ANTIFUNGAL AND ANTIBIOFILM ACTIVITY OF OZONIZED SUNFLOWER OIL AGAINST *Candida albicans* STRAINS: A PILOT STUDY AND CLINICAL PERSPECTIVES

ABSTRACT

Aims: This study aimed to assess the antifungal and antibiofilm activities of ozonized (OGOZ) and non-ozonized (OG) Helianthus annuus (sunflower) oil against *Candida albicans* strains.
Study Design: Laboratory-based study.
Place and Duration of Study: Microbiology Laboratory of the Federal University of Ceará and State University of Vale do Acaraú, from August 2023 to Decembrer of the same year.
Methodology: Seven clinical strains and one reference strain (ATCC 90028) were examined. Initially, antimicrobial susceptibility testing (AST) was performed using a modified disk diffusion method with both OGOZ and OG. Strains that displayed sensitivity were then subjected to a broth microdilution assay to determine the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC). The biofilm assay was conducted specifically with the *C. albicans* reference strain.
Results: AST results showed that all strains exhibited inhibition zones with OGOZ, while non-ozonized oil did not produce inhibition zones. MIC values ranged from 0.15 to 2.50 mg/mL, with one clinical strain (LABMIC H10) demonstrating an MIC value 16 times lower than other strains. None of the strains reached MFC at the tested concentrations. In terms of biofilm formation, application of MIC and twice the MIC resulted in a 48,6% and 85% reduction in mature biofilms, respectively.
Conclusion: The findings indicate that OGOZ has fungistatic and antibiofilm effects, highlighting its potential as a promising agent for bioprospecting. Further clinical trials are essential to evaluate its therapeutic potential for managing infectious diseases such as oral candidiasis.

Keywords: Candida albicans; Ozonized Sunflower Oil; Antifungal Activity; Antibiofilm.

1. INTRODUCTION

Species of the genus *Candida* are among those most frequently associated with fungal infections affecting humans, causing candidiasis [1]. *Candida* is a yeast often found in the mouths of healthy individuals and is considered a member of the normal oral microbiome. The incidence of oral candidiasis in the general population has been traditionally reported between 35% and 80%, depending on the specific population studied [2, 3]. *C. albicans* is the most prevalent *Candida* species in the oral cavity of healthy individuals and is estimated to account for over 80% of oral fungal isolates [4].

The incidence of fungal infections has increased in recent decades [5]. A concerning issue frequently discussed in the literature is the increasing virulence mechanisms related to this microorganism. The virulence of *Candida* in the oral cavity is strongly correlated with certain predisposing factors such as wearing dentures, hyposalivation, prolonged antibiotic or immunosuppressive therapy, local trauma, malnutrition, endocrine disorders, increased longevity, and any elements that compromise the immune system [6]. Additionally, *C. albicans*, which is often the cause of oral candidiasis, is believed to be an extremely heterogeneous species, with strains differing phenotypically and genotypically. Strain variation may contribute to pathogenesis through the elevated expression of virulence factors, affecting the nature of host immune responses [7]. Moreover, studies have reported the expression of genes associated with fluconazole resistance [8].

In this context, discovering new antifungal agents capable of treating candidiasis caused by these yeast species is essential. Ozonized vegetable oils are known to possess antibacterial activity, with studies proving their efficacy against gram-positive bacteria [9, 10]. However, research on the antifungal activity of these ozonized oils is still lacking.

Sunflower oil is derived from the plant's seeds and is rich in linoleic (48–74%) and oleic acids (14–39%) [11, 12]. Like other vegetable oils, its composition is diverse and varies based on factors like harvest, plant genotype, climate, production method, and storage [13, 14]. The ozonization reaction of O_3 occurs at the unsaturation of hydrocarbon chains in the oil, leading to the formation of cyclic ozonized species [15]. The ozonolysis mechanism is called the Criegee oxidation reaction, in which ozone chemically reacts at an unsaturated bond, forming an initial unstable primary ozonide. This quickly decomposes into carbonyl fragments that can combine to generate cyclic trioxolanes in anhydrous environments [16]. The products of this reaction include ozonides, hydroperoxides, aldehydes, peroxides, diperoxides, and polyperoxides, which promote the antibacterial, fungicidal, and antiviral properties of ozonized oils, making them applicable in cosmetics and pharmaceuticals [17]. After the ozonization process, the chemical composition of vegetable oils changes drastically, leading to alterations in physical appearance, with slight changes in taste and odor and increased viscosity [18].

Ozone (O_3) is a highly reactive molecule composed of three oxygen atoms that acts as an oxidant [11]. When bacteria are exposed to ozone in vitro, the phospholipids and lipoproteins of the bacterial cell envelope are oxidized. This mechanism disrupts the integrity of the cytosolic membrane, allowing ozone to infiltrate microorganisms and oxidize glycoproteins and glycolipids, blocking enzymatic functions. Evidence has also shown that ozone interacts with fungal cell walls similarly to bacteria [19, 20].

Another interesting aspect of using ozonides in medical sciences is their lack of cytotoxicity. Cell culture assays showed no cytotoxic effects on fibroblasts or keratinocytes, and they induced fibroblast migration, potentially aiding in wound healing [21].

Biofilm formation is one of the most critical survival strategies of *C. albicans*, enabling it to adhere to biotic and abiotic surfaces and form resistant clusters. These clusters are highly organized, distinct from planktonic life, and protected by an exopolysaccharide matrix layer that envelops the entire biofilm [22].

Given the above, the objective of this study was to analyze the antifungal activity of ozonized Helianthus annuus (sunflower) essential oil (OGOZ) against different strains of *C. albicans* in their planktonic and biofilm forms.

2. MATERIAL AND METHODS

2.1 Studied compound and ozonation process

To investigate the antifungal activity of ozone, ozonized sunflower oil (OGOZ) was chosen for the study due to its physicochemical qualities and the limited amount of research on its application. The OGOZ ozonization process was carried out in Philozon stainless steel reactors (Camboriú, Santa Catarina, Brazil) in a 26 kg (28 L) oil reactor over various time periods. The reaction ends when the product reaches the desired viscosity, measured using a Ford cup. For scientific rigor and comparison purposes, samples of the same non-ozonized sunflower oil (OG) were also tested to clarify whether the ozone activity would be inert, additive, or synergistic.

2.2 Analyzed strains

Seven clinical isolates of *C. albicans* were randomly selected from the microbiology laboratory collection of the State University of Vale do Acaraú, and the reference strain ATCC 90028 were analyzed. Table 1 details the clinical isolates analyzed and the origin of isolation of each fungal strain.

Table 1. Clinical isolates of *C. albicans* analyzed and the source of isolation

Strain	Source
LABMIC 0102	Tissue lesion
LABMIC 0104	Tracheal aspirate
LABMIC 0105	Blood culture
LABMIC 0133	Isolated from patient with Acquired Immunodeficiency Syndrome
LABMIC 0134	Isolated from patient with Acquired Immunodeficiency Syndrome
LABMIC 0136	Isolated from patient with Acquired Immunodeficiency Syndrome
LABMIC 0137	Isolated from patient with Acquired Immunodeficiency Syndrome

Source: Authors, 2024.

2.3 Antimicrobial susceptibility testing (AST)

Initially, all strains were subjected to sensitivity profile analysis using the qualitative modified disk diffusion method. The test was performed in triplicate by applying a 10 µL drop of ozonized sunflower oil (OGOZ) and non-ozonized sunflower oil (OG) at 100% concentrations onto Petri dishes containing Mueller-Hinton agar. The medium was pre-inoculated with each microorganism suspension adjusted to the 0.5 McFarland scale. Plates were incubated for 48 hours at 37°C [23, 24].

Only strains with positive results in the disk diffusion method were subjected to quantitative evaluation using broth microdilution methodology to determine the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) [25]. For better analysis, the reference strain and a randomly selected clinical strain were subjected to the assay using a standard antifungal agent. Thus, ATCC 90028 and the LABMIC0102 variant were tested with Nystatin 100 at a concentration of 100 IU.

2.4 Determination of MIC and MFC

In sterilized 96-well plates, 100 µL of RPMI medium was added to each well. Then, 10 mg/mL of OGOZ was added to the wells in the first column, followed by serial dilutions. Subsequently, 100 µL of the inoculum adjusted to the 0.5 McFarland scale was added to each well [26]. A 0.12% chlorhexidine digluconate solution (Colgate, São Paulo, Brazil) was used as the positive control. OGOZ was tested in concentrations ranging from 0.002 to 2.5 mg/mL. The plates were incubated at 37°C, and visual readings were taken after 24 hours. All assays were performed in duplicate. The MIC is defined as the lowest concentration of the extract capable of inhibiting 100% of visible fungal growth [26, 27]. The Minimum Fungicidal Concentration (MFC) was determined by subculturing, following the methodology of Fontenelle et al. (2008) with modifications [27].

2.5 Evaluation of the antibiofilm activity of OGOZ

ATCC 90028 reference strain was used in assays to inhibit *C. albicans* biofilm formation. The crystal violet technique was employed to quantify the total biomass of the biofilm. Flat-bottom polystyrene plates were used to assess the effect of OGOZ on the eradication of mature biofilm. 200 µL of fungal suspension, adjusted to 10^6 CFU/mL in RPMI medium, was added to the plate for biofilm formation. The plate was incubated at 37°C for 48 hours and then washed to remove planktonic cells.

After this period, 200 µL of the OGOZ compound, at concentrations of MIC, 2x MIC, 4x MIC, and 8x MIC for ATCC 90028 (2.5 mg/mL, 5.0 mg/mL, 10 mg/mL, and 20 mg/mL, respectively), were added to the formed biofilm. For negative controls, only 200 µL of medium was added. The plates were incubated at 37°C for 24 hours. After incubation, the plates were washed three times with 200 µL of saline solution to remove planktonic cells. The biofilm was fixed with 200 µL of 99% methanol for 10 minutes. Once the plates were air-dried, 200 µL of 1% (w/v) crystal violet was added for 5 minutes. The plates were then washed three times with sterile distilled water. To solubilize the dye in the adhered cells, 200 µL of 33% (v/v) acetic acid was used for 10 minutes [28]. The plate readings were taken with a spectrophotometer at 590 nm. The percentage of biofilm formation inhibition at each concentration was calculated using the formula: % biofilm inhibition = 100 - (OD of treated sample/OD of untreated positive control) x 100 [28].

3. RESULTS AND DISCUSSION

The ozonized sunflower oil (OGOZ) exhibited antifungal activity against the seven clinical isolates and the reference strain of *C. albicans* tested. Based on this sensitivity result, all specimens underwent a broth microdilution assay analysis. The average diameters of the inhibition zones obtained with antimicrobial susceptibility testing (AST) for each strain are presented in Table 2.

Table 2. Average Inhibition Zone Diameters (AST) of Undiluted OGOZ Against the Tested *C. albicans* Strains

Strain	Inhibition Zone Diameter (mm)
ATCC 90028	16.66
LABMIC 0102	18.00
LABMIC 0104	18.00
LABMIC 0105	17.33
LABMIC 0133	18.00
LABMIC 0134	18.00

LABMIC 0136	18.83
LABMIC 0137	18.00

Source: Authors, 2024.

For comparison purposes, nystatin, an antifungal agent commonly used in treating oral candidiasis, was tested at a concentration of 100 IU against the reference strain ATCC 90028 and a randomly chosen clinical strain (LABMIC0102). The average inhibition zone diameters were 12.33 and 14.33 mm, respectively, indicating that the tested compound exhibited an inhibitory action approximately 30% greater than that of nystatin. However, there have been reports of *Candida* spp. resistance to antifungals commonly used in clinical practice [29].

On the other hand, no inhibition zones were observed with sunflower oil alone (OG), demonstrating that the non-ozonized oil lacked antifungal activity. The MIC values for each strain are presented in Table 3, without visible fungal growth at any concentration tested, 0.12% chlorhexidine digluconate was used as a positive control.

Table 3. MIC obtained for the *C. albicans* reference strain and other clinical isolates analyzed

Strain	MIC (mg/mL)
ATCC 90028	2.5
LABMIC 0102	2.5
LABMIC 0104	2.5
LABMIC 0105	2.5
LABMIC 0133	2.5
LABMIC 0134	1.25
LABMIC 0136	0.15
LABMIC 0137	2.5

Source: Authors, 2024.

The subculture assay revealed positive growth, indicating that no MFC was observed for any of the strains at the tested concentrations. OGOZ exhibited significant differences in all treatments compared to the untreated control (Figure 1). The oil's MIC concentration reduced the total biofilm biomass by approximately half (48.6%). The concentrations of 2x MIC, 4x MIC, and 8x MIC did not show significant differences among themselves, reducing the total *C. albicans* biofilm biomass by 85%, 87%, and 84.4%, respectively.

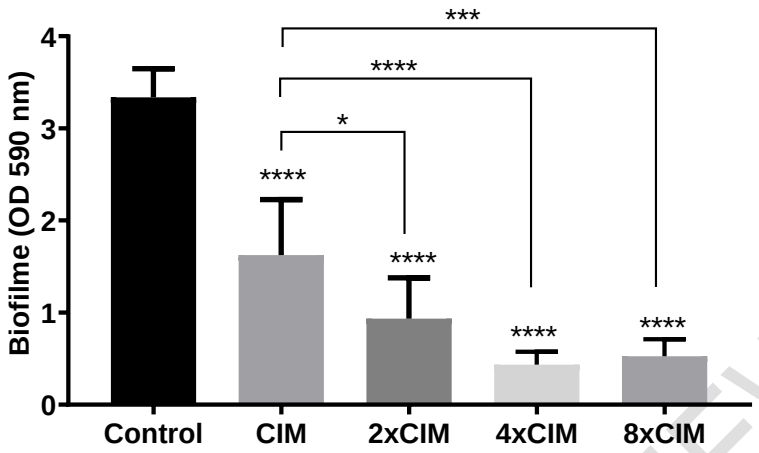

Fig. 1. Quantification of the Total Biomass of *C. albicans* treated with Ozonized Sunflower (Helianthus annuus) Oil.

*Test: * p < 0.1 e *** p < 0.001 e ****p < 0.0001.*

The analysis of the results obtained in this study demonstrated that ozonization of sunflower oil was effective, as the OGOZ compound showed antifungal activity against all tested *C. albicans* specimens, unlike the non-ozonized oil (OG). Therefore, we can infer that sunflower oil alone acts only as an inert vehicle for preserving and applying the ozonized particles [16]. Other studies have presented similar results with different ozonized compounds. For example, ozonized gel (GeliO3), composed of bio-ozonized olive oil and synthetic amorphous silica gel with a peroxide index of 20 mEq O2/kg, was found to inhibit cell division through an agar diffusion method when tested against *Candida* species (*C. albicans, C. parapsilosis, C. glabrata, and C. tropicalis*) [30]. However, one advantage of OGOZ over GeliO3 is the ability to perform quantitative assays to determine the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC), which was not possible with GeliO3 due to the bio-ozonized olive oil's insolubility in culture broth, making it highly hydrophobic. Even the addition of a detergent like Tween 80 [30] could not dissolve the gel. The possibility of performing MIC and MFC assays with OGOZ is facilitated by the lower hydrophobicity of the vehicle (sunflower oil) compared to olive oil.

Regarding the data obtained in the quantitative experiments of this study, a considerable variation in MIC values was observed. The clinical isolate LABMIC 0136 had an MIC 16 times lower than most other strains (ATCC 90028, LABMIC 0102, LABMIC 0104, LABMIC 0105, LABMIC 0133, and LABMIC 0137), as seen in table 3. Additionally, LABMIC 0134 had an MIC of 1.25 mg/mL. This variation can be attributed to the high genetic and phenotypic variability found in the species [7]. Using a similar methodology, a literature review found an MIC value of 5 mg/mL for C. albicans MTCC 227 using ozonized olive oil [32].

The subculture method revealed that OGOZ did not have fungicidal activity due to the growth of specimens at the MICs presented in this study. Elshinawy et al. [32] reported a value of 5 mg/mL as the MFC for *C. albicans* MTCC 227 using ozonized olive oil. However, the highest concentration of OGOZ used in this study was 2.5 mg/mL, which was insufficient to verify whether this compound possesses fungicidal properties. Nevertheless, within the MIC range, OGOZ demonstrated fungistatic action for all *C. albicans* specimens studied.

The antibiofilm activity analysis revealed positive results in this study. Using the lowest concentration (2.5 mg/mL) resulted in a 51% reduction in biofilm mass, while concentrations

above 10 mg/mL led to an approximate 99.9% reduction. It is essential to note that after biofilm formation, the MIC of *C. albicans* increases by 30 to 20,000 times compared to planktonic cells [33]. Moreover, fungal biofilms are widely resistant to available antifungal drugs [33, 34]. This scenario emphasizes the importance of this work, as it's crucial to explore the potential of new antimicrobial drugs with antibiofilm properties, like OGOZ. In a biofilm assay with premolars investigating the effect of ozonized extra virgin olive oil (Novox®, MOSS Srl, Lesa – Novara, Italy) containing active oxygen in the form of peroxide (560–590 mmol-equiv/kg) against endodontic pathogens, it was shown that this compound reduced the *C. albicans* MTCC 227 biofilm by 86%. However, the concentrations were not disclosed [32].

Ozone therapy has been explored in various aspects of health, particularly in dentistry. It has shown efficacy in treating wound healing, dental caries, oral lichen planus, gingivitis, periodontitis, halitosis, jaw osteonecrosis, post-surgical pain, bacterial plaque, biofilms, root canal therapy, dentin hypersensitivity, temporomandibular joint disorders, and tooth whitening [35]. This article provides clear evidence that OGOZ can be used to reduce *C. albicans* counts. From a clinical perspective, OGOZ could be a promising treatment option for oral candidiasis, making it a substance of relevance for studies in dentistry, especially stomatology. This prospect becomes even more significant when considering the lack of cytotoxicity of this compound [21] and the increasing prevalence of antifungal resistance [8].

Ozone is a potential antiseptic agent, and its aqueous form exhibits less cytotoxicity than gaseous ozone or established antimicrobial agents (2% and 0.2% chlorhexidine digluconate; 5.25% and 2.25% sodium hypochlorite; and 3% hydrogen peroxide) under most conditions. Therefore, ozone demonstrates ideal cellular biological characteristics in terms of biocompatibility for oral application [36].

The biological activity of ozone therapy in oral candidiasis was investigated in immunosuppressed rats. The animals received oral inoculations of *C. albicans* on the dorsal surface of the tongue and were treated with daily intraperitoneal injections of 1 cm^3 of an ozone-oxygen mixture with an ozone concentration of 70 $\mu g/cm^3$. Histological examination showed that ozone therapy gradually reduced lingual papillary atrophy and *Candida* invasion [37]. However, further studies, particularly *in vivo* models and clinical trials, are necessary to promote the bioprospecting of ozonized compounds like OGOZ.

4. CONCLUSION

The results of this study allow us to conclude that ozonized sunflower oil (OGOZ) exhibits antifungal and antibiofilm activities, and that ozone has additive effects. This emphasizes the importance of OGOZ as a product for bioprospecting, reinforcing the need for clinical studies to investigate the potential application of this compound in treating infectious diseases such as oral candidiasis.

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