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Combined effects of fungal β-glucan and *Zataria multiflora* essential oil on phagocytosis in Balb/C mice

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ABSTRACT

Introduction: Natural substances have been used since ancient times for treatment of a range of diseases and have represented stimulatory effects on the function of innate immunity. The purposes of this study were to prepare β-glucan from *S. cerevisiae* and to assess the efficacy of purified β-glucan, *Zataria multiflora* (*Z. multiflora*) essential oil and their complex on phagocytosis in Balb/c mice.

Methods: β-glucan was purified during three stages including alkaline-acid treatment (S1), DEAE sephacel chromatography (S2) and con-A sepharose chromatography (S3). *Z. multiflora* essential oil was extracted by water-distillation using Clevenger-type apparatus. The chemical composition of *Z. multiflora* essential oil was analyzed by a GC/MS system. β-glucan (15 mg/kg), *Z. multiflora* essential oil (100 mg/kg) and their complex (the same doses) were injected into Balb/c mice intraperitoneally (IP). The blood samples were collected at days 4 and 7 after injection and phagocytic activity was assayed by chemiluminescence method.

Results: The results showed that the ratios of mannan to β-glucan were 70.3 to 29.7 for S1, 71.9 to 28.1 for S2 and zero to 100 for S3 (purified β-glucan). The major components of *Z. multiflora* were carvacrol (61%) and thymol (25%). Phagocytosis index means exhibited significant increases at day 4 (246%, 165% and 367%) and day 7 (242%, 235% and 309%) in mice treated with purified β-glucan, *Z. multiflora* essence, and their complex when compared to control mice, respectively ($P<0.05$).

Conclusion: The results suggest that the complex of β-glucan and *Z. multiflora* oil might be used to immunize individuals as prophylactic and/or therapeutic adjuvant in immunocompromised subjects.

Implication for health policy/practice/research/medical education:
The results suggest that β-glucan is a phagocytic cell activator. Hence, it could be used as a prophylactic and/or therapeutic agent alone or in combination with *Z. multiflora* essential oil in subjects with immune disorders.

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Introduction

Saccharomyces cerevisiae (*S. cerevisiae*) is genetically well-defined yeast that has traditionally provided a good model system for yeast cell wall study. In *S. cerevisiae*, the cell wall makes up 15% to 30% of the cell dry weight, which protects it from osmotic pressure and environmental stress (1). The cell wall of *S. cerevisiae* is organized into two layers that are made up of mannoproteins, β(1,3)-glucan, β(1,6)-glucan and chitin. These components are all interconnected by covalent bonds (2). In recent years, increasing attention has been paid to β-glucans isolated from the cell wall of yeasts. In respect to the high incidence of life-threatening infections among cancer patients, transplant recipients, patients with AIDS and patients receiving broad-spectrum antibiotic, corticosteroid and cytotoxic drugs, widespread efforts have been made to identify immune-modulatory agents (3). Numerous studies have demonstrated that β-glucans, either soluble or particulate, isolated from the yeast, exhibit antitumor, antimicrobial and radio-protective activities (4). Clinical studies revealed that β-glucan stimulated neutrophil degranulation and respiratory bursts and the secretion of IL_1, TNF_α and IL_6 from macrophages (5).

Herbal essential oils have been used since ancient times as drugs for the treatment of a range of diseases and represented stimulatory effects on the function of innate and

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acquired immunity (6). Among various herbal plants, *Zataria multiflora* Boiss. is a plant belonging to the Lamiaceae family that geographically grows only in Iran, Pakistan and Afghanistan (7). This plant has the local name of Avishan Shirazi (in Iran) and traditional uses such as antiseptic, anesthetic, antispasmodic, antinociceptive, antioxidant and antibacterial (8). To our best knowledge, there are little available data concerning the influence of *Z. multiflora* essential oil on phagocytosis in vivo (9,10). The aims of this study were to evaluate the effects of purified β-glucan, *Z. multiflora* essence and their combination on the phagocytic activity in Balb/C mice.

Materials and Methods

Plant materials and extraction of essential oil

The aerial parts of *Z. multiflora* plant were collected from Shiraz, Iran. A total of 100 g of the plant powder were subjected to hydrodistillation using a Clevenger-type apparatus for 3 hours. The collected essential oil was dried with anhydrous sodium sulphate, filtered and stored at 4-6°C in the dark (11). The plants were identified by Iranian Forest Organization and voucher specimens (no. 1106) were kept at Herbarium Collection Center.

Gas chromatography/mass spectroscopy analysis of essential oil

The Gas chromatography/mass spectroscopy (GC/MS) analysis was carried out on a Varian 3400 equipped with a DB-1 column. The transfer line temperature was 260°C. The ionization energy was 70 eV with a scan time of 1 s and mass range of 40-300 amu. The percentages of compounds were calculated by the area normalization method, without considering response factors. The components of oil were identified by comparison of their mass spectra with those of a computer library or with authentic compounds. Data obtained were confirmed by comparison of their retention indices (12).

Yeast strain and cultivation

S. cerevisiae (*PTCC 5052* strain) was obtained from Industrial-Scientific Researches Organization (Tehran, Iran). *S. cerevisiae* yeasts were grown in Sabouraud dextrose agar containing chloramphenicol at 30°C for 3 days. The yeast suspension was prepared with 500 ml of YPG broth (Yeast extract 1%, Peptone 2%, and Glucose 2%) and agitated in the shaker (150 rpm) at 30°C for 48 hours. At the end of incubation, cells were harvested at 4°C by centrifugation at 4500 g for 5 minutes and washed three times with sterile distilled water, weighed, lyophilized by means of Freeze-dryer (Labconco, USA) and stored at –20°C until used for the preparation of the walls (13).

Purification of β-glucan

Yeast cells were disrupted by sonication (UP 200 s, Dr. Hielscher Sonicator set, Germany) method using 60% amplitude for 48 minutes (2:4 minutes pulse on: off basis) to achieve maximum disruption of the cells. After cell disruption, the cell walls were separated by centrifugation at 1000 g at 4°C for 20 minutes and incubated in a boiling water bath for 2.5 minutes to inactivate endogenous wall lytic enzymes. For β-glucan purification, the cell wall was treated with 2% sodium hydroxide (NaOH) at 90°C for 5 hours. After cooling, the suspension was centrifuged at 3000 g for 10 minutes, and the resulting supernatant was neutralized with 2M acetic acid and treated with 3 volumes of ethanol to precipitate β-glucan (S1). Subsequently, S1 sample (40 mg/175 µl of distilled water) was loaded into a DEAE sephacel column (12 × 1.5 cm, Bio-Rad Laboratories, USA) to remove the residual proteins (S2). Finally, S2 sample (30 mg/40 µl of binding buffer) was applied into a con-A sepharose column (7 × 0.7 cm, Bio-Rad Laboratories, USA) to remove mannan from the mixture. The unbound fractions in the column containing water soluble β-glucan (S3) were collected by eluting the column with 7 mL of the binding buffer (20 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl, 1 mM $MgCl_2$, 1mM $MnCl_2$ and 1 mM $CaCl_2$), dialyzed against double distilled water (dialysis bag, cut off: 10 KD, Sigma), and then lyophilized (14).

Animal model

Female, 6-week old BALB/c mice were purchased from Razi Institute (Karaj, Iran). Animals were divided 8 per group (A, B, C, and control groups) in cages and were fed under specific pathogen-free conditions. β-glucan powder was suspended in physiological saline solution (0.9% NaCl). Mice were intraperitoneally (IP) administered with β-glucan (single dose, 15 mg/kg) in group A, *Z. multiflora* essence (single dose, 100 mg/kg) in group B, complex of β-glucan and *Z. multiflora* essence (the same doses) in group C, and distilled water in control group, respectively. Then, 4 mice from each group at days 4 and 7 were anesthetized with chloroform. The bloods were collected by cardiac puncture and poured into Eppendorf centrifuge tubes containing 5 units of heparin to examine phagocytic activity.

Phagocytosis assay

Chemiluminescence method was used to study phagocytic activity. The blood was diluted with dextran (Pharmacia) in ratio of 2:1 (dextran: blood) and then stood at room temperature for 45 minutes to allow the erythrocytes to sediment. The supernatant containing neutrophils was poured to a fresh tube, and 1 mL of Ficoll-Hypaque (1083, Sigma) was added to the supernatant, and then resuspended in 10 mL of phosphate buffer saline (PBS, pH 7.2). The suspension was centrifuged at 1500 g for 10 minutes. Subsequently the supernatant was harvested, brought to a final volume of 1 mL with PBS and neutrophils were counted. The PBS (500 µl), luminol (Sigma) (200 µl), phorbol 12-myristate 13-acetate (PMA, Sigma) (200 µl) solutions, and neutrophils (200 µl) were added to both sample and control tubes and their values were read by the luminometer (BioOrbit, Finland) (15). In this study, all general chemical materials were purchased from Merck Co. (Tehran, Iran).

Statistic analysis

Phagocytic activity in leukocytes of mice challenged with purified β-glucan, *Z. multiflora* essence, their complex and control group was compared by means of *t* test. *P* value less than 0.05 was considered as significant difference.

Results

The results showed that the ratios of mannan to β-glucan were 70.3 to 29.7 in S1, 71.9 to 28.1 in S2 and zero to 100 in S3 (purified β-glucan). The monosaccharide analysis of the sample showed that the only free carbohydrate present in S3 sample was glucose (14.3 mg/dl).

The composition of the oil from *Z. multiflora* was shown in Table 1. Carvacrol was the major component constituting 61%, followed by thymol (25%) and linalool (2%).

The effect of IP administration of purified β-glucan, *Z. multiflora* essence, and their complex on phagocytosis in Balb/c mice was shown in Table 2. Results showed that in mice treated with β-glucan (group A), the phagocytic responses exhibited a significant increase at day 4 (246%, $P=0.002$) and day 7 (242%, $P=0.0005$) when compared to control group. Phagocytosis index mean in mice treated with *Z. multiflora* essence (group B) showed an increasing effect at day 4 in amount of 165% ($P=0.002$) and at day 7 in amount of 235% ($P=0.001$) when compared to control mice. As shown in Table 2, IP administration of the combined β-glucan and *Z. multiflora* essence (group C) had more stimulatory effects on phagocytic activity of neutrophils at day 4 (367%, $P=0.0005$) and day 7 (309%, $P=0.0005$) when compared to control mice. Statistical analysis did not show significant differences in phagocytic activity of neutrophils between days 4 and 7 in treatment (A and C) and control groups except group B.

Table 1. The chemical compositions of *Zataria multiform* essential oil identified by GC/MS

No.		*Zataria multiflora*
1	61%	Carvacrol
2	25%	Thymol
3	2%	Linalool
4	2%	ρ-cymene
5	2%	β-caryophyllene
6	2%	β-phellandrene
7	1%	Carvacrol methyl ether
8	1%	Thymol methyl ether
Total	97%	-

Discussion

The yeast *S. cerevisiae* is an object of extensive research during some decades, but in the last years the use of methods for purification of cell wall components has been described in detail. The general composition of the cell wall of *S. cerevisiae* is consisted of mannoproteins, alkali-soluble β-glucan, alkali-insoluble β-glucan and small amounts of chitin (2). The purposes of this study were to purify β-glucan and combined effects of purified β-glucan and *Z. multiflora* oil on phagocytosis in Balb/C mice.

In this study, the ratios of mannan to β-glucan were 70.3 to 29.7 in S1, 71.9 to 28.1 in S2 and zero to 100 in S3 (purified β-glucan). Glucose (14.3 mg/dl) identified as the only free carbohydrate present in S3 sample. This finding is in agreement with those values reported by Lee et al (1) and Ha et al (16).

The present study exhibited carvacrol (61%), thymol (25%) and linalool (2%) as the major components of *Z. multiflora* essence. In a study conducted by Sharififar et al (17), *Z. multiflora* possessed carvacrol and thymol as main phenolic compounds and p-cymene as main non-phenolic compound. Rahimabadi et al (18) showed that the main phenolic and non-phenolic active compounds in *Z. multiflora* essential oil consist of thymol (59.50%), p-cymene (13.60%), carvacrol (5.6%) and γ-terpinene (4.3%). It has been reported that the quantity of these compounds can be vary due to harvesting season, plant age, soil, climate, geographical sources, herb drying method and extraction method (19).

In this study, the efficacy of purified β-glucan, *Z. multiflora* essence, and their complex on phagocytosis in Balb/c mice was determined. The phagocytic responses of mice treated with β-glucan (group A) exhibited a significant increase at day 4 (246%, $P=0.002$) and day 7 (242%, $P=0.0005$) when compared to control group. β-glucan is known to possess antimicrobial and antitumor activities by enhancing the host immune function, and activates macrophages, neutrophils and NK cells to triggers phagocytosis, respiratory burst and secretion of cytokines. Therefore, phagocytosis, as one of the most important host defense mechanisms, is an important measurement of the phagocytic cells function. In accordance with our findings, Ohno et al (20) showed significant activation of neutrophils by IP injection of β-glucan in mice. Also, several clinical studies have reported modulatory effects of glucans on priming of phagocytic cells function in mice (21,22).

In this study, we also evaluated the immunostimulatory effect of one of the Iranian herbal essence, *Z. multiflora*,

Table 2. Results of phagocytic index between treatment and control mice.

Day	Mouse			
	Treatment Mean ± SD (Phagocytic index)			Control Mean ± SD (Phagocytic index)
	A	B	C	
Day 4	181.63± 64.17	122.2± 29.47	270.88±49.95	73.75±18.79
Day 7	175.38±50.56	170.62±49.09	309.25±52.59	72.38±17.74

Abbreviation: SD, Standard deviation.

A: Mice treated with β-glucan; **B:** Mice treated with *Z. multiflora* essence; **C:** Mice treated with combined β-glucan and *Z. multiflora* essence.

on innate immune response. Phagocytosis index mean in mice treated with *Z. multiflora* essence (group B) showed an increasing effect at day 4 in amount of 165% ($P=0.002$) and at day 7 in amount of 235% ($P=0.001$) when compared to control mice. As shown in Table 2, IP administration of the combined β-glucan and *Z. multiflora* essence (group C) had more stimulatory effects on phagocytic activity of neutrophils at day 4 (367%, $P=0.0005$) and day 7 (309%, $P=0.0005$) when compared to control mice. Statistical analysis did not show significant differences in phagocytic activity of neutrophils between days 4 and 7 in treatment (A and C) and control groups except group B. It has been proven that the phagocytic cells obtain maximum effectiveness after 3 days of administering immunostimulators and then decrease phagocytic activity after day 6 (23). In contrast to β-glucan, the essence developed enhancing phagocytic activity at day 7 in comparison to day 4 (1.4 folds, $P=0.03$), indicating an increasing stimulation period. Only few studies have been focused on the innate immune responses of *Z. multiflora* in some species of terrestrial animal models. In rabbit, intracutaneous injection of *Z. multiflora* essential oil affected antibody titer against *Candida albicans*, neutrophil phagocytosis plus significant difference in lymphocyte transformation (9). Soltani et al (24) showed that *Z. multiflora* essential oil has some immunostimulatory effect on immunological factors such as antibody titers, total white blood cells and the phagocytic index of macrophages isolated from rainbow trout. The biological activity of essential oils is complex and depends on the oil's overall composition. The highest compositions of the essential oil from *Z. multiflora*, which was previously analyzed by other research groups, were shown to be phenolic compounds (thymol and carvacrol) (8). The potent immunostimulatory activity of the essential oil could be related to the levels of thymol and carvacrol (25). In a study conducted by Kavoosi et al (26), *Z. multiflora* essential oil, carvacrol and thymol showed strong radical scavenging and phagocytic activity. So, we speculated that *Z. multiflora* essence as an adjuvant in combination with β-glucan act as immunostimulatory agents in mice. In this regard, it has been proven that intraperitoneally injection of the complex of essence and β-glucan has more stimulatory effect on phagocytosis than the essence ($P=0.0005$ at day 4 and $P=0.0005$ at day 7) and purified β-glucan ($P=0.008$ at day 4 and $P=0.0005$ at day 7) when they used alone. To our best knowledge, there are no previous studies on the efficacy of the combination of β-glucan and essential oils against immune functions.

Conclusion

In summary, this study suggests that β-glucan is a phagocytic cells activator. Hence, it could be used as a prophylactic and/or therapeutic agent alone or in combination with *Z. multiflora* essential oil in subjects with immune disorders at the future.

Authors' contributions

All authors contributed to the conception of the study, confirmed the final version of the article and approved all aspects of the study.

Conflict of interests

The authors declare no competing interests.

Ethical considerations

All the experiments were performed based on the Veterinary Research Ethics and this study was approved on Society Protection of Cruelty to Animals (SPCA) in Iran. Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission and redundancy) have been completely observed by the authors.

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