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Antifungal Activity of Olive Oil and Ozonated Olive Oil Against *Candida* Spp. and *Saprochaete* Spp.

Kemal Varol ^a, Ayşe Nedret Koc^b, Mustafa Altay Atalay^b, and Ihsan Keles^a

^aDepartment of Internal Medicine, Faculty of Veterinary Medicine, Erciyes University, Kayseri 38100, Turkey; ^bDepartment of Microbiology, Medical Faculty, Erciyes University, Kayseri 38100, Turkey

ABSTRACT

Ozonated olive oil was investigated for their capacity to inhibit growth of 38 yeast strains of *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, and *Saprochaete capitata*. Two different ozonated olive oil (OZO1, OZO2) and two different olive oil (OL1, OL2) samples having different biochemical parameters were assessed in terms of their antifungal ability and comparison was made. Fluconazole was chosen as control antifungal agent. Each sample's antifungal activity decreased in the following order: OZO1 > OZO2 > OL1 ≥ OL2. This study demonstrated that ozonated olive oil may help to control some fluconazole-resistant and dose-dependent sensitive fungal strains.

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Ozone; Antifungal Activity; *Candida* Spp; Fluconazole Resistance; Olive Oil; Ozonated Olive Oil; Ozone Therapy; *Saprochaete* spp

Introduction

Fungal infections are important problems and frequently occur worldwide (Koc et al. 2011, 2016). In recent years, presence of opportunistic fungi that cause common invasive fungal infections in hospitals have been reported to increase (Garcia-Ruiz et al. 2013; Ulu Kilic et al. 2016).

Although in invasive fungal infections, *Candida* (C) spp. and *Aspergillus* spp. are more common, yeast species, such as *Trichosporon*, *Saccharomyces*, *Saprochaete* (S), fungal genera; such as *Zygomycetes*, or *Fusarium* can also be seen. *S. capitata* is also the reason of some fungal infections (Garcia-Ruiz et al. 2013; Koc et al. 2016; Pfaller and Diekema 2010; Richardson and Lass-Flörl 2008). *C. albicans* and rarely *C. parapsilosis* and *C. tropicalis* cause cutaneous candidiasis, which is a skin infection occurring acutely or chronically (Wagner and Sohnle 1995). Again, *S. capitata* invade skin and cause generalized maculopapular erosion (Garcia-Ruiz et al. 2013; Koc et al. 2016).

In the treatment of fungal infections, polyenes and some imidazole derivatives have been used (Fernández Torres et al. 2006). Long periods of drug usage or short-term but inadequate treatments results in drug resistance. Furthermore, long-term usage of ketoconazole, fluconazole (FLU), itraconazole and their derivatives cause side effects. For all these reasons, new treatments options have been investigated alternatively to the antimicrobial agents

(Garcia-Ruiz et al. 2013; Geweely 2006; Koc et al. 2016; Ouf et al. 2016; Silici and Koc 2006). One of the treatment choice is ozone therapy (Ouf et al. 2016). Ozone (O₃) is the high-energy form of atmospheric oxygen (O₂) (Bocci 2011). Despite the stable oxygen molecules, ozone is an unstable molecule. Ozone gas; has antiviral, antibacterial, antiparasitic and antifungal activity (Lezcano et al. 2000; Ouf et al. 2016; Rodrigues, Cardoso, and Caputo 2004). Furthermore, in toxicological studies, ozone also found to be a reliable molecule and had no genotoxic activity (Rodrigues, Cardoso, and Caputo 2004).

Olive oil (OL) is an essential oil obtained from olive fruit that contains a high percentage (65–85%) of oleic acid and is also comprised of different fatty acids (Geweely 2006). OL is known to inhibit reproduction of microorganisms (Cicerale, Lucas, and Keast 2012). In recent years, ozone in the form of ozonated olive oil (OZO) or ozonated water have widely been used for medical indications (Fernández Torres et al. 2006; Geweely 2006; Ouf et al. 2016; Sakazaki et al. 2007; Skalska et al. 2009). Nowadays, essential oils and ozonated oils have been used for the treatment of bacterial, viral and fungal infections of skin, otitis, intraocular infections, dental disease and vaginitis (Bocci 2006; Geweely 2006; Helal et al. 2006; Kim et al. 2009, 2013; Kutlubay et al. 2010; Sechi, Lezcano, and Nunez 2001).

Ozone has strong antimicrobial and germicidal activity against virus, bacteria, parasites, and fungus.

The interaction of ozone molecule against cellular components, especially having double bonds sulfhydryl groups and phenolic rings cause an oxidation reaction that prevents their growth. Therefore, membrane phospholipids, intracellular enzymes and genomic materials are attacked by ozone. As a result of this, cell damage and death of microorganisms occur (Arana et al. 1999; Gonçalves 2009; Ouf et al. 2016; Perez, Poznyak, and Chairez 2015). Approximately 80% carbon hydrate and 20% of protein and glycoproteins are the components of cell walls of fungi, which are multi-layered. Because there are several disulphide bonds in the site, this promotes oxidative inactivation by ozone. Ozone has the ability to diffuse through the fungal wall, then enters into the cytoplasm and disturbs the vital cellular functions. Ozone also has an inhibitory effect on spore germination and production (Antony-Babu and Singleton 2009; Ouf et al. 2016).

The effect of ozone combined with olive oil occurs with almost all of the carbon-carbon double bonds that are present in unsaturated fatty acids, which cause different toxic products such as many oxygenated compounds, ozonides, aldehydes and peroxides. These compounds can also account for the wide antimicrobial activity of OZO (Pryor and Uppu 1993; Ouf et al. 2016). The safety of ozonated sunflower oil (OSO) was reported by Gundarova et al. (1996) and Alvarez et al. (1997).

The aim of the present study was to determine antifungal activity of OZO and OL in vitro against different strains of yeast using guidelines of Clinical Laboratory Standards (CLSI) (2008) document M27-A3. Results presented in this study will assist in future studies to help focus more studies on OZO as a potential natural drug to control most fungal pathogens in medical mycology.

Material methods

Study design

In this study, two different olive oil [Olive oil 1 (OL1) and Olive oil 2 (OL2)] samples having different acidity, iodine, peroxide, *p*-anisidine, pH and viscosity values and two Ozonated olive oil samples [Ozonated olive oil 1 (OZO1) and Ozonated olive oil 2 (OZO2)] obtained after ozonation of the olive oils (OL1 and OL2) were used. Their antifungal activities were then compared with the effects of FLU.

Identification and antifungal susceptibility

Thirty-eight non-repetitive strains isolated from blood cultures at the Medical Microbiology Department of Erciyes

University, Gevher Nesibe Hospital were included in the study. All strains tested were considered as infection agents. Again, all strains were collected during a 6-month period in the Mycology Laboratory. They were comprised of 10 strains of *C. albicans*, 10 strains of *C. glabrata*, 10 strains of *C. parapsilosis*, 4 strains of *C. krusei* and 4 strains of *S. capitata*. One standard *C. albicans* ATCC 90028 and one standard *C. parapsilosis* ATCC 22019 were also used. Strains were identified as follows: according to carbon hydrate assimilation of with API AUX C 20 (bioMérieux, France) kits, the macroscopic and microscopic morphology, capability of growing at 37 °C on corn meal agar, germ tube test, sensitivity for cycloheximide and urea hydrolysis. The isolates used in this study were kept at –20 °C in tryptic soy broth having 10% glycerine. Before their examination, they were melted and sub-cultured on Sabouraud glucose agar plates at least twice. Quality control was made according to suggestions of CLSI document M27-A3 by testing *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019 (CLSI 2008).

FLU was obtained as a powder from the manufacturer (Fako Co., Istanbul, Turkey). CLSI (2008) (M27-A3), microdilution broth methods were used to determine MICs. Antifungal activity of OL, OZO and FLU, *in vitro* were examined by the guidelines given (M27-A3). FLU dissolutions were made in sterile distilled water. Final drug content in the microdilution plates ranged between 0.125 to 64 µg/mL for FLU, and from 0.1 to 50% (v/v) for all of the olive oils and ozonated olive oils (OZO1, OZO2, OL1, and OL2) and its' dilutions were made in RPMI 1640 broth medium (Sigma Chemical Co., Madrid, Spain) with L-glutamine but without sodium bicarbonate and buffered at pH 7.0 with 0.165 mol/L morpholinepropanesulfonic acid (Sigma Chemical Co.).

Inoculum suspensions of the yeast were formulated as explained in the CLSI M27-A3 document using sterile saline solution (0.85%). Cell density was adjusted. To do this a spectrophotometer was used. At a 530-nm wavelength, an adequate saline was added to match the transmittance generated by a 0.5 McFarland density standard which resulting in a concentration of $0.5 \times 10^3 - 2.5 \times 10^3$ cells/mL. MICs were determined visually at 24 and 48 h of incubation at 35 °C. The plates were then investigated for the presence or absence of growth at 24 and 48 h.

The MIC for olive oils and ozonated olive oils were described as the lowest concentration that optically observed. For the fluconazole, the MIC was expressed as the lowest concentration in which 50% decrease in turbidity as observed visually. For all of the OL and OZO the lowest concentration was 100%. MIC₅₀ and MIC₉₀, minimal inhibitory concentration at which 50% and 90%, respectively, of the isolates were inhibited.

Ozonation procedure

OL and OZO were obtained from a certified commercial company (Aktifoks, Isık Cosmetics/Turkey) as required for experimental study. According to commercial firm, Hansler brands (Ozonosan Alpha Plus, Germany) ozone device (which produce 95% oxygen and 5% ozone mixture) was used to prepare 10 L of OL; this device reportedly produced 25 mg/L of ozone per min in at 18–20 °C. This procedure was applied for 10 days.

Characterization of oil and ozonated olive oil

Acidity value

Acidity value of OL and OZO were determined according to American Oil Chemists' Society (AOCS) (1998a). Acidity index expressed as the quantity of potassium hydroxide that are necessary to neutralize the free fatty acid in 1 g. of OZO (Diaz et al. 2005, 2006; Geweely 2006; Sechi, Lezcano, and Nunez 2001; Travagli et al. 2010).

Iodine value

Iodine values of OL and OZO were determined according to AOCS (1998b) defined as the number of grams of iodine that is a measure of unsaturation rate of OZO (Diaz et al. 2006; Geweely 2006; Molerio et al. 1999; Sechi, Lezcano, and Nunez 2001; Travagli et al. 2010).

Peroxide value

Peroxide value (PV) of OL and OZO were determined according to AOCS (1998c), which suggests the amount of peroxide within the OZO. It is expressed as the quantity of active oxygen per kilogram of OZO (mmol/kg) and defined as milliequivalent (Cirlini et al. 2012; Diaz et al. 2005; Geweely 2006; Molerio et al. 1999; Moureu et al. 2016, 2006; Sechi, Lezcano, and Nunez 2001; Tellez, Lozano, and Gomez 2006; Travagli et al. 2010).

P-anisidine value

P-anisidine value of OL and OZO were determined according to AOCS (2011). It is about the aldehyde ratio, which is determined by adding free hydroxylamine to the aldehyde carboxylic group. The result is expressed as in mmol/g. (Sechi, Lezcano, and Nunez 2001)

pH value

pH values of OL and OZO were determined at room temperature (24 °C).

Viscosity

Viscosity of OL and OZO were determined by vibrating viscometer device (AND SV-10 Japan) at 24–40 °C. To provide a swift quality control assessment during the entire ozonation process, a typical trend can be a useful tool, and also deciding on the process time for obtaining the desired ozonation level for the sample is important (Diaz et al. 2005; Sechi, Lezcano, and Nunez 2001; Travagli et al. 2010).

Results

OZO and OL peroxide, acidity, iodine, p-anisidine, viscosity and pH values that were used in this study are given in Table 1. Antimicrobial sensitivities are given in Table 2. According to the data all microorganisms used in the present study OL had no antifungal activity but FLU, OZO1 and OZO2 had antifungal activity (Table 2 and Table 3).

Each fungal strains sensitivity against OZO1, OZO2, OL1 and OL2 were tested. Their MIC values were also summarized in Table 2. When all strains are taken into consideration together at 24 h, MICs of geometric mean values of OZO1, OZO2, OL1, OL2 and FLU were 0.437% (v/v), 0.678% (v/v), 48.11% (v/v), 50% (v/v) and 1.924 µg/mL, respectively. When all strains are taken into consideration together at 48 h, MICs of geometric mean values of OZO1, OZO2, OL1, OL2 and FLU were 1.193% (v/v), 1.493% (v/v), 50% (v/v), 50% (v/v) and 1.771 µg/mL, respectively (Table 2).

The samples showed antifungal activity in terms of the geometric mean in all strains as follows: OZO1 > OZO2 > OL1 > OL2 at 24 h and as follows: OZO1 > OZO2 > OL1 ≥ OL2 at 48 h (Table 2). All antifungal agents, MIC ranges, geometric mean, and the MIC₅₀, MIC₉₀ values for *Candida* and *S. capitata* were summarized in Table 3. The MIC₅₀ values were lowest for OZO1, OZO2, and FLU.

In this study, at 24 h when all strains were considered together, MIC range values of OZO1, OZO2, OL1, OL2 and FLU were between 0.1 to 1.56% (v/v), 0.1 to 6.25% (v/v), 50% (v/v), 50% (v/v), and 0.25 to 32 µg/mL, respectively. At

Table 1. Olive oil, ozonated olive oil analysis results.

	Olive oil 1	Olive oil 2	Ozonated olive oil 1	Ozonated olive oil 2	Unit
Peroxide value	392	370	1352	1053	mmol—mEq/kg
Acidity value	0.7281	2.0079	8.9951	8.4948	unit
Iodine value	64.8067	62.8731	2.2040	4.3750	unit
P-Anisidine value	0.155	0.100	0.516	0.738	mmol/g
Viscosity (24–40 °C)	81.7	85.5 cp	1000	1630	centipoise
pH (24°C)	4.7	4.4	2.1	1.3	-

Table 2. Minimum inhibitory concentrations of olive oil 1, olive oil 2, ozonated olive oil 1, ozonated olive oil 2 and fluconazole.

Number	Strain	Material	Incubation time (24 h)					Incubation time (48 h)				
			OL 1%	OL 2%	OZO 1%	OZO 2%	In µg/mL	OL 1%	OL 2%	OZO 1%	OZO 2%	In µg/mL
							FLU					FLU
1	<i>C. albicans</i>	Blood	50	50	1.56	6.25	0.25	50	50	3.125	12.5	0.25
2	<i>C. albicans</i>	Blood	50	50	1.56	6.25	0.25	50	50	3.125	6.25	0.25
3	<i>C. albicans</i>	Blood	50	50	1.56	3.125	0.25	50	50	3.125	6.25	0.25
4	<i>C. albicans</i>	Blood	50	50	1.56	3.125	0.25	50	50	3.125	6.25	0.25
5	<i>C. albicans</i>	Blood	50	50	1.56	3.125	0.25	50	50	3.125	6.25	0.25
6	<i>C. albicans</i>	Blood	50	50	1.56	3.125	0.25	50	50	1.56	3.125	0.25
7	<i>C. albicans</i>	Blood	50	50	1.56	1.56	0.5	50	50	1.56	1.56	0.5
8	<i>C. albicans</i>	Blood	50	50	0.4	1.56	0.5	50	50	1.56	1.56	0.5
9	<i>C. albicans</i>	Blood	50	50	0.4	0.8	0.5	50	50	0.8	1.56	0.5
10	<i>C. albicans</i>	Blood	50	50	0.1	0.8	1	50	50	0.4	0.8	1
11	<i>C. glabrata</i>	Blood	50	50	1.56	0.4	8	50	50	6.25	3.125	8
12	<i>C. glabrata</i>	Blood	50	50	1.56	0.2	8	50	50	6.25	3.125	8
13	<i>C. glabrata</i>	Blood	50	50	0.8	0.1	16	50	50	6.25	1.56	16
14	<i>C. glabrata</i>	Blood	50	50	0.8	0.1	16	50	50	3.125	0.8	16
15	<i>C. glabrata</i>	Blood	50	50	0.8	0.1	16	50	50	3.125	0.4	16
16	<i>C. glabrata</i>	Blood	50	50	0.8	0.1	16	50	50	3.125	0.4	16
17	<i>C. glabrata</i>	Blood	50	50	0.2	0.1	16	50	50	3.125	0.4	32
18	<i>C. glabrata</i>	Blood	50	50	0.1	0.1	16	50	50	1.56	0.2	32
19	<i>C. glabrata</i>	Blood	50	50	0.1	0.1	32	50	50	1.56	0.2	64
20	<i>C. glabrata</i>	Blood	50	50	0.1	0.1	64	50	50	0.4	0.1	64
21	<i>C. parapsilosis</i>	Blood	50	50	1.56	3.125	0.5	50	50	6.25	6.25	0.5
22	<i>C. parapsilosis</i>	Blood	50	50	1.56	3.125	0.5	50	50	6.25	3.125	0.5
23	<i>C. parapsilosis</i>	Blood	50	50	0.8	1.56	0.5	50	50	6.25	3.125	0.5
24	<i>C. parapsilosis</i>	Blood	50	50	0.4	1.56	0.5	50	50	3.125	3.125	0.5
25	<i>C. parapsilosis</i>	Blood	50	50	0.4	1.56	0.5	50	50	3.125	3.125	0.5
26	<i>C. parapsilosis</i>	Blood	50	50	0.4	1.56	1	50	50	3.125	3.125	1
27	<i>C. parapsilosis</i>	Blood	50	50	0.4	0.8	1	50	50	3.125	1.56	1
28	<i>C. parapsilosis</i>	Blood	50	50	0.4	0.8	1	50	50	3.125	1.56	1
29	<i>C. parapsilosis</i>	Blood	50	50	0.4	0.8	1	50	50	1.56	1.56	1
30	<i>C. parapsilosis</i>	Blood	50	50	0.1	0.8	1	50	50	0.4	1.56	1
31	<i>C. krusei</i>	Blood	50	50	0.2	0.4	8	50	50	0.4	0.8	8
32	<i>C. krusei</i>	Blood	50	50	0.1	0.4	16	50	50	0.2	0.8	16
33	<i>C. krusei</i>	Blood	50	50	0.1	0.1	16	50	50	0.1	0.1	16
34	<i>C. krusei</i>	Blood	50	50	0.1	0.1	32	50	50	0.1	0.1	32
35	<i>S. capitata</i>	Blood	-	-	-	-	-	50	50	0.2	3.125	0.25
36	<i>S. capitata</i>	Blood	-	-	-	-	-	50	50	0.2	3.125	0.5
37	<i>S. capitata</i>	Blood	-	-	-	-	-	50	50	0.1	1.56	0.5
38	<i>S. capitata</i>	Blood	-	-	-	-	-	50	50	0.1	1.56	0.5
39	<i>C. albicans</i> ATCC 90028	Standart	25	50	0.1	3.125	0.25	50	50	0.1	3.125	0.25
40	<i>C. parapsilosis</i> ATCC 22019	Standart	25	50	0.1	1.56	0.5	50	50	0.1	3.125	1
GM of Total strains			48.11	50	0.437	0.678	1.924	50	50	1.193	1.493	1.771

GM, geometric mean; MIC, minimal inhibitory concentration.

48 h, when all strains were considered together, MIC range values of OZO1, OZO2, OL1, OL2 and FLU were between 0.1 to 6.25% (v/v), 0.1 to 12.5% (v/v), 50% (v/v), 50% (v/v), and 0.25 to 64 µg/mL, respectively (Table 3).

At 24 h, two strains of *C. glabrata* were resistant against fluconazole. Nine strains of *C. glabrata* and 10 strains of *C. krusei* were dose-dependent sensitive against fluconazole. At 48 h, 2 strains of *C. glabrata* were resistant against fluconazole. Eight strains of *C. glabrata* and 10 strains of *C. krusei* were dose-dependent sensitive against fluconazole. (Table 2). At 24 h, *C. glabrata* and *C. krusei* had the lowest MIC values against OZO1 and OZO2. At 48 h, *C. krusei* had also the lowest MIC values against OZO1 and OZO2. But, *C. glabrata* had only the lowest MIC values against OZO2. Furthermore, *S. capitata* had also the lowest MIC values against OZO1 (Table 3).

On the other hand, at 24 h, *C. albicans* had the highest MIC values against OZO1 and OZO2. At

48 h, *C. albicans* had the highest MIC values against only OZO2. In addition, *C. parapsilosis* had the highest MIC values against OZO1 (Table 3).

For the resistant strains against FLU MIC range values of OZO1, OZO2, OL1 and OL2 were 0.1 and 6.25% (v/v), 0.1 and 3.125% (v/v), 50% (v/v) and 50% (v/v), respectively (Table 3). Results of this study, at 24-h and 48-h incubation, sensitive strains, dose-dependent sensitive strains and resistant strains for FLU are shown in Table 4.

Discussion

Mycoses are important problems in our daily lives. Even though we know they may threaten our life, research to combat their survival is still neglected (Ansari et al. 2013). Human and animal populations have been increasing, mycoses are getting more widespread; and, at the same time, antimicrobial resistance also develops. Thus, scientists are searching for

Table 3. Susceptibility to olive oil 1, olive oil 2, ozonated olive oil 1, ozonated olive oil 2 and fluconazole as determined by the Clinical Laboratory Standards Broth Microdilution Methods.

Antifungal agent	MIC values							
	Incubation time (24 h)				Incubation time (48 h)			
	Range	GM	MIC ₅₀	MIC ₉₀	Range	GM	MIC ₅₀	MIC ₉₀
Olive oil 1 (%(v/v))								
<i>C. albicans</i> (n:10)	50	50	50	50	50	50	50	50
<i>C. glabrata</i> (n:10)	50	50	50	50	50	50	50	50
<i>C. parapsilosis</i> (n:10)	50	50	50	50	50	50	50	50
<i>C. krusei</i> (n:4)	50	50	50	50	50	50	50	50
<i>S. capitata</i> (n:4)	-	-	-	-	50	50	50	50
Olive Oil 2 (%(v/v))								
<i>C. albicans</i> (n:10)	50	50	50	50	50	50	50	50
<i>C. glabrata</i> (n:10)	50	50	50	50	50	50	50	50
<i>C. parapsilosis</i> (n:10)	50	50	50	50	50	50	50	50
<i>C. krusei</i> (n:4)	50	50	50	50	50	50	50	50
<i>S. capitata</i> (n:4)	50	-	-	-	50	50	50	50
Ozonated olive oil 1 (%(v/v))								
<i>C. albicans</i> (n:10)	0.1–1.56	0.90	1.56	1.56	0.4–3.125	1.80	1.56	3.125
<i>C. glabrata</i> (n:10)	0.1–1.56	0.40	0.8	1.56	0.4–6.25	2.72	3.125	6.25
<i>C. parapsilosis</i> (n:10)	0.1–1.56	0.44	0.4	1.56	0.4–6.25	2.9	3.125	6.25
<i>C. krusei</i> (n:4)	0.1–1.56	0.35	0.1	0.1	0.1–0.4	0.17	0.1	0.2
<i>S. capitata</i> (n:4)	-	-	-	-	0.1–0.2	0.14	0.1	0.2
Ozonated olive oil 2 (%(v/v))								
<i>C. albicans</i> (n:10)	0.8–6.25	2.38	3.125	6.25	0.8–12.5	3.35	3.125	6.25
<i>C. glabrata</i> (n:10)	0.1–0.4	0.12	0.1	0.2	0.1–3.125	0.56	0.4	3.125
<i>C. parapsilosis</i> (n:10)	0.8–3.125	1.37	1.56	3.125	1.56–6.25	2.54	3.125	3.125
<i>C. krusei</i> (n:4)	0.1–0.4	0.2	0.1	0.4	0.1–0.8	0.28	0.1	0.8
<i>S. capitata</i> (n:4)	-	-	-	-	1.56–3.125	2.20	1.56	3.125
Fluconazole (µg/mL)								
<i>C. albicans</i> (n:10)	0.25–1	0.35	0.25	0.5	0.25–1	0.35	0.5	0.5
<i>C. glabrata</i> (n:10)	8–64	17.15	16	32	8–64	21.11	16	64
<i>C. parapsilosis</i> (n:10)	0.5–1	0.70	0.5	1	0.5–1	0.70	0.5	1
<i>C. krusei</i> (n:4)	8–32	16	16	16	8–32	16	16	16
<i>S. capitata</i> (n:4)	-	-	-	-	0.25–0.5	0.42	0.5	0.5

GM, geometric mean; MIC, minimal inhibitory concentration; MIC₅₀ and MIC₉₀, minimal inhibitory concentration at which 50% and 90%, respectively, of the isolates were inhibited.

compounds that are more effective compared to older ones, have wide spectrum, do not cause resistance, and are natural products (Arif et al. 2009). These new compounds are natural products and are tolerated better by patients and have several beneficiary effects to them (Di Santo 2010; Fernández Torres et al. 2006; Hammer, Carson, and Riley 2002; Lima et al. 1993, 2006). Among the natural products having antimicrobial activity; besides wild plants, essential oils, ozone and ozonated oils formed by ozonation of essential oils (Arif et al. 2009; Di Santo 2010; Fernández Torres et al. 2006, 2013; Kutlubay et al. 2010).

Ozonated vegetable oils have antibacterial and fungicide activity (Geweely 2006). Wu, Doan, and Cuenca (2006) also reported that ozone gas has an inactivating effect on fungi. Furthermore, Menendez et al. (2002) used OSO to treat onychomycosis and found it effective without side effects. In addition, Daud et al. (2011) used OSO to treat dermatomycoses caused by *Microsporidium* (*M*) *canis*, and is reported to be effective. Moreover, Leopoldina et al. (1998) stated that oleozone was effective against *Trichophyton* (*T*) *rubrum*, *T. mentagrophytes*, *C. albicans* and *M. canis*. Leopoldina et al. (1998) used OSO to treat superficial mycoses in 1,000 patients and found 91% success in

these cases. OZO was also found to have germicidal activity against *Tinea pedis* (Menendez et al. 2002).

In the present study, OZO was also found to have effective antifungal capacity. Each yeast pathogen examined in the present study had different sensitivity responses. These results were in parallel with the results reported by other workers which investigated infections caused by *Microsporum*, *Trichophyton*, *Epidermophyton* and *C. albicans* (Gewely 2006). Furthermore, Leopoldina et al. (1998) also found OZO effective against *T. rubrum*, *T. Mentagrophytes*, *C. albicans* and *M. canis*. Additionally, Geweely (2006) found MIC values of OZO against *C. albicans*, *M. canis* ve *T. rubrum* as 0.78–0.53 mg/mL, which is in agreement with our results.

In a study carried out by Tara et al. (2014) on vulvovaginal candidiasis, using OZO and clotrimazole for treatment, they found OZO as effective as clotrimazole in reducing clinical symptoms of vulvovaginal candidiasis and also resulted in negative specimen cultures. Similarly, in the present study, OZO reduced *C. albicans* cultures in vitro.

Ouf et al. (2016) investigated the five most common dermatophytes (*M. canis*, *M. gypseum*, *T. rubrum*, *T. mentagrophytes*, and *T. interdigitales*), and the effects of different concentrations of gas ozone and ozonated oils

Table 4. Comparison of inhibitory activity of fluconazole, olive oil 1, olive oil 2, ozonated olive oil 1 and ozonated olive oil 2 samples according to sensitivity and resistance to fluconazole of the strains.

Sample concentration % (v/v)	24-h incubation			48-h incubation		
	Sensitive strains	Dose-dependent sensitive strains	Resistant strains	Sensitive strains	Dose-dependent sensitive strains	Resistant strains
Olive oil 1						
≤50	22	14	1	24	12	2
25	-	-	-	-	-	-
12.5	-	-	-	-	-	-
6.25	-	-	-	-	-	-
3.125	-	-	-	-	-	-
1.56	-	-	-	-	-	-
0.8	-	-	-	-	-	-
0.4	-	-	-	-	-	-
0.2	-	-	-	-	-	-
0.1	-	-	-	-	-	-
Olive Oil 2						
≤50	22	14	1	24	12	2
25	-	-	-	-	-	-
12.5	-	-	-	-	-	-
6.25	-	-	-	-	-	-
3.125	-	-	-	-	-	-
1.56	-	-	-	-	-	-
0.8	-	-	-	-	-	-
0.4	-	-	-	-	-	-
0.2	-	-	-	-	-	-
0.1	-	-	-	-	-	-
Ozonated olive oil 1						
≤50	-	-	-	-	-	-
25	-	-	-	-	-	-
12.5	-	-	-	-	-	-
6.25	-	-	-	3	3	-
3.125	-	-	-	10	4	-
1.56	9	2	-	4	1	1
0.8	1	4	-	1	-	-
0.4	8	-	-	2	1	1
0.2	-	2	-	2	1	-
0.1	4	5	1	4	2	-
Ozonated olive oil 2						
≤50	-	-	-	-	-	-
25	-	-	-	-	-	-
12.5	-	-	-	1	-	-
6.25	2	-	-	5	-	-
3.125	7	-	-	10	2	-
1.56	7	-	-	9	1	-
0.8	6	-	-	1	3	-
0.4	-	3	-	-	3	-
0.2	-	1	-	-	1	1
0.1	-	9	1	-	2	1
Total strains	22	13	1	26	12	2

FLU sensitivity = resistance was classified as follows: sensitive strain, MIC <8 µg/mL; dose-dependent sensitive strain, MIC 8–32 µg/mL; and resistant strains, MIC >64 µg/mL.

on these dermatophytes' growth and germination were examined. Furthermore, they pointed out that OZO has a better fungicidal effect compared to gaseous ozone and they explained the possible reason for this being a result of long-term ozonation, gradual decrease of fatty acid chain unsaturation, formation of ozonide, and increases in peroxide and acid values.

PV is generally used as an indicator of the progression or controlling ozonation process due to its simplicity, rapidity, and low cost. In addition, the PV may be sufficient for the stability evaluation of vegetable oil ozonides, and it seems to be very considerable for trading distribution as well as determining better storage modalities. Differences obtained in the present study with concern to PV; believed to be due to ozonation time and acid number which had been reported to be essential for a

validated PV (Sechi, Lezcano, and Nunez 2001; Geweely 2006; Travagli et al. 2010).

Diaz et al. (2006) compared the antibacterial efficacy of OZO and OSO with different PVs and found that OZO and OSO with high PVs demonstrated better antimicrobial and germicidal activity.

Fernández Torres et al. (2006) have used antifungal activity on ozonated theobroma oil against *C. albicans* ATCC 10231 standards strain and used four different PVs of ozonated theobroma oil. Ozonated The obroma oil showed inhibitory effects on *C. albicans*: when PV was 1200 mmol-Eq/kg, MIC value was 3.75 mg/mL, when PV was 1002, MIC value was 5.78 mg/mL, when PV was 572 mmol-Eq/kg, MIC value was 15 mg/mL, when PV was 260, MIC value was 25 mg/mL. They also determined that the MIC concentration decreased as PV increased. Our

findings were in parallel with the preceding results (Table 1 and Table 3). Furthermore, *C. albicans* ATCC 90028 at 24 and 48 h, we found that, when PV was 1352 mmol-Eq/kg, MIC value was 0.1% (v/v) in OZO1, when PV was 1053; MIC value was 3.125% (v/v) in OZO2. As the PVs increased, antifungal activity also increased as reported in the literature. In addition, it was determined that OZO1 showed a better (with concern to geometric range) antifungal effect against all strains compared to OZO2.

Kawamura et al. (1986) reported that when *C. parapsilosis* exposed to 0.23 mg/L ozone and *C. tropicalis* exposed to 0.02 mg/L ozone for 20 sec, 2 log reduction in the number of yeast cells observed. Thomson et al. (2011) investigated the effect of ozonated oil on dermatophytes, and they found the MIC values of OZO on *C. parapsilosis* ATCC 22019 as 0.25–2% and they found MIC₅₀ and MIC₉₀ values as 1%. They also investigated *C. krusei* ATCC 6258 and found same MIC values as given previously. In the present study, it can be seen from Table 3 that MIC values of OZO1 and OZO2 were in accordance with the literature values, with regard to *C. parapsilosis* standard (ATCC 22019) and clinical isolates and *C. krusei* clinical isolates. Of these, OZO1 against *C. parapsilosis* ATCC 22019 was much more effective compared to Thomson et al.'s (2011) findings, but it was less effective against the same culprit compared to Thomson et al.'s (2011) findings when OZO2 was the case.

Essential oil of plants has been known to have antimicrobial activity against a wide range of bacteria and fungus. In fact, extra virgin olive oil was reported to have antibacterial character, thereby inhibiting recruitment of microorganisms. It was reported to be used as an adjunctive therapeutic agent for some diseases (Cicerale, Lucas, and Keast 2012; Helal et al. 2006). Furthermore, Markin, Duek, and Berdicevsky (2003) examined water extract of olive leaf against some microorganisms and found that *C. albicans* was killed within 24 h. In contrast, in the present study, OL had no antifungal activity against yeast tested.

According to our findings, in general, strains that were susceptible against FLU were less susceptible against OZO1 and OZO2 or vice versa, meaning that strains resistant against FLU were much more susceptible against OZO1 and OZO2. McIntyre and Galgani (1989) studied the effect of pH on clotrimazole antifungal activity according to broth dilution method and they tested 3, 4, 5, 6, 7.4 pH values against *C. albicans* (C17; ATCC 64546), *C. tropicalis* (F26), *C. parapsilosis* (3288), and *C. lusitanae* MIC values. They found against these fungi that susceptibility was better at pH 7.4 compared to pH 3. On the other hand, they also examined *C. glabrata* (R87) strain at pH 3, 4, 5, 6, 7.4 and found MIC values as 5 µg/mL, 10 µg/mL, 5 µg/mL, 10 µg/mL and 5 µg/mL, respectively. According to the preceding

results, the *C. glabrata* (R87) strain responded to different pH values differently, which is not easy to explain. In our study, *C. glabrata* strains were more sensitive against OZO2 compared to OZO1 that OZO1 had higher PV and had higher pH values compared to OZO2 as seen in Table 1, for which we also could not explain the behavior of *C. glabrata* strains. For these reasons, this issue should be investigated separately in future studies.

There is insufficient information about antifungal effects of *S. capitata* (Miceli, Diaz, and Lee 2011). However, in some studies, as in our study, FLU was an active drug for this fungus. Reduced susceptibility to caspofungin with an MIC range value of 0.25–8 g/mL in all species given (Koc et al. 2016). In literature studies, there is insufficient information about susceptibility of *S. capitata* against OZO. In our study, OZO1 and OZO2 were found to be effective against four strains of *S. capitata*. Additionally, OZO1 was more effective compared to OZO2 against 4 strains of *S. capitata*. Furthermore, sensitivity to *S. capitata* strains could not be determined at 24 h; yet, sensitivity at 48 h was obvious in the present study and should be evaluated further.

In conclusion, antifungal activity was demonstrated in OZO products. This study indicates that OZO can help control some fungal pathogens. In addition, this study has proved that some yeasts resistant to certain antifungal agents are susceptible to OZO. Furthermore, PV of OZO is very important in its antifungal activity and increased proportionally with the PV apart from *C. glabrata*. Especially, when OZO is prepared for use, their PVs need to be analyzed. Those with low PVs should not be used for yeasts. It has been concluded that the data should be supported by further in vitro and in vivo studies.

ORCID

Kemal Varol  <http://orcid.org/0000-0002-3057-2865>

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