"COMPARISION OF ANTI-BACTERIAL EFFICACY OF CALCIUM HYDROXIDE, DICLOFENAC WITH CALCIUM HYDROXIDE AND IBUPROFEN WITH CALCIUM HYDROXIDE AGAINST *ENTEROCOCCUS FAECALIS* IN AN ENDODONTIC MODEL-AN *IN VITRO* STUDY"

By

Dr. DEEPTI ANCY CHACKO

REG.NO. IE0218003

Dissertation

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(BRANCH – IV)

Under the guidance of

Dr. NEHA DHADED M.D.S

DEPARTMENT OF CONSERVATIVE DENTISTRY AND ENDODONTICS

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KLE Academy of Higher Education & Research , Belagavi

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Place : Belagavi

Dr. DEEPTI ANCY CHACKO
REG.NO. IE0218003

KLE Academy of Higher Education & Research , Belagavi

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Date:

Place: Belagavi

Dr. NEHA DHADED M.D.S
Reader
Department of Conservative Dentistry
and Endodontics,
KAHER V.K. Institute of Dental Sciences,
Belagavi.

KLE Academy of Higher Education & Research , Belagavi

Karnataka

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Dr. JYOTI NAGMOTI **M.D., P.hD**
Professor
Department of Microbiology,
Jawaharlal Nehru Medical College,
Belagavi.

Date:

Place: Belagavi

KLE Academy of Higher Education & Research, Belagavi

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Head of Department

Dr. (Mrs.) PREETI DODDWAD M.D.S
Professor & Head,
Department of Conservative Dentistry and Endodontics,
KAHER V.K. Institute of Dental Sciences, Belagavi.

Date:

Place: Belagavi

Principal

Dr. (Mrs.) ALKA KALE M.D.S, Ph.D
Principal,
KAHER V. K. Institute of Dental Sciences, Belagavi

Date:

Place: Belagavi

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This dissertation is dedicated to

ALMIGHTY GOD,

My Parents,

&

My Brother

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Date: ***Dr.Deepti Ancy Chacko***
Place: Belagavi

ABSTRACT

Aim and Objectives: The purpose of this *In vitro* study was to evaluate and compare the antibacterial efficacy of Calcium hydroxide, Diclofenac with Calcium Hydroxide and Ibuprofen with Calcium Hydroxide against *E. faecalis*.

Study design: Sixty extracted human permanent maxillary central incisors which fulfilled the inclusion and exclusion criteria were selected for the study. De-coronation of samples was done at CEJ using a diamond disc to standardize the root length to 14 mm with continuous water coolant. A size 10 K-file was used and working length was established 1mm short of the length where 10 K-file exited the apical foramen. Cleaning and shaping were performed using Protaper Universal (DENTSPLY) nickel-titanium rotary instrument up to F3/F4, depending upon the apical gauging. 2ml of 3% Sodium Hypochlorite (NaOCl) was used as an irrigating solution after each instrument followed by 2 mL of 17% EDTA. The final irrigation was done using 5 ml of distilled water. After drying the canals with size 30 absorbent paper points, all external surfaces were made impermeable with nail varnish, except for coronal access. Teeth were then autoclaved at 121°C for 20 min. The prepared samples were placed in an Eppendorf tube for 14 days to inoculate the samples with the test organism i.e. *E.faecalis.* The first root canal sample S-1 was taken immediately after the inoculation of the specimen.

According to the placement of the medicaments, the samples were divided into three groups (n=20) -

GROUP 1: Calcium Hydroxide.

GROUP 2: Diclofenac with Calcium Hydroxide.

GROUP 3: Ibuprofen with Calcium Hydroxide.

After one week of the medicament placement, the canals were rinsed and cleaned with passive ultrasonic irrigation along with 10 ml of 17% EDTA, followed by 10 ml of 3% NaOCl and a final rinse with distilled water. Sample(S-2) was collected in the same manner as sample 1 for microbiological analysis.

Results: There was a statistically significant difference between groups (Calcium Hydroxide, Diclofenac with Calcium Hydroxide, and Ibuprofen with Calcium Hydroxide) based on the reduction in the log CFU in the post medicament samples ($p<0.05$) at 5% level of significance.

It was found that Diclofenac with Calcium Hydroxide group was superior to Calcium Hydroxide group and Ibuprofen with Calcium Hydroxide group.

Conclusion: Within the limitations of this *In vitro* study, Diclofenac with Calcium Hydroxide has shown better antimicrobial activity as compared to Calcium Hydroxide, and Ibuprofen with Calcium Hydroxide. Thus, Diclofenac with Calcium Hydroxide has shown promising results suggesting that it could be used as an alternative intracanal medicament.

Keywords: Antibacterial agents; anti-inflammatory nonantibiotics; Calcium Hydroxide; Diclofenac; Ibuprofen.

LIST OF ABBREVIATIONS

SR.NO	ABBREVIATIONS	FULL FORM
1	RCT	Root Canal Treatment
2	$Ca(OH)_2$	Calcium hydroxide
3	PUI	Passive ultrasonic irrigation
4	*et al*	Additional persons involved in the same study
5	*E. faecalis*	*Enterococcus faecalis*
6	Ni-Ti	Nickel Titanium
7	CEJ	Cemento-Enamel Junction
8	NSAIDs	Nonsteroidal Anti-Inflammatory Drugs
9	° C	Degrees Celsius
10	DW	Distilled Water
11	DMSO	Dimethyl sulfoxide
12	MIC	The mean minimum inhibition concentration
13	US scaler	Ultra-Sonic scaler
14	OSHA	Occupational Safety and Health Administration
15	ANOVA	Analysis of Variance
16	SD	Standard Deviation
17	N	Number of specimens
18	p-value	Probability of obtaining a test statistic at least as extreme as the one that was actually observed
19	WL	Working Length
20	MBC	The mean Minimum Bactericidal concentration
21	NaOCl	Sodium Hypochlorite

22	EDTA	Ethylene Diamine Tetra-acetic Acid
23	<	Less than
24	>	Greater than
25	CFU/ml	Colony-forming units per milliliter
26	mm	Millimetre
27	**%**	Percentage
28	ATCC	American type culture collection
29	BHI	Brain heart infusion
30	pH	power of hydrogen
31	w/w	Weight/Weight

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INTRODUCTION

Bacteria plays a pivotal role in the pathogenesis of apical periodontitis. It leads to pain and tenderness of the affected tooth, which subsequently requires root canal treatment RCT. For optimal treatment outcome complete elimination of bacteria is of utmost importance[1,2]. Cleaning and shaping along with antimicrobial agents lead to the reduction in the bacterial colonies present in the root canal system [3].

However, studies have shown chemo-mechanical instrumentation alone is unable to abolish the full bacterial colonies in the root canal system[4-7]. To achieve the above mentioned beneficial effect, intracanal medication is advocated which will lead to bacterial reduction [8.]

The gold standard intracanal medicament used during inter-appointment dressing is Calcium Hydroxide due to its therapeutic antibacterial along with its inherent biological properties. Despite having favourable clinical evidence, it has been reported that there is a correlation between $Ca(OH)_2$ placement with that of increased incidence of tooth fracture, principally reported in teeth with an open apex. Studies have even shown that $Ca(OH)_2$ is ineffective in recurrent and chronic infection cases [10,11].

The most habitually encountered bacteriain recurrent and chronic infection cases is *E.faecalis*. Various studies have shown their colonization inside the root canal system as isolated infections. This kind of colonization is most commonly seen in failed RCT cases. It has also been reported that they can infiltrate dentinal tubules to a deep magnitude with a predisposition to form biofilms. This enables it to escape endodontic instrumentation and irrigation. It also renders it impervious to

$Ca(OH)_2$ due to its potential to combat pH values on the higher side. *E.faecalis* possesses the abilitytoincrease its virulence by inheriting traits such as antibiotic resistance by plasmid transfer [12.]

As stated, the thorough elimination of bacteria is essential for a favourable prognosis following an RCT[13-15]. Studies have shown, that with a qPCR-negative result, healing is seen in up to 79% of RCT cases [15]. Contrary to this, canals which were qPCR-positive exhibited a decreased healing rate of 45% [15.] Hence, there is a need to improve the efficacy of $Ca(OH)_2$. To attain improvement in the anti-bacterial effect of $Ca(OH)_2$, additions such as NSAIDs and antibiotics have been advocated for a favourable treatment outcome [16].

The conventionally used class of analgesics NSAIDs– may possess additional therapeutic properties such as anti-bacterial efficacy through inhibition of bacterial DNA synthesis or impairment of membrane activity [17-20]; prevention of bacterial colonization and biofilm formation by interfering with quorum sensing of bacteria [21]; and anti-plasmid activity[22]. This finding is merely one in the long line of research investigating the antibacterial effects of non-antibiotic agents, commencing with Ehrlich's (1854–1915) discovery of the antibacterial effects of the Phenothiazine compound, methylene blue dye. The term non-antibiotics was coined by Kristiansen and Amaral (1997) to refer to drugs developed to treat noninfectious diseases but have shown to exhibit anti-microbial activity [23].

Other anti-inflammatory agents such as Corticosteroids (Ledermix) are already in use as inter-appointment intracanal medicaments. They have shown efficacy in reducing post-operative pain compared to $Ca(OH)_2$ [24], but they may compromise host immune response [25]. Further studies have shown that long term exposure of the

aqueous tetracycline component present in Ledermix causes enamel discolouration [26.] It has been established that 1-25 hrs of enamel exposure to this aqueous tetracycline component in long treatment protocols reduces the microhardness of enamel [26].

Till present there is no single antibiotic that is effective against all types of microorganisms present within the diseased tooth, therefore local antibiotics are not routinely recommended [27.] Studies have reported that routinely used antibiotics cause teeth discoloration due to the presence of minocycline, making it un-esthetic when it is used in the treatment of anterior teeth[28, 29]. Furthermore, it is also seen that in regenerative endodontics and non-surgical management of large periapical lesions, the longterm use of triple antibiotic paste causes a negative impact on radicular dentin by increased demineralization and reduction in microhardness of the dentin [26,30.]

Therefore, incorporating commonly prescribed NSAIDs such as Diclofenac or Ibuprofen as a component in inter-appointment dressing has the potential to utilize beneficial effects of NSAIDs i.e anti-inflammatory action, local analgesia, and potential anti-bacterial action, and also to improve on the properties of $Ca(OH)_2$ (limited anti-bacterial action).

There are very limited studies done which have evaluated the anti-bacterial efficacy of NSAIDs with $Ca(OH)_2$ against *E.faecalis*. Considering all the above-mentioned concepts, this study was formulated to evaluate and compare the antibacterial efficacy of NSAIDs with $Ca(OH)_2$ against *E.Faecalis*.

AIM AND OBJECTIVES

AIM

To evaluate and compare the antibacterial efficacy of three different intra-canal medicaments Calcium hydroxide, Diclofenac with Calcium Hydroxide and Ibuprofen with Calcium Hydroxide against *E. faecalis*.

OBJECTIVES

- To determine the minimum bactericidal concentration (MBC) of Diclofenac with Calcium Hydroxide and Ibuprofen with Calcium Hydroxide.
- To assess the antibacterial activity of Calcium Hydroxide, Diclofenac with Calcium Hydroxide and Ibuprofen with Calcium Hydroxide against *E.faecalis*using semi-quantitative analysis.
- To compare the antibacterial activity of Calcium Hydroxide, Diclofenac with Calcium Hydroxide and Ibuprofen with Calcium Hydroxide as an intra-canal medicament against *E. faecalis*.

HYPOTHESIS

NULL HYPOTHESIS: -

There is **no difference** in the antibacterial efficacy of Calcium Hydroxide, Diclofenac in combination with Calcium hydroxide and Ibuprofen in combination with Calcium Hydroxide when used as an intra-canal medicament.

ALTERNATE HYPOTHESIS: -

There is a **difference** in the antibacterial efficacy of Calcium Hydroxide, Diclofenac in combination with Calcium Hydroxide and Ibuprofen in combination with Calcium Hydroxide when used as an intra-canal medicament.

REVIEW OF LITERATURE

1. A clinical review on the current concepts of root canal infection and the consequences for endodontic treatment has emphasized the usage of intracanal medicament in cases of apical periodontitis. The review emphasizes the various fruitful and favourable properties of the medicament that renders the root canal system bacteria-free resulting in better treatment outcome [32.]

2. A study was done to evaluate the antibacterial effect of calcium hydroxide paste in distilled water as an intracanal medicament in twelve cases. It was found that 54.5 % of the cases became asymptomatic and sterile in 4 visits, 27.2 % in 7 visits, while 18.20 % were failures. It was concluded that calcium hydroxide could be successfully used as an intracanal medicament in highly infected canals [33].

3. Antibacterial activity of 2 formulations of calcium hydroxide pastes (Calen™ and Calen with p-chlorophenol- PMCC-Calen™) on *E.faecalis* grown in contact with dentinal walls of root canal systems was evaluated. The results of the study concluded that intracanal usage of Calcium Hydroxide pastes after chemo-mechanical preparation of root canals, were capable of eliminating *E. faecalis*, which is the most resistant bacteria known for re-treatment cases[34.]

4. An *In vivo* study was done to evaluate the antibacterial efficacy of Calcium Hydroxide when used as an intracanal medicament for a short period of time. The study showed that a 10-minute application of the dressing was ineffective, when the same was applied for seven-days, it was able to eliminate the bacteria that has survived the biomechanical preparation of the canal. After chemomechanical preparation of the root canals, the presence of bacteria was

seen in 9 out of the 18 canals which were dressed with Calcium Hydroxide for a period of seven days and in 6 of the 12 canals treated with Calcium Hydroxide for around 10 minutes. No bacteria were found in tests following the removal of the dressing among the 18 canals that had been dressed for one week, neither from samples which had been sealed without an antibacterial dressing for an extended week(1-5 weeks). Application of Calcium Hydroxide for 10 minutes was ineffective in 6 root canals since bacteria persisted at the second appointment [35.]

5. An *In-vitro* study was done to check the long term effect of Calcium Hydroxide dressing. The review tells us about the various beneficial antibacterial properties of $Ca(OH)_2$ when used in root canal treatment as an intracanal medicament. Furthermore, it explains the reason for the antimicrobial effect of $Ca(OH)_2$, which is due to the release of hydroxyl ions when it comes in contact with aqueous fluids. Even though $Ca(OH)_2$ shows effectiveness against a wide range of endodontic pathogens, but it's efficacy against *Enterococcus faecalis* and *Candida albicans*is effectively less. The studies end with the conclusion that the addition of vehicles or other agents might contribute to an increase in the antimicrobial effect of $Ca(OH)_2$[36].

6. A meta-analysis study was done in patients undergoing root canal treatment for apical periodontitis, to determine the antibacterial activity of Calcium Hydroxide when used as an intracanal medication. Eight studies were chosen and added in the review, covering 257 cases with a sample size of 18 to 60 cases. A statistically significant difference between pre and the post-medicated canal was seen among six studies. The study concluded that Calcium

Hydroxide when assessed by culture techniques, has limited efficacy in bacteria eradication from the root canal [37].

7. An *In-vitro* study was done which stated that the teeth treated with Calcium Hydroxide as an intracanal medicament were more susceptible to fracture. Edge chipping was the test of choice used to measure the resistance of brittle materials to fracture. Chip resistance of the teeth may depend on both the factors i.e. fracture resistance and the hardness of dentin. The study uses an edge chipping test to demonstrate the fracture resistance of human dentin exposed to calcium hydroxide for up to 60 days. Twelve teeth that were recently extracted were divided into three experimental groups and a control group with varying calcium hydroxide exposures. All the teeth used in this study underwent pulpectomy via a standard protocol. The study expected the $Ca(OH)_2$ treated teeth to be more susceptible to fracture in comparison to the control group but the results showed that the edge chip resistance was improved in all Calcium Hydroxide exposed test groups. The reason for the increase could be due to change in the molecular structure of the dentin which increased fracture resistance of the dentin. Another possible explanation could be that increase in chip resistance in the earlier study might be due to other forms of a mechanism other than chipping [38. .]

8. An *In-vitro* examination was done among Diclofenac, Ibuprofen, Calcium Hydroxide and Amoxicillin to evaluate their antibacterial effect against *E. faecalis*. The Agar diffusion test and tube dilution method were the tests of choice to evaluate the antibacterial activity of materials. Mixtures of 400 mg/ml of materials were prepared. 10 Muller-Hinton agar culture plates were

used onto which the bacteria were seeded. In each well punched in agar plates, 30 microliters of each test material were placed. Once incubation was done, the zone of bacterial inhibition was measured. The Agar dilution method was used to find Minimum inhibitory concentration (MIC) of the test materials. The results showed the greatest antibacterial activity by antibiotics (Amoxicillin, Gentamycin) followed by NSAIDs (Ibuprofen, Diclofenac) and $Ca(OH)_2$ failed to show any antibacterial activity. Distinct antibacterial activity was shown by Diclofenac and Ibuprofen against *E. faecalis* in concentrations of 50 µg/ml and above [39].

9. Another study was done in an endodontic model to investigate and compare the anti-bacterial effectiveness of intracanal medicaments Ibuprofen, Diclofenac, and Calcium Hydroxide against *E.faecalis*. The study included a total of 76 single-rooted mandibular premolar teeth which were decoronated and instrumented up to F4-ProTaper rotary. The roots were subjected to autoclave (121°C for 20 min), placed in Eppendorf tubes, and contaminated with *E. faecalis* for 14 days. Paper point sampling was used to record the Colony-forming unit (CFU) counts before (CFU-1), and after intracanal medication (CFU-2). Distilled water (1:1 w/v) was mixed with Group-1: Ibuprofen, Group-2: Diclofenac, Group-3: $Ca(OH)_2$, which was then placed into root canals, temporarily sealed and incubated (37°C; 7 days). Group-4 being the control group, received no medicament. The results demonstrated a greater antibacterial effect against *E.faecalis* by the anti-inflammatory nonantibiotics (Ibuprofen, Diclofenac) [40].

10. An *In-vitro* study was done to evaluate and compare the different combination of NSAIDs and whether they altered the pH of Calcium Hydroxide. The groups analyzed were group 1: Calcium Hydroxide paste with Propylene Glycol, group 2: Calcium Hydroxide paste with Propylene Glycol + 5% Diclofenac Sodium, group 3: Calcium Hydroxide paste with Propylene Glycol + 5% Ibuprofen, group 4: Calcium Hydroxide paste with Propylene Glycol + 5% Ciprofloxacin and group 5: positive control (without medication) The pH was measured with a calibrated pH meter, at time intervals of 3, 24, 72, and 168 hours. The biofilm was induced in 30 bovine dentin blocks for 21 days, for microbial analysis. The pastes were placed on the blocks with biofilm for 7 days. The specimens were analyzed with a laser scanning confocal microscope. Results demonstrated statistically significant differences ($P < .05$) in comparison with the positive control [41].

11. A review article stated that the process of the formulation was greatly influenced by the solubility properties of drugs. The problem of solubility could be solved by different approaches. To improve the solubility of poorly water-soluble drugs, the complexation technique had been employed. Cyclodextrin can interact with appropriate size drug molecules which lead to the formulation of inclusion complexes. To determine the rightful utilization of cyclodextrins as complexing, solubility enhancing agents, a comprehensive literature survey was made. This review discusses the various complexation techniques and highlights the applications with these approaches [42].

12. An *In-vivo* study was done on asymptomatic apical periodontitis cases to evaluate and compare the effectiveness of $Ca(OH)_2$paste against microorganisms when combined with Ibuprofen and Ciprofloxacin. The study comprises of 45 patients who were subdivided into three groups on the basis: $Ca(OH)_2$: 1 gm$Ca(OH)_2$ powder with 1 mL propylene glycol, $Ca(OH)_2$ + Ibuprofen: 50 mg of ibuprofen was added into 950 mg $Ca(OH)_2$ powder and mixed with 1 mL propylene glycol, $Ca(OH)_2$ +Ciprofloxacin: 50 mg of Ciprofloxacin was added into 950 mg $Ca(OH)_2$ powder and mixed with 1 mL propylene glycol. The microbiological samples were taken before (S1) and after chemo-mechanical procedures (S2). The intracanal medicaments were placed using K-file after canals were prepared. The second visit of patients was scheduled after 7 days post-medicament wherein the medicament was removed mechanically and the samples (S3) were collected. The bacterial counting was done using a quantitative real-time polymerase chain reaction. The quantitative reduction from S1 to S3 and from S2 to S3 of Ca(OH)2 + Ciprofloxacin was superior when compared to other groups($P < 0.05$)[43].

13. A Systematic review was done to study *Enterococcus faecalis,* which is the most common bacteria seen in re-treatment cases. Two databases, PubMed and Google Scholar were searched using specific inclusion and exclusion criteria in this Systemic review. Among 2943 studies that were found, only 11 met the inclusion criteria, and these 11 were included in the review for further analysis. The 11 studies showed a prominent distribution of *Enterococcus faecalis* within the root canal. The study investigated various aspects of *Enterococcus faecalis* and showed its association as the primary pathogen with

endodontic treatment. Moreover, the *Enterococcus faecalis* has the characteristic properties to escape the disinfection means[44].

14. An *In-vitro* study was done to evaluate and compare the dentinal tubule penetration of Calcium Hydroxide(CH) and triple antibiotic paste(TAP) when performed with distilled water (DW) or a low surface tension liquid (i.e. propylene glycol(PG)). A total of 40 premolars were taken and were standardized to 14 mm root length. The roots were randomly divided into 4 groups (n = 10) according to the root canal medicaments and the vehicles used: group 1:TAP + DW, group 2: TAP + PG, group 3: CH + DW, and group 4:CH + PG. Using a Lentulo spiral the root canal medicaments were applied into the root canals once they were labeled with 0.1% rhodamine. The specimens were placed into acrylic blocks, after which 1-mm sections were taken from the middle third of the root. The samples were studied under a confocal laser scanning microscope. The results showed that CH had a lower penetration area than TAP regardless of the vehicle used ($P < .05$)[45].

MATERIALS AND METHODS

SOURCE OF DATA:

The study was conducted in the Department of Conservative Dentistry and Endodontics,

V K Institute of Dental Sciences, KLE Academy of Higher Education & Research, Belagavi Karnataka.

Laboratory procedures were carried out in the College of Pharmacy, KAHER Belagavi Karnataka.

The microbiological study was done at:

- Jawaharlal Nehru Medical College, Department of Microbiology, KAHER Belagavi Karnataka.
- Dr.PrabhakarKore Basic Science Research Center, KLE Academy of Higher Education and Research, Belagavi.

INCLUSION CRITERIA:

- Extracted human single-rooted single canal maxillary anterior teeth with patent canals.
- Teeth with apical width corresponding to #20 K-file or less.
- Teeth with straight canals.

EXCLUSION CRITERIA:

- Teeth with Carious lesions.
- Teeth with apical width more than #20 K-file size.
- Teeth with calcified canals.
- Teeth with fracture/crack or a restoration.
- Teeth with anatomic variations.

ARMAMENTARIUM USED FOR THE STUDY

The following armamentarium was used in the study. The armamentarium used was divided into the following steps as follows:

[A] Armamentarium for MIC, MBC, and microbiological processing

- Diclofenac powder(College of Pharmacy, KAHER Belagavi)
- Ibuprofen powder(College of Pharmacy, KAHER Belagavi)
- ATCC strains of *Enterococcus faecalis* ((ATCC 29212)
- Weighing scale (Citizen CY220, Citizen Scale(I) Pvt. Ltd., Malad, Mumbai)
- Eppendorf tubes (Tarsons Products Pvt. Ltd., West Bengal)
- Pipette with micro pipette tips (Tarsons Products Pvt. Ltd., West Bengal)
- Brain heart Infusion (BHI) Broth(HiMedia laboratories Pvt Limited, Mumbai)
- Incubator (Yorco, York Scientific Industries, India)
- Blood Agar plates
- Platinum Inoculum loops
- Electric loop sterilizer (HiMedia laboratories Pvt Limited, Mumbai)
- Thioglycolate Broth ((HiMedia laboratories Pvt Limited, Mumbai)

[B] ARMAMENTARIUM FOR COMBINATION PREPARATION

- Beaker (Borosil Glass Works Limited, Mumbai)
- Whatman Qualitative filter paper (Sigma- Aldrich Co., USA)
- Petri dish
- Spatula
- Weighing scale (Citizen CY220, Citizen Scale(I) Pvt. Ltd., Malad, Mumbai.
- Diclofenac powder (College of Pharmacy, KAHER Belagavi)
- Calcium Hydroxide (College of Pharmacy, KAHER Belagavi)
- Ibuprofen powder (College of Pharmacy, KAHER Belagavi)
- β Cyclodextrin (College of Pharmacy, KAHER Belagavi)
- Ethanol (College of Pharmacy, KAHER Belagavi)
- Distilled water (BenzerMultitech India Private Limited, Pune, India)

[C]Armamentarium for *In-vitro* study

- Human permanent maxillary central incisors teeth (n=60)
- 0.1% Thymol solution (S D FINE-CHEMICALS LIMITED, MUMBAI)
- 3% Sodium hypochlorite (VISHAL DENTOCARE, AHMEDABAD)
- Distilled water (NICE LIF Intracanal medicament- Diclofenac with Calcium Hydroxide (1mg) Calcium Hydroxide (1mg) Ibuprofen with Calcium Hydroxide(1mg)
- 17% EDTA (CANALARGE,AMMDENT PUNJAB)
- Paper points (DIADENT GROUP INTERNATIONAL, KOREA)
- Cavit G - (3M, ESPE GERMANY)
- Endoaccess bur (DENTSPLY, SWITZERLAND)
- 20 K file (MANII INC, JAPAN)

- Protaper Universal nickel-titanium files (DENTSPLY MAILLEFER, SWITZERLAND)
- Airotor (NSK, JAPAN)and Endomotor (DENTSPLY X-SMART)
- Micromotor (NSK, JAPAN)
- Lentulospiral (No-25) (MANI INC, JAPAN)
- 5ml 27-gauge syringe (DISPOVAN, HINDUSTAN SYRINGES LTD FARIDABAD)
- Diamond disc
- Autoclaved (Confident Dental EquipmentsPvt. Ltd., India)
- Pluggers (Sybron endo)
- Tweezer
- Cement spatula
- Contra angle micromotorhandpiece (NSK, Japan)
- RC Prep (Premier Dental)

METHODOLOGY:

To get an effective and validated concentration of the medicament Minimum inhibitory concentration and Minimum bactericidal concentration test were done against *Enterococcus faecalis*.

A) DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION -

BROTH DILUTION METHOD: [72]

- Minimum Inhibitory Concentration (MIC) was assessed against the ATCC 29212 strain of *Enterococcus faecalis*.
- Inoculum of standard strains of organisms was prepared as per 0.5 McFarland Standard.

Methodology:

Diclofenac and Ibuprofen were taken in combination with Calcium Hydroxide and weighed. These drugs were dissolved in 0.5 ml of Dimethyl sulfoxide(DMSO). Ten graduated micro-centrifuge tubes were taken for the study after being sterilized. These microcentrifuge tubes were designated with numbers from 1 to 10. All the autoclaved centrifuge tubes were arranged systematically following their designated number.

Vertical laminar flow was used to follow the Standard Operating Protocols. Minimum inhibitory Concentration procedure was carried out in the Vertical Laminar flow. For the MIC test, 200µL of plain Brain Heart Infusion (BHI) broth was added to all the 10 tubes with the help of micropipette, this was followed by the addition of 200µL of respective test medicament to the 1st tube. The broth and the test medicament were thoroughly mixed using the micropipette. From the 1st tube 200µL was pipetted and added to the 2nd tube. Again from the 2nd tube 200µL was pipetted

and added to the 3rd tube. This tube dilution method was repeated until the tube 9th tube. For the 9th tube, 200µL was pipetted and discarded. Here, the 1st tube is acting as a positive control and the 10th tube is a negative control. Finally, 100µL of inoculum of *E. faecalis* (culture) was added to all the 10 tubes.

All the graduated microcentrifuge tubes were incubated in a CO_2 jar for 48 hours. The MIC was taken as the lowest concentration that prevented the growth of the bacteria for both the groups. To confirm the inhibitory concentration, each of these serial dilutions was plated on blood agar culture plates under laminar airflow for both Group II (Diclofenac with $Ca(OH)_2$ group) and Group III (Ibuprofen with $Ca(OH)_2$ group). The culture plates were incubated for 24 hours. Colony-forming units were recorded after the respective times of incubation with the help of Eliza Reader along with manual counting of the colonies.

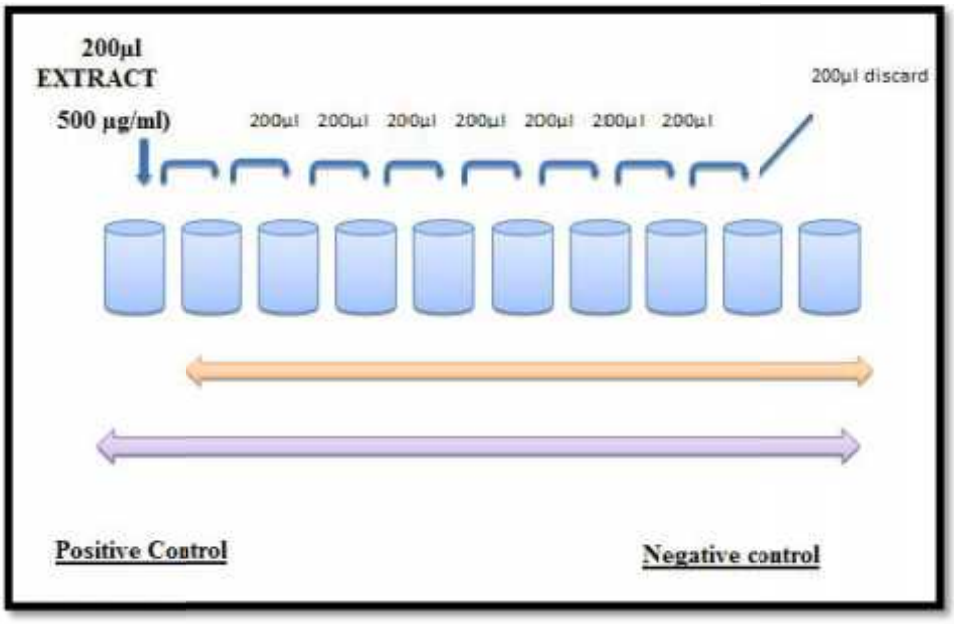


Fig.1 The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal concentration (MBC) for Diclofenac with Calcium Hydroxide (Group 2) and Ibuprofen with Calcium Hydroxide (Group 3) carried out against *E. faecalis*

MIC Results

E.faecalis	**200µl**	**100**	**50**	**25**	**12.5**	**6.25**	**3.125**	**1.56**	**0.78**	**0.39**
D+CH	S	S	S	R	R	R	R	R	R	R
I+CH	S	S	R	R	R	R	R	R	R	R

- S-Sensitive R-Resistant
- Sensitive- the organism is inhibited by the concentration of the drug that is achieved using the usual dosage.
- Resistant- the organisms are resistant to the usually achievable serum drug levels
- MIC was noted by the visual turbidity of the tubes both before and after incubation.
- MIC is the lowest concentration which prevents visible growth of the organism.
- Sensitive implies that the organism is inhibited by the concentration of the drug that is achieved using the usual dosage.

MBC Results

E.faecalis	200µg/ml	100	50	25	12.5	6.5	3.125	1.56	0.78	0.39
D+CH	00	00	00	10	30	50	80	120	150	200
I+CH	02	02	03	20	20	30	40	50	80	150

- MBC was noted by the absence of bacterial growth in agar plates both before and after incubation.

PREPARATION OF MEDICAMENT

The medicament was prepared in the Department of Pharmaceutics, College of Pharmacy, KLE Academy of Higher Education and Research, Belagavi

COMPLEXATION METHOD

The intracanal medicament that was taken for this study was not water-soluble, Therefore the complexation method was chosen to make the medicament's more water-soluble. [42]Complexation is an extensively used technique in the pharmaceutical field to improve the solubility of several pharmaceutical ingredients, to increase the bioavailability of poorly water-soluble drugs. Cyclodextrin is used to increase the solubility of the water-insoluble drug through inclusion complexes formulation. The mentioned compound has a hydrophobic cavity that produces inclusion complexes by trapping a variety of molecules within it. The advantages of drug complexes with cyclodextrin are increased solubility, enhanced bioavailability, improved stability, masking of bad test or odour, reduced volatility reduced side effect, the possibility of a drug release system. In the present study, test medicaments

were mixed with β cyclodextrin, which is an (α-1,4)-linked oligosaccharides of α-d glucopyranose and organic solvent. The obtained mixture was kept in a hot air oven for 2 hours for drying. The dried samples were scrapped and collected for use.

For the *In-vitro* study Diclofenac with $Ca(OH)_2$ and Ibuprofen with $Ca(OH)_2$ratio was decided based on the MIC and MBC values of the combination of Diclofenac and Calcium Hydroxide, Ibuprofen and Calcium Hydroxide.

The Diclofenac with $Ca(OH)_2$ was prepared in 33.3 %w/w.

The Ibuprofen with $Ca(OH)_2$was prepared in 58.3 w/w.

$Ca(OH)_2$ was prepared in 72.2%w/w.

Preparation of bacterial sample: Test Microorganism

- The facultative strain of *Enterococcus faecalis* (ATCC 29212) were grown on brain heart infusion agar plate (with 5% defibrinated sheep blood). Microorganisms were subcultured on nutrient agar medium to confirm their purity. The facultative strain of *Enterococcus faecalis* was inoculated individually into a tube containing 5ml of sterile 85% saline. The suspension was adjusted spectrophotometrically at 800nm to match the transmittance of 90T (equivalent to 0.5 McFarland scale =1.5x108 C.F.U).

METHODOLOGY

- Sixty permanent human single-rooted maxillary central incisors were chosen and maintained conferring to OSHA (Occupational Safety and Health Administration) guidelines. Calculus and soft tissue debris were removed with an ultrasonic scaler and 0.1% Thymol(S D FINE-CHEMICALS LIMITED, MUMBAI) was utilized as storage solution until use.

- **SAMPLE PREPARATION**

Decoration of samples was done at CEJ using a diamond disc to standardize the root length to 14 mm with continuous water coolant. A size 10 K file was used and working length was established 1mm short of the length where 10 K file exited the apical foramen. Cleaning and shaping were performed using Protaper Universal (DENTSPLY) nickel-titanium rotary instrument up to F3/F4, depending upon the apical gauging. 2ml of 3% Sodium Hypochlorite (NaOCl) was irrigated after each instrument followed by irrigation with 2 mL of 17% EDTA and was allowed to remain for 1 min. The final irrigation was done using 5 ml of distilled water. After drying with size 30 absorbent paper points, All external surfaces were made impermeable with nail varnish, except for coronal access. Teeth were then autoclaved 121°C for 20 min. The prepared samples were placed in an Eppendorf tube for 14 days to inoculate the samples with the test organism i.e*E.faecalis*.

Collection of Samples

The first root canal sample S-1 was taken immediately after the inoculation of the specimen. The procedure that was followed -Glucose broth was injected into the root canal and a size 10 or 15 K- H file was pumped circumferentially to 1mm short of the estimated working length. Test tubes containing samples were incubated at

37^0C for 30 minutes and shaken vigorously in a vortex mixer for 60 seconds. Before inoculation samples were subjected to serial ten-fold dilution in Brain heart infusion broth. 10µL of the sample was inoculated on blood agar and incubated aerobic chamber for 24 hours. The colony-forming units were counted.

The number of colonies was counted by semi quantitation [31]. The colony count was represented as

No. of colonies x 1000 = Colony forming units (CFU)/ml

For the intracanal medicament placed, 60 specimens were divided randomly divided into groupsof 20 each

Group 1- $Ca(OH)_2$

Group 2-$Ca(OH)_2$ + Diclofenac

Group3- $Ca(OH)_2$ + Ibuprofen

Mixing of Test Medicaments

The medicament powder obtained from the pharmacy was mixed with saline in a 1:1 ratio.The prepared pastes of the medicament were carried into the canal using lentulospiral (Mani Inc, Tachigi-ken, Japan) and condensed with hand pluggers (Sybron endo). Cavit G was placed to seal the specimen. The specimens were stored in a humidifier at 37^0C in 100% humidity for 7 days to simulate clinical conditions. After one week, the canals were rinsed with 10 ml of 17% EDTA followed by 10 ml of 3% NaOCl and final irrigation with distilled water and passive ultrasonic irrigation.

Sample(S-2) was collected in the same manner as sample 1 for microbiological analysis [31].

FLOWCHART DEPICTING THE STUDY DESIGN

Sixty permanent human single-rooted maxillary central incisors were chosen and maintained conferring to OSHA guidelines

↓

Calculus and soft tissue debris were cleaned using US scaler and immersed in 0.1% Thymol.

↓

De-coronation of samples was done at CEJ using a diamond disc to standardize the root length to 14mm with continuous water coolant

↓

WL was established 1mm short of the length where 10 K file exited the apical foramen.

↓

Cleaning and shaping were performed using Protaper Universal (DENTSPLY) NiTi rotary instrument till F3/ F4.

↓

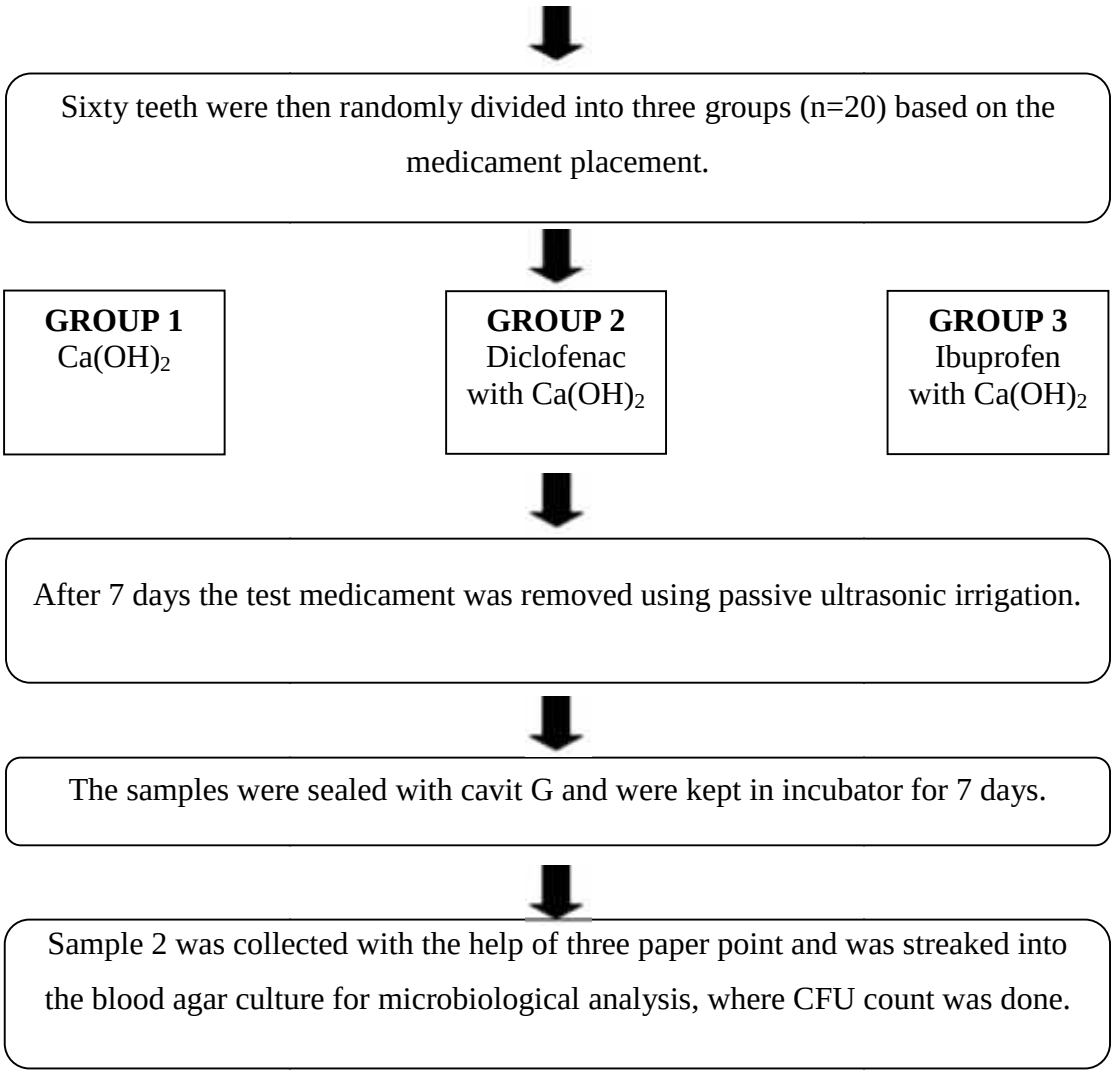
2ml of 3% Sodium Hypochlorite (NaOCl) was irrigated after each instrument followed by irrigation with 2 mL of 17% EDTA and allowing it to remain for 1 min.

↓

The final irrigation was done using 5 ml of distilled water and dried with size 30 absorbent paper points

↓

The root canal were inoculated with *E faecalis* for 14 days. Sample 1 collection was taken with the help of three paper point for microbiological analysis


Sixty teeth were then randomly divided into three groups (n=20) based on the medicament placement.
GROUP 1
Ca(OH)2
GROUP 2
Diclofenac with Ca(OH)2
GROUP 3
Ibuprofen with Ca(OH)2
After 7 days the test medicament was removed using passive ultrasonic irrigation.
The samples were sealed with cavit G and were kept in incubator for 7 days.
Sample 2 was collected with the help of three paper point and was streaked into the blood agar culture for microbiological analysis, where CFU count was done.

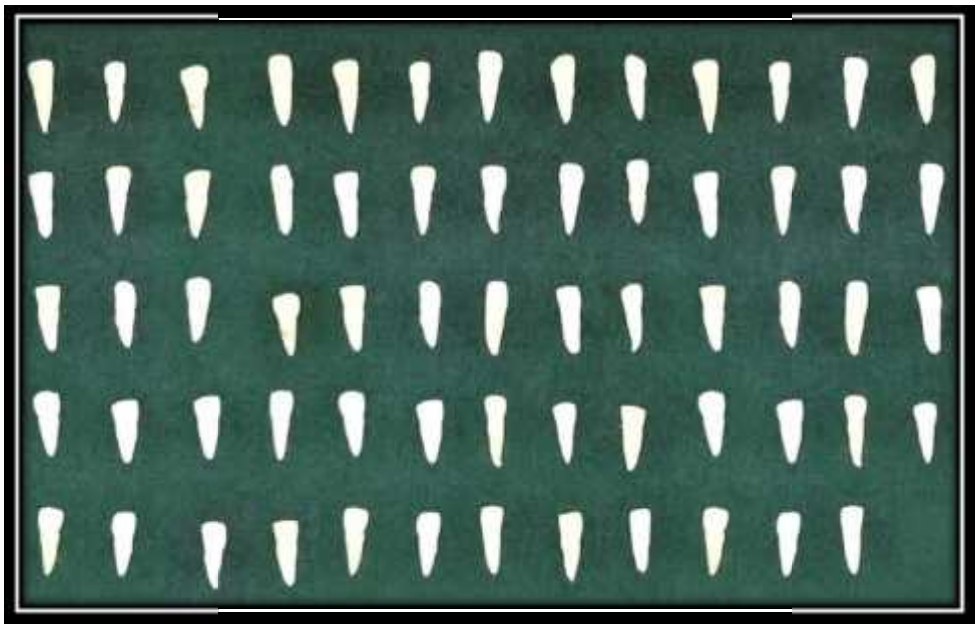


Fig 2:Total Sample Size (n=60)

Fig 3: Removal of debris and calculus using ultrasonic scaler

Fig 4: Decoronation of sample at the level of CEJ

MATERIALS AND ARMAMENTARIUM

Fig 5:Materials used for microbiological assessment

Fig 6: Hand and rotary instruments required for root canal preparation

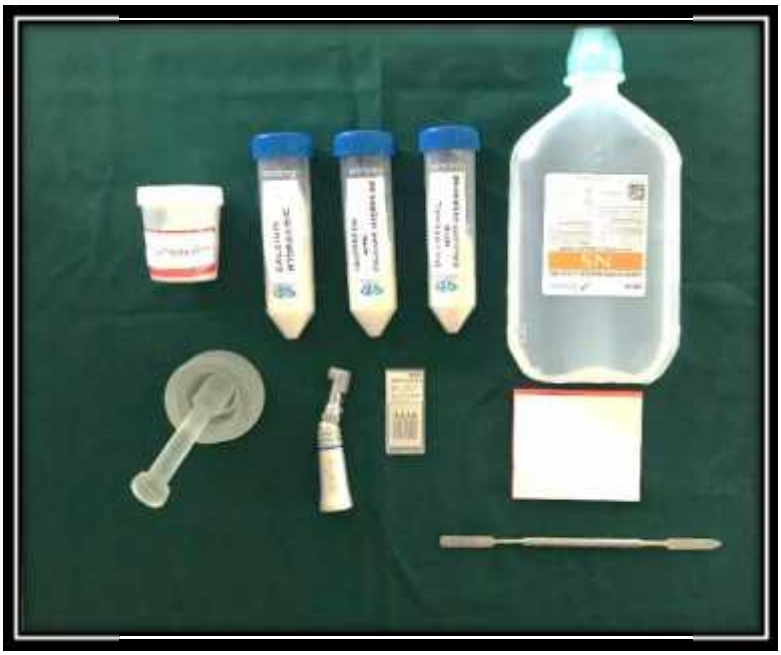


Fig 7: Materials required for medicament preparation and placement

Fig 8: Materials required for irrigation and medicament removal

Fig 9: MIC of group 1

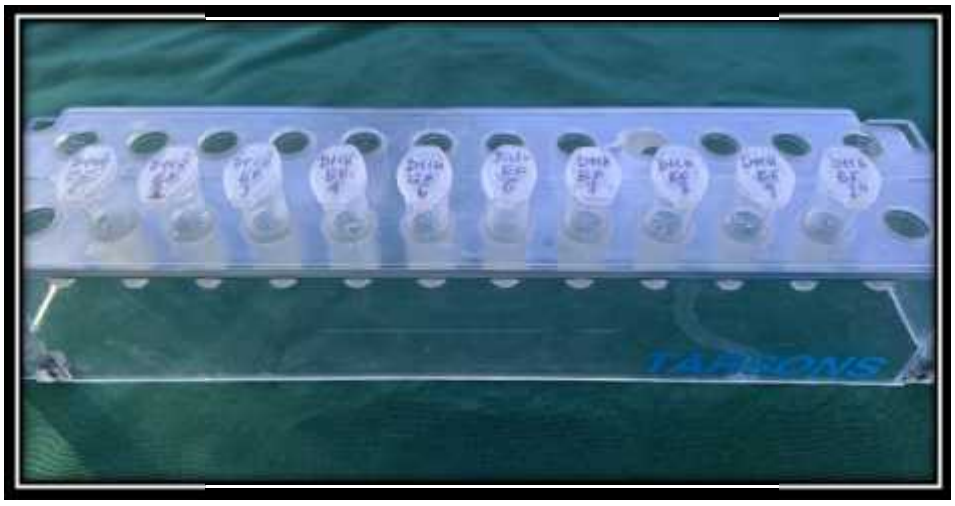


Fig 10: MIC of group 2

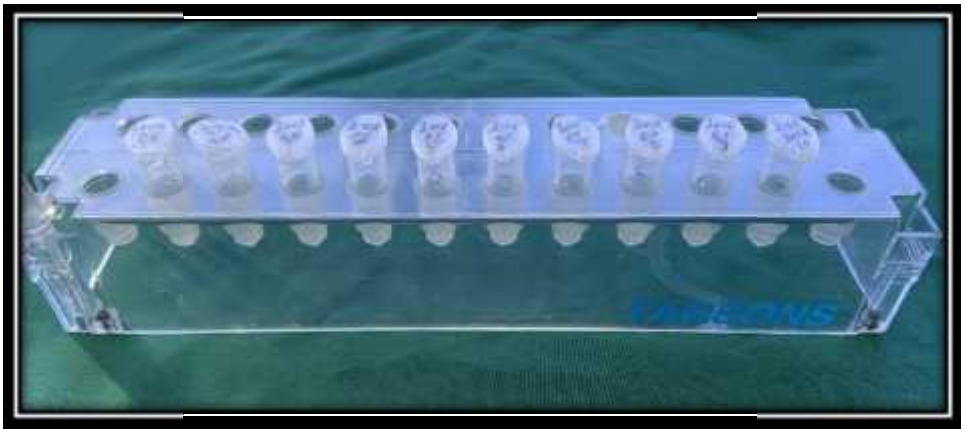


Fig 11: MIC of group 3

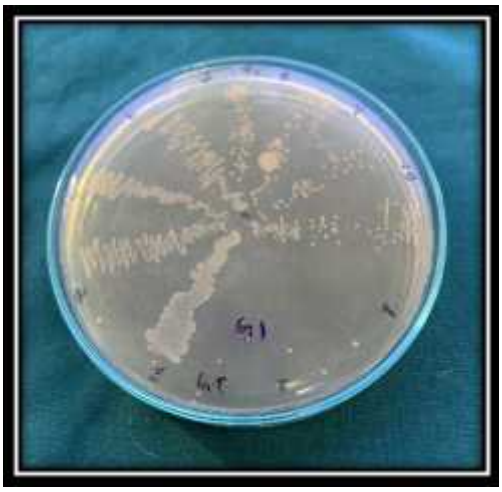


Fig 12: MBC of group 1

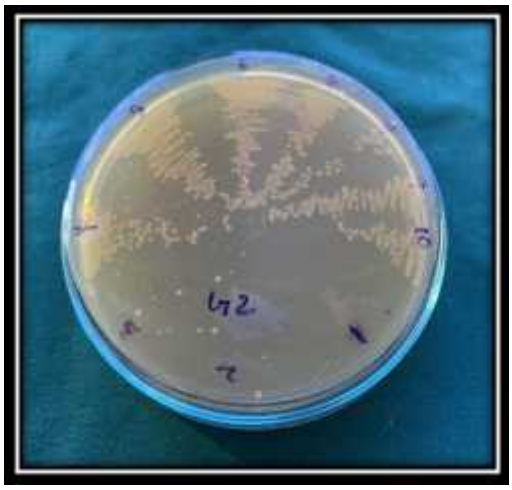


Fig 13: MBC of group 2

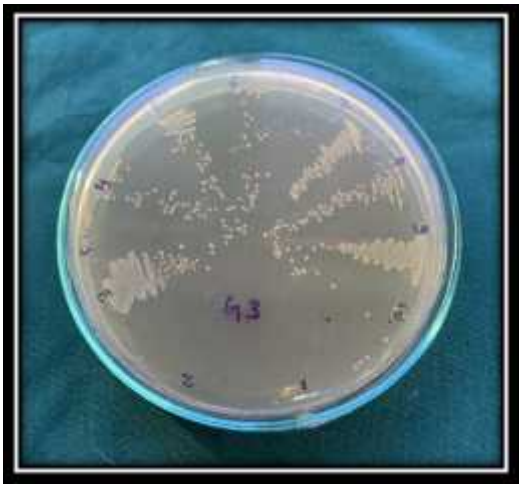


Fig 14: MBC of group 3

Fig 15: Working length determination

Fig 16: BMP using F4

Fig 17: Irrigation using 5 ml syringe

Fig 18: Test samples inoculated with *E. faecalis*

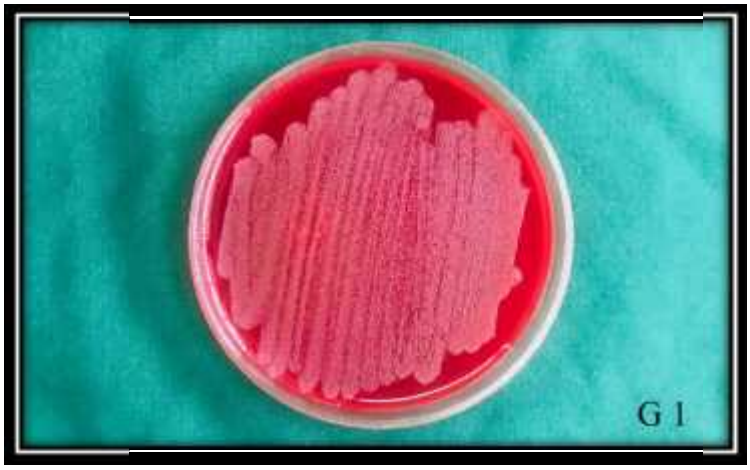


Fig 19: Pre-treatment *E. faecalis*colonies of G1

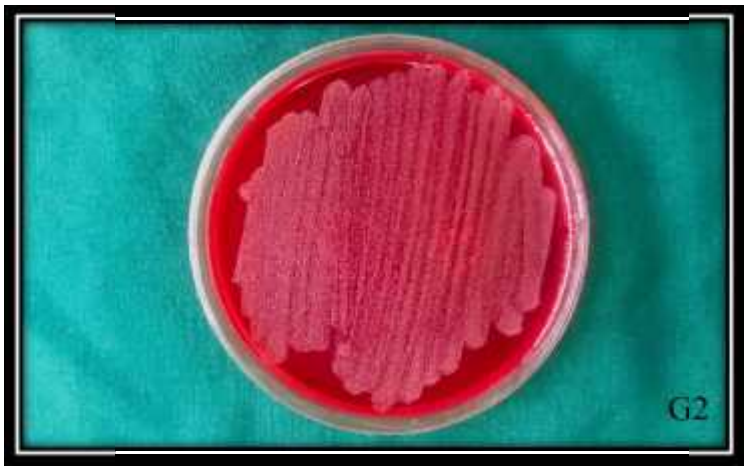


Fig 20: Pre-treatment *E. faecalis*colonies of G2

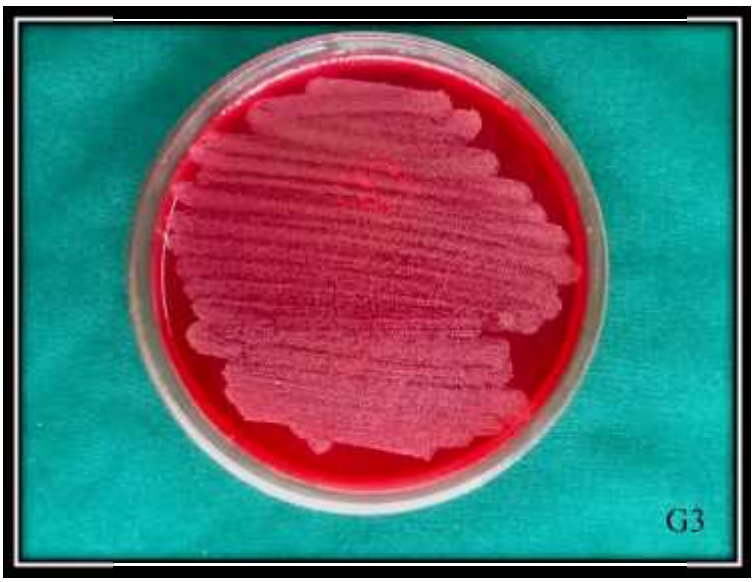


Fig 21: Pre-treatment *E. faecalis*colonies of G3

Fig 22: Incubator

Fig 23: Mixing of the Medicament

Fig 24: Medicament placement using lentulospiral

Fig 25: Test samples were kept in incubator for 7 days

Fig 26: Ultrasonic irrigation to remove the medicament

Fig 27: Ultrasonic irrigation to remove the medicament

Fig 28: Post medicament colonies seen in Group 1.

Fig 29: Post medicament colonies seen in Group 2.

Fig 30: Post medicament colonies seen in Group 3.

RESULTS

Statistical analysis and results

Table 1: Normality of MIC and MBC scores in the three groups (1, 2, 3) by Kolmogorov Smirnov test

Parameters	Group 1		Group 2		Group 3	
	Z-value	P-value	Z-value	P-value	Z-value	P-value
MIC	1.2430	0.0910	1.2430	0.0910	0.7980	0.5470
MBC	1.0680	0.2040	1.2430	0.0910	0.7980	0.5470

The MIC and MBC scores in the three groups (1, 2, 3) follow a normal distribution. Therefore, the one way ANOVA and followed by Tukeys multiple posthoc procedures is used.

Table 2: Summary statistics of MIC scores in the three groups (1, 2, 3)

Groups	N	Min	Max	Mean	SD	SE	95% CI for mean	
							Lower bound	Upper bound
Group 1	9	25.00	50.00	33.33	12.50	4.17	23.73	42.94
Group 2	9	12.50	25.00	16.67	6.25	2.08	11.86	21.47
Group 3	9	12.50	50.00	29.17	16.54	5.51	16.46	41.88

Table 3: Comparison of the three groups (1, 2, 3) with mean MIC scores by one way ANOVA

Sources of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F-value	p-value
Between groups	2	1354.17	677.08	4.3333	0.0247*
Within groups	24	3750.00	156.25		
Total	26	5104.17			

*p<0.05

Table 4: Pairwise comparison of the three groups (1, 2, 3) with mean MIC scores by Tukeys multiple posthoc procedure

Groups	Group 1	Group 2	Group 3
Mean	33.33	16.67	29.17
SD	12.50	6.25	16.54
Group 1	-		
Group 2	P=0.0244*	-	
Group 3	P=0.7618	P=0.1067	-

*p<0.05

Figure 1: Comparison of the three groups with mean MIC scores

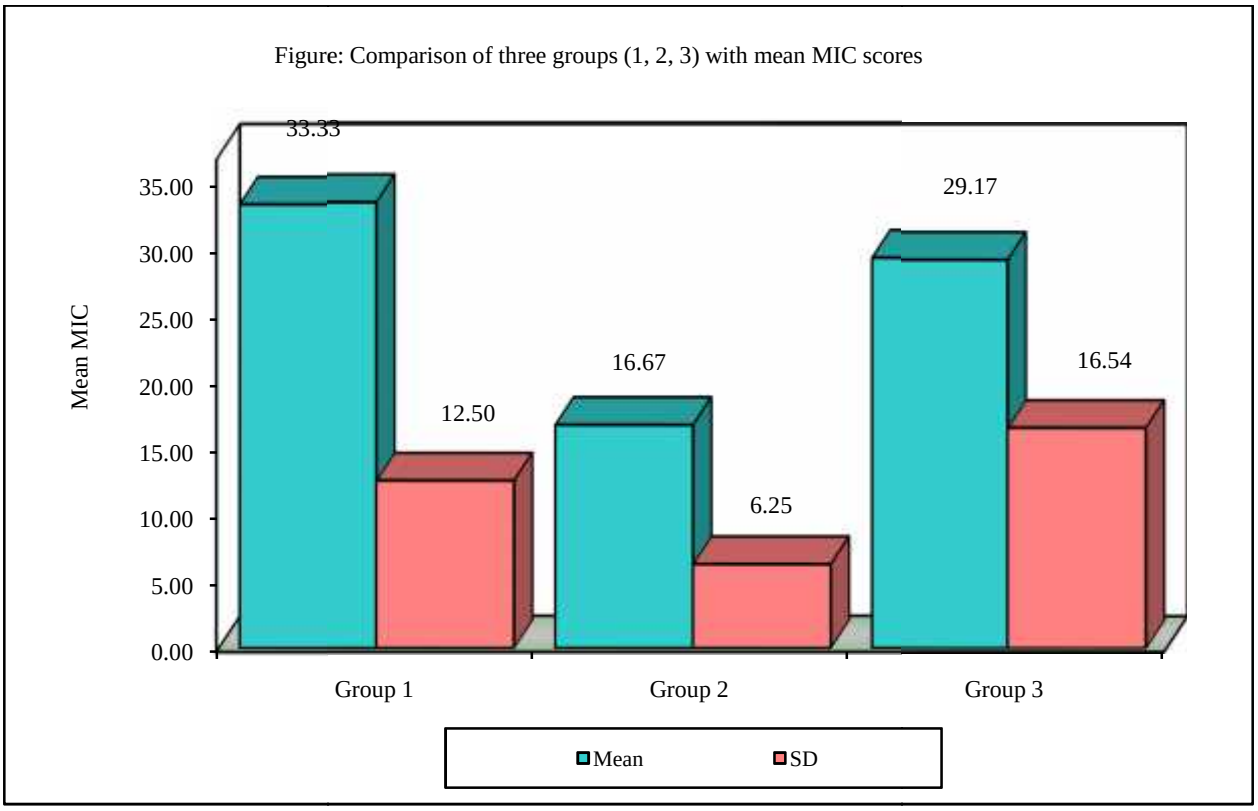


Table 5: Summary of MBC scores in the three groups (1, 2, 3)

Groups	N	Min	Max	Mean	SD	SE	95% CI for mean	
							Lower bound	Upper bound
Group 1	9	50.00	100.00	72.22	26.35	8.78	51.97	92.48
Group 2	9	25.00	50.00	33.33	12.50	4.17	23.73	42.94
Group 3	9	25.00	100.00	58.33	33.07	11.02	32.91	83.75

Table 6: Comparison of the three groups (1, 2, 3) with mean MBC scores by one way ANOVA

Sources of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F-value	p-value
Between groups	2	6990.74	3495.37	5.3929	0.0116*
Within groups	24	15555.56	648.15		
Total	26	22546.30			

*p<0.05

Table 7: Pairwise comparison of the three groups (1, 2, 3) with mean MBC scores by Tukey's multiple posthoc procedure

Groups	Group 1	Group 2	Group 3
Mean	72.22	33.33	58.33
SD	26.35	12.50	33.07
Group 1	-		
Group 2	P=0.0095*	-	
Group 3	P=0.4895	P=0.1147	-

*p<0.05

Figure 2: Comparison of the three groups with mean MBC scores

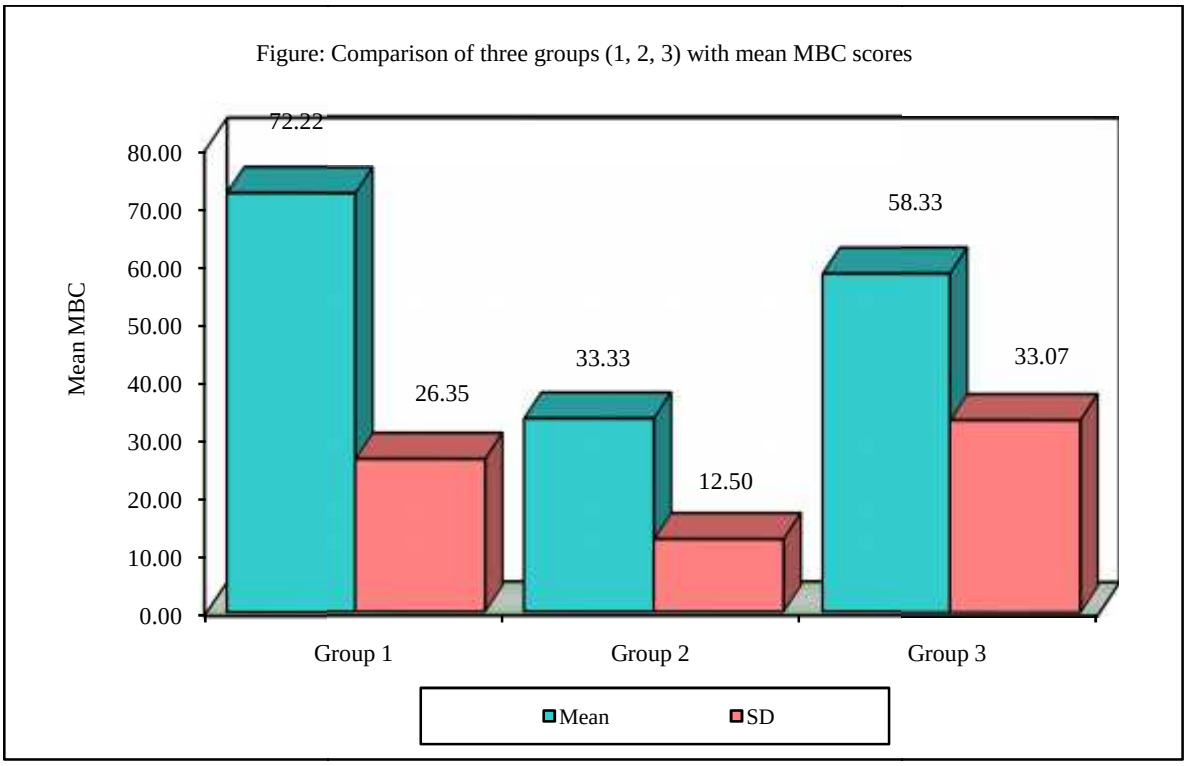


Table 8: Normality of before and after treatment CFU counts in three groups (1, 2, 3) by Kolmogorov Smirnov test

	Group 1		Group 2		Group 3	
	Z-value	P-value	Z-value	P-value	Z-value	P-value
Before	0.7660	0.6010	0.7170	0.6830	0.7370	0.6500
After	0.4810	0.9750	-	-	0.4630	0.9830
Difference	0.7660	0.6010	0.7170	0.6830	0.7360	0.6500

The before and after treatment CFU counts in three groups (1, 2, 3) follow a normal distribution. Therefore, the one way ANOVA and followed by Tukey's multiple posthoc procedure is used.

Table 9: Summary statistics of before and after treatment CFU counts in the three groups (1, 2, 3)

Treatment	Groups	N	Min	Max	Mean	SD	SE	95% CI for mean	
								Lower bound	Upper bound
Before	Group 1	20	220000	270000	243000	17502	3914	234809	251191
	Group 2	20	220000	270000	243000	18382	4110	234397	251603
	Group 3	20	220000	270000	251500	16944	3789	243570	259430
After	Group 1	20	10.0	20.0	15.4	3.3	0.7	13.8	16.9
	Group 2	20	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Group 3	20	50.0	59.0	54.8	2.6	0.6	53.5	56.0
Change	Group 1	20	219984	269983	242985	17501	3913	234794	251175
	Group 2	20	220000	270000	243000	18382	4110	234397	251603
	Group 3	20	219945	269950	251445	16944	3789	243515	259375

Table 10: Comparison of three groups (1, 2, 3) with mean before and after treatment CFU counts by one way ANOVA

Treatment	Sources of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F-value	p-value
Before	Between groups	2	963333333	481666667	1.5516	0.2207
	Within groups	57	17695000000	310438596		
	Total	59	18658333333			
After	Between groups	2	31903.63	15951.82	2703.6977	0.0001*
	Within groups	57	336.30	5.90		
	Total	59	32239.93			
Change	Between groups	2	952694904	476347452	1.5345	0.2243
	Within groups	57	17694667336	310432760		
	Total	59	18647362240			

*$p<0.05$

Table 11: Pairwise comparison of three groups (1, 2, 3) with mean CFU scores by Tukeys multiple posthoc procedure

Treatment	Groups	Group 1	Group 2	Group 3
Before	Mean	243000	243000	251500
	SD	17502	18382	16944
	Group 1	-		
	Group 2	P=1.0000	-	
	Group 3	P=0.2868	P=0.2868	-
After	Mean	15.35	0.00	54.75
	SD	3.28	0.00	2.63
	Group 1	-		
	Group 2	P=0.0001*	-	
	Group 3	P=0.0001*	P=0.0001*	-
Change	Mean	242985	243000	251445
	SD	17501	18382	16944
	Group 1	-		
	Group 2	P=1.0000	-	
	Group 3	P=0.2900	P=0.2913	-

*p<0.05

Figure 3: Comparison of three groups with mean before and after treatment CFU count

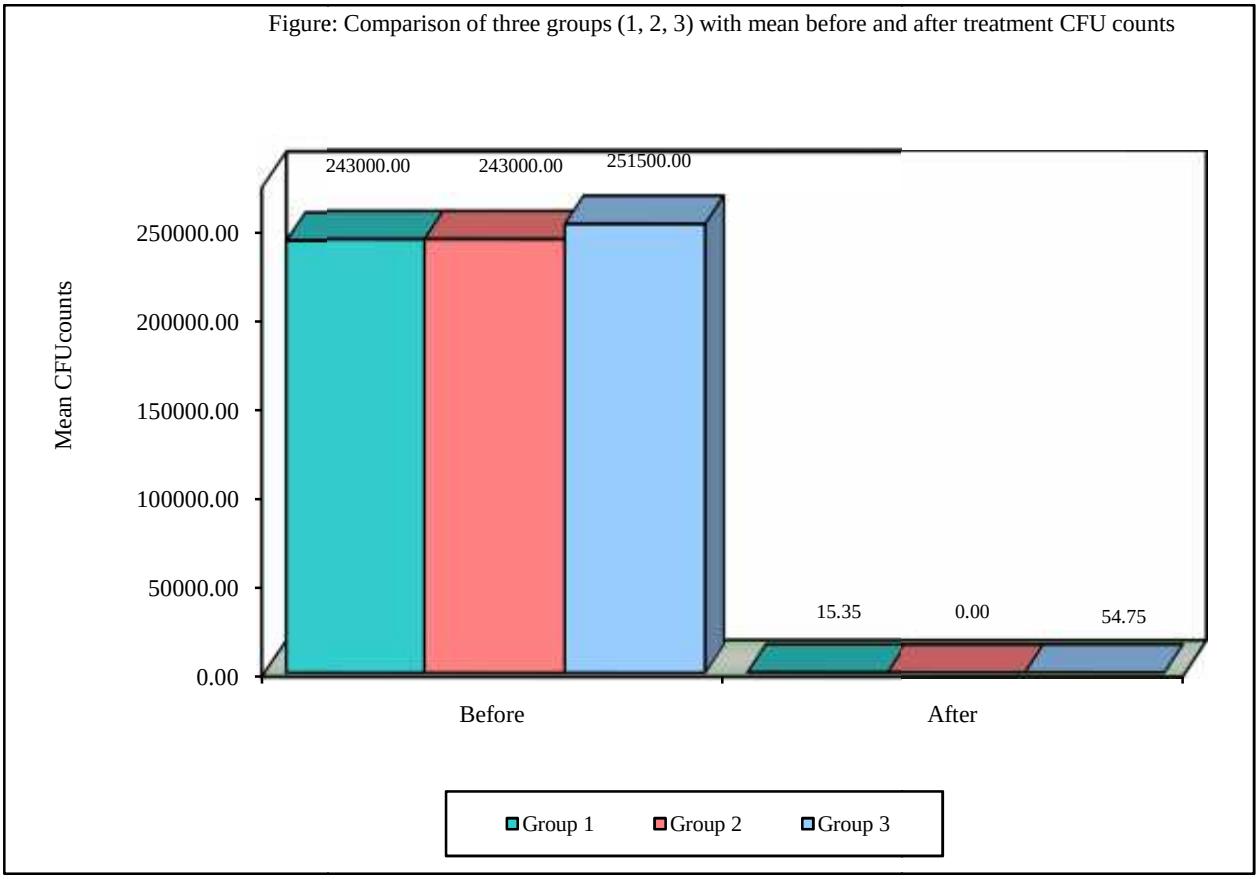
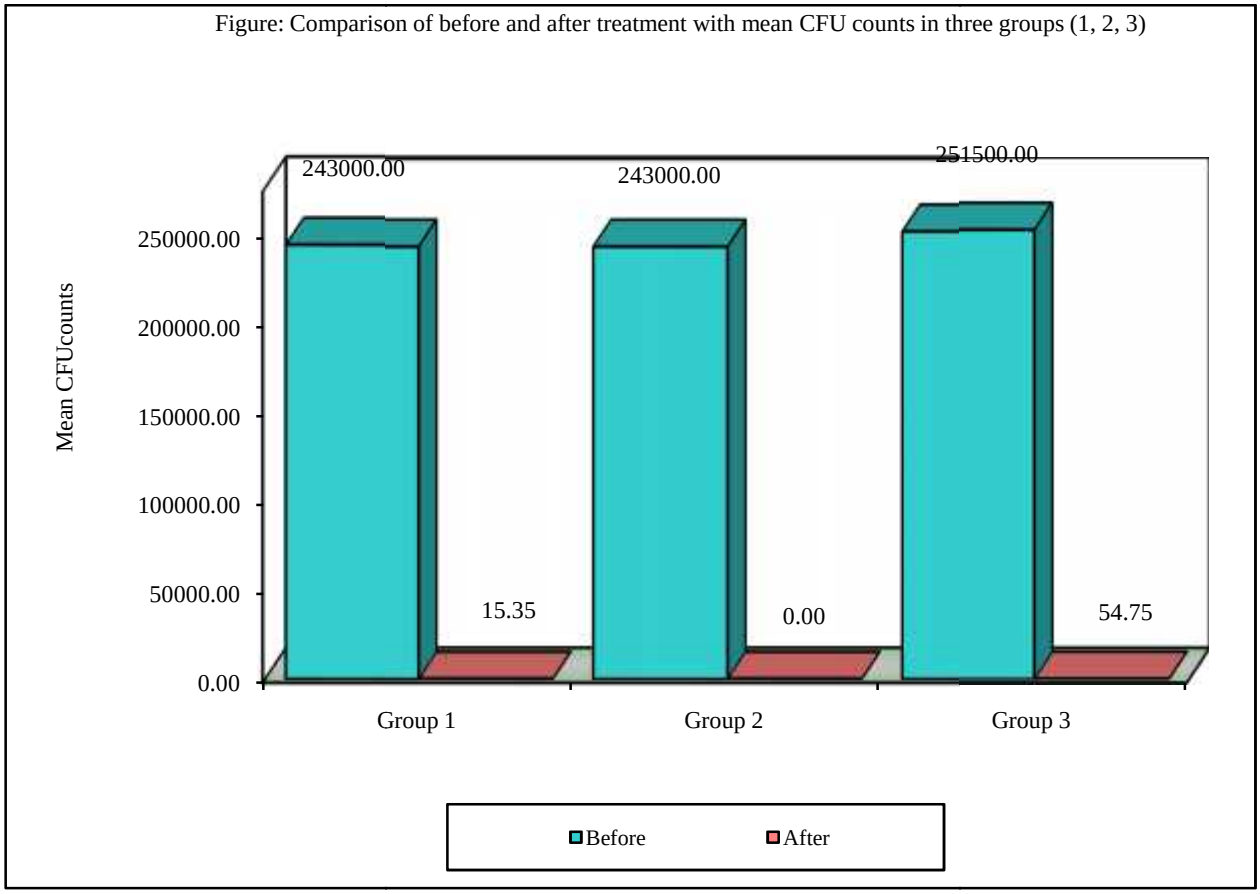


Table 12: Comparison of before and after treatment with mean CFU counts in three groups (1, 2, 3) by Dependent t-test

	Treatment	Mean	Std.Dv.	Mean Diff.	SD Diff.	Paired t	p-value
Group 1	Before	243000.00	17501.88				
	After	15.35	3.28	242984.65	17501.13	62.0909	0.0001*
Group 2	Before	243000.00	18381.91				
	After	0.00	0.00	243000.00	18381.91	59.1195	0.0001*
Group 3	Before	251500.00	16944.18				
	After	54.75	2.63	251445.25	16944.44	66.3638	0.0001*

*$p<0.05$

Figure 4: Comparison of before and after treatment with a mean CFU count in the three groups



Results:

The following hypothesis was taken for this study-

H0: The null hypothesis taken was that there is no difference in the antibacterial efficacy of Calcium Hydroxide, Diclofenac in combination with Calcium Hydroxide and Ibuprofen used in combination with Calcium Hydroxide when used as an intra-canal medicament.

H1: There is a difference in the antibacterial efficacy of Calcium Hydroxide, Diclofenac in combination with Calcium Hydroxide and Ibuprofen in combination with Calcium Hydroxide when used as an intra-canal medicament.

After checking for normal distribution of the tests, further statistical analysis was done to test the significance of the interaction in each of the groups.

The one way ANOVA has done using the MIC and MBC (Table 3 and Table 6) have shown statistically significant results ($p<0.05$). Since there is less than 5% probability of the success of the null hypothesis, the null hypothesis (H0) is rejected and the alternate hypothesis (H1) is accepted.

Among the three groups, pairwise tests by Tukey's multiple posthoc procedure (Table 4, Table 7 and Table 11) has shown that Group 2 is the most efficient when compared to Group 1 and Group 3 in terms of MIC, MBC and CFU counts (after treatment efficacy).

One way ANOVA performed using CFU counts for before and after treatment using medicament shows that after medicament is statistically significant with P=0.0001 (Table 10). Hence H0 is rejected and H1 is accepted i.e there is a difference

in the antibacterial efficacy of the three groups when used as an intra-canal medicament.

Tukeys multiple posthoc procedure is done using CFU mean counts, shows that post-treatment mean CFU count is statistically significant among the three groups 1, 2, 3. In post-treatment CFU counts among the 3 groups, **group 2** has the lowest mean CFU count (0.00) compared to group 1 and group 3 (15.35 and 54.75 respectively).

Therefore, in this study, **group 2** i.e. Diclofenac in combination with Calcium Hydroxide is accepted as having better antibacterial effectiveness when used as an intracanal medicament.

DISCUSSION

Anatomical irregularities are one of the prime factors for the harbouring of microorganisms and necrotic tissue debris, even after meticulous instrumentation and chemical disinfection [46]. Literature shows that in routine cases of an infected root canal, the CFU counts are in the range of $10^2 – 10^8$ [47]. Progressive enlargement of the canal was suggested by Dalton et al. for the reduction of bacterial colonies, however, the subsequent reduction obtained was only 28% of the total bacterial eradication [48].

Shuping et al. has reported bacterial reduction up to 61.9% with the usage of NaOCl and rotary instrumentation[49] .Law et al. has reported the re-emergence of bacterial level the same as that of pre-treatment in an instrumented unfilled canal within 2-4 days. In such situations, the usage of intracanal medicament is recommended [50].

E. faecalis an anaerobic facultative microorganism was taken for this study, due to its higher correlation in cases with re-infection. Furthermore, Baumgardener et al. reported in his study that when apical 5mm of the root was exposed to caries it was found that 34% of the bacteria found were facultative anaerobes [51]. Studies have shown the ability of *E. faecalis* to persist during chemo-mechanical preparation and also to resist the high pH of calcium hydroxide due to the presence of proton pump [52.]

Despite calcium hydroxide being the gold standard for intracanal medicament, a recent study done by Tanriverdi et al. and Lana et al., shows less effectiveness i.e only 70% bacterial elimination of *E. faecalis*[53,54]. Several medicaments such as chlorhexidine[55,56,57-58] iodine [59], camphorated paramonochlorophenol[60, 61], iodoform[62],

propolis[63], and linezolid [64] are added to overcome the drawbacks of calcium hydroxide.

The first antibacterial efficacy of NSAIDs with sodium salicylate was reported by Domenico et al. against Klebsiella pneumonia [65]. Recent studies [66-69] through an *In-vitro* and *In-vivo* animal study concluded that Diclofenac has anti-bacterial efficacy against *Salmonella typhimurium, Mycobacterium tuberculosis, and Listeria monocytogenes*. Moreover, Shirin et al. emphasized the usage of Ibuprofen as an antibacterial medicament against *Helicobacter pylori*[70]. Recently Freitas et al. proved increased anti-bacterial efficacy of 'NSAIDs' with Calcium Hydroxide on a bovine dentin block. Therefore, the potential advantages of combining NSAIDS with Calcium Hydroxide as an intracanal medicament are anti-inflammatory action, local analgesia, and possible antibacterial action with added benefits of Calcium Hydroxide[71].

The MIC results confining to the study were tested using the tube dilution method, whereas the MBC of the test medicament was detected by sub-culturing these on a medicament free media. The advantage of using this technique is that the examination of a substantial number of bacterial cells could be done by a large initial inoculum provided by the relatively large volume of broth in each of the tubes. The principal disadvantages of the same include the tedious manual task of preparing the two-fold solutions for each test, along with relatively large amounts of reagents and space required for such tests. The test also results in the possibility of making errors in the preparation of medicament concentrations[72.]

The inhibition produced by different brands of a medicament against a particular organism depends upon various extrinsic and intrinsic factors. As per Salem-Milani et al. the effective drug dose (MIC) of Diclofenac and Ibuprofen against *E. faecalis* is 50 µg/ml and above (RW). Similarly, Blanscet et al. showed antibacterial activity of Ca(OH)2 at 400 µg/ml and 600 µg/ml [99]. In this study, the mean MIC [Tab.2] against *E.faecalis* for Diclofenac with $Ca(OH)_2$ group was 16.6µg/l, whereas Ibuprofen with $Ca(OH)_2$ group was 29.17µg/l. The mean MBC [Tab.5] for Diclofenac with $Ca(OH)_2$ group was 33.3µg/l and Ibuprofen with $Ca(OH)_2$ group was 58.3µg/l. Also according to the present study, both MIC(33.3 µg/l) and MBC(72.2 µg/l) results of $Ca(OH)_2$ came out to be less effective than both, group 2(Diclofenac with $Ca(OH)_2$) and group 3(Ibuprofen with $Ca(OH)_2$).

In the present study, single-rooted, single canal, extracted maxillary central incisors were included to eliminate any anatomical complexities which would lead to any bias in the study[74].

To eliminate any variable in access preparation, the root lengths were adjusted to 14 mm by sectioning using a high-speed diamond bur and water spray [40]. This was also done to establish a level surface that would serve as a stable and equivocal reference for all measurements.

ProtaperNiTi rotary instruments were used to prepare the canal over stainless steel instruments because NiTiProtaper instruments are designed to cover the whole range of treatment with only a few sets of files. The file system has superior flexibility, unmatched efficiency, and improved safety[75]. The apical canal preparation was done to size F3/F4 depending upon the apical constriction of maxillary central incisors [40].

In the debridement and disinfection of the root canal system, irrigation plays a primary role. For the present study, 3% sodium hypochlorite (NaOCl), 17% Ethylene diaminetetracetic acid (EDTA), and 5 ml of distilled water were used according to the following protocol [76].

- In between each instrumentation: 2ml of 3% NaOCl per canal
- After shaping: 5 ml of 3% NaOCl followed by 5 ml of 17% EDTA per canal.
- After preparation, the canals were irrigated with 5 ml of distilled water and dried with sterile paper points.

For the present study, 3% sodium hypochlorite (Vishal DentocarePvt Ltd., India) was used because of its effective antimicrobial and tissue dissolution property [77]. Studies have shown, for removal of vital and necrotic tissue concentrations of NaOCl above 2% have the dissolving characteristics. Even at lower concentrations NaOCl has strong antimicrobial activity (Bystrom and Sundquist 1983) and kills bacteria very rapidly [78].

17% EDTA solution (Canalarge, Ammdent, Chandigarh, India) was used as an irrigant because it removes the debris by chelating the inorganic tissue. The smear layer refers to this debris, which includes soft tissue and dentine components and the former interrupts the penetration of the irrigation(Violich and Chandler 2010).NaOCl is used along with EDTA to increase the antibacterial effectiveness when used in the same irrigating regime. (Bystrom and Sundqvist 1985) [79].

5ml of distilled water (NICE LIFECARE, NEW DELHI) was used as a final flush to neutralize various chemicals of the irrigants by its flushing action [80].

To make the negotiation and movement of the file within the root easier 17% EDTA gel (Well Prep, Vericom, Korea), was used in this study as a lubricant to facilitate instrumentation of the root canal. It was used with stainless steel and nickel-titanium file systems in the early stages of canal negotiation and shaping [81].

Needle irrigation is the most common method for the delivery of irrigants into the root canal system. Large syringes can be used to deliver a large volume of irrigant, but these are difficult to control [82.] A study done by Gopikrishna et al. concluded that a 26-gauge needle provides a higher flow rate, but impairs the depth of penetration and a 30-gauge needle had the slowest flow rate. Hence a 5ml side vented syringe with a 27 gauge needle, which has a good depth of penetration as well as flow rate was used in study [82].

The endodontic model was inoculated with the test organism *E.faecalis* for 2 weeks. This *In-vitro* model stimulates an actual clinical situation resembling endodontic retreatment. The inoculation time for the adequate growth of the bacteria was kept under 2 weeks because it is difficult to maintain the viability of the test organism in an *In-vitro study*[83]. The usage of an endodontic model gives us a better understanding of dentin buffering action, which otherwise would not have been possible, had the study been done on a Petri dish [84].

A 4 mm thick coronal seal was placed to restrict the nutrient supply to the test organism [85]. The medicaments were applied for seven days to check their antimicrobial effect. It was observed by Sjogren et al. that a negative culture was attainable with the placement of a Ca(OH)2 for just 7 days [86].

In this study, a leptulospiral was used for the placement of intracanal medicament. The insertion of the paste was performed using small quantities of the medicament at a time. Lentulospiral propels the medicament centrifugally and helps in better placement [87.]

Passive ultrasonic agitation of irrigation solutions is more effective than syringe irrigant alone. The hydrodynamic phenomenon caused by vibration of the file in the canal filled with irrigant creates acoustic microstreaming. This microstreaming is responsible for the removal of root canal debris [88.] Therefore, PUI was also used for 3 minutes in the study for the removal of intracanal medicaments.

For the beneficial effect of the intracanal medicament, it is of utmost importance that the medicament to come in direct contact with the microorganism. Studies have shown that the penetration of the medicament is directly proportional to the penetration and solubility of the drug[89]. The complexation method was used to make the drugs water-soluble to increase their penetration in the dentinal tubules. The medicaments were prepared by mixing them with β cyclodextrin in a 1:1 ratio. βcyclodextrin (α-1,4)-linked oligosaccharides of α-d-glucopyranose is a solubility enhancer which clatharates the test medicament and helps in better dissolution in distilled water [84].

Instrumentation and microbiological analyses were done by a single operator, to eliminate inter-operator bias. The samples were attained by the circumferential pumping action of the file, to get a suspension of bacteria from root canal walls, dentin, and the apical delta. The pumping action also aids in the disruption of biofilms in these areas [74].

In the present study, thioglycolate broth was used to keep the viability of the microorganism during transportation. The sampling was repeated using additional paper points until all the glucose broth was absorbed. According to Fouad et al. the last paper point is most important because it will absorb the liquid from the most peripheral areas of the apical region [74].

In the present study, the first sample (S1) reading of all the three groups was in the range of 2.2×10^6 -2.7×10^6, marking the same CFU count in all the three groups. Post medicament it was observed that all the groups (Group I, Group II, Group III) showed reduced CFU counts, therefore the antibacterial effect was significant in all of them [Fig 3].

Comparing the medicaments with the control group $Ca(OH)_2$ (Group-1), Diclofenac with $Ca(OH)_2$ (Group-2) and Ibuprofen with $Ca(OH)_2$ (Group-3)showed very strong evidence against the null hypothesis (P = 0.0001) [Figure 3][Tab.10].

This is in favour of earlier research done by de Freitas et al. (2017) which documented enhanced antibacterial activity of $Ca(OH)_2$ in combination with NSAIDs [39]. But the present findings were not in accordance with their study in terms of the increased antibacterial efficacy of Ibuprofen in combination with $Ca(OH)_2$. This may be because Ibuprofen is lipophilic [101] i.e. it is not water-soluble as opposed to diclofenac which is water-soluble, [albeit sparingly] [101].

Besides when Diclofenac with Calcium Hydroxide (Group 2) was compared with Ibuprofen with Ca(OH)2 group 3 also showed strong evidence against the null hypothesis [$p<0.05$]. Thus, the null hypothesis was rejected.

Salem-Milani et al. , considered the first one to ever study the antibacterial efficiency of Ibuprofen, Diclofenac, and Ca(OH)2 by utilizing the agar diffusion test against *E. faecalis* and concluded that NSAIDs can be used as an intracanal medicament [37].

The results of this study partly concur with a study by Salem-Milani et al., where a greater antibacterial action was shown by diclofenac and Ibuprofen than Ca(OH)2.

In the present study when the intergroup i.e group 1($Ca(OH)_{2)}$, group 2(Diclofenac +$Ca(OH)_{2)}$, group 3(Ibuprofen+ $Ca(OH)_{2)}$ were compared strong evidence against the null hypothesis [$p<0.05$] was proven. Therefore once again, the null hypothesis was rejected. Several studies have documented the antibacterial activity of Diclofenac and Ibuprofen but the exact mechanism remains uncertain. However, studies have proposed the following mechanism(s) of action:

- Inhibition of bacterial DNA synthesis[69]
- Impairment of membrane activity [67,68]
- Anti-plasmid activity[101]
- Alteration in genes encoding transport/binding proteins,
- DNA synthesis and cell envelope[100]
- Down-regulation of efflux pumps [100]
- Reduced quorum sensing-controlled motility leading to reduced biofilm[102.]

Siqueira and Uzeda et al. demonstrated that 1 week of contact with Ca(OH)2 mixed with saline had limited effect in *E. faecalis*eradication [91.] Estrela et al. reported in her study that Ca(OH)2 had no antimicrobial effect on *E. faecalis, S. aureus,*

Bacillus subtilis, Pseudomonas aeruginosa[92]. In a Systematic review to assess the antibacterial efficacy of Ca(OH)2 by using culture techniques, Sathorn et al. reported that Ca(OH)2 had decreased efficacy in eradicating bacteria [93].

The probable mechanism for the antimicrobial action of Calcium Hydroxide is the release of hydroxyl ions in an aqueous environment which causes damage to the bacterial cytoplasmic membrane and also protein denaturation leading to the damage of DNA [90].

The limited antimicrobial effect of Calcium Hydroxide on facultative anaerobes could be attributed to the dentine buffering effect. The antibacterial activity of Calcium Hydroxide is driven by its high pH (12.5) due to which it has a destructive impact on bacterial cell membranes and protein structure. For medicament's efficacy, the hydroxyl ions from Calcium Hydroxide must penetrate and reach dentin sufficiently. This diffusion is made difficult since dentin hydroxyapatite has a strong buffering property that must be overcome by the hydroxyl ions leaving the root canal space. The buffering of alkaline substances in the dentin is due to the proton donors in the hydroxyl layer of the hydroxyapatite. It is seen that Calcium Hydroxide alkalizes the dentin, even so, it is unable to eradicate *E.faecalis*, which survives high pH up to 11.5 [94.]

One of the few mechanisms that explain the poor action of Calcium Hydroxide on *E.faecalis* is the ability of *E.faecalis* to penetrate deep into the dentin to the depth of 1000µm [95] which facilitates its protection from chemomechanical root canal preparations. Another possible mechanism is the formation of co-aggregates with other bacterial species.

Also, *E.faecalis* biofilms have exhibited the ability to calcify, which may further facilitate their stability. Studies have also shown that the virulence factors within the *E.faecalis* have the potential to adapt and survive, due to the presence of the transcripts of ftsZ, a gene involved in cell division that helps its multiplication. [96,97]. The presence of the above factors along with many other virulence traits such as enterococcus surface protein, a collagen-binding protein with the secretion of proteases (ex. Gelatinase) and lexin (ex. cytolysin) gives it an upper hand over the other bacterial species found in the root canal, all of which contributes to its survival. [98].

In this study, medicaments in their powder forms were mixed in 1:1 ratio (approx.) with distilled water. This was because of the difficulty in measuring such a small amount of the medicament as well as due to the complexity in delivering the medicament inside the root canal [38].

The complexation procedure which was done to enhance the solubility of the medicament did indeed enhance the solubility to an extent but didn't make the drug completely soluble [40]. It is well documented that the penetration is directly dependent on the solubility, which in turn helps the medicament to come in direct contact with the bacteria. Therefore in the present study, Ibuprofen with Calcium Hydroxide did not show comparable results as Diclofenac with Calcium Hydroxide which showed excellent results.

According to Sjögren et al, a 7-day placement of a $Ca(OH)_2$ was adequate in complete eradication of the root canal bacteria. Similarly, Shuping et al in 2000 reported that 92.5% of root canals showed negative culture in a 1 week after

placement of $Ca(OH)_2$. Prolong duration of placement of intra-canal medicament ($Ca(OH)_2$) causes has a negative effect on fracture resistance of the teeth.

The present study shows that the combination of diclofenac with $Ca(OH)_2$ is effective in complete eradication of root canal bacteria in 7 days, and thus minimizes any damage to the root fracture resistance of the treated tooth.

Therefore Diclofenac with $Ca(OH)_2$ with both anti-inflammatory and antibacterial activity can be a potential intracanal medicament and can be a replacement for Calcium Hydroxide with further research.

Further *In vivo* studies are to be carried out for the practical implementation in the clinical scenario of results obtained from the present study.

The incompetent testament of Ibuprofen could be attributed to the medicament preparation at a molecular level that might be one of the reasons for the lack of synergistic effect with $Ca(OH)_2$. Also, appropriate research should be carried out on the proportionating field of the combination of the medicament so that we can get the practically achievable value of the medicament that can be used in a clinical scenario. In our further research, we would want to study if this combination could decrease the placement time of intracanal medicament so that fracture resistance of the teeth can be maintained.

CONCLUSION

Under the limitations of the present research, it can be concluded that:

1) A combination of NSAIDS with Calcium Hydroxide is effective against *E.faecalis.*
2) Diclofenac with Calcium Hydroxide shows 100 % efficacy against *E.faecalis*and can be used in a clinical scenario.
3) Ibuprofen with Calcium Hydroxide could not eliminate *E.faecalis* due to decreased solubility and possibly less penetration into the dentinal tubules, therefore further research should be carried to improve the drug at the molecular level.
4) The better efficacy of all the combination could be attributed to the complexation method.

SUMMARY

The study was conducted in the Department of Conservative Dentistry and Endodontics, ViswanathKatti Institute of Dental Sciences, KAHER Belagavi with the aim for comparisons of anti-bacterial efficacy of Calcium Hydroxide, Diclofenac with Calcium Hydroxide and Ibuprofen with Calcium Hydroxide against *Enterococcus faecalis* in an endodontic model.

Bacteria play a pivotal role in the pathogenesis of apical periodontitis. For optimal treatment outcome complete elimination of bacteria is of utmost importance. Cleaning and shaping along with antimicrobial agents lead to a reduction in the bacterial colonies present in the root canal. However, studies have shown chemo-mechanical instrumentation alone is unable to abolish the full bacterial colonies in the root canal. To overcome this, intracanal medication is advocated. Despite favorable clinical evidence of commonly used $Ca(OH)_2$, it has been reported that there is a correlation between $Ca(OH)_2$ placement and increased incidence of tooth fracture, principally reported in teeth with an open apex. This led to the addition of newer medicament which could enhance the property of $Ca(OH)_2$ and act synergistically to them.

The conventionally used class of analgesics- Non-steroidal anti-inflammatory drugs (NSAIDs)– may possess additional therapeutic properties such as anti-bacterial efficacy through inhibition of bacterial DNA synthesis or impairment of membrane activity; prevention of bacterial colonization and biofilm formation by interfering with quorum sensing of bacteria; and anti-plasmid activity.

Thus, the present investigation was undertaken to evaluate and compare the antibacterial efficacy of $Ca(OH)_2$, Diclofenac with $Ca(OH)_2$ and Ibuprofen with $Ca(OH)_2$ against *Enterococcus faecalis*.

Sixty extracted human maxillary central incisors teeth were chosen which fulfilled the inclusion and exclusion criteria. De-coronation of samples was done at CEJ using a diamond disc with continuous water coolant for standardization. Biomechanical preparation was done using Protaper Universal NiTi files up to F3/F4. 2ml of 3% of NaOCl was irrigated between each instrument. After completion of chemo-mechanical preparation, sample S-1 was collected by introducing a sterile paper point. The paper point was immediately placed in test tubes containing glucose broth. Intracanal medicaments were placed according to the respective group Group1-$Ca(OH)_2$, Group 2- Diclofenac + $Ca(OH)_2$, Group 3- Ibuprofen + $Ca(OH)_2$. Intracanal medicaments were removed after 7 days by using passive ultrasonic irrigation and sample S-2 was collected.

The results were analyzed statistically by One way ANOVA, Tukey's multiple post hoc, Dependent t-test all of which indicated that there is a significant difference between all the three groups (p-value <0.05). Among all the three groups, Group 2 (Diclofenac + $Ca(OH)_2$) performed better, this could be because of the better synergistic action of both the medicament.

Therefore, the null hypothesis that there is **no difference** in the antibacterial efficacy of Calcium Hydroxide, Diclofenac in combination with Calcium Hydroxide and Ibuprofen in combination with Calcium Hydroxide was rejected.

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ANNEXURE – I

ETHICAL CLEARANCE CERTIFICATE

Research and Ethics Committee
KLE V K INSTITUTE OF DENTAL SCIENCES
KLE University
Accredited 'A' Grade by NAAC Placed in Category 'A' by MHRD (GoI)
Nehru Nagar, Belagavi - 590 010, Karnataka State
☎: 0831-2470362
FAX: 0831-2470840
Web: http://www.kledental-bgm.edu.in
E-mail:principal@kledental-bgm.edu.in

Sl. No.: 1221

CERTIFICATE

This is to Certify that the synopsis titled

Comparison of antibacterial efficacy of calcium hydroxide, diclofenac with calcium hydroxide and ibuprofen with calcium hydroxide against enterococcus faecalis in an endodontic model- an in vitro study

Submitted by

Dr. Deepti Ancy Chacko *P. G. Student /*

Staff, Guided by Dr Neha Shaded *from Department of* Conservative dentistry & endodontics *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date : 24/06/2019

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLE VK Institute of Dental Sciences
Belgaum

ANNEXURE – II

BIOSTATISTIC CLEARANCE CERTIFICATE

KLE V.K. Institute of Dental Sciences
(A Constituent unit of KLE Academy of Higher Education & Research
Deemed-to-be-University u/s 3 of the UGC Act, 1956)
Nehru Nagar, Belagavi-590 010 INDIA
Re-Accredited 'A' grade by NAAC (2nd Cycle) & Placed in Category 'A' by MHRD (GoI)
☎ 0831-2470362
FAX: 0831-2470640
Web: http://www.kledental-bgm.edu.in
E-mail: principal@kledental-bgm.edu.in

Biostatistics Clearance Certificate

This is to certify that the Biostatistics aspect of the Dissertation / Research work of DR. DEEPTI ANCY CHACKO

entitled COMPARISON OF ANTI-BACTERIAL EFFICACY OF CALCIUM HYDROXIDE, DICLOFENAC WITH CALCIUM HYDROXIDE & IBUPROFEN WITH CALCIUM HYDIOXIDE AGAINST E. FAECALIS-IN AN ENDODINTIC MODEL — AN IN VITRO STUDY

has been done under my guidance and considered satisfactory.

Place : Belagavi
Date : 22/9/21

Name & Signature of Biostatistician
(Dr. S. B. Javali)

ANNEXURE – III

PLAGIARISM CHECK CERTIFICATE

Scientific Correspondence and Review Committee

KLE VK Institute of Dental Sciences

A Constituent Unit of KLE Academy of Higher Education and Research
(Deemed-to-be-University u/s 3 of the UGC Act, 1956)
Nehru Nagar, Belagavi - 590 010, Karnataka State

Accredited 'A' Grade by NAAC (2nd Cycle) Placed in Category 'A' by MHRD (GoI)

☎: 0831-2470362
FAX: 0831-2470640

Web: http://www.kledental-bgm.edu.in
E-mail:principal@kledental-bgm.edu.in

Date : 28 / 9 / 2020 Serial No. : 046

PLAGIARISM CHECK REPORT

Name of the Applicant : Dr. Deepti Ancy Chacko
UG / PG / Ph.D / Staff : Post graduate
Batch & Year : 2018 - 2021
Department : Conservative dentistry and Endodontics

The soft copy of Research Work / Manuscript by ..... Dr. Deepti Chacko entitled "Comparison of antibacterial efficacy of Calcium hydroxide, diclofenac with Calcium hydroxide, ibuprofen with Calcium hydroxide against enterococcus faecalis in an endodontic model an invitro study" under the guidance of .....has been submitted for Anti-Plagiarism check to the Scientific Correspondence & Review Committee of KLE VK Institute of Dental Sciences using "Turn-it-in" software.

The scan has been carried out and the scanned output reveals a Similarity Index of .....3.....%, which is **within / not within** the acceptable limits of 10% as per the UGC guidelines.

Member Secretary
Scientific Correspondence and Review Committee
KLEVK Institute of Dental Sciences
KAHER-Belagavi

Chairman
Scientific Correspondence and Review Committee
KLEVK Institute of Dental Sciences
KAHER - Belagavi