ISSN: 2754-494X

Journal of Dermatology Research Reviews & Reports

SCIENTIFIC Research and Community

Research Article

Open Access

Standardization, Characterization and Potential Biological Activity of Ozonized Sunflower Vegetable Oil

Vieira MH[1*], Boreski D[1], Cacoci ESP[1], Martins CJ[1], Bauab TM[2] and Chin CM[1,3]

[1]Laboratory for Drug Design (LAPDESF), University of São Paulo, Araraquara, São Paulo, Brazil

[2]Laboratory of Microbiology, University of São Paulo, Araraquara, São Paulo, Brazil

[3]Advanced Research Center in Medicine (CEPAM), University of São Paulo, Araraquara, São Paulo, Brazil

ABSTRACT

Objective: The ozone molecule, composed of three oxygen atoms, possesses high oxidizing power and is widely utilized for various applications, including therapeutic treatments involving the administration of medicinal ozone to combat various diseases. The standardization of ozonized oil is essential to ensure patient safety. The objective of this study was to develop protocols for the standardization and characterization of ozonized oil using analytical techniques such as gas chromatography-mass spectrometry (GC-MS) and to evaluate its biological activity through microbiological and cytotoxic assays.

Methods: Standardization and characterization were carried using gas chromatography coupled to mass spectrometry (CG-MS), and microbiological tests including agar diffusion, time-kill and MIC and cytotoxic tests were employed in bacterial cells.

Results: The obtained results indicated that after 480 minutes of ozonization in 100 mL of sunflower oil (OG), a significant antimicrobial potential was observed, leading to the formation of ozonides. Antibacterial and antifungal activity assays, conducted using the 'time kill' method, agar diffusion, and Minimum Inhibitory Concentration (MIC) tests, demonstrated activity against Staphylococcus aureus, Escherichia coli, Salmonella choleraesuis, Pseudomonas aeruginosa, Candida albicans, Aspergillus brasiliensis, and Malassezia furfur.

Conclusion: Results demonstrate the therapeutic potential of the application of ozonized oil in the treatment of topic infectious diseases. In summary, the standardization of the ozonization process of sunflower oil showed to be promising, providing a product exerting antimicrobial activity and therapeutic potential, opening perspectives for its application in various areas of medicine.

***Corresponding author**

Vieira MH, Laboratory for Drug Design (LAPDESF), University of São Paulo, Araraquara, São Paulo, Brazil.

Received: December 12, 2024; **Accepted:** December 18, 2024; **Published:** January 20, 2025

Keywords: Ozonized Oil, Microbiology, Antimicrobial Activity, Chemical Analysis, Gas Chromatography, Safety Testing

Introduction

Ozone (O_3), although it was first produced in the laboratory in 1839 by Christian Friedrich Schönbein, is a natural substance. Its discovery was a result of Schönbein's interest in the odor produced by certain chemical and electrical processes [1].

Ozone therapy involves the medical application of ozone gas to treat various health conditions. Ozone, a form of oxygen with three atoms, is administered in precise concentrations and methods tailored to the specific condition. It is believed to exert therapeutic effects through its oxidative properties, enhancing oxygenation, modulating the immune system, and potentially aiding in the treatment of infections, chronic diseases, and tissue healing [2,3]. Despite ongoing research and clinical use, the therapy's efficacy and safety remain subjects of debate and further investigation within the medical community.

In ozone therapy, ozonized vegetable oils have been used due to their low cost and ease of topic application, yielding promising results [4]. Ozonized oil has garnered significant interest in scientific and clinical spheres because of its potential therapeutic applications. The transformation of vegetable oils by ozone results in the formation of ozonides and other compounds that slowly release ozone retained as ozonides [5,6].

However, the standardization and characterization of ozonized vegetable oils are crucial. Inadequate ozone concentration can render the oil ineffective, while excessive ozone can make it toxic, forming components such as formaldehyde, toxic to microorganisms and humans [7]. Therefore, this study aims to standardize ozonized vegetable oil and evaluate its antimicrobial potential, contributing to significant advances in the field of ozone therapy for safe medicinal use.

Material and Methods

Preparation of Ozonized Sunflower Oil (OGO)

The OGO (ozonized sunflower oil) was produced using a PHILOZON® MEDPLUS model ONE ozone generator, at a concentration of 60 µg/mL from medicinal oxygen supplied by the company Oxitab®. The working pressure was 2 Kgf/cm^2, with a gas flow of 1L/min. The ozone generated was immersed through a silicone hose attached to an Acquap® porous stone and

immersed in 100 mL of sunflower oil (OG) stored in a 250mL kit. The ozone immersion process was standardized in three distinct times, 100 minutes, 240 minutes and 480 minutes.

Gas Chromatography Coupled to Mass Spectrometry (GC-MS)
To analyze the oil samples, gas chromatography coupled to mass spectrometry in a GC-MS/MS series (Gas Chromatography Tandem Mass Spectrometry model GC-2010) was used, equipped with an automatic sampler model Combipal AOC 6000 and a mass spectrometer detector. with a triple quadrupole type analyzer model TQ 8050 Shimadzu. The carrier gas used was helium (99.999% purity) at a constant flow rate of 1.30 mL.min−1. Injector temperature of 250 °C and 1 µL were injected at a pressure of 300 kPa in split mode, with a sample split ratio of 1:10. To separate the compounds, an Rtx®-5MS capillary column (30 m × 0.25 mm × 0.25 µm) (Restek, Bellefonte, PA, USA) was used employing a temperature ramp starting at 80°C and remaining at this temperature for 1 min, followed by a heating rate of 10°C min−1 until 180°C

Finally, the oven was heated to 330°C at 7°C min-1, with a total analysis time of 32.43 min. The ionization of the compounds was carried out by electron impact (EI) with an energy of 70 eV. The interface and ion source temperatures were 280°C and 230°C, respectively. The determinations were carried out in scanning mode, in the range between 50 and 500 m/z. Equipment manipulation, data collection and processing were performed using the GC-MS solution software, version 4.45 SP1 (Shimadzu®).

To determine the fatty profile, an aliquot of 30 mg of oil was weighed on an analytical balance, followed by the addition of 500 mL of 0.1 mol.L-1 potassium hydroxide solution. The solution was left for 1h30m in a water bath at 60°C, followed by the addition of 1.5 mL of 1 mol.L^{-1} sulfuric acid. Then, room cooling was carried out and 2 mL of hexane was added, stirred and waited for the phases to separate. After the procedure, the hexane aliquot was injected into the GCMS/MS.

To analyze the unsaponifiable profile, derivatization was carried out to transform the fatty acids into esters, with better chromatographic properties. Weighing both 500 mg oils on an analytical balance, added 5 mL of 2 mol.L-1 potassium hydroxide in ethanol, keeping in a water bath for 60 minutes at 80°C. After cooling, 2 mL of water and 8mL of hexane were added, kept under agitation for 2 minutes in a vortex mixer and another 5 minutes in a centrifuge. After the entire procedure, the hexane aliquot was injected into the GC-MS/MS for analysis.

Physicochemical Evaluation
Acidity, peroxide and iodine index were performed according to Adolfo Lutz Institute, in triplicate [8].

Acidity Index
The sample 2 g were homogenized with 25 mL of ethanol for 1 min and phenolphthalein were used as indicator. The titration was carried out with sodium hydroxide (0.1 mol. L^{-1}) until the pink color appeared and persist for 30 min. The acidity index was calculated based on the formula:

$$Acidity\ index = \frac{v \times f \times 5.61}{P}$$

v = volume of 0.1 M sodium hydroxide solution titrated (mL).
f = sodium hydroxide solution factor
P = sample weight (g)

Peroxide Index
0.5 g of the sample was added, 10 mL of the acetic acid/chloroform solution (3:2) was added, homogenized for 1 minute. Afterwards, 1 mL of potassium iodide solution (14 grams of potassium iodide and 8 mL of deionized water) was added, stirring for 1 minute at room temperature and then for 30 min at 60°C. 25 mL of deionized water was added and homogenized for 1 minute. The solution was titrated with 0.1 N sodium thiosulfate solution until the yellow color disappeared under constant stirring.

Then, 1mL of 1% indicator starch solution was added (1:99 starch: water at 60°C) and the titration followed until the blue color disappeared. A blank test was prepared under the same titration conditions. The peroxide index was calculated based on the formula:

The samples 0.5 g were homogenized with 10mL of acetic acid/chloroform solution (3:2) during 1 min. Subsequently, 1 mL of potassium iodide solution (1,75g/mL) was added and stirred for 1 minute at room temperature and then for 30 minutes at 60°C. Following this, 25 mL of deionized water was added, and the mixture was homogenized for 1 minute. The solution was titrated with 0.1 N sodium thiosulfate solution until the yellow color disappeared under constant stirring. Then, 1 mL of 1% starch indicator solution was added, and the titration was continued until the blue color disappeared. A blank test was performed under the same titration conditions. The peroxide index was calculated using the following formula:

$$Peroxide\ index = \frac{(A - B) \times N \times f \times 1000}{P}$$

A = volume of 0.1 N sodium thiosulfate solution used in sample titration (mL)
B = volume of 0.1 N sodium thiosulfate solution used in the blank titration (mL) N = normality of the sodium thiosulfate solution f = factor of the sodium thiosulfate solution
P = sample weight (g)

Iodine Index
A 0.25 g sample was added to 10 mL of carbon tetrachloride. Afterwards, 25 mL of Wijs-solution (1.3% Iodine + 75% acetic acid + deionized water) was added. The bottle was capped and shaken carefully in rotation movements, after which it remained at rest protected from light and at room temperature for 30 minutes. Then, 10 mL of 15% potassium iodide and 100 mL of demineralized water were added. Afterwards, titration was carried out with 0.1 mol.L-1 sodium thiosulfate until a faint yellow color appeared. 2 mL of 1% starch indicator solution was added and the titration continued until the blue color completely disappeared. The iodine index was calculated based on the formula:

$$Iodine\ index = \frac{(VB - VA) \times M \times 12.68}{P}$$

M = molarity of the sodium thiosulfate solution
VB = volume in blank titration (mL)
VA = volume in sample titration (mL)
P = sample weight (g)

Microbiological Assays
Strains
Staphylococcus aureus ATCC 6538, Escherichia coli ATCC 8739, Salmonella choleraesuis ATCC 10708, Pseudomonas aeruginosa

ATCC 9027, Candida albicans ATCC 10231, Aspergillus brasiliensis ATCC 16404, and Malassezia furfur ATCC 14521.

Agar Diffusion (Pour-Plate)

They were incubated at a temperature of 35+/-2 °C for 24 hours for bacteria and 25+/-2°C for 5 days for yeast. After the incubation period, each microorganism was suspended in TSB broth, the yeasts were suspended in Sabouraud dextrose broth. 1.0 mL of the suspension was diluted until dilution 10^7, using the previously melted culture medium and kept in a water bath at a temperature of 45+/-2°C in the plates containing the dilutions. After solidification, the plates were incubated inverted for 48 hours in an oven according to the temperatures being 35+/-2°C for bacteria and 25+/-2°C for yeast [8].

Time-Kill

1 mL of the standard suspension of bacteria containing 10^8 to 10^9 UFC/mL and 10^6 to 10^7 yeasts were inoculated in 49 mL of sample. To control the inoculum, the same amount was taken and transferred to 49 mL of sterile water. Performed serial decimal dilutions and plated dilutions -3, -4, -5 and -6. Samples were collected at contact times of 30 and 60 seconds, homogenized vigorously and immediately retained an aliquot of 0.1mL of the inoculated sample, transferring to a tube containing 8.8 mL of neutralizing broth + 1.1 mL of water. At the end of the last time collection, the tubes are incubated at 35+/-2°C for 5 minutes. After this incubation period, serial dilutions were carried out for each of the samples, then the plates were incubated at 35+/-2°C for 48 hours for bacteria and 25+/-2 °C for 5 days for yeasts. After incubation, counts were carried out [9].

Strains Evaluated

Staphylococcus aureus ATCC 6538, *Escherichia coli* ATCC 8739, *Salmonella choleraesuis* ATCC 10708, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, *Aspergillus brasiliensis* ATCC 16404.

Minimum Inhibitory Concentration (MIC)

From the prepared suspensions, 1 mL of each suspension was inoculated in 10 mL of 0.85% saline solution and serial decimal dilutions were performed in 10-3, 10-4 and 10-5. Then, 1 mL of each dilution was transferred to sterile Petri dishes, where 25 mL of previously melted TSA Agar and kept in a water bath were inoculated for the bacteria strains and 25 mL of Sabouraud Dextrose Agar for the yeast strains. After solidification of the culture medium, the plates containing TSA were incubated at 35+/-2°C for 48 hours and the plates containing Sabouraud Dextrose Agar at 25+/-2°C. Colonies were counted.

As it is an OGO, we used the OG vehicle, but before using it as a vehicle, CIM was performed to verify that there was no inhibition. After the negative result, we continued with dilutions of ozonized sunflower vegetable oil in preserved sunflower vegetable oil at the following concentrations: 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%.

Strains Evaluated

Staphylococcus aureus ATCC 6538, Escherichia coli ATCC 8739, Candida albicans ATCC 10231 and Aspergillus brasiliensis ATCC 16404.

In Vitro Cytotoxicity Study

Balb.c 3T3 clone 31 cells (Rio de Janeiro Cell Bank, batch 001242) were maintained in culture with DMEM (Dulbecco's Modified Eagle's Medium) with the addition of supplements, in an oven at 37°C and 5% CO2. After thawing, the cells were distributed in 96-well plates maintained in culture under the same conditions described.

OGO was diluted in cell culture medium at the following concentrations 10; 1; 0.1; 0.001 and 0.0001 mg.mL^{-1}. Supplemented cell culture medium was used as a negative control group and diluted sodium lauryl sulfate in supplemented cell culture medium was employed on positive control group.

For analysis, 0.1 mL of sample was applied to the cell culture, per well, followed by incubation in an oven at 37°C and 5% CO2 for 24 hours. After washing with deionized water, cell viability was analyzed with the neutral red indicator, 0.1 mL per well. After incubation, the reaction was revealed, and absorbance was determined at 540 nm. The procedure was performed in quadruplicate.

Results and Discussion

Preparation of Ozonized Sunflower Oil (OGO)

Studies of ozonized vegetable oils use sunflower and olive vegetable oil due to the greater number of unsaturations present in these oils [10]. The research already carried out was standardized using reactors and different times and most of the tests involved characterization of physicochemical properties and antimicrobial activity restricted to bacteria: *Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa, Candida albicans, Salmonella typhimurium, Candida parapsilosis, Candida tropicalis, Trichosporum asaii* [11-13].

To define the standardization of the sample that we would follow with the proposed studies, it was determined that the sample would need to have microbial activity compared to the microbiological assay of the agar diffusion technique.

The sample ozonized at 100 minutes was not successful in the proposed tests, the sample at 240 minutes had a small halo, while the sample at 480 minutes had a larger halo compared to the one at 240 minutes and closer to the positive control. Based on the previous results, it was decided to follow the proposed studies with the standard OGO sample at 480 minutes of ozonation

Gas Chromatography Coupled to Mass Spectrometry (GC-MS)

Gas chromatography-mass spectrometry (GC-MS) is a powerful analytical technique used to identify and quantify substances in complex mixtures. It combines the separation capabilities of gas chromatography with the detection and identification capabilities of mass spectrometry. In GC-MS, a sample is first vaporized and injected into a gas chromatograph, where it is separated into its components based on their affinity for the stationary phase within the column. As each compound elutes from the column, it enters the mass spectrometer where it is ionized and fragmented into smaller ions. These ions are analyzed based on their mass-to-charge ratio (m/z), providing a unique profile for each compound [14]. In this way, the 480-minute OGO results showed the presence of the major components of great importance in ozonation, which are the unsaturation chains. The fatty profile of the control oil OG obtained the following concentrations: 33.10% oleic acid C18:1, 47.13% linoleic acid C18:2 and 0.41% linolenic acid C18:3 Table 1. Data that corroborate studies by and his collaborators, which describe that OG contains approximately 15% saturated fatty acids, 85% unsaturated fatty acids, 14 to 43% oleic acid and 44 to 75% linoleic acid in its unsaturated fatty acid content [15].

Table 1: Sunflower Oil (OG) Fat Profile

Pic	%	Components
1	0,06%	Ácido Mirístico - C14:0
2	0,07%	Ácido palmitoléico - C16:1
3	10,81%	Ácido palmítico - C16:0
4	0,07%	Ácido margárico - C17:0
5	47,13%	Ácido Linoleico - C18:2
6	33,10%	Ácido oleico - C18:1
7	0,41%	Ácido linolênico - C18:3
8	6,12%	Ácido esteárico - C18:0
9	0,26%	Ácido eicosenóico - C20:1
10	0,59%	Ácido araquídico - C20:0
11	1,06%	Ácido beénico - C22:0
12	0,32%	Ácido lignocérico - C24:0

After ozonization, a considerable reduction was observed in the content of C18 oleic acid, from 33.10% to 10.84%, C18:2 linoleic acid, from 47.13% to 5.12% for 480 minutes, and C18:3 linolenic acid of 0.41%, in the first ozonation time 100 minutes until its total consumption Table 2.

Table 2: Fat Profile Ozonized Sunflower Oil (OGO 480 minutes)

Pic	%	Components
1	0,46%	C8:0 - caprylic acid
2	1,77%	2-Nonenal, (E)-
3	3,65%	9-Decen-2-ol
4	5,70%	nonanoic acid, methyl ester
5	0,45%	2-Octanol, acetate
6	19,64%	Dimethyl acetal nonanal
7	7,31%	Nonanoic acid, 9-oxo-
8	1,40%	C12:0 - lauric acid
9	8,19%	Azelaic acid
10	7,92%	Dimethyl acetal decanal
11	1,11%	octanoic acid, 6,6-dimetoxi-
12	0,63%	C14:0 – myristic acid
13	1,10%	Methyl 12-oxo-9-dodecenoate
14	2,60%	Methyl 5,13-docosadienoate
15	9,91%	C16:0 – palmitic acid
16	5,12%	C18:2 – linoleic acid
17	10,84%	C18:1 – oleic acid
18	9,52%	C18:0 – stearic acid
19	0,68%	C20:0 – arachidonic acid
20	1,57%	C22:0 – docosanoic acid
21	0,43%	C24:0 – tetracosanoic acid

Vegetable oil include unsaponifiable components in their composition, which are substances dissolved in oil and fat that are not saponifiable, that is, they are not converted into fatty acid salts by the action of strong bases, being insoluble in aqueous solution, but soluble in common solvents of fats as per In Figure 1 we can see the OG chromatogram and in Figure 2 OGO.

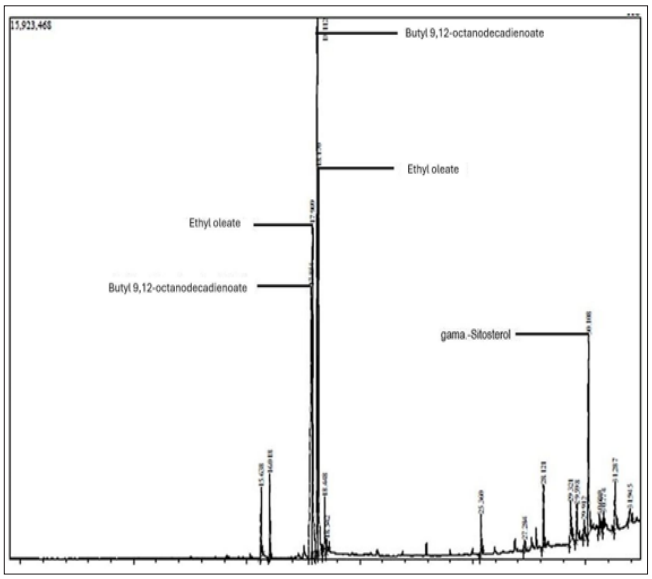

Figure 1: Chromatogram of Unsaponifiable Components in Sunflower Oil (OG)

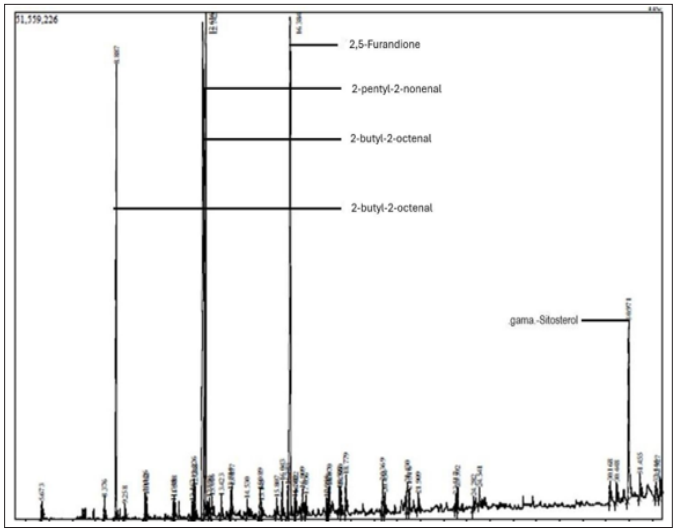

Figure 2: Chromatogram of Unsaponifiable Matter of Ozonized Sunflower Oil (OGO 480 minutes)

Physicochemical Profile

Acidity Value

The results showed an increase in the acidity index in line when compared with literature results due to the increase in acidic by-products such as aldehydes, peroxides and carboxylic acids generated during the ozonation process as shown in Table 3 [16, 17].

Table 3: OG and OGO Acidity Index

Sample	Acidity index
OG – 0 min	0,5
OGO - 100 min	0,8
OGO - 240 min	1,15
OGO - 480 min	2,15

Peroxide Index

The peroxide index (PI) is a measure used to assess the extent of oxidation in oils and fat samples. It is responsible to quantifiy the number of peroxides species present in the sample, which are formed as an early-stage product of lipid oxidation. The peroxide

index is typically measured using standard analytical methods, often involving titration with a solution that reacts specifically with peroxides present in the oil. Peroxides are formed when oils and fats react with oxygen, a process known as oxidation. Higher levels of peroxides indicate a higher degree of oxidation, which can negatively impact the quality of the oil. This is especially important in oils used for human consumption, as oxidized oils can lead to off-flavors and potential health risks. The peroxide index is used as an indicator of the freshness and stability of oils. Oils with low peroxide index values are considered fresher and more stable, whereas higher values suggest the oil has undergone oxidation, possibly due to exposure to light, heat, or air [18,19]. Studies point to the importance of determining limits in ozonation, due to chain reactions, which result in the formation of ozonide by-products, which can generate problems such as allergies, irritations, tissue destruction, among others [20]. The results showed a considerable increase in the peroxide index with increasing ozonation time Table 4.

Table 4: OG and OGO Peroxide Index

Sample	O_3 Dosage	Index peroxide
OG	0	120
OGO 100	60 µg/mL	1300
OGO 240	60 µg/mL	1660
OGO 480	60 µg/mL	3170

Iodine Index iodine index, is a measure of the degree of unsaturation in fats and oils. It
indicates the amount of iodine that can be absorbed by 100 g of oil. The iodine value is determined through a chemical reaction where iodine is added to the double bonds present in the unsaturated fatty acids. The excess iodine that is not consumed in the reaction is then titrated with a standard solution of sodium thiosulfate to determine the amount of iodine absorbed by the oil, according to Díaz [21]. Oils with higher iodine values contain more unsaturated fatty acids, such as oleic acid, linoleic acid, and linolenic acid, compared to oils with lower iodine values, which are more saturated.

The results showed a considerable drop in the iodine index with increasing ozonation time, suggesting that this process promotes the reduction of unsaturated compounds in the oil as shown in Table 5.

Table 5: Iodine Index of OG and OGO Samples

Sample	Iodine index
OG	145
OGO 100 minutes	81
OGO 240 minutes	71
OGO 480 minutes	53

Microbiological Assays

Agar Diffusion (Pour-Plate)

The agar diffusion (pour-plate) method is used to assess antimicrobial activity. It involves inoculating a bacterial culture on agar plates, creating wells or placing discs impregnated with test substances (e.g., antibiotics). After incubation, zones of inhibition around discs indicate antimicrobial efficacy. Widely used in clinical settings for antibiotic susceptibility testing, it screens natural and synthetic compounds, disinfectants, and preservatives. Thus, the OGO 480 minutes sample proved to be effective in the agar diffusion assay for the follow strains: Staphylococcus aureus ATCC 6538, Escherichia coli ATCC 8739, Salmonella choleraesuis ATCC 10708, Pseudomonas aeruginosa ATCC 9027), Candida albicans ATCC 10231, and Aspergillus brasiliensis ATCC 16404. In the Figure 3 it is possible to observe the difference in halo from the OGO 240 min sample to the 480 min sample, while the OGO 100 min sample did not have a growth inhibition halo.

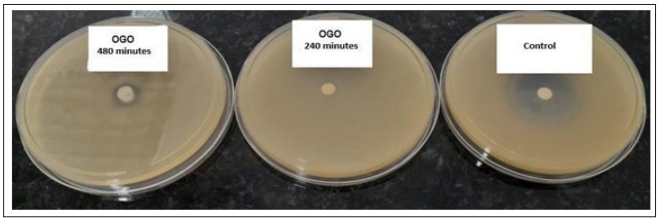

Figure 3: OGO Agar Diffusion vs Control with Staphylococcus Aureus ATCC 6538 Strains

Time-Kill

The time-kill method is a technique used to evaluate the bactericidal or bacteriostatic activity of antimicrobial agents over a specified period. The time-kill method provides information on the rapidity and extent of antimicrobial activity against specific bacterial strains. That helps characterize the kinetics of bacterial killing and differentiation between bactericidal and bacteriostatic effects. Figure 4 shows the plating of one of the strains evaluated. Ogo 480 minutes presented antimicrobial activity against the microorganisms Staphylococcus aureus ATCC 6538, Escherichia coli ATCC 8739, Salmonella Choleraesuis ATCC 10708, Pseudomonas aeruginosa ATCC 9027, Candida albicans ATCC 10231, and Aspergillus brasiliensis ATCC 1640 18. In Table 6 we can check the bacteria and yeast count (contact time and reduction factor).

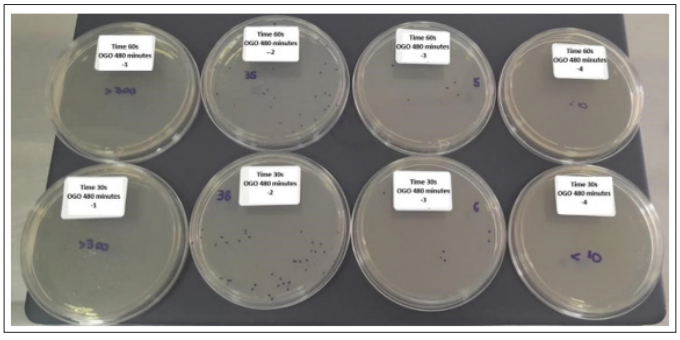

Figure 4: Strain Plating for the Time-Kill Assay

Table 6: Bacteria and Yeast Counts (Contact Time and Reduction Factor)

Bacterial count (UFC/g or mL)							
Microorganism		**Contact time**			**%RF (Reduction factor)**		
	T = 0	**T-30 seg.**	**T-1 min.**	**T-2 min.**	**T-30 seg.**	**T-1 min.**	**T-2 min.**
Pseudomonas aeruginosa ATCC 9027	1,2x107	3,2x103	1,5x103	9,5x102	>99,9	>99,9	>99,9
Escherichia coli ATCC 8739	1,9x107	1,1x105	8,5x104	5,5x103	>99	>99	>99,9
Sthaphylococcus aureus ATCC 6538	1,5x107	2,4x104	1,6x104	3,5x103	>99	>99	>99,9
Salmonella choleraesuis ATCC 10718	6,0x107	3,5x104	9,5x102	1,4x102	>99,9	>99,99	>99,999
Aspergillus brasiliensis ATCC 16404	5,0x105	<10	<10	-	>99,9	>99,99	-
Candida albicans ATCC 10231	2,7x105	4,2x102	<10	-	>99	>99	-

Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) method is a standard laboratory technique used to determine the lowest concentration of an antimicrobial agent that inhibits the visible growth of a microorganism. Thereby, the OGO sample were tested against the bacterial strains. The inhibition of the bacterium Escherichia coli had the lowest inhibition in the MIC assay and the yeast Candida albicans had the highest inhibition in the 6.25% OGO and 93.75% OGO dilutions.

In Figure 5, A inhibition of around 50% of Escherichia coli was observed in TTC 1% medium (triphenyltetrazolium chloride) and Figure B shows the inhibition pattern of *Candida albicans and Aspergillus brasiliensis.*

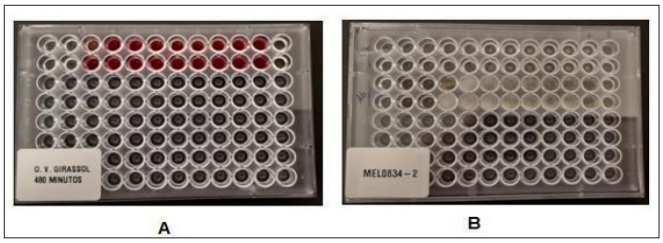

Figure 5: Microdilution with 96-well Microplate

The results demonstrate that the mixture of 50% OGO 480 minutes in OG inhibited all microorganisms studied. With 25% OGO 480 minutes, it still inhibited Staphylococcus aureus, Aspergillus brasiliensis and Candida albicans, but not E. coli. By further decreasing the concentration of OGO 480 minutes to 12.5%, inhibition occurred for Staphylococcus aureus and Candida albicans, while the 6.25% dilution of OGO 480 minutes inhibited only Candida albicans Table 7 [22].

Table 7: Minimum Inhibitory Concentration (MIC) of OGO - 480 min

100% - initial concentration									
		OGO	**OGO**	**OGO**	**OGO**	**OGO**	**OGO**	**+ Control**	**- control**
Strain	**OGO**	**50%**	**25%:**	**12,5%**	**6,25%:**	**3,12%:**	**1,56 %**		
		:OG	**OG**	**:OG**	**OG**	**OG**	**:OG**		
		50%	**75%**	**37,5%**	**93,75%**	**96,87%**	**98,43%**		
	Sample	**50%**	**25%**	**12,50%**	**6,25%**	**3,13%**	**1,56%**	**C+**	**CB**
Sthaphyloco ccus aureus ATCC 6538	**NA**	-	-	-	+	+	+	+	-
Escherichia coli ATCC 8739	**NA**	-	+	+	+	+	+	+	-
Aspergillus brasiliensis ATCC 16404	**NA**	-	-	+	+	+	+	+	-
Candida albicans ATCC 10231	**NA**	-	-	-	-	+	+	+	-

+ growth of the microorganism
- inhibition of microorganism growth
Standard Inoculum: Bacteria and Yeast: 106 a 107 UFC/mL and Molds: 104 a 105
UFC/mL.

Conclusion

In summary, the standardization and characterization of OGO for 120, 240 and 480 minutes at a concentration of 60 µg/ml of ozone, the physicochemical properties changed with the increase in ozonation time. The color changed, becoming lighter, the density increased, the odor changed, the peroxide and acidity levels increased, and the iodine index reduced. In ozonation, the formation of new ozonide compounds was characterized, GC-MS data pointed to the appearance of benzoic acid, 2,5 furandione, phytosterols, among others. The biological activity, OGO 480 minutes was effective in the following strains: Staphylococcus aureus, Escherichia coli, Salmonella choleraesuis, Pseudomonas aeruginosa, Candida albicans, and Aspergillus brasiliensis among the microbiological assays. logical as diffusion on agar, time-kill and CIM. In the CIM assay, it was noted that the yeast Candida albicans ATCC 16404 showed the greatest inhibition at the 6.25% dilution of OGO 480 minutes and 93.75% OG, while the bacterium Escherichia coli showed inhibition at the 50% dilution of OGO and 50% OG. In addition, the physical-chemical parameter peroxide index is a much talked about subject when it comes to ozonized vegetable oil, studies corroborate that this index is not the only determinant for the quality of the product, but the longer the ozonation time, the greater this will be. index. A predominant parameter is chromatography, as it is in this analysis that we will identify the components that will be effective and safe for the product.

Regarding the cytotoxic result in Balb/c 3T3 clone 31 cells in vitro, OGO 480 minutes proved to be cytotoxic at doses of 1 to 10 ug/mL. In summary, this work corroborates to the therapeutic use of OGO (O3 standardized, 480min 60 µg/ml) in bacterial and fungal model. Additionally, despite being a plant origin, free of preservatives and fragrance, it can characterize an antimicrobial for dermatological use option.

Acknowledgements

The authors would like to thank the Integrated Analysis Center - CIA-FURG and KAAPI FRANGRACE for the chromatography tests carried out. To the IPCLIN Group for the microbiological tests carried out. The company Ozon Solution for the vegetable oil sample provided and Melk Cosmetics for the chemical analyzes carried out.

References

1. Mcelroy CT, Fogal PF (2008) Ozone: From discovery to protection, Atmosphere-Ocean. T & F 46: 1-13.
2. Brito Júnior AA, Carneiro JKMP, Reis JVNA, Oliveira TJS, Dantas JBL (2022) Application of ozonized oils in human body and oral cavity systems. RGO, Rev Gaúch Odontol 70: e20220027.
3. Serra MEG, Baeza-Noci J, Abdala CVM, Luvisotto MM, Bertol CD, et al. (2023) The role of ozone treatment as integrative medicine. An evidence and gap map. Front Public Health 10: 1112296.
4. Uebele DTR, Soto CAT, Galvão NKAM, Tim CR, Sobrinho ASS (2022) Ozonizing sunflower oil using Fourier- transform infrared spectroscopy for product characterization, Vibrational Spectroscopy 123: 103460.
5. Moulydia F, Salsabila N, Dewi RK, Nirmala A, Bismo S (2018) Burn drug made from ozonated vegetable oil mixture with white tumeric and cassava leaves extract. MATEC Web of Conferences 154: 04002.
6. Ugazio E, Tullio V, Binello A, Tagliapietra S, Dosio F (2020) Ozonated oils as antimicrobial systems in topical applications. Their characterization, current applications, and advances in improved delivery techniques. Molecules 25: 334.
7. Sadowska J, Johansson B, Johannessen E, Friman R, Broniarz-Press L (2008) Characterization of ozonated vegetable oils by spectroscopic and chromatographic methods. Chem Phys Lipids151: 85-91.
8. (2008) Adolfo Lutz Institute (São Paulo). Physicochemical methods for food analysis / coordinators Odair Zenebon, Neus Sadocco Pascuet and Paulo Tiglea -- São Paulo: Instituto Adolfo Lutz.
9. (2023) ABIHPEC Microbiology GUIDE (Brazilian Association of the Toiletries, Perfumery and Cosmetics Industry). 1. ed. http://abihpec.org.br/guia- microbiologia/files/assets/basic-html/index.html#1 acesso 22 de novembro de 2023.
10. Adusei EBA, Adosraku RK, Oppong-Kyekyeku J, Amengor CDK, Jibira Y (2019) Resistance Modulation Action, Time-Kill Kinetics Assay, and Inhibition of Biofilm Formation Effects of Plumbagin from Plumbago zeylanica Linn. J Trop Med 2019: 21250645.
11. Díaz MF, Sánchez Y, Gómez M, Hernández FC, Veloso MCP et al. (2012) Physicochemical characteristics of ozonated sunflower oils obtained by different procedures. Grasas y Aceites 63: 466-474.
12. Kowalska-Krochmal B, Dudek Wicher R (2021) The Minimum Inhibitory Concentration of Antibiotics: Methods, Interpretation, Clinical Relevance. Pathogens 10: 165.
13. Rodrigues KR, Cardoso CC, Caputo LR, Fiorini JE, Schneedorf M (2004) Cicatrizing and antimicrobial properties of an ozonised oil from sunflower seeds. Inflammopharmacology 12: 261267.
14. Gerrer LV, Cunha KC, Nogueira MCL, Cardoso CC, Soares MMCN, et al. (2012) "In vitro" antifungal activity of ozonized sunflower oil on yeasts from onychomycosis. Brazilian Journal of Microbiology 43: 1315-1318.
15. Stauffer E, Dolan JA, Newman R (2008) Gas Chromatography and Gas Chromatography-Mass Spectrometry. Fire Debris Analysis 235-293.
16. Akkaya MR (2018) Fatty acid compositions of sunflowers (Helianthus annuus L.) grown in east Mediterranea region. Revista Italiana Delle Sostanze Grasse 95: 239-247.
17. Díaz MF, Hernández R, Martínez G, Vidal G, Gómez M (2006) Comparative study of ozonized olive oil and ozonized sunflower oil. Journal of the Brazilian Chemical Society 17: 403-407.
18. Amri Z, Dbeibia A, Ghorbel A, Mahdhi A, Znati M, et al. (2020) Physico-chemical Characterization and Antibacterial Activity of Ozonated Pomegranate Seeds Oil. Ozone: Science & Engineering 42: 531-538.
19. Cirlini M, Caligiani A, Palla G, Ascentiis A, Tortini P (2012) Stability Studies of Ozonized Sunflower Oil and Enriched Cosmetics with a Dedicated Peroxide Value Determination. Ozone-science & Engineering – Ozone Sci Eng 34: 293-299.
20. Slavinskienė G, Ivanauskas L, Juodžentė D, Ramanauskienė K, Daunoras G, et al. (2024) A Comparative Study of the Chemical Properties and Antibacterial Activity of Four Different Ozonated Oils for Veterinary Purposes. Vet Sci 11: 161.

21. Martínez SG (2021) Scientific rational for the medical application of ozonized oils, an up-date. Ozone Therapy Global Journal 11: 239-272.

22. Amri Z, Ben Hamida S, Dbeibia A, Ghorbel A, Mahdhi A, et al. (2020) Physico-chemical Characterization and Antibacterial Activity of Ozonated Pomegranate Seeds Oil. Ozone: Science & Engineering 42: 531-538.