In vitro Antibacterial Activity of *Mauritia flexuosa* and *Carapa guianensis* Oils and *Menta piperita* and *Eugenia caryophyllus* Essential Oils Added to Ozone

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Article

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Abstract: Ozonated oil has several benefits for humans, as it contributes to numerous interventions, maintaining the quality of life and the well-being of individuals. Being a natural product, it has few or almost no side effects. Thus, the study aimed to verify the sensitivity and/or resistance of fixed o "Ozonated" and "Non-Ozonated" oils from *Mauritia flexuosa* (Buriti), *Carapa guaianensis* (Andiroba) and essential "Ozonated" oils from *Menta piperita* (Mint), *Eugenia caryophyllus* (Clove) in different concentrations, against internationally known bacteria from the American Type Culture Collection – ATCC, comparing with the "Non-Ozonated" versions of the respective oils, using the Agar diffusion technique. Thus, it is evident that the ozonation of the fixed oils of Buriti and Andiroba, as well as the essential oils of Mint and Clove, provided significant results, although they were statistically different. The Buriti and Andiroba oil were ineffective in inhibiting microorganisms. On the other hand, the essential oil of Mint and Clove obtained similar activity of inhibition of microorganisms. With regard to Ozonation, the Essential Oil of "Ozonated" Clove provided greater activity than the "Non-Ozonated" one, which inhibited only one tested microorganism, in addition to producing changes in its chemical composition, requiring individualized studies for the ozonation of each essential oil.

Keywords: antibacterial activity; chemical analysis; essential and fixed oils; ozonation

1. Introduction

Although the contagiousness of many diseases has been established, humans have always used substances to fight infections, as human history attests, from 2500 BC to 3000 BC, when the Chinese, Hindus, Babylonians, Sumerians, and Egyptians used medicinal plants and their derivatives to fight infections, in addition to products of animal and/or mineral origin. However, it was only from the 16th century, with the development of "alchemy", that medicines began to be obtained by laboratory methods, but even before these, there were oils which are classified today as vegetable or fixed and essential, where they were already used to combat various diseases and infections (OLIVEIRA, 2022).

Vegetable oil is a source of energy produced by plants and is found in greater concentration in seeds and fruits. The majority (approximately 95%) of vegetable oils are composed of triglycerides. The remaining 5% are phospholipids, glycolipids, sulpholipids, waxes, hydrocarbons such as squalene, pigments in the form of carotenoids and chlorophyll, vitamin E, polyphenols and

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triterpenoids. The non-triglyceride fraction is called the "unsaponifiable fraction" (SARKIC; STAPPEN, 2018).

Essential oils are small, nonpolar lipophilic molecules, with volatility as one of their main characteristics. It receives this name because it has the "essence" of the olfactory characteristics of the plant from which it is extracted. They are usually extracted by steam distillation, and can be synthesized in different parts of the plant such as buds, flowers, leaves, stems, branches, seeds, fruits, roots, wood or bark. However, they can also be extracted by other methods such as hydrogenation and pressure diffusion (GUIDONI et al., 2019).

Both oils are derived from plants, but there are major differences between them, one of the main ones being the composition. Vegetable oils contain a high concentration of fatty acids in their composition, due to their properties, vegetable oils are used as emollients, which aim to hydrate and smooth the surface of the skin. There are no regulations regarding their use, but these ingredients are often used in high concentrations, depending on the product. Essential oils, on the other hand, have a high concentration of volatile molecules (mainly terpenes), which is why they are mainly used as fragrances in low concentrations (BRUNO; ALMEIDA, 2021).

Another widespread use of essential oils today is in aromatherapy, and can be utilized for their other properties, with anti-inflammatory, antibacterial and calming effects (GUIDONI et al., 2019; BRUNO; ALMEIDA, 2021).

Ozone, on the other hand, has its use described for the treatment of various pathologies, among which it can be cited as an example: Autoimmune, respiratory, dermatological, gastrointestinal, ophthalmological, cardiovascular and neurological diseases (ISCO3, 2020)

Ozonated vegetable oils are well tolerated by biological tissues and the germicidal activities of ozone are attributed to its ability to destroy bacterial membranes and the viral capsid through direct oxidation of phospholipids and lipoproteins. This causes changes in the chemical structure of the cell and inhibits exchange with the environment and causes disintegration of the cell envelope causing cell lysis and death (Aghaei et al., 2019; Basile et al., 2019). Thus, they have bactericidal and fungicidal properties and favor their use topically for skin infections (Moureu et al., 2015).

Due to the instability of ozone and its toxicity, vegetable oils have proven to be very useful in ozone therapy and are enjoyed more safely (RICCO; AQUINO JÚNIOR, 2022; LESCURA; BEGA, 2020). Thus, this article aims to evaluate the sensitivity and/or resistance of the fixed oils of Buriti and Andiroba and essential oils of Mint and "Ozonated" and "Non-Ozonated" Clove in different concentrations against internationally known bacteria from the ATCC collection using the agar diffusion technique.

2. Methods

Investigation studies of the antibacterial activity of vegetable oils from *Mauritia flexuosa* (Buriti) and *Carapa guaianensis* (Andiroba) and essential oils from *Menta piperita* (Peppermint) and *Eugenia caryophyllus* (Cravo) were carried out at the Bioprocess Laboratory of the Federal Institute of Tocantins, Campus Araguaína – TO.

The methodology used in this research was experimental and comparative, between ozonated and non-ozonated fixed and essential oils, in 05 concentrations tested in triplicates, on 04 bacterial strains, totaling 960 observations. The flowchart below represents the treatment performed for each oil tested in each microorganism.

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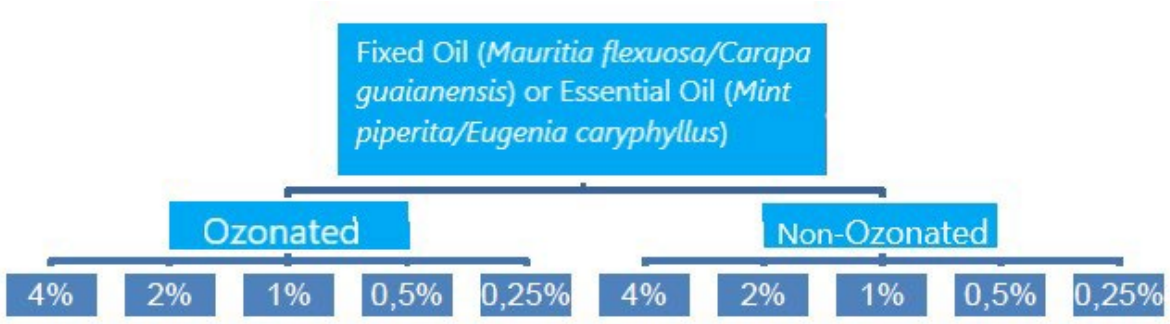

Figure 1. Flowchart of the Fixed or Essential Oil Processing Methodology that were executed for the 4 types of oils used in the microbiological analyzes of the 4 microorganisms. Source: Author.

2.1. Vegetable oils and essential oils

Vegetable oils from Mauritia flexuosa (Buriti) and Carapa guaianensis (Andiroba seed) and essential oils from Menta piperita (Mint) and Eugenia caryphyllus (Clove) were selected for analysis according to the vast literature reported for antimicrobial activity, as well as the use of these items (CARVALHO; ASSIS; SANTOS, 2020; SOARES, 2020), and purchased from companies specialized in selling these items, as shown in the table below, with batches of known origin and technical report, with specificities that prove their physical-chemical characteristics within the acceptability standard for oils according to the literature and legislation (Chart 1).

The vegetable oils, after acquisition, were stored at room temperature, in a cool place, with a maximum value of 24°C, while the essential oils were stored under refrigeration until use, being placed at the laboratory room temperature 30 minutes before the procedures of chemical and antimicrobial analysis **Chart 1:** Selected vegetable oils and essential oils with batch specifications and origin.

Chart 1. Selected vegetable oils and essential oils with batch specifications and origin.

Plant	Oil type	Batch	Supplier	Batch chemical specifications	Reference values of the oil.
Carapa guaianensis Albu. f. (Meliaceae)	Vegetable	135	FERQUIMA	- Appearance: Viscous Liquid; - Color: Yellow; - Impurities: Exempt; - Density (20º): 0.919; - IR* (20th) = 1.4665; - AGL/FFAa = 10.6%; - Iodine Index**: 64.41. - Saponification Index***: 195;	- Appearance = Viscous Liquid (may become pasty at low temperatures; - Color = Straw Yellow to Brown; - Impurities = Exempt; - Density (20º): 0.900 – 0.950; - IR* (20th): 1.460 – 1.470; - AGL/FFAa: Maximum 30%; - Iodine Index**: 50-70

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					- Saponification Index***: 180-200;
Mauritia flexuosa L. f. (Arecaceae)	Vegetable	111	FERQUIMA	- Appearance: liquid; - Color: Reddish yellow; - Impurity: exempt; - Density (20º): 0.9090; - IR* (20th): 1.4658; - Iodine Index**: 67.09; - Saponification Index***: 200	- Appearance: liquid; - Color: Reddish yellow; - Impurity: exempt; - Density (20th): 0.890 – 0.925; - Income Tax* (20th): 1.450 – 1.475; - Iodine Index**: 62 – 80; - Saponification Index***: 180 – 210
Eugenia caryophyllus Spreng. f. (Myrtaceae)	Essential	226	FERQUIMA	- Appearance: Clear liquid; - Yellow color; - Impurity: exempt; - Odor: Characteristic; - Density (20th): 1.0545; - IR*: (20th): 1.5337; - Optical Rotation: -0.4ºº	- Appearance: Clear liquid; - Color: Light yellow to Brown; - Impurity: exempt; - Odor: Characteristic; - Density (20th): 1.020 – 1.060; - IR*: 1.527 – 1.540; - Optical Rotation: -1.3º; +1.5º
Menta pipertita L. f. (Lamiaceae)	Essential	208	FERQUIMA	- Appearance: Clear liquid; - Color: Straw yellow; - Impurity: exempt; - Odor: Characteristic; - Density (20º): 0.9031; - IR*(20th): 1.4614; - Optical Rotation = -24.80º	- Appearance: Clear liquid; - Color: Colorless to Straw yellow; - Impurity: exempt; - Odor: Characteristic; - Density (20th): 0.890 – 0.911; - Income Tax*(20th): 1.459 – 1.465; - Optical Rotation: -32º; -18º

*IR: Refractive Index; ** (g I_2/100g); *** (mg KOH/g); [a] – FFA/FFA: Free Fatty Acid/free fatty acids; ****CAS No.: Chemical Abstracts Service. Source: Supplier data (2022).

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2.2. Ozonation of Oils

The vegetable oils of *Mauritia flexuosa* (Buriti) and *Carapa guaianensis* (Andiroba) and essential oils of *Menta piperita* (Mint) and *Eugenia caryophyllus* (Cravo) received ozonation through diffusion of O_3, administered directly into the oil as described below.

Ozone production was carried out using a generator device, white, model MOG003, from the brand Ozone Generator, which produces electrical discharges under medicinal O2 (Corona effect), coming from an industrial cylinder, from the brand AIR GÁS Health Solution, with specifications UN 1072 oxygen, compressed 2.2 (5.1) and concentration grade 99.5% vol./vol, which supplies the device, releasing O3 at a concentration of 20 µg/ml, according to the specifications of the equipment supplied by the manufacturer, converts medical Oxygen into Ozone.

The rich O_3 gas continuously produced by the device at a rate of ½ liters per minute is conducted through a silicone tube to a diffuser, which is immersed in vegetable oil or essential oil and perfused for 30 minutes. The temperature of the vegetable oils during perfusion was controlled at 25°C with an ultrathermotized bath. As for essential oils, it was perfused with ozone at a temperature of 5°C in order to reduce the loss by evaporation of essential oils.

2.3. Antimicrobial Activity

2.3.1. Bacterial Strains and Culture Medium

The antimicrobial assay was conducted with Gram-negative and Gram-positive bacteria in standardized strains from an international collection, according to the methodology of sensitivity tests for antimicrobial agents by dilution for aerobic growth bacteria (RENTES, 2022; BATISTA, 2008).

The bacteria, Table 1, were acquired from the Tropical Culture Collection of the André Tosello Foundation – FAT with authenticity and procedure reports (Annex B) and kept in the Bioprocesses laboratory of the IFTO Campus Araguaína – TO, as recommended by the FAT, in medium of Muelle Hinton culture. For the tests, the suspensions of the bacteria used came from the first passage (repeat) of the strains received by the IFTO laboratory.

Table 1. Strains of bacteria used in antimicrobial assays.

CCT	Microorganism	Reference	NB[1]	Batch
1457	*Escherichia coli*	ATCC 25922	1	T07/05/C
1476	*Pseudomonas aeruginosa*	ATCC 27853	2	T15/08J
1486	*Staphylococcus aureus subsp. Aureus*	ATCC 29213	2	T04/04/H
4295	*Staphylococcus aureus subsp. Aureus*	ATCC 6538	2	T03/02/A

Source: Lineage Catalog, Tropical Culture Collection – CCT, Tropical Research and Technology Foundation. André Tosello, 1st Ed., 1996.

The culture medium used was Mueller Hinton Agar, brand ION, Lot 0719/0354, the compositions of which is: Beef Infusion 300 g/L; Hydrolyzed acid casein 17.50g/L; Starch 1.5g/L; Agar 17g/L and pH 7.3 +- 0.2.

The preparation of the medium was carried out according to the instructions described by the manufacturer, in a concentration of 38 grams of the medium for 1 liter of distilled water. With the medium dissolved, it was sterilized in an autoclave at 121°C for 15 minutes and then taken for microbiological analysis.

2.3.2. Microbiological analysis of fixed and essential oils

[1] NB = Biosafety Level required for handling the strain

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The use of "in vitro" antimicrobial activity tests is a usual practice, standardized by Kirby and Bawer since 1966, being reviewed and modified by several authors. Currently, standardized methods are adopted by several organizations such as: NCCLS – National Committee for Clinical Laboratory Standards, such as "Methods for Determining Bacterial Activity of Antimicrobial Agents; Approved Guideline", standard M26-A, ISBN 1-56238-384-1 by James H. Jorgensen, 1999, which was followed by making minor adaptations as discussed below.

An aliquot of vegetable oil and essential oil was solubilized in a 1:1 solution of Dimethylsulfoxide (DMSO) in water and subsequently diluted, respectively obtaining a concentration of oils of 4, 2, 1, 0.5 and 0.25%. These diluted strata were submitted to microbiological tests, in sterile plates of 20x150mm, where 1mL of saline solution was deposited, containing microorganisms in suspensions (saline solution at 0.85% NaCl), standardized by tube 0.5 on the Mac Farland scale, set to 90% Transmittance (530nm). Then, 50mL of Mueller Hinton Agar solid medium was added per plate, prepared according to the manufacturer's standards (ION), melted at 50ºC, using the "Pour Plate" technique, homogenized by the "eight" technique (MC GINNIS, 1980; BAUER et al., 1966). The microbiological analysis process in its entirety was carried out in a BSTEC exhaust hood, intending to mitigate the dangers of ozone gas aspiration, thus following international safety standards (KUME, 2020).

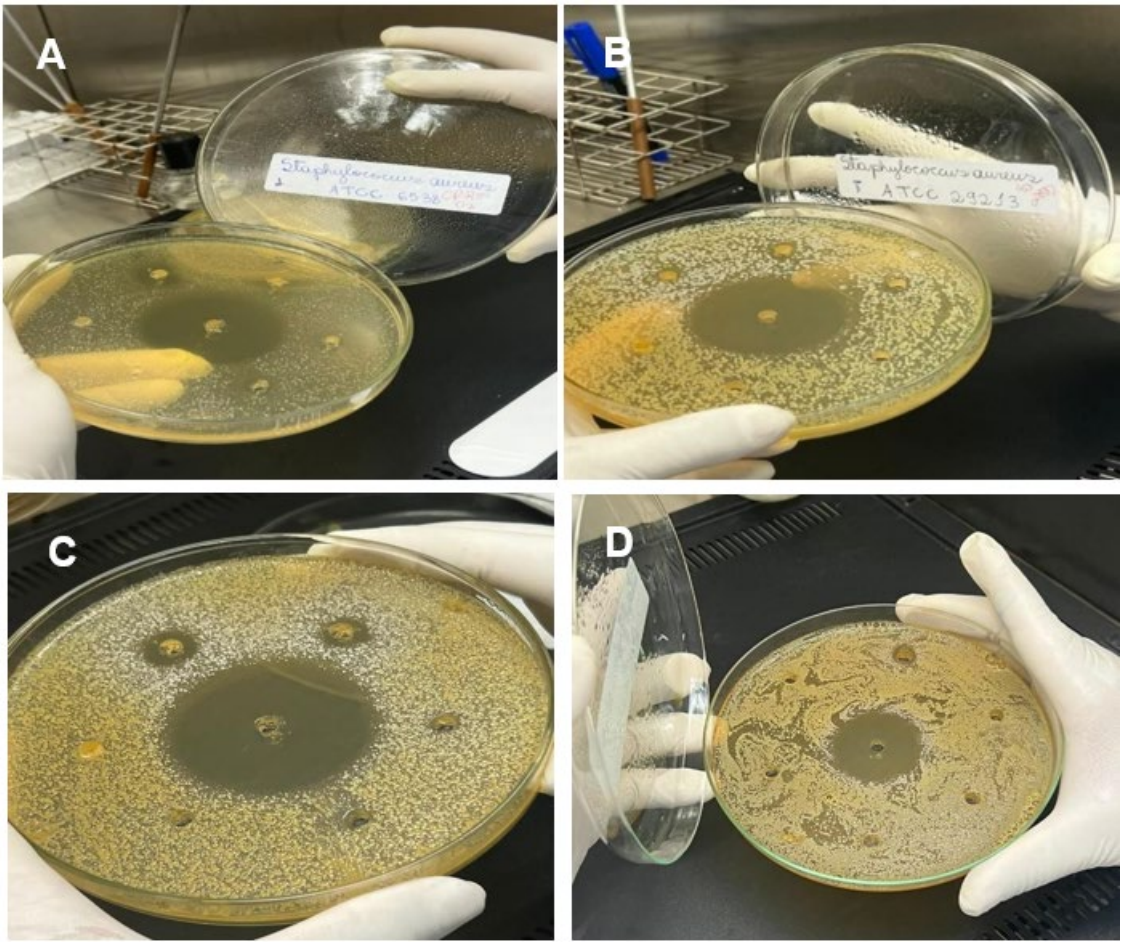

Figure 2. Plates with antimicrobial assays of microorganisms tested with fixed and essential oils. A – Microrganismo Staphylococcus Aureus ATCC 6538; B – Staphylococcus aureus ATCC 29213. C - Escherichia coli ATCC 25922; D- Pseudomonas aeruginosa ATCC 27853. Source: Author (2022).

2.4. Chemical Analysis of "Ozonated" and "Non-Ozonated" Essential Oils

The analyzes were carried out using a Gas Chromatography with Mass Spectrometer (GC-MS) apparatus, at the Chemistry Laboratory of the Federal University of Norte do Tocantins, Campus

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Araguaína – TO. The technique was performed in triplicate to obtain the identification of the major constituents in the essential oils of clove and mint and their centesimal composition.

For this purpose, from clove and mint oils, both ozonated and non-ozonated, a 5mg/mL solution in ethyl acetate was prepared. These samples were analyzed on an Agilent Technologies 7890B Gas Chromatograph hyphenated to a 5977B Mass Spectrometer (GC-MS). The chromatograph operated with HP-5MS capillary columns (5% Phenyl Methyl Siloxane), L = 30 m, ID = 0.25 mm and film = 0.25 µm and the carrier gas used was Helium (99.999%) with flow of 1.2 mL/min. The GC-MS was used with injector temperature at 270 ºC, transferline at 250 ºC, quadrupole at 150 ºC and source at 230 ºC, with purge flow at 100 mL/min.

The analysis mode was Split in "scan" mode with a split ratio of 80:1 and a scan from 40 to 500 Da. The GC oven temperature program was initially set at 40 °C for 2 min and increased by 5 °C per minute to 170 °C, followed by an additional 25 °C per minute to 270 °C, which was maintained in isotherm for 3 minutes. In total the races lasted 35 minutes.

Figure 3. Gas Chromatograph with Mass Spectrometer (GC-MS), Federal University North of Tocantins (UFNT). Source: Author (2022).

The mass spectra obtained were analyzed using Agilent software, MSD ChemStation F.01.03.2357 and compared with spectra from libraries: NIST 2014, NIST WEBBOOK and Adams (2017), along with the similarity index. The retention indices (RI) were determined using a homologous series of n-alkanes, C7 to C30, injected in the same method as the samples, using the equation of Van den Dool and Kratz (1963).

3. Results

3.1. Antimicrobial Activity

The design of this work consisted of an assessment of the sensitivity and/or resistance to fixed oils of Buriti and Andiroba and essential oils of Mint and Clove - "Ozonated" and Not "Ozonated" - in different concentrations against internationally known bacteria from the ATCC collection using the agar diffusion technique. Statistical analysis by ANOVA (analysis of variance) was significant for all possible variables used, according to PR>HR values reported in chart 1. Thus, it demonstrates that there are means with identical or approximate behaviors within the events, suggesting that the oils in the respective concentrations, ozonated or not, must be analyzed two by two, using the Tukey Test, also called Studentized Amplitude Test (MOTTA, 2006).

Chart 2. Analysis of Variance of fixed oils of Buriti and Andiroba and essential oils of Mint and Clove "Ozonated" and "Non-"Ozonated" in concentrations of 4, 2 1, 0.5 and 0.25%, against bacteria tested.

Variance Factors	GL	SQ	QM	Fc	Pr>Fc

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Ozonation	2	292,7	146,39*	13,5	<0,001
Plant	4	2449,2	612,3*	56,7	<0,001
Concentrations	5	2682,7	536,5*	49,7	<0,001
Bacterium	4	668,6	167,2	15,5	<0,001
Ozonation x Plant	2	1076,1	538,1*	49,9	<0,001
Error	898	9690,4	10.8		
Total Corrected	963	17916,85			

Source: (ANOVA, Tukey, P<0.05, CI:95%). If * significant at 5%. GL: Degree of Freedom, QM: Mean Square, Fc: Correction Factor, Pr>Fc: F calculated less than the tabulated critical value, there will be no significant difference between treatments. CV: 207.93%; Overall Average: 1.5799; Number of observations; 960.

The results revealed that the process of ozonation of the fixed oils of Buriti and Andiroba did not produce additional synergistic effects inherent to the inhibition of the tested bacteria. However, the essential oils of Peppermint and Clove showed statistically inverse results in the face of the ozonation process, shown below in Chart 3, thus confirming that each oil has a different behavior in the face of the ozonolysis process.

Chart 3. Results of antimicrobial sensitivity of fixed oils from buriti fruit and andiroba seed and essential oils from clove and mint efflorescence.

	Ozonated					**Non- Ozonated**				
	Carapa guaianensis Albu. (Andiroba oil) Mean ± Standard Deviation (CIM)	*Mauritia flexuosa L.* (buriti oil) Mean ± Standard Deviation (CIM)	*Eugenia caryophyllus spring.* (Clove essential oil) Mean ± Standard Deviation (CIM)	*Mentha piperta L.* (Peppermint essential oil) Mean ± Standard Deviation (CIM)		*Carapa guaianensis Albu.* (Andiroba oil) Mean ± Standard Deviation (CIM)	*Mauritia flexuosa L* (Buriti – fruit oil) Mean ± Standard Deviation (CIM)	*Eugenia caryophyllus* spreng. (Clove essential oil) Mean ± Standard Deviation (CIM)	*Mentha piperta* L. (Peppermint essential oil) Mean ± Standard Deviation (CIM)	Chloramphenicol 30µg/ml
Staphylococcus aureus ATCC 6538	-	-	18,8 ± 2,93^{Aa} (1%)	11,8 ± 0,98^{Ca} (2%)		-	-	-	13,3 ± 0,98Bab (4%)	31,4 ± 2,81[1]
Staphylococcus aureus	-	-	14,0 ± 4,00^{Aab} (1%)	11,2 ± 0,17^{Aa} (2%)		-	-	-	12,3 ± 1,86^{Ab} (2%)	34,0 ± 4,26[1]

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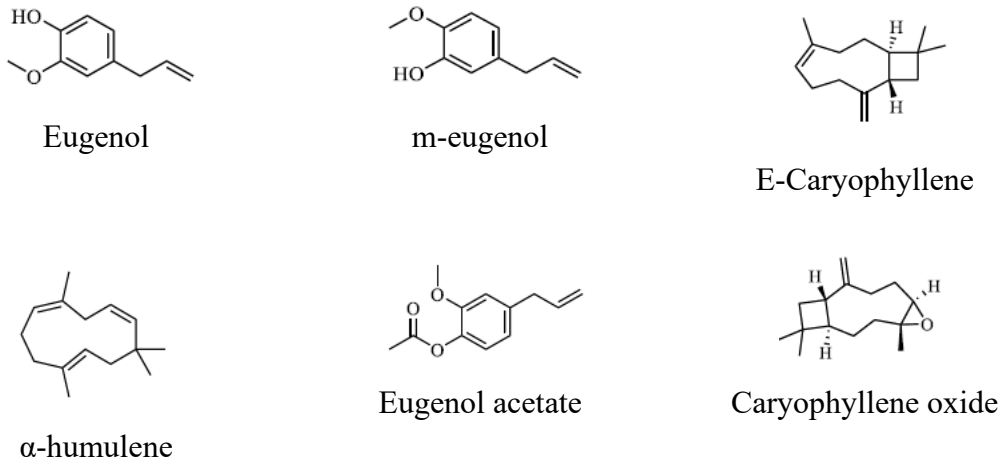
ATCC 29213										
Escherichia coli ATCC 25922	-	-	10,83 ± 0,98^{Bb} (4%)	11,2 ± 0,98 ^{Ba} (2%)		-	-	10,0 ± 0,71^{Ba} (4%)	19,67 ± 6,66 ^{Aa} (2%)	27,4 ± 2,68[1]
Pseudomonas aeruginosa ATCC 27853	-	-	-	-		-	-	-	-	25,4 ± 2,11[1]

[1]All positive results were significant when using the T test in comparison with Chloramphenicol, called positive control, including dilutions ($p<0.05$; CI:95%). Assays for the diluting liquid (solvent) did not demonstrate inhibition of the microorganisms tested. Means followed by the same lowercase letter in the column and the same uppercase letter in the row do not differ statistically, at a 5% probability level using Tukey's test.

3.2. Chemical Analysis of Essential Oils

3.2.1. Chemical Analysis of Clove Essential Oil

The identification of chemical compounds in samples of Clove essential oils -"Ozonated" and "Non-Ozonated" - was identical. It was carried out by GC-MS, obtaining the major compounds: Eugenol; m-eugenol; eugenol acetate; E-caryophyllene; α-humulene and caryophyllene oxide.



Source: ChemDraw Professional 16.0.

Figure 4. Chemical structure of the major compounds contained in "Ozonated" and "Non-Ozonated" Clove essential oils.

In Figure 5, the comparison chromatogram of Clove "Non-Ozonated" essential oils, is displayed, which is shown in black and "Ozonated" represented in blue. In addition, they have the indication of the major compounds.

Black: "Non-Ozonated"; Blue: "Ozonated"

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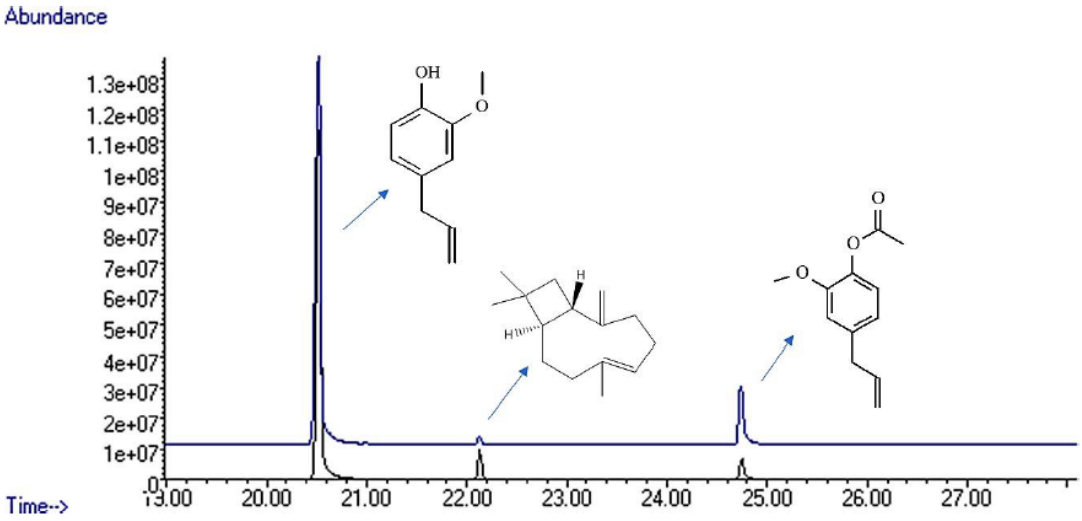

Source: Author (2022).

Figure 5. – Comparison chromatogram of Clove essential oils - "Ozonated" and "Non-Ozonated" - with indication of identified compounds.

After being recognized, the compounds were integrated into the chromatograms area, for the detection of each one of them by peaks in the graph (Figure 5), in order to identify the quantity and centesimal proportion of each chemical compound belonging to the essential oils of "Ozonated" and "Non-Ozonated Clove. The analysis determined that the essential oils have different proximate compositions, that is, ozonation modifies the composition of the essential oil, as shown in Table 2

Table 2. Identification of compounds present in "Ozonated" and "Non-Ozonated" Clove essential oils.

No.	TR	Component	IR exp	IR lit	Average % of peak areas		Ref.
					OCO	OCI	
1	20.529	Eugenol	1361	1356	85,35±0,98	86,32±1,35	Adams (2017)
2	21.002	m-eugenol	1379	-	0,56±0,33	0,44±0,06	Adams (2017)
3	22.134	E-Caryophyllene	1423	1417	1,18±0,11	5,79±0,16	Adams (2017)
4	22.987	α-humulene	1457	1452	-	0,30±0,03	Adams (2017)
5	24.749	Eugenol acetate	1530	1521	11,72±0,52	7,01±1,10	Adams (2017)
6	26.111	Caryophyllene oxide	1588	1582	0,29±0,04	0,13±0,01	Adams (2017)

No.: ordering of compounds by elution; TR: compound retention time; IRexp: Experimental retention index; IRlit = Literature Retention Index; %: Percentage area of compounds based on area normalization by GC-MS; OCO: Clove oil "Ozonated"; OCI: "Non-Ozonated" Clove Oil; Ref: reference.

When comparing the average peak areas of the "ozonated" and "non-ozonated" essential oil, which corresponds to the centesimal analyzes of each oil, it is observed that they have different proportions which, when compared by the T test, confirms the differences in the amount of each compound, as shown in Table 3.

Table 3. T-test result for compounds present in Clove essential oil "Ozonated" and "Non-Ozonated".

Compound	T.stat	p value	- log10(p)	FDR
E-Caryophyllene	40.601	2.1992e-06	5.6577	1.3195e-05
α-humulene	16.472	7.9536e-05	4.0994	0.00023861
Caryophyllene oxide	-7.4066	0.00177728	2.7513	0.0035457

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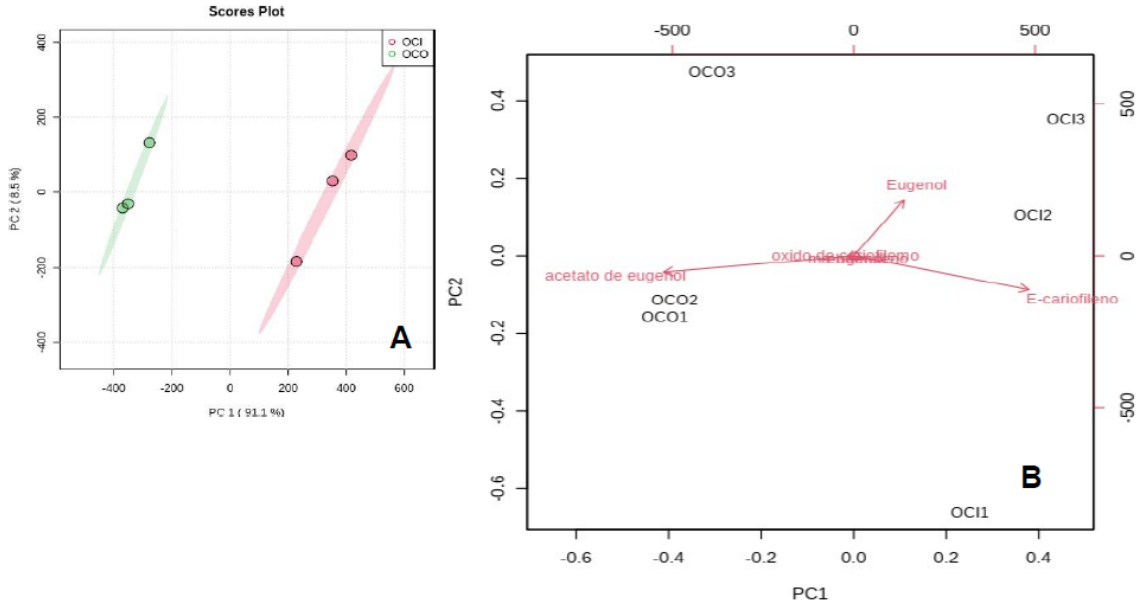
Eugenol acetate	-6.6728	0.0026214	2.5815	0.0039321

Source: Research results (2022).

The amount of eugenol and m-eugenol compounds did not statistically change with ozonation. The multivariate analysis of the main components - PCA showed that the essential "Ozonated" oil is statistically different from the "non-ozonated" when we observe all the compounds present, as shown in Figure 6 below.

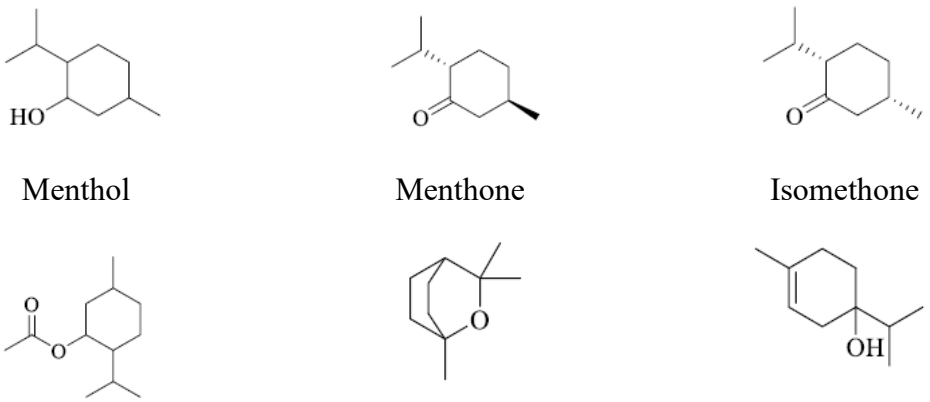


Source: Author (2022).

Figure 6. – Results of the analysis of the main components - PCA of Clove essential oil -"Ozonated" and "Non-Ozonated". Figure A and B respectively: Score of the values between the selected compounds with extended variance values and Plot of the central dispersion graph of the principal component analysis - PCA.

3.2.2. Chemical Analysis of Peppermint essential oil.

The amount of chemical compounds identified in the samples of "Ozonated and "Non-Ozonated" Peppermint oils was identical, according to analyzes carried out by GC-MS. The compounds were identified: Menthol; Menthone; Isomentone; Menthyl acetate; 1,8-cineol; terpinen-4-ol; α-thujene; Sabinene; trans-sabinene hydrate; iso-menthol; neomenthol acetate; isomenthol acetate; β-bourbonene; E-Caryophyllene and Germacrene D, whose structures are shown below:



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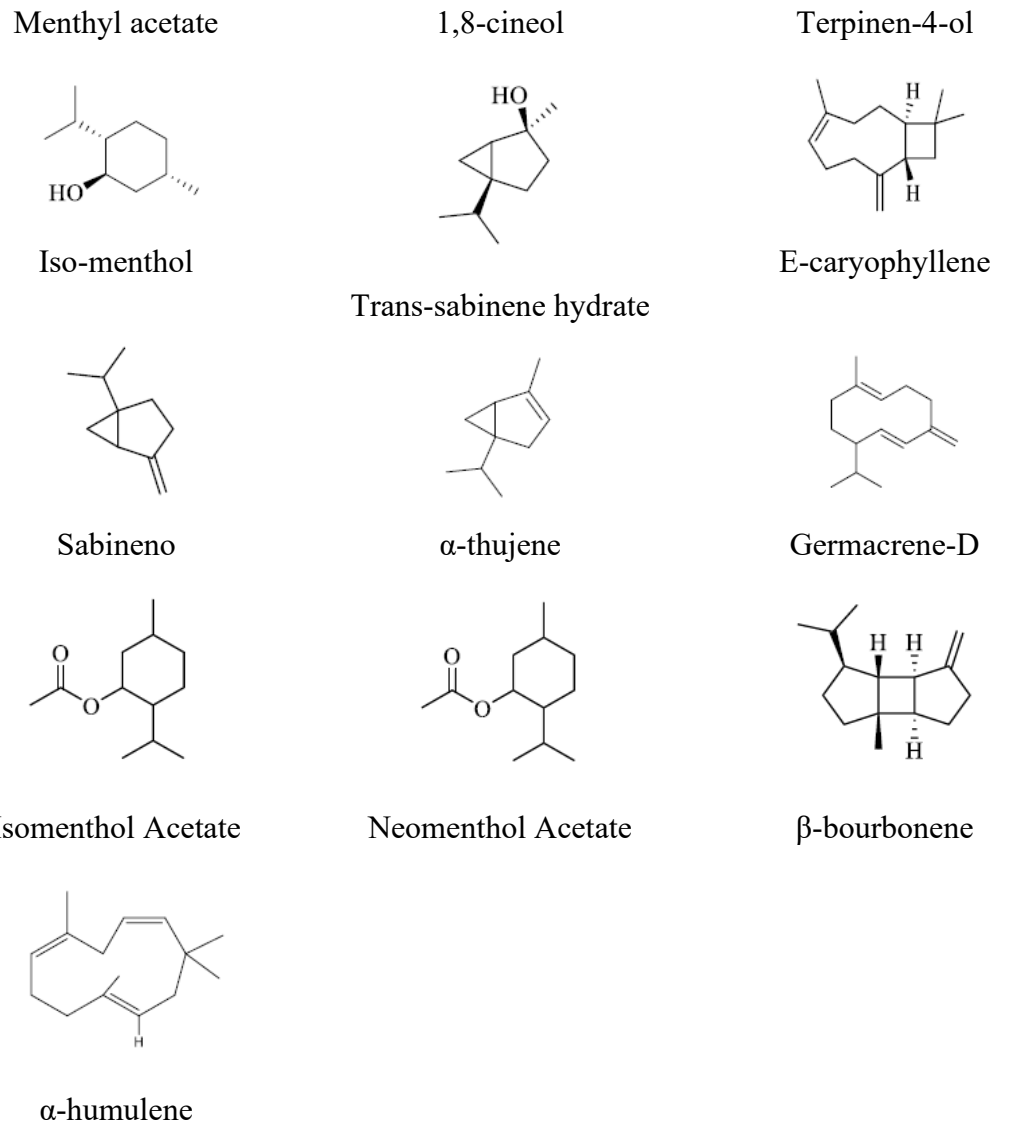

Source: ChemDraw Professional 16.0.

Figure 7. Chemical structure of the major compounds contained in "Ozonated" and "Non-Ozonated" Peppermint essential oils.

In Figure 8, the comparison chromatogram of the essential oils of Peppermint "Non-Ozonated" is displayed, which is shown in black and "Ozonated" represented in blue, in addition, they have the indication of the major compounds.

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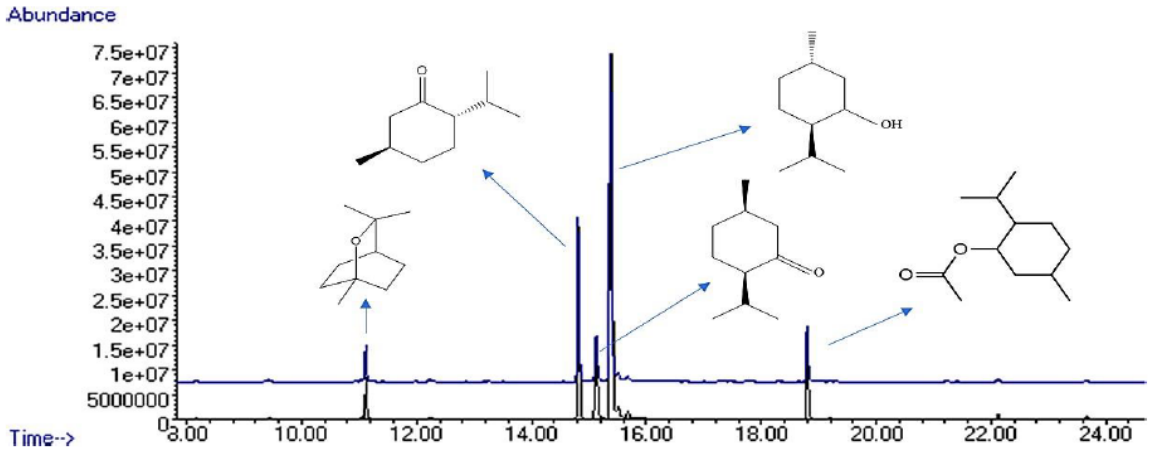

Source: Research results (2022).

Figure 8. Comparison chromatogram of "Ozonated" and "Non-Ozonated" Peppermint Essential Oils with indication of identified compounds.

Once identified, the compounds were integrated into the chromatogram area, identifying the peak area of each compound in order to identify the amount, centesimal proportion, of Ozonated and non-Ozonated mint essential oil. The analysis determined that the mint essential oils have different proximate compositions, that is, ozonation modifies the composition of the essential oil. As shown in Table 4.

Table 4. Identification of compounds present in "Ozonated" and "Non-Ozonated" Peppermint essential oils.

No.	TR	Component	IR exp	IR lit	Average % of peak areas		Ref.
					OCO	OCI	
1	8.160	α-thujene	925	924	0,20±0,02	0,20±0,01	Adams (2017)
2	9.437	Sabineno	970	969	0,29±0,02	0,31±0,02	Adams (2017)
3	11.114	1,8-cineol	1027	1026	4,64±0,31	4,57±0,14	Adams (2017)
4	12.230	trans-sabinene hydrate	1065	1065	0,44±0,04	0,41±0,01	Adams (2017)
5	14.815	Menthone	1153	1148	21,37±0,12	20,27±0,04	Adams (2017)
6	15.130	iso-menthone	1164	1158	7,60±0,17	9,86±0,13	Adams (2017)
7	15.385	Menthol	1173	1167	54,43±0,95	53,03±0,40	Adams (2017)
8	15.510	terpinen-4-ol	1177	1174	2,13±0,06	2,23±0,02	Adams (2017)
9	15.679	iso-menthol	1183	1179	1,00±0,03	1,01±0,01	Adams (2017)
10	18.289	neomenthol acetate	1277	1271	0,13±0,01	0,11±0,01	Adams (2017)
11	18.804	menthyl acetate	1295	1294	6,89±0,30	6,61±0,16	Adams (2017)
12	19.195	isomenthol acetate	1310	1304	0,16±0,01	0,14±0,01	Adams (2017)
13	21.236	β-bourbonene	1388	1387	0,11±0,02	0,11±0,00	Adams (2017)
14	22.124	(E)-Caryophyllene	1423	1417	0,44±0,03	0,73±0,04	Adams (2017)
15	23.661	germacrene D	1485	1480	0,19±0,01	0,41±0,02	Adams (2017)

No.: ordering of compounds by elution; TR: compound retention time; IRexp: Experimental retention index; IRlit = Literature Retention Index; %: Percentage area of compounds based on area normalization by GC-MS; OMO: ozonated mint oil; OMI: non-ozonated peppermint oil; Ref: reference.

When comparing the average of the peak areas of the essential oil of Peppermint "Ozonated " and "Non-Ozonated" Peppermint, which corresponds to centesimal analyzes of the ozonated and

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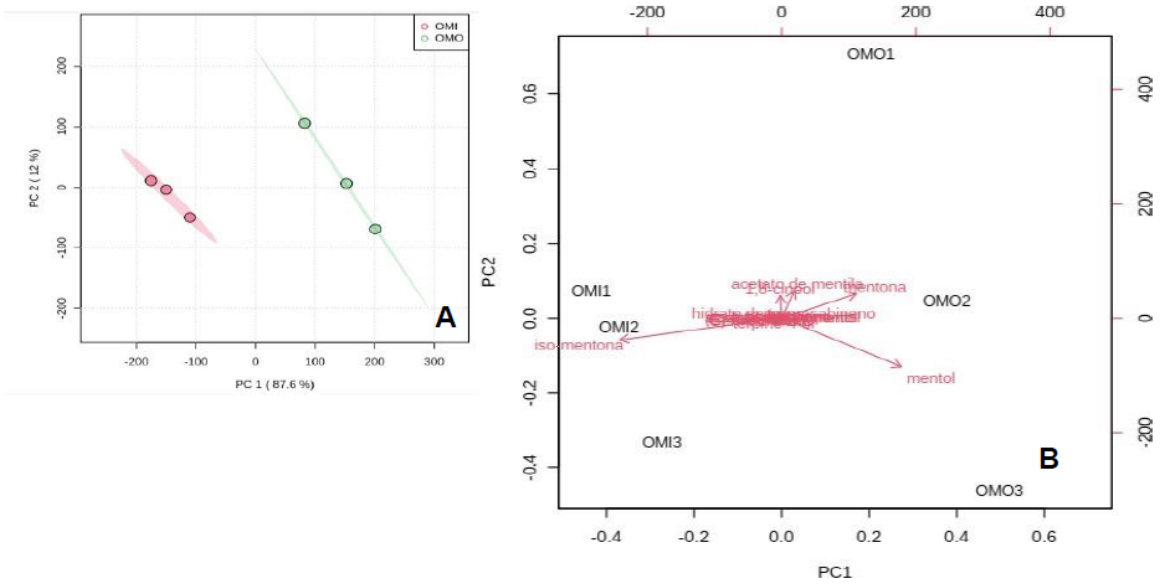
non-ozonated oil, it is observed that they have different proportions which, when compared by the T test, confirms the differences in the amount of each compound, according to Table 5.

Table 5. T-test result for compounds present in "Ozonated" and "Non-Ozonated" Peppermint essential oil.

Compound	T.stat	p value	-log10(p)	FDR
Iso-Menthone	17.943	5.6705e-05	4.2464	0.00052658
germacrene D	17	7.0211e-05	4.1536	0.00052658
Menthone	-15.126	0.00011137	3.9532	0.00055684
(E)-caryophyllene	10.325	0.00049649	3.3041	0.0018618

Data: Test results.

The amount of menthol compounds, 1-8 cineol and other compounds not described in the table above did not statistically change with ozonation. The multivariate analysis of the main components - PCA showed that the essential "Ozonated" oil is statistically different from the "Non-Ozonated" of Mint, when all the compounds present are verified, as shown in Figure 9.



Source: Research results (2022).

Figure 9. Results of principal component analysis - PCA of "Ozonated" and "Non-Ozonated" Peppermint essential oil, Figure A - Score of values between selected compounds, with extended variance values, Figure B - Plot of dispersion graph central component analysis - PCA.

4. Discussion

A priori, fixed Buriti oil was ineffective in inhibiting microorganisms, which corroborates a study carried out by Nunes et al. (2021), which aimed to determine the antimicrobial activity of fixed oils extracted from native fruits of Maranhão cerrado, where Buriti oil did not obtain satisfactory inhibition against pathogenic microorganisms *Escherichia coli, Staphylococcus aureus*; however, Nunes et al. (2021) explain that this oil may have antimicrobial activity against other pathogenic microorganisms, becoming antimicrobial agents.

In relation to Andiroba fixed oil, it also did not show any inhibitory activity against the microorganisms tested in this study and similar studies were not found in the literature related to this study.

The ozonation of the fixed oils did not provide antibacterial activity by the tested methodology, demonstrating that the ozone added to the oil may have dissipated during the dilution process of the

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fixed oils with the solvent used, since the proportion of this without ozone is greater than 96% by volume, significantly diluting the fixed ozone.

The essential oils of "Ozonated" and "Non- Ozonated" Clove showed similar inhibition activities of the microorganisms tested, however the ozonation of the oil showed greater activity in comparison to the non-ozonated one, showing inhibition capacity against 03 microorganisms, while the non-ozonated oil inhibited only one microorganism tested. Thus, the "Ozonated" essential oil of Clove was more effective to inhibit the growth of Staphylococcus aureus ATCC 6538 and Staphylococcus aureus ATCC 29213, which has statistically similar behavior, while it obtained less efficiency for *Escherichia coli* ATCC 25922. Results similar to those presented in the research are from Baima et al. (2017), Mahendran and Rahman (2019), Badea et al. (2019) and Haro-Gonzáles et al. (2021).

Baima et al. (2017) conducted a study to evaluate the antimicrobial activity by determining the minimum inhibitory concentration (MIC) of clove essential oil (Syzygium *aromaticum*) against strains of *Escherichia coli* – ATCC 25922 and *Staphylococcus aureus* – ATCC 25923 and, as a result, it was obtained that the essential oil of Cloves has antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. By determining the minimum inhibitory concentration (MIC), a MIC of 100 μg/mL was obtained for *Escherichia coli* and 200 μg/mL for *Staphylococcus aureus*.

Thus, reinforcing the result of this article, Haro-Gonzáles et al. (2021) explain that non-Ozonated Clove essential oil can inhibit gram-negative bacteria (*E. coli, Salmonella, Klebsiella pneumoniae, Erwinia carotovora, Agrobacterium, and Pseudomonas aeruginosa*) and gram-positive bacteria (*S. aureus, Streptococcus, and L. monocytogenes*), and Aspergillus fungi (*A. flavus, A. parasiticus, and A. ochraceus*), *Penicillium*, *C. albicans*, and yeast. It has also been observed that Clove essential oil inhibits Gram-positive bacteria to a greater extent than Gram-negative bacteria. This is attributed to a diffusible mucopeptide layer in Gram-positive bacteria that makes them susceptible to antimicrobial agents. In contrast, the complex lipopolysaccharide layer on the outer cell membrane of Gram-negative bacteria can significantly reduce the rate of diffusion of lipophilic antibacterial compounds across the cell membrane (Behbahani et al. 2019), as these pathogens have shown greater sensitivity to oil than probiotics and fungi (Shahbazi, 2019).

After the "Ozonated" and "Non-Ozonated" Clove essential oils are analyzed using the GC-MS method and compared in their respective chemical compositions, it is identified that both have the same major compounds; however, it is clear that the process of ozonation alters the centesimal composition of all of them, which may be a justifying factor in the difference in behavior in view of the inhibition of the analyzed bacterial strains.

As for the "Ozonated" and "Non-Ozonated" Peppermint essential oils, they statistically obtained the same activity for 03 of the 04 microorganisms tested (Staphylococcus aureus ATCC 6538, Staphylococcus aureus ATCC 29213 and Escherichia coli ATCC 25922). The "Ozonated" Peppermint oil had inhibition behavior for sensitive microorganisms and they were statistically similar in the formation of the inhibition halo and also in the minimum inhibitory concentration. The "Non-Ozonated" Peppermint oil had greater inhibition for *Escherichia coli* ATCC 25922, while for *Staphylococcus aureus* ATCC 6538, it obtained less efficiency, requiring a higher concentration (4%) of oil to inhibit this strain.

In a recent study, *Menta piperita* essential oil was observed as a potentiating agent for the antimicrobial action of ozone, with its synergistic activity at an inhibitory concentration of 2% of essential oil (Floare et al., 2023), corroborating the values obtained by the inhibition of the ozonated and non-ozonated essential oil of this study that varied between 2 to 4% for the same selected strains. The chemical analysis of the essential oil - "Ozonated" and "Non-Ozonated"- of Menta, has similar composition of oxygenated monoterpenes, but with different hundredths variations between the oils of this study in relation to the oil studied by Floare et al., 2023; which only analyzed the chemical composition of non-Ozonated oil. It appears that the proportion of Menthol present in the essential oil of the cited study is lower than the 60% of the one in this study, maintaining a similar proportion of Menthone. These 02 components represent 55% of the total composition of the essential oil in the

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study conducted by Floare, while in this study the proportion was 75% for the Ozonated oil compared to 73% for the non-Ozonated oil.

The results obtained by this study reinforce those expressed by Badea et al. (2019) who aimed to analyze the influence of peppermint essential oil (*Menta piperita*), absorbed on the surface of hydroxyapatite nanoparticles and its morphological, physicochemical and antimicrobial properties. Still in the study developed by Badea et al. (2019), they also investigated the antimicrobial activity against bacteria resistant to methicillin such as S. aureus (MRSA) 388, *S. aureus* ATCC 25923, and carbapenems *E. coli* C5 (carbapenemase-producing strain), as well as *S. aureus* ATCC 6538, *E. coli* ATCC 25922, which were also tested in this study, in addition to E. faecium DSM 13590. The *E. coli* strain ATCC 25922 in the Badea et al. (2019) presented the largest diameter of the inhibition halo with values between 20 and 22 mm, corroborating the largest inhibition halo found for *Menta piperita* oil at 4% with a mean value of 19.7 mm in this study. It should be noted that the authors' research did not use Ozonated essential oil.

Mahendran and Rahman (2019), explain in their study with Peppermint essential oil, that it showed antibacterial activity against different bacteria, including *E. faecalis*, *S. aureus* and *S. dysenteria* exposed the highest MIC (8.3 ± 0.2, 8.3 ± 0.1 and 5.8 ± 0.1 mg/ml) (NIKOLIC et al., 2014; BASSOLÉ et al., 2010). The study by Ceylan et al. (2014), demonstrated a maximum zone of inhibition against *Staphylococcus aureus* (15 mm) and *Pseudomonas aeruginosa* (15 mm) represented by in vitro disk diffusion/dilution method. Thus, the results showed that the higher concentration of oils produced larger inhibition halos in the inoculum, which helped in the process of identifying the minimum inhibitory concentration presented.

The highest concentration of oils produced in the inoculums larger halos of inhibition, helping in the process of identifying the minimum inhibitory concentration. None of the "Ozonated" and "Non-Ozonated" oils inhibited the microorganism Pseudomonas aeruginosa ATCC 27853. Thus, Peppermint essential oil has significant antimicrobial activity and ozonation can be a synergistic factor contributing to therapeutic and/or control antimicrobial needs

5. Final Considerations

It was evidenced in the study that Andiroba and Buriti oils in the "Ozonated" and "Non-Ozonated" versions were ineffective in inhibiting microorganisms.

On the other hand, the "Ozonated" Clove essential oil showed a better performance when compared to the "Non-Ozonated" version of the same, which may be related to the difference in the centesimal composition of the oils.

Peppermint essential oils, "Ozonated" and "Non-Ozonated", showed similar antimicrobial activities, and different chemical composition after the ozonation process, although the major constituents are oxygenated monoterpenes.

Thus, the ozonation of essential oils can contribute to the synergism of antimicrobial activity and there is a need for individualized study of each type of oil produced and the species of extraction.

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