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Original Article

Evaluation of immune responses induced by hydroalcoholic extract of *Allium ampeloprasum. L subsp iranicum* in mice

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Abstract

The extract of some plants can play a role in modulating the function of the human immune system. Several studies have shown that *Allium* species can stimulate the immune system through different cellular mechanisms. In this study the effect of the hydroalcoholic extract of *Allium ampeloprasum. L subsp iranicum* on the immune responses of NMRI mice was investigated. Mice were randomly divided into three groups and each group was divided into four subgroups: normal saline, levamisole (20 kg/mg), *A. Iranicum* (150 kg/mg), and *A. Iranicum* (300 kg/mg). To evaluate immune responses, body and spleen weight, white blood cell count, delayed hypersensitivity response (DTH), antibody titer (HA), and lymphocyte proliferation test were used.

A. Iranicum provoked several parameters including spleen weight, spleen cellularity, proliferation response to lipopolysaccharide (LPS) and phytohemagglutinin-A (PHA), HA titer, and DTH response. While *A. Iranicum* did not affect body weight and white blood cell counts. Considering the obtained results, it seems that the *A. Iranicum* can play an effective role in activating the immune system. To better understand the effect of the *A. Iranicum* on different cellular mechanisms of the immune system, further research is needed.

Keywords: Allium iranicum, Cellular immune system, Herbal extract, Humoral immune system, Levamisole, NMRI mice.

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1. Introduction

In recent years, the use of medicinal plants has gained a lot of popularity in the world and extensive research has been done on special species of medicinal plants that have appropriate effects on many human diseases [1]. Therefore, new research introduces the use of complementary medicine, especially herbal therapy, as a low-cost treatment solution with low side effects [2].

The immune system is a highly complex cellular and molecular machinery that has evolved to protect the host from infectious agents (viruses, bacteria, fungi, and parasites) [3]. Defects in the immune system are related to
important diseases such as allergies, autoimmunity, immunological resistance, inflammation, and cancer [4].
Studies show that different medicinal plants can have a positive effect on different components of the immune system due to their bioactive compounds. The various plant-derived compounds, including alkaloids, quinones, flavonoids, saponins, terpenoids, phenol carboxylic acids, and polysaccharides have immunostimulatory activity [5, 6].
Allium is one of the most diverse genera of *Amaryllidaceae*. This genus includes important horticultural crops such as garlic, shallots, onions, chives, leeks, and other species with unknown medicinal, vegetable, or ornamental properties. Due to the beneficial effect of plants of this genus on human health, much scientific research has been conducted on their biochemical and biological properties [7, 8]. *Allium ampeloprasum. L subsp iranicum* is a plant with ten long flower stalks and relatively wide leaves with a tuberous part. It has various sulfurous compounds, phenolic and flavonoid components, and steroidal saponin in its different parts. The results of studies show that this plant can prevent the growth of some bacteria, especially those of food origin [9, 10]. Considering the high importance of the immune system and related diseases, in this study the effect of *A. iranicum* was investigated on some components of the immune system.

2. Materials and Methods

2.1. Plant material

A. iranicum was collected from Chaharmahal VA Bakhtiari province of Iran and identified by the botanists of Islamic and Complementary Medicine, Iran University of Medical Sciences, Tehran, Iran. Total extraction was done using the maceration procedure. 100 mL of ethanol: water (80:20) was added to 10 g of the shade-dried ground powder of whole parts of *A. iranicum* for 24 hours. the mixture was filtered and the fresh solvent was added. This process was repeated three times. All extracts were concentrated using a rotary evaporator and then a freeze dryer [11].

2.1.1. Determination of total content of phenolic compounds

The total phenolics content of the hydroalcoholic extract of *A. iranicum* was measured according to the Folin-Ciocalteu assay described previously. Briefly, the calibration curve was plotted using different concentrations of gallic acid. 400 µL methanolic solution of the hydroalcoholic extract of *A. iranicum* was mixed with 3 mL Folin-Ciocalteu's reagent and incubated at 22℃ for 5 minutes. In the next step, the reaction mixture was further incubated for 90 min at room temperature by addition3 mL sodium carbonate (7%). Finally, absorbance was measured with a UV–Vis spectrophotometer at 750 nm. The total phenolic content was calculated as equivalent in milligrams per 100 g dried extract using a gallic acid calibration curve [12].

Animals

Male NMRI inbred mice (6–8 weeks; weighing 20–25 g) were purchased from the Faculty of Medical Sciences, Tarbiat Modares University, and acclimated in polystyrene cages for one week. The animals were maintained on a 12-h light/dark cycle at room temperature (20–25 ◦C), relative humidity of 50%, and allowed free access to food and water. All experiments carried out on animals were approved by the ethics committee of Tarbiat Modares University (approval ID: IR.MODARES.REC.1397.114)

2.2. Doses and exposure schedules

Sixty mice were randomly divided into three groups for use in the different investigations (n=20). Each group was further subdivided into four subsets (n=5): (i) Oral administration of 150 mg/kg *A. iranicum* for 14 days; (ii) Oral administration of 300 mg/kg *A. iranicum* for 14 days [13,14] (iii) Oral administration of 20 mg/kg levamisole as a positive control for 14 days; and (iv) Oral administration of normal saline as a negative control for 14 days [15].

2.3. Body and spleen weight

On the 14th day, 2 hours after receiving the last dose, mice were killed by cervical dislocation. Body and spleen weight were determined for each mouse.

2.4. White blood cell count

On the fourteenth day, 2 hours after receiving the last dose, 20 µL of blood was obtained from the retroorbital plexus in tubes soaked in EDTA-K2, and the number of white blood cells and their subclasses was measured.

2.5. Preparation of single-cell suspension

On the 14th day, two hours after the administration of the last dose, the mice were killed by cutting the spinal cord. Then the spleen was aseptically removed from the mice and placed in a 6-well plate. Then, using a needle and a syringe, they were made into a cell suspension in RPMI 1640 medium. The resulting suspension was passed through a cell strainer to separate the remnants of the spleen tissue. The cells were centrifuged at 224 g at 4°C for 10 minutes. The resulting supernatant was discarded. The remaining sediment was kept in the vicinity of 3 mL of RBC lysis solution for 3 minutes at room temperature. Again, the cells were centrifuged at 224 g at 4°C for 5 minutes. The resulting supernatant was discarded. Then the cells were washed three times using an RPMI 1640 culture medium. To determine the percentage of cell viability, a cell suspension was prepared in 5% PBS, and with 0.4% trypan blue, the percentage of cell viability was determined using an optical microscope [16].

2.6. Serum antibody titer hemagglutination (HA) titer:

One mL of washed sheep RBCs (SRBCs) was diluted using Freund's complete adjuvant to reach a dilution of 5×10^8. On day 11, mice were administered 5×10^8 SRBCs intraperitoneally. On day 14, serum samples were prepared from peripheral blood, and the serum was diluted one-to-one in phosphate buffer. After preparing serial dilutions of serum in different tubes, 25 µl of serum was exposed to 25 µl of red SRBCs suspension in phosphate buffer in each test tube. To observe the agglutination of the samples, the tubes were incubated for 1 h at 37°C. Antibody titer was recorded based on the highest dilution in which agglutination was observed [17].

Delayed-type hypersensitivity (DTH) response

The delayed hypersensitivity response test in this study was performed based on Fararjeh et al.'s method with some changes. The preparation of red blood cell suspension was done in the way described previously, with the difference that the dilution continued using Freund's complete adjuvant until reaching a dilution of 1×10^9. On the 11th day, the animals were sensitized to IP with 1×10^9 SRBC. On the 14th day, a booster dose equal to 1×10^8 SRBC was injected into the left hind footpad. A similar volume of Freund's incomplete adjuvant was injected in the right hind footpad to remove the effect of non-specific swelling. After 24 and 48 hours, the volume increase in two paws was measured using a Vernier caliper (Mitutoyo, Kawasaki, Japan), and the difference between left and right hind footpad volumes was calculated [16].

2.7. Lymphocyte proliferation

One hundred μL of spleen cell suspension was transferred into a 96-well plate. Either no mitogen or PHA or LPS at final concentrations of 5 and 1 μg/ mL, respectively, was added to each well. The plate was placed in an incubator at 37°C and 5% CO2 for 48 hours to ensure that the cells were attached to the substrate and stimulated. After the mentioned time, the proliferation of cells was determined using the MTT assay. The proliferation index (PI) was calculated:

PI = absorbance of stimulated cells⁄ and absorbance of unstimulated cells [16].

2.8. Statistical analysis

All data were presented as the mean ± SD and were analyzed using one-way ANOVA, followed by Tukey's multiple comparison tests using PRISM, version 6.00 (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significantly different.

3. Results

3.1. Body and spleen weight

There was no significant alteration in the average body weight of the mice before and after treatment ($P>0.05$). Also, the comparison of spleen weight in different groups indicated a significant difference between mice exposed to levamisole and 300 kg/mg of *A. Iranicum* compared to the normal saline group ($P<0.0001$) (Table 1).

Table 1. Effects of subacute orally exposure of mice to *A. Iranicum* for 14 day son body and spleen weight

Experimental groups	Spleen weight (g)	Body weight (g) (Before)	Body weight (g) (after)
A. iranicum (150 mg/kg)	0.20±0.01	24.10±1.02	25.15±1.12
A. iranicum (300 mg/kg)	0.28±0.02***	24.72±0.40	25.12±0.03
Normal saline	0.13±0.01	25.07±1.40	25.97±1.15
Levamisole	0.27±0.02***	24.90±1.2	25.90±1.3

Data are the mean ± SD. *** P < 0.001: significant changes compared to the control group (NS).

3.2. White blood cell counts

The number of white blood cells, lymphocytes, and neutrophils in the levamisole group is more than in the other groups (P<0.0001). There was no meaningful difference in the number of monocytes between the levamisole and *A. Iranicum* extract group (P>0.05) (Table 2).

3.3. Spleen cell numbers

The results of counting live cells in the spleen in different groups showed that there is a significant increment in the number of live cells between the normal saline group and both groups receiving *A. Iranicum* plant extract (P<0.05) (Figure 1).

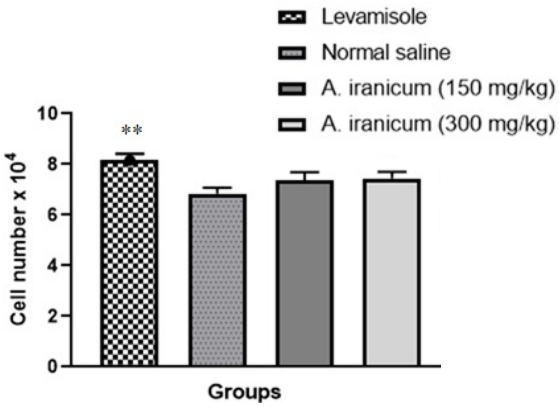

Figure 1. Effects of orally exposure of mice to *A. Iranicum* for 14 days on spleen cell number. Data are the mean ± SD. * P < 0.05 and ** P < 0.01: significant changes compared to the control group (NS).

Table 2. Effects of subacute orally exposure of mice to *A. Iranicum* for 14 days on white blood cell counts

	White blood cell counts			
Experimental groups	White Blood Cells (Cell/µl × 10^3)	Lymphocytes (Cell/µl × 10^3)	Neutrophils (Cell/µl × 10^3)	Monocytes (Cell/µl × 10^3)
A. iranicum (150 mg/kg)	6.89± 0.31	4.48± 0.82	2.29± 0.5	0.12± 0.7
A. iranicum (300 mg/kg)	6.93± 0.12	4.43± 0.40	2.27±0.13	0.14± 0.13
Normal saline	6.85± 0.42	4.44± 0.40	25.97± 1.15	0.12± 0.08
Levamisole	8.12± 0.10**	5.03± 0.14*	2.97± 0.10*	0.12± 0.04

Data are the mean ± SD. * P < 0.05 and** P < 0.01: significant changes compared to the control group (NS).

3.4. Serum antibody titer hemagglutination (HA) titer

Serum anti-SRBC titer demonstrated a considerable enhancement in *A. Iranicum* treatment groups compared to the normal saline group. There was no considerable difference between *A. Iranicum* groups treated groups (Figure 2).

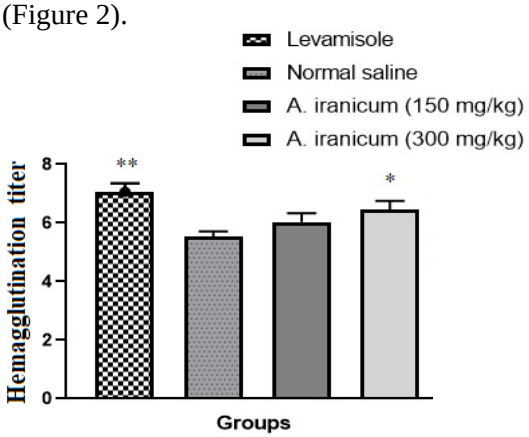

Figure 2. Effects of orally exposure of mice to *A. Iranicum* for 14 days on serum antibody titer hemagglutination (HA) titer. Data are the mean ± SD. * P < 0.05 and** P < 0.01: significant changes compared to the control group (NS).

3.5. Delayed-type hypersensitivity (DTH) response

Regarding the evaluation of DTH response, there was a significant enhancement in the 24h and the 48h-DTH response of mice exposed to either 150 or 300 mg/kg *A. Iranicum* groups compared to negative controls. Also, the levamisole group showed a remarkable increase in DTH response (P<0.001) (Figure 3).

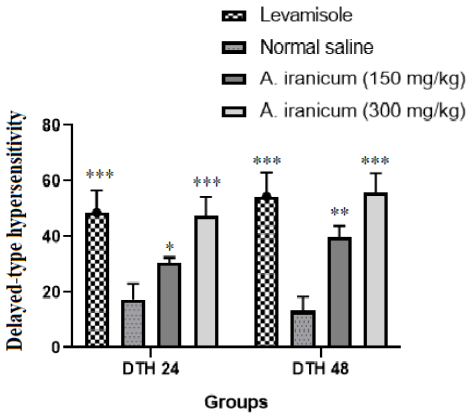

Figure 3. Effects of orally exposure of mice to *A. Iranicum* for 14 days on DTH. Data are the mean ± SD. * P < 0.05, ** P < 0.01 and *** P < 0.01: Significant changes compared to the control group (NS).

3.6. Lymphocyte proliferation

Exposure of mice to 150 or 300 mg/kg *A. Iranicum* increased the proliferative responses of splenocytes stimulated with LPS (Figure 4A). While PI (after PHA stimulation) of splenocytes exposed to 300 kg/mg of *A. Iranicum* was significantly different from the control group, there were no meaningful differences between mice dosed at 150 mg/kg of *A. Iranicum* and control groups (Figure 4B). The enhanced mitogen-induced proliferation of splenocytes occurred in cells of mice treated with the levamisole control (Figure 4).

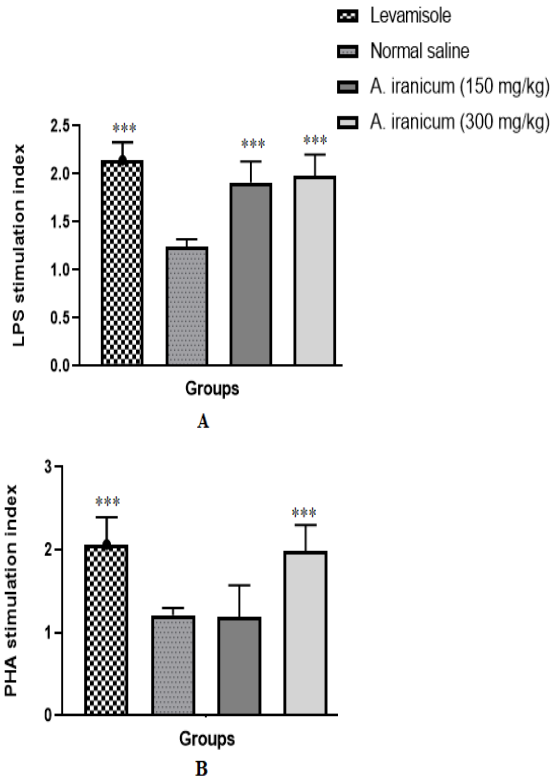

Figure 4. Effects of orally exposure of mice to *A. Iranicum* for 14 days on lymphocyte proliferation response to LPS (A) and PHA (B). Data are the mean ± SD. *** P < 0.01: significant changes compared to the control group (NS).

4. Discussion

The extract of some plants can play a role in modulating the function of the human immune system [18]. As one of the most diverse genera of the *Aliaceae* family, the genus *Allium* contains more than 900 species worldwide. Several studies show that *Allium* species can stimulate the immune system through different cellular mechanisms [19, 20]. The evidence from different investigations shows that

different doses have different effects on immune responses, so the dosage of herbal extracts is another important point. For example, a study by Mohamed et al showed that different doses of garlic produced completely different effects on immune system responses [21]. The study of Farhadi et al., 2014 showed that an increase in the dose caused a decrease in the response of the immune system against SRBC as the DTH response in mice that received a dose of 100 mg/kg of *A.ascalonicum* was higher than the groups receiving doses of 1000 and 2000 mg/kg [22]. In the present study, increasing the dose from 150 mg/kg to 300 mg/kg caused an increase in the strength of the immune response, so finding an optimal dose has critical importance in this regard.

This study confirmed the stimulatory effects of oral exposure to *A. Iranicum* for 14 days on the mice's immune system. Treatment with *A. Iranicum* led to a higher spleen weight, possibly because of an overall higher spleen cell number. Our findings showed that there is a significant difference in the number of splenocytes between both groups receiving *A. Iranicum* plant extract and the normal saline group which is consistent with the results of Radjabian et al., 2019 which found that *A. iranicum* had the most impact on the proliferation of immune system cells compared to other species [23].

The results of the DTH response showed a robust immune response in mice receiving hydroalcoholic extract *A. Iranicum*. Because by measuring the DTH, it is possible to indirectly understand the stimulation of Th2, Th1, and Th17 cells, so it seems that *A. Iranicum*, like the other *Allium* species, may affect the balance of Th1/Th2 and even affect the activity of Th17 cells. Different studies have proven that flavonoids increased the DTH and stimulated the secretion of IFNγ from Th1 cells. As the number of flavonoids in the hydroalcoholic extract of *A. Iranicum* was 334.66 mg/g of dry extract based on quercetin, the observed strengthening effect can be attributed to this compound [22].

Hanieh et al., 2010 proved that the addition of *A. sativum* and *A. cepa* in low doses to the diet of birds immunized with different antigens increased the production of related antibodies. They concluded that *A. sativum* and *A. cepa* may directly or indirectly increase the proliferation and differentiation of B lymphocytes [24]. The HA test in the present study shows a significant increase in the production of antibodies against SRBC in the *A. iranicum* group (300 kg/mg) compared to the normal saline group (Figure 2). On the other hand, the exposure of mice to *A. Iranicum* increased the proliferative responses of splenocytes exposed to LPS and PHA mitogenic compounds. The results of the lymphocyte proliferation test showed the direct effects of *A. Iranicum* on the normal function of T and B lymphocytes, which have been resulting in stimulating effect responses of DTH and HA.

The literature review reveals the different compounds in *Allium* species can have diverse effects on the immune system components [23]. Phytol is one of the compounds found in some species of *Allium* such as *A. iranicum*. Today, this compound is used as an adjuvant to enhance immune responses caused by vaccines. The conducted research shows the safety and high efficiency of this combination as an immune system stimulant in the combination of different vaccines [25, 26].

Moreover, most of the medicinal effects of this species and other Allium members are related to their organosulfur compounds the most bioactive compound in *A. iranicum* is dipropyl trisulfide, which accounts for 34.77% of the total chemical compounds in this plant. The results of the Arsenijevic et al., 2021 study showed that the solution containing dipropyl polysulfide compounds (contained 8.48% dipropyl trisulfide) can change the balance of Th17/Treg cells in favor of Treg (regulatory T) cells [27-29].

5. Conclusion

The results of this research showed that *A. iranicum* could strengthen the both cellular and humoral immune systems by probably affecting the balance of Th2/Th1 and Th17/Treg and the activation of B lymphocytes, respectively. To better understand the effect of the A. iranicum plant on different mechanisms of the immune system, further research is needed.

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Conflicts of interest

The authors declare that they have no conflicts of interest

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