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BASIC RESEARCH:

Antibacterial and Antifungal Effects of Ozonated Essential Oil of Sacha Inchi, Calcium Hydroxide, and the Combination of Both Against *Enterococcus Faecalis* and *Candida Albicans*: An *In Vitro* Study

Efecto antibacteriano y antifúngico del aceite esencial ozonizado de sacha inchi, hidróxido de calcio y la combinación de ambos frente al *Enterococcus faecalis* y *Candida albicans*: estudio *in vitro*

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ABSTRACT: The biggest challenge in root canal treatment is the elimination of microorganisms; therefore, new techniques are continually sought to achieve success. The agar diffusion method was used to determine the antibacterial and antifungal effect of ozonated Sacha Inchi essential oil, calcium hydroxide paste, and the combination of both. Brain heart infusion and sabouraud agar were the culture media for *Enterococcus faecalis* and *Candida albicans* respectively. The antibacterial and antifungal effects were determined using the inhibition halos that formed around the wells containing the drugs at 24 hours and 48 hours. From the study, the antibacterial and antifungal effects of the ozonized oil was greater after 6 hours, proportional to the higher concentration of peroxide. This study showed that the antibacterial effect of ozonated Sacha Inchi oil is superior to that of calcium hydroxide and the combination of both substances.

KEYWORDS: *Enterococcus faecalis*; *Candida albicans*; Sacha Inchi; Ozonated oil; Calcium hydroxide; Antibacterial effect; Antifungal effect.

RESUMEN: El desafío más grande en el tratamiento de conductos es la eliminación de los microorganismos; por lo que se busca continuamente nuevas técnicas para alcanzar éxito en el tratamiento. Se determinó el efecto antibacteriano y antifúngico del aceite esencial ozonizado de sacha inchi, hidróxido de calcio en pasta y la combinación de ambas; se utilizó el método de difusión en agar, siendo el medio de cultivo para los *Enterococcus faecalis* infusión cerebro corazón y para la *Candida albicans* agar sabouraud, para determinar el efecto antibacteriano y antifúngico se midieron y se registraron los halos de inhibición que se formaron alrededor de los pocillos que contenían los medicamentos a las 24 horas y 48 horas. Obteniendo como resultado que el efecto antibacteriano y antifúngico del aceite ozonizado es mayor a las 6 horas proporcional a la mayor concentración de peróxido. El presente estudio mostró el efecto antibacterial del aceite ozonizado de sacha inchi que es superior frente al hidróxido de calcio y la combinación de ambas sustancias.

PALABRAS CLAVE: *Enterococcus faecalis*; *Candida albicans*; Sacha inchi; Aceite ozonizado; Hidróxido de calcio; Actividad antibacterial; Actividad antifúngica.

INTRODUCTION

Endodontic treatment involves the removal of diseased root tissue, including stages of instrumentation, irrigation, application of medications, and filling of canals; the eradication of microorganisms and the prevention of reinfection is one of the main goals of root canal treatment (1). Between 40% to 70% of microorganisms survive (2, 3), among these are *Enterococcus faecalis* and *Candida albicans*, which are commonly isolated and are resistant to medications. A successful treatment being the reduction or elimination of bacteria both in the root canals and in places where the chemical-mechanical preparation could not reach (4-6).

Enterococcus faecalis are facultative anaerobes and gram-positive cocci which can exist individually, in pairs or in short chains (7). They invade the dentinal tubules, persisting and surviving due to morphogenetic characteristics that help tolerate systemic antibiotics and procedures (8-11). Similarly, *Candida albicans*, microorganisms classified within the yeasts of the fungal kingdom, binds to the dentin, forms biofilms, and invades the dentinal tubules with a prevalence that ranges between 0.5% and 55% and has virulence factors that can influence the appearance of endodontic pathologies, resisting medications. Mergoni *et al*. (12) found that fungi with a predominance of Candida albicans are frequently involved in infected root canals.

In addition to this, without procedures to counteract microorganisms, medications must be used as auxiliary resources, capable of improving diffusion through the ducts and increasing the antibacterial effect (13, 14). Several substances have been investigated including calcium hydroxide, which reduces tissue inflammation and microbial activity; it has a high alkaline pH (12, 4), giving it lethal properties on bacteria. It is a primary alternative due to its wide use as an intracanal medication; however, research shows it is inefficient against *Enterococcus faecalis*, which remained in the dentinal tubules (13-16).

Further research showed that ozone had strong antimicrobial and germicidal activity against viruses, bacteria, parasites, and fungi (17). Due to their viscosity, ozonated oils can be used as intracanal medicine to achieve disinfection (3, 18). There are few previous *in vitro* studies on ozona-

ted oil where its antimicrobial effect is confirmed, especially after 10 minutes; these studies show it can be used in root canal treatment because it remains in contact for a long period and has potential success in pulp therapy (19).

In the School of Dentistry, the use of ozonated Sacha Inchi oil is very attractive and interesting for research. In an *in vitro* study of the effect of ozonated sesame oil, calcium hydroxide, and the combination of both substances against *Enterococcus faecalis* carried out by Kishore *et al*. (3), ozonated oil proved to be effective as an intracanal medication against these bacteria.

Mohamed *et al*. (20) researched the antibacterial effect of ozonated olive oil and 1% sodium hypochlorite, measuring the inhibition zones; the result showed that ozonated olive oil has a superior effect.

Sacha Inchi (plukenatia volubillis) is an oleaginous plant from the Amazon Jungle that is rich in protein and vegetable oil. It is extracted from the pressing of its seeds and has a high content of polyunsaturated fatty acids (omega 3, 6, and 9) (21, 22). The ozonation study of Sacha Inchi oil is a hopeful alternative aimed at the treatment of various diseases. In the same way, it is rich in antioxidants and contains anti-inflammatory properties. The compounds of the ozonized oil are ozonides, peroxides, and aldehydes, which are substances that have germicidal (allowing them to reach places where other aqueous antibiotics do not reach), immuno-stimulating, and tissue-repairing properties (23-25).

Therefore, the objective of the study was to evaluate the *in vitro* antibacterial and antifungal action of ozonated essential oil of sacha inchi, calcium hydroxide, and the combination of both against *Enterococcus faecalis* and *Candida albicans* at 24 hours and 48 hours.

MATERIALS AND METHODS

In the present *in vitro* experimental study, strains of *Enterococcus faecalis* ATCC® 29212 and those of *Candida albicans* ATCC® 2091 were used. The sample size was obtained with the formula for comparing means, replacing the values and the variance of 0.20 obtained in the pilot test; 10 plates per group were included in the study.

EXPERIMENTATION GROUPS

- Ozonated Sacha Inchi essential oil 6 hours
- Ozonated Sacha Inchi essential oil 5 hours
- Calcium hydroxide + propylene glycol
- Calcium hydroxide + ozonated Sacha Inchi oil

Authorization for the development of the project was requested after acceptance and approval from the Ethics Committee of the Cientifica del Sur University where the experimental study was carried out, as well as approval for the use of the laboratory facilities of the same university.

HOW TO GET AND MAKE MEDICINE

The ozonated Sacha inches oil: Extra virgin Sacha Inchi oil was purchased from the NutriOmega commercial brand with a total unsaturated fat of 93%, obtaining ozonation with the help of the certified ozone therapy center Ozone & Life (it has medical generators ozone) at different times like 1.5 hours, 3 hours, 4 hours, 5 hours, and 6 hours; subsequently, the pilot study was carried out. According to the results of the halos, the five-hour and six-hour ozonized oil were selected (the meaning of ozonized is expressed as the amount of peroxides present. The greater the number of hours of ozonation, the higher the peroxide, and therefore, the antimicrobial properties). The peroxide measurement was carried out in the chemistry laboratory of the Scientific University of the South, where the results of the five-hour

ozonized oil with peroxide of 286,429 meq/kg and the six-hour ozonized oil with peroxide of 391,312 meq/kg were obtained.

Calcium hydroxide paste: It was purchased in 10 gram powder from the biodynamic brand BDP (Biodinámica Química y Farmacéutica Ltda, Brazilian industry) to prepare the paste; a small amount of calcium hydroxide was placed on a sterilized glass plate, and a few drops propylene glycol were added. It was mixed slowly with the spatula until it had a creamy and homogeneous consistency.

Combination of ozonized Sacha Inchi oil with calcium hydroxide paste: The mixture of 25ul of ozonized oil + calcium hydroxide paste was used for each inoculation of the wells made in the petri dishes.

INSTRUCTIONS TO SET UP BACTERIA AND FUNGI

Obtaining the strains: *Enterococcus faecalis* strain ATCC® 29212 and *Candida albicans* strain ATCC® 2091 were obtained from the laboratory of the Universidad Cientifica del Sur; they were reactivated, and thus both antibacterial and antifungal susceptibility to the different medications tested was evaluated.

Setting up the culture medium: The culture medium for *Enterococcus faecalis* was the brain heart infusion (BIH) provided by the laboratory of the Scientific University of the South. 8.14g of the medium and 3.3g of Agar were weighed, mixed with 220ml of distilled water, then heated in the microwave to facilitate dissolution. Likewise, the medium for *Candida albicans*, sabouraud agar, was obtained from the Gen Lab laboratory from Peru, and 14.3g was weighed and mixed with 220ml of distilled water. Each of the media was immediately autoclaved for approximately 1 hour. Afterwards, we waited for the temperature to decrease so that 25ml of the culture media could be placed in each plate.

Strain Activation: The strains of *Enterococcus faecalis* and *Candida albicans* were removed from the freezer and allowed to reach room temperature before being cultured on the agar plates-in sterile BHI agar medium (brain heart infusion) in the case of *Enterococcus faecalis* and in sabouraud agar medium for *Candida albicans*. With the help of the sowing handle, it expands in a zig zag shape. Incubation was carried out for 24 hours to achieve colony growth.

Preparation of the inoculum: Part of the colonies was taken with the help of the sowing loop and placed in test tubes with sterile physiological saline until a turbidity of 0.5Mc Farland was reached.

Inoculation of agar plates: The strains were deposited with the help of a micropipette, 100ul to each plate, and with the help of the drigalsky loop, it was spread in all directions until it was completely dry. In brain heart agar medium for *Enterococcus faecalis* strains and in dextrose agar medium for *Candida albicans*, 6 wells were subsequently made in each plate with 5mm diameter and 4mm depth at equal distances; in these, 50ul of each medication was added with the help of sterile micropipettes in aseptic conditions. One well was used for each experimental solution: ozonized Sacha inchi oil for 6 hours, ozonized sacha inchi oil for 5 hours, calcium hydroxide paste and the

combination of both, for both the *Enterococcus faecalis* and *Candida albicans* groups. For positive control, in the case of *Enterococcus faecalis*, 2% chlorhexidine was used, and for *Candida albicans*, the positive control was given by Nystatin and distilled water as a negative control. The plates were then incubated at 37 °C for 24 hours.

Measurement of inhibition zones: The measurement of inhibition zones followed the Duraffourd scale and thus determined the antibacterial effect *in vitro* (Figure 1).

After that, the bacterial inhibition zones were measured and recorded in millimeters using the digital caliper; where the existence of antibacterial and antifungal activity of both *Enterococcus faecalis* and *Candida albicans* were evaluated against the medications used at 24 hours and 48 hours.

Descriptive statistics were performed, and the means and standard deviations of the antibacterial and antifungal responses of the experimental mixtures were obtained; then, normality tests were carried out where it was established that the variables meet the assumptions of normality, for which a parametric ANOVA test was applied and the p value was obtained with a confidence level of 95%. After that, a Tuckey's post hoc test was performed.

For the statistical analysis, only the experimental substances were considered. The positive control group for the bacteria was 2% chlorhexidine (average equal to 18.22 at 24 hours and 18.16 at 48 hours), and for candida albicans (average equal to 23.21 at 24 hours and 23.7mm after 48 hours), it was nystatin, while the negative control group in both cases was distilled water.

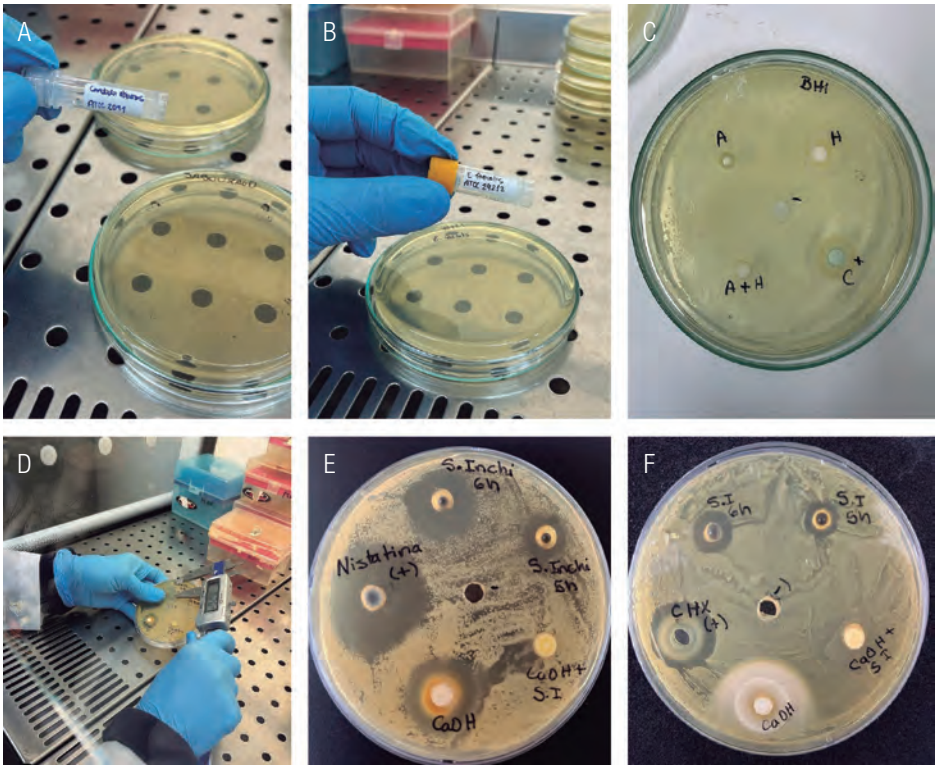

Figure 1: A, B: activation of the *Enterococcus faecalis* and *Candida albicans* strain. C: preparation of the wells. D: measurement of the inhibition zones. E, F: bacterial inhibition zone around the drugs.

RESULTS

The average inhibition zones of *Enterococcus faecalis* (Table 1): It is observed that the antibacterial effect of the ozonated oil is greater at 6 hours proportional to the highest concentration of peroxide, with the average halo size being 13.43mm at 24 hours. Likewise at 48 hours, the average halo with ozone oil at 6 hours is equal to 13.39mm.

At 24 hours and 48 hours, significant differences with lower p were found between all the test substances.

The average inhibition zones of Candida albicans (Table 2): we can observe that the antifungal effect of the ozonized oil is greater at 6 hours proportional to the higher concentration of peroxide and that the antifungal effect of the 5-hour ozonized oil is similar to that of the calcium hydroxide paste. The average halo at 24 hours with ozonated oil at 6 hours is equal to 18.97mm. At 48 hours, the average halo with ozonated oil at 6 hours is equal to 18.85mm.

At 24 hours and 48 hours, significant differences with lower p were found between all the test substances.

Table 1. Description and comparison of the antibacterial effect of ozonated Sacha Inchi oil at 6 hours and 5 hours and experimental substances against *Enterococcus faecalis*.

Time	Groups	N	Median	Standard deviation	Minimum	Maximum	F	P
24 hours	Ozonated oil 6 hours[a]	10	13.43	0.46	12.3	13.9		
	Ozonated oil 5 hours[b]	10	10.63	0.44	10.16	11.56		
	Calcium hydroxide[c]	10	0	0	0	0		
	Calcium hydroxide plus Ozonated oil[c]	10	0	0	0	0	4856.58	<0.001
48 hours	Ozonated oil 6 hours[a]	10	13.39	0.53	12.52	13.93		
	Ozonated oil 5 hours[b]	10	10.78	0.41	10.19	11.5		
	Calcium hydroxide[c]	10	0	0	0	0		
	Calcium hydroxide plus Ozonated oil[c]	10	0	0	0	0	4453.31	<0.001

Applying ANOVA
Tukey Test
(a,b) $p<0.01$
(a,c) $p<0.001$
(b,c) $p<0.001$

Table 2. Description and comparison of the antifungal effect of ozonated Sacha inchi oil at 6 hours and 5 hours and experimental substances against *Candida albicans*.

Time	Groups	N	Median	Standard deviation	Minimum	Maximum	F	P
24 hours	Ozonated oil 6 hours[a]	10	18.97	0.64	17.84	19.91		
	Ozonated oil 5 hours[b]	10	15.12	0.72	13.83	15.95		
	Calcium hydroxide[b]	10	14.92	0.82	13.74	16.74		
	Calcium hydroxide plus Ozonated oil[c]	10	0	0	0	0	1754.47	<0.001
48 hours	Ozonated oil 6 hours[a]	10	18.85	0.53	17.82	19.64		
	Ozonated oil 5 hours[b]	10	14.89	0.88	13.84	16.89		
	Calcium hydroxide[b]	10	14.53	0.61	13.31	15.31		
	Calcium hydroxide plus Ozonated oil[c]	10	0	0	0	0	1929.75	<0.001

Applying ANOVA
Tukey Test
(a,b) $p<0.01$
(a,c) $p<0.001$
(b,c) $p<0.001$

DISCUSSION

In the present investigation, *E. faecalis* and *C. albicans* were evaluated for their persistence and resistance to drugs in previously filled root canals, thus causing failure and reinfection. Bacteria and their byproducts are considered the main etiological agents of pulpal and periapical pathologies (26). Given the need to find new therapeutic means in root canal treatment, various studies analyzed how to inhibit or eliminate the growth of the said bacteria and fungi to the neutralization and inactivation of these (27). Ozone was an alternative, with evidence of its use in dentistry since 1930 by the German Edward Fisch in surgeries (28). It was used in the form of gas, water, and oil; however, better results were obtained when it was used in the form of oil due to its prolonged contact with the tooth surface and its long duration of action (3). It had great potential in endodontics; it is active against bacteria, fungi, and viruses. In bacteria, it acts by inducing the loss of nuclear membrane integrity through the oxidation of lipoproteins and phospholipids, with loss of organelle function. In fungi, it prevents cell growth at certain stages. In viruses, it damages the viral capsid and alters the reproductive system (29).

Da Silva *et al*. (2020) in a literature review concluded that medicinal ozone had great potential in relation to endodontic treatment, finding antimicrobial power with reduction of periapical flora and stimulation of apical bone regeneration: it reduced the need for periapical surgical procedures (27). These findings are consistent with those of the present investigation, where it was verified that the ozonized Sacha Inchi oil inactivated the strains of *Enterococcus faecalis* and *Candida albicans*; thus, these bacteria show less resistance to the drug, and this is evidenced by the size of the inhibition zone. On the other hand, Mohamed *et al*. (2017) chose to investigate ozonated oil that is unstable in the gas form and has a very short useful life in ozonated water (20). Similarly, Pratyusha *et al*.

(2017) mentioned that ozone dissolved in water is extremely unstable and cannot be stored; On the other hand, there is a big difference when it is dissolved in oil because it has a useful life that could be measured in years. Finding permanence in the oral cavity, it has adequate penetration, high efficacy, and acceptability (26). In agreement with this research, it was therefore decided to study ozonated oil as it has slower viscosity and ozone degradation; It can be stored at low temperatures (for months) with a long shelf life and has proven to be very effective with the aforementioned bacteria.

In another way, Tiwari *et al*. (2016) pointed out that ozonated oils can be used as an intracanal dressing, reducing the marked anaerobic odor emanating from infected teeth (28). Similarly, research carried out demonstrated a high antibacterial potential due to the interaction of fats and unsaturated oil with ozone, as well as peroxide concentrations at the different ozonation hours of 5 hours and 6 hours, which led to greater effectiveness.

There are few studies of ozonated oils that show that they are biocompatible with oral cells, do not cause damage to the periapical tissue, have low cytotoxicity and can help in pulp therapy, having sesame, sunflower, and olive as antecedents, demonstrating good results against bacteria and fungi. No research was found regarding Sacha Inchi oil, so the present study focused on investigating it, due to its high unsaturated fatty acid content, similar to sunflower oil; it was acquired from the NutriOmega commercial brand, proceeding to ozonate for different times 1.5 hours, 3 hours, 4 hours, 5 hours, and 6 hours, and according to the results of our pilot study, we selected the ozonation of 5 hours and 6 hours which presented the best results.

Satisfactory results were found with respect to the use of ozonized Sacha Inchi oil at both 6 hours and 5 hours, reducing the number of bacteria and fungi that are reflected in the size of the inhibition zone. It could also be demonstrated that its antibacterial effect was greater at higher concentrations of ozone, with higher peroxide in the case of the oil ozonated for 6 hours compared to that of the oil ozonated for 5 hours. Thus, the effect of ozone against resistant microorganisms in endodontic treatments was confirmed. Likewise, we were able to observe that calcium hydroxide paste and the combination of calcium hydroxide with ozonated oil had no inhibitory effect with respect to *Enterococcus faecalis*; On the other hand, it could be observed that calcium hydroxide paste had an inhibitory effect against *Candida albicans* but not the combination of calcium hydroxide with ozonated oil.

The results obtained in the present study showed the antibacterial effect of ozonated sacha inchi oil against *Enterococcus faecalis*, confirming the findings of Mohamed *et al*. (2017) who investigated the effect of ozonated olive oil versus that of sodium hypochlorite on *Enterococcus faecalis*. Their results showed a superior antibacterial effect of ozonated oil with respect to sodium hypochlorite. On the other hand, Varol *et al*. (2017) determined the antifungal activity of olive oil and ozonized olive oil against *Candida spp*. and *Saprochaete spp.*, demonstrating that ozonized olive oil can help control some fungal pathogens. These results are corroborated by the present investigation with the antifungal effect of ozonated sacha inchi oil against *Candida albicans*.

Based on the above, it can be stated that ozone diluted in oil has an antibacterial and antifungal effect with respect to the bacteria and fungi mentioned, which is proportional to the concentration of peroxide used. Thus, future research is required for better understanding.

Along the same lines, Gonçalves *et al*. (2020) confirmed the antimicrobial property of

ozonated sunflower oil, especially after 10 minutes of application, suggesting that it could be used as intracanal medication in endodontic treatment.

The results of the present study showed that calcium hydroxide paste with propylene glycol were not able to produce a halo of inhibition for *Enterococcus faecalis*, which is in accordance with what was mentioned by De Araujo *et al*. (2017) in their *in vitro* antibacterial effectiveness study of different formulations of hydroxide paste (30). Similarly, Mohammad *et al*. (2014) (31) and Martínez *et al*. (2021) had similar results in their literature reviews: although calcium hydroxide has excellent antimicrobial properties, it was not able to eliminate *Enterococcus faecalis* in its entirety (32), thus corroborating what was found in the present study-it is not effective against *Enterococcus faecalis*.

When performing the statistical tests, it was observed that for both *Enterococcus faecalis* and *Candida albicans*, the null hypothesis was rejected since the ozonized sacha inchi oil at 6 hours presented a greater halo of inhibition than the other experimental substances, which marks the start of future research.

This research is important because it begins a line of research with ozonated sacha inchi oil, as a possible therapeutic option or resource due to the antibacterial and antifungal properties it presents. However, more studies are suggested to obtain safe protocols in the application of the medication.

CONCLUSION

The present study showed that the antibacterial effect of ozonated Sacha Inchi oil is superior to that of calcium hydroxide and the combination of both substances.

Likewise, the superior antifungal effect of ozonated oil compared to that of calcium hydroxide and the combination of both substances was demonstrated.

A greater effect is found in the oil that has a higher ozonation time as well as a higher peroxide concentration.

INTEREST CONFLICT

The authors declare they have no conflicts of interest.

AUTHOR CONTRIBUTION STATEMENT

Preparation of the research project and reports on the progress of the research. Data collection. Statistical analysis. Preparation of the scientific article: M.I.LL.B.
Preparation of the research project and reports on the progress of the research. Collaboration in the preparation of the scientific article: C.R.G.R.

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