

## Specific IgG Antibodies (Total and Subclasses) against Saffron Pollen: A Study of Their Correlation with Specific IgE and Immediate Skin Reactions

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### ABSTRACT

Saffron (Zaafaran), botanical name *Crocus sativus*, is the most expensive spice in the world. It is derived from the dried stigma and pistil of the purple saffron crocus flowers. Iran is the largest saffron producer accounting for more than 80% of the world's production. Saffron contains an aeroallergen that causes reactive respiratory allergic reactions in atopic subjects. IgG antibody to allergens in the serum of allergic patients is not routinely measured. In this study in order to find out more about mechanism of allergy against saffron pollen, specific antibodies (IgE and IgG, total and subclasses) in atopic subjects were assayed.

We used an ELISA assay for measuring specific IgE and IgG against saffron pollen extract in the sera of 38 atopic subjects (test group) and 20 non allergic subjects (control group). The optical densities were compared between allergic subjects and non-allergic individuals. The prick test with saffron pollen extract was used to evaluate the cutaneous and specific antibody responses in the allergic subjects. The correlation was determined by statistical analysis.

Specific saffron pollen IgE and IgG subclasses were found significantly higher in the allergic subjects than the control group. The immediate skin reaction was found positive in 70% of the test group. We report here, the existence of a positive correlation between specific IgE and skin reaction by prick test in atopic subjects ( $R=0.433$ ). A negative correlation between specific IgE and IgG4 subclass was also found ( $R=-0.576$ ).

These data may be useful to understand the mechanism of allergy to saffron and may help in clarifying clinical manifestations and to prevent IgE production as well as therapeutic application.

**Key words:** Allergy; Saffron pollen; Specific IgE; Specific IgG; Specific IgG subclasses; Skin prick test

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## INTRODUCTION

Exposure to plant pollens, aeroallergens, is known to cause respiratory symptoms and allergic rhinitis in people who are exposed to them.<sup>1,2</sup> This allergy can be defined as occupational allergy which is one of the most frequent causes of asthma.<sup>3</sup>

*Crocus sativus* (Saffron plant), whose stigma and style of flowers are an expensive food additive and therapeutic substance, has long been cultivated in the Middle East, Eastern Mediterranean, the Balkans, India, Spain, France, Italy and Iran (Figure 1). Khorasan province in Iran is one of the most important cultivation areas of saffron in the world.

Symptoms in the nose (sneezing, blockage, and running), eyes (itching, redness, tears, and swelling), mouth and throat (itching and dryness), and chest (breathlessness, cough, wheezing, and tightness) are very common during Saffron flowering in atopic people who are exposed to saffron flowers.

Sensitization to the saffron flower and its clinical significance as an occupational allergy were studied, previously.<sup>4,5</sup> The outbreak of Influenza-like- epidemic during saffron flowering in saffron farm areas in Iran has been reported, which confirms the involvement of saffron pollen as an aeroallergen. Regarding the positive skin prick tests with saffron extract in atopic subjects, this reaction was considered immunologic and IgE-mediated.<sup>5</sup> The presence of the serum saffron-specific-IgE was confirmed in previous studies with RAST.<sup>5</sup> Although elevated levels of IgE antibodies against pollens have been found in the sera of sensitized people, pollen-specific IgG was not determined.<sup>5</sup> In contrast to IgE antibody, IgG antibody is not usually measured in the serum of pollinosis patients, because its detection has little diagnostic value and there are no simple methods for assaying small amounts of specific IgG in the serum.<sup>6</sup> Furthermore, it has not been assayed in the serum of healthy individuals (non-patients). Pollen specific IgG has been found in human serum but its roles in the development of allergic reactions is still unclear.<sup>7</sup>

In this study, Specific IgG antibodies (total and subclasses) as well as specific IgE against Saffron pollen were evaluated by ELISA. The correlation between various data was determined in order to understand more about the mechanism of allergy to aeroallergens.



Figure 1. *Crocus sativus* (Saffron plant)

## MATERIALS AND METHODS

### Participants

Thirty-eight subjects (age  $38 \pm 8.5$  years) who were living in a saffron crop area (south of Khorasan province) with clinical manifestation of saffron pollen allergy in the nose (sneezing, blockage, and running), eyes (itching, redness, tears, and swelling), mouth and throat (itching and dryness), and chest (breathlessness, cough, wheezing, and tightness) were included in this study. Twenty subjects (age  $36 \pm 6.5$  years) were selected randomly as a negative control group without any signs and history of allergy, who were living in Mashhad. All subjects gave written informed consent.

**Preparation of Pollen Extract**

Saffron pollen extract was used for the ELISA. We used a modified method of Kwaasi *et al.*<sup>8</sup> Pollens were collected from pistils of saffron. The samples were defatted by adding cold acetone (1/10 w/v) and shaking for 16 h. After removing the acetone by suction, phosphate-buffered saline (PBS, pH=7.4, 0.01 M) containing 20 mM EDTA was added to the samples and shaken for 18h. The supernatant of this mixture was obtained by centrifuging at 5600 g for 30 min and dialyzed in PBS for 48 hours. All of extraction steps were performed at 4° C. This extract was used for ELISA test. For using in skin prick tests, it was sterilized by passing through 0.2-µm Whatman filter and then sterilized glycerin (50% v/v) was added.<sup>9</sup> The protein concentration of the extract was measured by the Bradford assay.<sup>10</sup>

**Saffron Pollen Skin Test**

A 20 µl of saffron pollen extract (100 µg/ml) was placed on the forearm skin of the atopic participants, and then the skin was pricked with a sterile needle at the placement of the drop, allowing the allergens to penetrate into the skin.

The wheal and flare sizes were followed for 20 minutes. Histamine hydrochloride (10 mg/ml) and PBS (pH 7.2) containing glycerol (50% v/v) were used as positive and negative controls, respectively. A wheal of less than 3 mm was considered to be a negative reaction (grade=0) and the positive reaction was graded according to the Table 1.<sup>11</sup>

**Table 1. Grading scale for skin prick testing.**

Grade	Wheal diameter (mm)
0	<3
1	3-8
2	8-12
3	12-20
4	>20

**Table 2. Results of skin Reactions in allergic group.**

Grade of skin reaction	Number of subjective
0	10
1	9
2	9
3	6
4	4

**Assessment of Saffron Pollen Specific Antibodies by ELISA****Antigen coating on microtiter plates**

Microtiter plates (Nunc company, Covalink) were coated with the extract of saffron pollens in a volume of 100 µl/well at a protein concentration of 1 mg/ml in phosphate buffer (pH=8.2, 0.015 M) and incubated at 4°C overnight. After washing step, blocking was done using 2% BSA in PBS (pH=7.2) for 2 hours. The plates were kept at -20° C until use.

**ELISA for detection of saffron pollen-total IgG antibodies**

ELISA was performed using the saffron pollen extract coated plates. Serum samples were added in a volume of 100 µl/well at a dilution of 1:100 in 0.05% bovine serum in phosphate-buffered saline, and the plates were incubated at 37 °C for 2 hours. After incubation and washing, conjugated goat anti human IgG-HRP (Biogene-Iran) was added at a dilution of 1:400 and incubated for 1 hour at room temperature. The plates were washed and dried. The substrate solution, 50 µl of 0.3 mg/ml 3,3', 5,5' tetra methyl Benzedine (TMB) in Dimethyl Sulfoxide (DMSO) was added to 5 ml of sodium acetate buffer pH=5.5, 0.16 M and 10 µl of 37% H2O2, was used. The enzyme reaction was stopped by adding 50 µl of 2 N HCl after 30 min. The absorbency was measured at 450 nm using an ELISA reader.

**ELISA for detection of Saffron pollen specific- IgG subclasses antibodies**

Serum samples were added to the saffron pollen extract coated plates in a volume of 100 µl/well in PBS containing 0.05% BSA, and the plates were incubated at 37° C for 2 hours. After incubation and washing, mouse anti human IgG1, IgG2, IgG3 and IgG4 antibodies (Sigma, USA) were added in optimum dilution. After incubation at 37°C for 1 hour, and washing, peroxidase conjugated anti mouse IgG (Sigma, USA) at dilution of 1:10000 was added and incubated at 37°C for 1 hour. The next steps were done as described previously.<sup>12</sup>

**ELISA for detection of Saffron pollen- IgE specific antibodies**

ELISA was performed according to the protocol described previously.<sup>5</sup> Undiluted sera were added to the

Saffron pollen extract coated plates and after incubation and washing steps, conjugated goat anti human IgE-HRP (Sigma, USA) was added. The enzyme reaction was detected with TMB substrate solution and absorbencies were read at 450 nm.

### Statistical Analysis

Between-group comparisons were performed using the Mann-Whitney U test. Correlation coefficients were obtained using Spearman's rank method. Within group comparisons were performed using the Wilcoxon matched pairs signed-rank test. All tests were two tailed and values of  $p < 0.05$  were considered to be statistically significant. SPSS software, (Version 11.1.1; Statistical Product and Service Solutions, Inc., Chicago, III) was used for these analysis.

## RESULTS

### Skin Reactions

Immediate reactions to saffron pollens were evaluated in allergic subjects. Twenty eight of thirty eight, 70%, tested subjects had immediate positive skin reactions to saffron. The immediate reaction was considered as a wheal with a surrounding erythematic or flare appearing within 20 minutes. Based on the table of grading (Table 1), 9 subjects showed immediate reaction with grade 1, 9 subjects showed grade 2, 6 subjects showed grade 3 and 4 subjects showed grade 4 (Table 2).

### Specific Saffron Pollen IgE Levels

The optical density of saffron specific total IgE was significantly higher in the test group than the control group, ( $p < 0.05$ ) (Figure 2a).

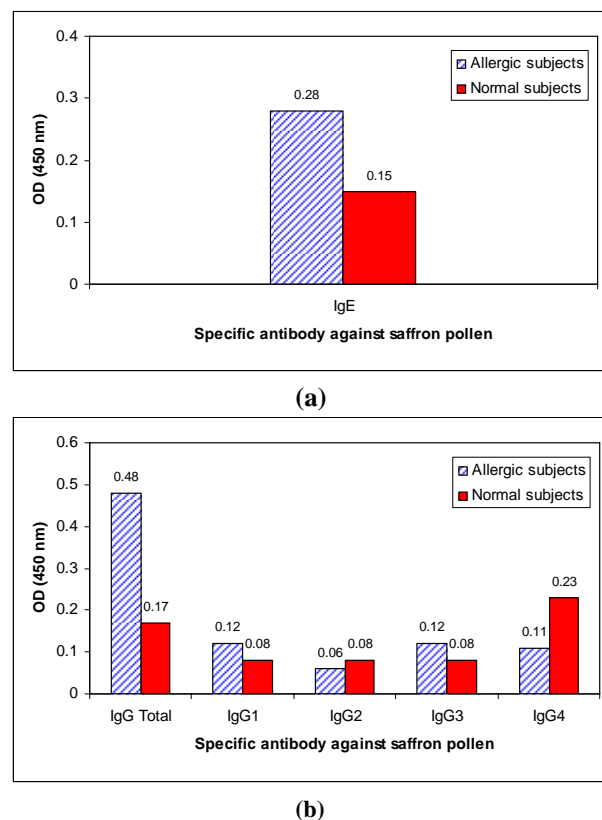
### Specific Saffron Pollen IgG Levels

The optical density of saffron specific total IgG was significantly higher in the test group than the control group, ( $p < 0.05$ ) (Figure 2b). However, there was no difference in the total serum IgG subclass levels between two groups.

### Correlation Study between Specific Saffron Pollen Antibodies

A significant reverse correlation between specific IgE and IgG4 subclass antibodies was also found ( $R = -0.576$ ,  $p < 0.01$ ). The specific IgE antibody levels were also correlated with specific IgG1 and IgG2 subclasses and total IgG antibodies (Figure 3). Analysis

of data showed a positive correlation between skin reactions and specific IgE antibodies ( $R = 0.433$ ,  $p < 0.05$ ) (Figure 4).



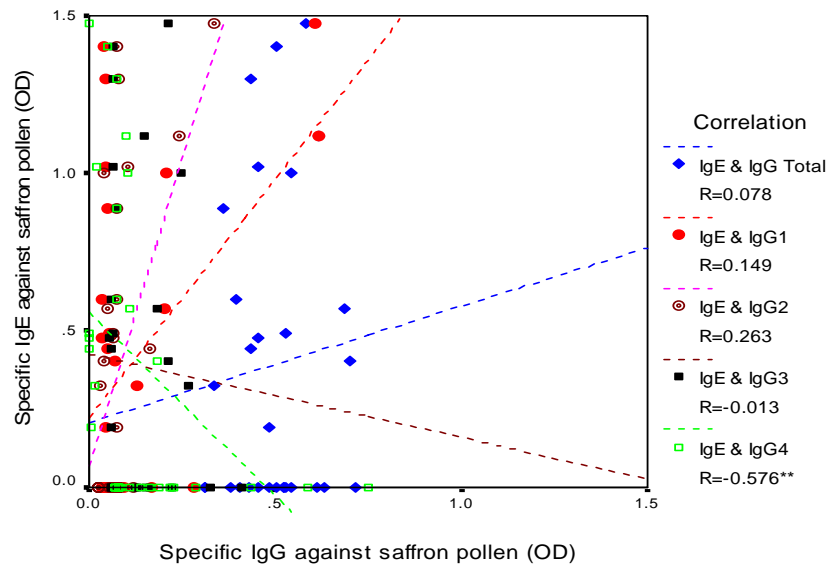
**Figure 2.** Comparison of optical densities of saffron pollen specific antibodies (allergic and normal groups), mean  $\pm$  SD of duplicate values, a: Saffron pollen specific IgE, b: Saffron pollen specific total IgG and its subclasses

## DISCUSSION

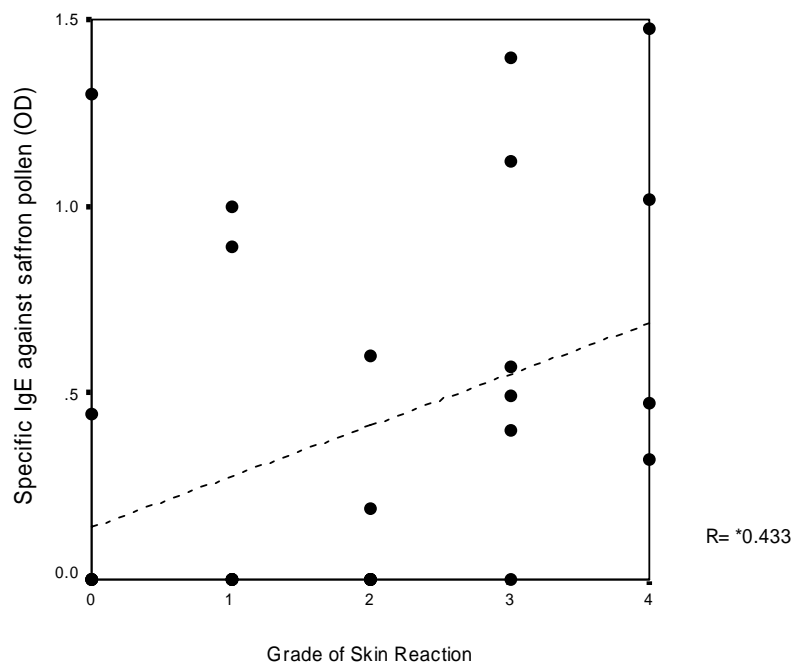
Saffron (*Crocus sativus*) belongs to the Iridaceae family and its dried pistils and stigma is an expensive food additive. Iran is the largest saffron producer accounting for more than 80% of the world's production with production about 150 tones, annually. Khorasan province is the most important cultivation region for cultivation of saffron in Iran.

Farid *et al* reported initially the involvement of the saffron plant components as an allergen in people who were living in saffron cultivation areas in Iran and suffered from influenza-like symptoms in saffron flower picking season. The allergenicity of saffron pollen was confirmed by demonstration of positive cutaneous tests with an extract of saffron pollen.<sup>13</sup>

## Specific IgG and IgE Antibodies against Saffron



**Figure 3.** The correlation of saffron specific IgE with saffron specific total IgG and IgG subclasses



**Figure 4.** The correlation of skin reaction with saffron pollen specific IgE in allergic group

Wuthrich *et al* reported anaphylactic reaction in a saffron worker.<sup>14</sup> Feo *et al* studied sensitization to the saffron flower and evaluated the cutaneous and specific antibody reactions in saffron workers. In that study the involvement of saffron components was demonstrated by RAST and skin reaction and allergenic properties of saffron protein were analyzed by immunoblotting.<sup>4</sup>

In the present study, we have investigated the presence of specific IgG and IgE antibodies in a group who were living in saffron cultivation area, exposed to saffron pollen, and manifested allergic symptoms. The test group was compared to the control group who was not exposed to saffron pollen and had no allergic manifestation.

Skin reactions to saffron pollen consisted of an immediate wheal and flare which was compatible with IgE-mediated hypersensitivity. A direct correlation between immediate skin reactions and serum saffron pollen-specific IgE levels was detected. The antibody levels against saffron pollen were higher in the test group compared to the other group. Based on statistical analysis, the difference of specific IgE and also total IgG and IgG4 subclass antibodies between two groups were significant ( $p < 0.05$ ). Specific IgE were also showed weak correlation with specific total IgG, IgG1, and IgG2 subclasses antibodies. Another interesting finding was the significant reverse correlation between levels of specific IgE and IgG4 subclass antibodies ( $R = -0.576$ ).

In contrast to normal subjects, allergic patients develop specific IgE directed against sensitizing allergens that play a key role in the pathophysiology of allergic diseases.<sup>15</sup> In parallel to the IgE response, allergic patients usually produce high levels of total and allergen-specific IgG4 antibody.<sup>16,17</sup> However, a potential role for IgG4 antibody in allergic diseases has not yet been clearly established. In allergic patients, the finding of serum IgG4 antibody directed against allergens to which the patients were not sensitive suggests that they are not sensitizing antibody.<sup>15</sup> On the contrary, the existence of a positive correlation between a successful hymenoptera venom desensitization and the high levels of specific IgG4 antibody generated suggests that IgG4 may be protective.<sup>17,18</sup> Therefore, it may be of potential therapeutic interest to be able to modulate selectively the production of IgE versus IgG4. While IgE and IgG4 synthesis *in vitro* require identical signals, *vivo* observations suggested that these isotypes may be produced independently.<sup>19</sup> Moreover, in response to an immunization with keyhole limpet hemocyanin, atopics develop a specific IgG4 response but no IgE.<sup>16</sup> Both of these observations suggest that IgG4 antibody, in contrast to IgE, are not sensitizing antibody. Moreover, the development of a potent and specific IgG4 antibody response during hymenoptera venom desensitization has been associated with a positive outcome.<sup>20</sup> Consequently, in IgE-mediated diseases such as allergic disorders, it could be of clinical interest to prevent IgE production without affecting IgG4 response.<sup>21</sup> Although it has not been determined the exact reasons for the difference, one possibility may be that there are more B cell clones committed for IgG production than for IgE production.

Another reason may be that the immunologic memory of IgG4 antibody is longer than that of IgE antibody.

It has been suggested that IgE antibody has shorter memory than IgG4 antibody.<sup>7</sup> The role of IgG and its subclasses were studied in the immunopathology of allergic reactions, previously.<sup>22,23</sup> It was found that the elevated IgG antibody levels were not directly related to the pathogenesis of the disease.<sup>24</sup> Michils et al found a relationship between allergic status and the specificity of IgG antibody to inhaled allergens.<sup>25</sup> Pulmbo et al showed a difference in the IgG isotype distribution depending on their sensitivity and duration of allergic exposure.<sup>26</sup> Nordwall and Ruffin also found out that the specific IgG antibody levels vary with pollen season.<sup>27,28</sup> The presence of serum specific IgG may either be primarily involved in the pathogenesis of the allergy or may be produced as a result of the complex allergic reactions. Biological activities of the IgG subclass antibodies may explain the clinical and pathophysiological features for allergic disease.<sup>29</sup> But there is little information about what happens in airborne allergic diseases. Reverse correlation between IgE and IgG4 antibody levels in prolonged exposure, which can be explained by the results in an IgG4-restricted response.<sup>18</sup> It has been shown that the increase of specific IgG4 during hyposensitization reflects a possible influence of this subclass as the blocking antibodies on the induction of tolerance towards allergens.<sup>7,22,30</sup>

We conclude that specific IgG antibodies seem to play a critical role in allergic response. We suggest that other factors including age, sex, exposure time, clinical manifestation, persistence of other pollens in picking season of saffron flower, familial and immunotherapy histories should be considered in further studies.

Evaluation of saffron-specific IgG antibodies with ELISA is useful for large sero-epidemiologic studies on the prevalence of non-IgE antibodies in general population. Moreover, the assessment of specific IgG level that is expected to increase with successful immunotherapy in pollen allergy is useful for monitoring the efficacy of hyposensitization.<sup>7</sup>

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