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Purified Aged Garlic Extract Modulates Allergic Airway Inflammation in Balb/c Mice

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ABSTRACT

Garlic is known as a potent spice and a medicinal herb with broad therapeutic properties ranging from antibacterial to anticancer and anticoagulant. Our previous studies have shown some immunoregulatory effects for aged garlic extract, suggesting a key role for 14-kD glycoprotein of garlic in shifting the cytokine pattern to T helper-1.

In present study, we investigated the effect of 1, 2, and 3 times intraperitoneal injections of aged garlic extract on an established allergic airway inflammation in murine model (BALB/c mice). The garlic extract, isolated by biochemical method, includes proteins precipitation by ammonium sulfate. After injection of the aged garlic extract, IFN- γ , anti allergen specific IgE and IgG₁ were measured in lavage and serum by ELISA and histological assessment was performed on the lung tissues.

The results indicated that three-time intra peritoneal injections of the aged garlic extract caused a significant decrease in the hallmark criteria of allergic airway inflammation levels which included eosinophil percentage in lavage, peribronchial lung eosinophils, IgG₁ level in lavage and serum, mucous producing goblet cells grade and peribronchial and perivascular inflammation.

Our findings in the present research suggested that aged garlic extract has the potential of attenuation of inflammatory features of allergic airway inflammation in murine model.

Key words: Aged Garlic extract; Allergic airway inflammation; IFN γ ; IgG₁; IgE; Murine model

INTRODUCTION

In the past decades, the prevalence of asthma increased worldwide. Some clinical symptoms of

asthma are attributed to the increases in serum total IgE and allergen specific IgE due to the synthesis of IL-4 and IL-5 cytokines from allergen specific CD4⁺ Th 2 cells.^{1,2} It has been suggested that shifting of the allergen-specific response away from a Th2-polarized response towards a Th1 response may furnish a therapeutic effect in asthma.³⁻⁵ The medical uses of garlic (*Allium sativum*) have been known for centuries.^{6,7} Various researches have indicated that

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garlic modulates immune responses.⁸⁻¹⁰ Our previous studies demonstrated that garlic enhances natural killer (NK) activity¹¹ and T-lymphocyte function.¹² Recently, it has been found that garlic extract induced a shift in cytokine pattern in *Leishmania Major* infected Balb/c mice.¹³

Murine models of allergic airway diseases and asthma have been used in order to elucidate the mechanisms and treatment of allergic airway inflammation. In these models, we can follow hallmark criteria of asthma such as increase in goblet cell proportion in airway epithelium¹⁴ allergen-specific IgE and IgG₁ and eosinophil accumulation in and around the airways¹⁵⁻¹⁸ and peribronchial and perivascular cellular infiltration without alveolar inflammation and granulomas.^{19,20} In this study, we used a murine model to examine the immunomodulatory effect of aged garlic extract in the animals with established airway disease.

MATERIALS AND METHODS

Preparation of Aged Garlic Extract

Fresh garlic bulbs were obtained from Hamadan, Iran. Garlic bulbs were peeled and kept in freezer (-20 °C) for 6-24 months. Five hundred grams of the obtained aged garlic was homogenized with two parts of distilled water in a varying blender. The homogenized blend was filtered under vacuum through Whatman paper No.1 and the filtrate was centrifuged at 4500 G for 30 minutes. The cleared supernatant was collected and sufficient water was added to make a one liter solution. Then, Twenty-seven grams of NH₄SO₄ was added (a 5% solution) and the obtained solution was centrifuged at 3600 G for 30 minutes. The pellet was resuspended in saline and dialyzed against buffer saline. The fraction was sterilized through 0.22µ Millipore filter and stored at 4 °C.²¹ Protein concentration was determined by Bradford method using bovine serum albumin as standard. The Garlic extract was diluted in distilled water and 20mg/kg¹³ of the obtained dilution was administered intraperitoneally.

Polyacrylamide gel (12% w/v) was used to evaluate the purity of the molecules and to estimate the molecular mass with standard protein. After electrophoresis, the gel was fixed with methanol, acetic acid formaldehyde for 60 minutes and stained with coomassie blue (Figure 1).

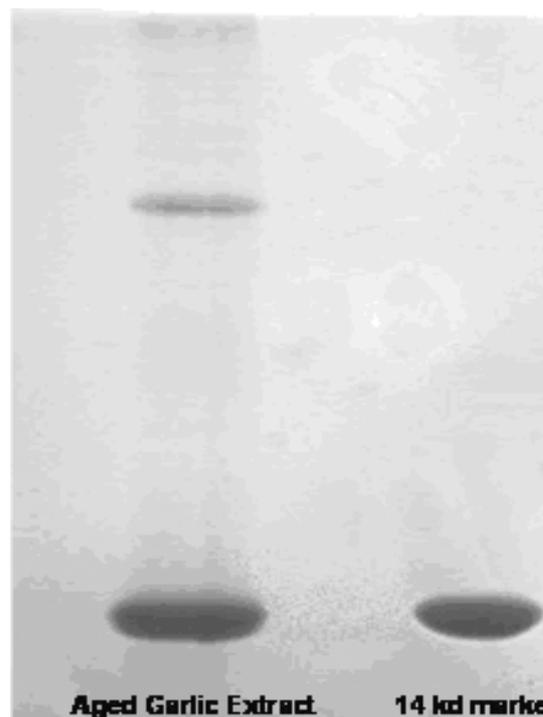


Figure 1. Aged garlic 14 kD fraction band evaluated by SDS-PAGE electrophoresis

Animals

Six to eight weeks old male BALB/c mice were purchased from Pasteur Institute (Tehran, Iran). Once arrived, the mice were quarantined for at least 1 week before use. They were kept in a controlled environment regarding temperature, humidity and light, with filtered airflow, sterilized instruments and free access to standard rodent autoclaved chow and water *ad libitum*. The animals were then sacrificed under euthanasia.

Experimental Design

Induction of Allergic Airway Inflammation Model

Four groups of mice each group consisting of 10 mice were intraperitoneally sensitized on the days 0 and 14 with 20 µg of Ovalbumin adsorbed to alum adjuvant in a total volume of 100 µl. Subsequently, on the days 24, 26, 28 and 30, they were exposed to 8 ml of 1% (wt/vol) aerosolized Ovalbumin in saline for 30 min/day. Aerosol administration was performed with constant pressure through an ultrasonic nebulizer (NE – UO7, Omron Co, Tokyo, Japan) in a chamber of 5500 cm² volume. On the day 31, the mice were sacrificed and their Blood, bronchoalveolar lavage and lung tissue were collected for subsequent analyses.

Modulation of Allergic Airway Inflammation by Aged Garlic Extract

Treatment Protocol

To examine the effect of garlic extract fraction on the mice sensitized with Ovalbumin, five groups of mice were planned as follows:

First group: Ten mice were sensitized with Ovalbumin only and no treatment was used (positive control group).

Second group: Ten mice were sensitized with Ovalbumin and treated with one dose of 14 KD garlic fractions (on the day 27).

Third group: Ten mice were sensitized with Ovalbumin and treated with two doses of 14 KD garlic fraction (on the days 25 and 27).

Fourth group: Ten mice were sensitized with Ovalbumin and injected with three doses of 14 KD garlic fractions (on the days 25, 27 and 29).

Fifth group: Ten mice (not sensitized with Ovalbumin) were injected with saline and alum adjuvant on the days 0 and 14. Then, on the days 24, 26, 28 and 30, they were exposed to aerosolized saline (negative control group).

Each animal was injected intraperitoneally with 20 mg/Kg of 14 KD garlic fractions.

Preparation of the Samples

Preparation of the samples was done by the same method described by Farzaneh *et al.*²² In brief, the mice were anaesthetized; the blood sample were collected and their sera were separated. The mice were sacrificed and divided into two groups: The first group included five mice for bronchoalveolar lavage (BAL) sample preparation. Their trachea were cannulated and BAL fluid containing cells were recovered. The BAL fluid was collected for subsequent cytokine and Immunoglobulin analysis. The second group also included five mice, In order to obtain histopathology samples of the lung tissue the lower lobe of their left lung was cut into three slices and fixed in 10% buffered formalin and paraffin embedded. The samples were cut into five μm and stained with haematoxylin-chromotrop 2R for evaluation of eosinophilia, and with periodic acid Schiff (PAS) to visualize mucous production and goblet cells.

Histology and Eosinophils:

The BAL cell slides were stained with Wright-Geimsa. At least 200 cells were counted per mouse and the cells were identified by morphologic criteria to determine the percentage of eosinophils in each sample.

Histological assessment was performed in the same method described by Farzaneh *et al.*²² and the total lung sections were scored at 100X or 400X final magnifications. In order to evaluate the criteria of eosinophilic inflammation in the lung, a semi-quantitative scoring system was used to grade the histopathologic lung changes. Peribronchial inflammatory cell infiltrates were graded as follows:

0 = lack of any infiltrate;

1+ = bronchiole with scattered infiltrates;

2+ = bronchiole with an infiltrate of upto 2 cells per section;

3+ = bronchiole with an infiltrate of up to 5 cells per section;

4+ = bronchiole with an infiltrate of more than 5 cells per section;

The sum of the airway scores from the lung slide was divided by the number of airways examined (20–30 per mouse) and expressed as peribronchial inflammatory score in a semi quantitative unit. The same scoring method was used for perivascular inflammation grading. Alveolar septa cellularity was graded as follows:

0 = no infiltrate of the inflammatory cells or widening of the septa;

1+ = minimally increased cellularity without significant widening of septa;

2+ = obvious cellular infiltrates with moderate widening of the septa; and

3+ = markedly increased cellularity with thickened septa.

Interstitial alveolar space cellularity was scored as follows:

0 = no infiltrate;

1+ = scattered infiltrates of the inflammatory cells in alveolar spaces,

2+ = the alveolar spaces full of inflammatory cells.

The sum of the alveolar scores from each lung section was divided by the number of sections examined and expressed as septal or interstitial alveolar inflammatory scores in semi-quantitative units. The proportion of goblet cells within the airway epithelium was scored at 400x final magnification as follows:

0=<5% goblet cells;

1= 5–25%;

2=25 –50%;

3 = 50–75%,

4=>75% goblet cells.

The summary of the airway scores from each lung slide was divided by the number of airways examined (20–30 per mouse) and expressed as mucous score in a semi-quantitative term. The eosinophils were visualized at 1000X final magnification and counted using a rectangular eyepiece (Nikon, HWF10X-F) calibrated with stage micrometer slide. At least 10–20 fields of views around the airways were counted for each slide. The airways with inflammatory cells were randomly selected from all the sections in the slide. The eosinophils were counted within an area of 0.1 mm² of epithelium and subepithelium and multiplied by 10. The ratio of the total number of the eosinophils to total area of each mouse lung was calculated and expressed as the number of eosinophils per mm². Different cell types were defined as follows:

small mononuclear cells = lymphocytes;

larger mononuclear cells with moderate basophilic cytoplasm and large coarse nucleoli = transformed cells;

largest cells with generous eosinophilic cytoplasm and small nucleoli = histiocytes;

cells with segmented nuclei and red cytoplasm on chromotrop 2R stain = eosinophils;

cells with segmented nuclei without red cytoplasm on chromotrop 2R stain = neutrophils.

Determination of Allergic Responses in the Serum and Lavage:

In addition to BAL eosinophils, specific anti Ovalbumin IgE and IgG₁ levels in the BAL and serum samples and IFN- γ level in BAL samples were measured. Anti-allergen IgE and IgG₁ levels were detected using ELISA according to the same method described by Farzaneh *et al.*²² IFN- γ was analyzed in the BAL fluid samples using standard sandwich ELISA. (BMS606, Bender Medsystems GmbH, Austria).

Statistical Analysis

The data were expressed as means \pm SEM. They were considered statistically significant when $p < 0.05$. The differences were determined using the Kruskal-Wallis test. For comparison of the pairs, non-parametric comparison test was used.

RESULTS

Isolation of the Proteins from the Garlic Extract

The molecules were purified from garlic extract by means of Ammonium sulfate precipitation and the fractions were collected by centrifugation. In order to evaluate the purity of a 14 kD fraction, it was run in SDS/PAGE electrophoresis and the results indicated the presence of one band of 14 kD molecule (Figure 1).

The Allergic Responses in the Lavage and Serum

In order to evaluate the effect of garlic fraction on the allergic response, the mice were divided into five groups and the protocol described in figure 2 was employed. The results indicated that the serum level of IgG₁ significantly decreased at three doses treated with 14 kD fraction of garlic, while at one and two doses, no significant decrease was observed in the serum level of IgG₁ comparing with the positive control group (Figure 2b). In addition, the results indicated that the 14 kD fraction of garlic could significantly decrease the IgG₁ response of lavage in the three doses in treated animals comparing with the positive control group (Figure 3b). 14 kD fraction of the garlic extract did not induce significant changes in IgE levels comparing with positive control group (Figures 2a, 3a).

The Effect of Garlic Extract on the Shifting of Th1/Th2 Immune Response

The results indicated that IFN- γ showed a significant difference in all the allergen treated mice comparing with the negative control group (Figure 3c). Moreover, no significant differences were noticed in terms of the levels IFN- γ comparing with the positive control group.

The percentages of lavage eosinophils significantly decreased in all the groups of garlic fraction treated mice comparing with the positive control group (Figure 3d).

The Histopathologic Status of the Allergic Responses:

The first step was to emphasize the induction of an allergic airway model. The negative control mice showed normal lung cellular architecture. Histologic analysis of the tissue sections revealed no inflammatory signs in the interstitial alveolar septa and spaces in all the groups of mice. However, significant peribronchial and perivascular infiltrates and goblet cells metaplasia and hyperplasia were seen in all allergic Ovalbumin sensitized mice. These signs are in agreement with an allergic airway model induction (Figure 4).²²

Modulation of Allergic Airway Inflammation by Aged Garlic Extract

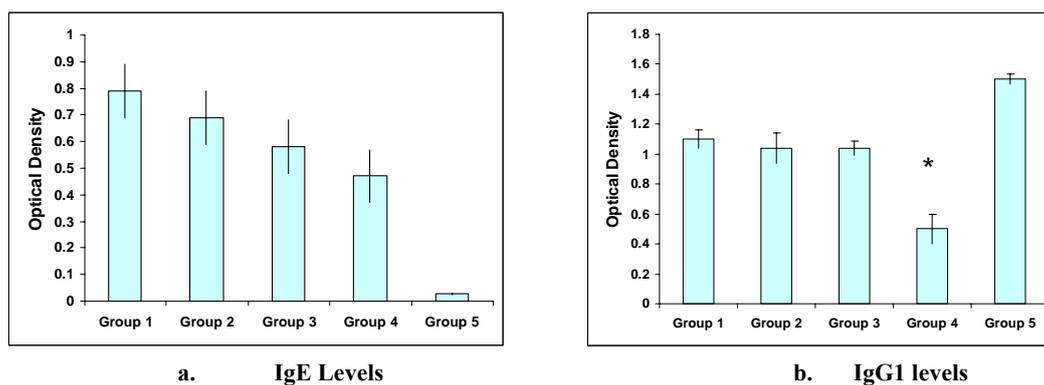


Figure 2. The level of IgG1 and IgE in the serum of the animals sensitized with Ovalbumin and treated with 14kD fraction of aged garlic. a: IgE and b: IgG₁. The significance is indicated by *= $p < 0.05$, compared with positive control group.

- 1, Positive control, animals sensitized with Ovalbumin allergen, without treatment.
- 2, Ovalbumin allergic animals treated with one dose of 14kD fraction of aged garlic extract
- 3, Ovalbumin allergic animals treated with two doses of 14kD fraction of aged garlic extract
- 4, Ovalbumin allergic animals treated with three doses of 14kD fraction of aged garlic extract
- 5, Negative control animals injected and challenged with only saline

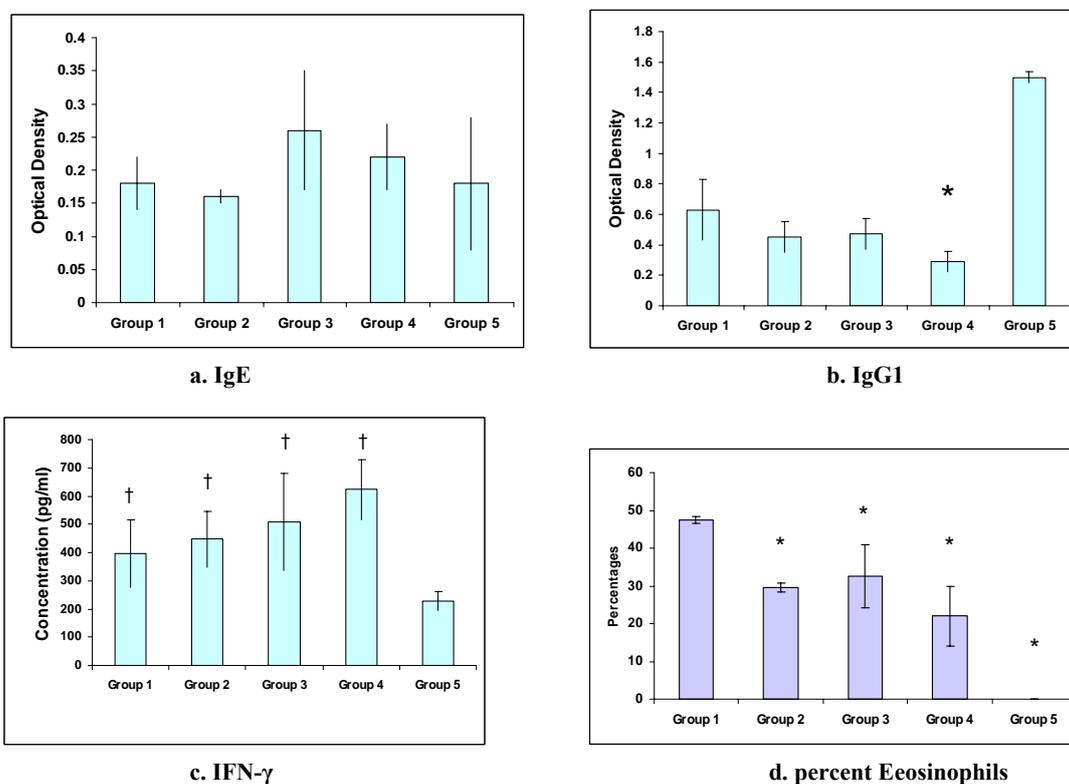


Figure 3. The level of IgG₁, IgE and IFN- γ and the percentages of eosinophils in the lavage in the animals sensitized with Ovalbumin and treated with 14kD fraction of aged garlic a: IgE, b: IgG₁, c: IFN- γ and d: the percentages of eosinophils. The significance is indicated by *= $p < 0.05$, compared with positive control group and [†]= $p < 0.05$, compared with negative control group

- 1, Positive control, animals sensitized with Ovalbumin allergen, without treatment
- 2, Ovalbumin allergic animals treated with one dose of 14kD fraction of aged garlic extract
- 3, Ovalbumin allergic animals treated with two doses of 14kD fraction of aged garlic extract
- 4, Ovalbumin allergic animals treated with three doses of 14kD fraction of aged garlic extract
- 5, Negative control animals injected and challenged with saline

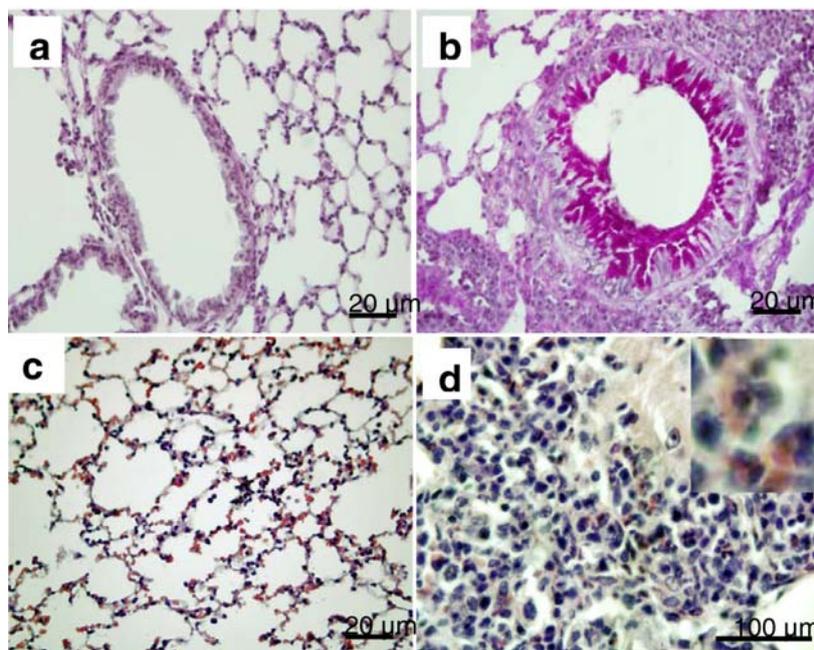


Figure 4. Representative lung sections of sensitized mice compared with negative control mice. (a) Negative control mice that were not sensitized comparing with (b) Ovalbumin sensitized mice with peribronchial inflammatory cell infiltration and mucous producing goblet cells, (c) Normal structure of interstitial alveolar septa and spaces in Ovalbumin induced models. (d) Eosinophils in inflammatory areas of lung sections of sensitized mice. Eosinophils were identified by specific staining (red cytoplasm) and morphological criteria. (a) and (b) stained with Periodic acid Schiff. (c) and (d) stained with haematoxylin-chromotrop 2R.

Significant decreases in the number of mucous producing goblet cells in the airways were also observed at all doses of the treatments with 14 kD fraction of the garlic extract comparing with the positive control of mice (Table 1). Significant decreases in the number of eosinophils in the peribronchial inflammatory sites in all doses of

treatments of 14 kD garlic fraction were seen comparing with the positive control of mice (Table 1).

Significant decreases in the perivascular and peribronchial inflammatory grades were also seen after treatment of mice with the 14 kD fraction of the garlic extract comparing with the positive control group (Table 1).

Table 1. Mean (\pm SEM) of pulmonary inflammatory changes.

Groups	Goblet Cells	Perivascular Infiltrates	Peribronchial Infiltrates	Peribronchial Eosinophil Density (1)
Group 1	3.9 \pm 0.2	3.6 \pm 0.2	3.4 \pm 0.4	4.2 \pm 0.8
Group 2	3.0 \pm 1.1*	2.5 \pm 0.8*	2.4 \pm 0.9**	1.7 \pm 0.7
Group 3	1.6 \pm 0.4**	2.4 \pm 0.6**	2.0 \pm 0.2**	0.24 \pm 0.2
Group 4	0.9 \pm 0.3**	1.6 \pm 0.4**	1.5 \pm 0.2**	0.0 \pm 0.0
Group 5	0.0 \pm 0.0	0.06 \pm 0.05	0.14 \pm 0.1	0.0 \pm 0.0

Stained sections were scored as described in materials and methods and visualized by staining with periodic acid Schiff and Hematoxylin-Chromotrop 2R. n=5 each group.

(1); Number of eosinophils in 1 mm² area of bronchiolar epithelium and subepithelium with inflammatory infiltrates.

- 1, Positive control, animals sensitized with Ovalbumin allergen, without treatment
- 2, Ovalbumin allergic animals treated with one dose of 14kD fraction of aged garlic extract
- 3, Ovalbumin allergic animals treated with two doses of 14kD fraction of aged garlic extract
- 4, Ovalbumin allergic animals treated with three doses of 14kD fraction of aged garlic extract
- 5, Negative control animals injected and challenged with saline

The significance is indicated by *= p <0.05, **= p <0.01 compared to positive control group.

DISCUSSION

Since the discovery of allicine, much progress has been made in identifying the various unusual organosulfur compounds formed when garlic extract is prepared.²³ Despite this progress, many other compounds remained to be identified in allium extracts. Various researchers have shown that garlic modulates immune responses.^{10,24,25} Our previous immunopharmacological studies on garlic were assessed by two stages. The first was identification and purification of the molecules involving in augmentation of immune responses; and the second was the pharmacological effect of the purified molecules on immune response.^{11,13,26,27} Accordingly garlic enhances natural killer (NK) activity²⁰ and T-lymphocyte function.²⁶ Recently, we found that garlic extract induces a shift in cytokine pattern in Leishmania Major infected Balb/c mice¹³ and the outcome of the immune response with regard to Th1 (IFN- γ , IL-2).

Previous studies have shown that garlic contains two major proteins constituting 96% of the total garlic proteins.^{26,28} Ghazanfari *et al* isolated two major proteins in their studies: 14 and 47 kD. The latter was responsible for immunomodulatory activity and shift in cytokine pattern.^{13,26} In our laboratory, we found that freezing of garlic for more than six months (6-24 months) causes an increase of 14 kD to 47 kD ratio (up to 98%, unpublished data). Our findings in the present research showed that aged garlic extract, which contains a 14 kD protein administered at 1-3 doses, could reduce the allergic responses in mice at the doses of 20 mg/kg compared with the control group.

Although, some pharmacologic, biological and immunomodulatory effects of garlic and its derivatives have been considered and reviewed previously,^{25,29-32} there are few studies in the field of allergy and asthma, the published results are mainly on occupational asthma and the adverse effects of garlic and its dust.³³

Kyo *et al*³⁴ reported anti-allergic properties for garlic extract. In their rodent basophile cell line model, addition of garlic extract reduced histamine release (anti-histamine effect). They showed suppression of IgE-mediated antigen-specific skin reaction in murine model too. The authors of the present research concluded that garlic extract could beneficially balance, or modify the function of mast cells, basophile, and activated T lymphocyte factors, which all play a leading role in allergic cascade reactions and inflammation.

In present research, we isolated a protein that can modulate the airway inflammation. Our findings showed that the ability of 14 kD fraction of aged garlic extract in the reduction of allergic airway inflammation hallmarks was accompanied by an increase in the bronchoalveolar lavage IFN- γ level in this model. Although there are several studies about the interaction and the role of IFN- γ and other cytokines in allergic airway inflammation process,³⁵⁻⁴¹ but in our study, 14 kD fraction treatment did not lead to a significant altering in the BAL fluid IFN- γ level but further investigation may help to do up these points.

The controlled manipulation of immune response by pharmacological means is also a highly sought goal of clinicians because of its potential for application of those drugs to the patients with cancer, immunodeficiency disorders and infectious diseases, where immunomodulation may be of value. Garlic may prove useful in these aspects, but the extrapolation of animals, studied in a clinical situation, must be treated with caution.

High doses of garlic may probably cause a toxic effect on animals. LD50 values of garlic extract by intraperitoneal and subcutaneous administration have been estimated to be over 30 g/kg in both male and female mice.⁴²

In summary, this study showed that 14 kD fraction of aged garlic extract is able to reduce the allergic airway inflammation hallmarks in murine model accompanied by increase in the bronchoalveolar lavage IFN- γ level.

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