Research Article:
The Immunoregulatory Effects of Four *Allium* Species on Macrophages and Lymphocytes Viability

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**ABSTRACT**

**Background:** Immune cells perform unique functions against infections and imbalances in immunity associated with various diseases. Applying natural products may control immune responses. Among herblas, much attention has been paid to the immunoregulatory functions of *Allium sativum*. However, the effects of other *Allium* species on the immune system have remained undiscovered. Therefore, the current study investigated the effects of *A. sativum*, *A. Iranicum*, *A. elburzense*, and *A. asarense* bulb extracts on macrophage and lymphocyte viability.

**Materials and Methods:** The different concentrations of aqueous extracts of the aged bulb samples were prepared and used for the incubation of examined immune cells. Macrophages and lymphocytes were isolated and cultured in the presence of different concentrations of bulb extracts of *Allium* species. Macrophages and lymphocytes viability was followed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

**Results:** The applied *Allium* species had stimulatory or inhibitory effects on the examined immune cells at different concentrations. *Allium sativum* demonstrated the highest effects on macrophage viability indices at 1 mg/mL. The similar effects were found for *A. Iranicum* extract on macrophage viability indices at 0.01 mg/mL. The bulb extract of *A. sativum* at the most used concentrations stimulated lymphocyte viability indices. The bulb extracts of all 4 *Allium* species inhibited viability indices of lymphocytes at 1 mg/mL. The bulb extracts of *A. elburzense*, at all of the applied concentrations, slightly affected viability indices of the macrophage and lymphocyte.

**Conclusion:** Besides *A. sativum*, wild growing *Allium* species could be introduced to study their immunomodulatory effects on various diseases.

**Keywords:** *Allium* species, Aged bulb extract, Macrophage, Lymphocyte, Cell viability

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Introduction

The immune system is a finely balanced network that eliminates foreign substances through interacting between cellular and humoral protective responses [1, 2]. The innate and adaptive immunity play key roles in the immune system [3]. Macrophages, as a key cellular mediator in innate immunity, do phagocytosis and produce cytokine to eradicate pathogens, besides other immunity functions [4, 5]. The effector cells of adaptive immunity are lymphocytes, consisting of several subsets, which specifically respond to antigens [4, 6, 7]. Immunomodulation contributes to control of the immune-related diseases. Thus, the regulation of immunological responses is the objective of natural or chemical therapeutic products. Immunomodulatory drugs have numerous side effects, including pulmonary toxicity, neurotoxicity, hepatic fibrosis, hypertension and so on [2]. Exploring changes in immune functions revealed the beneficial immunomodulatory activities of plants and their components [8].

Among the plants of the genus Allium L. (family Amaryllidaceae), garlic (Allium sativum L.) [9], is one of the most important species of this genus. Its effects have been reported on the immune system [10, 11]. For many centuries, garlic has been used as a therapeutic and culinary agent [9, 12]. The important bio-pharmacological features of garlic are attributed to its organosulfur compounds [8, 13].

The antibacterial, antiviral, antifungal, anticancer and immunomodulatory effects of garlic have been widely reported [9, 10, 12]. It has been suggested that the immunomodulatory effects of garlic are due to macrophage phagocytosis, i.e., the infiltration of macrophages and lymphocytes and their proliferation [14]. In addition, a~14 kD purified glycoprotein from raw garlic increases T lymphocyte proliferation [15], and Natural Killer (NK) cells activity [16]. Based on the results of Morioka et al. [17], the protein fraction of aged garlic extract revealed NK cells activity and the cytotoxicity of macrophages against cancer cells.

Allium species have widespread distribution in Central Asia. Furthermore, there are around 115 Allium species and more than 40 endemic ones in Iran [18, 19]. In spite of the benefits attributed to some Allium genus, including garlic and onion [20, 21], on immunostimulation or immunosuppression, there is not enough information about the effects of other members of Allium. The present study conducted a screening approach on the impact of 4 Allium species from Iran on macrophage activation and lymphocyte proliferation.

Materials and Methods

Plant materials

Cultivated A. sativum was purchased from a field in Hamedan Province, Iran, and wild plants of A. Iranicum, A. elburzense, and A. asarense were collected from their natural habitats in different regions of Iran. The obtained plants were taxonomically identified by Dr. Shahin Zarre (College of Science, University of Tehran) (Table 1). The bulbs were peeled and stored at -21°C for 4-6 months until used.

The aged bulbs of all collected species were cut into small pieces and 1 g of each bulb sample was homogenized in 1 mL distilled water using a blender. The homogenized mixtures were filtered on Whatman filter paper and the extracts were diluted in distilled 10 mL water as stock solutions. Finally, different concentrations (0.0001, 0.001, 0.005, 0.01, 0.05, 0.1 and 1 mg/mL) of the extracts were prepared. The different concentrations of aqueous extracts of bulb samples were prepared and used for the incubation of the examined immune cells.

Table 1. The scientific names and geographical locations of collection sites of the examined Allium species

<table>
<thead>
<tr>
<th>Allium Species</th>
<th>Collection Site</th>
<th>Latitude (N)</th>
<th>Longitude (E)</th>
<th>Altitude (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. sativum L.</td>
<td>A field in Hamedan Province</td>
<td>34° 47’ 49”</td>
<td>48° 30’ 51.4”</td>
<td>1737</td>
</tr>
<tr>
<td>A. Iranicum (Wendelbo)</td>
<td>Meshkin Shahr, Ardabil Province</td>
<td>38° 22’ 51.2”</td>
<td>47° 41’ 29”</td>
<td>1127</td>
</tr>
<tr>
<td>A. elburzense (Wendelbo)</td>
<td>Damavand, Tehran Province</td>
<td>35° 42’ 09.6”</td>
<td>52° 03’ 21.2”</td>
<td>2103</td>
</tr>
<tr>
<td>A. asarense R.M. Fritsch &amp; Matin</td>
<td>Asara, Alborz Province</td>
<td>36° 02’ 11.03”</td>
<td>51° 11’ 41.4”</td>
<td>1896</td>
</tr>
</tbody>
</table>
Study animals

Inbred strains of 6- to 8-week-old male BALB/c mice were purchased from the Pasteur Institute of Iran (Tehran, Iran).

Macrophages preparation

Peritoneal exudate cells were isolated using the intraperitoneal injection of cold Phosphate Buffered Saline (PBS) and washed. After that, the cells were seeded into RPMI 1640 (Sigma-Aldrich, USA), 10% FBS supplemented (Sigma-Aldrich, USA), and maintained in a humidified incubator with a 5% CO₂ atmosphere for 2 h, then non-adherent cells were eliminated. The adherent cells were washed with warm PBS. Finally, the macrophages were cultured in 96-well plates in RPMI 1640 containing 10% FBS, in the presence of different concentrations of bulb extract of *Allium* species (0.0001-1 mg/mL), except controls that were maintained at 37°C in a 5% CO₂ humidified incubator.

Lymphocyte preparation

Splenocytes were separated from mouse spleen using sterile needles and RPMI 1640 medium. After centrifugation, the cell suspension was washed with RPMI 1640. Then, the red blood cells were removed by treating with erythrocyte lysis buffer and the cells were centrifuged. Next, the cells were cultured on 96-well plates in the RPMI 1640 containing 10% FBS in the presence of the different concentrations of *Allium* species bulb extract (0.0001-1 mg/mL). However, the controls were kept at 37°C in a 5% CO₂ humidified incubator.

Cell viability and proliferation assay

The effects of *Allium* species extracts on macrophage and lymphocyte cells viability were measured by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. Macrophages and lymphocytes were incubated with the *Allium* species bulb extracts at different concentrations (0.0001-1 mg/mL) and media. After the incubation of macrophages for 24 h and lymphocyte for 48 h, MTT (20 µL of 5 mg/mL) (Sigma, USA) was added to each well and incubated for 4 h at 37°C and 5% CO₂. Then, the medium was removed and acidified isopropanol dissolved purple formazan crystals produced by viable cells. The absorbance was measured at 492 nm using Enzyme-Linked Immunosorbent Assay (ELISA).

Statistical analysis

The following formula was used to calculate the viability index of each treatment group:

$$Viability \ index = \frac{Mean \ estimated \ value \ for \ the \ controls - Mean \ estimated \ value \ for \ the \ samples}{Mean \ estimated \ value \ for \ the \ controls}$$

The obtained data were presented as mean±SE (Standard Error). Analysis of Variance (ANOVA) was followed by Tukey’s test to detect the mean score differences. P≤0.05 was considered significant. All statistical analyses were performed in SPSS.

Results

Macrophage viability index

After the treatment of macrophages with aqueous bulb extracts of *Allium* species, cell viability was determined by MTT assay. Then, the cell viability indices were obtained and analyzed statistically (Table 2). The obtained results revealed considerable differences between the extracts of 4 *Allium* species on the viability indices. There was no direct positive or negative correlation between the changes in the concentration of bulb extracts and their effects on viability indices. The bulb extract of *A. sativum* at most concentrations, except 0.0001 mg/mL, stimulated macrophage viability indices. Moreover, the bulb extract of *A. Iranicum* increased the macrophage viability indices at all applied concentrations, especially at higher concentrations (0.01-1 mg/mL). The bulb extracts of *A. Iranicum* at a 100-fold lower concentration (0.01 mg/mL), demonstrated the same stimulatory effect as *A. sativum* (1 mg/mL), on cell viability index of macrophages.

The bulb extract of *A. elburzense* induced the macrophage viability indices, except at 1 mg/mL. Macrophage viability indices reduced after the treatment at all concentrations of the bulb extracts of *A. asarense*, especially at 0.005, 0.1 and 1 mg/mL.

Lymphocyte viability index

After the treatment of lymphocytes with aqueous bulb extracts of *Allium* species, cell viability was determined by MTT assay. Then, cell viability indices were obtained and analyzed statistically (Table 3). The obtained results indicated considerable differences between the 4 *Allium* species extracts on viability indices. No direct positive or negative correlation was observed between the concentrations of bulb extracts and their effects on viability indices.
Table 2. The cell viability indices of macrophages after incubation with the bulb extracts of *Allium* species

<table>
<thead>
<tr>
<th>Species (Concentration mg/mL)</th>
<th>Macrophage Viability Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. sativum (1)</td>
<td>1.12 ±1</td>
</tr>
<tr>
<td>A. Iranicum (0.01)</td>
<td>1.1 ±1</td>
</tr>
<tr>
<td>A. Iranicum (1)</td>
<td>0.89 ±1</td>
</tr>
<tr>
<td>A. sativum (1)</td>
<td>0.77 ±1</td>
</tr>
<tr>
<td>A. sativum (0.001)</td>
<td>0.62 ±1</td>
</tr>
<tr>
<td>A. Iranicum (0.1)</td>
<td>0.6 ±1</td>
</tr>
<tr>
<td>A. elburzense (0.01)</td>
<td>0.57 ±1</td>
</tr>
<tr>
<td>A. sativum (0.05)</td>
<td>0.55 ±1</td>
</tr>
<tr>
<td>A. elburzense (0.0001)</td>
<td>0.39 ±1</td>
</tr>
<tr>
<td>A. sativum (0.005)</td>
<td>0.32 ±1</td>
</tr>
<tr>
<td>A. elburzense (0.05)</td>
<td>0.32 ±1</td>
</tr>
<tr>
<td>A. elburzense (0.005)</td>
<td>0.3 ±1</td>
</tr>
<tr>
<td>A. elburzense (0.001)</td>
<td>0.3 ±1</td>
</tr>
<tr>
<td>A. sativum (0.01)</td>
<td>0.23 ±1</td>
</tr>
<tr>
<td>A. Iranicum (0.005)</td>
<td>0.2 ±1</td>
</tr>
<tr>
<td>A. Iranicum (0.0001)</td>
<td>0.18 ±1</td>
</tr>
<tr>
<td>A. Iranicum (0.001)</td>
<td>0.18 ±1</td>
</tr>
<tr>
<td>A. elburzense (0.1)</td>
<td>0.17 ±1</td>
</tr>
<tr>
<td>A. asarense (0.001)</td>
<td>0.14 ±1</td>
</tr>
<tr>
<td>A. asarense (0.01)</td>
<td>0.14 ±1</td>
</tr>
<tr>
<td>A. Iranicum (0.05)</td>
<td>0.1 ±1</td>
</tr>
<tr>
<td>A. asarense (0.0001)</td>
<td>0.08 ±1</td>
</tr>
<tr>
<td>A. asarense (0.05)</td>
<td>0.02 ±1</td>
</tr>
<tr>
<td>A. asarense (1)</td>
<td>-0.02 ±1</td>
</tr>
<tr>
<td>A. asarense (0.005)</td>
<td>-0.04 ±1</td>
</tr>
<tr>
<td>A. asarense (0.1)</td>
<td>-0.05 ±1</td>
</tr>
<tr>
<td>A. elburzense (1)</td>
<td>-0.21 ±1</td>
</tr>
<tr>
<td>A. sativum (0.0001)</td>
<td>-0.33 ±1</td>
</tr>
</tbody>
</table>

The difference in the mean scores is significantly defined with non-identical letters at P<0.05.
indices. The lymphocytes incubation with *A. sativum* bulb extract at most applied concentrations, except at 1 mg/mL, significantly increased viability indices. The best effects of this species on viability indices were observed at lower concentrations. However, no noticeable changes were detected on lymphocytes viability indices after the treatment with bulb extracts of *A. Iranicum*.

The bulb extract of *A. elburzens* had no considerable influence on cell viability indices. The activity pattern of bulb extract of *A. asarense* on cell viability indices was different. The bulb extract of this species revealed insignificant stimulatory effects on cell viability of the lymphocytes at some concentrations. This is while it had nonsignificant inhibitory effects at 1 and 0.1 mg/mL. Interestingly, lymphocyte viability indices declined by the bulb extracts of all 4 *Allium* species at 1 mg/mL.

### Discussion

The immune system responds to pathogen infections through innate and adaptive immunity [3]. *Allium sa-

tivum possesses antitumor immune responses, as well as anti-inflammatory and immunomodulatory activities [22]. Moreover, most of the medicinal effects of this species and other Allium members (onion, shallot), are related to their organosulfur compounds [10, 14].

The literature review reveals a large body of research on the immunomodulatory properties of A. sativum and A. cepa. However, there are not sufficient reports on other Allium species. To introduce new medicinal plants with powerful immunomodulatory effects, in addition to A. sativum, the effects of bulb extracts of 3 wild grown Allium species on macrophages and lymphocytes viability were assessed in this research. After the incubation of macrophages with the bulb extracts of A. sativum, A. Iranicum, and A. elburzense, cell viability indices increased at most applied concentrations. Conversely, the bulb extract of A. asarense declined macrophage viability indices.

Sung et al. (2015) reported that the water extracts of A. sativum bulbs had no mitogenic effects on murine macrophage cell line J774A.1 [23]. Furthermore, the metabolic activity of macrophage declined down to 10% in the absence of LPS at 1:40 extract dilution. Another research reported various concentrations of LPS failed to influence RAW264.7 macrophage viability. In contrast, fresh and heated raw A. sativum bulb extracts remarkably decreased cell viability at 1% extracts [12]. Conversely, the obtained results indicated that A. sativum and A. Iranicum, especially at higher concentrations, significantly increased macrophage viability indices, and A. asarense decreased the indices.

Kim et al. (2017) claimed that after the treatment of RAW 264.7 cells with black A. sativum bulb-derived components, their cell viabilities did not significantly change [24]. A 14 kD protein derived A. sativum bulb had no impact on J774A.1 macrophage viability at 5-30 µg/mL [25]. According to Lee et al. A. sativum bulb-derived organosulfur compound of Diallyl Trisulfide (DATS) had no effect on murine RAW 264.7 macrophage cell viability at lower concentrations (10-30 µM) [26]. However, the cytotoxic effect of that compound was observed at ≥30 µM concentrations. Whereas, DATS failed to act as a cytotoxic organosulfur compound on LPS-incubated macrophages at ≤30 µM. Raw A. sativum bulb extract had more toxic effects (at concentrations ≥250 µg/mL), on macrophage viability, compared with the aged black A. sativum bulbs (up to 2000 µg/mL) [27]. This is while in this study, a noticeable enhancement was observed by the aqueous extracts of A. sativum on macrophage viability indices, especially at higher concentrations. In addition, there were progressive effects of A. Iranicum and A. elburzense on macrophage viability indices. However, the aqueous extract of A. asarense decreased the indices in particular, at higher concentrations (0.1 and 1 mg/mL).

A comparative study on the effects of Fresh Garlic Polysaccharides (FGPs) and Black Garlic Polysaccharides (A. sativum) (BGPs) bulbs demonstrated the stronger effect of FGP's than BGP's on the cell proliferation of RAW264.7 macrophages. Although various concentrations of fresh A. sativum polysaccharides (1.6-200 µg/mL), significantly raised the cell viability of RAW264.7 macrophages, BGP's had no effect on cell viability [1]. The present study findings are to some extent consistent with the above-mentioned reports. In other words, increasing or decreasing effects of A. sativum bulb extracts and the other used 3 Allium species were observed on macrophage viability indices.

The available evidence on A. sativum suggests that not only the applied bulb extract concentration or the assessed compound, but also the type of treated cell line are effective factors on the experiment outcomes. In this research, the aqueous extracts of A. sativum and A. Iranicum had remarkable and identical effects on macrophage viability indices, especially at higher concentrations. Also, the aqueous extracts of A. elburzense slightly induced the viability indices of treated macrophages. A. asarense decreased macrophage viability indices at most applied concentrations. Considering the exceptions, the bulb extract of A. sativum increased lymphocytes viability indices, while the bulb extract of A. asarense showed various impacts on viability indices. A. Iranicum and A. elburzense at all of the used concentrations had no considerable effects on viability indices.

Administration of A. sativum tablet increased ConA-induced murine splenic lymphocyte proliferation and macrophages phagocytic activity [28]. Isolated ~13KD proteins from raw A. sativum bulbs, had mitogenic effects on human peripheral blood lymphocytes, murine splenocytes, and thymocytes [29]. Kyo et al. [30] reported two-fold increases in proliferation after the treatment of mouse splenocytes with aged A. sativum bulb extract. Incubation of spleen isolated mononuclear cells at various concentrations (5-40 µg) of 14 kD protein derived A. sativum increased cell proliferation [31]. When murine thymocytes and splenocytes were treated with 50 µg/mL A. cepa fructooligosaccharides, 2.5 to 3.5 fold increase was observed in proliferative responses [20].

Based on our results, A. sativum bulb extract has stimulatory effects on lymphocyte viability indices, which are
comparable to the aforementioned results. In the case of A. Iranicum and A. elburzens species, no significant changes were observed on lymphocyte viability indices after incubation with their bulb extracts. Treatment of lymphocytes with A. asarense bulb extracts increased viability indices at most concentrations, except the highest ones (1 and 0.1 mg/mL). The bulb extract of A. asarense, however, had less stimulatory effects on lymphocyte viability indices, compared to A. sativum.

The identification of harmful microorganisms and elimination of damaged cells are performed by the immune mechanism. Immune system malfunctions cause the pathogenesis of some disorders such as cancer and autoimmunity. In these situations, the regulation of the innate and adaptive immune responses is induced by immunomodulatory agents. They act as prophylactic or therapeutic substances and induce immune activity against tumors, infections, and immunodeficiency diseases [4]. However, they can suppress immune activity in various immune-related disorders [4, 32]. Natural or chemical products are candidates to modulate immune responses. The side effects of synthetic drugs and the positive effects of plants and their components have been previously reported [2, 33].

Among herbs, garlic has immunostimulatory and immunosuppressive effects. Therefore, A. sativum, A. Iranicum, and A. elburzense, which have stimulatory effects on macrophage and lymphocyte viability indices, can be considered as suitable natural sources for studies on the treatment of cancers, infectious and immunodeficiency diseases. The bulb extract of A. asarense differently impacts macrophage and lymphocyte viability indices at different concentrations. Additionally, the effects of this species should be investigated in immune disorders, including cancer and autoimmunity. Further investigations on the effects of applied species on other immune cells and their cytokines and animal models of immune diseases are suggested.

All 4 examined Allium species demonstrated various stimulatory or inhibitory effects on viability indices of two immune cell types at different applied concentrations. The bulb extract of A. sativum had a stimulatory effect on macrophage viability indices at the highest concentration. In addition, A. Iranicum had the same stimulatory effect on macrophage viability indices at 100-fold lower concentration, compared to A. sativum. Therefore, A. Iranicum is suggested as an alternative wild source of more investigations on immunity and its related diseases. Among the 4 studied species, A. sativum as a prominent species had the highest effect on lymphocyte viability indices. The bulb extract of A. asarense differently affected the viability indices of macrophages and lymphocytes at various used concentrations. In addition to A. sativum and its compounds investigated on immunity, further studies on the effects of 3 other species on immune cells and immune-related diseases are recommended.

**Ethical Considerations**

**Compliance with ethical guidelines**

The present research was approved by the Ethics Committee of Research Ethics Board of Shahed University (code: 1394.103).

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**Authors contributions**

Conceptualization: All authors; Methodology: All authors; Invesigation: Tayebeh Radjabian, Zahra Hosseinpur Yektai, Tooba Ghazanfari, Shahn Zarré, Writing-original draft: Tayebeh Radjabian, Zahra Hosseinpur Yektai; Writing-review & editing: Tayebeh Radjabian, Zahra Hosseinpur Yektai, Tooba Ghazanfari; Resources: All authors; and Supervision: Tayebeh Radjabian.

**Conflict of interest**

The authors declared no conflict of interest.

**References**


[4] Wen CC, Chen HM, Yang NS. Developing phytochemicals from medicinal plants as immunomodulators. Advances in


