



## In vitro antifungal activity of aqueous-ethanolic extract of *Allium jesdianum* against fluconazole-susceptible and -resistant human vaginal *Candida glabrata* isolates

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### ABSTRACT

**Introduction:** About 50% of women are diagnosed with an episode of vulvovaginal candidiasis (VVC) during first 25 years of their lives. *Candida glabrata* is considered the second most prevalent non-*C. albicans* species associated with VVC. In this study, we examined the antifungal effect of a medicinal plant, *Allium jesdianum*, as a natural therapeutic agent against fluconazole-susceptible and -resistant human vaginal *C. glabrata* isolates, collected from 2 groups of volunteers; healthy women and women with VVC.

**Methods:** An aqueous-ethanolic extract of *A. jesdianum* was prepared by maceration method. Vaginal specimens were collected from 28 women diagnosed with VVC and eight healthy subjects. The specimens were cultured using fungal-specific media in optimum conditions. The antifungal susceptibility of clinical isolates of *C. glabrata* to the plant extract and fluconazole was evaluated according to the standard protocols.

**Results:** *Candida glabrata* was found to be the major cause of vaginal infection among 15.2% of women with VVC. We could identify the *Candida* spp. yeasts that colonized the vagina of 35% of healthy women while 19% of the isolated yeasts strains were detected as *C. glabrata*. Moreover, 7.1% of isolates obtained from VVC-patients were fluconazole resistant. The results showed the antifungal effect of *A. jesdianum* against all fluconazole resistant and susceptible *C. glabrata* vaginal isolates. The MIC<sub>90</sub> of aqueous-ethanol (A-EtOH) extract of *A. jesdianum* against *C. glabrata* isolates from both VVC-patients and healthy women was 3 mg/mL.

**Conclusion:** Our results showed the promising antifungal efficacy of aqueous-ethanolic extract of *A. jesdianum*. *A. jesdianum* extract might be used as an alternative choice to treat the VVC infections caused by fluconazole resistant *Candida* spp.

### Implication for health policy/practice/research/medical education:

Aqueous-ethanolic extract of *A. jesdianum* showed promising antifungal effect against both fluconazole-susceptible and -resistant vaginal isolates of *C. glabrata*, an important fungal pathogen. Hence, the *A. jesdianum* might be used to develop effective therapy against vaginal infections caused by *C. glabrata*.

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### Introduction

Several predisposing factors and microbial agents have been known to cause vaginitis. Vaginal infection by *Candida* spp., also known as vulvovaginal candidiasis (VVC), is the second most common cause of a vaginal infection after bacterial vaginosis (1). Among *Candida* species, *Candida albicans* is the most important cause

of VVC. Epidemiological studies have revealed that *C. glabrata* is the second most usual *Candida* spp. after *C. albicans* (2), and ~55% of women have at least one episode of VVC by age 25 years (3). The prevalence of culture-validated VVC has been reported to be ~40% in Iran (4). Most of the fungal microorganisms are classified as opportunistic. This implies that they can cause infections

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in the hosts with defective immune system. However, VVC infections are usually observed in immunocompetent healthy women. Over a decade ago, VVC was classified and now is internationally accepted and adapted. Uncomplicated VVC (UVVC) is characterized by mild to moderate symptoms in immunocompetent healthy women and *C. albicans* is often primary microbial cause of the infection. The disease usually can be well-controlled with a period of short/mid-term antifungal therapy. The complicated VVC (CVVC) is generally caused by non-*C. albicans* species and determined by severe symptoms. The CVVC is occurred among women who suffer from an immunosuppressing disease such as uncontrolled diabetes and pregnancy. A mid/long-term antifungal regimen is necessary for resolution of the disease (5).

In fact, ~20%-30% of healthy women experience an asymptomatic vaginal colonization by candida species in their lives (6). There is no quantitative definition for *Candida* growth in host's body to distinguish between colonization and infection. However, a cut-off of  $10^3$  to  $10^5$  CFU/mL has been introduced by researchers to characterize colonization and infection. The defined range is equal to 1-100 *Candida* countable colonies on the culture plates. Moreover, an index named as Pittet's *Candida* colonization index of  $\leq 0.35$  and  $\geq 0.4$  was defined for colonization and infection, respectively (7).

The azole resistance among non-*C. albicans* *Candida* species (NCC) is more frequent. In some cases, NCCs are acquired resistance when frequently exposed to azole drugs or treated with sub-lethal doses of azole antifungals. In a so-called acquired-resistance, *C. glabrata* can acquire rapidly resistance to azoles. The intrinsic resistance of *C. glabrata* to azoles has also been reported (2).

The antifungal effect of some novel natural-originated compounds has recently been investigated. These types of studies could help us to discover novel antifungal agents and extend our understanding of mechanisms of resistance to routine antifungals. *Allium jesdianum* with its known medicinal and antimicrobial properties has been used from ancient times in Iran. Typically, the plant grows in western and northern areas of Iran.

The aim of this study was to evaluate the antifungal effect of A-EtOH extract of *A. jesdianum* against fluconazole-susceptible and-resistant human vaginal *C. glabrata* isolates, collected from two groups of women; healthy women and women with VVC.

## Materials and methods

### Plant material and preparation of herb extracts

The aerial parts of *A. jesdianum* which is native to Iran, during the flowering stage were collected from Zard-Kuh mountains (Zagros range, Chaharmahal and Bakhtiari province, western part of Iran) in spring 2014. The plant materials were kept on ice in order to maintain their freshness. In the laboratory, the plant samples were

washed with distilled water. The fresh plant including leaves, flowers and stems were ground using pestle and mortar. The ground plant material was macerated by mixing 5 g of the material with 250 mL of aqueous ethanol (A-EtOH) (ethanol: water, 70:30 v/v) for 24 hours at room temperature. The obtained extract was then filtered through Whatman No.1 filter paper, and sterilized using a 0.45  $\mu$ m membrane filter. Afterwards, with a rotary evaporator at 30°C and under high vacuum, the excess alcohol solvent was removed. Finally, the semisolid extract was stored in a sterile dark glass container and kept at -20°C until use.

### Human subjects and specimen collection

All women with VVC referred to department of obstetrics and gynecology of Arad hospital in Tehran, Iran were selected to participate (between spring and summer 2014). Healthy women were considered those who had attended at the Hospital but not diagnosed with VVC, other vaginal infections, hormonal dysfunction, pregnancy, diabetes and other malignant diseases. Informed consent was obtained from all subjects. However, women currently receiving an antifungal treatment, and also women who have received a recent (<1 month) azole/antifungal therapy were all excluded from the study. All VVC-suspected subjects were examined by a gynecologist, validated with microscopy and culture of vaginal discharge. A sterile cotton swab was used for specimen collection from the VVC-confirmed patients and healthy women.

### Yeast culture and identification

The fungal cultures were prepared on BBL CHROMagar *Candida* medium (CCM)-contained plates by direct contact of swabs on the CCM surface. CCM is a chromogenic fungal specific medium that contains chloramphenicol to inhibit bacterial growth. In the case, different species of *Candida*, i.e. *C. albicans*, *C. tropicalis* and *C. krusei* were distinguished using CCM. Moreover, it has been shown that CCM can identify *C. glabrata* (8,9). The *C. glabrata* grows as dark pink colonies with pale edges on the CCM (8). Then, each group of homochromatic candida colonies was sub-cultured onto Sabouraud Dextrose agar (SDA) and investigated for their morphology under microscope. The identity of isolates was also confirmed by a polymerase chain reaction (PCR) detection method. The primer sequences used for PCR were as follows; forward: AAGAAGGCTGCCCGTTGTAATG and reverse: CACTTATCTAAACAACGGTGGC. The PCR reaction mixture and the protocol for programming of thermocycler machine were adapted from previous study (10).

### Disc-diffusion antifungal susceptibility testing

Neo-Sensitabs™ fluconazole 25  $\mu$ g discs (Rosco Diagnostica, Taastrup, Denmark) were used for disc-

diffusion in-vitro antifungal susceptibility testing (DAST) was done according to M44-A protocol (11). In brief, the yeast cell suspensions were prepared from all *C. glabrata* isolates then the suspensions were spread on the surface of Mueller-Hinton agar medium (supplemented with 2% glucose) using cotton swabs. After incubation at  $35 \pm 2^\circ\text{C}$  for 20-24 hours, the zones of inhibition around discs were measured using a ruler. The isolates were classified in one of following categories: resistant (R), susceptible dose-dependent (SDD) and susceptible (S) groups, based on the definitions of M44-A protocol. A blank disc (without any drug) was used as negative control. The standard strains were used for reference and quality control purposes.

#### Broth microdilution antifungal susceptibility testing

The broth microdilution antifungal susceptibility testing (MAST) was carried out according to CLSI's M27-A2 guideline. With preparing a broth medium (RPMI 1640 without sodium bicarbonate supplemented with 2% glucose), it was then buffered by morpholinepropanesulfonic acid (MOPS). Before being diluted with MOPS-buffered RPMI 1640 (MBR) broth medium, yeast suspensions were prepared using normal saline. A 0.5 McFarland standard was used as a reference to adjust the yeast concentration ( $10^3$ – $5 \times 10^3$  CFU/mL). A two-fold stock solution of fluconazole was made at concentration of 128  $\mu\text{g}/\text{mL}$ . Then, we prepared 10 two-fold concentrations of A-EtOH extract including 20, 18, 16, 14, 12, 10, 8, 6, 4, 2 mg/mL using alcohol as a solvent. The final concentration of alcohol in the inoculated solution was limited to 5%. The yeast suspension and drug or herbal extract was inoculated in 96-well flat-bottomed plates and incubated at  $35^\circ\text{C}$  for 24 and 48 hours then visually evaluated. As defined, the minimum inhibitory concentration (MIC) of fluconazole is the lowest drug concentration that inhibits yeast growth by 50%. Also, the MIC of herbal extracts is defined as the lowest drug concentration that completely inhibits yeast growth.

#### Results

##### 15.2% of VVC cases caused by *Candida glabrata*

Out of a total of 198 women with VVC and recurrent VVC,

vaginal positive culture for *C. glabrata* was 16.2% (32 patients). 14/198 (7.1%) of women were diagnosed with recurrent VVC while 184 (92.9%) of them were diagnosed with usual VVC. Among 32 women with a positive culture for *C. glabrata*, 28/184 (15.2%) did not experience a recent episode of *C. glabrata*-initiated VVC (CG-VVC) or experienced less than four episodes of CG-VVC during one year. This means that out of a total of 32 women positive for *C. glabrata*, 28 women had experienced VVC while four experienced the recurrent VVC. Therefore, 15.2% of all VVC infections (28/184) caused by *C. glabrata*. Out of a total of 120 healthy volunteer women, 42 (35%) of women were colonized by different species of *Candida*. And of these women (42/120), 8 of them (19%) were colonized by *C. glabrata*.

##### Fluconazole resistance was not seen in isolates obtained from healthy women but found in isolates from women with VVC

All *C. glabrata* isolates obtained from healthy women were identified as fluconazole-S. Only two isolates were identified as fluconazole-R among women with VVC. Two isolates (7.1%) obtained from patients with VVC were classified as fluconazole-R by both the DAST and MAST methods. However, the frequencies of fluconazole-S and SDD isolates were different by two methods. 24 (85.7%) and only 2 (7.1%) isolates were characterized as S and SDD, respectively by DAST method whereas 18 (64.3%) and 8 (28.6%) isolates were classified as S and SDD, respectively by MAST method (Table 1). All isolates in healthy group were identified as Flu-S by DAST method while only one strain was detected as Flu-SDD by MAST method. The MIC<sub>50</sub> and MIC<sub>90</sub> of fluconazole against isolates from healthy group were calculated as 2 and 4  $\mu\text{g}/\text{mL}$ , respectively (Table 1).

##### The A-EtOH extract could completely prevent growth of all fluconazole-R and fluconazole-non-R vaginal isolates of *Candida glabrata*

The A-EtOH extract showed fungicidal activity against *C. glabrata* isolates. The MIC<sub>90</sub> of A-EtOH extract against *C. glabrata* isolates obtained from VVC patients was 3

**Table 1.** MIC ranges, MIC<sub>50</sub> and MIC<sub>90</sub> of azoles and *Allium jesdianum* (aqueous-ethanol) extract and frequencies of azole-resistance in *Candida glabrata* isolates obtained from healthy individuals and patients with vulvovaginal candidiasis (VVC)

Participants	Antifungals	MIC*			R No. (%)	SDD No. (%)	S No. (%)
		50 (%)	90 (%)	Range			
VVC	Fluconazole	8	32	2-64	2 (7.1)	8 (28.6)	18 (64.3)
	AJA	2	3	1-3	-	-	-
Healthy	Fluconazole	2	4	1-16	0 (0)	1 (12.5)	7 (87.5)
	AJA	2	3	1-3	-	-	-

**Abbreviations:** VVC, patients with vulvovaginal candidiasis; AJA, *Allium jesdianum* aqueous-ethanol extract; MIC, minimum inhibitory concentration; No., number; R, resistant; SDD, susceptible dose-dependent; S, susceptible.

\*MICs of fluconazole have been presented in  $\mu\text{g}/\text{mL}$  scale; MICs of AJA have been presented in mg/mL scale.

mg/mL. The MIC values of herb extract against either the azole-resistant or non-resistant isolates obtained from both evaluated groups (healthy women and women with VVC) were in a range between 1 to 3 mg/mL (Table 1). The MIC range of A-EtOH extract against fluconazole-non-R isolates from patients with VVC was 1-3 mg/mL. Also, the MICs of A-EtOH extract against fluconazole-R isolates from patients with VVC were 3 mg/mL, being in the same MIC range of fluconazole-non-R isolates. Moreover, the MIC<sub>50</sub> and MIC<sub>90</sub> values calculated for isolates in the healthy group and VVC group were 2 and 3 mg/mL, respectively (Table 1). In other words, the MIC<sub>50</sub> and MIC<sub>90</sub> values of herb extract against both groups of isolates obtained from VVC-patients and healthy women were the same (Table 1).

### Discussion

The results of current study are consistent with previous studies reporting frequent isolation of *C. glabrata* from patients with VVC (2,12). *C. glabrata* is considered to be responsible for ~15.2% of VVC cases and the second prevalent cause of VVC after *C. albicans*. In our examinations, while 35.7% of isolates were classified as Flu-non-R (S or SDD) by MAST method, only 7.1% of *C. glabrata* isolates classified as the Flu-R. A previous study conducted in Iran showed that 10.8% of patients with vaginal infection were being diagnosed with an episode of VVC (13). Another similar research has also reported *C. albicans* as the most frequent *Candida* spp. in patients with VVC with frequency of 42.5%, and *C. glabrata* as the second most prevalent species with frequency of 21.9% (14). In our study, however *C. glabrata* was identified only in 19% of healthy women colonized by *Candida* spp., and out of a total of 120 healthy women, 42 (35%) were colonized by one species of *Candida*. In fact, 10%-55% of healthy adult women were involved in asymptomatic colonization by one species of *Candida* (15). Another study reported that ~15% of women carrying *Candida* in the vagina were colonized by *C. glabrata* (16). Furthermore, in another study, *C. albicans* and *C. glabrata* were ~71% and ~19% of *Candida* spp. isolates obtained from patients with vulvovaginitis, respectively. They found that 3.7% of all *Candida* spp. isolates were resistant to fluconazole (Flu-R). The occurrence of Flu-resistance among *C. glabrata* isolates was higher than the others (2). In fact, no correlation was found between previous exposure to the over-the-counter azole antifungals and emergence of azole-resistant *Candida* spp. in patients with VVC (17). However, it has been known an inducible or acquired azole-resistance phenomenon among *Candida* spp. exposed continuously to azoles (18,19). In our study, a higher number of Flu-S isolates were observed by the DAST method as compared to the MAST one. Therefore, out of a total 28 isolates obtained from VVC-patients, 8 isolates were classified as Flu-SDD by

the MAST method, but only 2 isolates classified as Flu-SDD by the DAST method. It has been shown that the disk diffusion method of antifungal susceptibility testing (AST) cannot appropriately distinguish between the susceptible and susceptible dose-dependent isolates of *C. glabrata* due to lack of sensitivity (20). The *C. glabrata* isolates tend to be categorized as susceptible to fluconazole in agar-based disk diffusion method of AST. However, the comparative evaluation of broth microdilution method of AST to disk diffusion method of AST showed a reliable detection of Flu-R isolates of *C. glabrata* by both methods (20). Furthermore, all isolates in healthy women were shown to be Flu-S by the DAST method with one as SDD by MAST. The absence of Flu-resistance among *C. glabrata* isolates from healthy women might be associated with the lack of pathogenicity and virulence of these strains and dominance of commensal bacteria in competence with *Candida* spp. to colonize the vagina.

In the current study, the antifungal effect of an endemic Iranian plant wildy-grown in northern, western and south-western regions of Iran; *A. jesdianum* (also known in Persian: Bon-e-Sorkh or Lizak, in Kurdish, Sourah Boneh) (21-23) was also examined. Obtained antifungal efficacy of the extract on both Flu-R and Flu-non-R isolates of *C. glabrata* was the same. According to these results, we could predicate that the herb extracts are active against both fluconazole-resistant and susceptible isolates of *C. glabrata*, independent of the known mechanisms of azole resistance emerged in *C. glabrata*.

In this study, good antifungal activity of A-EtOH extract of AJ was achieved. The antifungal activity of *Allium* species extracts or essential oils has previously been reported (22). Our results are in agreement with similar studies evaluating antifungal activity of *Allium* species extracts on different fungal species. For instance, MIC<sub>90</sub> of ethanolic extract of *Allium ascalonicum* against *C. albicans* was found to be 8.65 mg/mL (24). In our examinations, MIC<sub>90</sub> of A-EtOH extract against isolates collected from VVC-patients and healthy women was 3 mg/mL. Other studies found that the MFC of seven *Allium* species extracts against three filamentous fungi including *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus fumigatus* was between 35-1536 µg/mL (25). In other study, the anti-*C. albicans* activity of 70% ethanolic extract of *A. ascalonicum* prepared by a maceration method at low temperature was reported (24). Among a number of compounds present in the *Allium* spp., the thiosulfinate allicin has been known as the most abundant antimicrobial compound in garlic and most of the other *Allium* spp. Indeed, allicin and other members of organosulfur family of compounds have promising antimicrobial activities (26). Nonetheless, there is no allicin in fresh, raw garlic. Allicin is created by crushing the garlic. When cell lysis occurs, an endogenous enzyme called alliinase is released and converts the alliin to allicin (27). Allicin is a fragile compound sensitive to

temperature and drying. It can be readily degraded during high-temperature drying or even at low temperatures (28). A solution of allicin prepared in water and ethanol could be preserved for longer periods of time compared to other solvents. Also, considerable amounts of allicin could be preserved in water solution than in ethanol (29).

### Conclusion

In conclusion, *A. jesdianum* showed good anti-*C. glabrata* activities so it is recommended as an alternative medicine and/or combination therapy for treating antifungal infections. It is worth noting that this study investigated the *in vitro* antifungal activity of herb extract only, therefore *in vivo* research must be performed to determine the cytotoxicity and side-effects of the extract. Also, further studies may be necessary to characterize the active ingredients and precise mode of anti-*Candida* action of *A. jesdianum*.

### Authors' contributions

This study was coordinated and supervised by ARK. SS performed the experiments. Data analysis was done by GV. The manuscript was written under the supervision of ARK by GV. All authors commented on and approved the manuscript.

### Conflict of interests

Authors declare no conflict of interest.

### Ethical considerations

An informed consent was obtained from each participant before specimen collection. The protocol of specimen collection was approved by the Ethics Committee of the local center.

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