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# Evaluation of *Chenopodium album* allergenicity in atopic asthmatics

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## ABSTRACT

Pollen grains are considered as the main causative agent of the respiratory allergic disorders. Pollen grains of the family Amaranthaceae are predisposing agents in atopic allergy in arid and semiarid areas. This study aimed at identifying the antigenic properties of *C. album* pollen extracts in atopic asthmatic patients. Pollen grains of *C. album* were extracted and the antigenicity of the extract was primarily assessed by skin prick test (SPT). The protein content of the extract was quantified by modified Lowry assay and separated by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Specific immunoglobulin E (IgE) interaction with pollen extracts was determined by enzyme-linked immunosorbent assay (ELISA) and confirmed with immunoblot. In this study, 70 out of 300 atopic asthmatic patients (23.3%) were skin prick test-positive toward *C. album* pollen extracts. Specific IgE against *C. album* pollen grains was recorded among 78.5% of the positive skin patients with high levels in 15.7% of them as determined by ELISA. Four distinct protein bands with molecular weights 50, 57, 63 and 67 kDa were detected by SDS-PAGE. The antigenicity of the protein fractions was confirmed by the interaction of patients' sera on the immunoblot with IgE antibodies. Allergenicity to *C. album* pollen extracts was identified and confirmed among atopic patients. The results discussed here could attribute in the development of diagnosis processes and therapeutic strategies for allergic individuals.

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**KEYWORDS:** allergy, pollinosis, *Chenopodium*, pollen grains, immunoblot

## INTRODUCTION

Allergic disorders are the most common chronic diseases worldwide. Respiratory allergic reaction mainly involves bronchial asthma, sneezing, rhinitis, and coughing accompanied by inflammation of nose, throat and eyes. Atopic asthma begins before the age of 6, associated with a genetic predisposition for sensitization to allergens, and an increased severity of bronchial hyper responsiveness (atopy), which persists into adulthood [1]. Asthma can influence personal activities, work dynamics and even may cause mortality. In addition, it represents one of the main causes for school day-missing among children [2]. The contributing allergens include various types of fungal spores, pollen grains, mites and animal debris [3].

Pollen grains are major causative agents for respiratory allergic disorders and also a source of morbidity among atopic subjects [4]. Pollen grains of the family Amaranthaceae have been recognized as the main cause of pollinosis in the European countries, western areas of the United States and Asia [5]. It also causes allergic sensitization in Saudi Arabia, and Kuwait [6].

*C. album* belongs to the Amaranthaceae family and can grow in open habitats [7]. *C. album* inhabits gardens, lawns, roadsides, and waste areas. *C. album* can survive in diverse environmental conditions and can grow in salt soil or shade.

*C. album* pollen grains have been reported to be a predisposing cause of allergic disorders in the desert

and semi-desert regions [8]. *C. album* is distributed all over Egypt especially at roadsides, cultivated lands, Nile valley, Nile river banks and canals [9].

*C. album* pollen grains could exhibit a key feature in the sensitization of atopic asthmatics. This study was carried out to identify the antigenic properties of *C. album* pollen grains in allergic patients. Therefore, we prepared *C. album* pollen extract and evaluated its antigenic characters. Molecular characterization and immunological assay of protein allergens were also performed. Accurate identification of emerging allergenic sources might assist in the characterization of the predisposed patients and prescribing the appropriate treatment.

## MATERIALS AND METHODS

### Pollen collection

*C. album* pollen grains were collected from around Mansoura city, Egypt during the pollen season (October to April). Dried flowers were crushed gently to release pollen grains which were purified by passage through a mesh with 100, 200 and 300 $cm^2$ diameter pores. Morphological characterization of the fresh pollen grains was carried out under the scanning electron microscope (JSM-6510 LV). Dry pollen grains were metalized with gold and the structure of the exine was observed as well as any particles deposited on the surface.

### Protein extraction

Pollen grains were ground in a mortar and then were defatted with diethyl ether using 3-4 times the volume of pollen grains. The ether was repetitively changed with a fresh one till it became colorless. Delipidated pollen extracts were dried in desiccators containing calcium chloride for 24-48 h [10]. Protein extraction was carried out by suspending dry powder in 0.01 M phosphate buffer saline (PBS) pH 7 at ratio 1:10 (w/v) for 16 h at 4 °C with agitation. The pollen extracts were separated by centrifugation at 8.000 × g at 4 °C for 30 min. The clear supernatant was separated, extensively dialyzed against distilled water and passed through 0.22 μm Millipore filter (Seitz-filter, Germany) [11]. The total protein content of the prepared pollen extract was quantified by modified Lowry assay, (1951) [12] using bovine serum albumin (BSA) as a standard.

### Clinical studies and skin prick test

### Inclusion criteria

This study was approved by Medical Research Ethical Committee, Faculty of Medicine, Mansoura University, Egypt (No. 16.03.124), in accordance with World Association (Declaration of Helsinki). The consent of every patient was taken prior to skin testing and blood collection.

The present study was performed on 300 bronchial asthma patients who met the criteria of the global initiative for asthma (GINA) 2011 [13] and were attending the outpatient clinic of Allergy Unit, Chest Medicine Department, Mansoura University Hospital during the period October 2013 to April 2014. The criteria for their diagnosis were based on their clinical symptoms of asthma combined with positive reversibility of air flow limitation at spirometry and positive skin prick test for allergy. The level of asthma control was defined in the tested patients as controlled, partly controlled and uncontrolled asthma according to GINA 2011 [13].

### Exclusion criteria

Patients above 50 years, below 5 years, smokers, diabetics, auto immune disorders and pregnant women were excluded.

### Skin prick test (SPT)

Skin prick test using the extract of *C. album* pollen grains (1:10 w/v) and extracts of sixteen common allergens in Egypt was performed on all patients. SPT battery included pollen of *Phalaris paradoxa* (Bristle spiked canary grass), *Arundodonax* (Giant reed) and *Cynondactylon* (Bermuda grass). The battery also included mixed mites (*Dermatophagoide spteronyssinus* and *Dermatophagoides farina*), mold extracts of *Alternaria alternata* and *Aspergillus* species, animal epithelia of cats and dogs and some food allergens. Skin reaction was graded as compared to negative control (glycerinated PBS) and positive control (histamine hydrochloride 1 mg/ml) according to the criteria outlined by Singh *et al.*, (1992) [14].

### Serum sampling

Blood samples were collected from patients with positive skin test sensitized to *C. album* extracts. In addition, blood samples collected from sixteen healthy persons were included as negative control.

The collected samples were centrifuged and the sera were distributed as small aliquots and stored at -80 ºC.

**Enzyme-linked immunosorbent assay (ELISA)**

Levels of specific IgE to *C. album* antigens were measured in patients' sera by indirect ELISA [15]. In brief, polystyrene microtiter plates (Nunc, Maxisorp, Denmark) were coated with 100 µl of pollen extracts (20 µg/ml protein) in 100 mM carbonate buffer (15 mM $Na_2CO_3$ and 35 mM $NaHCO_3$, pH 9.6) and the plates were incubated overnight at 4 °C. The plates were washed with TBS-T (Tris-buffered saline containing 0.05% Tween 20, pH 7.6) and blocked with 1% BSA for 2 h at room temperature. The coated wells were washed with TBS-T and incubated with the diluted patient's sera (1:10) (in duplicate) overnight at 4 °C. Wells receiving buffer only were used as negative controls. Subsequent to five washes with T-TBS, 100 µl of diluted (1:1000) monoclonal antihuman IgE-alkaline phosphatase (Sigma-Aldrich, USA) were added to each well. The plates were incubated for further 2 h at room temperature. For color development, 100 µl of 4-nitrophenol phosphate (1 mg/ml) (Sigma-Aldrich, USA) was added to each well and the plate was incubated at room temperature in the dark for 30 min. Optical density ($OD_{405}$ nm) two times greater than the median values of the negative controls was assigned as a positive interaction. OD values of patients' sera were evaluated as estimated by Kauffman *et al*. (1983) [16] with slight modifications.

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

The protein profile of *C. album* pollen extract was performed by SDS-PAGE with separating gel 12.5% on Minigel electrophoresis apparatus (Bio-Rad, USA) [17]. Sample and the Protein Ladder Spectra™ Multicolor Broad Range (10-260 kDa) (Thermo Fisher Scientific, USA) were run together at 90 V. Finally, the gel was stained using Coomassie Brilliant Blue G-250.

**Immunoblot (Western blot)**

The protein bands separated by SDS-PAGE were blotted to nitrocellulose membrane with a SE 245 blotter (Hoefer, USA) using Tris-glycine buffer (25 mM Tris, 192 mM glycine, 20% methanol pH 8.3) at 250 mA for 1.5 h [18]. The unbound sites of the nitrocellulose membranes were blocked by 5% Amersham ECL blocking agent in TBS-T for 1 h. The membrane was washed three times in TBS-T buffer for 10 min each. The blot was incubated for 16 h at 4 °C with pooled sera of ELISA-positive patients. On the other hand, sera collected from healthy individuals were pooled and used as negative control. The membrane was washed three times and incubated for 1 h in diluted (1:1000) anti-human IgE horseradish peroxidase (Calbiotech, NL). The color was developed with 3, 3', 5, 5'-tetramethylbenzidine (Sigma, USA) substrate.

**Statistical analysis**

Statistical analysis was performed using MedCalc Version 16.2.1 software. Data wee expressed as the median ± SD. The t-test was used to assess the level of significance between groups with $p < 0.05$ considered significant.

## RESULTS

**Morphological characterization of *C. album* native to Egypt**

*C. album* plant grown in Egypt was characterized by small, greenish and inconspicuous flowers. Moreover, the seeds were shiny, green and disk shaped. The mature plant was 30-70 cm in height with a smooth and grooved stem. Leaves had alternate arrangement, lance-shaped and had dull green color with red stem (Figure 1A). Pollen grains were spheroidal, isopolar, and pantopolyporate, with a diameter of around 18-18.5 µm (Figure 1B). Pores were circular and the diameters (D) of the pores were about 1.5-1.7 µm. Pore ornamentations were granular and distinctly sunken. The distance between the centers of the adjacent pores (C) was 4.2 µm. Hence, the C/D ratio was 0.227. The total number of pores/pollen grain was about 80 pores.

**Skin prick test**

Out of the 300 asthmatic patients, 180 (60%) were females and 120 (40%) were males. The positive SPT rates among tested subjects in the rural and urban areas were 71% (213 patients) and 29% (87 patients), respectively. All patients gave various grades of positive SPT to one or more of the tested allergens. Prevalence of positive skin test to any allergen is shown in table 1.

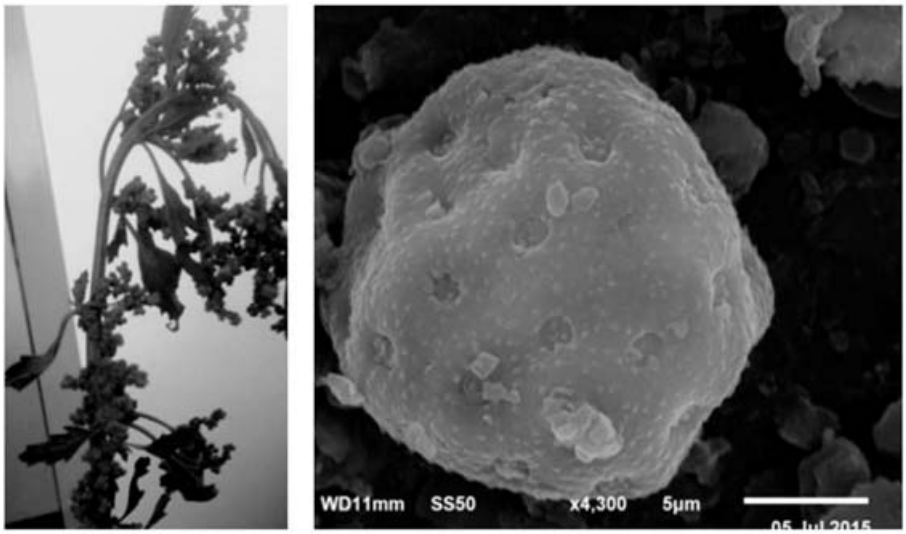


**Figure 1. A) Egyptian *Chenopodium album*:** Flowers are small, greenish and inconspicuous. Seeds are shiny, green and disk shaped. A mature plant is 30-70 cm high with tall, smooth, grooved stems. Leaves are alternate, lance-shaped, dull green, with red stem. **B) SEM micrograph of *C. album* pollen (X 4300):** Pollen grains are spherical and isopolar with a diameter of around 18-18.5 µm. Pores were circular and distinctly sunken granular around 1.5-1.7 µm in diameter. Numerous foreign particles attached to the surface of the grains were observed.

**Table 1.** Prevalence of positive skin prick test to different allergens. Number of patients is not cumulative.

| Skin prick test battery | Patients (%) |
|---|---|
| - Mixed Mites (*Dermatophagoide spteronyssinus* and *Dermatophagoides farina*) | 86 (28.5%) |
| - *Chenopodium album* pollen | 70 (23.3%) |
| - Bermuda grass pollen (*Cynondactylon*) | 49 (16.5%) |
| - Giant reed grass pollen (*Arundodonax*) | 69 (23%) |
| - Bristle spiked canary grass pollen (*Phalaris paradoxa*) | 63 (21.3%) |
| - *Aspergillus* species | 44 (14.8%) |
| - *Alternaria alternate* | 29 (9.8%) |
| - Hay dust | 23 (7.8%) |
| - Chicken feather | 9 (3.2%) |
| - Fish | 13 (4.5%) |
| - Milk | 11 (3.7%) |
| - Strawberry | 19 (6.4%) |
| - Lentils | 9 (3%) |
| - Pigeon Feather | 14 (4.6%) |
| - Cat hair | 8 (2.8%) |
| - Dog hair | 13 (4.2%) |
| - Rabbit hair | 14 (4.8%) |

Out of total 300 asthmatic patients, 70 patients were found positive toward the pollen extract of *C. album* (Figure 2). All of the cases sensitive to *C. album* were partly controlled asthma according to GINA guidelines 2011 [13]. Demographic characteristics of patients with positive skin test for *C. album* are presented in table 2. Eighteen percent of those patients were mono-sensitized to *C. album*.

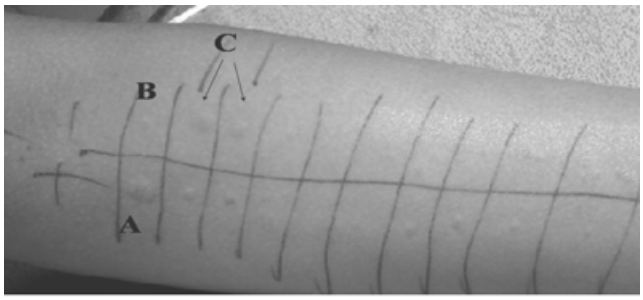


**Figure 2. Skin prick test (SPT).** Many allergens were used and reactions were assessed by the size of the interacting wheal and the degree of the surrounding erythema compared to the positive and negative control. **A)** Positive control solution (1 mg/ml histamine in glycerinated phosphate buffered saline, PBS). **B)** Negative control (the glycerinated PBS). **C)** The allergenic reaction to extract of *Chenopodium* pollen (1:10 w/v in PBS-glycerol) and the standard battery of antigen extracts used for skin prick tests.

**Table 2.** Characteristics of the *C. album*-positive patients with regard to age, sex, residence, pulmonary function and skin test.

| **Characteristics** | **No. of cases (%)** | **P value** |
|---|---|---|
| **All patients** | 70 (100%) | |
| **Gender** | | |
| Female | 45 (64.3%) | $p < 0.0001$ |
| Male | 25 (35.7%) | |
| **Residence distribution** | | |
| Rural area | Female: 37 (67.3%)<br>Male: 18 (32.7%)<br>Total: 55 (78.6%) | $p < 0.0001$ |
| Urban area | Female: 8 (53.3%)<br>Male: 7 (46.7%)<br>Total: 15 (21.4%) | |
| **Age (years): 25.5 ± 12.17*** | | |
| Group 1 (6-12 years) | Female: 8<br>Male: 7<br>Total: (9.0) ± 1.6* | $^{**}p_1 < 0.0001$<br>$^{***}p_2 < 0.0001$ |
| Group 2 (13-30 years) | Female: 24<br>Male: 9<br>Total: (21.0) ± 5.9* | |
| Group 3 (30-50 years) | Female: 14<br>Male: 8<br>Total: (38.5) ± 4.6* | |

*Values are expressed as median ± SD.
***$p_1$: between group 1 and 2.*
****$p_2$: between group 2 and 3.*

**ELISA assay**

Patients who showed positive for skin test were included in ELISA assay. Out of the tested 70 sera, IgE binding less than 15% is considered negative (15 patients), 11 patients had a significantly high absorbance range (>66%) of specific IgE antibodies against *C. album* pollen antigen. About 20 patients showed a moderate reaction (33%-66%) and 24 sera revealed mild reaction (<33%) against *C. album* pollen grains (Figure 3).

**Separation of proteins with SDS-PAGE**

The total protein content of the pollen extract as estimated by modified Lowry method was 1.21 mg/ml in 1:10 (w/v) of the crude extract. The proteins of *C. album* pollen extract were separated by SDS-PAGE. Four protein fractions were detected on the gel (Figure 4A) with molecular weights of 50, 57, 63 and 67 kDa. Among them, protein bands with molecular weights 50 and 67 kDa were more prominent than the middle two bands. Some stained protein bands with high and low molecular weights were detected on the gel and considered as non-prominent weakly diffused bands.

**Immunoblotting**

Western immunoblotting revealed IgE binding bands at a similar molecular weight to that produced in SDS-PAGE (50-70 kDa). Figure 4B shows the blot of *C. album* pollen extract after incubation with pooled sera of allergic patients who gave the highest ELISA absorbance range (11 patients). The allergenic profile of pollen extracts was mainly composed of two distinct reactive bands at 50 and 67 kDa. The two middle bands shown in SDS-PAGE appeared to be fused together on blotting of the membrane as they have very near sizes. Incubating the pollen extract with sera of the patients showed negative. ELISA was considered as the negative control for the immunoblotting (Figure 4C).

**DISCUSSION**

Atopic patients suffer from an increased tendency to mount IgE antibody responses against common environmental allergens [19]. The exposure to allergens such as pollen grains is one of the significant factors associated with the dominance of allergic diseases such as asthma and rhinitis [20].

According to the survey of Hasnain *et al*., 2016 [21], the most common outdoor allergens in the Middle East are pollen grains, especially *Amaranthus*, *Chenopodium* and *Salsola* which is in agreement with our study as indicated by the results of SPT (Table 1). The allergenic characteristics of pollen grains are due to some constitutes such as protein contents, organic materials and its sporoderm [22]. The pattern of sensitization to pollen grains varies with regional distinction. Various regions have a characteristic climate, humidity, geography, flowering seasons and blooming times [23]. Hence, the major allergenic agents should be identified in each area which will assist in the diagnosis of atopic patients and in prescribing appropriate treatment.

The present study was carried out to evaluate the role of pollen extract of *C. album* as an allergen in atopic patients. Morphological characterization and microscopic features of *C. album* native to Egypt were checked. The numbers of pores and the C/D ratio on periporate pollen were used for taxonomic characterization and analytical purpose in the genus *Chenopodium* [24]. These findings are harmonized with that for *C. album* [25].

In our study, several antigens such as common pollen grains, fungal spores, mixed mites, animal epithelia and some food allergens were tested along with *C. album* extracts for allergenicity. We found 70 out of 300 atopic asthmatic patients (23.3%) were skin prick test-positive toward *C. album* pollen extracts. This prevalence is lower than the rate in Kuwait which was 57.6% [26] and higher than that found in Turkey [27]. This could be attributed to the difference in weather between Egypt and other countries and this also denoted that *C. album* is more prevalent in the desert and in the hot climate areas than areas with low temperature.

In the conducted study, it was noted that asthmatics sensitized to *C. album* from rural areas (78.6%) were significantly higher than those from urban areas (21.4%), $p < 0.0001$) which is in agreement with Yazicioglu *et al*., 2004 [27] (5.7% in rural areas and 4.4% in urban areas; although not significant but meets with our results). This may be explained by the high pollen concentration of *C. album* in the rural atmosphere where the plant mainly inhabits.

With regard to gender, many studies concluded that asthma prevalence is higher in boys than girls

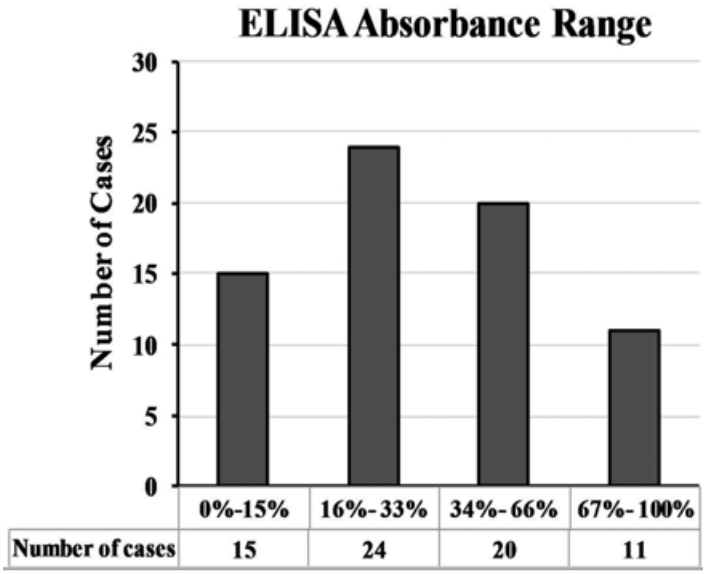


**Figure 3. ELISA results.** The absorbance range was plotted against the number of patients. IgE binding less than 15% was considered negative.

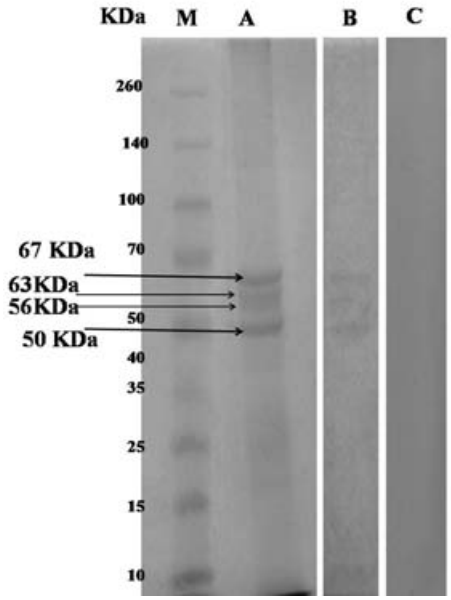


**Figure 4. SDS-PAGE and immunoblotting profile of *C. album*. A) SDS-PAGE:** Showing multiprotein fractions in molecular weight range between 50-70 kDa. Protein bands with a molecular weight of 50 and 67 kDa were more prominent than the middle two 57 and 63 kDa. **B) Immunoblotting:** Specific binding of *C. album* antigens with IgE probed in pooled sera from atopic patients. **C) Negative control:** Blotting of *C. album* extract against healthy individuals. The molecular weight marker (M) ranged 10-260 kDa.

in childhood and then reverses in adolescence, with a higher prevalence of asthma in adult women than men [28] which agree with our findings. In the studied asthma cases, females were more predominant (64.3% of cases, $p < 0.0001$) and the median age was 25.5 years. This could be explained according to Almqvist *et al*., 2008 [29] who concluded that sex hormones are likely to play an important role in the development and outcome of the allergic immune response, and asthma in particular.

Skin prick test is taken as the primary diagnostic tool to substantiate the allergy diagnosis [30]. To confirm IgE-mediated hypersensitivity, ELISA was performed to estimate levels of specific IgE in sera against *C. album* pollen extract. Results of ELISA test proved allergenicity of these pollens among 78.5% of the reactive skin patients (Figure 3). This could be explained by the presence of proteins in the pollen extract which are considered as allergenic substrates. This is in agreement with the previous studies carried out on other members of the family Amaranthaceae, such as *Salsola kali* and *C. botrys* [31] and on *C. album* [7] where proteins are the accused substances. These proteins induce allergy in atopic cases as revealed by the presence of blood eosinophilia and the increase in neutrophilia count [32]. However, low IgE was detected among 21.5% patient with positive SPT (Figure 3). This may be attributed to low or mixed exposure to

*C. album* pollen grains and other grains with cross-reactivity to *Chenopodium* sp. Hence those patients exhibited positive SPT and low levels of specific IgE to *C. album* extracts. Also, in our study all skin test positive cases toward *C. album* were partly controlled asthma, which could be due to exposure of patients to pollen in the season of flowering during the period of the study.

The total soluble proteins in the pollen extract of *C. album* were estimated as 1.21 mg/ml in 1:10 (w/v). Similar protein content of 1.32 mg/ml of *Mallotus phillipensis* pollen grains could induce allergy in atopic patients [10].

The stained polyacrylamide gel showed four distinct protein bands from *C. album* pollen extract. These proteins are probably responsible for the allergic sensitization to *C. album* extracts as indicated by immunoblot (Figure 4). Other studies showed that the allergenic molecules of *C. album* pollen extract have three bands (10-18 kDa) with a different binding capacity to human IgE. They are called Che a 1, Che a 2 and Che a 3 [33] where Che a 1 and Che a 2 are the major allergens. Variation in protein allergens of *C. album* pollen grains has been approved in various publications. Wurtzen *et al*., 1995 [34] reported that *C. album* has three important allergens with molecular masses 14, 35 and 42. The antigenic profile of *Chenopodium* pollen extracts at Northern Portugal indicates reactive bands at 40, 53 and 70 kDa [31]. Moreover, in the study of Bianchimano *et al*., 2014 [11], the immunoblot shows a conserved pattern of bands for three urban zones in Argentina, demonstrating two allergens around 76 kDa in one studied zone, and several bands between 52 and 38 kDa in the other two zones. Bianchimano *et al*., 2014 [11] could not detect any band of molecular weight less than 35 kDa. Actually, in our study the band with molecular weight of 57 kDa could be analogous to that with a molecular weight around 53 kDa detected by Bianchimano *et al*., (2014) [11] and Sousa *et al*., (2011) [31].

The difference in the bands detected in various studies may be attributed to the difference in the strains according to the geographical area. Industrial and environmental contaminates and soil components may affect plant development and vegetation. These pollutants induce stress to plants, and change the expression of the proteins present in their pollen grains [35]. This is also accompanied by a change in the morphological features of the pollen grains, especially pollen diameter and pore size. Our pollen grains had a diameter of around 18-18.5 μm [34]. *C. album* pollen grains from urban areas at Iran exhibit similar diameter and collapsed features [25]. Hence, IgE reactivity to *C. album* pollen extracts was parallel with the results of immunoblotting. These results collectively implied that protein components with molecular weights from 50 to 67 kDa of these pollen extracts could play a greater role in induced allergy to *C. album*. This finding revealed diverse sensitization profiles among our patients, relative to previous studies. Hence, purification and characterization of these proteins will be of significance to determine a possible cross-reactivity with other types of pollen grains.

## CONCLUSION

The current study indicated that *C. album* pollen grains are accused allergens in Egypt. This platform characterization of *C. album* pollen extracts is a prerequisite for the management of patients sensitive to *C. album*. Moreover, it could be utilized in the diagnosis and as a therapeutic agent for atopic allergy to *C. album*.

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## CONFLICT OF INTEREST STATEMENT

The authors state no conflict of interest.

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