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In vitro antifungal activity of *Zataria multiflora* essential oil, fluconazole and ciclopirox olamine against *non-albicans Candida* species isolated from recurrent vulvovaginal candidiasis

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Abstract:

The aim of the present study was to investigate the antifungal activity of *Zataria multiflora* essential oil, fluconazole and ciclopirox olamine against 44 *non-albicans Candida* isolates from patients with recurrent vulvovaginal candidiasis. A set of *C. glabrata* (29 strains), *C. krusei* (3 stains) and *C. patapsilosis* (2 strains) were studied using Clinical and Laboratory Standards Institute (CLSI) broth macrodilution method. The results revealed that 33 isolates were resistant (MIC=64 µg/ml), 4 isolates were susceptible (MIC≤ 8 µg/ml) and 7 isolates had dose-dependent susceptibility (MIC= 16, 32 µg/ml) to fluconazole, respectively. With regard to fluconazole, high resistance rate was observed in *C .glabrata* and *C. krusei*. However, ciclopirox olamine was found to inhibit the growth of all non-*albicans Candida* species (MIC≤8 µg/ml). In this study, favorable antifungal activity against *non-albicans Candida* species was obtained by *Z. multiflora* despite having a wide range of MICs (34875-139500 µg/ml). The results indicated that ciclopirox olamine and *Z. multiflora* might be promising in the treatment of recurrent vulvovaginal candidiasis.

Keywords: ciclopirox olamine, fluconazole, *non-albicans candida* spp., recurrent vulvovaginal candidiasis, *Zataria multiflora.*

Introduction

Candida species are the etiologic agents of 20 to 25% of vulvovaginal infections. Vulvovaginal candidiasis (VVC) is characterized by purities, burning, vaginal discharge, dyspareunia and dysuria. This

condition is uncommon before puberty, and at 25 years of age, approximately 50% of females must have had at least one episode of VVC and 75% of women must have experienced at least one episode of VVC during their lifetime (Trama *et al*., 2005). Recurrent vulvovaginal candidiasis (RVVC) is defined as four or more episodes of *Candida* vaginal infection during a year and is estimated to occur in 5 to 8% of women during their fertility period (Sobel, 2006). Various epidemiologic surveys revealed that *C.albicans* is the most common etiologic agent of VVC, accounting for up to 85% of cases. However, some reports suggest that non-*albicans Candida* (NAC) species are responsible for 10 to 30% of episodes in specified geographical areas (Kennedy and Sobel, 2010). The evidence is slowly being aggregated on the increasing incidence of VVC owing to NAC species, especially *C.glabrata*, *C.tropicalis* and *C. krusei* (Faro, 1996).

Ciclopirox olamine (CPO) is a synthetic antifungal agent. It is a hydroxypyridone derivate and is different in mechanism of action and structure from the other recognized antifungal drugs. Its action may be via the chelation of trivalent metal cations (Fe^{3+}). CPO also prevents metal-dependent enzymes, such as peroxidase and catalase, which play an important role in the intracellular destruction of poisonous peroxides. Furthermore, CPO shows some antibacterial activities (Leem *et al*., 2003). *Z. multiflora* (family: Laminaceae) is a plant that usually grows in Iran, Afghanistan and Pakistan. The major components of the essential oil of this plant are phenolic compositions such as carvacrol and thymol (Khosravi *et al*., 2009).

Since women with RVVC present a difficult management problem and these patients often suffer from depression and psychosexual problems, therefore specific attention to diagnosis and management of these patients are important. This study was conducted to evaluate the anti *Candida* activities of *Z. multiflora* essential oil (ZMEO) and CPO against NAC species isolated from patients with RVVC in order to find an effective drug for the treatment of RVVC.

Materials and Methods

Fungal isolates: A total of 44 NAC species (*C.glabrata*; 29, *C.kefyer*; 10, *C.krusei*; 3 and *C. parapsilosis*; 2) from patients with RVVC were included in this study. *C. parapsilosis* ATCC22019 (fluconazole MIC ranges: 2–8 µg/ml) and *C.krusei* ATCC6258 (fluconazole MIC ranges: 16–64 µg/ml) were used as standard control species.

***In vitro* antifungal susceptibility testing:** Antifungal susceptibility tests were performed using the Clinical Laboratory Standards Institute (CLSI) M27-A2 broth macrodilution method (CLSI, 2008). RPMI 1640 (Gibco, USA) culture medium buffered at pH=7.0 with MOPS (Sigma-Aldrich, USA) was used for broth susceptibility testing. Water and 100% dimethyl sulfoxide (Merck, Germany) were used to dissolve fluconazole (Sigma-Aldrich, USA), ciclopirox olamine (USP, USA) and ZMEO (Barijessence, Iran), respectively. Two-fold serial dilutions of Flu, CPO and ZMEO were prepared in RPMI 1640 to achieve a concentration of 0.03125-64 µg/ml for Flu, 0.00195-8 µg/ml for CPO and 1089.8-558000 µg/ml for ZMEO. For yeast inoculums preparation, two to three colonies (diameter >1 mm) of an overnight culture of *Candida* spp. on Sabouraud agar (Merck, Germany) at 35-37°C were suspended in 2 ml normal saline. The cell density was adjusted to $1\text{-}5\times10^4$ CFU/ml using hemocytometer slide. According to M27-A2 standard procedure of CLSI, each macroplate had two drug free controls, one with the media alone and the other with the media containing an equivalent amount of suitable solvent used to dissolve the drug. Plates were incubated at 35-37˚C and results were read visually after 48 h and susceptibility levels were defined according to the MIC breakpoints suggested by CLSI. All

tests were carried out at least twice.

Results

Based on the results of this study, it was demonstrated that 28 out of 29 isolates of *C.glabrata* (MIC=64 µg/ml), 3 out of 10 isolates of *C. kefyer* (MIC=64 µg/ml) and 2 out of 3 isolates of *C. krusei* (MIC=64 µg/ml) were resistant to Flu. Susceptibility to Flu was seen in a few number of isolates including 1 isolate of *C.glabrata* (MIC=4 µg/ml), 2 isolates of *C. kefyer* (MIC= 4 and 8 µg/ml) and 1 isolate of *C. parapsilosis* (MIC=8 µg/ml). From all 44 NAC isolates, dose dependent susceptibility to Flu was seen in 5 isolates of *C. kefyer* (MIC=16-32 µg/ml), 1 isolate of *C.krusei* (MIC=16 µg/ml) and 1 isolate of *C.parapsilosis* (MIC=16 µg/ml) (Additional information is shown in Table 1).

In addition, in almost all 44 NAC isolates, MIC of CPO was clearly lower than Flu. However, MIC was 8 µg/ml for 42 out of the 44 isolates that includes *C.glabrata* (29 isolates), *C. kefyer* (8 isolates), *C.krusei* (3 isolates) and *C.parapsilosis* (2 isolates). For the 2 remaining *C. kefyer* isolates, the MIC of CPO was 4 µg/ml.

The growth of 30 isolates, which includes *C. glabrata* (17 isolates), *C. kefyer* (8 isolates), *C. krusei* (3 isolates) and *C. parapsilosis* (2 isolates), were inhibited at 69750 µg/ml of ZMEO. In the case of four (4) *C. glabrata* and one (1) *C. kefyer* isolates, the MIC of ZMEO was 34875µg/ml, and for the other remaining eight (8) *C. glabrata* and one (1) *C. kefyer* isolates, MIC was 139500 µg/ml.

Table 1. GM, MIC 50, MIC 90 and MIC ranges of fluconazole, Ciclopirox olamine and *Zataria multiflora* essential oil against *C.glabrata*, *C.kefyer*, *C.krusei* and *C.parapsilosis* isolates from recurrent vulvovaginal candidiasis

***Candida* species** (number of isolates)		**Antifungal concentration (µg/ml)**		
		Flu	CPO	ZM
C. glabrata (29)	GM	58.16468	8	76747.7391
	MIC 50	64	8	69750
	MIC 90	64	8	139500
	MIC range	4-64	8	34875-139500
C. kefyer (10)	GM	21.1121	6.9644	69750
	MIC 50	16	8	69750
	MIC 90	64	8	69750
	MIC range	4-64	4-8	34875-139500
C. krusei (3)	GM	40.3175	8	69750
	MIC 50	64	8	69750
	MIC 90	64	8	69750
	MIC range	16-64	8	69750
C. parapsilosis (2)	GM	11.3137	8	69750
	MIC 50	8	8	69750
	MIC 90	16	8	69750
	MIC range	8-16	8	69750

Flu: Fluconazole - CPO: Ciclopirox olamine – ZM: *Zataria multiflora* – GM: Geometric Mean - MIC 50, MIC 90: Minimum Inhibitory Concentration which inhibits 50 and 90 % of isolates, respectively.

Discussion

Treatment of RVVC still faces difficulties and is yet to be determined. The incidence of RVVC due to NAC species is increasing significantly in recent years. These species have also shown significant drug resistance (Sobel, 2006; Watson and Pirotta, 2011).

The results of the present study revealed a high resistance to Flu among NAC species isolated from RVVC. On the other hand, high antifungal activity of CPO presented its potency as a good alternative choice for the treatment of patients with Flu resistant NAC species infections. Also growth inhibition of 30 out of 44 NAC isolates by ZMEO was good evidence to prove the notable antifungal activity of this medicinal plant.

Furthermore, the result of the present study revealed high resistance to Flu among *C. glabrata* isolates (28 out of 29) with 4-64 µg/ml MIC range. This outcome is inconsistent with the results of Padua, Asticcioli and Pfaller with MIC ranges of 0.25-4, 0.125-64 and 0.5-128 µg/ml, respectively (Pádua *et al*., 2003; Asticcioli *et al*., 2009; Pfaller *et al*., 2011). However, the results of Richter (MIC range=2-64 µg/ml), and Badiee (MIC range=8-64 µg/ml) are almost similar to the findings of this study (Richter *et al*., 2005; Badiee *et al*., 2010).

However, as shown in this study, the MIC range of Flu against *C. Kefyer* isolates (4-64 µg/ml) was considerably higher than those achieved by Badiee and Ozcelik (0.25-1 µg/ml and ≤0.03-4 µg/ml, respectively) (Badiee *et al*., 2010; Ozçelik *et al*., 2006).

In addition, the susceptibility *of C. krusei* and *C. parapsilosis* to Flu in this study's results were not similar to the studies conducted by Richter, Ozcelik, Satana and Swinne (Richter *et al*., 2005; Ozçelik *et al*., 2006; Satana *et al*., 2010; Swinne *et al*., 2005).

CPO is a synthetic drug with broad-spectrum anti-fungal properties (Gupta and Plott, 2004). The results of this study revealed lower MIC ranges of CPO against all previous studied NAC-species in comparison to Flu. This could be a therapeutic efficacy indicator of CPO in the treatment of RVVC owing to the NAC-species. In spite of this, the values of MICs for CPO in the present study were considerably higher than those reported by Hanel, Czaika and Gupta (Hanel *et al*., 1988; Czaika *et al*., 2000; Gupta and Kohli, 2003). Broad spectrum antimicrobial activity, high mucosal absorption, multiple mechanisms of antifungal activity and fewer side effects are some of CPO advantages in the treatment of vaginal infections (Zaneveld *et al*.).

The results of the present study revealed generally higher MIC ranges for Flu and CPO against NAC-species (*C. glabrata*, *C. kefyer, C.krusei* and *C. parapsilosis*). This may be attributed to the higher drug resistance of investigated isolates leading to recurrent vulvovaginal symptoms in patients with RVVC.

In traditional medicine, *Z. multiflora* has been used as a medicinal plant in the treatment of dysmenorrhea, spasms, respiratory infections and digestive disorders (Simbar *et al*., 2008). Also, anti inflammatory and analgesic effects of this plant have been reported (Hosseinzadeh *et al*., 2003). Owing to limited geographical distribution of *Z. multiflora*, there are few studies on its in *vitro* antifungal activity against NAC-species. Mahmoudabadi evaluated the *in vitro* anti *Candida* activity of aqueous, ethanolic and methanolic extract of *Z. multiflora* against *albicans* and non- *albicans Candida* species. Their results showed a notable anti *Candida* activity for methanolic followed by ethanolic extract. Aqueous extract has no anti *Candida* activity, and *C. parapsilosis* was the most

sensitive species (Mahmoudabadi *et al*., 2006). Moreover, Zomorodian showed a considerable anti *Candida* activity for *Z. multiflora* with MIC value ranging from 0.07 to 0.5 µl/ml (Zomorodian *et al*., 2011). Gandomi reported that *Z. multiflora* mainly affects the cell membrane and cell wall of fungi. This may be a mechanism for the antifungal activity of *Z. multiflora* (Gandomi *et al*., 2011). Khosravi studied the efficacy of 0.1% *Z. multiflora* cream in the treatment of acute vaginal candidiasis in comparison to 1% clotrimazole. The results showed 90 and 74.8% recovery in patients that received *Z. multiflora* cream and clotrimazole, respectively (Khosravi *et al*., 2008). The results of the present and previous studies reveals the powerful potency of *Z. multiflora* as a natural antifungal agent. Therefore, more studies need to be conducted in order to identify the effective compound of this medicinal plant.

Conclusion

The results of numerous studies conducted across the globe on the drug susceptibility of NAC species, revealed different patterns of drug resistance, in various geographical areas. Therefore, more studies should be conducted in different parts of the world. The favorable activity of CPO and ZMEO against NAC isolates in this study implies their efficacy in the treatment of RVVC in Iranian patients.

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