Comparative Analysis of *In Vitro* Antibacterial Effectiveness of Ozonized and Non-Ozonized Vegetable Oils on *Neisseria gonorrhoeae*

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Abstract: The bacterium *Neisseria gonorrhoeae*, responsible for bacterial infection known as gonorrhea, have become a public health problem because of its increasing therapeutic resistance. To evaluate the antibacterial effectiveness of ozonized vegetable oils and non-ozonized oils (sunflower, coconut, palm and olive) on *Neisseria gonorrhoeae*. The standard strain of *Neisseria gonorrhoeae* ATCC 49226 was evaluated against ozonized and non-ozonized sunflower, coconut, palm and olive oils. The antibacterial activity of the oils, in concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.562% and 0.781, was obtained by disk diffusion and broth microdilution. Positive controls comprised ceftriaxone and ciprofloxacin, and negative controls, dimethylsulfoxide and Tween 20. The minimum inhibitory concentration and minimum bactericidal concentration were evaluated. The experimental design was completely randomized. Ozonized sunflower oil showed greater bactericidal action, followed by olive and palm oils. Multivariate clustering approach made it possible to confirm that sunflower oil was more effective against *Neisseria gonorrhoeae*, followed by palm and olive oils, and the coconut oil was the least efficient. In the comparative analysis of the antibacterial effectiveness of ozonized vegetable oils on *Neisseria gonorrhoeae*, although the palm and olive oils present antibacterial activity, the ozonized sunflower oil showed high efficiency in reduced minimum inhibitory concentration, and thus has the potential to be a promising treatment against gonorrhea.

Keywords: *Neisseria gonorrhoeae*, Bactericidal agents, Ozone, Oxigen-reactive species, Alternative treatment.

1. INTRODUCTION

Neisseria gonorrhoeae or *gonococcus* is a bacterium, gram-negative diplococci responsible for the sexually transmitted disease known as gonorrhea. This infection is considered a serious public health problem, due to its ability to develop bacterial resistance to major antibiotics, currently the subject of extensive research [1-3].

In its initial treatment, the substances used are: ceftriaxone, cefixime, ofloxicin and ciprofloxacin, all of them in a single-dose [1,4]. However, indiscriminate use of antibiotics has resulted in the emergence of micro-organisms with multiple resistance to conventional drugs, making it difficult to control and thus favoring the spread of the disease [5,6].

Due to bacterial resistance, many studies were developed using vegetable oils and ozone for therapeutic purposes, which are indicated as promising alternative treatments for the resistant strains, including Gram-negative bacteria such as *Neisseria gonorrhoeae* [7-9].

In this context, the aim of this study was to assess the antibacterial activity of ozonized sunflower, coconut, olive and palm oils, and determine which of these oils is more effective in the treatment of "*in vitro*" *Neisseria gonorrhoeae*.

2. MATERIALS AND METHODS

2.1. Study Design

The *Neisseria gonorrhoeae* strain was studied in microbiology laboratory at Universidade Brasil with completely randomized design. The treatments of *N. gonorrhoeae*, consisted of two groups of vegetable oils "one group of ozonized and non-ozonized oil" (not ozonated or *in natura*, it means control group).

2.2. Vegetable Compounds

The vegetable oils used were: sunflower oil (Liza®), olive oil (Borges®), coconut oil (COPRA®) and palm oil (Cepera®), bought at natural product markets. The oils

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used are food products, whose physical-chemical quality has not been evaluated.

2.3. Ozonation of Vegetable Oils

Ozone was produced by a corona generator (Ozon & Life) and taken by a silicone tube to the diffuser, and pure oxygen was introduced via the oxygen cylinder. One liter of each oil was exposed to ozone directly through the diffuser for 6 hours in a controlled temperature of 25°C, according to the methodology described by Kozusny-Andreani [10]. All ozonation procedure was conducted in a fume hood (Quimis model 216.11).

The same procedure was used for the olive oil and palm oil. The coconut oil, before the ozonation, was incubated at a temperature of 67°C for 20 minutes to complete liquefaction and ozonation was conducted for 10 hours.

After ozonation, 0.1 mL of each oil was taken and inoculated on Petri plates containing trypticase soy agar, incubated at 37°C for 24-48 hours. It was considered a sterile oil those that showed no colony.

The ozonized oils were stored in amber bottles and kept under-cooling (8°C), controlled by a fume thermometer, with the temperature being measured and recorded daily in the morning.

2.4. Bacterial Strain

It used a standard strain of *Neisseria gonorrhoeae* ATCC 49226 (American Type Culture Collection), and reactivated in Thayer-Martin Agar (Oxoid®) and incubated under anaerobic conditions for 24 hours at 37°C.

2.5. Inoculum Preparation

Inocula were prepared by taking three to four colonies of the strain of *N. gonorrhoeae* isolated in Thayer-Martin Agar, being inoculated in Brain-Heart Infusion Broth (BHI, Oxoid®), and incubated under anaerobic conditions for 24 hours at 37°C. Subsequently, the suspension was diluted in saline solution (0.85% NaCl) until reaching the turbidity corresponding to the 0.5 MacFarland scale [11], equivalent to the concentration of $1,5x10^8$ CFU/ml. This bacterial suspension constituted the inoculum for evaluation of antibacterial oils by disk diffusion techniques of minimum inhibitory concentration, minimum bactericidal concentrations and bacterial survival.

2.6. Preliminary Evaluation of the Antibacterial Activity of *in natura* and Ozonized Vegetable Oils

The preliminary tests were performed by disk diffusion technique following the Kirby-Bauer method [12], and broth dilution for standardization of the methodology. All oils showed antibacterial activity and were used for further experiments.

2.7. Disk Diffusion Method

Initially, in the disc diffusion method, the bacterial suspension was inoculated (in duplicate) with the aid of a disposable swab over the entire surface of Petri plates containing Mueller-Hinton Agar (Oxoid®). The next step was to add dry sterile filter paper discs of 6mm diameter with no antimicrobial agents (Whatman - type 3), which were uniformly distributed, with a distance among them up to 25mm, avoiding interactions among the oils. With the aid of micropipettor, 10 µL of each oil concentration (100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.562% and 0.781) were transferred to the discs, dissolved in dimethyl-sulfoxide (DMSO, Sinth®) and 0.1% Tween 20 (Sigma-Adrich®). The experiment was conducted in triplicate.

In order to proof test the disk diffusion, positive controls consisted of disks containing ceftriaxone (30µg, CT0417- Oxoid®) and ciprofloxacin (10mg, CT1615- Oxoid®) were used, while the negative controls were prepared with 10 µL of 0.1% Tween 20.

The plates were inverted and incubated at 37°C for 24 hours under anaerobic conditions. After this period, growth inhibition halos formed around the discs were measured with a millimeter ruler. The halo equal to or larger than 10 mm in diameter was considered susceptible and halos with diameters between 5 and 10mm were considered as moderate susceptibility [13].

2.8. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Testing

By using the broth microdilution method, 100 µL of Brain-Heart Infusion Broth was distributed in the cavities of the microplates. In the first ones, 100 µL of oils was added, obtaining a dilution of 50% (1/2). Subsequently, serial dilutions were performed in six subsequent wells, removing 100 µL from the higher concentration well, until obtaining a concentration of 0.781% (1/128). Thus, the obtained serial dilutions corresponded to 50% (1/2) 25% (1/4), 12.5% (1/8) 6.25% (1/16) 3.125% (1/32), 1.562% (1/64) and 0.781% (1/128).

Bacterial suspensions of *Neisseria gonorrhoeae* ATCC 49226 in Brain-Heart Infusion Broth (0.5 MacFarland scale) were added to each cavity with oils dilutions (in triplicate). The experiment was controlled by a series of dimethylsulfoxide dilution with bacterial suspensions and oils without bacterial suspensions similar to those mentioned (positive control) and in the negative control, the viability of the strain was observed, inoculated with the bacterial suspension in the Brain-Heart Infusion Broth; tests were also conducted in triplicate.

The microplates were incubated at 37°C for 24-48 hours in anaerobic conditions. At the end of this period, minimum inhibitory concentration was determined, defined as the lowest concentration of antibiotic agent capable of inhibiting bacterial growth.

The presence of viable bacterial cells in non-inhibitory concentration was determined by adding 50 µL of 2,3,5 -Triphenyltetrazolium Chloride staining. It was then possible to distinguish the live samples, which were colored red, from those that are dead retaining their color [14,15].

To determine the minimum bactericidal concentration, an aliquot (100 µL) of the minimum inhibitory concentration and two previous and one subsequent to this concentration, besides the positive control, was inoculated by the use of Thayer-Martin Agar, with no antimicrobial agents performed in triplicate. After incubation at 37°C for 24 hours under anaerobic conditions, the bacterial growth was evaluated, and the minimum bactericidal concentration was the lowest concentration of the oil which had a negative subculture or average of 0.1 CFU [16].

2.9. Survival Curve of *Neisseria gonorrhoeae* using *in natura* and Ozonized Oils

Once the minimum inhibitory concentration is determined, it was used to assess the growth of bacteria in the presence of oils as a function of time. The survival curve was established in accordance with the methodology described by Sforcin *et al*. [17] in order to observe the incubation period of *Neisseria gonorrhoeae* ATCC 49226 in the different oils. The assays were developed in triplicate.

Thus, bacterial suspensions of *Neisseria gonorrhoeae* ATCC 49226 in the Heart-Brain Infusion Broth (0.5 MacFarland scale) were inoculated into Brain-Heart Infusion Broth along with the oils in concentrations corresponding to the minimum inhibitory concentration previously obtained. Then, every 15 minutes for a period of four hours incubation (37°C under anaerobic conditions), 100µL aliquots of each culture were removed and plated onto Thayer-Martin Agar. After 24 hours incubation at 37°C in anaerobic conditions, the colony forming units were counted (CFUs.).

2.10. Data Analysis

The data were submitted to descriptive analysis of the minimum inhibitory concentrations and bactericidal of each of the ozonated and non-ozonized oils. The Mann-Whitney test and the Kruskal-Wallis test were used to analyze the percentage variation (%) of the microbial count in relation to the evaluated plant extracts. Statistical tests were applied with a 5% significance level ($P < 0.05$).

3. RESULTS

3.1. Results of the Univariate Analysis

Taking into consideration the results in Table **1**, it is possible to suggest that the inhibition halos of ozonized oils were greater in size when compared to the inhibition halos of oils that did not undergo ozonation.

However, the difference between the measures of the halos was not significant ($P > 0.05$). Thus, the effect of oil ozonation to optimize microbial inhibition did not produce a significant effect.

We could determine the minimum inhibitory and minimum bactericidal concentrations from the measurements of microbial inhibition halos. The minimum bactericidal concentration was applied in each oil in order to observe the survival of *Neisseria gonorrhoeae* in times that vary between 0 and 105 minutes of exposure.

In order to evaluate the time required to reduce the microbial load to null values, microbial counts were analyzed according to their respective percentage changes in each study period. The period related to the analysis of the microbial count ranged from 0 to 105 minutes. The percentage variation of the microbial count consisted of the following relationship:

$$Microbial\ Count_{0-10\,\min}(\%) = \frac{(Count_{10\,\min} - Count_{0\,\min})}{Count_{10\,\min}} \times 100$$

According to the formula above, negative changes show a decrease in microbial counts and positive

Table 1: Mean of Inhibition Halos (mm) for each of the Concentrations Tested

Concentration of vegetable oils (%)	Oils							
	Sunflower	OZ Sunflower	Olive	OZ Olive	Palm	OZ Palm	Coconut	OZ Coconut
100	16.67	24.33	7.00	11.33	16.67	28.00	11.67	16.00
50	16.33	19.00	5.00	8.00	13.67	25.33	8.00	10.00
25	15.00	18.00	3.00	6.00	10.00	20.33	5.00	5.00
12.5	12.67	16.00	0.00	5.00	8.33	14.67	0.00	0.00
6.25	8.33	11.33	0.00	0.00	5.33	10.33	0.00	0.00
3.125	5.00	8.00	0.00	0.00	3.00	8.00	0.00	0.00
1.563	3.00	4.00	0.00	0.00	0.00	5.00	0.00	0.00
0.781	0.00	2.67	0.00	0.00	0.00	3.00	0.00	0.00
0.390	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.195	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MIC (%)	3.125	1.563	25	6.25	3.125	1.563	25	25
MBC (%)	6.25	3.125	25	6.25	6.25	1.563	100	25
Mean (Median)	7.70 (6.67)	10.33 (9.66)	1.50 (0.00)	3.03 (0.00)	5.70 (4.17)	11.47 (9.16)	2.47 (0.00)	3.10 (0.00)
Valor P[1]	0.520		0.477		0.220		0.962	

[1]P-value related to the Mann-Whitney test P <0.05. P: Probability OZ (Oil ozonized); MIC (Minimum Inhibitory Concentration); MBC (Minimum Bactericidal Concentration).

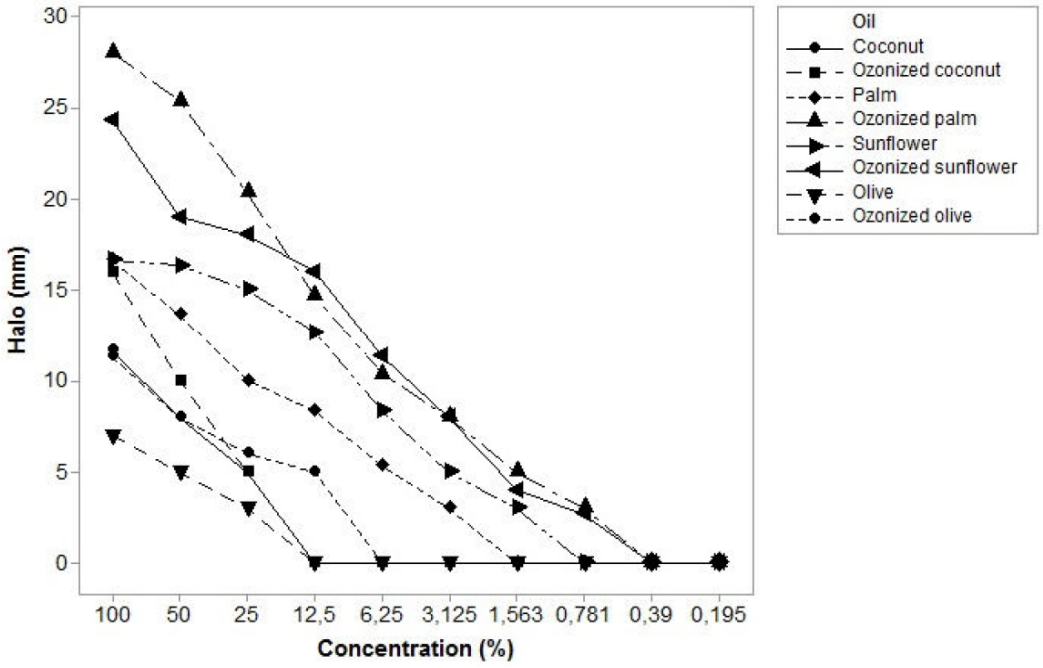

Figure 1: Inhibition halos (mm) for each concentration and evaluated oil, ozonized or non-ozonized.

variants show an increase in microbial counts throughout the evaluated time.

Two types of results are shown in Table **2**, one of them shows the existence of significant differences (P=0.006) in the variation of the microbial count when all the oils are compared. Thus, the multiple comparison test showed that the difference is between ozonized sunflower oil, which presented the highest reduction of microbial load, and the sunflower, olive and coconut oils, because they had the lowest reduction of microbial load. In this context, the

Table 2: Descriptive Statistics of Percentage Change (%) of the Microbial Count in Relation to the Vegetable Extracts Evaluated

Oil	N	Mean±SD	Median (Md)[1]	P-value[2]
Sunflower	15	-86.19±16,89	-88.00 b	0.014
Ozonized Sunflower	6	-100.0±0,01	-100.0 a	
Olive	21	-81.64±24,46	-91.67 b	0.011
Ozonized Olive	12	-96.60±3,09	-96.90 ab	
Palm	18	-91.67±6,31	-91.83 ab	0.187
Ozonized Palm	12	-95.10±5,92	-97.30 ab	
Coconut	21	-80.41±21,68	-88.33 b	0.413
Ozonized Coconut	18	-89.88±8,30	-92.15 ab	
P-value[1]	0.006			

[1]P-value relating to the Kruskal-Wallis test P<0.05. Different letters in the same column differ significantly by the Dunn's multiple comparison test P<0.05. [2]P-value related to the Mann-Whitney test P<0.05. **P:** Probability and **N**: Numbers plates valued at each oil.

ozonized sunflower oil showed the greatest effectiveness in reducing microbial load.

When the same type of oil was compared between the control and ozonized treatments, we noted that the ozonization procedure had only a positive effect in reducing the microbial load for sunflower (P=0.014) and olive oils (P=0.011). For both oils mentioned, the reduction of microbial load was significantly higher when we used the ozonized oil in comparison to the control oil control (non-ozonized). For palm and coconut oils, ozonation had no significant effect on reducing microbial load. Thus, despite the reduction of the microbial load was higher in all cases using the ozonized oils, such reduction was significant only for the sunflower oil and olive oil.

The results in Table **2** and Figure **2** show that the use of the ozonized sunflower oil was extremely effective to reduce the microbial load of *Neisseria gonorrhoeae*, since this microorganism showed null load in just 30 minutes of contact with the oil.

3.2. Multivariate Approach Results

The results indicate the formation of five different clusters: cluster 1 formed by reducing microbial load obtained by sunflower oil; cluster 2 formed by ozonized sunflower, olive and palm oils; cluster 3 formed by ozonized coconut and palm oils; cluster 4 formed only by olive oil and cluster 5 formed by coconut oil. Oils belonging to cluster 2 showed greater reduction in microbial load, being considered the most effective

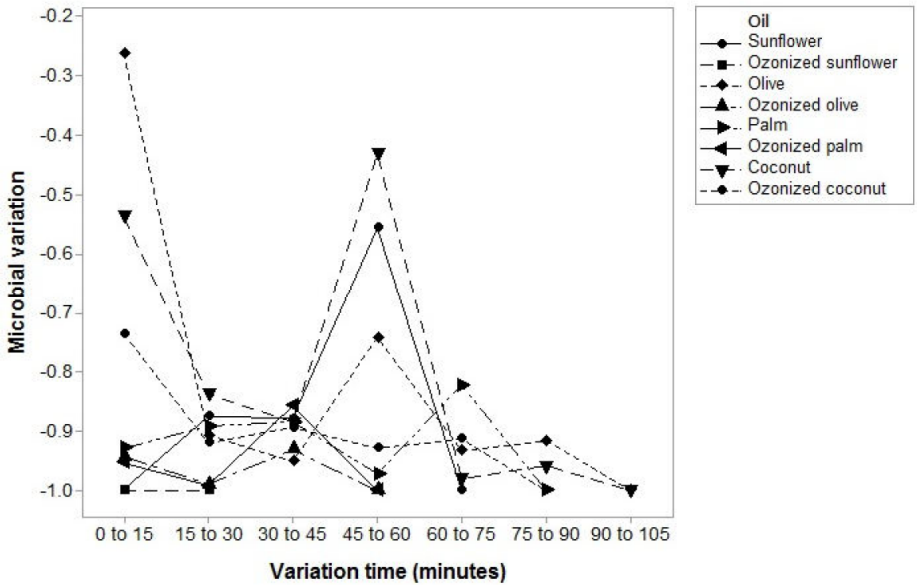

Figure 2: Percentage change of microbial count in relation to the studied oils.

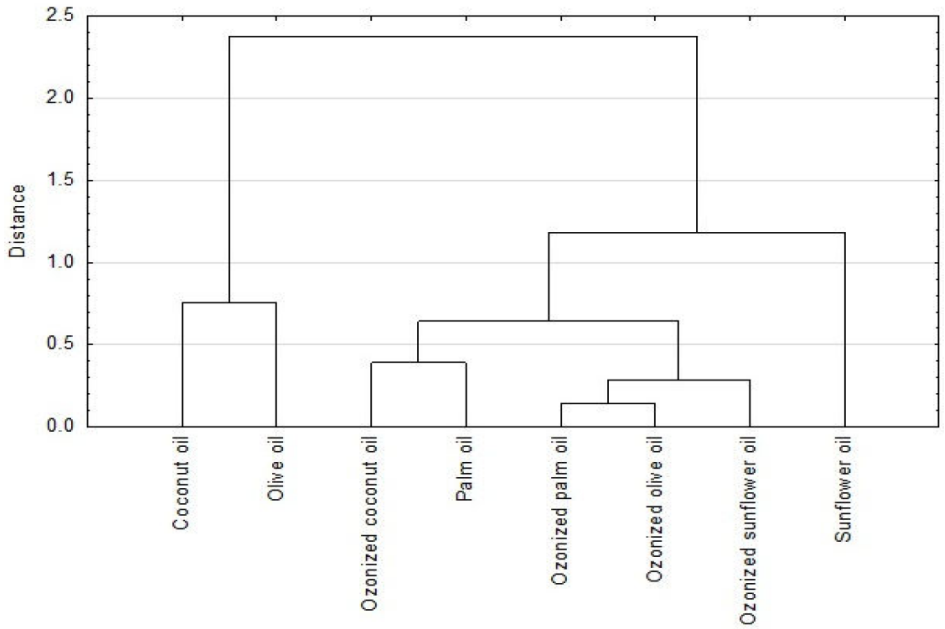

Figure 3: Cluster analysis of the evaluated oils in relation to the reduction of microbial load.

ones. The oils from cluster 3 showed the greatest reduction after the oils from cluster 2, followed by olive oil (cluster 4), coconut oil (cluster 5) and sunflower oil (cluster 1). In this context, we could note that not only the ozonized sunflower oil showed great efficacy in reducing microbial load as seen by the results in univariate analysis (Table **2**), however, besides the ozonized sunflower oil, it was possible to observe the relevant efficacy of ozonized palm and olive oils, therefore, they were also considered effective on reducing *Neisseria gonorrhoeae* load.

4. DISCUSSION

The mechanisms of antibiotic resistance in *Neisseria gonorrhoeae* are diverse, and the emergence and spread of resistant strains is a threat to global public health. In this context, surveillance and monitoring of the antimicrobial sensitivity of *N. gonorrhoeae* should be continuous to reduce the effect of antimicrobial resistance on antibiotic therapy for gonorrhea [6].

Different aspects, in addition to sensitivity to antibiotics, are considered in the therapeutic decision, as the pharmacokinetic characteristics of the agent, toxicity, convenience of administration, cost and potential effectiveness of the agent for concomitant infections [18]. These factors, along with the high incidence of adverse reactions to current antibiotics used to treat gonorrhea, are urging the search for alternative sources, such as some species of plants, whose secondary metabolites are considered potential antibacterial drugs [19].

Medicinal plants in the form of extracts and vegetable oils, such as the oils used in this study, are used worldwide for the treatment of various infectious diseases including gonorrhea [20-23].

Tabassum and Vidyasagar [24], using the agar diffusion method, they found that sunflower vegetable oil was efficient in controlling Escherichia coli, Trichophyton rubrum and Candida albicans, with minimum inhibitory concentrations ranging between 0.62 and 40 mg/ml. The authors state,that vegetable oils may be used for the treatment of dermatological diseases. In our study, sunflower oil presented antimicrobial activity against *N. gonorrhoeae*, with minimum inhibitory concentration of 3.125%, minimum bactericidal concentration of 6.25% and a survival time of these bacteria of 75 minutes in this oil.

Boukhebti *et al*. [25] also found that olive oil has exercised effective antibacterial activity against Staphylococcus aureus, Pseudomonas aeruginosa and Citrobacter freundii with low inhibitory concentration; however, it did not show any antibacterial activity against Escherichia coli and Bacillus subtilis. Our results demonstrated that olive oil showed moderate antibacterial activity against *N. gonorrhoeae*, with both minimum inhibitory concentration and minimum bactericidal concentration of 25%, and survival of cells of 105 minutes, while the minimum inhibitory

concentration and minimum bactericidal concentration of the palm oil were 3.125% and 6.25% with a survival time of 90 minutes. According to Mohamed [26], the palm oil showed broad antibacterial and antifungal activity, especially against Staphylococcus aureus and Candida albicans.

The results obtained by the agar diffusion method, minimum inhibitory concentration, minimum bactericidal concentration and the survival test of *N. gonorrhoeae* showed that coconut oil showed a slight antibacterial activity, conflicting with *in vitro* studies by Verna *et al*. [27] on the antimicrobial properties of coconut mesocarp on Salmonella typhi and Escherichia coli, that showed efficacy in the control of these bacteria, as well as their potential application as pharmaceutical products.

Another alternative antibacterial method is to use ozone. This is an antimicrobial agent that inactivates the microorganisms by reaction with intracellular enzymes, the nucleic material and with the cell envelope components, resulting in oxidation processes effective against resistant microorganisms [28-30].

Ozonized solutions show bactericidal effect against several microorganisms. According to Peretyagin *et al*. [31], the bactericidal activity of the ozone on Proteus, Staphylococcus aureus, Pseudomonas, Streptococcus and Neisseria cultures was observed after 24 hours of incubation in the presence of ozonized olive oil. In our study, we used ozonized vegetable oils and found that ozonation of sunflower, palm and olive oils enhanced the antibacterial activity against *N. gonorrhoeae*, which did not occur with the coconut oil.

Schwartz [32] proved the effectiveness of ozonized solutions in the treatment of recurrent Candida albicans vulvovaginitis in patients resistant to the usual drug treatment . They were employed to ozonized water, ozone gas and ozonized olive oil (Oleosan®). During the study, a synergistic action of ozone treatment in its different application forms was observed (ozonized water, ozone gas and ozonized oil) with adopted measures of nutrition. There was a reduction of symptoms and negative results of cultures and vaginal studies. Similar results were obtained by Tara *et al*. [33] with the effectiveness of ozonized olive oil on clinical candidiasis symptoms. The authors state that olive oil can be suggested as an effective topical treatment for patients.

In the present study, the ozonized sunflower oil showed antimicrobial effectiveness against *in vitro Neisseria gonorrhoeae* with minimum inhibitory concentration of 1.563%, minimum bactericidal concentration of 3.125% and a survival time of the bacteria of 30 minutes.

5. CONCLUSION

In this study, the ozonized palm and olive oil effectively presented antibacterial activity against *Neisseria gonorrhoeae*, however, the higher efficiency was confirmed with the ozonized sunflower oil, due to the low values of the minimum inhibitory concentration of 1.563% and minimum bactericidal concentration of 3.125%, respectively showing the beginning of growth inhibition of Neisseria during ozonation and elimination of the bacteria in the shortest time interval between the ozonized oils analyzed, proving to be a promising alternative treatment for gonorrhea. However, *in vivo* studies are needed.

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DECLARATION OF POTENTIAL CONFLICT OF INTEREST

The authors declare no conflict of interest.

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