



Ethnopharmacological communication

The effect of the extract of *Crocus sativus* on tracheal responsiveness and plasma levels of IL-4, IFN- γ , total NO and nitrite in ovalbumin sensitized Guinea-pigsGoltaj Byrami^a, Mohammad Hossein Boskabady^{a,*}, Sedigheh Jalali^b, Tahereh Farkhondeh^c^a Applied Physiology Research Centre and Department of Physiology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, IR Iran^b Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, IR Iran^c Department of Toxicology, Faculty of Veterinary Medicine, University of Tehran, Tehran, IR Iran

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ABSTRACT

Ethnomedical relevance: Anti-inflammatory, anti oxidant and effect of *Crocus sativus* (*C. sativus*) on Th₁/Th₂ balance were described previously.**Aim of the study:** The preventive effects of the extract of *Crocus sativus* on tracheal responsiveness and plasma levels of IL-4, IFN- γ , total NO and nitrite were examined on sensitized guinea pigs.**Materials and methods:** Five groups of sensitized guinea pigs to ovalbumin (OVA), were given drinking water containing three concentrations of the extract of *Crocus sativus*, dexamethasone (S+D) or alone (group S). Tracheal responses (TR) of control animals (group C) and sensitized guinea pigs ($n=6$, for each group) to methacholine, OVA and the levels of IL-4, IFN- γ , total NO and nitrite in serum were examined. **Results:** The TR to both methacholine and OVA, the levels of serum IL-4, total NO and nitrite in S guinea pigs were significantly increased but that of IFN- γ and IFN- γ /IL-4 ratio (Th₁/Th₂ balance) were decreased compared to the controls ($p < 0.05$ to $p < 0.001$). In the treated animals with dexamethasone and all concentrations of the extract, TR to both methacholine and OVA, IL-4, total NO and nitrite were significantly decreased but IFN- γ and IFN- γ /IL-4 ratio increased compared to S group ($p < 0.05$ to $p < 0.001$). The effects of the highest concentration of the extract was greater than those of other concentrations and the effect of dexamethasone ($p < 0.05$ to $p < 0.01$).**Conclusions:** These results not only showed a preventive effect of *C. sativus* extract on tracheal responses and serum levels of inflammatory mediators in sensitized guinea pigs but also showed increased Th₁/Th₂ balance.

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1. Introduction

Airway inflammation (Dodig et al., 2011) and airway responsiveness (AHR) (Meurs et al., 2008) are main characteristic feature of asthma. T helper 2 (Th₂) is activated in asthma disease, and its mediators cause airway inflammation (Cohn et al., 2004; Wegmann, 2009) but Th₁ can inhibit Th₂ and thus one goal of asthma therapy should be focused on increasing the activity of Th₁ (Randolph et al., 1999). Increased nitrite, nitrate, and nitrotyrosine concentrations in asthma (Robroeks et al., 2007) was shown which could be contribute in physiopathology of the airways inflammation in asthma (Ricciardolo et al., 2004).

Crocus sativus L., commonly known as saffron, is a small perennial plant from the iridaceae family which is widely cultivated in Iran and some other countries. The medicinally used part

of the plant is its stigma (central part of the flower, female sexual organ). Constituents of the plant include; picrocrocin and its derivatives including safranal, flavonoid derivatives and crocin (Srivastava et al., 2010).

Saffron is used in folk medicine as; antispasmodic, eupeptic, gingival sedative, anticatarrhal, nerve sedative, carminative, diaphoretic, expectorant, aphrodisiac and emmenagogue (Rios et al., 1996). Pharmacological effects including; anti-inflammatory (Hosseinzadeh and Younesi, 2002), anti-oxidant (Abe et al., 1999), antitumor, radical scavenger effects (Rios et al., 1996; Abdullaev and Espinosa-Aguirre, 2004; Tavakkol-Afshari et al., 2008), chemopreventive, genoprotective and protection from genotoxins-induced oxidative stress (Premkumar et al., 2003;) have shown for this plant.

Our previous study also showed the relaxant effect of saffron on tracheal smooth muscle (Boskabady and Aslani, 2006), inhibitory effect on histamine (H₁) receptor (Boskabady et al., 2010) and its stimulatory effect on β -adrenoceptors (Nemati et al., 2008).

Crocus sativus (saffron) is widely used as a traditional medicinal plant for treatment of various diseases. In addition, a wide range of

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pharmacological effects including anti-inflammatory, antioxidant properties and relaxant effect on tracheal smooth muscle were demonstrated. In the present study the effects of *Crocus sativus* on tracheal responsiveness (main characteristic of asthma), serum IL-4/IFN- γ ratio (as an indicator of Th1/Th2 balance), total NO and nitrite levels (important inflammatory mediators in asthma) in sensitized guinea pigs were examined.

2. Material and methods

2.1. Plant, extract and drugs

Crocus sativus was collected from Torbat Heydarieh (northeast Iran), and its stigmas were dried at room temperature in the absence of sunlight. The plant was identified by botanists in the herbarium of Ferdowsi University of Mashhad (specimen number 293-0303-1). For preparation of hydro-ethanolic extract, three grams of chopped *Crocus sativus* stigmas were mixed with 50 ml ethanol 70% for 72 h at room temperature and the solution was separated by maceration method which was repeated for three times. The solutions were dried in room temperature and stored at -4°C away from light. The chemical characteristics of the same plant were identified previously (Hadizadeh et al., 2007; Boskabady et al., 2011).

2.2. Animal sensitization and animal Groups

Sensitization of animals to OVA was performed according previous method (McCaig, 1987; Boskabady and Adel-Kardan, 1999). Briefly, guinea pigs were sensitized to 10 mg OVA (Sigma Chemical Ltd, UK) and 100 mg aluminum hydroxide ($\text{Al}(\text{OH})_3$) dissolved in 1 ml saline (i.p.) in day one and further 2 mg OVA and 100 mg $\text{Al}(\text{OH})_3$ (i.p.) in day 7. From day 14 sensitized animals were exposed to an aerosol of 4% OVA for 18 ± 1 days, 5 min daily. The aerosol was administered in a closed chamber, dimensions $30 \times 20 \times 20$ cm. Control animals were treated similarly but saline was used instead of OVA solution. The study was approved by the ethical committee of the Mashhad University of Medical Sciences.

The study was performed in control animals (group C, treated the same as the sensitized group but normal saline was used instead of OVA and were given drinking water alone) and five different groups of sensitized animals which were given drinking water containing various substance during sensitization period as follows ($n=6$ for each group); 1) Drinking water alone (group S, an animal model of asthma), 2) 10 mg/kg/day dexamethasone (group S+D), 3) 20 mg/kg/day extract (group S+CS1), 4) 40 mg/kg/day extract (group S+CS2), 5) 80 mg/kg/day extract (group S+CS3).

2.3. Tissue Preparations

Guinea pigs were sacrificed by a blow on the neck, and trachea was removed. Each trachea was cut into 10 rings (each containing 2–3 cartilaginous rings). All the rings were then cut open opposite the trachealis muscle, and sutured together to form a tracheal chain (Boskabady et al., 2004). Tissue was then suspended in a 20 ml organ bath (Schuler organ bath type 809, March-Hugstetten, Germany) containing Krebs-Henseliet solution of the following composition (mM): NaCl 120, NaHCO_3 25, MgSO_4 0.5, KH_2PO_4 1.2, KCl 4.72, CaCl_2 2.5 and dextrose 11. The Krebs solution was maintained at 37°C and gassed with 95% O_2 and 5% CO_2 . Tissue was suspended under isotonic tension of 1 g and allowed to equilibrate for at least 1 h while it was being washed with Krebs solution every 15 min.

In all experiments the contractions responses were measured using an isometric transducer (MLT0202, AD Instruments,

Australia) which was connected to a powerlab system (PowerLab 8/30, ML870, AD Instruments, Australia).

2.4. Assessment of tracheal response to methacholine

In each experiment a cumulative log concentration-response curves of methacholine hydrochloride (Sigma Chemical Ltd, U.K.) induced contraction of tracheal chain was obtained. Consecutive concentrations (including 10^{-7} to 10^{-3} M) were added every 2 min, and the contraction due to each concentration was recorded at the end of 2 min, and the effect reached a plateau in all experiments. The percentage of contraction of the tracheal smooth muscle due to each concentration of methacholine in proportion to the maximum contraction obtained by its final concentration was plotted against log concentration of methacholine. The effective concentration of methacholine, causing 50% of maximum response (EC_{50}) using methacholine response curve in each experiment, was measured.

2.5. Measurement of tracheal response to ovalbumin

The tracheal response to 0.1% solution of OVA was measured as follows: 0.5 ml of 4% OVA solution was added to the 20 ml organ bath and the degree of tracheal chain contraction was recorded after 15 min and was then expressed as proportion (in percentage) to contraction obtained by 100 μM methacholine.

2.6. Evaluation of plasma Levels of inflammatory Mediators

Five ml of blood samples was taken by cardiac puncture immediately after sacrificing and exposing the animals chest and were collected into test tube and placed at room temperature for 1 h. The samples were then centrifuged at $3500 \times g$ at 4°C for 10 min. The supernatant was collected and immediately stored at -70°C until analyzed.

Serum IL-4 (platinum ELISA BMS628/BMS628TEN, Bender Med Systems GmbH, Austria) and IFN- γ (platinum ELISA BMS621/BMS621TEN, Bender MedSystems GmbH, Austria) were measured using Elisa sandwich method according to the manufacturer's instructions. The ratio of IFN- γ /IL-4 as an index of Th1/Th2 was also calculated.

Serum NO level was determined by Nitric Oxide Calorimetric Assay kit (nitric oxide colorimetric assay kit k262–200, BioVision Research Products, USA) according to the manufacturer's instructions. Nitrate in the samples and standards were converted to nitrite by adding nitrate reductase and Griess reagent in the each well plate. Absorbance was determined at 540 nm. The concentration of total NO (nitrite+nitrate) was calculated according to a standard curve of known nitrite concentrations. For measuring nitrite, a nitrite standard curve was prepared in the absence of Nitrate Reductase in the standard and assay samples.

2.7. Statistical analysis

The data were quoted as mean $\pm$ SEM. According to the Kolmogorov Smirnov test the data had normal distribution. The data of sensitized group were compared with control guinea pigs using unpaired "t" test. The data of three groups of animals treated with the extract and dexamethazone were also compared with sensitized guinea pigs using unpaired "t" test. Statistical comparison between three treated groups with the extract was performed using one way ANOVA with Tukey Kramer hoc post test. Significance was accepted at $p < 0.05$.

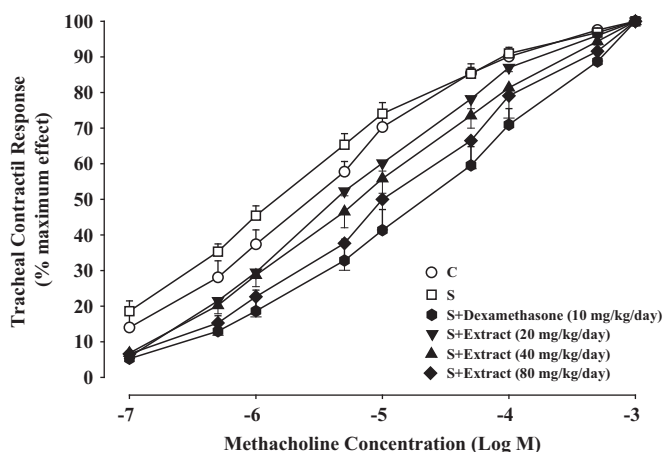


Fig. 1. Cumulative log concentration-response curves of methacholine induced contraction of isolated trachea in control (C), sensitized (S), S treated with dexamethasone and three concentrations of *Crocus sativus* extract (S+Extract) guinea pigs (for each group, $n=6$).

3. Results

3.1. Tracheal response to methacholine

Concentration response curves to methacholine showed leftward shift of in group S compared to group C but the curves of treated groups with dexamethasone and all concentrations of the extract were shifted to right compared to group S (Fig. 1).

The mean value of EC_{50} in tracheal chains of group S was significantly lower than group C ($p < 0.01$, Fig. 2a). The mean values of EC_{50} in treatment groups with all three concentrations of the extract and dexamethasone were significantly improved compared to the group S ($p < 0.05$ to $p < 0.001$, Fig. 2a).

3.2. Tracheal response to ovalbumin

Tracheal response to OVA in group S was significantly higher than group C ($p < 0.001$, Fig. 2b). Tracheal response to OVA in treatment groups with all three concentrations of the extract and dexamethasone were significantly improved compared to group S ($p < 0.05$ to $p < 0.001$, Fig. 2b). However, tracheal response to OVA in treatment groups were still significantly higher than group C ($P < 0.001$ for all treated groups, Fig. 2b).

3.3. Serum cytokine Levels and Th1/Th2 balance

Serum level of IL-4 in group S was significantly higher ($P < 0.001$) and the value of IFN- γ (0.06 ± 0.01) was significantly lower ($p < 0.01$, Fig. 3a) than group C. However, treatment of sensitized animals with all three concentrations of the extract as well as dexamethasone led to significant reduction in IL-4 and increase in IFN- γ levels ($p < 0.01$ to $p < 0.001$, Fig. 3a). In addition, the levels of IFN- γ in all treated groups were significantly higher than control group ($p < 0.001$, Fig. 3a).

The ratio of IFN- γ /IL-4 (Th1/Th2 balance) was also significantly decreased in sensitized compared to control group ($p < 0.001$, Fig. 3b). However, treatment of sensitized animals with dexamethasone and the extract led to increase in Th1/Th2 balance ($p < 0.05$ for two lower concentration of the extract and $p < 0.001$ for high concentration of the extract and dexamethasone, Fig. 3b).

3.4. Serum total NO and nitrite Levels

Serum levels of total NO and nitrite in group S were significantly higher than that of group C ($P < 0.01$ for total NO and $p < 0.05$ for nitrite, Fig. 4). Treatment of sensitized guinea pigs with all three

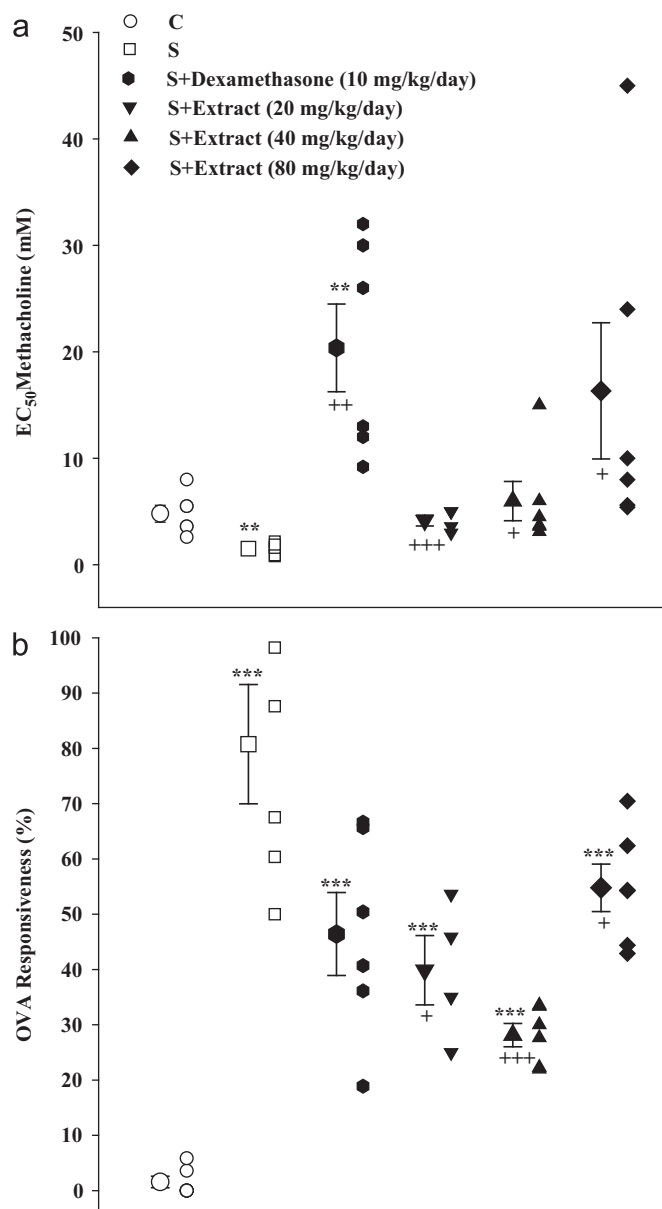


Fig. 2. Individual values and mean $\pm$ SEM (big symbols with bars) of (a) tracheal response to methacholine (EC_{50}) and (b) tracheal response to ovalbumin (percent contraction due to ovalbumin 0.1% in proportion to contraction obtained by 100 μ M methacholine) in control (C), sensitized (S), S treated with dexamethasone and three concentrations of *Crocus sativus* extract (S+Extracts) guinea pigs (for each group, $n=6$). Statistical differences between control and different groups: **, $p < 0.01$, ***, $p < 0.001$. Statistical differences between treated vs sensitized group: +: $p < 0.05$, ++: $p < 0.01$, +++: $p < 0.001$.

concentrations of the extract and dexamethasone led to significant reduction in total NO and nitrite levels ($p < 0.05$ to $p < 0.001$, Fig. 4). In addition, the levels of total NO and nitrite in treated animals with high concentration of the extract were significantly lower than control group ($p < 0.01$ for total NO and $p < 0.05$ for nitrite, Fig. 4).

3.5. Differences between the effects of three Concentrations of the extract

The mean values of EC_{50} in tracheal chains of treated groups with two lower concentrations of *Crocus sativus* extract (20 and 40 mg/kg/day) were significantly lower than the effect of higher concentration (80 mg/kg/day, $p < 0.05$ and $p < 0.01$ respectively, Table 1). In addition, tracheal response to OVA in treated group with medium concentration

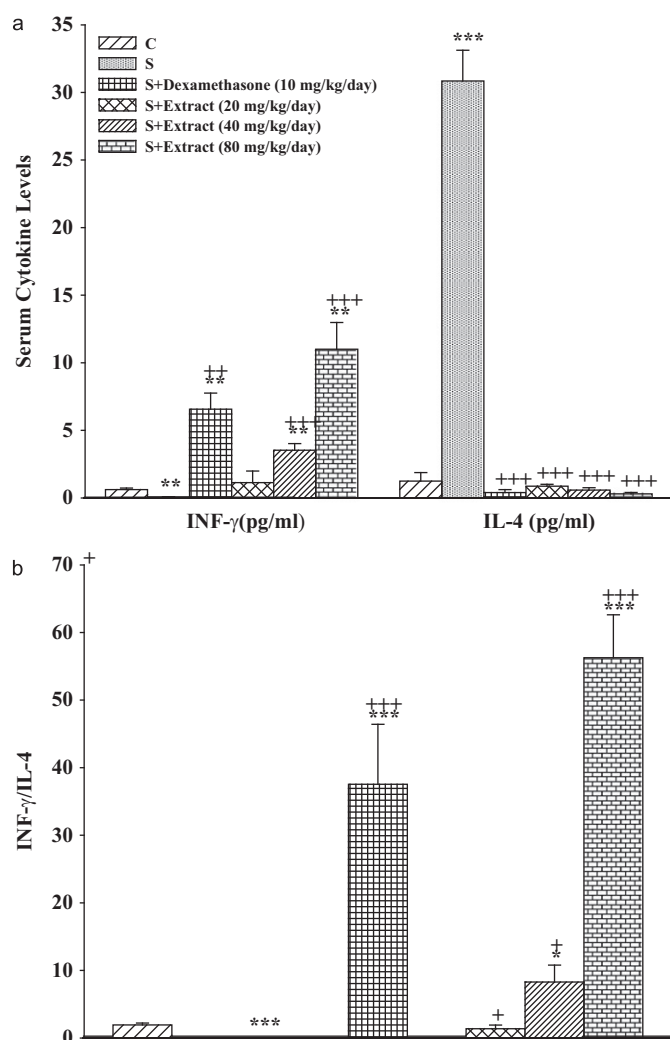


Fig. 3. The levels of serum INF- γ and IL-4 (a) and The INF- γ /IL-4 ratio (Th1/Th2 balance), (b) in control (C), sensitized (S), S treated with dexamethasone and three concentrations of *Crocus sativus* extract (S+Extract) guinea pigs (for each group, $n=6$). Statistical differences between control and different groups: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. Statistical differences between treated animals vs sensitized group: +, $p < 0.05$, ++, $p < 0.01$, +++, $p < 0.001$.

of the extract (40 mg/kg/day) was greater than those of higher concentrations (80 mg/kg/day, $p < 0.05$, Table 1). The effect of higher concentration of the extract (80 mg/kg/day) on IFN- γ level and IFN- γ /IL-4 ratio was significantly higher than the effect of two lower concentrations ($p < 0.01$ for IFN- γ and $p < 0.001$ for IFN- γ /IL-4 ratio, Table 1). The effect of higher concentration of the extract (80 mg/kg/day) on total NO and nitrite levels was significantly higher than the effect of two lower concentrations ($p < 0.01$ for total NO and $p < 0.05$ for nitrite, Table 1).

3.6. Differences between the effect of dexamethasone and the extract

The effects of two lower concentrations of the extract on TR to methacholine and IFN- γ /IL-4 ratio and the effect of its low concentration on IFN- γ level were significantly lower than that of dexamethasone ($p < 0.05$ to 0.01, Table 1). However, the effect of higher concentration of the extract (80 mg/kg/day) on IFN- γ level, IFN- γ /IL-4 ratio and total NO was significantly higher than the effect of dexamethasone ($p < 0.05$ for all cases, Table 1).

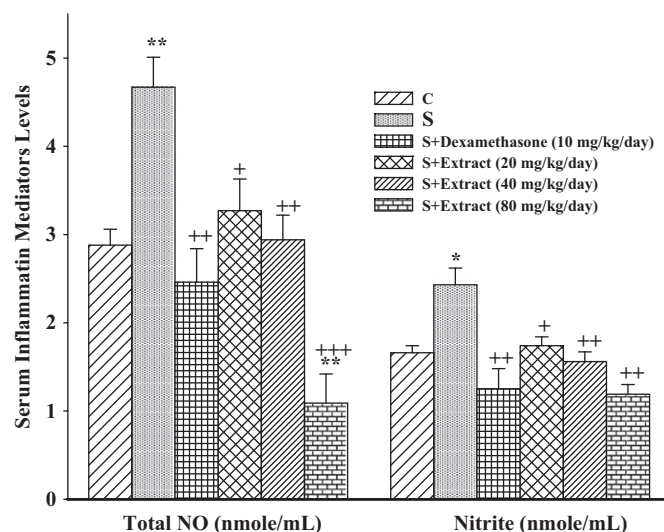


Fig. 4. The values of serum total NO and nitrite (mean $\pm$ SEM values) in control (C), sensitized (S), S treated with dexamethasone (S+D), S treated with three concentrations of the extract (S+Extract), (for each group, $n=6$). Statistical differences between control vs sensitized group: *, $p < 0.05$, **, $p < 0.01$. Statistical differences between sensitized and treated groups: +, $p < 0.05$, ++, $p < 0.01$, +++, $p < 0.001$.

4. Discussion

The results of the present study showed that treatment of sensitized animals with *Crocus sativus* caused significant reduction in tracheal responsiveness to both methacholine and OVA as well as IL-4, NO and nitrite levels but caused increase IFN- γ level and IFN- γ /IL-4 ratio. The effects of the extract on all measured values seem to be concentration dependent. In addition the effects of the extract on tracheal responsiveness and serum cytokines were comparable with the effect of dexamethasone.

All prophylactic drugs used in the treatment of asthma should focus on reduction of airway inflammation and airway hyperresponsiveness. The preventive effect of long term administration of the extract of *Crocus sativus* on tracheal responsiveness (which is mainly due to airway inflammation) of sensitized animals is perhaps due to its suppressing effect on airway inflammation. In fact, anti-inflammatory and anti-oxidant effects of *Crocus sativus* have been shown previously (Hosseinzadeh and Younesi, 2002; Abe et al., 1999; Papandreou et al., 2006). The inhibitory effect of this plant on histamine (H_1) receptor (Boskabady et al., 2010) and its antitussive effect (Hosseinzadeh and Ghenaati, 2006) can also contribute to its anti-inflammatory effect.

The other therapeutic effect of the extract on sensitized animals seen in the present study is its reduction effect on IL-4 and increasing effect on IFN- γ levels which indicate the effect of the extract on regulation of Th1 and Th2 balance. The inflammatory condition of asthmatic lung is regulated by the balance of CD4+ helper T cells. Th1 cells produce interleukin 2 (IL-2) and interferon- γ (IFN- γ), Th2 cells produce IL-4 and IL-10 (Randolph et al., 1999). Th2 cells promote the activity of macrophages and regulate the pro-inflammatory response, whereas Th1 cells inhibit the activity of macrophages directly or indirectly by inhibiting Th2 activity and thus regulate the anti-inflammatory response (Romagnani, 1999). The reduction in IL-4 and increase in IFN- γ in serum of sensitized animals treated with the extract of *Crocus sativus* may indicate its stimulatory effect on Th1 and a suppressive effect on Th2 cells. The results also showed a significant increased in IFN- γ /IL-4 ratio (Th1/Th2 balance) in sensitized animals treated with the extract. Our previous study also showed increased Th1/Th2 balance effect for *Crocus sativus* on human

Table 1
Values of tracheal response to methacholine (EC₅₀) and response to ovalbumin (OVA), serum cytokine, total NO and nitrite levels in control guinea pigs (C), sensitized animals (S), S treated with three concentrations of the extract (S+CS1, S+CS2 and S+CS3) and S treated with dexamethasone (S+D), (for each group, n=6).

	C	S	S+D	S+CS1	S+CS2	S+CS3
EC ₅₀ (μM)	4.80 ± 0.79	1.51 ± 0.23	20.36 ± 4.12	4.15 ± 0.50¶	5.98 ± 1.85¶¶	16.33 ± 6.39
OVA (%)	1.57 ± 1.03	80.76 ± 10.80	46.42 ± 7.51	39.87 ± 6.26	28.13 ± 2.10¶	54.78 ± 4.29 ★★★
IFN-γ (pg/ml)	0.62 ± 0.11	0.06 ± 0.01	6.57 ± 1.19	1.13 ± 0.86 ¶, ★★	3.53 ± 0.49 ★★	11.00 ± 1.98¶
IL-4 (pg/ml)	1.25 ± 0.63	30.85 ± 2.27	0.40 ± 0.22	0.87 ± 0.14	0.58 ± 0.18	0.30 ± 0.1
IFN-γ/IL-4	1.93 ± 0.22	0.002 ± 0.0002	37.54 ± 8.88	1.37 ± 0.54 ¶¶, ★★★	8.29 ± 2.50 ¶, ★★★	56.28 ± 6.35¶
Total NO (mM)	2.88 ± 0.18	4.67 ± 0.34	2.46 ± 0.38	3.27 ± 0.36 ★★	2.94 ± 0.28 ★★	1.09 ± 0.33¶
Nitrite (mM)	1.66 ± 0.08	2.43 ± 0.19	1.25 ± 0.23	1.74 ± 0.10	1.56 ± 0.11	1.19 ± 0.11★

Values are presented as mean ± SEM. Three concentrations of the extract were 20 (S+CS1), 40 (S+CS2) and 80 (S+CS3) and that of dexamethasone was 10 mg/kg/day.

Statistical **significance** for the **difference** between the data of S+D vs S+CS1, S+CS2 and S+CS3: ¶; p < 0.5, ¶¶; p < 0.01.

Statistical **significance** for the **difference** between the data of S+CS1 and S+CS2 vs S+CS3: ★; p < 0.05, ★★; p < 0.01, ★★★; p < 0.001.

lymphocytes (Boskabady et al., 2011) which supports the results of the present study. Although, phenotyping of lymph node T cell, IgE/IgG2 ratio and measurement of other cytokines could be used for evaluation of Th1/Th2 balance, in the present study, the ratio of IFN-γ/IL-4 was used for this purpose which is the same as several previous studies.

The effect of saffron on serum levels of total NO and nitrite may also suggest an antioxidant effect for the plant. Inducible NOS is inhibited by glucocorticoids which supports the findings of the present study (Saleh et al., 1998).

The comparable effect of the extract on measured variables of sensitized animals with the effect of dexamethasone also confirmed anti-inflammatory effect of the extract of *Crocus sativus*. However, the results showed greater effect for highest concentration of the extract on serum levels of total NO, IFN-γ, and Th1/Th2 balance which indicated more potent effect of higher concentration of the extract than dexamethasone. The concentration dependency effect of the extract also confirmed its anti-inflammatory effect.

In a previous study, the effects of *Crocus sativus* and its constituent safranal, on lung pathology, total and differential white blood cells (WBC) were studied (Boskabady et al., 2012). However, in the present study the effect of the plant on specific and non specific tracheal responsiveness (responsiveness to methacholine and OA respectively) in sensitized guinea pigs, the main characteristic feature of asthma was evaluated. In addition the effects of the plant on the levels of IL-4, IFN-γ and their ratio as an indicator of Th1/Th2 balance which is an important immunological feature of asthma was also evaluated in sensitized animals, in the present study. Although in our other previous study the effects of the plant on IL-4 and IFN-γ in human lymphocyte were examined, in the present study effects of the plant on IL-4 and IFN-γ in sensitized guinea pigs were examined which was more compatible with real asthma. The effect of the extract on serum total NO and nitrite levels, which are important inflammatory mediator was also evaluated in the present study.

Our previous study also showed the relaxant effect of saffron on tracheal smooth muscle (Boskabady and Aslani, 2006) which indicates the bronchodilatory effect of the plant and its possible mechanism(s) including; inhibitory effect of the plant on histamine (H₁) receptor (Boskabady et al., 2010) and its stimulatory effect on β-adrenoceptors (Nemati et al., 2008). Therefore, *Crocus sativus* could affect asthma treatment by both bronchodilatory and reducing airway inflammation and airway responsiveness.

In conclusion, these results indicated a preventive effect of *Crocus sativus* in sensitized animals. The possible mechanism effects of the plant are its anti-inflammatory effect, increasing Th1/Th2 balance and anti-oxidant effect. Therefore the results may suggest a preventive effect for *Crocus sativus* (saffron) on asthma disease.

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