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RESEARCH ARTICLE

EVALUATION OF ANTIBACTERIAL EFFICACY OF OZONATED OLIVE OIL AND COLD PRESSED NEEM OIL AGAINST ENTEROCOCCUS FAECALIS: AN AGAR WELL DIFFUSION STUDY

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ABSTRACT

Background: Successful endodontic treatment depends on effective disinfection and complete sealing of root canal. Various medicaments are advised for disinfecting root canal, such as herbal and non-herbal medicaments.

Aim: This in vitro study was done to evaluate the antibacterial effect of ozonated olive oil and cold pressed neem oil against *Enterococcus faecalis (E. faecalis) (ATCC 29212)*.

Materials and Method: Agar well diffusion method was used to evaluate the antibacterial action of Group 1: Ozonated olive oil, Group 2: cold pressed neem oil, Group 3: 2% Chlorhexidine, Group 4: Calcium hydroxide and Group 5: Normal saline. The petri plates were incubated aerobically at 37° for 48 hours. The inhibition zones against *E. faecalis* were recorded. The data was statistically analyzed using one way analysis of variance (ANOVA) and Post Hoc test ($p< 0.05$).

Results: Ozonated olive oil showed the maximum mean value for the zone of inhibition after 48 hrs followed by 2% CHX and then cold pressed neem oil. Least mean value for the zone of inhibition was obtained for calcium hydroxide. There was a statistically significant difference between the groups. ($p<0.001$)

Conclusion: Ozonatedolive oil was most effective against *E.faecalis* when compared to other experimental groups and can be used as an alternative intracanal medicament.

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INTRODUCTION

Microorganisms and their by-products are considered to be the major etiologic agents in endodontic diseases. Failure, during and after endodontic treatment, are associatedwith the presence of bacteria in the root canal. *Enterococcus faecalis* (*E. faecalis*) is known to be important resistant species in infected root canals, and they may cause treatment failures (Dubey *et al*., 2012). As it is a facultative anaerobe, it can endure high pH. In such cases, inter-appointment dressing with an intracanal medicament is essential. Calcium hydroxide [$Ca(OH)_2$] has been the most commonly used intracanal medicament. Nevertheless even $Ca(OH)_2$ could not entirely eradicate *E. faecalis*. The complete elimination of *E. faecalis*is essential from the root canal system for successful endodontic outcome (Reddy *et al*, 2015). At present, ozone therapy is gaining acceptance as a modern non-invasive method of treatment.

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Ozone is a gaseous, energized form of oxygen O_3, which is unstable and dissociates back into oxygen (O_2), thus liberating so-called singlet oxygen (O_1), which is a strong oxidizing agent. Thus ozone has a high antibacterialpower against oral pathogens, without resistance development for both gaseous state as well as aqueous state. It is used in various treatment modalities in field of medicine, dentistry, veterinary, food industry, and water treatment (Tiwari *et al*, 2017). Ozone inactivates bacteria and viruses by cell lysis. It disrupts theintegrity of the bacterial cell envelope by oxidation of phospholipids and lipoproteins (Nagayoshi *et al*, 2004). Ozone furthermoreacts as a super-oxygenator, carrying oxygen to tissues and supporting the body in its natural healing process (Holland, 2010). Various forms of Ozone used for root canal disinfection comprise- Ozone gas, aqueous solution, oil or ozonated water. These have been used as irrigant & intracanal medicament. Amongst these ozonated water is less cytotoxic than gaseous ozone and other irrigants like NaOCl, CHX and H_2O_2, but these forms lack residual effect (half-life 40min, 20ºc) and need to be freshly prepared (Raiyani, Arora and Bhayya, 2015) (Mohammadi *et al*., 2013). Ozonated oil due to

its viscosity remains in the root canal for extended periods, thus facilitating its use as an intracanal medicament (Bocci *et al*., 2009). Ozonated oils are still not widely used in dentistry and very few studies have been done on its use.(Farac *et al*., 2013) Ozonated olive oilis a cold pressed olive oil that has undergone ozonization using a steady flow of ozone oxygen mixture in the ratio of 5:95% until olive oil transforms from the greenish-colored liquid status to the whitish gel status (Shoukheba *et al*., 2014). It is recognized that neem leaves have antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, antipyretic, and analgesic effects without any side effect. Neem extract has several active constituents which are responsible for its antibacterial action. The oil from the leaves, seeds and bark has a wide spectrum of antibacterial action against gram positive and gram negative bacteria. (Kumar *et al*., 2013) The present study was aimed to explore the antibacterial efficacy of commercially available ozonated olive oil (NaturO3, Lightning in a jar, AZ Healthcare, Bangalore) (Fig.1) and cold pressed neem oil (Allin exporters, Delhi) (Fig.1) against E. facaelis in comparison to 2% chlorhexidine.

Fig.1. Cold pressed neem oil(Allin exporters, Delhi) (Left), ozonated olive oil (NaturO$_3$, Lightning in a jar, AZ Healthcare, Bangalore)(right)

Methodology

This study was conducted in the Microbiology department of Krishnadevaraya College of dental sciences, Bangalore.

Preparation of Microbial Inocula

The antibacterial activity was carried out against standard strain of American Type Culture Collection (ATCC). *Enterococcus faecalis* ATCC 29212 strain was used for the study. *E. faecalis* was freshly sub-cultured in Brain Heart Infusion (BHI) (HI Media, Mumbai) broth incubated at 37^0 C for 24 hours. The culture turbidity was adjusted to McFarland opacity standard 0.5.

Determination of Antibacterial Susceptibility using Agar Well-Diffusion Method

BHI agar was freshly prepared and 30 ml was dispensed in conical flask and sterilized by autoclaving for 15 lbs for 15 mins. The medium was allowed to cool to 50^0 C and 30 microlitre of the adjusted *E. faecalis*culture was inoculated into the medium. It was mixed well and poured into a sterile petri plate (90mm diameter) (Tarson, Mumbai).The medium was allowed to set completely for 15 mins. Using sterile templates 5 wells of 6 mm diameter and 4 mm depth were made which were placed equally distributed in the petri plate. 50 microliter of the undiluted reagents was inoculated into the wells with the help of sterile microtips under aseptic conditions.

The reagents used:

Group 1: Ozonated olive oil
Group 2: Cold pressed Neem oil
Group 3: 2%Chlorhexidine (CHX)
Group 4: Calcium hydroxide
Group 5: Saline (negative control)

The plates were incubated at 37^0 for 48 hrs. The petri plates were observed for the zone of inhibition, which was measured in millimeters using Vernier Caliper. The experiment was conducted in 3 replicates to minimize the errors.

Statistical Analysis

The data was compiled using Microsoft excel and was subjected to statistical analysis using SPSS 20.1. Descriptive and analytical statistics was done. ANOVA with Tukey's post hoc test was used to check the mean differences between groups where appropriate.

RESULTS

For *E. faecalis*, ozonated olive oil showed the maximum mean value for the zone of inhibition after 48 hrs followed by 2% CHX and then cold pressed neem oil. Least mean value for the zone of inhibition was obtained for calcium hydroxide (Fig 2). There was a statistical difference between the groups. ($p<0.001$) (Table 1)

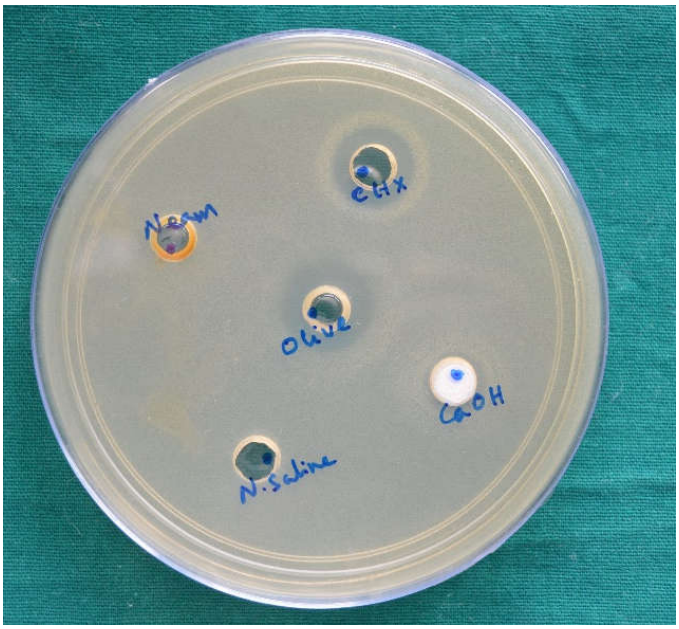

Fig.2. Petri plate with Brain heart infusion agar with 5 equally spaced wells depicting the zone of inhibiton for the experimental and control groups

Table 1. Intergroup comparison of mean zone of inhibition

Groups	N	Mean zone size (mm)	Std. Deviation	F value	p value*
Group 1 (Ozonated olive oil)	3	17.33	1.155		
Group 2 (Cold pressed Neem oil)	3	8.67	.577		
Group 3 (2% Chlorhexidine)	3	13.00	1.000		
Group 4 (Calcium Hydroxide)	3	7.00	1.000		
Group 5 (Saline)	3	0.00	0.000	174.227	<0.001††

*ANOVA was used, †Significant p<0.05; ††Significant p<0.01

Table 2. The inter group comparison of antibacterial efficacy (Zone of inhibition) of ozonated olive oil, Cold pressed neem oil, 2% CHX, $Ca(OH)_2$ and saline

Variables	Comparison Groups	Std. Error	Mean Difference	95% Confidence Interval(lower bound – upper bound)	p-value<0.05
Ozonated olive oil	Cold pressed neem oil	0.699	8.667*	6.37-10.97	<0.001††
	2%CHX	0.699	4.333*	2.03-6.63	0.001††
	$Ca(OH)_2$	0.699	10.333*	8.03 - 12.63	<0.001††
	Saline	0.699	17.333*	15.03 -19.63	<0.001††
Cold pressed neem oil	Ozonated olive oil	0.699	-8.667*	-10.97 - (-6.37)	<0.001††
	2%CHX	0.699	-4.333*	-6.63- (-2.03)	0.001††
	$Ca(OH)_2$	0.699	1.667	-.63- 3.97	0.197
	Saline	0.699	8.667*	6.37-10.97	<0.001††
2%CHX	Ozonated olive oil	0.699	-4.333*	-6.63- (-2.03)	0.001††
	Cold pressed neem oil	0.699	4.333*	2.03- 6.63	0.001††
	$Ca(OH)_2$	0.699	6.000*	3.70 - 8.30	<0.001††
	Saline	0.699	13.000*	10.70 - 15.30	<0.001††
$Ca(OH)_2$	Ozonated olive oil	0.699	-10.333*	-12.63 - (-8.03)	<0.001††
	Cold pressed neem oil	0.699	-1.667	-3.97 - .63	0.197
	2%CHX	0.699	-6.000*	-8.30 - (-3.70)	<0.001††
	Saline	0.699	7.000*	4.70 - 9.30	<0.001††
Saline	Ozonated olive oil	0.699	-17.333*	-19.63 - (-15.03)	<0.001††
	Cold pressed neem oil	0.699	-8.667*	-10.97 - (-6.37)	<0.001††
	2%CHX	0.699	-13.000*	-15.30 - (-10.70)	<0.001††
	$Ca(OH)_2$	0.699	-7.000*	-9.30 - (-4.70)	<0.001††

*Post Hoc was used, †Significant p<0.05; ††Significant p<0.01

Post hoc test was performed to check the inter-group mean difference. Ozonated olive oil showed better antibacterial efficacy against *E. faecalis* than other groups. The difference with each group was statistically significant. (p<0.05) Similarly when cold pressed neem oil was compared it was observed that 2% CHX was better than cold pressed neem oil. The difference was statistically significant.But when cold pressed neem oil was compared to $Ca(OH)_2$ the difference was not significant (p=0.197). 2% CHX was better than $Ca(OH)_2$ andthe difference was statistically significant (p<0.005). (Table 2)

DISCUSSION

E. faecalis was taken in the present study because it has been identified as the most common species in root canal diseases and represents an organism that is commonly isolated from the root canals of teeth that have been previously root filled. *E. faecalis* shows high bacterial resistance to several irrigation solutions and medicaments used in endodontics, especially when a high pH is not maintained. Thus, *E. faecalis* can survive for a long time in the root canals (Adl, Shojaee, and Motamedifar, 2012). Several studies have been focused towards finding an effective way to eliminate and/or inhibit *E. faecalis* from gaining entry to the root canal space. *E. faecalis* can gainentry into the root canal system during the course of treatment, in between appointments, or even after the treatment has been completed (Rôças, Siqueira and Santos, 2004). Thus, it is essential to consider treatment proceduresdirected at eliminating or preventing the infection of *E. faecalis* during each of these phases.One of the new generations of the disinfectant agents is ozone; a powerfuloxidizing agentused to eliminate bacteria in root canals. There are several ways of delivering ozone, when it dissolves in water, it becomes highly unstable and rapidly decomposes through a complex series of chain reactions, so it cannot be stored. In contrast, when it is dissolved in an oil base, it has a life span that could be measured in years. It chemically reacts with oil, and forms long complex molecules. Hence, in this study ozonated olive oil gel was selected over ozonated water because the application of gels was found to provide a long stay in the oral cavity, adequate drug penetration, high efficacy and acceptability. Ozone reacts with various chemical compounds in two different and coexisting modes, one involving direct reactions of molecular ozone and the other a free radical-mediated reaction. Both these mechanisms may be involved in the destruction ofbacteria by ozone (Shoukheba *et al*, 2014) (Nagayoshi, Kitamura, Fukuizumi, Nishihara, and Terashita, 2004) Extracting oil through cold-pressing technique involves the crushing of the seed or nut and forcing out the oil. The seeds are dropped into a cylinder that comprisesof a rotating screw, which grinds and crushes the seeds until the oil is extracted. Small holes in the bottom of the cylinder permit the oil to escape into a collection container. The amount of heat produced by friction, as the screw breaks down the seeds, isn't enough heat to damage the extracted oil (Bachmann, 2001).

Ozonization of olive oil is carried out by bubbling ozone through cold pressed olive oil in the presence of magnetic field for eight weeks which is a redox reaction. When olive oilis completely ozonated, it turns in to a clear, gel substance holding the ozone within. The smell of ozone being emitted from the olive oil is obvious. The final product must be refrigerated at all times. When refrigerated, this gel will hold

the ozone for years (Geweely, 2006). The reaction of ozone with olive oil occurs exclusively with the carbon-carbon double bonds present in the un-saturated fatty acids producing different toxic products such as several oxygenated compounds, hyperoxides, ozonides, aldehydes, peroxides, diperoxides and polyperoxides and these compounds could also be responsible for the wide antibacterial activity of ozonized olive oil (Shoukheba *et al*., 2014) (Geweely, 2006).
Azadirachta indica (Neem) is perhaps the most useful traditional medicinal plant. Each part of the tree has been used as traditional medicine for household remedies against various human ailments. Most of the parts of the plant such as fruits, seeds, leaves, bark and roots contain compounds with proven antiseptic, antiviral, antipyretic, antiinflammatory, antiulcer and antifungal properties. Antibacterial effects of neem extract have been demonstrated against *Streptococcus mutans* (*S. mutans*) and *E. faecalis (*Ambareen and Chinappa, 2014). In a study by Nayak *et al*, they showed that aqueous and alcohol extract of neem leafhad significant inhibitory effect against *S. mutans, E. faecalis and Candida albicans* (Nayak *et al*., 2013). In a study by Datta *et al*, they showed that neem oil had no efficacy against *E. faecalis* (Dutta and Kundabala, 2013). The present study is the first of its kind wherein Cold pressed Neem oil is being tested for its antibacterial efficacy against *E. faecalis.*

The results of the current study shows maximum inhibitory effect against *E. faecalis* by ozonated olive oil followed by 2% chlorhexidine, cold pressed neem oil and Calcium hydroxide and no effect was seen with saline. Ozonated olive oil had the highest antibacterial effect with a mean zone of inhibition of 17.33mm which was statistically significant when compared to the other experimental groups (Table 1). The mean zone of inhibition for chlorhexidine was 13±1mm which was statistically significant when compared to calcium hydroxide which had a mean zone of inhibition of 7±1mm. This result was in agreement with results obtained by Bhandari *et al*. (2014) and Attia *et al*. (2015) who found that chlorhexidine had a better antibacterial efficacy when compared to calcium hydroxide. Since it is the first study using these novel herbal medicaments, the literature review is scarce to compare the results of theantibacterial efficacy of Ozonated Olive oil and cold pressed neem oil. However further research is required to brand the materials as safe and non-cytotoxic.

Conclusion

Ozonated Olive oil has shownsignificant inhibitory effect against *E. faecalis* when compared to cold pressed neem oil, 2% CHX and $Ca(OH)_2$. Hence, it can be concluded that Ozonated Olive oil can be consideredas an alternative endodontic intracanal medicament.

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