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 1-5×10$^8$ 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 34875 μg/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

<table>
<thead>
<tr>
<th>Candida species (number of isolates)</th>
<th>Antifungal concentration (µg/ml)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Flu</td>
</tr>
<tr>
<td><em>C. glabrata</em> (29)</td>
<td></td>
</tr>
<tr>
<td>GM</td>
<td>58.16468</td>
</tr>
<tr>
<td>MIC 50</td>
<td>64</td>
</tr>
<tr>
<td>MIC 90</td>
<td>64</td>
</tr>
<tr>
<td>MIC range</td>
<td>4-64</td>
</tr>
<tr>
<td><em>C. kefyer</em> (10)</td>
<td></td>
</tr>
<tr>
<td>GM</td>
<td>21.1121</td>
</tr>
<tr>
<td>MIC 50</td>
<td>16</td>
</tr>
<tr>
<td>MIC 90</td>
<td>64</td>
</tr>
<tr>
<td>MIC range</td>
<td>4-64</td>
</tr>
<tr>
<td><em>C. krusei</em> (3)</td>
<td></td>
</tr>
<tr>
<td>GM</td>
<td>40.3175</td>
</tr>
<tr>
<td>MIC 50</td>
<td>64</td>
</tr>
<tr>
<td>MIC 90</td>
<td>64</td>
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<tr>
<td>MIC range</td>
<td>16-64</td>
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<tr>
<td><em>C. parapsilosis</em> (2)</td>
<td></td>
</tr>
<tr>
<td>GM</td>
<td>11.3137</td>
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<tr>
<td>MIC 50</td>
<td>8</td>
</tr>
<tr>
<td>MIC 90</td>
<td>16</td>
</tr>
<tr>
<td>MIC range</td>
<td>8-16</td>
</tr>
</tbody>
</table>

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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**References**


