The effect of safranal, a constituent of *Crocus sativus* (saffron), on tracheal responsiveness, serum levels of cytokines, total NO and nitrite in sensitized guinea pigs

Mohammad Hossein Boskabady *, Goltaj Byrami, Azadeh Feizpour

Neurogenic Inflammation Research Centre and Department of Physiology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

**Abstract**

**Background:** The effect of safranal (one of the constituents of *Crocus sativus*) on ovalbumin (OVA) sensitized guinea pigs was examined.

**Methods:** One group of sensitized guinea pigs were given drinking water alone (group S), three groups drinking water containing three concentrations of safranal and one group contain dexamethasone (S + D). Tracheal responses (TR) of the animals to methacholine as effective concentration causing 50% of maximum response (EC50 M), TR to 0.1% OVA, relative to contraction induced by 100 µM methacholine, IL-4, IFN-γ, total NO and nitrite levels in serum were measured.

**Results:** The TR to both methacholine and OVA, the level of total NO, nitrite and IL-4 significantly increased but IFN-γ and IFN-γ/IL-4 ratio was decreased in group S compared controls (p < 0.05 to p < 0.001). The TR to both methacholine and OVA in treated animals with dexamethasone and all concentrations of safranal were significantly decreased compared to S group (p < 0.01 to p < 0.001). The level of serum IL-4 in treated guinea pigs was significantly decreased but IFN-γ and IFN-γ/IL-4 ratio was increased compared to S group (p < 0.01 to p < 0.001). The levels of total NO and nitrite were significantly decreased in treated groups compared to sensitized group (p < 0.05 to p < 0.001).

**Conclusion:** These results showed a preventive effect for safranal on tracheal responses and serum cytokine, total NO and nitrite levels as well as increased Th1/Th2 balance in sensitized guinea pigs.

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well as elevation in the baseline activity and excitability of pulmonary C fibers, and the hypersensitivity [47]. Inducible NOS (iNOS), localized predominantly in inflammatory cells and epithelial cells, plays an important role in both migration of inflammatory cells and increase in microvascular permeability after allergen challenge [43]. OVA sensitization also leads to increase in [Ca2+] levels in leukocytes and tracheal smooth muscle cells, higher rates of 45Ca and 22Na influx in leukocytes and increased levels of lipid peroxides in plasma [22]. Increased Th2-type cytokine production but reduction in IL-10 and IFNgamma levels (decreased Th1/Th2 balance) were shown in thoracic lymph node cells isolated from OVA-sensitized animals [14]. Allergen inhalation has indeed been shown to cause an increase in IL-5-producing CD3+ cells in the bone marrow. It is well shown that IL-5 is crucial for the induction of airway eosinophilia resulting in an increased production of eosinophils [27]. It was also shown that the expression of Th2 cytokines and IgE from lymphocytes isolated from OVA-sensitized animals were increased [46].

To evaluate the preventive effect of safranal on sensitized guinea pigs, the effect of oral administered safranal on TR, serum cytokine, total NO and nitrite levels were examined.

Materials and methods

Guinea pigs were sensitized using 10 mg OVA (Sigma Chemical Ltd., UK) and 100 mg Al(OH)3 dissolved in 1 mL saline administered via i.p. injection on days 1 and 8. From day 14, sensitized animals were exposed to an aerosol of 4% OVA for 18 ± 1 days, 5 min daily [49]. The aerosol was administered in a closed chamber (dimensions: 30 cm × 20 cm × 20 cm). The study was approved by the ethical committee of the Masland University of Medical Sciences (MUMS). The ethical committee for the animal experiments issued permission (No. 88736) dated 10th of January 2011.

Adult Dunkin-Hartley guinea pigs (400–700 g, both sexes) were randomly divided into sex groups including control group, in which the animals were treated in the same way as the sensitized groups but normal saline was used instead of OVA and they were only given drinking water (group C) and five different groups of sensitized animals treated with drinking water alone (group S), drinking water containing 50 µg/mL dexamethasone (Sigma Chemical Ltd., UK, Catalogue No. D 4902, purity 97%) (group S + D), and treated with 4, 8 and 16 µg/mL safranal (Fluka, Italy, Catalogue No. C4915, purity 75%) (groups S + SA1, S + SA2, and S + SA3, respectively) (n = 6 for each group).

Guinea pigs were sacrificed by a blow on the neck; then trachea was removed and cut into 10 rings (each containing 2–3 cartilaginous rings). The rings were then cut open opposite the trachealis muscle and sutured together to form a tracheal chain [10]. The tissue was suspended in a 20 mL organ bath (Schuler organ bath type 809, March-Hugstetten, Germany) containing Krebs–Henseleit solution, with the following composition (mM): NaCl 120, NaHCO3 25, MgSO4 0.5, KH2PO4 1.2, KCl 4.72, CaCl2 2.5 and dextrose 11, under isotonic tension of 1 g and was allowed to equilibrate for at least 1 h. The Krebs solution was maintained at 37 °C and gassed with 95% O2 and 5% CO2. The tissue was washed with Krebs solution every 15 min. Contraction responses were measured using an isometric transducer, connected to a powerlab system (MLT0202 and PowerLab 8/30, ML870, AD Instruments, Australia).

A cumulative log concentration–response curve to consecutive concentrations (including 10^-7 to 10^-3 M, added every 2 min) of methacholine hydrochloride (Sigma Chemical Ltd., UK) for each tracheal chain was obtained by recording the contraction at the end of 2 min. The percentage of contraction of the tracheal smooth muscles 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 (EC50), was measured from methacholine response curve.

Tracheal response to 1% OVA solution was evaluated by measuring the degree of contraction 15 min after its addition to organ bath and was expressed as proportion (in percentage) to contraction obtained by 100 µM methacholine. The measurement of tracheal response to methacholine and OVA was performed in random order.

Blood samples of five mL were taken by cardiac puncture immediately after sacrificing and exposing the animal's chest and were collected into test tubes and placed at room temperature for 1 h. The samples were then centrifuged at 3500 × g at 4 °C for 10 min. The supernatant was collected and immediately stored at −70 °C for further analysis. 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 (Ab Sandwich) method according to the manufacturer’s instructions. The ratio of IFN-γ/IL-4 as an index of Th1/Th2 balance was also calculated. Serum NO levels were determined using Nitric Oxide Colorimetric 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 was converted to nitrite by adding nitrate reductase and Griess reagent in 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.

The data were quoted as mean ± SEM. According to the Kolmogorov–Smirnov test, all data had normal distribution. The data of sensitized group vs. control and those of each treated group vs. sensitized guinea pigs were compared using unpaired t-test. Statistical comparison between treated groups with three concentrations of safranal was performed using one way ANOVA with Tukey–Kramer post hoc test. Statistical significance was accepted at p < 0.05.

Results

Concentration–response curves to methacholine showed leftward shift in the group S compared to those in the group C. However, the curves of treated groups with only dexamethasone and those of all concentrations of safranal alone were shifted to right compared to group S (Fig. 1). The mean value of EC50 methacholine in group S (1.51 ± 0.23 µM) was significantly lower than group C (4.80 ± 0.79 µM, p < 0.01, Fig. 2a). The mean value of EC50 in treatment groups with all concentrations of safranal alone and only dexamethasone were significantly improved compared to group S (p < 0.01 for dexamethasone and p < 0.001 for all concentrations of safranal, Fig. 2a).

Tracheal response to OVA in group S (80.76 ± 10.80%) was significantly higher than group C (1.57 ± 10.3%, p < 0.001, Fig. 2b). Tracheal responses to OVA in treatment groups with all concentrations of safranal alone and only dexamethasone were significantly improved compared to group S (p < 0.05 for dexamethasone, Fig. 2b). However, tracheal responses to OVA in treatment groups were still significantly higher than group C (p < 0.01 to p < 0.001, Fig. 2b). Serum level of IL-4 in group S (30.85 ± 2.27 pg/mL) was significantly higher than that of group C (1.25 ± 0.63 pg/mL, p < 0.001) and the value of IFN-γ in group S (0.06 ± 0.01 pg/mL) was significantly lower than group C (0.62 ± 0.11 pg/mL, p < 0.001,
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 safranal (S + safranal) guinea pigs (for each group n = 6).

Fig. 3a). However, treatment with all three concentrations of the safranal alone and only 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 level of IFN-γ in all treated groups was significantly higher than control group (p < 0.01 to p < 0.001, Fig. 3a). The ratio of IFN-γ/IL-4 (Th1/Th2 balance) was also significantly lower in sensitized compared to control group (p < 0.001, Fig. 3b). However, treatment of sensitized animals with only dexamethasone and safranal alone led to increase in Th1/Th2 balance (p < 0.01 to p < 0.001, Fig. 3b).

Total NO (4.67 ± 0.34 mM) and nitrite in serum group S (2.43 ± 0.19 mM) were significantly higher than that of group C (2.88 ± 0.18 mM, p < 0.01 for total NO and 1.66 ± 0.08 mM, p < 0.05 for nitrite, Fig. 4). Treatment of sensitized guinea pigs with all concentrations of safranal alone and only dexamethasone leads to significant reduction in total NO and nitrite levels (p < 0.05 to p < 0.001, Fig. 4). The levels of total NO and nitrite in treated animals with low concentration of safranal were significantly lower than group C (p < 0.001 for total NO and p < 0.01 for nitrite, Fig. 4). The mean value of EC50 in treated groups with low concentrations of safranal (4 µg/mL) was significantly higher than the effect of two higher concentrations (8 and 16 µg/mL, p < 0.01 and p < 0.01, respectively, Table 1). The effect of lower concentration of safranal on IFN-γ, total NO and nitrite levels and IFN-γ/IL4 ratio was significantly higher than those of two higher concentrations (p < 0.05 to p < 0.001, Table 2).

The effect of two lower concentrations of safranal on TR to methacholine was higher than the effect of dexamethasone (p < 0.05 to p < 0.01, Table 1). However, effect of two higher concentrations of safranal on IFN-γ and IFN-γ/IL4 ratio was significantly lower than the effect of dexamethasone (p < 0.05 for all groups, Table 2).

Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C</th>
<th>S</th>
<th>S + D</th>
<th>S + SA1</th>
<th>S + SA2</th>
<th>S + SA3</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 (µM)</td>
<td>4.80 ± 0.79</td>
<td>1.51 ± 0.23</td>
<td>20.36 ± 4.12</td>
<td>42.00 ± 3.45</td>
<td>36.66 ± 4.15</td>
<td>22.33 ± 2.50</td>
</tr>
<tr>
<td>OVA (%)</td>
<td>1.57 ± 1.03</td>
<td>80.76 ± 10.80</td>
<td>46.42 ± 7.51</td>
<td>56.46 ± 2.57</td>
<td>51.27 ± 10.98</td>
<td>52.28 ± 7.61</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM. Three concentrations of safranal were 4 (S + SA1), 8 (S + SA2) and 16 µg/mL (S + SA3) and that of dexamethasone was 50 µg/mL.

Statistical significance for the difference between the data of S + SA1 vs. S + SA2 and S + SA3: p < 0.05.

Statistical significance for the difference between the data of S + SA1 vs. S + SA2 and S + SA3: p < 0.01.

Statistical significance for the difference between the data of S + SA3: S + SA1, S + SA2 and S + SA3: p < 0.05.

Statistical significance for the difference between the data of S + D vs. S + SA1, S + SA2 and S + SA3: p < 0.01.

Discussion

The results of the present study showed preventive effect of safranal and dexamethasone on increased tracheal responsiveness.
to methacholine and OVA. IL-4 level, total NO and nitrite. However, the level of IFN-γ was increased in sensitized animals treated with safranal. The effect of safranal on sensitized animals was very similar to dexamethasone.

The preventive effect of long-term administration of safranal, one of the main constituents of C. sativus, on tracheal responsiveness is perhaps due to its suppressing effect on the airway inflammation which is the main pathological feature of asthma. Antioxidant properties of safranal [20, 24], anti-inflammatory effect of safranal [16], and its inhibitory effect on histamine (H1) receptor [8] could be due to its anti-inflammatory effect and supports the results of this study.

The serum levels of total NO and nitrite were also decreased in sensitized animals treated with safranal which may suggest its antioxidant effect. In fact, increased nitrite, nitrate, and nitrotyrosine concentrations in the exhaled breath condensate of asthmatic children [36] and adults were shown [29] which indicate the role of these agents in pathophysiology of asthma. Inducible NOS is inhibited by glucocorticoids, and selective inhibitors of this enzyme may have therapeutic effects in asthma [39] and support the findings of the current study.

The results of the current study also showed reduction of IL-4 and increasing IFN-γ levels in the serum of sensitized animals treated with safranal which indicate its effect on regulation of Th1 and Th2 balance. These results could indicate the immunoregulatory effect of safranal which can contribute to its therapeutic effect on asthma disease. It has been shown that the lung inflammation in asthma is regulated by the balance of CD4+ helper T cells. Th1 cells produce interleukin 2 (IL-2) and interferon-γ (IFN-γ), whereas Th2 cells produce IL-4 and IL-10 [37]. Th2 cytokines, IL-5 is an essential factor for eosinophil inflammation [30], and has a pivotal role in IgE production [28]. However, IFN-γ (a Th1 cytokine) is able to inhibit both eosinophil recruitment and IgE synthesis [21]. Therefore, Th1 is able to regulate anti-inflammatory response [37]. Tolerance to allergens is a mechanism that normally prevents Th2-based immune responses [40, 41]. The reduction in IL-4 and increase in IFN-γ in serum of sensitized animals treated

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

Fig. 4. The value of serum total NO and nitrite (mean ± SEM values) in control (C), sensitized (S), S treated with dexamethasone (S + D) and S treated with three concentrations of safranal (S + safranal) (for each group n = 6). Statistical differences between control vs. sensitized group: *p < 0.05 and **p < 0.01. Statistical differences between sensitized and treated groups: ***p < 0.005, ****p < 0.001.

Table 2

Values of serum cytokine, NO and nitrite levels of control guinea pigs (C), sensitized animals (S), S treated with three concentrations of safranal (S + SA1, S + SA2 and S + SA3) and S treated with dexamethasone (S + D) (for each group n = 6).

<table>
<thead>
<tr>
<th>Mediators</th>
<th>C</th>
<th>S</th>
<th>S + D</th>
<th>S + SA1</th>
<th>S + SA2</th>
<th>S + SA3</th>
</tr>
</thead>
<tbody>
<tr>
<td>INF-γ (pg/mL)</td>
<td>0.62 ± 0.11</td>
<td>0.06 ± 0.01</td>
<td>6.57 ± 1.19</td>
<td>11.01 ± 1.40</td>
<td>3.14 ± 0.43</td>
<td>2.37 ± 0.41</td>
</tr>
<tr>
<td>IL-4 (pg/mL)</td>
<td>1.25 ± 0.63</td>
<td>30.85 ± 2.27</td>
<td>0.40 ± 0.22</td>
<td>0.17 ± 0.05</td>
<td>0.30 ± 0.10</td>
<td>0.61 ± 0.31</td>
</tr>
<tr>
<td>INF-γ/IL-4</td>
<td>1.93 ± 0.22</td>
<td>0.002 ± 0.0002</td>
<td>37.54 ± 8.88</td>
<td>73.44 ± 4.85</td>
<td>12.22 ± 2.22</td>
<td>6.39 ± 1.10</td>
</tr>
<tr>
<td>Total NO (mM)</td>
<td>2.88 ± 0.18</td>
<td>4.67 ± 0.34</td>
<td>2.46 ± 0.38</td>
<td>1.63 ± 0.27</td>
<td>2.89 ± 0.33</td>
<td>3.22 ± 0.31</td>
</tr>
<tr>
<td>Nitrite (mM)</td>
<td>1.66 ± 0.08</td>
<td>2.43 ± 0.19</td>
<td>1.25 ± 0.23</td>
<td>1.14 ± 0.09</td>
<td>1.72 ± 0.14</td>
<td>1.82 ± 0.14</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM. Three concentrations of safranal were 4 (S + SA1), 8 (S + SA2) and 16 μg/mL (S + SA3) and that of dexamethasone was 50 μg/mL.

* Statistical significance for the difference between the data of S + SA1 vs. S + SA2 and SA3: p < 0.05.
** Statistical significance for the difference between the data of S + SA1 vs. S + SA2 and SA3: p < 0.01.
*** Statistical significance for the difference between the data of S + SA1 vs. S + SA2 and SA3: p < 0.001.
**** Statistical significance for the difference between the data of S + D vs. S + SA1, S + SA2 and S + SA3: p < 0.005.
with safranal may indicate its stimulatory effect on Th1 and a suppressive effect on Th2 cells. The results also showed increased Th1/Th2 balance in treated animals with safranal. In fact our previous study showed increased Th1/Th2 balance effect for the extract of the C. sativus on human lymphocytes which support the results of the present study [11]. In fact, dysregulation of the T-helper 1 (Th1)/Th2 cytokine balance toward increased Th2 is well known phenomenon in the pathogenesis of asthma and atopic diseases [45] which support the preventive effect of safranal on Th1/Th2 imbalance in asthma seen in the present study. Our previous studies also indicated the same findings in OVA sensitized animals with similar methodology ant similar preventive effect for *Nigella sativa* [26], thymoquinone [8] and carvacrol [23] which may support the findings of the pretest study.

The results of the present study showed the preventive effect of safranal on tracheal responsiveness, serum levels of total NO and nitrite and increased Th1/Th2 balance in sensitized guinea pigs.

The results of our previous studies showed relaxant effect of saffron or its constituent, safranal on smooth muscles (bronchodilatory effect) and its other possible effect [5,7,31]. The results of the present study also showed the preventive effect of safranal on tracheal responsiveness, Th1/Th2 balance, NO, and nitrite. Our other study also showed the effect of safranal on human lymphocyte’s cytokines and Th1/Th2 balance [11]. The prophylactic effect of the extract of C. sativus and its constituent, safranal on lung pathology, total and differential WBC counts of lung lavage in sensitized animals were also demonstrated [6]. With regard to the results of these studies, it could be suggested that safranal may have a therapeutic effect on asthma by causing both bronchodilatation and anti-inflammatory effect on the lung. Therefore, as indicated in ancient Iranian medical books, safranal, the main constituent of *C. sativus*, could have therapeutic effects on respiratory diseases including asthma. However, further studies needed to be done to evaluate the effect of safranal on asthmatic patients.

The results of the present study showed greater effect of lower concentration of safranal on tracheal responsiveness, serum levels of total NO, nitrite, and cytokine profile than higher concentrations. These results may indicate that maximum effect of safranal was occurred in its lowest concentration used in this study. However, the effect of some lower concentrations of safranal should be examined in the future studies.

While the major quantities constituent of the plant is crocin and with regard to its anti-inflammatory and anti-oxidant property [23], the effect of this constituent of *C. sativus* should also be examined on lung inflammation of sensitized animals.

The results showed comparable effect of safranal and dexamethasone on tracheal responsiveness, serum levels of total NO, nitrite, and cytokine level of sensitized animals. In fact, the effect of low concentration of safranal was higher than the effect of dexamethasone. These results also confirmed anti-inflammatory effect of safranal in sensitized guinea pigs which was comparable to or even greater than the effect of dexamethasone.

Concentrations of safranal used in the present study were 4, 8, and 16 μg/mL. In previous studies, the same concentrations of safranal showed similar effect on total and differential WBC counts as well as pathological changes and serum histamine level in sensitized guinea pigs [3,6]. Therefore, the concentrations of safranal used in the present as well as our previous studies were 0.4% of the extract. Although the concentration used for safranal was higher than the HPLC results indicating that the extract of saffron contains only 0.026% safranal [15], these results may show that the effect of the extract of safron is not merely due to its constituent safranal.

The right-ward shift in concentration–response curves to methacholine obtained in the presence of safranal may indicate a possible stimulatory effect for safranal on β2 adrenoceptors and/or an inhibitory effect on histamine H1 receptors which both effects were shown for safranal and saffron extract in our previous studies [7,31]. However, in the present study safranal was administered with drinking water during sensitization and then methacholine concentration curves were obtained in isolated tracheal chains. Therefore, the more probable reason for the shift in methacholine concentration–response curves is a long term anti-inflammatory effect.

In conclusion, the results of the present study indicated a preventive effect of safranal on tracheal responsiveness, serum levels of total NO and nitrite and IL-4 but an enhancing effect on IFN–γ levels and Th1/Th2 balance. Therefore, safranal may have preventive therapeutic values in asthma treatment by reducing airway responsiveness immunoregulatory effect.

**Conflict of interest**

No conflict of interest.

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