Original article Casp. J. Vet. Sci., 2025; 2(2): 33-44

Amol University of Special Modern Technologies

**Caspian Journal of Veterinary Sciences**

**doi: 10.22034/CJVS.2025.545281.1045**

**Journal homepage: https://Cjvs.ausmt.ac.ir/**

# Evaluation of the effects of fungal beta-glucan and *Zataria multiflora* on the stimulation of respiratory burst and secretion of TNFα cytokine in Balb/c mice

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**Article Info**

**Article history:**

Submit Date: 5 September 2025

Accept Date: 4 October 2025

Online Date: 4 December 2025

**Keywords:**

Balb/c mice

Respiratory burst

Standard beta-glucan

TNFα cytokine

*Zataria multiflora*

## Abstract

Fungal beta-glucan is among the most important immune stimulant, which can enhance the immune system and be used as medication to treat a wide range of diseases. The aim of this study was to evaluate the effects of respiratory burst stimulation in neutrophils and the secretion of tumor necrosis factor alpha (TNFα) cytokine by standard beta-glucan and *Zataria multiflora* in Balb/c mice. Laboratory animals were divided into four groups (B, C, E, and F) and treated with beta-glucan, *Z. multiflora*, and distilled water. The blood samples were collected on fourth and seventh days after intraperitoneally injections and then subjected to chemiluminescence testing (to measure neutrophil respiratory burst) and enzyme-linked immunosorbent assay (ELISA) testing (to measure the TNFα cytokine). In the chemiluminescence, on the fourth and seventh days, the highest respiratory burst activities were in the combination of standard beta-glucan and *Z. multiflora* (262.5 millivolts and 283.75 millivolts), respectively. In the ELISA, the highest level of TNFα on the fourth day was observed in the combination of standard beta glucan and *Z. multiflora* (2.95), followed by standard beta-glucan (2.45), and *Z. multiflora* (1.64). On the seventh day, the highest levels were also in the combination of standard beta-glucan and *Z. multiflora* (2.56), followed by standard beta-glucan (2.27), and *Z. multiflora* (1.84). The combination of standardized beta-glucan and *Z. multiflora* has significant immunomodulatory effects and can be utilized as an adjuvant, either alone or in a complex form, for the prevention or treatment of patients with immune deficiencies.

## Introduction

Over the past 25 years, the prevalence of microbial infections has significantly increased among patients with cancer, those undergoing prolonged treatment with broad-spectrum antibiotics, corticosteroids, cytotoxic drugs, and organ transplant recipients. Numerous risk factors have been identified that can suppress the host's immune mechanisms, making them more susceptible to acute diseases. Therefore, extensive efforts have been made to identify immune-stimulating factors. The use of immune stimulants as a preventive regimen or as part of a therapeutic regimen may enhance the host's immune system's ability to combat infectious agents. Immune stimulants can activate non-specific immune cells, such as monocytes, macrophages, neutrophils, and natural killer (NK) cells. The activity of these cells serves as the body's first line of defense, destroying a wide range of infectious agents, such as bacteria, fungi, parasites, and to some extent, viruses. Additionally, by effectively presenting antigens, they activate the adaptive immune system, which includes cellular and humoral immune responses (Sell, 1987).

Beta-glucan antigen is one of the most important immune-stimulating factors that has been extensively studied. This antigen is a carbohydrate polymer composed of glucose units, found in the cell walls of yeasts, mushrooms, bacteria, algae, and grains. Beta-glucan from different sources has unique structures and properties that influence their biological activities (Bohn and BeMiller, 1995; Kubala *et al*., 2003). One of the most important sources of beta-glucan is the cell wall of the *Saccharomyces cerevisiae* fungus. Numerous studies have shown that beta-glucan derived from this fungus has a high affinity for specific receptors on the surface of phagocytic cells. By activating these cells, it leads to the release of bactericidal products, such as lysozyme, reactive oxygen species, and nitric oxide. Additionally, it promotes the release of effective cytokines, which in turn activate peripheral lymphocytes associated with adaptive immunity (Carrow, 1996; Brown and Gordon, 2003).

*Zataria multiflora*, a plant from the mint family, is a shrub-like herb with thin, stiff, and highly branched stems. Its flowers have a membranous calyx with triangular-shaped teeth and are clustered in a circular arrangement near the leaves. The aerial parts of the plant are used, and it is native to Iran, where it has been traditionally utilized in Iranian medicine (Paulsen, 2001; Khosravi *et al*., 2007). The plant contains different chemical compounds, including thymol, carvacrol, p-cymene, trans-caryophyllene, linalool, methyl ether carvacrol, thymol acetate, carvacrol acetate, aromadendrene, terpinen-4-ol, valencene, spathulenol, and borneol. *Z. multiflora* exhibits various properties, such as antispasmodic, antibacterial, antifungal, anthelmintic, antioxidant, anti-mutagenic, anti-allergic, anti-inflammatory, and analgesic effects. It also aids in relieving bloating and strengthens the immune system (Shokri, 2018). The study aimed to evaluate the stimulatory effects of standard beta-glucan and *Z. multiflora* on neutrophil respiratory burst activity and TNFα cytokine secretion in an animal model.

## Materials and Methods

### Standard beta-glucan

Beta-glucan derived from the cell wall of *S. cerevisiae* is a type of soluble fiber that dissolves in distilled water (DSM Firmenich Co., Switzerland).

### *Zataria multiflora*

In this study, the essential oil of *Z. multiflora* was purchased from Barij Essence Company (Kashan, Iran).

### Preparation of the combination of standard beta-glucan and *Z. multiflora*

A standard amount of 5 mg of beta-glucan powder was dissolved in 1 ml of sterile distilled water. Separately, 20 µl of *Z. multiflora* essential oil was diluted in 980 µl of distilled water and mixed in equal volume with the standard beta-glucan solution (1:1 ratio). The resulting solution was vortexed for two hours to ensure maximum dispersion and entrapment of the aqueous phase within the oil phase, creating a uniform mixture. The prescribed amounts were calculated based on the weight of the mice under study, dissolved in 120 µl of distilled water, and administered to the mice via injection.

### Mice immunization

Balb/c female mice, aged four to six weeks, were purchased from the Razi Institute (Karaj,

Iran). The animals were housed under standard conditions, with eight mice per cage, and provided with proper care and feeding. Four groups were formed for immunization of the mice as follows:

⁂ Group B: Eight mice were intraperitoneally injected with standard beta-glucan at a dose of 15 mg/kg.

⁂ Group C: Eight mice were intraperitoneally injected with *Z. multiflora* essential oil at a dose of 100 mg/kg.

⁂ Group E: Eight mice were intraperitoneally injected with a combination of standard beta-glucan and *Z. multiflora* essential oil at the aforementioned doses.

⁂ Group F: Eight mice were intraperitoneally injected with distilled water (control group).

To evaluate the immune-stimulating potential of these compounds, on the fourth and seventh days post-injection, four mice from each group were anesthetized using chloroform, and blood samples were collected via cardiac puncture. The collected blood was immediately divided into two portions: a portion was placed in microtubes containing five units of heparin for testing neutrophil respiratory burst activity and the remaining blood was transferred into non-heparinized microtubes for serum separation and ELISA testing to measure TNFα cytokine secretion. For serum separation, the blood was left undisturbed for 20 minutes to clot and then centrifuged at 2500 g for 20 minutes. The separated serum was stored in a freezer at -80°C until further analysis of the desired cytokine.

**Quantitative luminescence test for measuring the respiratory burst activity of neutrophils**

Initially, 0.5 ml of heparinized blood in a syringe was mixed with 0.25 ml of 6% dextran solution in saline. The syringe was placed vertically at room temperature for 30 minutes. During this time, dextran aggregated the red blood cells, causing them to sediment. The plasma enriched with leukocytes collected above the sediment was carefully transferred to a plastic tube containing 0.19 ml of Ficoll by gently bending the needle tip and pressing the syringe piston, ensuring it layered over the Ficoll without mixing. The tube was centrifuged at 1800 g for 10 minutes. This process resulted in neutrophils and red blood cells settling at the bottom, while a distinct white ring of mononuclear cells appeared between the upper layer and the Ficoll layer. The supernatant was carefully removed using a Pasteur pipette, leaving only the sediment containing neutrophils and red blood cells. The sediment was washed once with Phosphate-buffered saline (PBS) and centrifuged at 1800 g for 10 minutes. After discarding the supernatant, 1 ml of distilled water was added to lyse the remaining red blood cells for 30-40 seconds while gently shaking the tube. PBS buffer was then added to neutralize the hypotonic effect of the distilled water. The tube was immediately centrifuged again at 1800 g for 10 minutes, and the supernatant was discarded. The washing process with PBS was repeated once more, resulting in neutrophils with a purity of over 96%. The viability of the neutrophils was assessed using eosin Y dye. The dye was mixed in equal proportions with a portion of the cell suspension solution and examined under a standard microscope at low magnification. The viability of neutrophils was found to be over 95%. The remaining sediment, containing pure neutrophils, was diluted with PBS buffer to achieve a concentration of $5 \times 10^6$ neutrophils per ml. For measuring neutrophil chemiluminescence activity, 0.1 ml of the neutrophil suspension, 0.2 ml of prepared luminol solution, 0.2 ml of prepared PMA (Phorbol-12-myristate-13-acetate) solution, and 0.5 ml of PBS buffer were added sequentially into a specialized polystyrene cuvette for the luminometer. After thorough mixing, the cuvette was immediately placed in a luminometer pre-set to 37°C. The light intensity produced was recorded in millivolts until it reached a maximum value and subsequently began to decline. At this point, the cuvette was removed, and the next experiment was commenced (Steele, 1991).

**ELISA test for measuring TNFα cytokine**

In this method, the RF51017 ELISA kit (GIBCO Company) was used. Serum samples from the treatment and control groups of mice were collected and used to measure the desired cytokine.

a) Preparation of the standard (recombinant murine TNFα): The standard was mixed with 400 µl of diluent solution A, resulting in a standard solution with a concentration of 50 ng/ml. Then,

80 µl of this solution was mixed with 586.7 µl of diluent solution A, producing the main standard solution with a concentration of 6000 pg/ml. For serial dilution, the following steps were taken: 200 µl of the main standard solution (6000 pg/ml) was added to the first tube containing 400 µl of diluent solution A, thoroughly mixed, and a concentration of 2000 pg/ml was achieved. Next, 200 µl from the first tube was transferred to the second tube containing 400 µl of diluent solution A, resulting in a concentration of 666.7 pg/ml. This process was repeated up to the sixth tube, yielding concentrations of 222.2, 74.07, 24.69, and zero pg/ml in sequence. The tube containing only diluent solution A was considered the zero standard, and the tube with the main standard solution (6000 pg/ml) was considered the highest standard.

b) Test procedure: 100 µl of the standard and test samples were added to the wells of the plate and gently shaken in a circular motion for one minute. The wells were then covered and incubated at room temperature for two hours. The contents of the wells were emptied by inverting the plate firmly. Subsequently, 200 µl of wash buffer was added to each well (using multi-channel pipettes to dispense the wash buffer with pressure), and the plate was inverted again. This washing step was repeated four times. After the final wash, the plate was firmly tapped on clean white paper to ensure no residual wash buffer remained in the wells. To confirm this, the plate was held up to light to check for any remaining liquid in the wells. Next, 100 µl of biotinylated anti-murine TNFα antibody was added to all wells, covered, and incubated at room temperature for one hour. The washing step was repeated as described previously. Then, 100 µl of HRP-Streptavidin solution was added to all wells and incubated at room temperature for 45 minutes. The washing step was repeated again. Afterward, 100 µl of substrate solution (TMB) was added to all wells, and the plate was gently shaken to ensure thorough mixing of the well contents. The plate was incubated in a dark place at room temperature for 30 minutes. Finally, 50 µl of stop solution was added to all wells. The optical density (OD) of the samples was read at 450 nm (color stability lasts for 30 minutes).

**Statistical analysis**

The findings from the respiratory burst of neutrophils and the secretion levels of the cytokine TNFα were expressed as mean ± standard deviation (Mean ± SD) in the control and treatment groups using descriptive analysis and SPSS software version 12 (USA). Subsequently, the differences between each treatment group and the control group were statistically analyzed using the t-student test, with significance levels set at $p < 0.05$.

## Results

**Neutrophil respiratory burst assay**

**A) The respiratory burst responses in mice treated with standard beta-glucan (Group B)**

The study examined the effect of standardized beta-glucan on respiratory burst activity in mice, revealing significant findings. On the fourth day, treated mice exhibited the lowest and highest respiratory burst levels at 98 and 298 millivolts, respectively, with an average of 186.12 millivolts. This represents a 252% increase compared to the control group. Although the respiratory burst response of treated mice decreased by the seventh day, this reduction was not statistically significant. However, compared to the control group, there was still a 234% increase on the seventh day, with an average of 169.75 millivolts. Statistical analysis confirmed significant differences in respiratory burst activity between treated and control groups on both the fourth day ($p = 0.003$) and the seventh day ($p = 0.001$), as illustrated in Figs. 1 and 2.

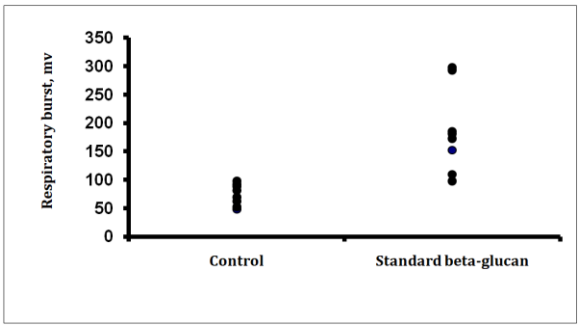


**Fig. 1.** Comparison of the respiratory burst activity of neutrophils between mice treated with standard beta-glucan and the control group on the fourth day.

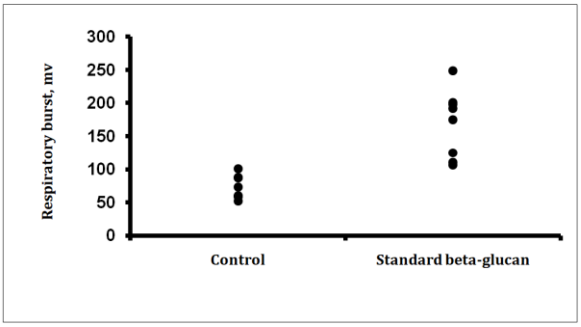


**Fig. 2.** Comparison of the respiratory burst activity of neutrophils between mice treated with standard beta-glucan and the control group on the seventh day.

### B) The respiratory burst responses in mice treated with *Z. multiflora* (Group C)

As shown in Figs. 3 and 4, the respiratory burst activity of stimulated neutrophils in the treatment group varies from 95 to 171 millivolts on day four and from 95 to 245 millivolts on day seven. Analysis of the results indicated that the mean response in the treatment group was approximately 122.2 millivolts on day four and 170.62 millivolts on day seven, representing a 165% and 235% increase, respectively, compared to the control group. Statistical tests revealed significant differences in respiratory burst activity between treated and control mice on both day four ($p$ = 0.002) and day seven ($p$ = 0.001). Furthermore, the respiratory burst activity of neutrophils in treated mice was higher on day seven than on day four, showing a 140% increase, with a statistically significant difference between these two time points ($p$ = 0.03).

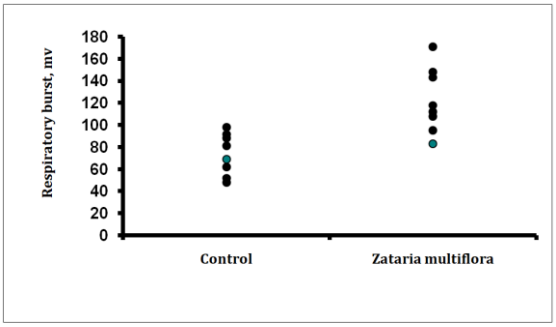


**Fig. 3.** Comparison of the respiratory burst activity of neutrophils between mice treated with *Zataria multiflora* and the control group on the fourth day.

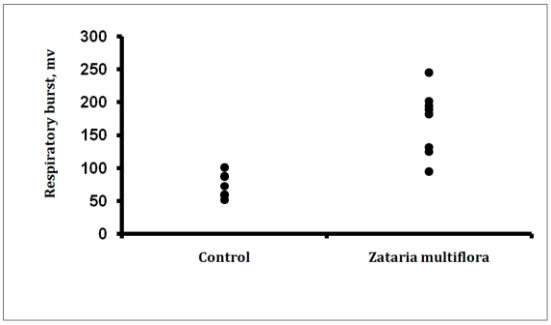


**Fig. 4.** Comparison of the respiratory burst activity of neutrophils between mice treated with *Zataria multiflora* and the control group on the seventh day.

### C) The respiratory burst responses in mice treated with standard beta-glucan + *Z. multiflora* (Group E)

The statistical analysis results of the respiratory burst index in mice treated with the standardized beta-glucan + *Z. multiflora* combination were presented in Figs. 5 and 6. As observed, the highest and lowest stimulation responses in the treatment group on the fourth day were 413 and 161 millivolts (mean 262.5 millivolts), respectively, and on the seventh day, 395 and 193 millivolts (mean 283.75 millivolts), respectively. The use of this combined solution resulted in an increase in respiratory burst stimulation by 355% on the fourth day and 392% on the seventh day compared to the control group. Statistical tests indicated a significant difference between the two groups ($p$ = 0.0005 on the fourth day, $p$ = 0.0005 on the seventh day). Although respiratory burst activity was higher on the seventh day compared to the fourth day, no significant difference was observed between these two days (Fig. 7).

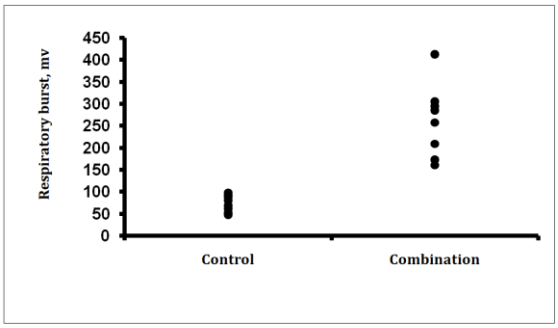


**Fig. 5.** Comparison of the respiratory burst activity of neutrophils between mice treated with beta-glucan+ *Zataria multiflora* (combination) and the control group on the fourth day.

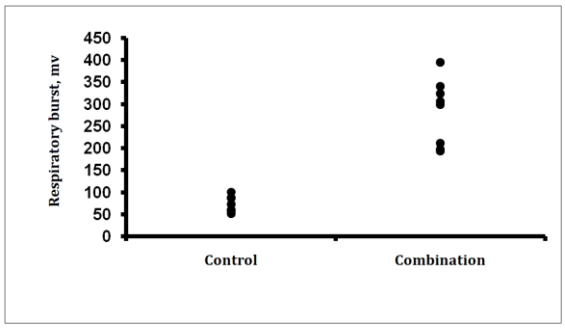


**Fig. 6.** Comparison of the respiratory burst activity of neutrophils between mice treated with beta-glucan+ *Zataria multiflora* (combination) and the control group on the seventh day.

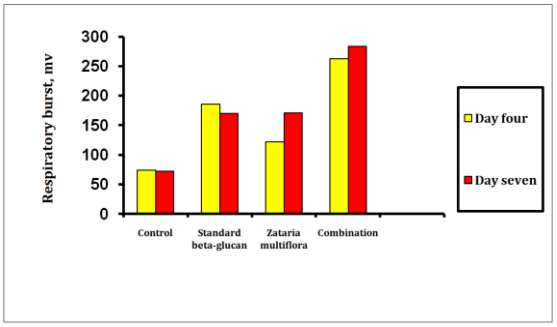


**Fig. 7.** Comparison of the respiratory burst activity of neutrophils on the fourth and seventh days in different groups understudy.

## ELISA test for measuring TNFα cytokine

### A) TNFα cytokine measurement in mice treated with standard beta-glucan (Group B)

Figs. 8 and 9 illustrated the quantitative distribution of TNFα secretion levels in treated and control mice. Statistical analysis indicated that the mean secretion levels of TNFα in treated mice on both day four (2.44) and day seven (2.24) show a significant difference compared to the control group. Specifically, there was a 500% increase on day four ($p$ = 0.0005) and a 436% increase on day seven ($p$ = 0.0005) relative to the control group. The highest and lowest secretion levels of this cytokine were observed as 3.1 and 1.57 on day four, and 2.8 and 1.5 on day seven, respectively. Although the mean secretion of TNFα on day seven was lower than on day four, no statistically significant difference was observed between the two days in this regard.

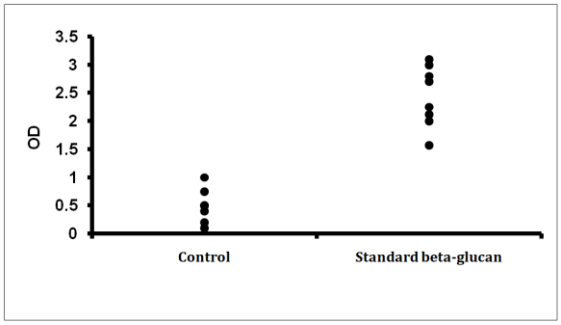


**Fig. 8.** Comparison of TNFα cytokine levels between mice treated with standard beta-glucan and the control group on the fourth day.

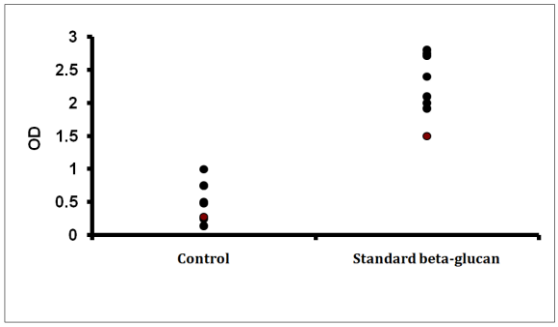


**Fig. 9.** Comparison of TNFα cytokine levels between mice treated with standard beta-glucan and the control group on the seventh day.

### B) TNFα cytokine measurement in mice treated with *Z. multiflora* (Group C)

The results of using *Z. multiflora* to evaluate one of the innate humoral immunity factors (TNFα cytokine) were presented in Figs. 10 and 11. The findings indicated that mice treated with doses of 2.5 and 0.75 exhibited the highest and lowest levels of TNFα secretion, respectively, on the fourth day, while doses of 2.9 and 0.75 showed similar patterns on the seventh day. Statistical analysis revealed a significant increase of 334% (mean 1.64, $p$ = 0.001) on the fourth day and 353% (mean 1.84, $p$ = 0.0005) on the seventh day compared to the control group. Unlike the results obtained from standard beta-glucan, the average TNFα secretion induced by *Z. multiflora* was higher on the seventh day than on the fourth day; however, this difference between the two days was not statistically significant.

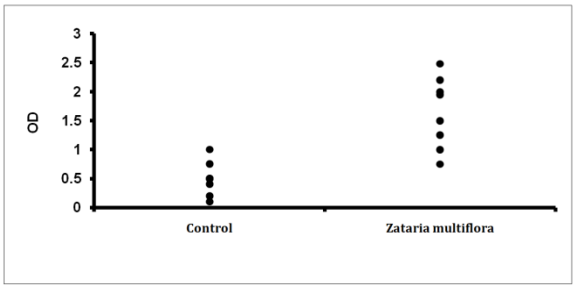


**Fig. 10.** Comparison of TNFα cytokine levels between mice treated with *Zataria multiflora* and the control group on the fourth day.

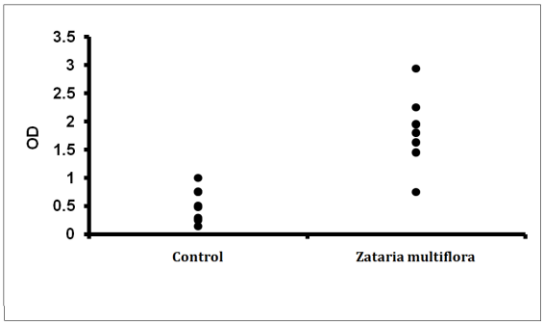


**Fig. 11.** Comparison of TNFα cytokine levels between mice treated with *Zataria multiflora* and the control group on the seventh day.

### C) TNFα cytokine measurement in mice treated with standard beta-glucan + *Z. multiflora* (Group E)

According to Figs. 12 and 13, the average secretion level of TNFα cytokine on the fourth day was approximately 2.95 (a 602% increase compared to the control group), and on the seventh day, it was around 2.56 (a 492% increase compared to the control group). Although the secretion level of this cytokine decreased on the seventh day, the reduction compared to the fourth day was not statistically significant. Statistical analysis of the results indicated a significant difference between the treatment and control groups in terms of TNFα secretion levels on both the fourth day ($p$ = 0.0005) and the seventh day ($p$ = 0.0005) (Fig. 14).

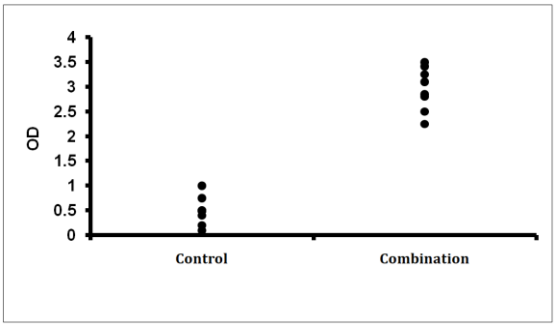


**Fig. 12.** Comparison of TNFα cytokine levels between mice treated with beta-glucan+ *Zataria multiflora* (combination) and the control group on the fourth day.

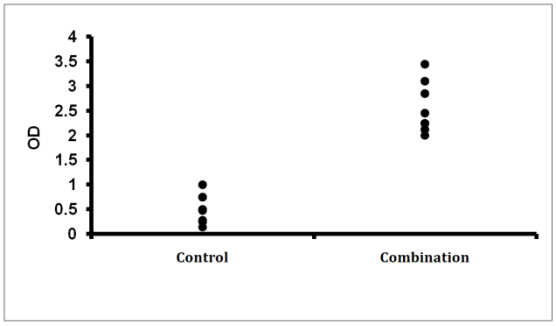


**Fig. 13.** Comparison of TNFα cytokine levels between mice treated with beta-glucan+ *Zataria multiflora* (combination) and the control group on the seventh day.

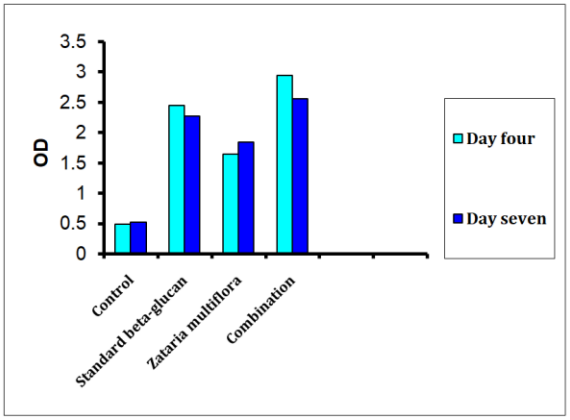


**Fig. 14.** Comparison of TNFα cytokine levels on the fourth and seventh days in different groups understudy.

### Discussion

*S. cerevisiae* is a eukaryotic, unicellular fungus widely used as a research model in various fields of science, medicine, and biotechnology (Magnelli

*et al*., 2001). Its cell wall consists of three main layers: an outer glycoprotein layer (35-40%), a middle layer of alkali-soluble beta-glucan (15-20%), and an inner layer of alkali-insoluble beta-glucan (35%) (Lipke, 1998). Beta-glucan is one of the most important immune-stimulating molecules, and extensive studies have been conducted on its properties. Approximately 60 years ago, Pillemer and Ecker (1941) enzymatically digested *S*. *cerevisiae* to isolate a compound that reacted with serum components and facilitated the elimination of pathogenic microorganisms. In 1957, Benacerraf and Sebastian (1957) published a study on the immunogenic effects of a crude, insoluble product derived from the cell wall of *S. cerevisiae*, called zymosan, on macrophage cells. Later, in 1979, Di Luzio *et al*. (1979) in New Orleans demonstrated that the active component in zymosan was beta-glucan, which could enhance host resistance to diseases and inhibit tumor growth.

In recent years, due to humans and animals being exposed to numerous risk factors, such as microbial pathogens and environmental toxins like mycotoxins, their innate immunity has gradually diminished, making them more susceptible to acute diseases. Therefore, extensive efforts are being made worldwide to identify various immune-stimulating agents that can be used as therapeutic or preventive regimens to address immune deficiencies or to stimulate and enhance immune activity. In this study, an attempt was made to use intraperitoneal administration of beta-glucan in mice, as it is a simpler method requiring a smaller amount of beta-glucan. The present study aimed to evaluate the innate immune-stimulating potential of standard beta-glucan, assess the immune-stimulating capability of *Z. multiflora* essential oil, and develop an appropriate combination of standard beta-glucan and *Z. multiflora* essential oil. In this regard, two factors of innate immunity, cellular and humoral, were assessed, including neutrophil respiratory burst and the secretion levels of TNFα cytokine.

The study discusses the effects of beta-glucan on respiratory burst responses and immune activity in mice. Neutrophils in mice treated with standard beta-glucan showed significant responses on day four (2.5 times) and day seven (2.3 times) compared to control mice. Researchers suggest that variations in biological and immunological responses to different beta-glucans depend on factors like side chain presence, molecular weight, spatial structure, and polymer charge (Bohn and BeMiller, 1995; Adachi *et al*., 1998; Kubala *et al*., 2003; Rice *et al*., 2004). Another study demonstrated that phagocytic cells reach peak activity around 72 hours post-beta-glucan administration, with a gradual decline after 144 hours (Carrow, 1996). The results of respiratory burst stimulation in beta-glucan-treated mice indicated that while activity levels were higher on day four than day seven, the difference was not statistically significant. This aligns with findings from Cleary *et al*. (1999), who observed changes in macrophage morphology, increased intracellular acid phosphatase, nitric oxide production, and superoxide generation three days after intraperitoneal beta-glucan administration. Further studies, such as those by Mucksova *et al*. (2001), showed that a single intraperitoneal dose of 15 mg/kg beta-glucan significantly increased peritoneal macrophage peroxidase activity and nitric oxide production. Ohno *et al*. (1996) found that nitric oxide synthesis peaked three to seven days after administering 250 µg beta-glucan intraperitoneally. William *et al*. (1988) highlighted beta-glucan's protective role in mice with *Escherichia coli*-induced peritonitis, observing significant increases in peritoneal and blood neutrophils ($p$ = 0.05 and $p$ = 0.001, respectively) and enhanced respiratory burst activity. Lejeune *et al*. (1979) reported a single 15 mg/kg beta-glucan dose increased peritoneal macrophage acid phosphatase levels on day three and quintupled peritoneal monocytes rich in peroxidase by day ten. In another study by Al Tuwaijri *et al*. (1987), mice received 450 µg beta-glucan intraperitoneally over four doses before being treated with *Leishmania major* promastigotes. Results showed a significant reduction ($p$ = 0.01) in amastigote proliferation in the liver and spleen four weeks post-injection. Overall, these findings indicated that beta-glucan stimulates respiratory burst responses in animals, with minor variations likely due to differences in beta-glucan type, dosage, administration method, frequency, and the health status of the animals studied.

The study focuses on evaluating the immunogenic potential of *Z. multiflora* essential oil, a native plant of Iran's mountainous regions. Key compounds in this plant include thymol, carvacrol, p-cymene, trans-caryophyllene, and

linalool, which exhibit properties, such as antispasmodic, antibacterial, antifungal, anthelmintic, antioxidant, antimutagenic, anti-allergic, and immune-boosting effects (Shokri, 2018). To assess its effects, 100 mg/kg of *Z. multiflora* was administered intraperitoneally to animals. Results showed a significant increase in respiratory burst responses in treated mice compared to the control group, with a 1.6-fold rise on day four and a 2.3-fold increase on day seven. Comparing *Z. multiflora* with standard beta-glucan revealed that the respiratory burst response on day four was significantly lower (152% decrease) in mice treated with the plant than those treated with beta-glucan. However, by day seven, the responses between the two groups were nearly identical, with no statistically significant difference. Interestingly, the respiratory burst response in *Z. multiflora* -treated mice increased by 1.4-fold from day four to day seven, suggesting a delayed but meaningful effect. The delay in peak stimulation of neutrophil respiratory bursts by *Z. multiflora* compared to beta-glucan could be attributed to several factors: 1) The oil-based composition of *Z. multiflora* may slow its absorption and access to immune cells compared to water-soluble beta-glucan; 2) Beta-glucan has numerous receptors on leukocytes that effectively stimulate immune responses, whereas *Z. multiflora* may have fewer or less diverse specific receptors (Xia *et al*., 1999; Ozinsky *et al*., 2000; Brown *et al*., 2003); 3) The complex mixture of compounds in *Z. multiflora* might include elements that both stimulate and suppress immune activity. Further extensive studies are needed to determine how long after administration of *Z. multiflora* essential oil maximally stimulates innate immune cells and when their activity returns to baseline levels. Additionally, research is required to explore the cellular and molecular mechanisms of its immune-stimulating properties. A prior study in Iran by Khosravi *et al*. (2007) found that subcutaneous administration of essential oils from *Z. multiflora*, geranium, myrtle, and lemon peel in rabbits significantly enhanced respiratory burst responses and both cellular and humoral immunity. Among these oils, *Z. multiflora* exhibited the most robust immune-boosting effects. However, many aspects of its mechanisms remain unexplored, necessitating further investigation.

The study aimed to create an optimal combination of standard beta-glucan and *Z. multiflora* to stimulate the immune system, based on the hypothesis that combining these two substances would enhance innate immunity more effectively than each one alone. A significant challenge was the insolubility of *Z. multiflora* essential oil in water, as it remained as oily spots on the water's surface. To address this, equal amounts of beta-glucan and *Z. multiflora* essential oil were mixed in a 1:1 ratio, forming a suspension that was vortexed for two hours. This process ensured maximum dispersion of the aqueous phase within the oily phase, creating a uniform suspension. The prepared suspension was then intraperitoneally injected into mice. The results demonstrated that the respiratory burst response induced by the beta-glucan + *Z. multiflora* combination increased approximately 3.6-fold on day four and 3.9-fold on day seven compared to the control group. These increases were statistically significant on both days. The findings further indicated that *Z. multiflora* in the combination enhanced the respiratory burst response triggered by standard beta-glucan and prolonged its duration. Statistical analysis comparing the respiratory burst response between the beta-glucan + *Z. multiflora* combination and beta-glucan alone showed a 1.4-fold increase on day four, which was not statistically significant. However, on day seven, there was a significant 1.7-fold increase. Additionally, comparing the beta-glucan + *Z. multiflora* combination with *Z. multiflora* alone revealed a 2.1-fold increase on day four and a 1.7-fold increase on day seven. Overall, it can be concluded that while both standard beta-glucan and *Z. multiflora* essential oil independently stimulate innate immune phagocytic cell activity significantly, their combination not only enhances the intensity but also extends the duration of the respiratory burst stimulation in the body.

To assess the level of TNFα cytokine secreted by stimulated phagocytic cells exposed to the studied compounds, blood samples were collected from treated and control mice on the fourth and seventh days post-injection. After isolating their serum, the presence of TNFα cytokine was analyzed using the ELISA method. TNFα plays a critical role in the body's defense mechanisms by stimulating other inflammatory cytokines, increasing vascular endothelial cell permeability, and recruiting other immune effector cells. Additionally, in macrophages and neutrophils, TNFα triggers respiratory bursts and enhances phagocytic activity, leading to the effective

elimination of pathogenic microbes.

In the measurement of TNFα cytokine levels secreted by stimulated phagocytes in mice treated with standard beta-glucan (Group B), a fivefold and 4.4-fold increase in TNFα secretion was observed on the fourth and seventh days, respectively, compared to the control group. Vetvicka *et al*. (2002) measured TNFα cytokine levels in the blood of mice three days after oral administration of beta-glucan (20-2 mg/kg). The results of this study indicated a 2.2-fold increase in TNFα secretion compared to the control group. Although beta-glucan significantly induced TNFα secretion in this study, the lower secretion levels compared to our study with standard beta-glucan may be attributed to differences in the chemical structure of the beta-glucan molecules under investigation (number of side chains, molecular weight, spatial structure, and polymer charge), the dosage of beta-glucan administered, method of administration, and the timing of blood collection from treated mice. In another study conducted by Kubala *et al*. (2003), the effect of beta-glucan on human leukocytes under *in vitro* conditions was evaluated in terms of respiratory burst and TNFα secretion. Eighteen hours after incubation, it was observed that beta-glucan increased TNFα secretion; however, this increase was not statistically significant compared to the control group. Additionally, the respiratory burst response in the treated group (94.4 ± 13.1) showed an increase compared to the control group (57.4 ± 0.4) (Kubala *et al*., 2003). Lee *et al*. (2001) assessed the immunomodulatory effects of beta-glucan on TNFα secretion in a cell culture environment containing Raw 264.7 cells. The analysis of the supernatant obtained from cells treated with 50, 200, and 500 µg/ml beta-glucan after 24 hours showed that concentrations of 50 and 200 µg/ml increased TNFα secretion, whereas at a concentration of 500 µg/ml, TNFα secretion was approximately 80% of that observed at 200 µg/ml. In a similar study conducted by Olson *et al*. (1996), rabbit alveolar macrophages were incubated with 100, 200, 500, and 1000 µg/ml beta-glucan. The highest TNFα secretion was observed after 12 hours of incubation at a concentration of 200 µg/ml ($p$ = 0.0003) compared to unstimulated macrophages. This study demonstrated that high concentrations of beta-glucan (above 500 µg/ml) significantly reduced TNFα secretion, while low to moderate concentrations (below 200 µg/ml) increased TNFα secretion. Researchers hypothesize that this suppression of TNFα activity is due to the presence of large amounts of non-phagocytosed beta-glucan particles that bind to the lectin domain of soluble TNFα, inhibiting its activity (Hoffman *et al*., 1993; Olson *et al*., 1996). Overall, the results of this study align with findings from other research in this field, which indicate that beta-glucan molecules stimulate TNFα secretion, with variations in response potentially attributable to the type of beta-glucan studied, dosage, and experimental conditions (*in vitro* or *in vivo*). For the first time in Iran, the effect of *Z. multiflora* essential oil on TNFα secretion levels was studied in laboratory animals. The results indicated a 3.3-fold increase in TNFα secretion on day four and a 3.5-fold increase on day seven, both showing significant differences compared to the control group on day four. Additionally, TNFα secretion on day seven was higher than on day four, although this increase was not statistically significant. As noted in the analysis of respiratory burst stimulation by *Z. multiflora*, a statistical comparison of the mean OD of TNFα between mice treated with *Z. multiflora* (Group C) and those treated with standard beta-glucan (Group B) revealed that TNFα secretion in group C mice was reduced by 1.5-fold on day four and by 1.2-fold on day seven (not statistically significant) compared to group B mice.

The study conducted on the immunogenic potential of the combination of standard beta-glucan and *Z. multiflora* revealed that the mean OD of the TNFα cytokine on the fourth day in mice treated with this combination was six times higher compared to the control group. On the seventh day, this increase was 4.9 times higher than the control group. Although the mean OD of TNFα in mice treated with the beta-glucan and *Z. multiflora* combination showed a 1.2-fold increase on the fourth day and a 1.1-fold increase on the seventh day compared to mice treated with standard beta-glucan alone, statistical analysis did not indicate any significant differences between these two groups at these time points. Nevertheless, *Z. multiflora* essential oil demonstrated a positive and supportive effect on TNFα levels induced by standard beta-glucan. According to the results, a significant increase in TNFα secretion was observed in mice treated with the beta-glucan and *Z. multiflora* combination compared to those treated with *Z. multiflora* essential oil alone on the fourth day (1.8-fold increase) and the seventh day (1.4-fold increase).

The findings of this research indicated that fungal beta-glucan and *Z. multiflora* essential oil, both individually and in combination, play a significant role in enhancing the innate immune system, particularly by stimulating respiratory burst and the secretion of TNFα cytokine.

## Acknowledgment

This study was supported by the Faculty of Veterinary Medicine at the University of Tehran, Tehran, Iran.

### Conflict of Interest

There is no conflict of interest among the authors.

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