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A comparative evaluation of antimicrobial efficacy of calcium hydroxide and Sesame oil based intracanal medicaments on enterococcus faecalis: An *in vitro* study

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Abstract

AIM: The aim is to compare the antimicrobial efficacy of calcium hydroxide, combination of sesame oil with chitosan paste combination and combination of sesame oil with polyethylene glycol paste intracanal medicaments on Enterococcus Faecalis at 72 hours, 8 days and 14 days respectively.

Materials and Methodology: A total of 72 single-rooted anterior teeth were selected, root canal preparation was done, and teeth were divided into three groups and contaminated with *E. faecalis*, which were further divided into four test groups each according to intracanal medicaments used.

After 21 days, the 72 samples were randomly divided into three groups (n=18) according to the medicaments used and are as –

Group 1: Negative Control, No medicament used

Group 2: Calcium Hydroxide intracanal medicament paste

Group 3: Sesame oil with chitosan paste

Group 4: Sesame oil with polyethylene glycol

The specimens were then incubated at 37 °C in an incubator until evaluation. After 72 hours, 6 samples per group were retrieved from the incubator and analyzed.

Values on continuous variable (CFU) were shown as mean \pm SD across four study groups. The inter-group statistical comparisons were done using one-way analysis of variance (ANOVA) with Bonferroni's correction for multiple group comparisons.

Results

- 1) All the medicaments showed reduction in the bacterial load of Enterococcus Faecalis. However, Calcium hydroxide medicament with Sesame oil and chitosan medicament showed better reduction in the colonies than Sesame oil with polyethylene glycol.
- 2) Significant reduction of bacterial load was also observed at 14 days when compared after 72 hrs and 8 days in all the three medicaments.
- 3) Calcium hydroxide medicament and Sesame oil with Chitosan compared to sesame oil with chitosan.

Conclusion: In this study, all the three groups showed reduction in the bacterial count of Enterococcus Faecalis, however complete eradication of this stubborn organism was not achieved which is still a drawback of intracanal medication of root canal therapy. However, Calcium hydroxide medicament with Sesame oil and chitosan medicament showed better reduction in the colonies than Sesame oil with polyethylene glycol.

Keywords: Sesame oil, chitosan, polyethylene glycol, calcium hydroxide, intracanal medicament, endodontics

Introduction

The microenvironment of root canal presents excellent conditions to establish microbial growth. Successful endodontic treatment depends upon the removal of microbes from the root-canal system which leads prevention of reinfection^[1]. Thorough irrigation of root canals with any antimicrobial solution might not be sufficient to eliminate all microorganisms from the root canal.

As root canal infections have a polymicrobial nature hence, anaerobic and facultative anaerobic microorganisms are usually found together in endodontic flare-ups and cases with post-treatment diseases [1, 2].

Enterococcus faecalis (*E. faecalis*) is a bacterium commonly found in endodontic infections, particularly in retreatment cases. *E. faecalis* can be very easily eliminated in planktonic forms in-vitro. However, it appears to turn out to be more resistant while it is present in an infected root canal system [3]. The resistance may be caused by the activation of virulence factors, biofilm formation, or invasion of dentinal tubules. *Enterococcus faecalis* has been reported to remain viable, proliferate, and establish a biofilm phenotype that is highly resistant to phagocytosis, antibodies and antimicrobials than non-biofilm producing organisms [4].

Intracanal medicament is generally recommended when treatment cannot be completed in one appointment; there are chances that surviving intracanal bacteria often proliferate between appointments. To curtail bacterial regrowth and possibly even improve bacterial suppression, an intracanal medication can be advantageous and successfully used to eliminate the bacterial flora [5].

Calcium hydroxide ($\text{Ca}(\text{OH})_2$) is widely used as an intracanal medicament in endodontic therapy. The high pH of $\text{Ca}(\text{OH})_2$ destroys the bacterial cell membrane and protein structures. In spite of various advantages and indications of calcium hydroxide, it do have some limitations. However, its low diffusibility and solubility makes it difficult to cause an increase in pH and gets neutralized by buffering system of the dentin itself and acids present in deeper layers of dentin and thus decrease its bioavailability [6].

Sesame (*Sesamum indicum* L.) is a flowering plant in the genus *Sesamum* and the family of Pedaliaceae which is annual plant and native to tropical areas. 50% of sesame seeds consist of oil, which contain Sesamol, Sesamolin, Sesamin, Sesaminol, γ -tocopherol and polyphenols. Sesamin and sesamolin are used as active ingredients in antioxidants, antiseptics, bactericides, viricides, disinfectants, moth repellents and antitubercular agents [7, 8]. Sesame oil downregulates the expression of Matrix Metalloproteinase-9 responsible for dental caries, pulp and periapical inflammation which play an important role in the degradation of the collagenous structure and spread of the pathology [8].

Chitosan is a natural cationic polysaccharide derived by N-deacetylation of chitin obtained from shells of shrimps and crabs [9]. Chitosan exhibits adhesiveness, biocompatibility and biodegradability and is widely used in biomedical and pharmaceutical applications. There is increasing scientific evidence regarding the role of chitosan on *C. albicans* and *E. faecalis* particularly as members of secondary or persistent infections associated with failed endodontic therapy and their resistance against commonly used intracanal medicaments [10].

Glycols are usually used as a carrier for triple antibiotic paste in endodontics. Evidence has proved that Polyethylene glycol shows some antibacterial qualities against *E. Faecalis*, *Streptococcus mutans* and *E.coli* [11].

The purpose of this study is to compare the antimicrobial efficacy of calcium hydroxide, sesame oil with chitosan paste combination, sesame oil with polyethylene glycol paste combination based intracanal medicaments on *E. Faecalis*.

Methodology

After obtaining the Institute Ethical clearance, the study was conducted in the Department of Conservative Dentistry and Endodontics in association Department of Microbiology of the same institute. Seventy-Two human extracted single-rooted teeth with single canal and well-formed root apices, extracted for orthodontic reasons were collected from Department of Oral and Maxillofacial Surgery with informed consent of the donor. The criteria for selection of samples were as follows:

Inclusion Criteria

- a) Sound single canal teeth with well-formed root apices (Vertucci Type 1)

Exclusion Criteria

- a) Multi-rooted teeth
- b) Teeth with canal variations
- c) Carious teeth
- d) Teeth with development anomalies
- e) Teeth with more than on root canal
- f) Teeth with resorption
- g) Teeth with calcifications
- h) Apically curved teeth
- i) Teeth with cracks

The teeth were stored in thymol solution at room temperature until use. The crowns were decoronated at the cemento-enamel junction (CEJ) using a rotating diamond disc (Carbodont; Gysi S.A, Buenos Aires, Argentina) at 20000 rpm under water spray and the canal lengths were standardized to 14mm. Each tooth was buccolingually and mesiodistally radiographed to verify the presence of a single canal and a single apex at the apical third (Vertucci type 1). The root specimens were then thoroughly ultrasonically scaled for any deposits or remnant periodontal tissues. The roots were then sterilized in an autoclave at a temperature of 121°C and a pressure of 15lbs for 20 min. The sterility of the specimens was indicated using Autoclave colour indicator and subjected to bacterial contamination.

Shaping and Cleaning of Samples

A size 10 K-file were placed into the canal until it was visible at the apical foramen to ensure that the canals were patent. (Figure 1). Root canals was instrumented 0.5 mm short of the apex up to size 25 instrument. The samples were then instrumented 1 mm short of their apices up to Pro Taper universal size F3 files by using the crown-down technique. 2ml of 5.25% sodium hypochlorite was used for irrigation after each instrument use. (Figure 2) 5 mL of 17% ethylenediamine tetra acetic acid (EDTA) for 3 minutes for each specimen to remove the residual debris. Final irrigation was done with 5 ml saline to remove the residual irrigants from the canals. The canals were dried by using Pro Taper Universal paper points of size F3 before placement of medicaments. The samples were then autoclaved at 121°C for 20 minutes at 20 psi.

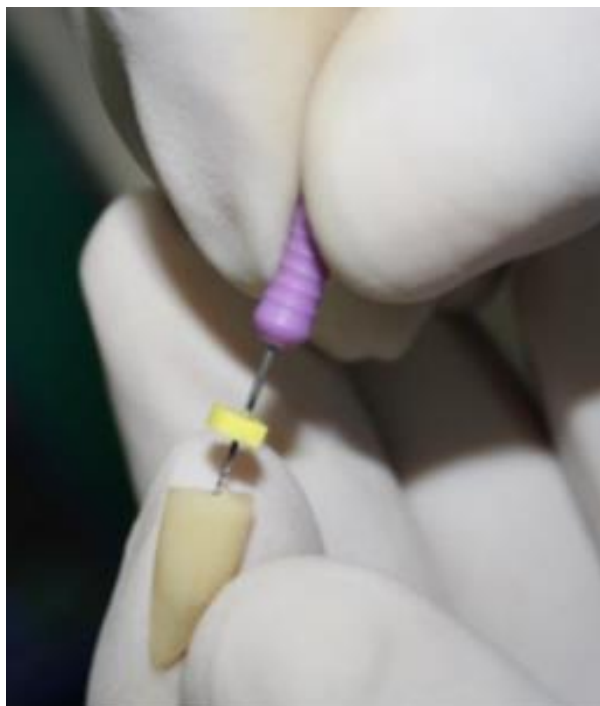


Fig 1: Working Length Determination using 10-K file

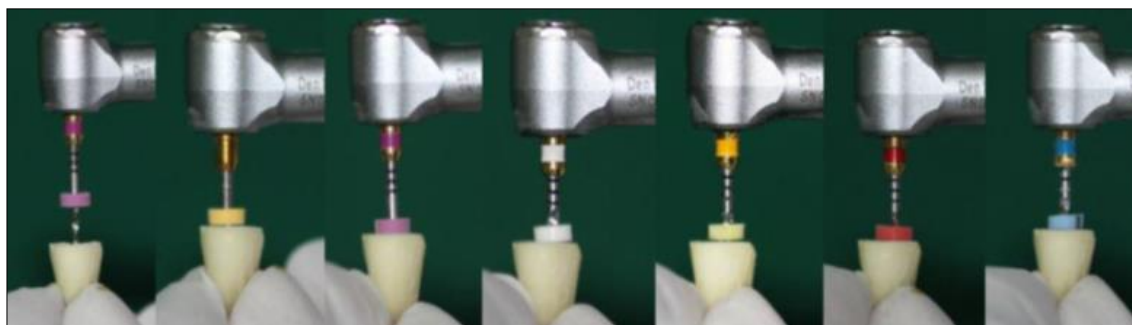


Fig 2: Protocol for Shaping the Root Canals

Bacterial strain and microbial growth preparation

Enterococcus faecalis (ATCC 29212; Microbiologists, Medimark Europe) was incubated in Trypticase soy agar plate (HiMedia Laboratory Private Limited, Mumbai, Maharashtra, India) to improve their growth. (Figure 3) Observation under stereomicroscope (BX-63 Olympus DIC) was performed to check the 100% purity of culture. The culture was incubated

anaerobically at 37°C for 7 days. Enterococcus Faecalis was then incubated in Trypticase soy broth at 36.5°C for 24 hrs (Figure 4) (HiMedia Laboratory Private Limited, Mumbai, Maharashtra, India) to obtain a turbid suspension of Enterococcus Faecalis and the turbidity was spectrophotometrically adjusted to 1.5×10^8 organisms/ml (equivalent to 0.5 McFarland standard).(Figure 5)

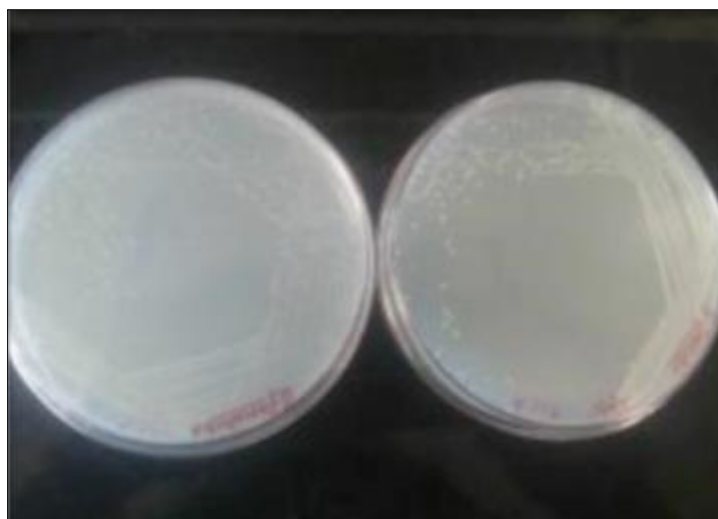


Fig 3: Trypticase Soy agar plate containing Enterococcus Faecalis

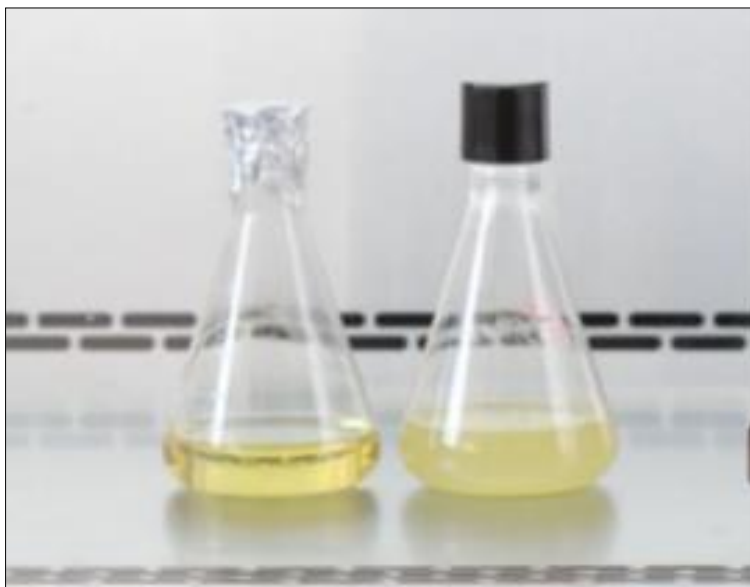


Fig 4: Trypticase Soy Broth containing Enterococcus



Fig 5: Inoculation of Enterococcus faecalis in Tooth Specimen

Specimen Contamination: The autoclaved root samples were placed in Eppendorf tubes and inoculated with 15 ml of 1.5×10^8 organisms/ml of Trypticase soy broth. The Eppendorf tubes were incubated under anaerobic conditions for 3 weeks

at 37°C and after every 3 days, the Eppendorf tubes with roots specimens were changed with fresh bacterial broth to ensure the penetration of Enterococcus Faecalis into dentinal tubules. The samples were then incubated for 21 days. (Figure 6)

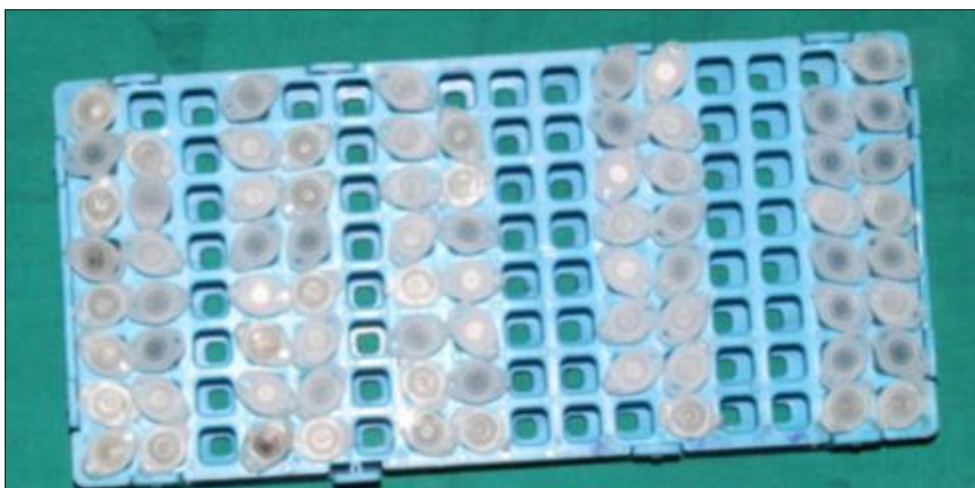


Fig 6: Samples after 21 days incubation

Medicament Preparation Sesame oil and Chitosan
 Medicament: 1) Sesame oil and Chitosan paste (100 gm)
 Material: Sesame Oil: Chitosan (1:1)
 Ingredient Quantity Sesame oil 50 ml Chitosan 50 gm
 Method: The size of Chitosan was reduced in mortar pestle -
 Oil was added and triturated to a get smooth paste
 2) Sesame oil and Polyethylene Glycol Medicament: Sesame

oil and PEG 400 paste (100 gm) Material: Sesame Oil: PEG 400 (1:1)
 Ingredient Quantity Sesame oil 50 ml PEG 400 50 gm
 Method: Peg 400 was melted in a beaker, on water bath Oil was added and cooled with stirrer. Powder was scraped out and reduced in size with mortar and pestle to get smooth paste. (Figure 7)

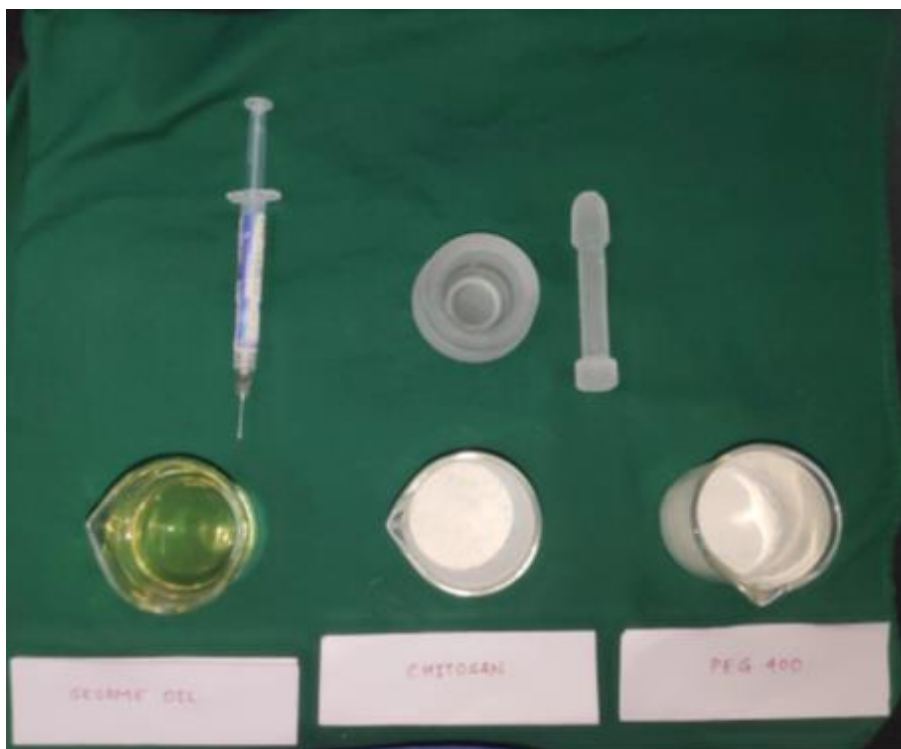


Fig 7: Armamentarium for Medicament Preparation

Specimen Treatment After 21 days, the 72 samples were randomly divided into three groups (n=18) according to the medicaments used and are as –

- Group 1: Negative Control, No medicament used
- Group 2: Calcium Hydroxide intracanal medicament paste
- Group 3: Sesame oil with chitosan paste
- Group 4: Sesame oil with polyethylene glycol

To Standardize the amount of intracanal medicaments that were to be loaded into each root, 0.8 mg of each medicament were weighed on an electronic scale and were syringed into each canal. The teeth were sealed with Intermittent Restorative Material. The specimens were then incubated at

37°C in an incubator until evaluation. After 72 hours, 6 samples per group were retrieved from the incubator and analyzed. After removing the restoration Gates Glidden drill no.4 were used to harvest the dentinal shaving from the canals. Autoclaved Paper points (size F3) were placed inside each root canal for 5 min to collect the dentinal shavings from the canals. After sampling, each paper point was transferred in Eppendorf tubes containing 1 mL of Tryptic soy broth. Decimal series of dilutions of the broth was made up to 10^{-3} . A 0.1 mL aliquot of this microbial suspension were incubated on a Blood agar for 24 h at 37°C. The above procedures were repeated at time intervals of 8 days and 14 days.



Fig 8: Placement of Medicament in Respective Samples

Values on continuous variable (CFU) were shown as mean ± SD across four study groups. The inter-group statistical comparisons were done using one-way analysis of variance (ANOVA) with Bonferroni’s correction for multiple group comparisons. The intra-group comparisons were done using repeated measures analysis of variance (ANOVA) in each study group. The underlying normality assumption were tested before subjecting the study variable to ANOVA. P-values less than 0.05 would be considered to be statistically significant. The entire statistical analysis was performed using a statistical software called statistical package for social sciences (SPSS version 21) for MS Windows

Results

Inter-group comparison of mean bacterial count (CFU).

Table 1 summarizes the Inter-Group Colony Forming Units (CFU)

Distribution of mean bacterial count at 72-Hrs is significantly

higher in Group 1 compared to Group 2, 3 and 4 (P-value0.05 for all).

Distribution of mean bacterial count at 8-Days is significantly higher in Group 1 compared to Group 2, 3 and 4 (P-value0.05 for all).

Distribution of mean bacterial count at 14-Days is significantly higher in Group 1 compared to Group 2, 3 and 4 (P-value0.05). Distribution of mean bacterial count at 14-Days did not differ significantly between study groups 3 and 4 (P-value>0.05)

Table 1: Inter-group comparison of mean bacterial count (CFU).

CFU (x 103)	Group 1 (n=6)		Group 2 (n=6)		Group 3 (n=6)		Group 4 (n=6)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
72-Hrs	18.33	3.01	10.17	0.75	10.67	1.63	11.00	0.89
8-Days	18.33	3.01	7.83	0.75	8.17	0.75	9.50	1.05
14-Days	19.17	2.14	3.67	0.82	4.00	0.89	6.17	0.75

Table 2: Inter-group statistical comparison of mean bacterial count (CFU).

P-value	Group 1 v Group 2	Group 1 v Group 3	Group 1 v Group 4	Group 2 v Group 3	Group 2 v Group 4	Group 3 v Group 4
72-Hrs	0.001***	0.001***	0.001***	0.999 ^{NS}	0.999 ^{NS}	0.999 ^{NS}
8-Days	0.001***	0.001***	0.001***	0.999 ^{NS}	0.608 ^{NS}	0.999 ^{NS}
14-Days	0.001***	0.001***	0.001***	0.999 ^{NS}	0.018*	0.051 ^{NS}

P-value by one-way analysis of variance (ANOVA) with Bonferroni’s correction for multiple group comparisons. P-value<0.05 is considered to be statistically significant. *P-value<0.05, ***P-value<0.001, NS-Statistically non-significant.

Intra-group comparison of mean bacterial count (CFU)

Table 2 summarizes the Intra-group mean CFU of E. Faecalis.

In Group 1: (Control Group)

Distribution of mean bacterial count at 72-Hrs did not differ significantly compared to mean bacterial count at 8-Days and 14-Days (P-value>0.05 for both). Distribution of mean bacterial count at 8-Days did not differ significantly compared to mean bacterial count at 14-Days (P-value>0.05).

In Group 2 (Calcium Hydroxide Group)

Distribution of mean bacterial count at 72-Hrs is significantly higher compared to mean bacterial count at 8-Days and 14-Days (P-value<0.001 for both).

Distribution of mean bacterial count at 8-Days is significantly higher compared to mean bacterial count at 14-Days (P-value<0.001).

In Group 3 (Sesame oil Chitosan Group)

Distribution of mean bacterial count at 72-Hrs is significantly higher compared to mean bacterial count at 8-Days and 14-Days (P-value<0.001 for both).

Distribution of mean bacterial count at 8-Days is significantly higher compared to mean bacterial count at 14-Days (P-value<0.001).

In Group 4 (Sesame oil Polyethylene Glycol Group)

Distribution of mean bacterial count at 72-Hrs did not differ significantly compared to mean bacterial count at 8-Days (P-value>0.05).

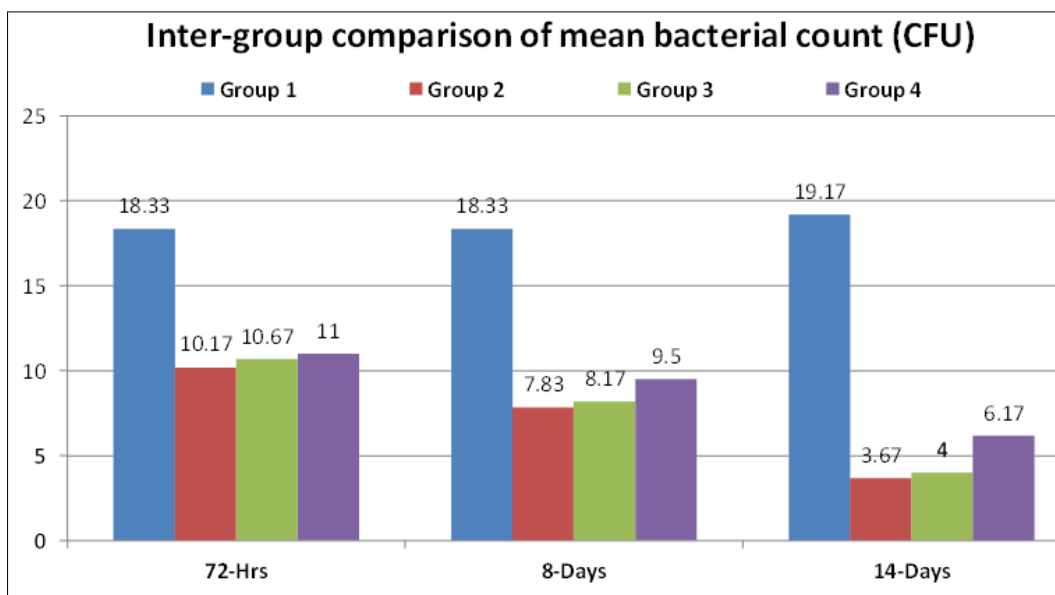
Distribution of mean bacterial count at 72-Hrs is significantly higher compared to mean bacterial count at 14-Days (P-value<0.001).

Distribution of mean bacterial count at 8-Days is significantly higher compared to mean bacterial count at 14-Days (P-value<0.01).

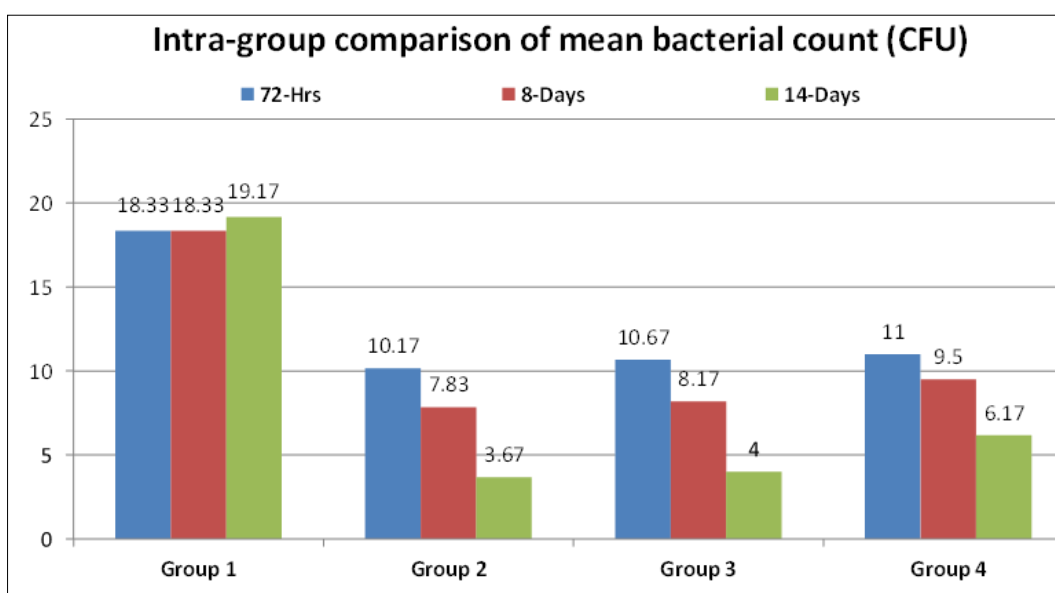
Table 2: Intra-group comparison of mean bacterial count (CFU).

CFU (x 103)	Group 1 (n=6)		Group 2 (n=6)		Group 3 (n=6)		Group 4 (n=6)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
72-Hrs	18.33	3.01	10.17	0.75	10.67	1.63	11.00	0.89
8-Days	18.33	3.01	7.83	0.75	8.17	0.75	9.50	1.05
14-Days	19.17	2.14	3.67	0.82	4.00	0.89	6.17	0.75
P-value (Intra-Group)								
72-Hrs v 8-Days	0.999 ^{NS}		0.001***		0.007**		0.076 ^{NS}	
72-Hrs v 14-Days	0.363 ^{NS}		0.001***		0.001***		0.001***	
8-days v 14-Days	0.363 ^{NS}		0.001***		0.001***		0.003**	

P-value by repeated measures analysis of variance (RMANOVA). P-value<0.05 is considered to be statistically significant. **P-value<0.01, ***P-value<0.001, NS-Statistically non-significant.



Graph 1: Bar Graph showing Inter-group comparison of mean bacterial count (CFU).



Graph 2: Bar Graph showing Intra-group comparison of mean bacterial count (CFU).

Discussion

This study evaluated the antimicrobial efficacy of calcium hydroxide and prepared sesame oil intracanal medicaments on *Enterococcus Faecalis*. It also evaluated the time taken for these medicaments for the reduction in the bacterial count of *Enterococcus Faecalis* on blood agar.

The Various etiological factors for endodontic diseases are:

1. Physical eg. Trauma, pathological wear, thermal changes
2. Chemical eg erosion, acids in dental materials
3. Bacterial eg bacteria, bacterial toxins ^[1].

Microorganisms and their products are considered to be the primary etiological agents in endodontic diseases. Success of endodontic treatment relies on complete elimination of bacteria and their toxic byproducts from the root canal system. Hence it is necessary to completely eliminate bacteria from the root canal system and prevention of recolonization or propagation of residual micro-organisms. This is done effectively by means of chemo-mechanical preparation and irrigation. In spite of the most technically demanding procedures and thorough cleaning and shaping, residual microorganisms are left behind especially in the apical third

of the canal because it has lateral canals and ramifications ^[2]. Studies which have investigated the occurrence of *Enterococcus Faecalis* in root canal treated teeth with periradicular lesion confirm that the microorganism’s antimicrobial resistance and the ability to adapt to changing environment help it to survive in root canal and cause re-infection ^[3].

Amongst these bacteria *Enterococcus faecalis* is the most dominant bacteria since it is the most commonly detected species in root filled teeth with persistent periapical lesions ^[3]. *Enterococcus faecalis* is Gram positive cocci that occur singly in pairs or in short chains. It accounts of 9.6% in the entire microflora of the oral cavity. It is a facultative anaerobe present in small proportion of the flora of untreated canal as a part of polymicrobial flora. It is a predominant bacterium implicated in root canal failures & persistent infections. Prevalence ranges from 24% to 77%. in apical periodontitis. It has been reported that enterococci are frequently isolated from obturated root canals of teeth that exhibit chronic periapical pathology (Sundqvist *et al.* Molander *et al.* 1998). *Enterococcus Faecalis* possesses lytic enzymes, cytolysin, aggregation substance, pheromones and lipoteichoic acid

which aids in its survival. It utilizes serum as the nutritional source [4].

Enterococcus Faecalis resists intracanal medicaments i.e. calcium hydroxide by maintaining pH haemostasis. It can colonize root canal and survive without the support of other bacteria and competes with other cells. It forms a biofilm that renders it more resistant to host immune response such as phagocytosis, antibodies & antimicrobial agents. *Enterococcus faecalis* is known to colonize dentinal tubules, isthmus, rami, lateral & accessory canals which are inaccessible sites during instrumentation. It is present in "mushroom shaped" micro colonies. The highly complex nature of the organism poses a great challenge for endodontists [5].

Action of intracanal medicaments

The purpose of intracanal medication is to kill the bacteria inside the root canals. Intracanal medicaments in modern endodontics have a different rationale [16, 17]. To curtail bacterial regrowth and possibly even improve bacterial suppression, an intracanal medication can be advantageous and successfully used to eliminate the bacterial flora. [14] Interappointment antimicrobial medication acts by inhibiting proliferation of bacteria and further eliminates surviving bacteria, as well as minimizes ingress of pathogens through a leaking restoration [5].

Calcium Hydroxide was widely used for root canal treatment during 1970s and is now regarded as one of the first choices as a multiple-visit root canal medication.

Ca (OH)₂ applied for 7 days eliminated bacteria in canal systems even up to 5 weeks later. Thus, Calcium Hydroxide is considered as the gold standard of all the intracanal medicaments.

In this study, Ca (OH)₂ showed reduction in the colonies of *Enterococcus Faecalis* to 10.17 x 10³ CFU/ml in 72 hrs, 7.83 x 10³ CFU/ml in 8 days and 3.67 x 10³ CFU/ml in 14 days.

Over the years, studies are being done in various parts of the world to find an alternative to Calcium hydroxide for the eradication of *Enterococcus Faecalis*. In this study, sesame oil was used as the antimicrobial agent and a vehicle for its combination antimicrobial agent [6].

According to Reza-Heidari *et al.*, sesame oil has antibacterial effects on *E.coli* and *Staphylococcus aureus*. Ozonated sesame oil has been also effective against *Streptococcus mutans* and *Lactobacillus acidophilus*, *Bacillus Subtillis*, *S. Thyphi*. According to Periasamay *et al.*, Sesame oil also downregulates Matrix Metalloproteinase-9 expression responsible for dental caries, pulp and periapical inflammation [7, 8, 13].

In this study, one of the groups was sesame oil in combination with chitosan as an intracanal medicament. Chitosan is a natural polysaccharide produced by N-deacetylation of the chitin shells of shrimp and crab, which are by-products of sea food processing industries.

Medicinal applications of chitosan are artificial skin dressings, chitin and chitosan dressings for open wounds, as a drug delivery system. In dentistry, chitosan is widely used in dentifrices, in adhesive restoration material, implant surface modifier, as a dentin collagen substitute [9].

Chitosan has been said to have anti-bacterial properties. The positively charged amino groups in chitosan interact with negatively charged microbial cell surfaces and which results in the loss of barrier function of the microbial cell wall and the leakage of proteinaceous and other intracellular material [10, 11, 12].

In a study, Chitosan in combination with chlorhexidine, enhanced the properties of Chlorhexidine when compared to Chlorhexidine alone [15].

In this study, sesame oil with chitosan intracanal medicament showed reduction in the colonies of *Enterococcus Faecalis* to 10.67 x 10³ CFU/ml in 72 hrs, 8.17 x 10³ CFU/ml in 8 days and 4.00 x 10³ CFU/ml 14 days.

Also, in this study sesame oil was combined with Polyethylene glycol (PEG 400).

Polyethylene glycol has been widely used in the field of endodontics, especially in intracanal medication as a vehicle for antibiotics for local drug delivery in the root canals.

Various studies on the antimicrobial efficacy of Polyethylene glycol have been performed.

In this study, sesame oil with Polyethylene glycol intracanal medicament showed reduction in the colonies of *Enterococcus Faecalis* to 11.00 x 10³ CFU/ml in 72 hrs, 9.50 x 10³ CFU/ml in 8 days and 6.17 x 10³ CFU/ml 14 days. [11,12]

In this study, all the three groups showed reduction in the bacterial count of *Enterococcus Faecalis*, however complete eradication of this stubborn organism was not achieved which is still a drawback of intracanal medication of root canal therapy. Thus, sesame oil with different antibacterial agents can be considered as a research project in near in the near future.

Conclusion

Within the limitations of this study it can be concluded that antimicrobial efficacy of intracanal medicament is of utmost importance for better elimination of *Enterococcus Faecalis* within the dentinal tubules thereby more efficient reduction of the bacterial load within the canals.

1) All the medicaments showed reduction in the bacterial load of *Enterococcus Faecalis*. However, Calcium hydroxide medicament with Sesame oil and chitosan medicament showed better reduction in the colonies than Sesame oil with polyethylene glycol.

2) Significant reduction of bacterial load was also observed at 14 days when compared after 72 hrs and 8 days in all the three medicaments.

3) Calcium hydroxide medicament and Sesame oil with Chitosan compared to sesame oil with chitosan.

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