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Molecular identification of *Candida* species isolated from vulvovaginal candidiasis and evaluation of antifungal effects essential oil of *Mentha aquatica* L.

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ABSTRACT

Mentha is a genus from the family *Lamiaceae*, whose essential oils has long been used in different forms. This herbal plant has traditionally been used as an alternative medicine to treat candidiasis. So, it seems crucial to find new antimicrobials that have fewer side effects. In this study, we investigated the antifungal effects of *Mentha aquatica L* essential oil on pathogenic *Candida* spp. This descriptive cross-sectional study was performed on 137 *Candida* spp isolated from vulvovaginal candidiasis. These yeasts were confirmed by Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). *Mentha aquatica L* essential oil was prepared by water distillation and Clevenger apparatus. The antifungal activity of *Mentha aquatica L* essential oil and fluconazole versus *Candida* spp was determined by microbroth dilution method using CLSI guidelines. The most common species were identified that *Candida albicans* (63.5%), *Candida glabrata* (28.5%) and *Candida krusei* (8%), respectively. MIC50, MIC90 and geometric mean (GM) of fluconazole were 0.5 µg/ml, 4 µg/ml and 0.573 µg/ml and for *Mentha aquatica L* essential oil 1 µg/ml, 4 µg/ml and 0.931 µg/ml, respectively. The antifungal effect of fluconazole on *Candida* spp was higher than that of essential oil of plant. It seems that the inhibitory effect of essential oil of *Mentha aquatica L* has shown that this plant can be considered as a potential candidate for the development of antifungal drug in the treatment of vulvovaginal candidiasis.

1. Introduction

In recent years, opportunistic fungal infections caused by *Candida* species have also increased significantly due to an increase in predisposing factors and immunodeficiency (Kullberg and Arendrup, 2015). Although *Candida albicans* is an important as the most common cause of candidiasis, other *Candida* spp such as *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, and *C. krusei* are also they have become very an important due to their resistance to antifungal drugs (Colombo *et al*., 2017). Vulvovaginal candidiasis (VVC) is one of the most common infections of the female

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genital tract that is seen daily in clinical clinics (Sobel,2016). In addition, the need for long-term use of antifungal drugs, which in turn leads to side effects, and the identification of several genetic factors associated with drug resistance to fluconazole that limit the use of such developed antifungal compounds, so different studies have been conducted to in order to find effective antifungal compounds of natural origin and fewer side effects (Zaidi and Dahiya, 2015). The high occurrence of the various forms of candidiasis in an increasing number of compromised cases and the development of resistance versus these conventional antifungal agent's point to the crucial need to determine and develop novel therapeutics versus infections caused by *Candida* spp (Bonifacio *et al*., 2019). A plant products have historically characterized the main starting point of compounds for therapeutic use and a majority of antifungals have been conventionally obtained from natural sources (Swamy *et al*., 2016). Several plants have revealed important antifungal activities, justifying even more the intense search for traditional medicine focused on antifungal classification of plants (Rajkowska *et al*., 2017). *Mentha aquatica L* is one of the plants from the family *Lamiaceae* that has six species. This plant is one of the species of this genus that has spread widely in the Caspian region in the provinces of Gilan, Mazandaran and Golestan *(*Esmaeili *et al*., 2006). According to research, the most important constituents of essential oil of peppermint are beta-caryophyllene, viridiflorol, 1,8-cineole, piperitone oxide and trans-caryophyllene (Boz *et al*., 2013). Among the antifungal drugs, fluconazole is more widely used in the treatment of localized and disseminated forms of the candidiasis due to its proper distribution in most tissues of the host body (Morace *et al*., 2014). In recent years, studies on the susceptibility of *Candida* spp to antifungal drugs, especially fluconazole, have shown various molecular mechanisms to express the reasons for the drug resistance of *Candida* strains (Berkow and Lockhart, 2017). The identification of *Candida* spp using traditional methods including culture, biochemical tests, in addition to being time consuming and costly, are less sensitive (Gharanfoli *et al*., 2019). The nucleic acid-based methods have higher speed, sensitivity and accuracy than conventional methods, so that they can identify the genus and species in a few hours (Kord *et al*., 2017). A PCR-RFLP method is one of the precise molecular methods that has been used to identify *Candida* spp in various studies (Shokohi *et al*., 2010; Ayatollahi *et al*., 2012). The aim of this study was to molecularly identify *Candida* spp isolated from VVC and antifungal activity of *Mentha aquatica L* essential oil on these strains.

2. Materials and Methods

2.1. Sample collection

This descriptive study was performed on 137 yeasts isolated from VVC during one year (2020-2021). Vaginal sampling of the participants performed by using a sterile swab. All isolates were stored at -20 °C.

2.2. Molecular identification of Candida spp

The genomic DNA was extracted from the new colonies using the glass-beads method previously described (Yamada *et al*., 2002). The DNA genomic amplification with ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers (Xie *et al*., 2008) were used in PCR reactions that containing 10 µl of PCR buffer, 0.5 µl primer. 0.5 µl of dNTP, 0.25 µl of Taq DNA polymerase (CinnaGen, Iran), 2 µl of DNA that the sterile distilled water was increased to 25 µl. PCR reaction was performed using a thermal cycler (Rad-Bio USA). Based on the method previously described by Mirhendi et al., the PCR product were digested by MSPI and BLNI enzymes (Fermentas, Germany). Then, 5 µl of PCR product with 0.5 µl of MSPI/BLNI enzyme, 1.5 µl of enzyme buffer and 8 µl of distilled water were mixed in 200 µl microtubes and placed at 37°C for 10 minutes. PCR product was electrophoresed on 1% gel and RFLP product on 2% gel.

The gels were observed after staining with ethidium bromide solution with Gel Doc device (Cambridg Uvidoc, UK Gel Documentation System).

2.3. Essential oil of Mentha aquatica L

First, 100 grams of leaves of *Mentha aquatica L* (Marzanabad, Babol, Mazandaran, Iran) were collected and dried. For the extraction of essential oils from *Mentha aquatica L* by hydrodistillation under optimal operating situations, a quantity of 100 g of *Mentha aquatica L* was added to 800 ml of distilled water in a 2-liter flask. The set was placed in a balloon heater attached to a refrigerator to warrant concentration of essential oils for 4 hours. At the end of the distillation, two phases were detected, an aqueous phase (aromatic water) and an organic phase (essential oil), less dense than water. The essential oil of *Mentha aquatica L* was obtained, dried under anhydrous sodium sulphate, and stored in sealed vials in the dark, at 4°C, until used (Elyemni *et al*., 2019).

2.4. Determination of Minimum Inhibitory Concentration

Antifungal activity of *Candida* spp to fluconazole and essential oil of *Mentha aquatica L* were performed using broth microdilution method according to CLSI-M27-S4 guidelines. Then, different *Candida* spp of were cultured on Sabouraud dextrose agar (Merck, Germany) and incubated for 48 hours at 37 °C. Subsequently, a suspension of fresh *Candida* spp was suspended in distilled water with concertation of 0.5×10^3 to 2.5×10^3 cfu/ml.

Lastly, the fluconazole and essential oil of *Mentha aquatica L* were diluted in RPMI-1640 buffered with morpho linepro panesulfonic acid (MOPS). Then 100 µl of the fungal suspension was added to each well and pipetted. Drug dilutions of fluconazole and plant essential oil in the presence of fungi were 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 µg/ml. The minimum drug concentration, which showed 50% growth inhibition compared to the control well, was considered as MIC. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 was used as standard strains for performance accuracy and quality control (Santos et al., 2014).

2.5. Statistical analysis

The SPSS software of version 16 was used for statistical analysis of results. One-way analysis of variance (ANOVA) with Tukey test was used to compare data.

3. Results

The mean age of patients was 37.4 ± 8.6 years, with the highest and lowest ages being 83 and 18 years, respectively. The highest number of patients was in the age range of 41-33 years. 24 patients (17.51%) had recurrent infection (RCCV) and the rest did not. Out of 137 yeast isolates identified by PCR-RFLP molecular method, 87 were *C. albicans* (63.50%), 39 were *C. glabrata* (28.5%), and 11 were *C. krusei* (8%).

According to the results of drug susceptibility evaluation of *Candida* spp, the MIC50, MIC90 and geometric mean (GM) of fluconazole for all isolates tested were 0.5 µg/ml 4 µg/ml and 0.573 µg/ml. However, the value for essential oil of *Mentha aquatica L* was 1 µg/ml, 4 µg/ml and 0.931 µg/ml, respectively. The results of statistical analysis showed that there was no significant relationship between different species of *Candida* with fluconazole and essential oil of *Mentha aquatica L* (Pvalue <0.05).

4. Discussion

Vulvovaginal candidiasis is caused by overgrowth of *Candida* spp in the genital mucosa and has become very challenging in recent years (Mohamadi *et al.,* 2015). PCR-RFLP is used to identify yeast isolated from patients with VVC. The frequency of VVC in reports in Iran and abroad varies from 5.4 to 84% (Lakshmi, 2019; Roshan *et al.,*2014; Lopes *et al.,* 2012). The present study shows a high rate of VVC in people aged 30 to 41 years, which the study (Mohamadi *et al.,* 2015) agrees with, while the study of (Rezaei-Matehkolaei *et al.,* 2016) disagrees. The current results showed a high prevalence of *C. albicans* in VVC cases.

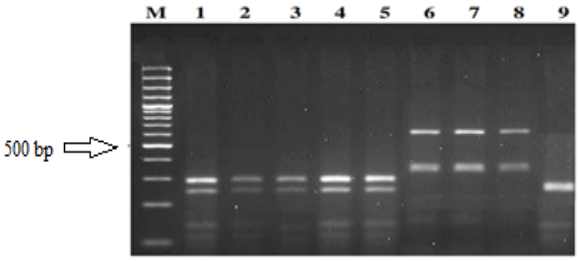

Figure 1. Agarose gel electrophoresis of restriction fragments length polymorphism ITS gene from Candida species isolated from vulvovaginal candidiasis with *Candida albicans* (lanes 1 - 5), *C. glabrata* (lanes 6 - 8), *C. krusei* (lanes 9) and Lane M represents a 100 bp size molecular marker.

Table 1. Antifungal sensitivity profile of Candida isolates to fluconazole and Mentha aquatica L

Number of *Candida* spp	Compounds	MIC rang (µg/ml)	MIC $_{50}$ (µg/ml)	MIC $_{90}$ (µg/ml)	GM	P-value
Candida albicans (87)	Fluconazole	0.125 - 4	0.5	4	0.537	P < 0.05
	Essential oil	0.125 - 8	1	4	0.923	
Candida glabrata (39)	Fluconazole	0.125 - 4	0.5	8	0.685	
	Essential oil	0.125 - 8	1	8	1	
Candida krusei (11)	Fluconazole	0.125 - 8	0.5	16	0.785	
	Essential oil	0.125 - 16	1	16	2.1	
Total isolates (137)	Fluconazole	0.125 - 16	0.5	4	0.573	
	Essential oil	0.125 - 16	1	4	0.931	

This finding is in accordance with the studies of Roshan et al. (86.2%) (Roshan *et al.,* 2014), Roudbary et al. (82.2%) (Roudbary *et al.,* 2013), Rezaei-Matehkolaei et al. (88.2%) (Rezaei-Matehkolaei *et al.,* 2016), Mahmoudi Rad 65.1% (Mahmoudi Rad *et al.,* 2012) and Fan et al. (89.5%) (Fan *et al.,* 2008). The frequency of non-*albicans Candida* has increased over the last decade.

The present study had a prevalence of 36.49% for non-*albicans Candida*, while this is in contrast to other studies, for example (Lakshmi, 2016; Lakshmi, 2019) showed a significant increase in non-*albicans Candida* spp with a prevalence of 59.2% in all *Candida* spp. (Richter *et al*., 2005) reported that 24% of the 593 symptomatic patients were non-*albicans Candida* spp from 1998 to 2001 (Richter *et al.,* 2005). The second most common species isolated in this study was *C. glabrata* (28.46) which was consistent with similar studies (Roshan *et al.,* 2014; Roudbary *et al.,* 2013) but Lakshmi and Budhani reported *C. tropicalis* as a common non-*albicans Candida* spp (Lakshmi, 2019; Mahmoudi Rad *et al.,* 2012; Mohammadi *et al.,*2013; Budhani *et al.,* 2016). The third an etiological agent of VVC in the present study was *C. krusei* (8%), while the study conducted by Mahmoudi Rad et al. Reported a high 4% (Mahmoudi Rad *et al.,* 2012). The results of the present study are consistent with the study of (Gharaghani *et al*. 2018) in terms of etiological factor (Gharaghani, 2018 #863). Given the increasing resistance to antifungal drugs in *Candida* spp, finding new plant-based compounds is attractive to researchers (Sanguinetti, 2015 #864). *Mentha aquatica L* is a perennial plant that grows in swamps and wetlands from the

southwestern cape to the tropics of Asia, Africa and Europe (Jäger, 2007 #865). In the present study, the MIC50, MIC90 and geometric mean for geranium essential oil against Candida isolates isolated from vulvovaginal candidiasis were 1 µg/ml, 4 µg/ml and 0.931 µg/ml, respectively. Few studies have examined the antifungal effects of this type of plant.

Antibacterial results in the study of (Getahun et al., 2008) MIC values essential oil of *Mentha aquatica L* varied from 5-100 µg/ml. The lowest MIC (5 µg/ml) was observed against *Staphylococcus aureus* 29737, *Staphylococcus aureus* ML267, *Sarcina luteus* 9341, *Bacillus pumilus* 8241 and *Shigella sonnei* BCH 217, while the highest MIC (100 µg) were seen in *Shigella flexneri* and *Shigella boydii* (Getahun, 2008 #867). (Movaghari et al.,2016) the mean diameter of growth inhibition zone around discs containing aqueous and alcoholic *Origanum vulgare* extract against *C. albicans* isolated from the mouth in all concentrations (10, 20, 40, 80 and 100% used was significantly less than nystatin (P <0.001) (Movaghari Pour, 2018 #868). (Al-Bayati et al., 2009) identified antimicrobial compounds of alcoholic (methanolic) extract of *Mentha* leaves (*Mentha longifolia L*) against seven types of microorganisms. The antifungal activity of this extract was reported versus *C. albicans* with a MIC of 125 µg/ml (Al-Bayati, 2009 #869). The difference in the amount of MIC with our research can be due to the difference in the type of plant species and the difference in the type of composition (Stagos, 2018 #870). (Dzamic *et al.,* 2010) investigated the antifungal and antioxidant properties of essential oil of *Mentha* (*Mentha longifolia L*) by well dilution method (Džamić, 2010 #871). The MIC reported in this study differs from the result of our work, which may be due to the use of essential oils and fatty acids in the plant instead of the *Mentha* plant itself. (Razavi *et al.,* 2012) investigated some of the biological activities of *Mentha longifolia L*. The antifungal properties of the plant have been reported during this experiment. The reported MIC was 30.5 µg/ml on *C. albicans* (Razavi, 2012 #872). Avijgan and Mahboubi (2021) compared the antifungal effect of medicinal products containing the hydroalcoholic extract of *Echinophora platyloba* DC and fluconazole in women with chronic recurrent vaginitis caused by *C. albicans*. After management, culture of vaginal discharge was positive for 13 (43.3%) and 6 (20%) cases with a recurrence rate of 17 and 8 (56.7% vs. 26.7%) in fluconazole and *Echinophora platyloba DC* cream plus fluconazole, respectively (Avijgan, 2015 #873).

Mentha longifolia L does not seem to have more antifungal properties than fluconazole. Therefore, it is suggested that further studies be performed on the effects of other fractions and compounds of the *Mentha longifolia L* on the clinical isolates of *C. albicans* isolated from VVC. By conducting more comprehensive research, the practical goals of such research can be achieved, which is the production of effective and efficient herbal mouthwashes with minimal side effects.

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Refereces

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