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Immunomodulatory effect of characterized extract of *Zataria multiflora* on Th_1, Th_2 and Th_{17} in normal and Th_2 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), Sensetized animals treated with dexamethasone or three concentrations of *Z. multiflora* extract (200, 400 and 800 μg/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 μg/ml, significantly upregulated IFN-γ gene expression ($P < 0.01$) and its 400 and 800 μg/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 μg/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.

1. Introduction

T cells as a part of adaptive immune system, are divided to effectors and regulator subdivisions. Type 2 T helper (Th_2) 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 Th_2 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 (Th_1) cells, also known as inflammatory cells, can antagonize Th_2 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, Th_2 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 shirazi") 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-μm-pore-size anti-microbial filter. Considering the anti-bacterial effect of DMSO and also by using 0.2 μm 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 μg/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 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 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 μg/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 μg/mL) were added and incubated for 18 h (at 37°C with P_{CO2} 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 μl 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 μl 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