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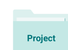


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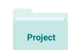


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# Antifungal Activity of Ozonized Olive Oil (Oleozone)

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## ABSTRACT

The effect of ozonized olive oil (Oleozone) on some pathogenic fungi (*Aspergillus fumigatus, Candida albicans, Epidermophyton floccosum, Microsporum canis* & *Trichophyton rubrum*), were tested. The olive oil was ozonized at Ozomaxe, Egypt, OZO- 3 VTT Cairo, Egypt by an ozone generator. Minimum inhibitory concentrations (MICs) were determined by the agar dilution method. Oleozone showed antimicrobial activity against all species analysed, with an MIC ranging from 0.53 to 2.0 mg $mL^{-1}$. *Candida albicans, Microsporum canis* and *Trichophyton rubrum* were more susceptible to oleozone than other species tested. *Microsporum canis* showed the minimum decrease in dry weight (0.03 g/100 mL) under oleozone effect. The inhibition effect of oleozone on nucleic acid content was correlated to the extent of fungal growth reaching the lowest value (0.02 & 0.13 mg $g^{-1}$ dry weight) in *Microsporum canis* for DNA and RNA, respectively. Application of oleozone leads to a significant reduction in amylase, lipase, keratinase and urease enzyme activities for all tested fungal species.

**Key Words:** Antifungal; Ozone; Olive; Oil; Pathogens; Fungi

## INTRODUCTION

Onychomycoses are infections of the nails by fungi. Human infections, particularly those involving dermatophytes (*Trichophyton, Epidermophyton floccosum & Microsporum canis*) and non-dermatophytic fungi (*Aspergillus* & *Candida albicans*) constitute a serious problem, especially in tropical and subtropical developing countries (Raza, 1998). Dermatophytes are keratinophilic fungi capable of causing dermatophytosis (commonly known as tinea or ringworm) in human and animals (Liu *et al*., 1997). Conant (2004) reported that the most common species of dermatophytes were *Trichophyton*, *Microsporum* and *Epidermophyton*, which cause superficial fungal infections of skin.

The overuse of antibiotics in the treatment of infectious disease and the appearance of multi-drug resistant fungal strains (resistant to two or more antibiotics) accompanied with lack of efficacy and side effects, has driven the research towards the study of antimicrobial agents from essential oils (Cox *et al*., 2000; Dorman & Deans, 2000). Resistance to drugs as well as limiting toxic effects has stimulated the search for new groups of antimycotic agents (Sternberg, 1994). Much attention was drawn to plant-derived fungicides, based on the knowledge that plants have their own defense against fungal pathogens (Gurgela *et al*., 2005). Plants are capable of sensing the presence of potential phytopathogens (fungi, bacteria & viruses) and can produce antifungal compounds to protect themselves from biotic attack that could be essential for fungal infection resistance (Wojtaszek, 1997). In traditional medicine, many plants were claimed to be effective against fungal pathogens and research on these may yield potential leads for the development of drugs effective against human pathogenic fungi (Kim *et al*., 2003; Navarro *et al*., 2003; Souza *et al*., 2003; Rajendraprasad *et al*., 2004). Olive oil is obtained from the fruit of olive, which contains different fatty acids, high proportion of oleic acid (65 - 85%) (Gunstone *et al*., 1994). Maoz and Neeman (2000) recorded the effect of plant extract on dermatophytes and *Candida albicans*.

Ozone does not contaminate the atmosphere and no fungal resistance to this substance has been reported so far. Application of this system can be more extensive, ranging from the treatment of deep infections to infections of the epidermis (Alvarez & O'Brien, 1982). Ozone is a powerful oxidant, principally applied as a disinfectant in many fields (Arana *et al*., 1999). Ozone inactivation of pathogenic fungi (*Candida albicans & Aspergillus niger*) was studied by Coronel *et al*. (2002).

As a natural preparation, ozonized oil is now available in several countries, but information with regards to the chemical data, standard preparations and antimicrobial activity is limited. In many countries the pure olive oil is ozonized by two days until it solidified (Bocci, 2002). Ozonized olive oil was widely used for its therapeutic effects and valuable antimicrobial activity against bacteria, virus and fungi (Lezcano *et al*., 2000; Sechi *et al*., 2001). The knowledge of the physicochemical properties of ozonized olive oil has a great importance for its characterization (peroxide, acidity & iodine values) are used to follow up the ozonization process and for determining the quality of ozonized olive oil (Díaz *et al*., 2005). Ozonized olive oil has germicidal action and used in the treatment of tinea pedis (Menendez *et al.,* 2002).

The reaction of ozone with olive oil occurs almost

exclusively with the carbon-carbon double bonds present in un-saturated fatty acids producing different toxic products such as several oxygenated compounds, hydroperoxides, ozonides, aldehydes, peroxides, diperoxides and polyperoxides and these compounds could be also responsible for the wide antimicrobial activity of ozonized olive oil (Pryor & Uppu, 1993; Ledea, 2003). Different ozonized solutions have been used successfully against different infections (Finch *et al*., 1993). The safety of oleozone was reported by Gundarova *et al*. (1996) and Alvarez *et al*. (1997).

The purpose of this study serves as the foundation for the susceptibility testing method for some pathogenic fungi against oleozone. The effect of oleozone on the pathogen growth and nucleic components was determined. Some enzymatic activities of the tested species were estimated under the effect of oleozone.

## MATERIALS AND METHODS

**Ozone apparatus.** Ozone was generated via a controlled flow of oxygen through a corona discharge in the ozone generator (Ozomaxe, Egypt, OZO- 3 VTT) Cairo, Egypt. The ozone was fed into both chambers, where the ozone measurement and ozone treatment were done. Ozone measurement was done by an ozone analyzer (model, INUSA, H1, VER 5.73) with a detection limit of 1.0 ppb.

**Olive oil ozonization.** Ozonated oil was carried out by bubbling ozone through pure olive oil in the presence of a magnetic field for eight weeks. Ozonated oil is actually created by a redox reaction. The ozone literally burns the oil. Once olive oil is completely ozonated, it will actually turn into a nearly clear, gel substance holding the ozone within. The smell of ozone being emmited from the olive oil will be noticeable. The final product must be kept refrigerated at all times. When kept refrigerated, this gel will hold the ozone for years.

**Standardization of the preparation was carried out according to the following parameters.** Peroxide Index (IP), which indicates the quantity of peroxide within the oleozone. It is defined as the quantity of active oxygen per kilogram of oleozone (mmol $kg^{-1}$). A range of IP between 500 and 800 (mmol $kg^{-1}$) was considered. The best antimicrobial activity was seen with an IP of 650 (mmol $kg^{-1}$). Acidity Index, which indicates the free fatty acids in the oleozon, is defined as the number of milligrams of potassium hydroxide that are necessary to neutralize the free fatty acids in 1 mg of oleozone (Panreac, 1992). The aldehyde concentration is measured by adding free hydroxylamine to the aldehyde carboxylic group. The results are expressed in mmol $g^{-1}$ of oleozone, the interval must range between 0.4 and 0.9 mmol $g^{-1}$. Iodine Index, which is a measure of the un-saturation rate of olive oil was expressed as the number of grams of iodine that react with 100 gram of olive oil. In olive oil, the rate varies between 125 and 135 units (Vajdia & Saenz 1976); whereas, in oleozone, the value is between 50 and 90 units (Molerio *et al*., 1999).

**Fungal strains.** Five pathogenic fungal species were tested: (*Aspergillus fumigatus, Candida albicans, Epidermophyton floccosum, Microsporum canis* & *Trichophyton rubrum*), were obtained from the faculty of veterinary, Cairo university. The isolates were tested for their susceptibility to oleozone and kept refrigerated on sabouraud dextrose agar (SDA) slants until used.

**Inoculum preparation.** Cultures were grown on sabouraud dextrose agar slants. Sterile saline solution (0.85%) was added to the slants and the culture was gently swabbed with a cotton-tipped applicator to dislodge conidia from the hyphal mat. The suspension was transferred to a sterile tube and the volume was adjusted to 5 mL with sterile phosphate buffered saline solution pH 7. The resulting suspension was used for the next experiments.

**MIC determination.** MICs were determined by the agar dilution method according to the NCCLS (1993) guidelines, the final inoculum was $10^4$ cfu $mL^{-1}$. Doubling concentration of oleozone in the sabouraud dextrose agar (SDA) medium were used (20, 16, 12, 8, 4, 2 & 0.5 mg $mL^{-1}$). The MIC of oleozone was determined on sabouraud dextrose agar (SDA) medium by the micro drop agar proportion test. An aliquot (5 µL) of spore suspension of each fungal culture was spotted into plates containing Tween-80 (to enhance oil solubility) and oleic acid albumin dextrose citric acid (OADC) as a supplement and a series of dilutions from 20 - 0.5 mg $mL^{-1}$ of oleozone. The plates were incubated at 30°C for 5 days for *A. fumigatus* and *M. canis* and 10 days for *T. rubrum* and *E. floccosum*, respectively while the plates for *C. albicans* were incubated at room temperature (from 25 - 27°C) for 10 h in a reciprocating shaker (100 strokes/min). The number of fungal colonies was counted. The MIC was defined as the lowest concentration resulting in a 99% reduction of the number of colonies on that plate compared with controls (sabouraud dextrose agar alone, plus Tween-80 & non-ozonized olive oil).

**Effect of oleozone on the dry weight and nucleic acid components of pathogenic fungi.** Five sterile Erlenmeyer flasks containing 100 mL of MIC of oleozone for each tested species in the sabouraud dextrose media were inoculated with discs (10 mm diameter) of ten days old cultures of the tested fungal species. Five flasks inoculated with un-treated media were used as control. The flasks were incubated at 30°C for 5 days for *A. fumigatus* and *M. canis* and 10 days for *T. rubrum* and *E. floccosum*, respectively while the flasks for *C. albicans* were incubated at room temperature (from 25 - 27°C) for 10 h. All flasks were incubated in a reciprocating shaker (100 strokes/min). The produced mats were collected, washed several times with distilled water and oven dried at 80°C till constant weight. The fungal growth was expressed as g/100 mL. Determination of DNA was carried out quantitatively according to the method of Burton (1968) by measuring the

colour developed after treating the extracted DNA with diphenylamine reagent and the absorbance was measured at 600 nm. The colorimetric analysis of ribose sugar using orcinol reaction (Ashwell, 1957) was applied for quantitative determination of RNA.

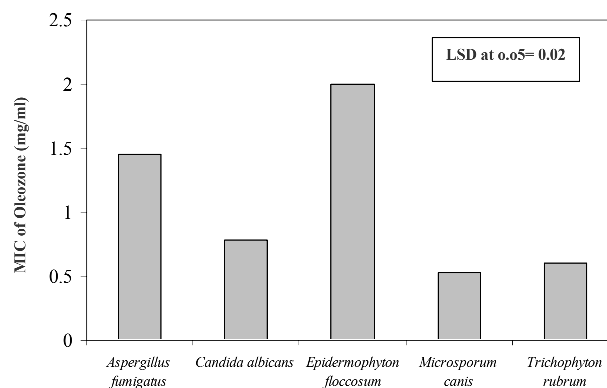
**Effect of oleozone on keratinase, amylase, urease and lipase enzymes of pathogenic fungi.** Fungal cultures of the MIC flasks were applied for measuring the enzyme activities. Keratinase enzyme activity in the growth medium was determined according to Yu *et al*. (1968) method and its modification by Siesenop (1993), while amylase enzyme was estimated by Somogyi (1960) method and Weatherburn (1967) method was applied for urease enzyme determination and lipase enzymes were estimated according to Hellgren and Vincent (1980).

## RESULTS AND DISCUSSION

**Antifungal activity.** Under the test conditions, oleozone showed an antifungal activity against all dermatophytic tested species. These results were in agreement with Sartori (2003), who stated that ozonized olive oil is helpful in suppresing fungal infections including ringworm (dermatophytes), caused by *Microsporum, Trichophyton* and *Epidermophyton* species and yeast infections of the skin including candidiasis (caused by *Candida albicans*). The tested species (*C. albicans, M. canis* & *T. rubrum*), were susceptible to oleozone with MIC ranging from 0.78 - 0.53 Fig. 1. This concentration may seem high if compared with the amount of antibiotics, expressed in µg/mL, necessary to inhibit microbial growth. This is due to the dilution of the active compounds in the ozonized oil that has not been altered in the ozonization process. Lincheta *et al*. (1998) stated that oleozone was effective against *T. rubrum, T. mentagrophytes, C. albicans* and *M. canis*. The tested ozonized olive oil was effective against *A. fumigatus* and *E. floccosum,* generated an MIC of 1.45 and 2.0, respectively. Benger *et al.* (2004) stated that *E. floccosum* was the only dermatophytic strain resistant to *Melaleuca alternifolia.* The resistance of *Aspergillus spp*. may refer to the morphology of spores of *Aspergillus* species is pigmented, large and more resistant to the inhibitory action of ozone and this finding agree with that obtained by Hibben and Stotzky (1969), who stated that spores most sensitive to ozone were, in general, relatively small and hyaline, whereas the most resistant spores were large, pigmented. *Aspergillus niger* spores were more resistant to ozone (Restaino *et al*., 1995). *Aspergillus fumigatus* spores were more resistant to ozone gas (Whistler & Sheldon, 1988).

The fact that most MICs were clustered around 2.0 mg $mL^{-1}$ and no species was found with an MICs higher than 2.0 mg $mL^{-1}$, may suggest that the activity is due to toxicity rather than to metabolic interruption, as is the case for traditional antimicrobial agents. On the other hand, Dyas *et al.* (1983) showed that *A. fumigatus* colonies seem to be relatively sensitive to ozonation. Diaz *et al*. (2006) stated

**Fig. 1. Susceptibility of different species of pathogenic fungi to Oleozone**




that the higher antimicrobial power of ozonized olive oil refers to its higher peroxide value, where peroxide values in ozonized olive oil sample have a notable increase, which was responsible for the germicidal effect of these oils and an increase of peroxide values has been observed due to formation of peroxidic substances, where ozone reacts with un-saturated compounds through the known Criegee mechanism (Criegee, 1975). This behavior might be due to formation of polymeric peroxides, which are responsible for viscous mass achieved in ozonized oil. The population of *Candida parapsilosis* decreased by exposing to 0.23 mg $L^{-1}$ ozone. Counts of *C. tropicalis* decreased by 2 log when the yeast cells were exposed to ozone at 0.02 mg $L^{-1}$ for 20 seconds (Kawamura *et al*., 1986). Research efforts in the field of ozonized oil chemistry have been concerned with the oxygenized products elucidation that could be related with their germicidal effect (Diaz *et al.,* 2001). Ozone with olive oil occurs almost exclusively with the carbon-carbon double bonds present in un-saturated fatty acids (Bailey, 1978). Topical ozone therapy is capable of inactivating *Trichophyton* and *Epidermophyton floccosum* (Gérard & Sunnen, 1998).

**Effect of oleozone on the dry weight and nucleic acids components of pathogenic fungi.** Data revealed that there are a significant decrease in the dry weight and nucleic acid components coupled with application of MIC of ozonized olive oil of all tested organisms reaching to the minimum value (0.03 g/100 mL dry weight, 0.02 & 0.13 mg $g^{-1}$ dry weight for DNA & RNA, respectively) in *Microsporum canis*, respectively. This finding is in accordance with the fact that the primary mode of inactivation by ozone appears to be nucleic acid damage (Roy *et al.,* 1981). RNA of microorganisms is degraded into protein subunits by ozonation (Kim e*t al.,* 1980). Ozone alters the protein capsid first to liberate RNA and that the naked RNA may be secondarily inactivated by ozone (Gwy & Sobsey, 2003). Ozone degradation of nucleic acids was studied by Shinriki *et al*. (1981). Ozonized oil considered as one effective therapy for antifungal action (Grillo *et al.,* 2006; Table I).

**Effect of oleozone on keratinase, amylase, urease and**

**Table I. Effect of the minimum inhibitory concentrations (MICs) of the tested ozonized olive oil for each species on dry weights (g/100 mL) and nucleic acid components of the biomass (mg/g dry weight) of the tested pathogenic organisms**

| Fungal species | Control | Dry weight | LSD | Control | DNA | LSD | Control | RNA | LSD |
|---|---|---|---|---|---|---|---|---|---|
| *Aspergillus fumigatus* | 1.34 | 0.37 | 0.97 | 0.20 | 0.18 | 0.01 | 0.42 | 0.30 | 0.12 |
| *Candida albicans* | 1.20 | 0.22 | 1.00 | 0.19 | 0.15 | 0.02 | 0.35 | 0.22 | 0.14 |
| *Epidermophyton floccosum* | 0.79 | 0.10 | 0.32 | 0.23 | 0.17 | 0.05 | 0.40 | 0.25 | 0.13 |
| *Microsporum canis* | 0.81 | 0.03 | 0.78 | 0.09 | 0.02 | 0.07 | 0.20 | 0.13 | 0.07 |
| *Trichophyton rubrum* | 0.95 | 0.08 | 0.87 | 0.11 | 0.09 | 0.02 | 0.26 | 0.16 | 0.09 |
| LSD at 0.05 | 0.43 | 0.15 | | 0.03 | 0.16 | | 0.23 | 0.17 | |

**Table II. Effect of the minimum inhibitory concentrations (MICs) of the tested ozonized olive oil on the production of amylase and lipase enzymes (determined as diameter of the clear zone, mm) liberated by the tested dermatophytes**

| Fungal species | Control | Amylase enzyme | LSD | Control | Lipase enzyme | LSD |
|---|---|---|---|---|---|---|
| *Aspergillus fumigatus* | 20.0 | 7.5 | 14.6 | 12.0 | 0.12 | 9.0 |
| *Candida albicans* | 11.0 | 1.4 | 7.8 | 2.2 | 0.9 | 1.1 |
| *Epidermophyton floccosum* | 8.3 | 1.0 | 5.1 | 3.0 | 2.2 | 0.8 |
| *Microsporum canis* | 14.0 | 2.2 | 11.8 | 9.2 | 3.4 | 5.6 |
| *Trichophyton rubrum* | 22.0 | 8.0 | 12.5 | 0.0 | 0.0 | 0.0 |
| LSD at 0.05 | 2.10 | 0.40 | | 1.70 | 0.35 | |

**Table III. Effect of the minimum inhibitory concentrations (MIC) of the tested ozonized olive oil on the production of keratinase enzyme (unit/ml) and urease enzyme (determined as a change in pH of the media) liberated by the tested dermatophytes**

| Fungal species | Control | Keratinase enzyme | LSD | Control | Urease enzyme |
|---|---|---|---|---|---|
| *Aspergillus fumigatus* | 5.6 | 0.9 | 4.7 | +ve | -ve |
| *Candida albicans* | 2.0 | 0.2 | 1.1 | +ve | -ve |
| *Epidermophyton floccosum* | 9.5 | 2.0 | 5.4 | +ve | -ve |
| *Microsporum canis* | 15.0 | 6.2 | 8.8 | +ve | -ve |
| *Trichophyton rubrum* | 10.4 | 3.9 | 6.5 | +ve | -ve |
| LSD at 0.05 | 2.1 | 0.7 | | | |

**lipase enzymes of dermatophytic fungi.** Data in Table II revealed that amylase enzyme was liberated by all examined fungi. Application of oleozone leads to a significant decrease in amylase activity except, *A. fumigatus,* which recorded the only non-significant amylolytic inhibition. It was found that *T. rubrum* had the highest amylase activity (22.0 mm) and this result was in accordance with that obtained by El-adly (2002), who found that *Trichophyton*, *Microsporum* and *Epidermophyton* were able to liberate amylase enzyme and *Trichophyton spp.* was the pioneer in amylase production. Not all tested pathogens have the ability to liberate lipase. A less enhancement of lipase production was associated with addition of oleozone for all species. *T. rubrum* lack the ability to produce this enzyme. The tested pathogens (*A. fumigatus & M. canis*), were the higher producer of lipase enzyme (12.0 & 9.2 mm, respectively). The highest lipolytic activity was demonstrated by *Microsporum canis* followed by *Trichophyton mentagrophytes* and *Epidermophyton floccosum* (Hellgren & Vincent, 1980).

The production of different enzymes by keratinophilic fungi is of immense value for their successful survival and subsequent hydrolysis of keratin (Azariah *et al*., 1997). The enzymes have a role in pathogenesis causing dermatophyte infection (Samdani & Al-Bitar, 2003). Table III indicate that there are a significant drop in the release of keratinase for all tested species reaching to the maximum value (0.2 unit/mL) for *C. albicans* compared with control. Many keratinophilic fungi are pathogenic and linked with the incidence of dermatophytosis (Qin *et al.,* 1992). Keratinases and other enzymes released by dermatophytes make it possible for the fungi to invade deeper in the stratum corneum (Lesher & Zember, 2004). All tested pathogens were negatively producers for urease under the effect of oleozone. Helmy (1983) obtained variable results when tested some dermatophytic species for their ability to hydrolyze urea. The protective effect of ozone on important enzymes was studied by León and Ajamiech (2004). Ozone pretreatment leads to inhibition of superoxide dismutase, catalase and glutathione peroxidase enzyme activities (González *et al.*, 2004).

The fungicidal effects of ozone are due to its high reactivity toward c = c on biological compounds *in vivo*, attention have been paid to changes in enzyme activities because enzyme activities are easily detected, when enzymes are damaged by ozone (Bockman & Heppel, 1968). In addition, it was easy to measure their activities, because they could be accumulated in cells by specific growth

conditions. This suggestion is supported by speculation that aldehydes, peroxides and hydroxyl radicals are produced by ozone in oxidizing process of polyunsaturated fatty acids in the cytoplasmic membrane and that they attack enzymes in the cytoplasm (Mehlman & Borek, 1987). Oxidation of sulfhydryl groups (SH to S-S) in the enzyme is the principal cause of death (Barron, 1954).

The degradation of nucleic acid was parallel to that for enzymatic activities, which may suggest the role played by nucleic acids in the synthesis of enzymes. Franco (2005) found that ozonization of RNA causes its denaturation and RNA molecules display enzymatic activities and has a role in synthesis of biological molecules.

The wide availability of olive oil makes oleozone a competitive antimicrobial agent. The therapeutic advantage of ozonized olive oil (Oleozone), as an alternative treatment for dermatophytosis or in conjunction with other antimycotics, can avoid problems such as side effects and/or resistance.

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