte in the present study, it is well known that immunization with OVA and animal sensitization induce the systemic activation of immune system leading to a shift in Th₁/Th₂ balance toward Th₂ activity which is present in asthma and allergic disease. Moreover, spleen is one of the main sources of lymphocytes including T helper cells. In addition, preparation of lymphocytes from peripheral immune system is too difficult with huge variations in responses.

Treatment of sensitized splenocytes with the highest concentration of the extract of *Z. multiflora* led to increased IFN- γ gene expression, and the two higher concentrations increased FOXP3 gene expression. However, all three concentrations of the extract decreased TGF- β and the highest concentration reduced IL-17 genes expression. IFN- γ /IL-4 ratio was also significantly increased in group treated with two higher concentrations of the extract. Therefore, treatment with the extract of *Z. multiflora* caused reduction of pro-inflammatory and potentiating of anti-inflammatory cytokines genes expression in splenocytes of sensitized mice. These results indicated a preventive therapeutic effect of the extract of the plant on inflammatory conditions associated with immunological disorders such as asthma. Increase in serum level of IFN- γ and reduction of IL-4 in sensitized guinea pigs and human lymphocytes as well as increase in IFN- γ /IL4 cytokine (Th₁/Th₂) balance following treatment with the extract of *Z. multiflora* was previously shown which supports the findings of the present study (Boskabady et al., 2013). The preventive effect of the extract of the plant on tracheal responsiveness (Boskabady et al., 2014a,b), pathological changes (Boskabady et al., 2014a,b) and inflammatory mediators (Boskabady et al., 2014a,b) in sensitized guinea pigs were also shown in previous studies. The results of the previous studies as well as the findings of the present study support the possible preventive therapeutic effect of the plant on asthma. In addition, the effects of the extract on all studied genes expression as well as IFN- γ /IL4 ratio were concentration-dependent which could be considered as another evidence for its preventive therapeutic effect on asthma.

Also, in our study, *Z. multiflora* increased FOXP3 gene expression in sensitized animals but dexamethasone had no significant effect on FOXP3 gene expression. CD⁴⁺CD²⁵⁺ Tregs were shown to suppress Th₂ cytokine production from both atopic and non-atopic

donors (Bellinghausen et al., 2003). These findings suggest that the extract of the plant may suppress Th₂ cytokine production which will help treatment of asthma.

IL-17 gene expression was also decreased in sensitized splenocytes treated with the extract of *Z. multiflora*. It was shown that recruitment of neutrophils into the lung in response to allergens or gram-negative bacteria was markedly diminished in mice deficient in IL-17RA or IL-17A (Ye et al., 2001; Nakae et al., 2002). Transfer or antigen-specific IL-17 producing Th₂ cells triggered influx of neutrophils, eosinophils, macrophages and lymphocytes, resulting in distinct pathophysiological features of severe asthma was shown in animal models of asthma (Wang et al., 2010). IL-17 producing Th₂ cells (Th₁₇) may represent the key pathogenic on promoting exacerbations of allergic asthma (Wang and Wills-Karp, 2011). The plant therefore, may also have therapeutic effect on asthma by suppressing IL-17 gene expression.

Treatment of splenocytes of sensitized mice with the plant extract also decreased TGF- β gene expression. The role of TGF- β in airway remodeling in asthma as well as its effect during the late phase of lung inflammation was shown previously (Duvernelle C and Frossard, 2003; Magnan et al., 1996). TGF- β gene expression was also increased in asthmatic airways after repeated exposure to low doses of cat allergen which was accompanied by asthma symptoms (Duvernelle C et al., 1998). Therefore, the results of the present study may also suggest that the extract of *Z. multiflora* could be of therapeutic value in asthma by reducing TGF- β gene expression and prevention of airway remodeling.

In fact, preventive effects of the plant extract on systemic inflammation, tracheal responsiveness and lung pathology in guinea pigs model of COPD were shown previously (Boskabady and Gholami Mahtaj, 2015; Gholami Mahtaj et al., 2015; Boskabady and Gholami Mahtaj, 2014) which may also support the anti-inflammatory effect of the plant and support the findings of the present study. The results of characterization of the extracts of the plant by HPLC showed that carvacrol is one of its main constituents.

In a previous study, the effects of carvacrol at the concentrations of 75, 150 and 300 μ g/mL on gene expression of IL-4, IFN- γ , IL-17, TGF- β and Foxp3 were examined. The results showed approximately equal effect of the carvacrol with those of the extract seen in the present studies on most cytokine gene expression (Kianmehr et al., 2016). The studied concentrations of carvacrol were 37.5% of the extract concentrations (75, 150 and 300 μ g/mL for carvacrol compared to 200, 400 and 800 μ g/mL for the extract). However, as indicated in the results section, the amount of carvacrol in the studied extract was 0.18% (w/w). Therefore, the studied concentrations of carvacrol in the previous study are much higher than the actual carvacrol content in the extract. Therefore, the immune modulatory effect of *Z. multiflora* might be only partially due to its constituent, carvacrol. Therefore, the effect of other constituents of the plant such as thymol on cytokine gene expression should be

examined in further studies. The extract concentration administered to guinea pigs were 0.2, 0.4 and 0.8 mg/mL in drinking water (Boskabady et al., 2013). Considering the daily consumption of water by guinea pigs (about 150 mL/day) and the extracellular fluid of the animal (approximately 200 mL), the concentrations of the extract in the extracellular fluid would be 600 µg/mL which is nearly similar to the concentration used in the present study. The dose of a given drug for humans is about one tenth of that used in animals. Therefore, the human dose should be around 10 mg/kg for clinical studies.

The cell viability was not evaluated in this study. However, the extract of *Z. multiflora* decreased IL-4, TGF-β and IL-17 but increased IFN-γ and FOXP3 genes expression in treated splenocytes of sensitized mice indicating that the extract do not induce cell toxicity at studied concentrations.

Dexamethasone treatment only decreased IL-4 and TGF-β genes expression which are pro-inflammatory cytokines, but had no significant effect on IFN-γ and FOXP3 genes expression in sensitized splenocytes. The significant increase in IFN-γ/IL-4 ratio following dexamethasone treatment seen in this study is mainly due to the suppressive effect on pro-inflammatory rather than anti-inflammatory cytokines. However, the extract affected both anti-inflammatory and pro-inflammatory cytokine genes expression. In fact, dexamethasone treatment only inhibited the activities of Th2 cells but *Z. multiflora* extract inhibited Th2 activities and upregulated Th1 cytokines gene expression. Dexamethasone treatment also did not affect IL-17 gene expression. Therefore, the plant has more specific therapeutic effect in disorders (such as asthma) associated with pro/anti-inflammatory cytokine imbalance as compared to dexamethasone. Similar results for *Z. multiflora* extract were observed on serum IFN-γ/IL4 cytokine (Th1/Th2) ratio in sensitized guinea pigs as well as the effect of the extract on human lymphocytes (Boskabady et al., 2013). In the present study, IFN-γ gene expression was decreased, IL-4 gene expression was increased and Th1/Th2 balance was decreased in the splenocyte of sensitized animals, but all these changes were reversed in sensitized animals treated with the plant extract. In human lymphocyte also IFN-γ gene expression was increased, IL-4 gene expression was decreased and Th1/Th2 balance was increased following *Z. multiflora* treatment in both non-stimulated and stimulated cells (Boskabady et al., 2013). In sensitized guinea pigs, serum concentration of both IFN-γ and IL-4 were increased and Th1/Th2 balance was decreased. However, in sensitized groups treated with *Z. multiflora* extract, serum concentration of IFN-γ was increased but that of IL-4 was decreased and Th1/Th2 balance (Boskabady et al., 2013) was increased which were in line with the findings of the present study.

The inhibitory effects of *Z. multiflora* essential oil on nitric oxide (NO) and hydrogen peroxide (H₂O₂) production in human monocytes cultured in the presence of 20 mM glucose showed that the plant can reduce oxidative stress (Kavoosia and Teixeira Da Silva, 2012) which also support the findings of the present study. Therefore, the results of the present study as well as several previous studies indicate that even short term administration of *Z. multiflora* can be used in the therapy of oxidative damage in inflammatory conditions.

5. Conclusions

In conclusion, these results indicated that the extract of *Z. multiflora* decreased IL-4, TGF-β and IL-17 but increased IFN-γ and FOXP3 genes expression in splenocytes of sensitized mice. These results may indicated the more specific therapeutic value for the extract of the plant compared to dexamethasone in allergy, autoimmunity and infectious diseases as it both potentiated anti-

inflammatory and suppressed pro-inflammatory cytokines. However, it should be noted that long-term pro-inflammatory potentiating effect of IFN-γ in asthma may result in some undesirable consequences.

Conflict of interest

The authors declare that they have no conflict of interest.

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