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An In-Vitro Evaluation Of Anti Microbial Efficacy Of Chlorhexidine Gel And Copaiba Oil As An Intracanal Medicament Against E.Faecalis By Real Time Polymerase Chain Reaction

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Abstract

INTRODUCTION: The aim of this in-vitro study was to evaluate the anti-microbial efficacy of chlorhexidine gel and copaiba oil as an intra-canal medicament against enterococcus faecalis using real-time polymerase chain reaction (PCR)

METHODOLOGY: Extracted **40** human mandibular first premolars were taken and were divided into 3 groups; GROUP 1(n=10) -2% Chlorhexidine gel, GROUP 2(n=10) – 10% Copaiba oil and GROUP 3(n=10) -combination of 2% CHX+ 10% copaiba oil and1control group. The *E. faecalis* was incubated in in-vitro conditions for 7 days at 37°C. After which the samples were subjected to the molecular assay. Real time polymerase chain reaction test was done by placing a standardized suspension of Enterococcus faecalis on the test materials in a 96 well microtiter plate and the quantification of E.faecalis was done.

RESULTS: The results derived from kruskal Wallis test indicate comparison of bacterial counts in group1, 2, 3 and 4 were 0.0807, 3.1501, 0.2260 and 8.2793 respectively, and this showed a reduction in microbial load in both groups i.e. group 1 and group 3 and comparison of mean CFU's between MIC and MBC of copaiba oil at 40µl/10ml was 1455 CFU and at 150µl/10ml was 628 CFU and this showed that MIC of copaiba oil at 40µl was significantly higher where a significant amount of E.faecalis was reduced.

CONCLUSION: Under the conditions of this study, it can be concluded that, out of three groups, CHX group showed lowest bacterial count compared to the other groups followed by the combination of CHX and copaiba oil against E. faecalis. However, there is no significant difference between chlorhexidine gel and its combination with copaiba oil on E. faecalis. This shows that copaiba oil could represent a potential phytotherapeutic agent to be used against microorganisms causing endodontic infection

Keywords: Hyperhomocystenemia, ischaemic stroke, Atherosclerosis, Diabetes

INTRODUCTION

Microorganisms and their toxic metabolites play a major role in pulp and periradicular pathosis.[1] Hence, the aim of root canal treatment is to prevent reinfection and to eliminate bacteria from the infected root canal system.[2] In spite of the relatively high success rates of endodontic procedures, failures occur. The occurrence of failure in endodontic treatment is multifactorial. The common factors associated with failure are

persistence of bacteria, overextensions of root filling materials, inadequate obturation of the canal, improper coronal seal and procedural errors such as poor access cavity design, perforations, separated instrument, ledges, missed canals and untreated accessory canals. Among these causes, persistent microbial infection is one principle causative factor.[3] The high incidence of failure is attributed to microbial reinfection by facultative anaerobic microorganisms and E. faecalis is the predominant microorganism considered in secondary infections.

Enterococcus Faecalis (E. Faecalis) has been found in 38% of the failed root canal-treated teeth.4 The ability to tolerate the rough environmental changes which is believed to be due to its high alkali tolerance 5 and tubular invasion ability of this cocci which protects it from intracanal endodontic medicaments,has made E. Faecalis a treatment-resistant microorganism.6 Many researchers have shown that significant portions of the root canal walls remain untouched after mechanical instrumentation. Consequently, chemical irrigators and intracanal medicaments seem necessary for eradication of infected tissues and microorganisms in addition to mechanical debridement. 7

Another alternative root canal medication other than calcium hydroxide is chlorhexidine (CHX) gluconate with well-known broad-spectrum antimicrobial effects.8 CHX has been shown to be more effective in eliminating microorganisms like E. Faecalis which resisted against CH inside the dentinal tubules.9-11Several researchers have pointed to the potential advantage of chlorhexidine gluconate (CHX) as an antimicrobial medicament in endodontic therapy.

In the recent years, the search for new substances with pharmacological potential and biocompatibility, highlights the growing number of studies regarding the use of natural products in dentistry, with the development of materials with active biological properties for managing oral diseases. 12 Among them, copaiba oil increases the interest for its reported working biological properties. Copaiba oils are produced by exudation from the trunks of trees belonging to the Copaifera genus. They have anti-inflammatory, healing, anti-edematogenic, antitumor, trypanocidal, and bactericidal activities.13 The chemical composition of the copaiba extract varies according to the species, but it is basically composed of β-caryophyllenes, the main bicyclic sesquiterpene found which exhibits ample antifungal and antibacterial activities against all Streptococcus spp. strains found in previous studies among them,14 as well as copalic acid, caurenoic acid, covalenic acid and chlorhequinic acid, with the latter being natural diterpenes.

Culture based materials have been useful in identifying the bacteria or bacterial combinations that are responsible for various types of root canal infection.16 To overcome the limitations of culture-based analyses, molecular analyses have been adopted for investigating the microbial flora. Recent advances in cellular and molecular biological methods revealed real- time quantitative Polymerase Chain Reaction (PCR) is 10-100 times more sensitive.

Although previous studies have proven the antibacterial effect of chlorhexidine and copaiba oil, the action of concentration of copaiba oil and chlorhexidine gel against E.faecalis has not been explored. To our knowledge, there are no microbiological studies comparing the efficacy of copaiba oil and CHX gel alone and in combination. So the present study is designed to evaluate the antibacterial efficacy of the novel combination of copaiba gel and chlorhexidine gel as an intracanal medicament against E. faecalis through quantitative Real-time PCR technique.

MATERIALS AND METHOD

Forty freshly extracted, single rooted and single canaled human mandibular first premolars with fully formed apices, without curvatures that had been extracted for periodontal/prosthetic reasons were included in the study. The present study was conducted in the Department of Conservative Dentistry and Endodontics, The Oxford Dental College, Bangalore. Modified direct contact test was conducted in the Dextrose laboratories, Bangalore. The experimental strain used is ATCC29212, and was obtained from the Department of Microbiology, The Oxford Medical College, Bangalore.

TESTED MATERIALS

Two available root canal medicaments, 2%Chlorhexidie digluconate gel (cerkamed polland) and copaiba oil (Sigma Aldrich) were used in this study. Copaiba oil was prepared into 10% copaiba gel by mixing it with 0.2gm of methyl cellulose to obtain a gel consistency and was standardized to 10% by

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adding 30 mL of alcohol 96º, 20 mL of propylene glycol, 20 mL of polyethylene glycol, 20 mL of glycerin and 10 mL of copaiba oil. The, medicaments thus prepared were stored and used for the further study.

These medicated samples were incubated at 37°C for 7 days. After 7 days, the intracanal medicaments were neutralized from canals using neutralizers (Tween 80 and alpha lecithin). Bacterial samples from dentinal tubules was collected by drilling the apical ⅓ of the roots using #4 Gates Glidden drill to the depth of 400um.[10] Dentinal shaves was removed using sterile round burs and was sent to the lab to analyze the quantitative amount of live bacteria present in the dentinal tubule through q-Real time PCR technique After extracting the teeth, Bone, calculus and soft tissues on the root surfaces was slightly removed by means of a periodontal curette. These teeth were placed in 5.25% NaOCl for 1 hr in order to disinfect the root surfaces. The samples were then be stored in 0.9% physiological saline. The crowns were cut at the CEJ with a diamond disc in conjugation with the physiological saline irrigation and were standardized to 16mm. Apical patency of the canal was evaluated using # 15k file. The roots were instrumented 0.5 mm beyond the apex using the ProTaper Rotary system with 5.25% NaOCl irrigation, to size F3. The samples were placed in 17% EDTA ultrasonic bath for 4 mins to remove smear layer and flushing was done with 5.25 % NaOCl. To remove the remnants of both EDTA and NaOCl each tooth was rinsed in 10ml of physiological saline. Each tooth specimen was then placed in a microtube containing 2ml of Tryptic soy broth (TSB) and was autoclaved twice at 121°C, 15lb for 30 mins. After this, samples were stored in an incubator at 37°C for 24hrs. Sterile TSB was inoculated with 1ml of E.faecalis suspension. The tubes were then closed and incubated at 37°C for 48 hrs. Bacterial viability and purity was checked in randomly picked sample tubes. After that, samples were inoculated with 1x 10^8 cells/ml concentration of E. faecalis and were incubated for 48 hrs at 37°C.

Each sample was randomly divided into 3 groups and 1 control group, n=10 and each prepared medicament was injected into the infected sample roots from coronal to apical till it gets extruded from the apex. The experimental design employed the following experimental groups

Group I - 2% CHX gel (Cerkamed Polland)

Group II - 10% copaiba oil (Sigma Aldrich)

Group III - 2% CHX gel and 10% copaiba oil

Group IV - Control group (saline).

PCR PROTOCOL

Real-time PCR was performed using a StepOne, Applied Biosystems. Each PCR was performed in a total volume of 20 µl containing 2 µl of 10X FastStart DNA Master SYBR Green, 0.5µM each of HPLC purified forward and reverse primers (Table 2), and 1µl of template DNA. Real-time PCR was carried out with an initial incubation of 10 min at 95ºC followed by 35 cycles consisting of denaturing at 95ºC for 10 seconds; annealing at 60 ºC for 5 sec followed by amplification at 72ºC for 30 seconds. Amplification and detection were carried out in optical-grade 48 well plates in an ABI Prism Sequence Detection System. After amplification, a melting curve analysis was performed to determine the specificity of the PCR products. For melting curve analysis, PCR products were incubated for 15 seconds at 5ºC below the annealing temperature for the respective primers and the temperature was increased to 95ºC with a ramp rate of 0.1/s. All reactions were performed in duplicates against a serially diluted standard.

Amplicons of Efrt cloned into the plasmid was used as a standard for the quantification of the sample. Threshold cycle (Ct) analysis of all samples were either set at 0.5 relative fluorescence units or left to automatic detection by the system. Quantification analysis was performed using StepOne Plus software.

STATISTICAL ANALYSIS

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Kruskal Wallis test followed by Mann Whitney's post hoc analysis was performed to compare the mean RT-PCR values of E. faecalis between 4 study groups.

Wilcoxon Signed Rank test was used to compare the mean CFUs of E. faecalis between MIC & MBC concentrations of Copaiba oil.

The level of significance was set at P<0.05.

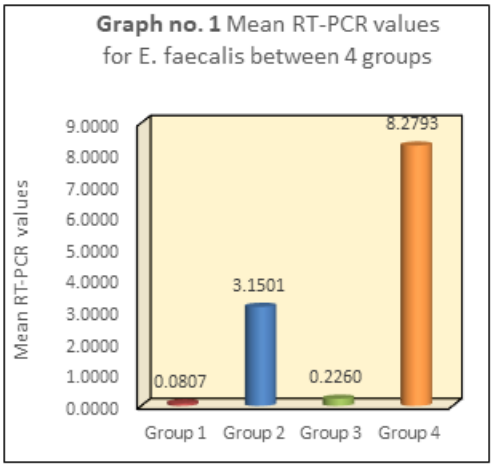
RESULTS

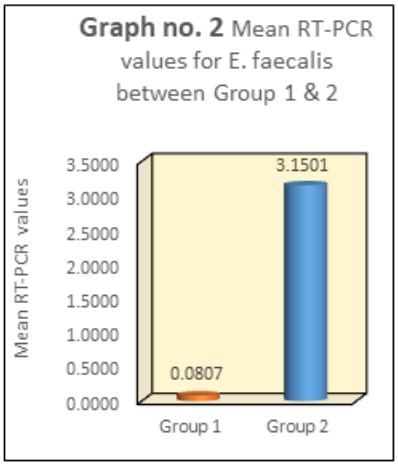
The test results demonstrate that the mean RT-PCR values for Group 1 was 0.0807 ± 0.0802, for Group 2 was 3.1501 ± 0.7807, for Group 3 was 0.2260 ± 0.1543 and for Group 4 was 8.2793 ± 0.0040. This mean difference in the RT-PCR values between 4 groups was statistically significant at P<0.001 (Refer graph no.1 and Table no. 1). Table no.1 illustrates the comparison of mean RT-PCR values for E. faecalis between 4 groups.

The test results showed that Group 1 showed significantly least mean RT-PCR values as compared to Group 2, 3 & 4 at P ≤ 0.001. This was followed next by group 3 showing significantly lesser mean RT-PCR values as compared to group 2 & 4 at P<0.001 and finally group 2 showing significantly lesser mean RT-PCR values as compared to group 4 at P<0.001. This infers that group 1 showed significantly least mean RT-PCR values, followed group 3 & 2 and highest with group 4. [Refer Graph no. 2 and Table no. 2].

Table no. 1 Comparison of mean RT-PCR values for E. Faecalis between 4 groups using Kruskal Wallis Test

Groups	N	Mean	SD	Min	Max	P-Value
Group 1	10	0.0807	0.0802	0.000	0.279	<0.001*
Group 2	10	3.1501	0.7807	1.542	4.660	
Group 3	10	0.2260	0.1543	0.041	0.613	
Group 4	10	8.2793	0.0040	8.271	8.286	





The test results demonstrate that the mean RT-PCR values of Group 1 [0.0807 ± 0.0802] was significantly lesser as compared to Group 2 [3.1501 ± 0.7807]. This difference was statistically significant at P<0.001

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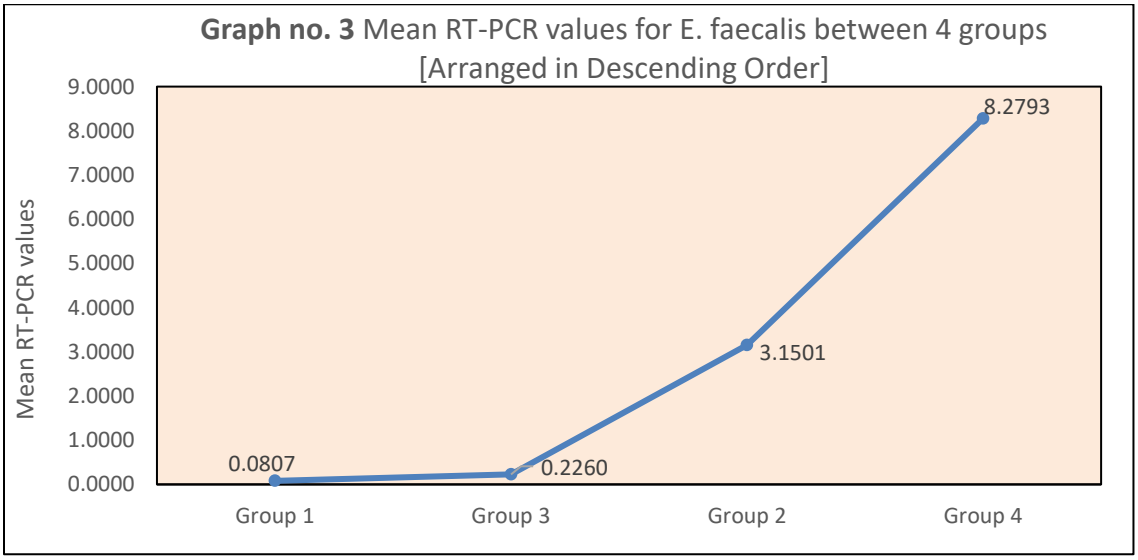
Table no. 2 Multiple comparison of mean difference in RT-PCR values for E. faecalis b/w 4 groups using Mann Whitney's Post hoc Test

(I) Groups	(J) Groups	Mean Diff. (I-J)	95% CI for the Diff.		P-Value
			Lower	Upper	
Group 1	Group 2	-3.0694	-3.4016	-2.7372	<0.001*
	Group 3	-0.1453	-0.4775	0.1869	0.001*
	Group 4	-8.1986	-8.5308	-7.8664	<0.001*
Group 2	Group 3	2.9241	2.5919	3.2563	<0.001*
	Group 4	-5.1292	-5.4614	-4.7970	<0.001*
Group 3	Group 4	-8.0534	-8.3856	-7.7212	<0.001*

Table no.2 illustrates the multiple comparison of mean differences in RT-PCR values for E. faecalis between 4 groups.



The mean CFUs of E. faecalis observed for Minimum Inhibition Concentration of Copaiba oil at 40 µl/10ml was 1455.00 ± 2285.92 & was significantly higher as compared to Minimum Bactericidal Concentration at 150

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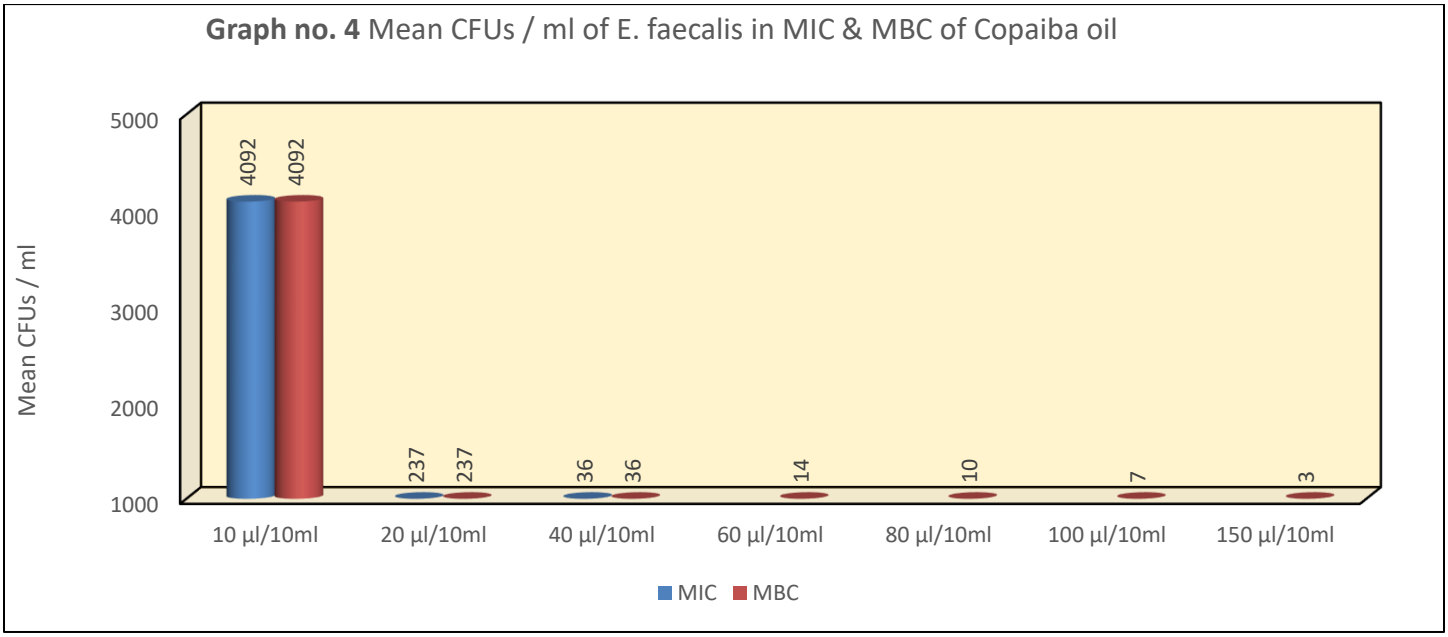
µl/10ml [628.43 ± 1529.59]. And the mean difference was statistically significant at P<0.001. [Refer Graph no. 4 and table no.3]. This infers that the MIC of Copaiba oil was at 40 µl/10ml and MBC was at 150 µl/10ml, where a significant amount of CFUs of E. faecalis was reduced by Copaiba oil.

Table no. 3 Comparison of mean CFUs between MIC & MBC concentrations of Copaiba oil using Wilcoxon Signed Rank Test

Parameter	Mean	SD	Min	Max	Mean diff	P-Value
MIC	1455.00	2285.92	36	4092	826.57	<0.001*
MBC	628.43	1529.59	3	4092		



The test results demonstrate that the mean RT-PCR values for Group 1 [0.0807 ± 0.0802] was significantly lesser as compared to Group 3 [0.2260 ± 0.1543]. This difference was statistically significant at P=0.00.**(Refer graph no. 5)**

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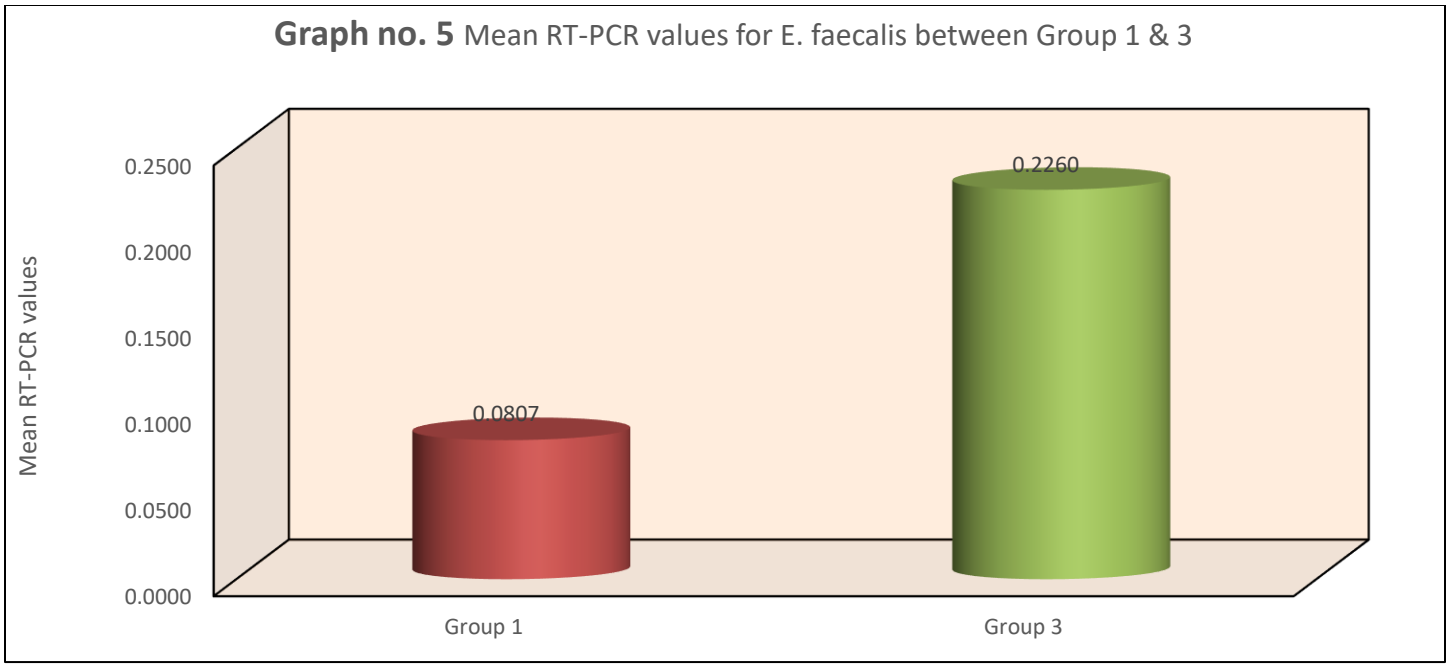

DISCUSSION

Endodontic failures can be attributable to inadequacies in shaping, cleaning and obturation, and also reinfection of the root canal system when the coronal seal is lost after completion of root canal treatment .18 To increase the efficiency of instrumentation, root canal irrigating solutions and intracanal medicaments are used to eliminate the bacteria from the root canals. 19 This can be ascribed to the usual inability of instruments and irrigants in cleaning and disinfecting anatomical variables, which are common in the apical portion of the root canals.20, 21

E. Faecalis is associated with persistent apical periodontitis and resists elimination from root canals.22 It has the capacity to proliferate in the deeper layers of dentine.23 Thus the penetration of medicaments into dentinal tubules was evaluated by polymwase chain reaction.The results showed that the most effective medicament against E. Faecalis was CHX gel. Studies have suggested that CHX gel is an effective intracanal medicament due to its broad antimicrobial spectrum, which is in agreement of the findings of the present study. Other studies also demonstrated that, 2% CHX gel was effective against E. Faecalis even 21 days after root dentine treatment.23 In the study by Dametto et al.24 2% CHX gel and 2% CHX liquid significantly reduced the number of E. Faecalis colonies.

In this study, 2 % CHX gel was used as it is demonstrated that 2% concentration is nearly 17 times stronger than 0.12% concentration.25 CHX in gel formulation has low toxicity on the periapical tissues, solubility in water as well as viscosity that keeps the active agent in contact with the root canal walls and dentinal tubules.26, 27. Even at the highest concentrations, it has very low toxicity. Also, it absorbs onto dental tissues and mucous membranes resulting in its prolonged gradual release at therapeutic levels.28 Since it is a positively charged hydrophobic and lipophilic molecule, it interacts with negatively charged phospholipids and lipopolysaccharides on the cell membrane of microorganism and enters the cell through some type of active or passive transport mechanism, which alters the osmotic equilibrium of the cells. This increases the permeability of the cell wall, allowing the CHX molecule to penetrate into the micro-organism, followed by leakage of intracellular constituents, particularly phosphate entities such as adenosine triphosphate and nucleic acids. It binds to hydroxyapatite and soft tissues, changing their electrical field to compete with microbial binding, thus decreasing microbial adherence. The result of the present study was similar to that of, Gomes et al.29, Krithikadatta et al.30 Basrani et al.31 and Vaghela et al.32, which showed that 2% Chlorhexidine gel produced a better antimicrobial action as compared to 0.2% Chlorhexidine gel or Calcium hydroxide mixed with 0.2%chlorhexidine.

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Copaiba oil-resin has been widely used and especially found in neotropical regions where bees of the Apis mellifera species are the main pollinating agents. There are records of copaiba oil-resin being used for almost 400 years, with several studies proving its innumerable biological activities, as it is effective against several microorganisms and commonly used in traditional medicine against various diseases.33 In dentistry, copaiba oil-resin is important due to its strong activity against oral bacteria and it can be used in appropriate formulations since the main oral diseases, caries and periodontal disease are strongly related to the dental biofilm.34 The antibacterial activity of copaiba oil appears to be related to the combination of sesquiterpenes and diterpenes, affecting the integrity of the bacterial cell wall.35,36 This action has been demonstrated in many pathogens, including gram negative and, mainly, gram-positive bacteria, such as Staphylococcus species and Streptococcus spp. Moreover, copalic acid (diterpene), extracted from Copaifera langsdorffii demonstrated bacteriostatic activity against P. gingivalis in the first 12 h and bactericidal activity between 12–24 h. Moreover, nanostructured emulsions based on the resin and essential oil of this species were shown to improve antimicrobial activities.37

In this study 10% copaiba oil was used as it demonstrated bacterial activity against S. aureus in MBC of 0.3125 mg/Ml109 and in an MIC of 0.78 ml/mL respectively.47 Moreover, 10% of copaiba oil was bacteriostatic (1.56-12.5%) against isolates of Paenibacillus spp., such as P. alginolyticus, P. pabuli, P. azotofixans, P. borealis, P. gluconolyticus, P. validus, P. thiaminolyticus, and P. larvae. Transmission electron microscopy revealed disruption and damage to the cell wall, resulting in the release of cytoplasmic compounds, alterations in morphology, and a decrease in cell volume, indicating that copaiba oil affects the cell wall.38

In this study a comparative analysis was done to evaluate the efficacy of intracanal medicaments like CHX and its combination with copaiba oil on E.faecalis, the results derived from Kruskal Wallis test indicate bacterial counts (Mean rank) in group 1 was 0.0807 and for group 2 was 3.1501, group 3 was 0.2260 and for group 4 it was 8.2793 respectively, this showed a reduction in microbial load in both groups i.e group 1 and group 3. Although the protocol used reduced E. faecalis levels, the bacterium was not totally eliminated from the canal. Siqueira JF, stated that This is due to the persistence of E. faecalis after intacanal medicament placement, and its ability to invade dentinal tubules, ability to survive in nutrient deprived environmental state and flourish when nutrient sources are regained, makes it hardtop completely eradicate the organism.39

The present study showed the comparison of mean CFU's between MIC and MBC of copaiba oil at 40µl/10ml was 1455CFU and at 150µl/10ml was 628 CFU and concluded that MIC of copaiba oil at 40µl was significantly higher as compared to MBC at 150µl where a significant amount of E.faecalis is reduced. (Table. 3 and Graph 5)This can be due to the presence of sesquiterpenes and diterpenes, affecting the integrity of the bacterial cell wall. This is in accordance with the study done by Santos et.all 35 and Gomes et.all36 about the antibacterial efficacy of copaiba oil on different microorganisms.

A multiple comparison of mean RT-PCR values was done using Mann Whitney's Post hoc test and the results showed that CHX showed significantly least mean RT-PCR values as compared to other groups. This was followed next by the group 3 i.e., the combination group which has CHX and copaiba oil in 1:1 ratio, which showed significantly lesser mean RT-PCR values as compared to the group 2 i.e., copaiba oil group and control group. Finally group 2 showed lesser mean RT-PCR values as compared to the control group (Table 2 Graph 2).In a study by Lima et al. (2006), he stated that the presence of the terpenoid in copaiba oil was responsible for its activity against Gram-positive bacteria, which is in accordance with the present study.40

Culture procedures have traditionally been used in the assessment of the microbiota associated with infections of endodontic origin. However, these procedures have some drawbacks such as underestimation of` the microorganisms living in a given ecosystem and it is difficult to simulate environmental conditions required for cultivation of fastidious microrganisms.41 PCR based detection methods enable rapid identification of both uncultivable and cultivable microbial species with high specificity and sensitivity.42 Thus, a more sophisticated and sensitive molecular technique like PCR was used in this study to assess the effect of intracanal medicaments against E. faecalis.

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Till date there is no sufficient data on the antimicrobial effect of the combination of these two medicaments i.e. Copaiba oil and CHX, and the present study has proved that the combination is effective against the tested microorganism. However, further in vivo studies have to be done, to rule out the unwanted effects when placed inside the root canal.

CONCLUSION

Within the limitation of this study it can be concluded that:

- The use of copaiba oil in combination with chlorhexidine gel in the ratio 1:1 has its effect on antibacterial activity.
- 2% chlorhexidine gel was more effective medicament followed by the combination of chlorhexidine with copaiba oil and then followed by then
- 10% copaiba oil alone against Enterococcus faecalis.
- The antibacterial efficacy of copaiba oil was less than chlorhexidine but its combination showed no significant difference with the chlorhexidine group against E. faecalis.

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