

Research Paper:

Immunomodulatory Impacts of Bulbs Extracts From Five *Allium* Species on IFN- γ , IL-4, and IL-17 Cytokines



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ABSTRACT

Background: Nowadays, the impacts of medicinal plants on immune-related diseases are one of the main pharmacological approaches. Thus, here, the modulatory potential of some *Allium* species on T helper cell cytokines were investigated.

Materials and Methods: The different concentrations of aqueous extract of the aged bulbs of five *Allium* species were prepared. Lymphocytes were then isolated and cultured in the presence of the bulb extracts. The amounts of IFN- γ , IL-4 and IL-17 were assessed using ELISA.

Results: The results demonstrated that *A. sativum* bulbs extract increased IFN- γ production at all concentrations, especially at 0.0001 and 0.01 mg/mL. After treatment with all doses of *A. asarensis* bulbs extract, IFN- γ production by lymphocytes was dropped, and this effect was remarkable at the lowest concentrations (0.0001, 0.001 and 0.005 mg/mL). The bulbs extract of *A. asarensis* enhanced IL-4 production by the treated cells, whereas the bulbs extracts of *A. sativum*, *A. jesdianum*, and *A. lenkoranicum* had inhibitory effects on the production of IL-4. Treatment with the bulbs extracts of *A. sativum* and *A. asarensis* caused increases in the secretion of IL-17 by the lymphocytes to some extent; however, the cytokine production decreased somewhat after incubation with *A. jesdianum* bulbs extracts. IFN- γ /IL-4 ratio was raised after treatment of lymphocytes with *A. stipitatum* and *A. sativum* bulbs extracts, while incubation with *A. asarensis* bulbs extracts decreased it.

Conclusion: Since the bulbs extracts of the studied *Allium* species demonstrated immunomodulatory features, with further research, they would be considered as useful candidate for clinical purposes.

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1. Introduction

Allium L. belongs to the Alliaceae family [1, 2]. Iran is the main center of *Allium* species diversity in central and southwest Asia, with ≥115 known species, including >40 endemic ones [3]. *Allium sativum* (garlic), as a prominent species of the genus, was cultivated for culinary practices and a therapeutic potency [4, 5] on account of its constituents, including sulfur compounds, enzymes, amino acids, lectins, and minerals [6, 7]. Garlic bulbs and their components were beneficial in combating various diseases in traditional medicine. For instance, in the Al-Qanoon Fi-al Tib (The Canon of Medicine) book, Avicenna wrote, garlic has been recommended for its potential therapeutic efficacies for arthritis, toothache, chronic cough, constipation, parasitic infestation, and infectious diseases [7, 8]. Currently, garlic’s antimicrobial, antioxidant, anti-carcinogenic, and anti-inflammatory traits have attracted particular attention from modern medicine [2, 8-11]. The medicinal influences of garlic have been attributed to the presence of sulfur compounds, namely allicin, diallyl disulfide, S-allyl cysteine, and diallyl trisulfide [12, 13].

Cytokines secreted by various cells mediate immunoregulatory activities and the modulation of immune-related disorders, such as cancer, allergy, and autoimmune diseases [14, 15]. Furthermore, they play critical roles in controlling immune responses through their specific receptors and activating cell signaling [15, 16]. Due to their diverse activities, the modulation of cytokine secretion was a pharmacological target to manage the diseases. Despite administering cytokines in the clinic, the primary concern for using these drugs is their side effects, e.g. transient lymphopenia or monocytopenia. Owing to the lower side effects, the administration of herbal medicines may be helpful for the regulation of cytokines [17]. Many scholars have reported garlic effects regarding the immunomodulatory features and bioactive constituents [9, 18-21]. Kang et al. [22] reported a considerable increase in TNF-α (Tumor Necrosis Factor-

alpha) production by the treated macrophages at 10 and 100 ng/mL of allicin.

Accordingly, there was a slight rise in IL-1 (Interleukine-1) and IL-6 (Interlukine-6) secretion by macrophages at 100 ng/mL of this metabolite. Clement et al. [23] separated 3 proteins (QR-1, QR-2, & QR-3) from 30 kDa ultrafiltrate of raw garlic extract. The 3 immunomodulatory proteins had mitogenic effects on human peripheral blood lymphocytes, murine splenocytes, and thymocytes, especially QR-2. Despite several reports on the impacts of garlic extracts on immunity, Information about whether other *Allium* species have immunomodulatory features remains scarce. Considering that several domesticated and wild *Allium* species grow in Iran, there are no reports about their biological activities. Therefore, conducting some research on the immunomodulatory effects of their extracts seems to be necessary. In the present study, to introduce some new plant species with potent immunomodulatory features, in addition to garlic, the impact of some wild *Allium* species on T helper cell cytokines was investigated for the first time.

2. Materials and Methods

Plant extract preparation

Cultivated *A. sativum* bulbs were purchased from a field in Hamedan (Iran), and wild plants of *A. lenkoranicum*, *A. jesdianum*, *A. asarense*, and *A. stipitatum* were collected from their natural habitats from different regions of Iran. The *Allium* species were taxonomically identified by Dr. Shahin Zarre (School of Biology, College of Science, University of Tehran), and a voucher specimen of each plant species was deposited in the Central Herbarium of the University of Tehran (TUH) (Table 1).

The aqueous extracts of the bulbs were prepared using the method of Wang et al. [19] with a slight modification. The peeled bulbs (100 g) of each examined spe-

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

<i>Allium</i> Species	Collection Site	Latitude (N)	Longitude (E)	Altitude (m)	Voucher No.
<i>A. sativum</i> L.	A field in Hamedan province	34° 47'	48° 30'	1737	-
<i>A. asarense</i> R.M. Fritsch & Matin	Asara- Alborz Province	36° 02'	51° 11'	1896	35782
<i>A. lenkoranicum</i> Miscz. ex Grossh	25 km from Khalkhal road to Asalem- Ardebil Province	37° 38'	48° 32'	1500	37322
<i>A. jesdianum</i> Boiss. & Buhse	20 km from Kandovan Village- East Azerbaijan Province	37° 47'	46° 14'	1950	35026
<i>A. stipitatum</i> Regel	Zagros mountain- Lorestan Province	33° 55'	48° 24'	2010	34799

cies, after storage at -20°C for 4-6 months, were crushed and homogenized with 100 mL of distilled water. Then, aqueous extracts were collected and added at different concentrations (0-0.1 mg/mL) to the culture media.

Animals

Six-to-eight-week-old male BALB/c mice were purchased from the Pasteur Institute of Iran (Tehran, Iran). They were kept under pathogen-free conditions.

Isolation and culture of lymphocytes

Lymphocytes were isolated from the mouse spleen using sterile needles and RPMI 1640 medium. After centrifugation, the cell suspension was washed with RPMI1640, and RBCs were excluded by Erythrocyte lysis buffer. The pellet was cultured into 96-well plates using RPMI 1640 containing 10% Fasting Blood Sugar (FBS) in the presence of the different concentrations of the bulb extract of *Allium* species (0-0.1 mg/mL), except controls, at 37 °C in a 5% CO₂ humidified incubator.

Cytokines assay

IFN-γ (Interferon-gamma), IL-4 (Interlukine-4), and IL-17 (Interlukine-7) are representative of Th1 (T-helper 1), Th2, and Th17, respectively. Therefore, lymphocytes were treated with Allium bulbs extracts to determine their concentrations. Then, the supernatants were collected and stored at -70 °C, and the cytokine levels in the supernatants were assayed using an ELISA kit (R&D Systems). All tests were performed according to the manufacturer’s guidelines.

All statistical analyses were performed by SPSS. An index production for cytokines assessing for each sample was calculated as follows:

$$\text{The index of cytokine production} = \frac{\text{The average values of controls} - \text{The values of samples}}{\text{The average values of controls}}$$

All tests were performed in triplicate, and the results were expressed as Mean±SE. One-way Analysis of Variance (ANOVA) was performed to determine statistically significant differences between mean scores. Tukey’s multiple-comparison test was used to compare the group means, and significant differences were considered at P≤0.05.

3. Results

The levels of IFN-γ production

The cytokine level was assessed to evaluate the effect of aqueous bulbs extracts of five *Allium* species on IFN-γ production by lymphocytes. As shown in Figure 1, all concentrations of *A. sativum* bulbs extract significantly increased the IFN-γ level, especially at 0.0001-0.01 mg/mL. However, it was noticeable that the inhibitory effects of *A. asarense* bulbs extract at all concentrations (0.0001-0.1 mg/mL) were remarkable compared to *A. sativum*. Furthermore, the bulbs extract of *A. stipitatum* slightly suppressed IFN-γ production. In contrast, those of *A. jesdianum* and *A. lenkoranicum* induced the IFN-γ production by lymphocytes at different concentrations.

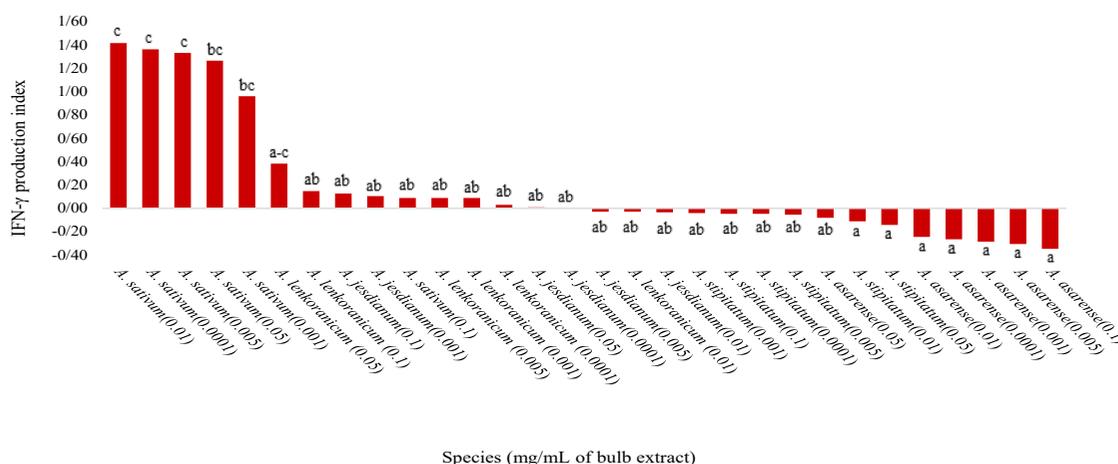


Figure 1. The effects of aqueous bulb extracts of the examined *Allium* species on IFN-γ production in lymphocytes. Values followed by the same letter (s) are not significantly different at P<0.05 based on Tukey’s multiple-comparison test.

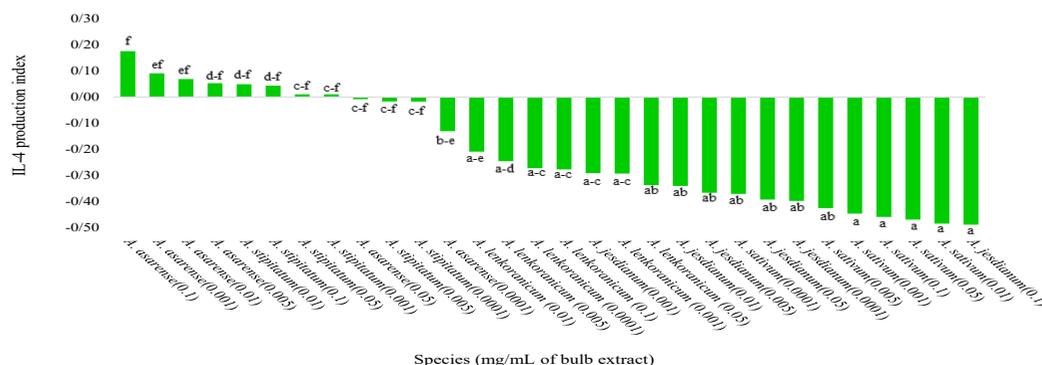


Figure 2. The effects of aqueous bulbs extracts of the examined *Allium* species on IL-4 secretion in lymphocytes. Values followed by the same letter (s) are not significantly different at $P < 0.05$ based on Tukey's multiple-comparison test.

The levels of IL-4 production

Data analysis indicated the dose-dependent effects of the five studied *Allium* species on IL-4 production at some concentrations. As per Figure 2, the treatment with the bulbs extract of *A. asarense* raised IL-4 production in the lymphocytes at most of the applied doses, remarkably at 0.1 mg/mL, although incubation at 0.0001 and 0.05 mg/mL led to a slight decrease in the IL-4 production. The bulbs extract of *A. sativum* and *A. jesdianum* at all used concentrations substantially dropped the production level compared to that of *A. asarense*. The suppressing effects of different concentrations of *A. lenkoranicum* bulbs extract, and the lowest dosages of *A. stipitatum* bulbs extract (0.0001 and 0.005 mg/mL) on IL-4 production were insignificant. At other concentra-

tions (0.001-0.1 mg/mL), *A. stipitatum* bulbs extract stimulated lymphocyte marginally for producing IL-4.

The levels of IL-17 production

The cytokine assays demonstrated a similar impact of *A. sativum* and *A. asarense* bulbs extracts in the regulation of IL-17 production. Thus, the incubation with bulbs extracts of both species caused insignificant increases in the levels of this cytokine in lymphocytes at all applied concentrations, except for 0.1 mg/mL of *A. asarense* extract. Moreover, compared to other species, the secretion of IL-17 by lymphocytes was decreased gradually in a dose-independent manner after treatment with bulbs extract of *A. jesdianum*. Moreover, the lowest inhibitory and stimulatory effects on IL-17 production were ob-

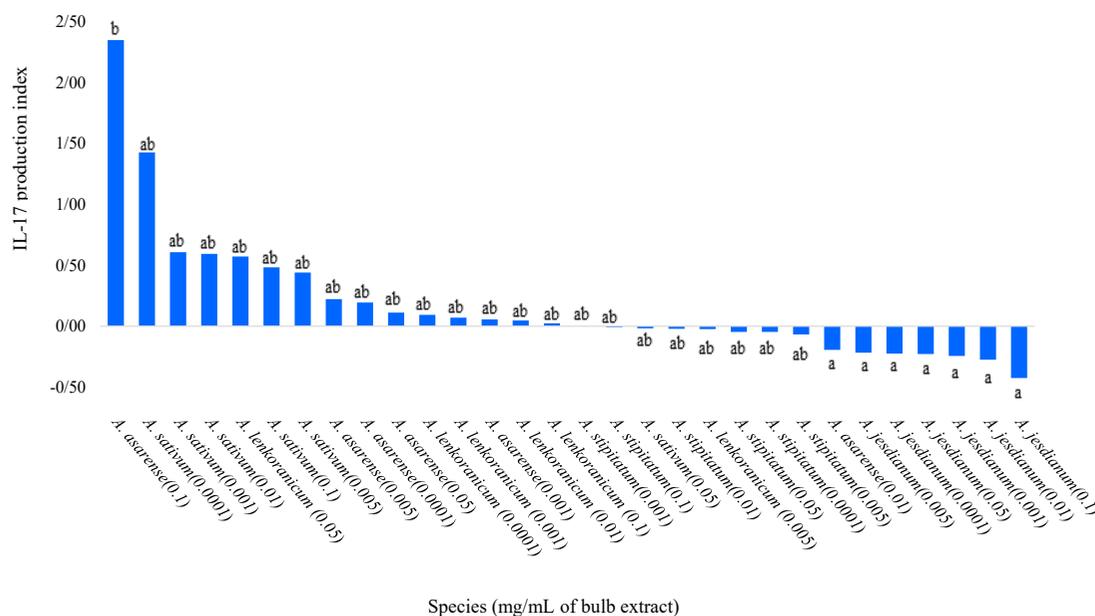


Figure 3. The effects of aqueous bulb extracts of the examined *Allium* species on IL-17 production in lymphocytes. Values followed by the same letter (s) are not significantly different at $P < 0.05$ based on Tukey's multiple-comparison test.

lymphocytes at 400 mg/kg dose. In another study, after 24h of the incubation of murine thymocytes with *A. cepa* (onion) agglutinin, high levels of IL-2 and IFN- γ with negligible effect on IL-4 level were detectable. Besides, in the mentioned research, the stimulatory effects of this lectin were higher at 0.01 $\mu\text{g}/\text{well}$, as a lower dose, than those of 10 $\mu\text{g}/\text{well}$, as a higher dose [27]. Here, the bulbs extract of *A. sativum* and *A. jesdianum* reduced the IL-4 produced by lymphocytes in a dose-independent manner. The bulbs extract of *A. lenkoranicum* also had a moderating effect on the decline of IL-4 levels at all concentrations. Contrary to our results, Zamani et al. [28] reported that the intake of garlic by rats caused increases in the IL-4 levels and stimulated Th2 responses; however, the production of IFN- γ decreased considerably. In other words, they suggested that administering garlic could switch on Th2 responses. Peyer's patch cell treated orally with garlic extract led to a substantial rise in the IFN- γ and IL-4 levels [29]. Based on our findings, different concentrations of bulb extract of *A. asarense* increased IL-4 levels.

Moutia et al. [30] assessed the effects of various doses of garlic extract on cytokine production by human Peripheral Blood Mononuclear Cells (PBMCs). They reported that producing IL-17 and its gene expression was significantly enhanced in PBMCs after treatment with garlic; however, the gene expression level of IL-4 was unchanged. A similar result of the present research indicated that treatment with *A. sativum* and *A. asarense* bulbs extracts was conducted to Th17 cytokine profile in protein level. However, the incubation of lymphocytes with *A. jesdianum* bulb extract resulted in a marginal decrease in IL-17 production. Interestingly, in the report of Moutia et al. [30], garlic extract suppressed IL-17 gene expression in the stimulated PBMCs with PHA as a mitogen. Another research reported a synergistic anti-inflammatory effect after co-treatment with *A. hookeri* and *Curcuma longa* extracts (3:7) in an air pouch model. The levels of IFN- γ , IL-1 β , IL-6, IL-13, and IL-17 declined after co-treatment with the extracts; although 500 mg/kg co-treatment consisted 350 mg/kg *A. hookeri* extract had a suppressor impact on IFN- γ and TNF- α secretion [31].

Similarly, in our work, treatments with *A. asarense* and *A. jesdianum* bulbs extract suppressed IFN- γ and IL-17 production, respectively, as inflammatory cytokines. Shirgholami et al. [32] reported substantial increases in the IFN- γ levels in serum of infected BALB/c mice with cystic echinococcosis after administering 20 mg/kg and 80 mg/kg methanolic extract of *A. sativum*. Several factors may influence the production of cytokines in the treated lymphocytes with extracts of *Allium* species.

They might include normal or pathologic conditions in the study, the type of administered species and their various preparations, the concentrations of used extract, and *in vitro* or *in vivo* studies.

The IFN- γ /IL-4 ratio represents the ratio of Th1/Th2 in lymphocytes. This project detected the highest stimulatory effects on the IFN- γ /IL-4 ratio in the lymphocytes after treatments with *A. stipitatum* (0.05 mg/mL) and *A. sativum* bulbs extracts. The incubation of lymphocytes with bulbs extract of *A. asarense* caused a decrease in the ratios of these cytokines. In a study designed by Liu and co-workers [33], administering garlic oil to mice at 100 mg/kg revealed an increase in IL-2 and IFN- γ levels, followed by a marginal decrease at 200 mg/kg. IL-4 and IL-10 production increased dramatically at the same doses, especially 200 mg/kg. The maximum ratio of IFN- γ /IL-4 was achieved at the dose of 50 mg/kg, and after that, the ratio experienced a decline at 100 mg/kg and 200 mg/kg doses. They concluded that garlic oil is involved in Th1 polarization at the lower doses; however, it could induce Th2 responses at the higher doses. Shamluo et al. [26] indicated that the extract of shallot suppressed Th1 and Th2 responses and their cytokines produced by lymphocytes. Here, the bulbs extracts of *A. stipitatum* and *A. sativum* regulated the immune system toward Th1 responses. Still, incubation with the bulbs extract of *A. asarense* dominantly led to Th2 response, especially at the lower concentrations. The studied species and their components and the used doses seemed to have had various effects on IFN- γ /IL-4 ratios.

Since cytokines are secreted in response to pathological conditions, regulating their levels is more likely to be a therapeutic strategy in immune-associated disorders, such as cancer, asthma, and autoimmunity. Furthermore, aged garlic extract had anti-cancer activity in mice by stimulating natural killer cells to secrete IFN- γ , IL-2, and TNF- α [34]. Moreover, the intra-peritoneal injection of black garlic extract boosted NK activity and the generation of IFN- γ , IL-2, and TNF- α secretion from Th1 cells, whereas IL-4 production was suppressed by T helper type 2 cells [35]. Following the increasing impacts of *A. stipitatum* and *A. sativum* bulb extracts on IFN- γ /IL-4 ratio and enhancing Th1 responses in our attempt, the effect of two species on the animal models of cancers can be assessed. There are pieces of evidence to support that tissue specific and systemic autoimmune diseases are the consequences of IFN- γ overexpression. Multiple Sclerosis (MS), Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis (RA), and Type1 Diabetes (T1D) are examples of autoimmune diseases in which IFN- γ and Th1 cells have a substantial role [36]. Regard-

ing the obtained results, treatment with bulbs extract of *A. asarense*, especially at the lower doses, decreased IFN- γ production in the lymphocytes; IFN- γ /IL-4 ratios were also declined. The impacts of the extracts should be examined to determine whether they would relieve the mentioned autoimmune diseases in animal models or not. The secretion of IL-17 by Th17 participates in various inflammatory diseases, such as MS, RA, and Inflammatory Bowel Diseases (IBDs). Based on the findings of Hodge et al. [37], garlic extract at ≥ 10 $\mu\text{g/mL}$ diminished the production of TNF- α , IL-1 α , IL-6, and IL-8 by monocytes and the secretion of IFN- γ , IL-2, and TNF- α by T cells isolated from patients with IBD. IL-17, as an inflammatory cytokine, plays an essential role in the indication of IBD; thus, treatment with bulbs extract of *A. jesdianum* is more prone to be an appropriate option to investigate its effects on the animal model of IBD.

In the case of allergic diseases, the effects of intraperitoneal injections of aged garlic extract were assessed by Zare et al. [38] in a murine model of allergic airway inflammation. Their results indicated that three-time intraperitoneal injections of the extract led to significant decreases in the signs of allergy, including the percentage of eosinophil in lavage, IgG1 levels in the serum and lavage, the number of mucus-producing goblet cells in the airways, and perivascular inflammation. Additionally, based on their suggestion, an isolated 14 kDa glycoprotein from extract had a crucial role in T helper-1 responses and immunomodulatory effects of garlic extract. Since the bulbs extracts of *A. sativum*, *A. jesdianum*, and *A. lenkoranicum* played inhibitory functions in the production of IL-4, the impacts of the bulbs extracts of these three species can reduce or delay inflammatory reactions in allergic diseases. However, further investigations are required in this respect.

5. Conclusion

This investigation evaluated the possible immunoregulatory effects of bulbs extracts of some selected *Allium* species. Besides *A. sativum*, the bulbs extracts of other examined *Allium* species showed stimulatory or suppressive effects on IFN- γ , IL-4, and IL-17 production under normal conditions. Interestingly, *A. asarense* bulbs extract inhibited IFN- γ production while stimulated IL-4 production. The bulbs extract of *A. jesdianum* also suppressed IL-17 production; however, *A. sativum* and *A. asarense* extracts stimulated this cytokine production. Therefore, the extracts of *A. asarense* and *A. jesdianum* are considered potential anti-inflammatory responses. Our findings revealed that different concentrations of *A. sativum* bulb extract enhanced IFN- γ /IL-4 ratios, while *A.*

asarense bulbs extract had suppressive effects on IFN- γ production by lymphocytes. Thus, the immunoregulatory features of *Allium* species look promising in clinical applications, and more studies are needed to identify their therapeutic roles on immune-related diseases.

Ethical Considerations

Compliance with ethical guidelines

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

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Authors' contributions

Conceptualization, methodology, resources: All authors; Investigation: Tayebeh Radjabian, Zahra Hosseinpour Yektaei, Tooba Ghazanfari; Writing original draft: Tayebeh Radjabian, Zahra Hosseinpour Yektaei; Writing, review, and editing: Tayebeh Radjabian, Zahra Hosseinpour Yektaei, Tooba Ghazanfari; and Supervision: Tayebeh Radjabian.

Conflicts of interest

The authors declared no conflict of interests.

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