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Antifungal and immunomodulatory activity of *Allium jesdianum* Boiss extracts

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ABSTRACT

Introduction: Macrophages are one of the key phagocytes against various pathogenic fungi, particularly *Candida* species killed by various mechanisms such as nitric oxide (NO) agents. The purposes of this research were to investigate the anti-*Candida* and immunomodulatory effects of the extracts from *Allium jesdianum* on mouse peritoneal macrophages.
Methods: The antifungal assay of amphotericin B and nystatin, as well as hydroalcoholic extract from *A. jesdianum* was carried out using disk diffusion and broth macrodilution methods against *Candida albicans* (ATCC 10231). Furthermore, microculture tetrazolium (MTT) and nitrite assays (Griess test) were applied to study the influence of the aqueous extract from *A. jesdianum* on macrophage viability indices and NO production, respectively.
Results: The results showed inhibition zone values of 8, 16, 28 mm for *A. jesdianum*, amphotericin B and nystatin against the organism tested, respectively. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *A. jesdianum* were found to be 330 and 663 µg/mL, respectively. Aqueous extract of *A. jesdianum* induced a significant decrease (2.8-fold at concentration of 5 mg/mL and 4.3-fold at concentration of 10 mg/mL) in macrophage viability indices in comparison with control group ($P<0.001$) but there was no toxic effect at 1 and 0.5 mg/mL. In addition, the aqueous extract of *A. jesdianum* resulted in a significant increase in NO production at non-toxic concentrations (77.6 µM nitrite at concentration of 1 mg/mL and 79.4 µM at concentration of 0.5 mg/mL) by macrophages ($P<0.01$).
Conclusion: The extract of *A. jesdianum* showed an *in vitro* anti-*C. albicans* and NO stimulatory effect. More studies with purified immunomodulatory components of *A. jesdianum* should be performed in future to shed light on the exact mechanisms of this activity.

Implication for health policy/practice/research/medical education:
Allium jesdianum revealed good antifungal effect against *C. albicans*, so it is suggested as an alternative medicine to treat disseminated candidiasis. In addition, *A. jesdianum* can be used to increase macrophage functions.
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Introduction

It is believed that macrophages, dendritic cells, and natural killer cells mediate the innate immune system (1). Macrophages are the key components of the mononuclear phagocytes consisting of closely related cells of bone marrow origin, involving blood monocytes and tissue macrophages. Macrophages are involved in all stages of the immune response and are capable of releasing numerous compounds like nitric oxide (NO) (2). NO has cytotoxic features, produced during host defense against invasive organisms and immunologic reactions, and is involved in numerous physiologic processes of mammals such as neurotransmition, blood pressure control and inflammation (3).

As the number of immunocompromised patients is on the rise and broad-spectrum antifungal agents, abdominal surgery, indwelling central venous lines, parenteral nutrition and cytotoxic chemotherapy are being used greatly; disseminated candidiasis has paved its way as an important cause of mortality and morbidity (4). In

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spite of recent advances in antifungal therapy, the cure rate of disseminated candidiasis is still not satisfactory due to lack of nontoxic effective antifungal agents having satisfactory pharmacokinetic features (5). It is argued that developing azoles provide new options to treat or prevent disseminated candidiasis. Yet, the development of resistance to antifungal azoles is a new challenge to the restricted therapeutic strategies (6). New agents with potent antifungal effectiveness, enhanced safety and high levels of tissue penetration are obviously required.

Many natural compounds obtained from herbal plants are believed to modify the biological response and modulate immune response (7,8). It is believed that herbal extracts/essential oils boost immune response by up-regulating NO from peritoneal macrophages acting as effective immunomodulator and antifungal substances (9). Among the plants of the genus Allium (family Amaryllidaceae), *Allium jesdianum* Boiss (Bon-e-Sorkh or Lizak in Persian, Sourah Boneh in Kurdish) is one of the main species of this genus. It is an endemic Iranian plant naturally grown in northern, western, and south-western regions of Iran. Iranians have used its medicinal and antimicrobial features from ancient times (10). Since studies on the pharmacological features of *A. jesdianum* are scarce (11), the current study aims to explore the effect of *A. jesdianum* extracts on growth inhibition of *Candida albicans* and NO production by applying murine peritoneal macrophages to reveal the possible immunomodulatory features.

Materials and Methods

Plant

The aerial sections of *A. jesdianum* were harvested from Chaharmahal and Bakhtiari province, western part of Iran, in 2018. Botanical identification was performed at the Herbarium of Pharmacognosy Department, Faculty of Medicine, Shahed University, Iran. All experiments were performed at Faculty of Medicine, Shahed University, Tehran, Iran.

Preparation of hydroalcoholic and aqueous extracts

Aerial parts of the plant were crushed into fine powder. The hydroalcoholic and aqueous extracts from *A. jesdianum* were prepared in this study. For hydroalcoholic extract preparation, 100 g of the plant powder was extracted with 50% ethanol + 50% water employing maceration method (12). Whatman paper (No. 42) was used to filter the extract and the solvent was distilled at 60°C. The remaining extract was ultimately put to dry in an oven at 30°C for 3 hours for removing any residual solvent, and was kept at 4°C until further analysis. For aqueous extract preparation, 100 g of the plant powder was mixed with 400 mL of water and boiled for 10 min. The resulting solution was frozen and lyophilized for 96 hours at –50°C and 0.04 mbar (Snijder scientific Ltd, the Netherlands).

Candida albicans strain

Sabouraud dextrose agar (Mk Co., Darmstadt, Germany) was used to culture *C. albicans* (ATCC 10231) at 35°C for 3 days, which was then harvested and kept at 4°C until further analysis.

Antifungal assays

We conducted a pilot study to determine the antifungal properties of different extracts from *A. jesdianum*. Based on *in vitro* study, only the hydroalcoholic extract from *A. jesdianum* had anti-*Candida albicans* effect. Therefore, the hydroalcoholic extract was selected for antifungal assays.

(A) Disk diffusion method

Test to assess the antifungal activity was performed using disk diffusion method based on the CLSI-M44-A2 standard for yeasts (13). Briefly, agar plates (90-mm diameter) containing Mueller-Hinton agar (Merck Co., Darmstadt, Germany) accompanied by glucose (2%) and methylene blue (0.5 mg) was used. Sterile cotton swabs dipped in yeast suspensions, adjusted to 1×10^6 cells/mL, were inoculated on the agar surface. Hydroalcoholic extract of *A. jesdianum* (30 µL of stock solution), amphotericin B (10 µg/disk) and nystatin (100 unit/disk) disks (Master Group, London, England) were placed on the agar surfaces. Subsequently, the media were kept at 35°C and read at 24 hours. After the colonies grew, the zones of inhibition around the disks were measured and recorded. All experiments were performed in duplicate. The interpretation of antifungal assay of standard drugs was done based on manufacture guideline: zone diameters of ≥16 mm of the drugs as susceptible; zone diameters of 11 to 14 mm as susceptible dose dependent; and zone diameters of ≤ 10 mm as resistant.

(B) Broth macrodilution method

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of hydroalcoholic extract of *A. jesdianum* was determined by broth macrodilution method (14). Briefly, sterile Sabouraud dextrose broth was used to prepare stock solution of hydroalcoholic extract. Serial dilutions of stock solution of extract were prepared for a final concentration ranging from 15 to 4000 µg/mL at 12 × 75 mm glass tubes. Also, the specific concentrations of amphotericin B and nystatin (Sigma-Aldrich Chemicals Co., St. Louis, MO, USA) ranged from 0.016 to 16 µg/mL and 0.25 to 128 µg/mL, respectively. After adding 50 µL of the yeast suspension (2.5×10^3 cell/mL) to each tube, all tubes were put for incubation at 35°C for 48 hours. Tubes containing only the Sabouraud dextrose broth with no microorganisms were applied as controls. The lowest hydroalcoholic extract concentration inhibiting fungal growth was recognized as MIC. For determining MFC, a loopful of broth was removed from each individual tube and spot-inoculated on individual Sabouraud dextrose

agar plates. The plates were put for incubation at 35°C for 48 hours, and MFC was set as the corresponding concentrations needed for killing 99.9% of the cells.

Macrophages preparation

The Animal Breeding Laboratory of the Faculty of Medicine, Shahed University, Tehran, Iran provided the study with male Balb/c mice (8 to 10 weeks of age, weighting 18-25 g). All animals were housed and handled according to institutionally recommended guidelines. The animals were sacrificed and peritoneal exudates cells were harvested as described by Naeini et al (9). Briefly, the macrophages were obtained by lavage using 5 mL of cold PBS (5 mg/mL, pH 7.2) and poured in sterile plastic tubes. Cells were pooled, re-suspended in RPMI 1640 containing 5% FBS (GIBCO, Grand Island, NY, USA) and cultured in 96-well flat-bottom microtiter plates at a final concentration of 4×10^5 cells per well. After 2 hours, the debris and non-adherent cells were taken away from the wells.

In *in vitro* pilot study, only the aqueous extract from *A. jesdianum* showed the immunomodulatory effect. Therefore, the aqueous extract was selected for MTT and nitrite assays. The monolayer macrophages were incubated once more at 37°C for 20 hours along with various concentrations of the aqueous extract (0.5, 1, 5 and 10 mg/mL).

MTT assay

The effect of the aqueous extract from *A. jesdianum* on macrophage viability was measured by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay (15). Macrophages were incubated with the *Allium* aqueous extract at different concentrations (10, 5, 1 and 0.5 mg/mL) and media. The 96-well plates were used to carry out the MTT assay. The complete medium was used to wash the wells followed by the addition of 180-µL aliquots of medium and 20-µL aliquots of MTT solution (5 mg/mL of PBS) to each well at the pre-defined time. Incubation lasted for 2 hours at 37°C and 5% CO_2 for exponentially growing cells and 15 minutes for steady-state confluent cells; then, the media were taken away followed by solubilizing the formazan crystals with 175 µL of DMSO. A microplate reader (Model 450) was used to read the plates on (Bio-Rad Laboratories, Hercules, CA, USA) at 540-nm wavelength.

NO production

With the method specified by Pertile et al, NO production was determined (16). NO discharged into the supernatants of mouse macrophages was specified through Griess reaction by addition of 50 µL of the aqueous extract from *A. jesdianum* to 96-well flat-bottomed plates containing 50 µL of Griess reagent [1% sulfanilamide/0.1% N-(1-naphthyl) ethylenediamine dihydrochloride/2.5% H_3PO_4]. The samples were assayed in quadruplicate. After 15 minutes at room temperature, a Multiskan MS microplate reader (Labsystems Oy, Helsinki, Finland) was used for measuring the absorbance of each well at 540 nm and the nitrite concentration was determined from a standard curve of sodium nitrite.

Statistical analysis

One-way analysis of variance (ANOVA) was used for data analysis and the results were shown as Mean + Standard deviation (SD). The P values < 0.05 were considered as significant differences.

Results

In disk diffusion method, the diameters of growth inhibition zones for *A. jesdianum* hydroalcoholic extract, amphotericin B and nystatin were 8, 16 and 28 mm, respectively. In broth macrodilution method, *C. albicans* isolate was sensitive to the hydroalcoholic extract from *A. jesdianum* and this activity was found to be with MIC of 330 µg/mL (Table 1).

As shown in Table 2, macrophage viability indices reduced after the treatment of peritoneal macrophages at all concentrations of the aqueous extract from *A. jesdianum*, especially at 5 and 10 mg/mL concentrations. Aqueous extract from *A. jesdianum* resulted in a significant decrease of 280% at concentration of 5 mg/mL and 430% at concentration of 10 mg/mL in macrophage viability indices in comparison with control group ($P<0.001$). Significant difference was not observed between the activity of the aqueous extract from *A. jesdianum* and control group at 1 and 0.5 mg/mL concentrations.

Regarding the effect of the aqueous extract of *A. jesdianum* on NO production, a progressive effect was observed (Figure 1). As shown in Table 2, the aqueous extract from *A. jesdianum* at the concentrations of 1 and 0.5 mg/mL stimulated a considerable increase in NO production in comparison with control group ($P<0.01$ and $P<0.001$, respectively). The concentrations of NO produced by macrophages treated with aqueous extract were 77.6 µM at 1 mg/mL and 79.4 µM at 0.5 mg/mL. The best effect of the aqueous extract from *A. jesdianum* on NO production was observed at lower concentrations. In

Table 1. Antifungal susceptibility of *Allium jesdianum* hydroalcoholic extract, amphotericin B and nystatin against *Candida albicans*

Groups	Method		
	Disk Diffusion	Broth macrodilution	
	Inhibition zone (mm)	MIC (µg/mL)	MFC (µg/mL)
Allium jesdianum	8	330	663
Amphotericin B	16	2	4
Nystatin	28	4	8

MIC: Minimum inhibitory concentration; MFC: Minimum fungicidal concentration.

Table 2. The cell viability and nitric oxide (NO) production of murine peritoneal macrophages treated with the aqueous extract from *Allium jesdianum*

Groups	Dose (mg/mL)	MTT assay			NO assay (µM)		
		Mean	SD	*P* value	Mean	SD	*P* value
Treatment groups	10	0.083	0.001	0.001[a]	42.0	6.4	-
	5	0.129	0.039	0.001[a]	37.2	4.4	-
	1	0.213	0.024	-	77.6	13.2	0.01[a]
	0.5	0.198	0.016	-	79.4	15.0	0.001[a]
Control	-	0.359	0.048	-	48.8	6.6	-

MTT, Microculture tetrazolium; SD, standard deviation.

[a] Significant differences were observed between treatment and control groups.

addition, no significant differences were observed between the aqueous extract from *A. jesdianum* and control group at 5 and 10 mg/mL concentrations.

Discussion

In this study, we investigated the influence of hydroalcoholic extract from *A. jesdianum* on growth inhibition of *C. albicans,* as well as the effect of the aqueous extract from *A. jesdianum* on viability and NO production of macrophages. The present study showed inhibitory effect of hydroalcoholic extract from *A. jesdianum* against *C. albicans*. The hydroalcoholic extract showed an 8 mm/30 µL inhibition zone against the tested strain, while the growth inhibition zones of amphotericin B and nystatin were 16 and 28 mm, respectively. In broth macrodilution method, the hydroalcoholic extract from *A. jesdianum* also exhibited an antifungal activity against tested strain with MIC value of 330 µg/mL. Our antifungal assays demonstrated the fungistatic and fungicidal effects of hydroalcoholic extract from *A. jesdianum* against *C. albicans*. Earlier studies have reported the antifungal activity of *Allium* species hydroalcoholic extract (17-19). Our findings are consistent with similar studies assessing antifungal activity of *Allium* species hydroalcoholic extract on different fungal species (20). Shahrokh et al (11) indicated the antifungal influence of hydroalcoholic extract from *A. jesdianum* against all fluconazole resistant and susceptible *Candida* isolates. The MIC_{90} of hydroalcoholic extract from *A. jesdianum* against *Candida* strains from patients with candidiasis was 3 mg/mL. In a study by Razzaghi et al (21), the MIC values of hydroalcoholic extract of *A. jesdianum* against *Trichophyton mentagrophytes* isolates were between 6.1 and 49.3 mg/mL (mean MIC: 20.3 mg/mL), whereas the MFC values of the isolates were between 24.6 and 49.3 mg/mL (mean MFC: 40.6 mg/mL). In another study by Moghim et al (22), the MIC_{90} of ethanolic extract of *Allium ascalonicum* against *C. albicans* was 8.65 mg/mL. Diba and Alizadeh (20) revealed that hydroalcoholic extract of *Allium hirtifolium* strongly inhibited the activity of *Candida tropicalis* isolates at a concentration of 100 mg/mL. Other studies reported that the MFC values of various extracts from *Allium* species against *Aspergillus niger*, *A. flavus* and *A. fumigatus* were between 35-1536 µg/mL (23). The chemical composition and bioactive compounds of *Allium* plants have been reported to be organosulfur compounds, which include diallyl trisulfide, diallyl-dithiosulfinate (allicin), diallyl disulfide and S-allylcysteine (24,25). Additionally, *Allium* plants contain polyphenolic compounds like flavonoids (26). Most of the medicinal effects of *Allium* genus are related to their organosulfur compounds (27). Organosulfur compounds downregulate the expression of hypha specific gene *HWP1*, which possibly explain the inhibitory effect of

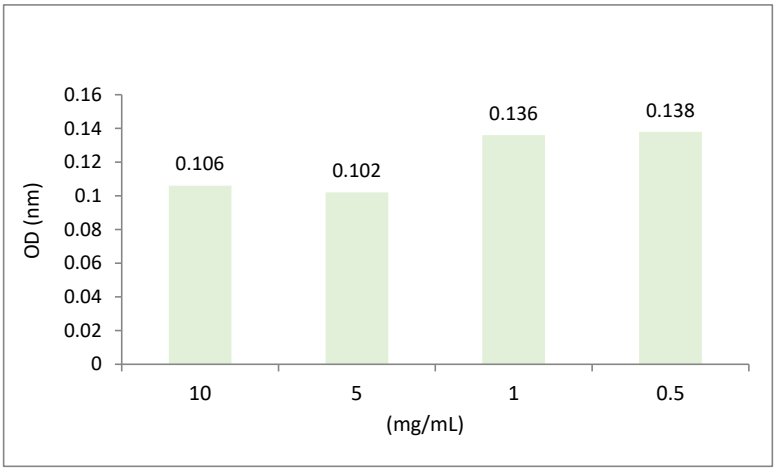

Figure 1. Nitric oxide (NO) production of peritoneal macrophages stimulated with the aqueous extract of *Allium jesdianum*. OD: optical density.

Allium hydroalcoholic extract on hyphae and biofilm formation in *C. albicans* (5).

Phagocytic cells, particularly macrophages, are believed to be involved significantly in resistance to *Candida* infections. They are active in the nonspecific first line of defense due to their capability of engulfing and degrading the invading *Candida* yeasts (28). In the current study, we explored the influence of the aqueous extract from *A. jesdianum* on different aspects of macrophages. Our results showed that macrophage viability indices reduced after the treatment of peritoneal macrophages at all concentrations of the aqueous extract from *A. jesdianum*, especially at 5 and 10 mg/mL concentrations. Aqueous extract from *A. jesdianum* resulted in a significant decrease of 2.8-fold at concentration of 5 mg/mL and 4.3-fold at concentration of 10 mg/mL in macrophage viability indices in comparison with control group (P <0.001). The literature review revealed a large body of research on the immunomodulatory properties of *Allium* species. However, there are not reports on *A. jesdianum*. In a study conducted by Radjabian et al (29), the tested *Allium* species had stimulatory or inhibitory effects on macrophages at different concentrations. *Allium sativum* and *Allium iranicum* aqueous extracts demonstrated the highest effects on macrophage viability indices at 1 and 0.01 mg/mL, respectively, whereas the aqueous extract of *Allium elburzense*, at high concentrations, slightly affected viability indices of the macrophage. In agreement with our results, *Allium asarense* decreased macrophage viability indices at most applied concentrations (29). The available evidence on *Allium* species suggests that not only the applied aqueous extract concentration or the assessed compound, but also the types of treated cell line are effective factors on the experiment outcomes. In a previous study by Naeini et al (30), the aqueous extract of *Ziziphora tenuior* at the concentrations of 10 and 20 mg/mL stimulated a considerable increase (24% and 21%, in the respective order) in macrophage viability as well.

The present study demonstrated that the aqueous extract from *A. jesdianum*, in a dose-dependent manner, on NO production had a progressive effect. Aqueous extract from *A. jesdianum* at the concentrations of 1 and 0.5 mg/mL stimulated a considerable increase in NO production in comparison with control group ($P<0.01$ and $P<0.001$, respectively). The concentrations of NO produced by macrophages treated with aqueous extract from *A. jesdianum* were 77.6 µM at 1 mg/mL and 79.4 µM at 0.5 mg/mL. The best effect of the aqueous extract from *A. jesdianum* on NO production was observed at lower concentrations. This activity could be due to the presence of flavonoids in *Allium* aqueous extract, which can augment the macrophage responses (31). To the best of our knowledge, no study has focused on influence of the aqueous extract from *A. jesdianum* in NO production; however, similar works have been done with various herbs. Previous studies demonstrated that the aqueous extracts from *Ziziphora tenuior* (30) and *Heracleum persicum* (9) had significant immunostimulatory activity in NO production by macrophages. In agreement with previous studies, our research demonstrated stimulatory activity of the aqueous extract from *A. jesdianum* in production of NO by peritoneal macrophages implying that these natural substances are activators of macrophages. The presence of NO produced by macrophages in suitable concentration during infections results in immunomodulatory functions of host defense. Therefore, the induction of NO may contribute in the immunoprevention of fungal infections, especially disseminated candidiasis (32).

Conclusion

Allium jesdianum hydroalcoholic extract exhibited an anti-*C. albicans* activity; thus, so it is suggested as an alternative medicine to treat disseminated candidiasis. In addition, *A. jesdianum* aqueous extract can be used to increase macrophage functions. Unfortunately, the role of various components of *A. jesdianum* aqueous extract on the macrophage function is still unknown. Therefore, more studies should be done on the influence of the components on immune cells.

Authors' contribution

AN, RY and HS conceived and designed experiments and analyzed the data; HS wrote the paper. All authors read and confirmed publication of the paper.

Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

The protocol of this study was confirmed by the Ethical Committee of Amol University of Special Modern Technologies, Amol, Iran (ir.ausmt.rec.1398.04.17). Ethical issues (including plagiarism, data fabrication and double publication) have been completely observed by the authors.

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