

Full length research paper

Comparing the effect of ozonized olive oil with clotrimazole on three *Candida* species *C. albicans*, *C. glabrata*, *C. krusei*

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One of the most important pathogenic fungi in immune compromised patients is *Candida*.spp. Ozone is an allotropic form of oxygen with high oxidation power; in addition it has fungicidal effects. Considering highly prevalence of *Candida* infections and drug resistance, it is important to find a low cost medicine with high effects and with low adverse effects for treating these kinds of infections. In this study, the effect of ozonated olive oil compared with clotrimazole on three species of *candida* (*C. albicans*, *C. glabrata*, and *C. krusei*) on saboraaud dextrose agar (SDA) media was evaluated. Different concentrations of ozonated olive oil (166.66, 200, 233.33, 266.66, 300 mg/ml) in culture media were prepared and poured in some plates separately. Plates without ozonated olive oil were used as negative control. Plates containing different amount of clotrimazole (1, 2, 3, 6, and 8µg/ml) were considered as positive control. After inoculation of different *candida* spp. in all media, the plates were incubated at 37°C for 72 hours and observed for fungal growth in their status every 24 hours. Our study showed that the minimum inhibitory concentration (MIC) of ozonated olive oil for *Candida krusei* was 166.66, and for *Candida albicans* and *Candida glabrata* were 233.33, 200 mg/ml respectively. Clotrimazole inhibited all *candida* species in concentration much lower than ozonated olive oil. Considering that the ability of ozonated olive oil to inhibit *candida* growth in the media, authors hope that future researches will be performed based on this study and it can be a new product for topical treatment of *candidiasis*.

Keywords: *Candida albicans*; *Candida krusei*; *Candida glabrata*; Olive oil; ozone; Clotrimazole.

Introduction

Candidiasis is a primary and secondary infection which is caused by *Candida* species especially *Candida albicans*. Its clinical symptoms are acute, sub acute or chronic and sporadic. Infection may be limited to mouth, throat, skin, vagina, fingers, nails, trachea, lung or digestion system. It may cause septicemia, endocarditis, meningitis, systemically. Since *Candida* species are endogenous the resultant disease demonstrates as an opportunist infection (Zeini *et al.*, 2009). More than 90% individuals with defect in immune system are affected to oral *candidiasis* and almost 75% of women are affected at least one time to vaginal *candidiasis* in her life.

Approximately 60% of healthy individuals and more than 65% of children are affected to *candida* infections without clinical symptoms (Ying *et al.*, 2010). Clotrimazole is an antifungal medicine from azole group, which blocks ergosterol synthesis by suppressing fungus demethylase cytochrome enzyme and causes growth stop with effecting on its membrane (Mahmoudabadi, 2002). Ozone is a very strong oxidization material and it is mainly applied as a disinfectant in different fields. Therapeutic ozone is presented in National Institutes of Health (NIH) in the United States as a method in supplementary medicine and also it is stated that excessive use for ozone can be poisonous like other medicines (Viebahn, 1985). Different ozonized oils are used successfully against different infections (Geveely, 2006). One of these materials is olive oil that in many countries, ozonized

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olive oil is used because of therapeutic effects and efficient antimicrobial activity against bacteria, virus and fungi (Lezcano *et al.*, 2000, Sechi *et al.*, 2001). Ozone with oxidization properly can convert olive oil to strong oxidizing material. Alvarez and Gundarova have reported safety of this material (Gundarova *et al.*, 1996; Alvarez *et al.*, 1997). Generally, excessive use of antibiotics for treating infectious diseases and appearing fungus strains resistance to some medicines along with inefficiency and side effects, driven the research towards the comparing therapeutic effects of ozone with clotimazole. (Shaschova, 1995). In this study antifungal effect of ozonized olive oil studied on three *Candida* species, including *Candida albicans*, *Candida glabrata* and *Candida krusei* on sabouraud dextrose agar medium.

Materials and Methods

Ozone apparatus

Ozone was produced by controlled oxygen flow in zone generator device (made in the USA with production power 13.5 g/h).

Ozoning olive oil

This procedure is elaborated by ozone generator device. In this method, ozone is passed as bubbling through olive oil for three weeks and olive oil is become ozonic cream gel material. This material is maintainable in environment temperature for three years and it is melted in higher temperature and it is returned by dropping temperature (Geveely, 2006).

Standardization of the preparation was carried out according to the following parameters: olive oil Acid Index determination

Base on definition, Acid Index, is value KOH in term of gram which is necessary to neutralize free fatty acid in one gram of fat. To determine the Acid Index of olive oil, 10 g of weighed olive oil transmit to an erlen containing 50 ml of alcohol and ether, then add 1 ml of phenolphthalein this mixture and titrate it to normal hydroxide potassium 0.1 until the mixture is become colorless pink to stabilize for 30seconds, indicating titration end. Acid Index with used alkali mass neutralize free fatty acid in 10 g olive oil with this formula is achieved: $56.11V \times N/W$ Where, N is hydroxide potassium normality, and W is tested sample mass, and V is alkali acid and 56.11 is the atomic mass of hydroxide potassium.

Olive oil Iodine Index determination

Base on definition, Iodine Index, is value Iodine base on gram which is absorbed by 100 g of fatty sample. This index indicates dual links numbers in test sample. In fats with non saturated fatty acid, oxidation occurs in dual links, this cause to produce hydroperoxide and consequently forming aldehyde. To determine olive oil Iodine Index, 1 g of weighed olive oil was transferred to flask 250 ml and it was solved by chloroform 19 ml, and then 250 ml of normal iodobromide 0.2% was added to mixture. After closing the cap of flask, it was put in dark place for 30 minutes and the flask was shaken one time per 10minutes. Then 30ml of potassium iodine 15% and 100 ml of water were added to the materials the mixture was titrated by normal thiosulphate 0.1% when the color changes to colorless pink, 3 ml of amylum solution was added to the materials and titration was continued until blue color was appeared. This work was also performed simultaneously on blank sample then Iodine Index achieved by this formula: $[126.9 (V_b - V_s) N] / 10W$. Where, 126.9 is Iodine atomic weight, V_b , V_s are normal thiosulphate sodium 0.1, N is thiosulphate sodium normality and W is olive oil weight

Fungal strains

Three pathogenic fungi species, *C. albicans* (PTCC: 5027), *C. glabrata* (PTCC: 5297), *C. krusei* (PTCC: 5295) were provide as lyophilized from Iranian Research Organization for Science and Technology. These isolations were tested for determining their sensitivities to ozonized olive oil.

Preparing medium containing ozonized olive oil

65 g of medium solid powder (sabouraud dextrose agar) was added in one liter of distilled water by heating. Then 3 ml of prepared medium was poured into each testing tube, and the tubes were autoclaved in 121°C for 15 minutes. After cooling tubes to 50°C, 50 µl tween 80 was added as emulsifier to each tube, and they were vortex mixed to uniform. Then ozonized olive oil in different values was added to each tube (500 mg, 600 mg, 700 mg, 800 mg, 900 mg), and therefore different concentration of ozonized olive oil medium were prepared: (166.66 mg/ml, 200 mg/ml, 233.33 mg/ml, 266.66 mg/ml, and 300 mg/ml).

Preparing fungi, determining species, and controls

0.5ml of prepared sabouraud dextrose agar medium was added to ampoule containing lyophilized powder of three fungi species and prepared fungal suspension, some of this suspension was cultured on sabouraud

Table 1: Different ozonized olive oil concentrations effects at mg/ml on *C. albicans*, *C. glabrata*, *C. krusei* in 24, 48 and 72 hours after culture

Ozonized olive oil mg/ml	<i>C.albicans</i>			<i>C.glabrata</i>			<i>C.krusei</i>		
	24h	48h	72h	24h	48h	72h	24h	48h	72h
166.66	+	+	+	+	+	+	-	-	-
200	+	+	+	-	-	-	-	-	-
233.33	-	-	-	-	-	-	-	-	-
266.66	-	-	-	-	-	-	-	-	-
300	-	-	-	-	-	-	-	-	-

Table 2: Different clotrimazole concentrations effects at mg/ml on *C. albicans*, *C. glabrata*, *C. krusei* in 24, 48, and 72 hours after culture

Clotrimazole	<i>C.albicans</i>			<i>C.glabrata</i>			<i>C.krusei</i>		
	24h	48h	72h	24h	48h	72h	24h	48h	72h
1 µg/ml	+	+	+	+	+	+	+	+	+
2 µg/ml	+	+	+	-	+	+	-	-	-
3 µg/ml	+	+	+	-	-	-	-	-	-
6 µg/ml	+	+	+	-	-	-	-	-	-
8 µg/ml	-	-	-	-	-	-	-	-	-

dextrose agar with chloramphenicol (SC) and the results is cream color colonies after 48-72 hours after incubation at 37°C, which indicates fungi growth. To confirm the presence of *Candida* species one colony was removed from SD medium and put on glass slide and a drop of KOH15% was added to it, and it covered with glass slide and examined under microscope for pseudohypha and blastoconidia that indicate the presence of *Candida* species. For determining species, tests used were germ tube test, Chlamydoconidium production, and culture on Chrom agar medium and Corn meal agar medium. Germ tube test and Chlamydoconidium production were used to identify *Candida albicans*.

For determining species, one colony was removed from SC medium and it was cultured on chrom agar plate (CHROM agar *Candida*, France) and after incubation at 37°C for 48-72 hours, the results were read based on changing the color of medium. Changing the color of medium to green indicates *Candida albicans*, changing to white: *Candida krusei* and changing to pink means *Candida glabrata*.

MIC determination

MIC determination was performed by agar dilution (Geveely, 2006). Clotrimazole was used as positive control and the antifungal efficacy of it compared with ozonated olive oil. A serial of concentrations 1, 2, 3, 6, 8µg/ml was produced from vaginal cream clotrimazole 1%. Then three *Candida* species from standard species were cultured on medium. The plates were incubated at 37°C and the growth or non growth results were read after

24, 48 and 72hours. As negative control concentrations 166.66 mg/ml, 200 mg/ml, 233.33 mg/ml, 266.66 mg/ml and 300mg/ml olive oil non- ozonized medium were produced.

Results

After 24, 48, and 72 hours incubation at 37°C, the results were observed and recorded for all *Candida* species. In addition the results of the control plates were examined. The presence of the white convex colonies indicates fungus growth. The condition was uniform when the experiments were performed and the experiment was repeated three times to accuracy confirmation condition in each concentration. To equalize inoculate amount in each plate, the culture was performed in two places. After 24hour reading was done for plates without fungi growth and growth inhibition confirmed against ozone and/or clotrimazole. From Table 1: *C. albicans* and *C. glabrata* growth in concentration 166.66 mg/ml ozonated olive oil was observed. *C. albicans* growth in concentration 200 mg/ml of ozonated olive oil was observed. In concentration 233.33, 266.66 and 300 mg/ml ozonated olive oil, There was not any growth for all species. Minimum ozone concentration (MIC) was 233.33 mg/ml for *C. albicans*, 200 mg/ml for *C. glabrata* and 166.66 mg/ml for *C.krusei* to suppress their growth (Table 1).From Table 2: The minimum clotrimazole concentration for suppressing growth (MIC) was 8 µg/ml for *C. albicans*, 3 µg/ml for *C. glabrata* and 2 µg/ml for *C. krusei*.

Discussion

Diseases resulted from genus *Candida* yeasts have a wide spectrum and generally have greater prevalence in immunocompromised patients. These fungi have various species, including *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. lusitanae*, *C. guilliermondii* and *C. dubliensis* (Zeini *et al.*, 2009). *Candida* species are among the most important opportunistic fungi that in recent decades, systemic and local infections as well as new *Candida* species have been increased (Rani *et al.*, 2002). Among *Candida* species, *C. albicans* has been more prevalent as an etiologic agent of *candidiasis*. This fungus habituates in digestion system, mouth and vagina and human acquires it on birth time while passing from vagina. Other *Candida* species are part of natural fungi dermis and mucosa, some exist in nature, in soil and different materials which have much less pathogenic power and they enter to body through external sources and under particular conditions they can be pathogenic with dominating natural defensive mechanisms of host (Shadzi, 2009). Generally, there are many factors which disorder natural equilibrium between *Candida* and host causing pathologic symptoms. These factors are age, physiologic changes, continuous use of antibiotics, breaking natural defensive barrier, disabling diseases, job, overweight, and alcoholism and avitaminosis for vitamins A, B, C (Zeini *et al.*, 2009). Considering high prevalence of *Candida* infections and recent performed studies and also reported drug resistances in these fungi, it is important to find a low cost medicine with high effects and without any consequences for treating fungal infections (Ying *et al.*, 2010).

In this study, we surveyed antifungal ozonated olive oil effects on *Candida* species. Ozone has strong fungicide effects. Geveely in a study showed the fungicide effect of the ozonized olive oil on Dermatophytes, *Candida albicans* and *Aspergillus fumigates* (Geveely, 2006). In addition, Menendez used successfully ozonized olive oil for treating ring worm of the foot (Menendez *et al.*, 2008). Since ozone doesn't exist in atmosphere, yet, fungus resistance to this material hasn't been reported (Geveely, 2006). Ozone reacts exclusively with dual bands in nonsaturation olive oil fatty acids, thus this reaction results in different toxic products. These compounds include hydroperoxidases, ozonides, aldehydes, peroxides, diperoxides and polyperoxides, have high oxidizing power. In this study obtained results showed that all three studied species were sensitive to ozonized olive oil and sensitivity to species was different and it depended on concentration. In this study, ozonized olive oil had better suppression effect on *Candida krusei* than *Candida glabrata* and *Candida albicans*. Also according to the results, inhibitory effect of clotrimazole on *Candida krusei* has been better than *Candida glabrata* and *Candida albicans*. The amplitude of MIC clotrimazole and ozonized olive oil has been determined 2-8 µg/ml and

166.66-233.33 mg/ml respectively. These results show that ozonized olive oil and clotrimazole are able to inhibit growth of these three *Candida* species.

As Tara and colleagues in 2012 performed a clinical study on patients with vaginal *candidiasis*, ozonized olive oil and clotrimazole was effective in improving these patients (Tara *et al.*, 2012). In studies of other researchers, observations based on ozonized olive oil effect on these species were observed. Riechart in a study in 2007 showed that ozonized olive oil has a more suppressing effect on *Candida krusei* than *Candida albicans* (Reichart, 2007). The results of this study are in agreed with our study results. Generally there are few studies on olive oil effect on *Candida* species for growth suppression, but based on current studies and other performed studies by other researchers (Geveely, 2006), we recommend that ozonized olive oil has a suppressing effect on these three species of *Candida* and its effect depends on concentration, which was observed in higher concentration in short time.

Conclusion

According to the results of this study and comparing it with negative control results, it was proofed antifungal ozone effects. The minimal inhibitory concentration (MIC) for every species was revealed. Also it was observed that *Candida krusei* has a more sensitivity to ozonized olive oil and clotrimazole than *Candida albicans* and *Candida glabrata*. Considering obtained results in this study and mentioned clinical studies, ozonized olive oil is an effective medicine to treat *Candida* infections (e.g. dermal and mucosal infections). Authors hope that future researches will be performed based on this study. Eventually Ozonized olive oil could be a substitute product for treating fungal infections and can be suggested as a proper replacement for antibiotics and it can be a solution for overuse of antibiotics in the treatment of fungal disease and overcoming of multi-drug resistant of *candida* strains.

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