"COMPARATIVE EVALUATION OF ANTIMICROBIAL EFFICACY OF A COMBINATION OF N-ACETYL CYSTEINE AND LEVOFLOXACIN WITH TRIPLE ANTIBIOTIC PASTE AGAINST *ENTEROCOCCUS FAECALIS* BIOFILM: AN IN-VITRO STUDY"

By

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REG.NO. IE0219003

Dissertation

Submitted to
KLE Academy of Higher Education and Research
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In

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(BRANCH – IV)

Under the Guidance of

Dr. PREETI DODDWAD M.D.S

DEPARTMENT OF CONSERVATIVE DENTISTRY AND ENDODONTICS
KAHER VK INSTITUTE OF DENTAL SCIENCES,
BELAGAVI, KARNATAKA

2019-2022

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This dissertation is dedicated to

Almighty God,

My Parents,

&

My Brother

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Date: ***Dr. MAHIMA GUPTA***

Place: Belagavi

LIST OF ABBREVIATIONS

SR.NO	ABBREVIATIONS	FULL FORM
1	TAP	Triple Antibiotic Paste
2	NAC	N-Acetylcysteine
3	LEV	Levofloxacin
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	SEM	Scanning Electron Microscopy
9	° C	Degrees Celsius
10	DW	Distilled Water
11	Micro CT	Micro Computed Tomography
12	CBCT	Cone Beam Computed Tomography
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	CH	Calcium Hydroxide
21	NaOCl	Sodium Hypochlorite
22	EDTA	Ethylene Diamine Tetra-acetic Acid
23	<	Less than
24	>	Greater than

ABSTRACT

Aim:

"Comparative evaluation of antimicrobial efficacy of a combination of N-acetyl cysteine and Levofloxacin with Triple antibiotic paste against *Enterococcus faecalis* biofilm : An In-vitro study"

Study design:

Sixty eight extracted human single rooted single canal teeth was used for the study. They were then cleaned of calculus and soft tissue and stored in 0.1% thymol solution till use. All teeth were radiographed and were selected as per the inclusion and exclusion criteria.

A diamond disc was used to section 4 mm of apical third of the root from the apex and then decoronation below the cementoenamel junction was done to obtain 6 mm of middle third of root. After this, a sterile Gates Glidden drill no. 3 in a slow speed handpiece was used to standardize the internal diameter of the root canal .

Following this, the specimens were placed in an ultrasonic bath of 17% EDTA for five minutes followed by 3% NaOCl for five minutes in order to remove the organic and inorganic debris.

In order to remove the traces of chemicals used , dentin specimens were then immersed in an ultrasonic bath containing distilled water for five minutes following which all the specimens were sterilized in an autoclave (20min at 121 ℃). Following this, five teeth were placed in a sterile BHI broth to serve as a negative control to check for absence of contamination.

Then for the contamination of the Specimens (63 specimens) : E. faecalis (MTCC 439) was grown in BHI agar for 24 hours. The culture was then suspended in 5 mL of BHI broth and incubated for 4 hours at 37°C and its turbidity was adjusted at 0.5 Mcfarland standard (1×10^{8} CFUmL−1)

Each dentin block was placed in a pre-sterilized microcentrifuge tubes containing 1 mL of the BHI broth. A 50 µL of the inoculums containing *E.faecalis* was transferred into each of the microcentrifuge tubes.

Following this, at the end of 24 hours, the dentin specimens were transferred into fresh broth containing *E.faecalis*. All procedures were carried out under laminar flow. Purity of the culture was checked by subculturing 5 µL of the broth from the incubated dentin specimens in BHI broth on BHI agar plates. Contamination of the dentin specimens was carried out for a period of 21 days.

For antimicrobial assessment, at the end of 21 days, the specimens were irrigated with 5 mL of sterile saline to remove the incubation broth. Then they were divided into 3 groups (n-21) -

GROUP 1: Triple Antibiotic paste

GROUP 2: N-Acetylcysteine plus Levofloxacin

GROUP 3: Positive Control - Saline

PREPARATION AND APPLICATION OF THE MEDICAMENT

Both the combinations that is, Triple antibiotic paste , N-acetylcysteine and Levofloxacin was prepared according to the values obtained after evaluating minimum inhibitory concentration with 1mL of propylene glycol.

For Minimum inhibitory concentrations evaluation: 9 dilutions of compound was done with Brain heart infusion (Broth).

In the initial tube only 500 µl of compound was added. For dilutions 500 µl of Brain heart infusion broth was added into the next 9 tubes separately. In the 1^{st} tube 500 µl of compound was added with 500 µl Brain heart infusion broth. This was considered as 10^{-1} dilution.

From 10^{-1} diluted tube 500 µl was transferred to second tube to make 10^{-2} dilution. The serial dilution was repeated up to 10^{-8} dilution for each compound. From the maintained stock cultures of required organisms, 20 µl was taken and added into 2 mL of Brain heart infusion broth.

In each serially diluted tube 500 µl of above culture suspension was added. The last tube will contain only the media and culture suspension. The tubes were kept for incubation for 24 hours at 37^0C in bacteriological incubator and observed for turbidity. After this minimum inhibitory concentration was evaluated.

The respective medicaments was placed in root canal and the specimens for positive control group was rinsed with saline and paraffin wax was used to seal both the ends; the specimens was then incubated in an anaerobic environment at 37°C for seven days.

Microbial cells assessment was carried out at the end of seven days of incubation. Harvesting of dentin was carried out at a depth of (400 µm) by preparing the root canal circumferentially using sterile Gates Glidden drills no.5 in a slow speed handpiece.

The fine dentin shavings were collected in a test tube containing 1 mL of sterile BHI broth and incubated in an anaerobic environment at 37°C for 24 hours. After 24 hours the contents were serially diluted 100 µL of broth in 100 µL of sterile saline five times.

About 50 µL of the dilution was then plated on BHI agar plates and incubated for 24 hours at 37°C. The colonies on the agar plates were counted and recorded by a blinded microbiologist, represented in colony-forming units (CFU) per mL and readings was tabulated. Formulae for evaluating the colony forming unit:

$$\text{Colony forming unit/mL} = \frac{\text{number of colonies formed}}{\text{Volume plated (mL)} \times \text{Total Dilution used(mL)}}$$

Results:

A statistically significant difference were seen between all the groups (<0.05). Combination of N-Acetylcysteine and Levofloxacin performed better than Triple Antibiotic paste in eradicating E. Faecalis.

Conclusion:

Within the limitations of present study, it can be concluded that none of the medicament could completely eradicate E faecalis ,(NAC+ LEV) showed the best result.

Key words: TAP, NAC, LEV , Intracanal Medicament

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INTRODUCTION

The primary steps in the treatment of teeth with pulpal and periapical disorders are diagnosis, instrumentation, obturation, and restoration. The elimination or significant decrease of irritants, as well as the prevention of root canal recontamination after treatment, are critical components for optimal outcomes[1]. Although significant breakthroughs in various elements of endodontics have been made in recent years to preserve natural dentition, the primary goal of this field remains the removal of bacteria from root canal systems and the prevention of recontamination after treatment[1] The presence of wide open apices, as well as divergent and thin dentinal walls that are vulnerable to fracture[2], makes treating infections in the root canal area of immature teeth with open apices a difficulty in endodontics. Because the root canal walls of such teeth are weak, mechanical instrumentation should be minimized, and irrigating solutions and intracanal medicaments should be prioritised[2]. Apexification with calcium hydroxide and apical barrier method with MTA are two traditional treatment options for teeth with an underdeveloped apex and necrotic pulp. Even though both of these ways have been utilized successfully over the years, both modalities have a few well-documented downsides[3]. These treatments do not assist in strengthening the root, and roots remain thin and weak in the absence of continuing root development[4]. This has prompted clinicians and academics to look for a different approach, one of which is pulp regeneration. The popularity of regeneration protocols in recent years has resulted in a move toward less intrusive therapeutic options in endodontics[5].

A sterile environment, a matrix for cellular growth, and a hermetic coronal seal to avoid re-contamination are all required for regenerative endodontic therapies[7].

Most regenerative endodontic procedures (REPs) use chemical debridement and intracanal medicaments to achieve disinfection rather than mechanical debridement[7].

According to research, Enterococcus faecalis is the most common colonizing microorganism in endodontic infections and the most common bacterial species in refractory root canal infections. The ability of E. faecalis to create biofilm is a distinguishing feature of this bacterium species and these biofilms of E. faecalis are resistant to antimicrobials[8].

Infections in the root canal system are thought to be polymicrobial, with bacteria from both aerobic and anaerobic environments[8].As a result, due to the canal's complexity, a single antibiotic treatment may not be enough to treat the infection. To address the various flora observed, a mix of antibiotics is necessary. It may also reduce the risk of resistant bacterial strains developing[10]. A triple antibiotic paste made up of three medicines Ciprofloxacin, Metronidazole, and Minocycline is an effective therapeutic option as an intracanal medicament for infected necrotic immature teeth [12]. However, this paste has certain drawbacks, including crown discoloration, root dentin demineralization, cytotoxic effects on cells, and lower biofilm potency[12,13].

Aiming at effective intracanal medicaments for regenerative endodontics, different chemotherapeutic agents were investigated. N-acetylcysteine (NAC) is a mucolytic agent that has reported to inhibit biofilm formation. The beneficial effects of NAC administration have been linked mostly to its mucus-dissolving characteristics, which disrupt disulfide bonds in the mucus, and its capacity to reduce biofilm formation, which results in considerable decrease in bacterial infections[14].

NAC has been found to suppress the development of Lipopolysaccharide-induced inflammatory mediators (IL-1, -6, and -8) in phagocytic cells and gingival fibroblasts during the inflammatory process in the context of oral illnesses. NAC has been proven in studies to suppress the growth of Enterococcus faecalis, a bacteria linked to endodontic treatment failures, and to destroy its biofilm. However, in studies, it has been found that a combination of NAC with fluoroquinolones has shown to have better antimicrobial efficacy than when NAC is used alone.[15,23,27]

Fluoroquinolones are highly strong, broad-spectrum antibiotics that penetrate bacterial cell walls and disrupt DNA gyrase activity, killing vulnerable cells quickly. The third generation Fluoroquinolones, such as Levofloxacin (LEV), can easily equilibrate across biofilms and hence appear to be useful in preventing biofilm formation[14,18].

Recently, a combination of N-acetyl cysteine (NAC) and levofloxacin (LEV) that has a larger disruptive effect on biofilm while having fewer cytotoxic effects on cells has been presented and found to be effective. In addition, investigations have indicated that when N-acetylcysteine and Levofloxacin are administered together, NAC increases fluoroquinolone's therapeutic action[14,15,18].

To the best of my knowledge, there is no data yet available on the effect of antimicrobial efficacy of Triple antibiotic paste vs N-acetyl cysteine and Levofloxacin against E. Faecalis biofilm.

As a result, the current study's goal is to compare the efficacy of biofilm removal using N-acetyl cysteine and Levofloxacin, a prospective intracanal medicament, to that of the gold standard Triple antibiotic paste.

OBJECTIVES OF THE STUDY

AIM

To evaluate the antimicrobial efficacy of a combination of N-acetyl cysteine and Levofloxacin with Triple antibiotic paste, against *E. faecalis* biofilm.

Group 1 – Triple antibiotic paste

Group 2 – N-Acetylcysteine and Levofloxacin

Group 3 – Positive control saline

OBJECTIVES

1. To determine the minimum inhibitory concentrations (MIC) of N-acetyl cysteine, Levofloxacin, and Triple antibiotic paste (Ciprofloxacin, Metronidazole, Minocycline)

2. To evaluate and compare the antimicrobial efficacy of a combination of N-acetyl cysteine and Levofloxacin with Triple antibiotic paste, against *E. faecalis* biofilm.

HYPOTHESIS

NULL HYPOTHESIS: -

There is no difference in the antimicrobial efficacy of a combination of N-acetyl cysteine and Levofloxacin with Triple antibiotic paste against E.faecalis biofilm.

ALTERNATE HYPOTHESIS: -

There is a difference in the antimicrobial efficacy of a combination of N-acetyl cysteine and Levofloxacin with Triple antibiotic paste against E. faecalis biofilm.

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REVIEW OF LITERATURE

1) A overview of the process for regenerative endodontic treatment. Apexification has traditionally been utilised to treat immature permanent teeth with no pulp life. This method encourages the construction of an apical barrier, which closes the open apex and confines the filling materials to the root canal. Because apexification does not allow for tissue regeneration, a novel approach termed regenerative endodontic treatment was recently introduced to treat immature permanent teeth. The goal of regenerative endodontic treatment is to replace injured pulp tissue with healthy tissue that restores the pulp-dentin structure's natural function. Under ideal circumstances, continuing root development and hard tissue deposition on the dentinal wall can occur after regenerative endodontic treatment. However, the outcome of regenerative endodontic treatment is difficult to predict. As a result, the goal of this study was to summarise the impacts of many factors on the outcome of regenerative endodontic treatment in order to produce more predictable outcomes. We compared the characteristics of regenerative endodontic therapy to those of other pulp treatment procedures and studied the elements that influence regenerative endodontic treatment in this study.

2) Antimicrobial Effects of Triple Antibiotic Paste and Calcium Hydroxide Mixed with 2% Chlorhexidine as Intracanal Medicaments Against Enterococcus faecalis biofilm were compared in an in vitro investigation. Sixty-five excised single-rooted human teeth were chosen, all of which had straight root canals. The cementoenamel junction (CEJ) was used to cut the crowns, and the canals were prepared using the step-back technique. EDTA (17%) and sodium

hypochlorite (5.25%) were used to dissolve the smear layer (NaOCl). Following that, the samples were gamma-ray sterilised before being placed in microtubes for a week. The teeth got infected with EF during this week. The canals were then filled with a TAP and Ca(OH)2 mixed with 2% CHX. The roots were sliced longitudinally, and dentin chips were collected using a round bur to a depth of 400 µm from the apical section of the roots. Counting colony-forming units (CFUs) was used to determine the vital bacterial load, and the study revealed that Ca(OH)2 combined with 2% CHX is more effective against EF biofilm than TAP.

3) Suzuki T et al, studied the minimum inhibitory concentrations of CXM and LVFX against endophthalmitis isolates were compared against conjunctival sac isolates. The average FIC indices of combined CXM and LVFX used in endophthalmitis isolates were significantly lower than those in conjunctival sac isolates ($P < 0.001$). The synergistic activities of CXM and LVFX combinations were observed in 50% of endophthalmitis isolates and no conjunctival sac isolates. No consistent additive or synergistic effect was observed in Enterococcus faecalis isolates from endophthalmitis cases.

4) An in vitro investigation was carried out to assess the cytotoxicity of poly-methylmethacrylate (PMMA)-based dental temporary filling resin to dental pulp cells, as well as the possibility of reducing the toxicity by using an anti-oxidant amino-acid, N-acetyl cysteine (NAC). The resin material was cultivated with or without dental pulp cells taken from rat maxillary incisors. Osteoblastic medium containing dexamethasone was given to the cultures. At 24 hours after planting, 45% of the cells on the PMMA dental resin were necrotic. By adding NAC into

the resin, this percentage was lowered to 27%, which was the same as the culture on polystyrene. On days 5 and 10, the culture on the untreated resin was found to be devoid of alkaline phosphate (ALP) activity, as well as von Kossa mineralized nodule development. Some sections of the cultures on NAC-incorporated resin substrates, on the other hand, tested positive for ALP and von Kossa. In day 7 culture on untreated resin, the collagen I and dentin sialoprotein genes were minimally expressed. Those genes, on the other hand, were expressed in the resin culture with NAC. These findings imply that by including NAC into the resin, the decreased cell viability and practically entirely suppressed odontoblast-like cell phenotype of dental pulp cells cultivated on PMMA dental resin can be saved to a clinically important degree.

5) The cytotoxicity of triple antibiotic paste and calcium hydroxide against cultured human dental pulp fibroblasts was tested in vitro. The dental pulp of two third molars was harvested for fibroblasts. TAP and CH doses of 0.1, 1, and 10 mg/mL were given to fibroblasts. For each medication, six samples were prepared, and fibroblast vitality was assessed after 72 hours. The cytotoxicity of the medicament was classified as severe (30 percent), moderate (30-60 percent), mild (60-90 percent), and non-toxic (>90 percent) based on the percentage of cell viability. The reduction in cell viability of fibroblasts caused by an increase in concentration was shown to be significant in this investigation.

6) An in-vitro investigation was conducted to assess the effects of N-acetyl cysteine alone and in conjunction with antibiotics on Prevotella intermedia, and it was discovered that N-acetyl cysteine had an antibacterial impact on Prevotella intermedia and considerably reduced biofilm formation. N-acetyl

cysteine (NAC) is an antioxidant with anti-inflammatory properties in the body's tissues. NAC has been shown to suppress the development of LPS-induced inflammatory mediators in phagocytic cells and gingival fibroblasts during the inflammatory process in the area of dentistry, however the effect of NAC on oral infections has been examined only infrequently. They looked at how NAC affected Prevotella intermedia planktonic and biofilm cells, a common oral pathogen. NAC had antibacterial action against planktonic P. intermedia with a MIC of 3 mg/m and dramatically reduced bacterial biofilm formation even at sub-MIC levels. Antibiotic susceptibiliy was not affected by NAC. In combination with ampicillin, ciprofloxacin, tetracycline, or metronidazole, the results were indifferent (fractional inhibitory concentration index of 0.5-4) against the bacterium. In the presence of NAC, however, the survivability of the pre-established bacterial biofilm exposed to antibiotics other than metronidazole was increased. NAC may be utilised to inhibit the production of bacterial biofilms by P. intermedia rather than eradicating pre-existing bacterial biofilms.

7) The minimal intracanal dressing time of triple antibiotic paste to eradicate Enterococcus faecalis (ATCC 29212) was evaluated in an Ex-Vivo investigation, as well as the minimum inhibitory and bactericidal concentrations. After instrumentation, E. faecalis was inoculated into the root canals of 34 removed human single canal teeth, and 4 g of TAP (ciprofloxacin, metronidazole, and doxycycline) was mixed with 4.5 mL of saline and used as an intracanal medicament. Dentin chips were obtained and analysed to assess the number of bacterial colonies in the teeth, which were sectioned longitudinally. TAP's MIC and MBC were determined using a micro-dilution

broth assay. The study found that E. faecalis was removed from the dentinal tubules of the apical half of the root canal up to 400µm depth after seven days of intracanal application of TAP. TAP's MIC and MBC were both found to be 16 g/mL at its original concentration.

8) An in-vitro investigation was conducted to assess the antimicrobial efficacy of Calcium Hydroxide and a Triple Antibiotic Paste Combination on E. faecalis Biofilm, with the conclusion that TAP was more effective than CH. When compared to the two treatments taken separately, the combination of CH and TAP showed to be more successful. Calcium hydroxide (CH) is an intracanal medicament that has been frequently utilised in endodontics and, due to its high alkalinity, can kill germs. E. faecalis, on the other hand, is resistant to CH. TAP is a combination of ciprofloxacin, minocycline, and metronidazole that is extremely effective against E. faecalis. The experiment was carried out using the agar diffusion method, in which three wells in Tryptone soya agar were punched and filled with CH, TAP, or a combination of both. The zone of inhibition values were recorded and statistical analysis was performed using SPSS. ANOVA with one way effects and post hoc analysis. The means were compared using the combination of CH and TAP performed significantly better than either CH or TAP alone (p-value 0.05).

9) Progress and Prospects in the Biological Activities and Potential Oral Applications of N-Acetylcysteine found that data on NAC's multiple biological properties, including antioxidant, anti-inflammatory, antibacterial, and anticarcinogenic capabilities has exploded in the last decade. The oral cavity has been subjected to a variety of environmental exposures that have the potential to

cause oxidative stress, inflammation, and even cancer. . NAC's biological and pharmacological actions, as well as its capacity to bypass disease development processes, make it a viable treatment agent for dental and oral illnesses. Still, because most of the outcomes in this field of research come from in vitro and in vivo studies, its clinical usefulness needs to be investigated further. The following should be the subject of future research: (i) develop novel dental and implantable materials with improved biocompatibility by incorporating NAC, (ii) investigate whether NAC could be used alone or in combination with other drugs to treat oral lichen planus, and (iii) investigate whether NAC could be used clinically as an intracanal medicament alternative in root canal therapy, (iv) to assess the clinical efficacy of NAC in the treatment of wound healing, and (v) to assess the clinical use of NAC as an anticancer adjuvant in the treatment of oral cancer.

10) A study of the effect of N-acetylcysteine in a combined antibiofilm treatment against antibiotic-resistant Staphylococcus aureus found that include NAC in a combination treatment is a potential technique for eradicating S. aureus biofilms. The intrinsic acidity of NAC has been found as a critical factor in biofilm breakup and matrix component degradation. NAC is a potent antioxidant that boosts glutathione production and scavenges free radicals in the body.Recent studies have used NAC to demonstrate successful disruption of multispecies biofilms, while also preventing biofilm formation on synthetic surfaces. The antibacterial activity of NAC is hypothesized to stem from the interaction between its thiol group (–SH) with bacterial cell wall proteins. Inhibition occurs because NAC can prevent cysteine utilization by bacterial cells, thereby inhibiting bacterial growth and proliferation. NAC by itself

showed a significant capacity to disrupt preformed biofilms and prevent adhesion of bacterial cells, thus impairing biofilm formation . There was no significant difference in the effect of 10 and 30 mm NAC in inhibiting adhesion, hence its effect is not concentration dependent . Thus, if successful in in vivo studies, this combination has potential to successfully treat existing S. aureus biofilms and prevent formation on synthetic surfaces such as catheters and implant devices.

11) Haapasalo M, Ørstavik D in 1987 conducted an in vitro model for dentinal tubule infection of root canals. Cylindrical dentin specimens, 4 mm high with a diameter of 6 mm and a canal 2.3 mm wide, were prepared from freshly extracted bovine incisors. The cementum was removed from all dentin blocks. The tubules were opened by four-minute treatments with 17% EDTA and 5.25% NaOCl before being infected with Enterococcus faecalis ATCC 29212 in yeast extract-glucose broth. Bacteria rapidly invaded the tubules. After three weeks of incubation, a heavy infection was found 400 micron from the canal lumen, and the front of the infection reached 1000 micron in some blocks. Camphorated para mono chlorophenol (CMCP) and a calcium hydroxide compound, Calasept, were tested for their disinfecting efficacy toward E. faecalis-infected dentin. Liquid CMCP rapidly and completely disinfected the dentinal tubules, whereas CMCP in gaseous form disinfected tubules less rapidly. Calasept failed to eliminate, even superficially, E. faecalis in the tubules. The method used in bacteriological sampling allowed for sequential removal of 100-micron-thick zones of dentin from the central canal toward the periphery. Control specimens were uniformly infected and yielded growth in

bur samples up to some 500 microns from the surface. The model proved quite sensitive and seems suitable for in vitro testing of root canal medicaments.

12) Kim D et al concluded that goal of endodontic treatment is the prevention and control of pulpal and peri radicular infections. Calcium hydroxide (Ca(OH)2) has been widely used in endodontics as an intra canal medicament to eliminate the remaining microorganisms after chemomechanical preparation. The purpose of this article is to review the antimicrobial properties of Ca(OH)2 as an intra canal medicament in root canal treatment. The first part of this review details the characteristics of Ca(OH)2 and summarizes the results of in vitro studies related to its antimicrobial effect. The antimicrobial effect of Ca(OH)2 results from the release of hydroxyl ions when it comes into contact with aqueous fluids. Ca(OH)2 has a wide range of antimicrobial effects against common endodontic pathogens, but is less effective against Enterococcus faecalis and Candida albicans. The addition of vehicles or other agents might contribute to the antimicrobial effect of Ca(OH)2.

13) Ridhalaksani, et al studied the antibacterial potential of NAC as an endodontic irrigant on the clinical isolates of the *Enterococcus faecalis* biofilm and concluded that the NAC pH 2.5 test group showed a reduction in the *E. faecalis* colonies, but this reduction was not statistically significant when compared to the 2% CHX group results. The NAC pH 11 test group showed the greatest reduction in bacterial colonies, and this reduction was statistically significant when compared to the NAC pH 2.5 and 2% CHX groups' results.

14) Quah SY et al studied the antibacterial and biofilm eradication efficacies of N-acetylcysteine (NAC) on Enterococcus faecalis and concluded that NAC was

most bactericidal at pH 11 with MIC and MBC of 1.56 mg/mL and 12.5 mg/mL, respectively. Although preincubation of calcium hydroxide with dentin powder abolished its antibacterial effects, NAC completely killed E. faecalis regardless of dentin powder preincubation. In addition, prolonged incubation of NAC with dentin powder (up to 3 weeks) did not significantly reduce its antibacterial activity on E. faecalis. Furthermore, NAC also effectively eradicated E. faecalis biofilms.

15) Jain P, et al studied the efficacy of triple antibiotic solution as a new endodontic irrigant that may possess superior antibacterial activity in comparison with chlorhexidine solution. And it was concluded that triple antibiotic solution can be used as an irrigating solution. The antibacterial action of triple antibiotic irrigating solution is comparable with chlorhexidine. Although saline may not be effective in the antimicrobial action but its flushing action may be able to decrease some microbial load.

MATERIALS AND METHODS

SOURCE OF DATA:

"The study was conducted in the "Department of Conservative Dentistry and Endodontics, KLE Academy of Higher Education & Research (KLE University), KLE VK Institute of Dental Sciences, Belagavi"

Evaluation of minimum inhibitory concentration (MIC) and colony-forming units was undertaken in Dr. Prabhakar Kore's Basic Science Research Centre (BSRC), KLE University, Belagavi.

Extracted human single-rooted single canal teeth were collected from the "Department of Oral and Maxillofacial Surgery, KLE Academy of Higher Education & Research, KLE VK Institute of Dental Sciences, Belagavi."

INCLUSION CRITERIA:

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

EXCLUSION CRITERIA:

- Teeth with apical width more than #20 K-file size.
- Teeth with calcified canals.
- Teeth with fracture/crack or a restoration.
- Teeth with internal and external root resorption.

- Teeth with the presence of anatomic variations
- Teeth with curvature
- Teeth with endodontic treatment
- Teeth with radiographically invisible canals or multiple canals

MATERIALS USED FOR THE STUDY:

68 extracted single-rooted single canal teeth

- 0.1% Thymol
- 3% Sodium hypochlorite (NaOCL) (Vishal dental care)
- 17% Ethylene diamine tetraacetic acid (EDTA) [CANALARGE]
- Normal Saline solution (Baxter NS Sodium Chloride IP 0.9% W/V)
- Propylene glycol
- Distilled water
- Triple antibiotic paste-Metronidazole (500mg) Ciprofloxacin (250mg) Minocycline (100mg)
- N- Acetylcysteine
- Levofloxacin
- Paraffin wax
- Cavit G (3M, ESPE)
- Brain heart infusion broth
- E.faecalis strain
- Brain heart infusion agar plates

ARMAMENTARIUM USED FOR THE STUDY

- Micromotor and straight handpiece (NSK PANA AIR)
- Gates Glidden Drill (MANI)
- Lentulospiral
- Carborundum Disks
- Eppendorf tubes
- Micropipettes and tips (100µm) (1000µm)
- Laminar air

METHODOLOGY

Sixty-eight extracted human single-rooted, single - canal teeth 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 was utilized as storage solution till use. All teeth were radiographed and selected as per the inclusion and exclusion criteria.

SAMPLE PREPARATION

A diamond disc was used to section 4 mm of the apical third of the root from the apex and then decoronation below the cementoenamel junction was done to obtain 6 mm of the middle third of the root. After this, a sterile Gates Glidden drill was used sequentially from no. 1-3 in a slow-speed handpiece to standardize the internal diameter of the root canal.

Following this, the specimens were placed in an ultrasonic bath of 17% EDTA for five minutes followed by 3% NaOCl for five minutes to remove the inorganic and organic debris.

To remove the traces of chemicals used, dentin specimens were then immersed in an ultrasonic bath containing distilled water for five minutes following which all the specimens were sterilized in an autoclave (20 min at 121 ℃). Five teeth from the sterilised specimens were placed in a sterile BHI broth to serve as a negative control to check for the absence of contamination.The apex of the remaining 63 specimens were sealed using paraffin wax.

CONTAMINATION OF SPECIMENS

E. faecalis (ATCC 439) was grown in BHI agar for 24 hours. The culture was suspended in 5 mL of BHI broth and incubated for 4 hours at 37℃ and its turbidity was adjusted at 0.5 Mcfarland standard (1×10^{8} CFUmL−1).

Each dentin specimen was placed in a pre-sterilized microcentrifuge tube containing 1 mL of the BHI broth. A 50 µL of the inoculums containing *E.faecalis* was transferred into each of the microcentrifuge tubes.

Following this, at the end of 24 hours, the dentin specimens were transferred into a fresh broth containing *E.faecalis.* All procedures were carried out under laminar flow. The purity of the culture was checked by subculturing 5 µL of the broth from the incubated dentin specimens in BHI broth on BHI agar plates. The contamination of the dentin specimens was carried out for 21 days.

At the end of 21 days, the specimens were irrigated with 5 mL of sterile saline to remove the incubation broth. Then they were divided into 3 groups with 21 specimens in each group.

- Group 1 – Triple antibiotic paste
- Group 2 – N-Acetylcysteine and Levofloxacin
- Group 3 – Positive control saline

MINIMUM INHIBITORY CONCENTRATIONS EVALUATION

For minimum inhibitory concentrations evaluation: 9 dilutions of the medicaments from Group 1 and 2 were done with Brain heart infusion broth.

In the initial tube, only 500 µl of the compound was added. For dilutions, 500 µl of Brain heart infusion broth was added into the next 9 tubes separately. In the 1st tube, 500 µl of the compound was added with 500 µl Brain heart infusion broth. This was considered as 10^{-1} dilution.

From 10^{-1} diluted tube, 500 µl was transferred to the second tube to make 10^{-2} dilution. The serial dilution was repeated up to 10^{-8} dilutions for each compound. From the maintained stock cultures of required organisms, 20 µl was taken and added into 2 mL of brain heart infusion broth.

In each serially diluted tube, 500 µl of above culture suspension was added. The last tube contained only the media and culture suspension. The tubes were kept for incubation for 24 hours at 37^0C in a bacteriological incubator and observed for turbidity. After this minimum inhibitory concentration was evaluated.

PREPARATION AND APPLICATION OF THE MEDICAMENT

Both the combinations that is Triple antibiotic paste, N-acetylcysteine with Levofloxacin were prepared according to the values obtained after evaluating minimum inhibitory concentration with 1mL of propylene glycol.

The respective medicaments were placed in the root canal and the specimens for the positive control group were rinsed with saline and paraffin wax was used to seal both ends, the specimens were then incubated in an anaerobic environment at 37°C for seven days.

ANTIMICROBIAL ASSESSMENT

Microbial cell assessment was carried out at the end of seven days of incubation. Harvesting of dentin was carried out at a depth of 400 μm by preparing the root canal circumferentially using sterile Gates Glidden drills no.5 in a slow-speed handpiece.

The fine dentin shavings were collected in a test tube containing 1 mL of sterile BHI broth and incubated in an anaerobic environment at 37°C for 24 hours. After 24 hours the contents were serially diluted 100 μL of broth in 100 μL of sterile saline five times.

About 50 μL of the dilution was then plated on BHI agar plates and incubated for 24 hours at 37°C. The colonies on the agar plates were counted and recorded by a blinded microbiologist, represented in colony-forming units (CFU) per mL, and readings were tabulated. Formulae for evaluating the colony-forming unit:

$$\text{Colony forming unit/mL} = \frac{\text{number of colonies formed}}{\text{Volume plated (mL)} \times \text{Total Dilution used(mL)}}$$

STATISTICAL ANALYSIS –

- Within the groups was done using "Tukeys multiple posthoc procedures"
- Between the Groups was done using "One Way ANOVA"

FLOWCHART OF THE STUDY

Sixty-eight extracted human single-rooted single canal teeth were chosen and maintained conferring to 'OSHA guidelines'

⇩

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

⇩

The decoronation was done using a diamond disc at 4mm from the apex to obtain 6 mm of the middle third of the root.

⇩

Root specimens were prepared with Sterile Gates glidden drill used sequentially from no. 1-3 (0.9mm)

⇩

Inorganic and organic debris from the specimens were removed by placing them in an ultrasonic bath of 17% EDTA for 5 min followed by 3% NaOCl for 5 min

⇩

Five teeth were placed in a sterile broth to serve as a negative control.

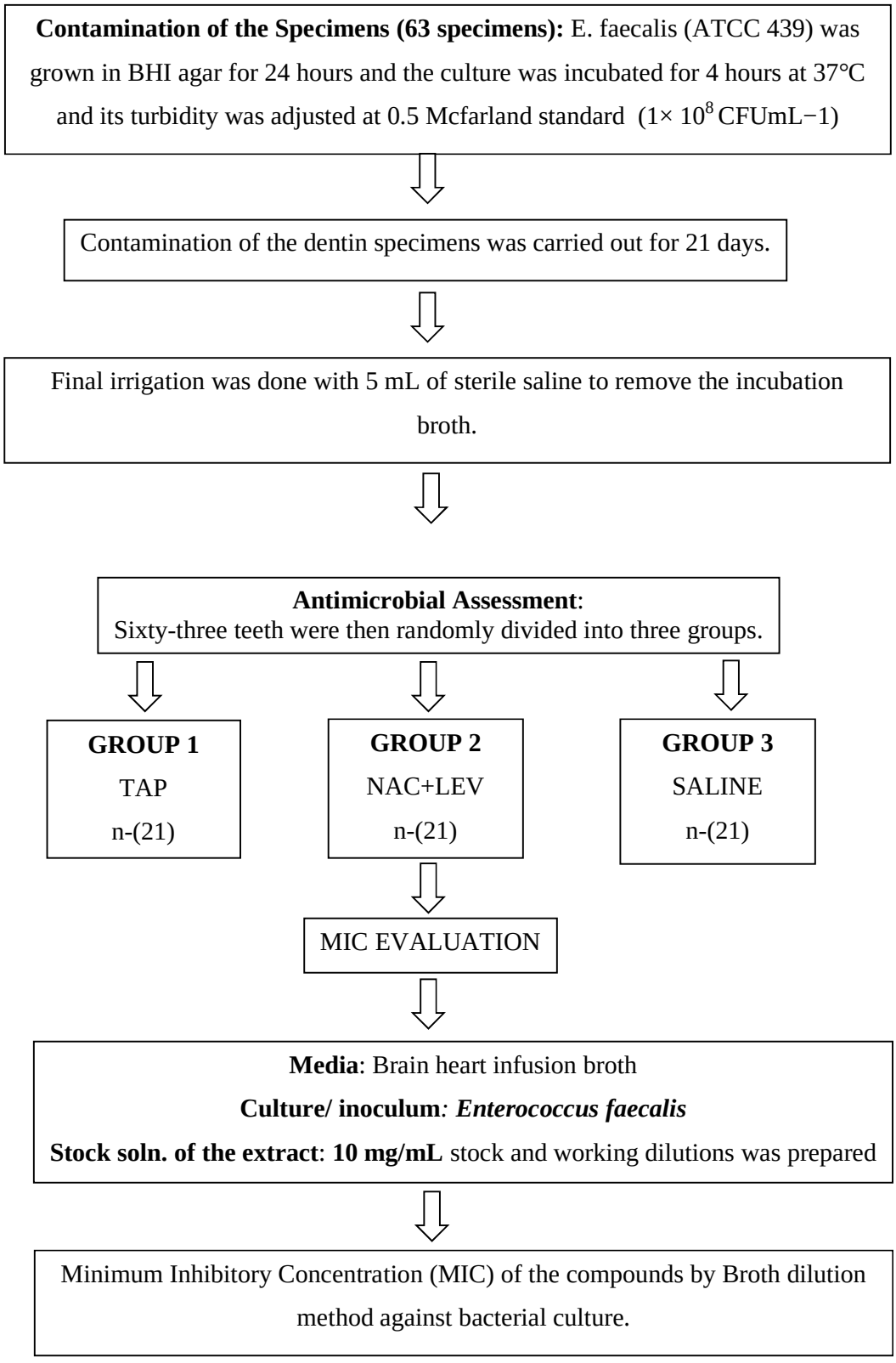
⇩

The apex of all the specimens were sealed with paraffin wax.

⇩

Chemicals were removed by immersing the dentin specimens in an ultrasonic bath containing distilled water for 5 min

⇩


Contamination of the Specimens (63 specimens): E. faecalis (ATCC 439) was grown in BHI agar for 24 hours and the culture was incubated for 4 hours at 37°C and its turbidity was adjusted at 0.5 Mcfarland standard (1× 10^8 CFUmL−1)
Contamination of the dentin specimens was carried out for 21 days.
Final irrigation was done with 5 mL of sterile saline to remove the incubation broth.
Antimicrobial Assessment:
Sixty-three teeth were then randomly divided into three groups.
GROUP 1
TAP
n-(21)
GROUP 2
NAC+LEV
n-(21)
GROUP 3
SALINE
n-(21)
MIC EVALUATION
Media: Brain heart infusion broth
Culture/ inoculum*: **Enterococcus faecalis***
Stock soln. of the extract: **10 mg/mL** stock and working dilutions was prepared
Minimum Inhibitory Concentration (MIC) of the compounds by Broth dilution method against bacterial culture.

9 dilutions of the compound (medicaments from Group 1 and 2) were done with Brain heart infusion Broth for MIC.
In the initial tube, only 500 µl of the compound was added.

In the 1st tube, 500 µl of the compound was added with 500 µl Brain heart infusion broth. This was considered as 10^{-1} dilution

From 10^{-1} diluted tube 500 µl was transferred to the second tube to make 10^{-2} dilutions. The serial dilution was repeated up to 10^{-8} dilutions for each compound

20 µl was taken from the maintained stock cultures of required organisms and added to 2 mL of brain heart infusion broth. In each serially diluted tube, 500 µl of above culture suspension was added.

The last tube contained only the media and culture suspension. The tubes were kept for incubation for 24 hrs at 37^0C in the bacteriological incubator and observed for turbidity

Preparation and application of the medicaments

Group 1- Triple antibiotic paste was prepared according to the values obtained from MIC with 1mL of propylene glycol.

Group 2- Combination of N-Acetylcysteine and Levofloxacin paste was prepared according to the values obtained from MIC with 1mL of propylene glycol.

GROUP 3- 21 samples were kept in saline and then inoculated with E.faecalis.

The respective medicaments were placed in the root canal and placed in an incubator for 7 days

Microbial cells assessment :

Harvesting of dentin was carried out at a depth of (400 μm) by preparing the root canal circumferentially using sterile Gates Glidden drills no.5 in a slow-speed handpiece.

The fine dentin shavings were collected in a test tube containing 1 mL of sterile BHI broth and incubated in an anaerobic environment at 37°C for 24 hours

About 50 μL of the dilution was then plated on BHI agar plates and incubated for 24 hours at 37°C. The colonies on the agar plates were counted and recorded, by a blinded microbiologist using the formulae:

$$\text{Colony-forming unit/mL} = \frac{\text{number of colonies formed}}{\text{Volume plated (mL)} \times \text{Total Dilution used(mL)}}$$

The readings were then tabulated.

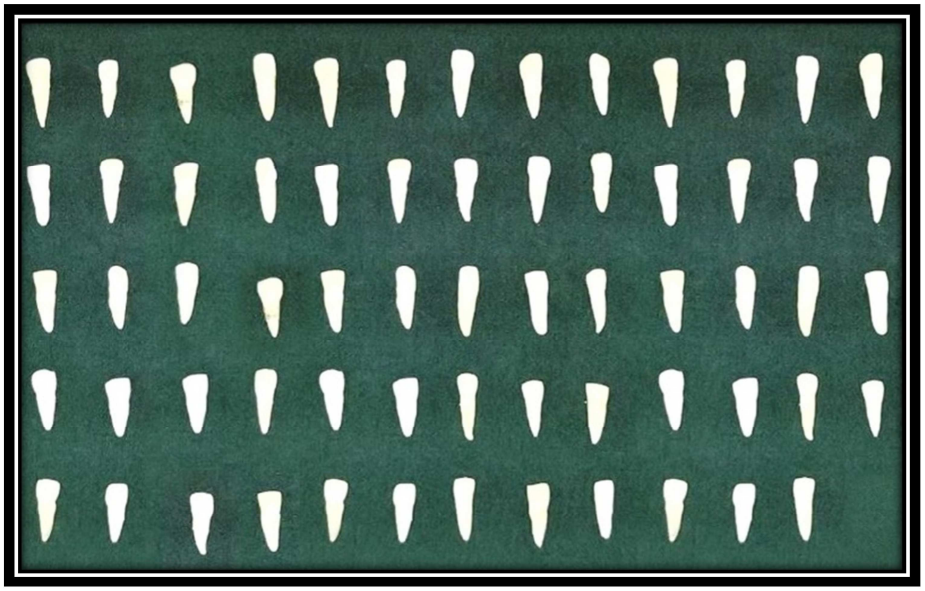


Fig 1: Total Sample Size (n=68)

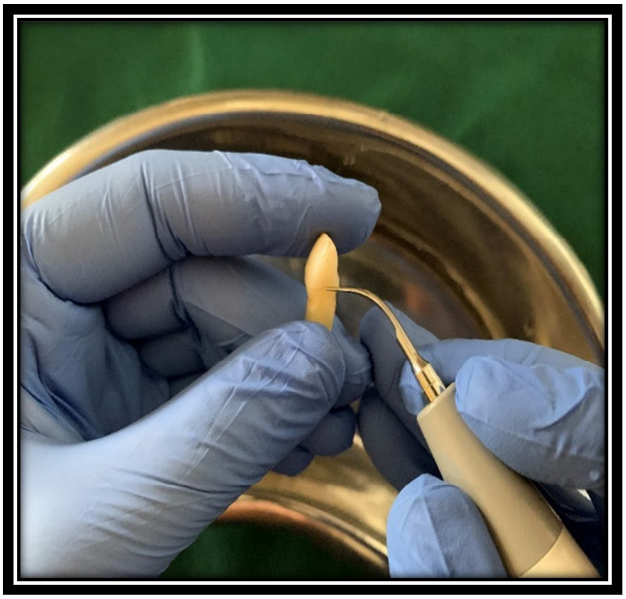


Fig 2: Removal of debris and calculus using ultrasonic scaler

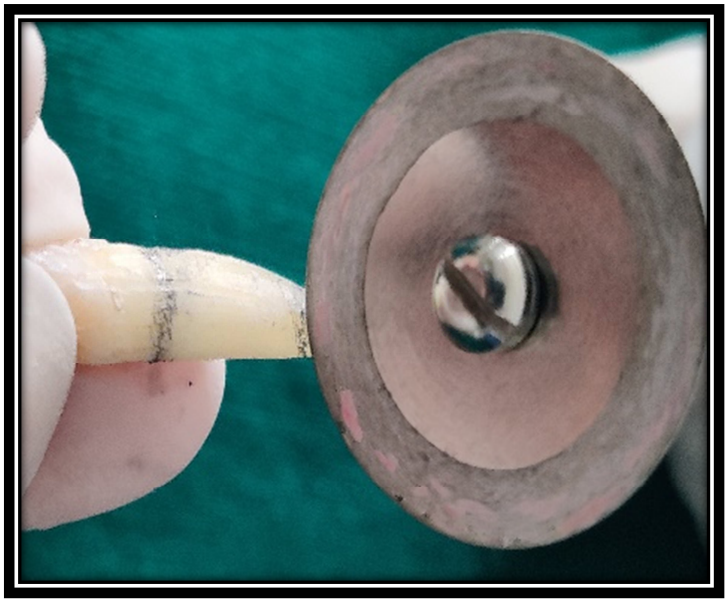


Fig 3: Decoronation of sample at 4mm from apex

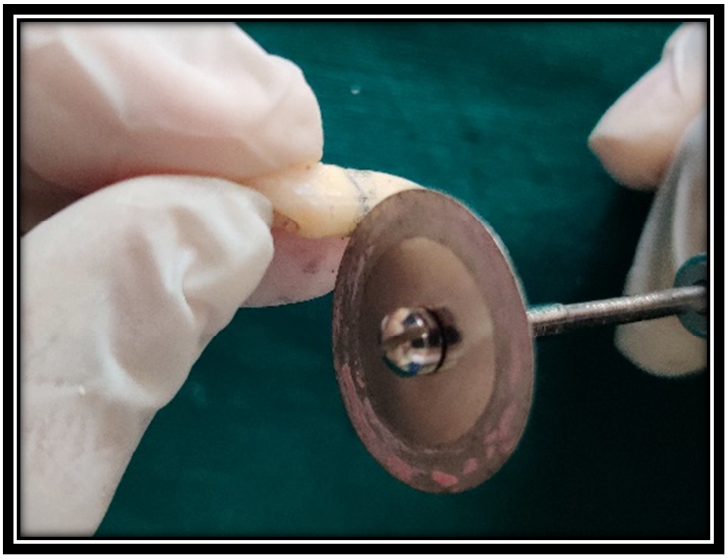


Fig 4: Decoronation of sample at 6mm below CEJ

Fig 5: Materials used for irrigation

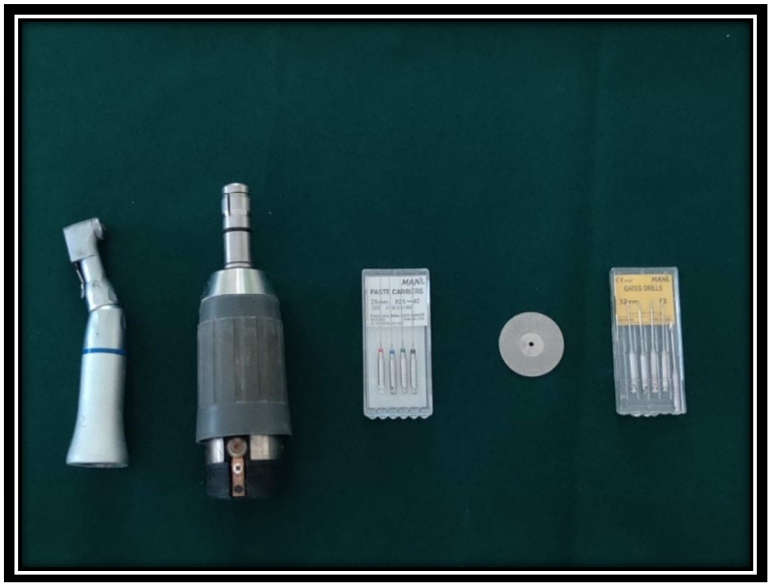
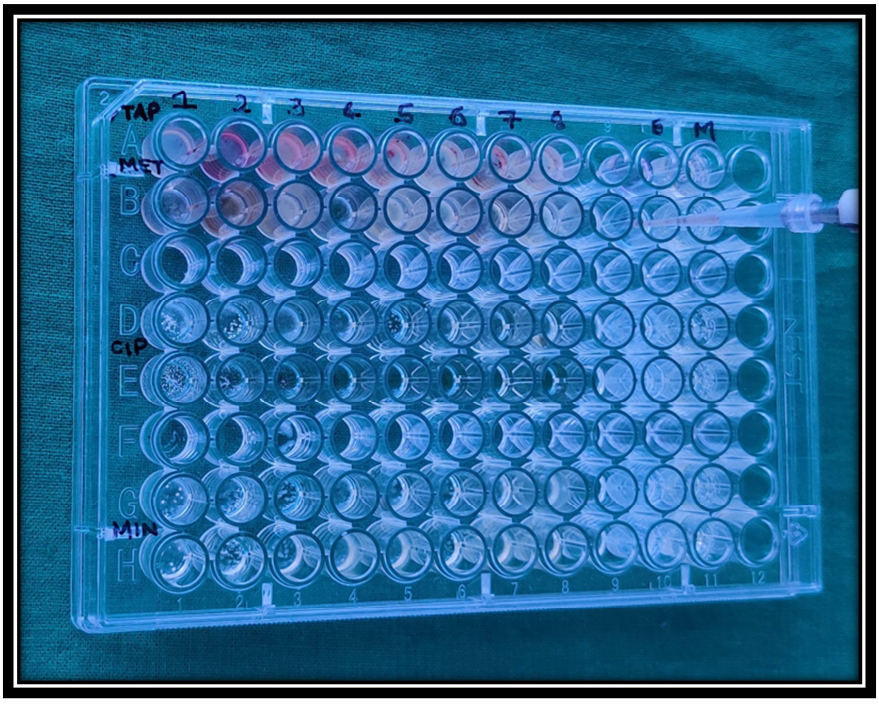


Fig 6: Material used for sectioning of crown and placement of medicament

Fig 7: Materials used for microbiological assessment



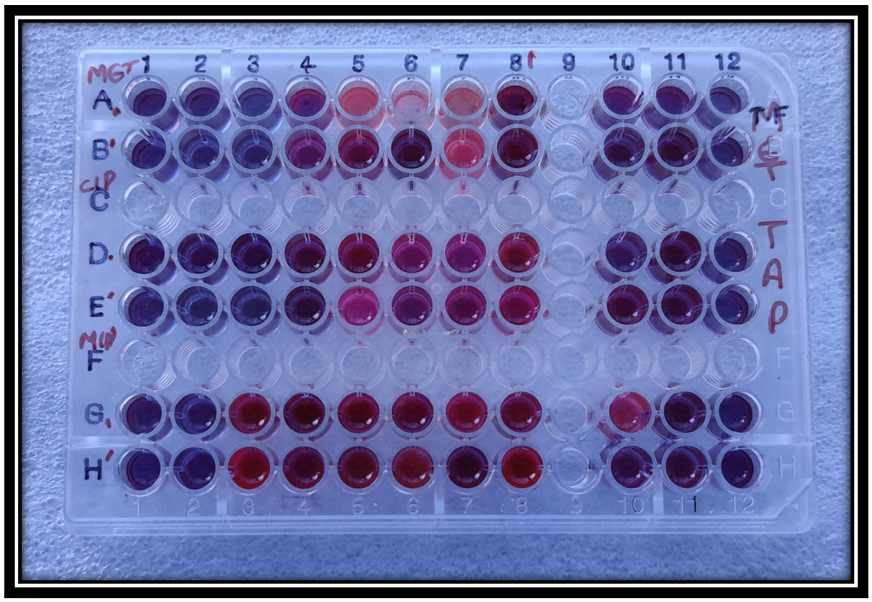
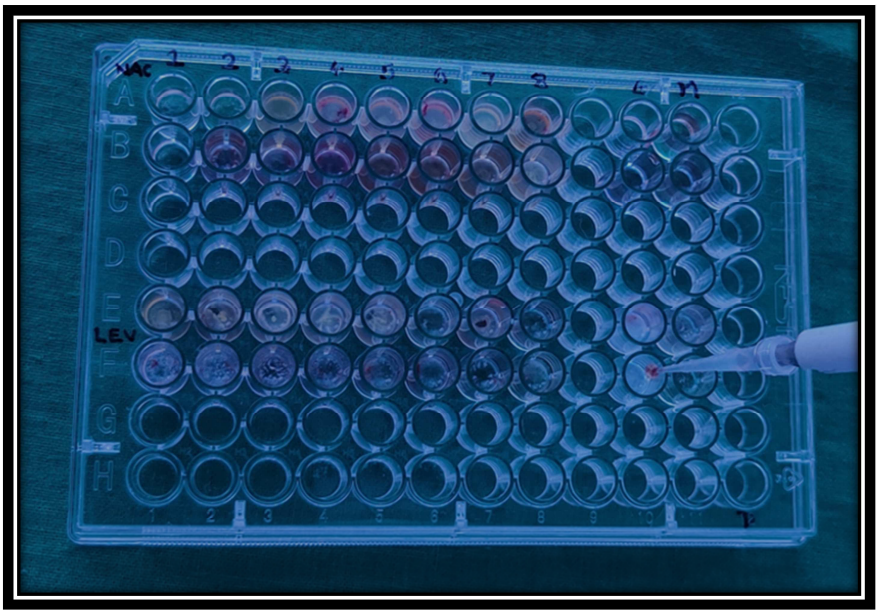


Fig 8 : MIC of Group 1 (TAP)



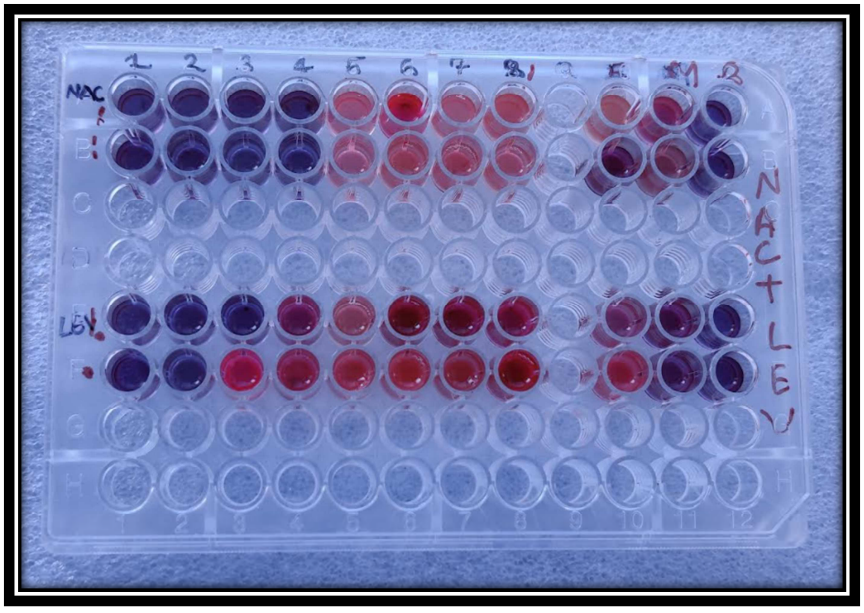


Fig 9: MIC of Group 2 (NAC And LEV)

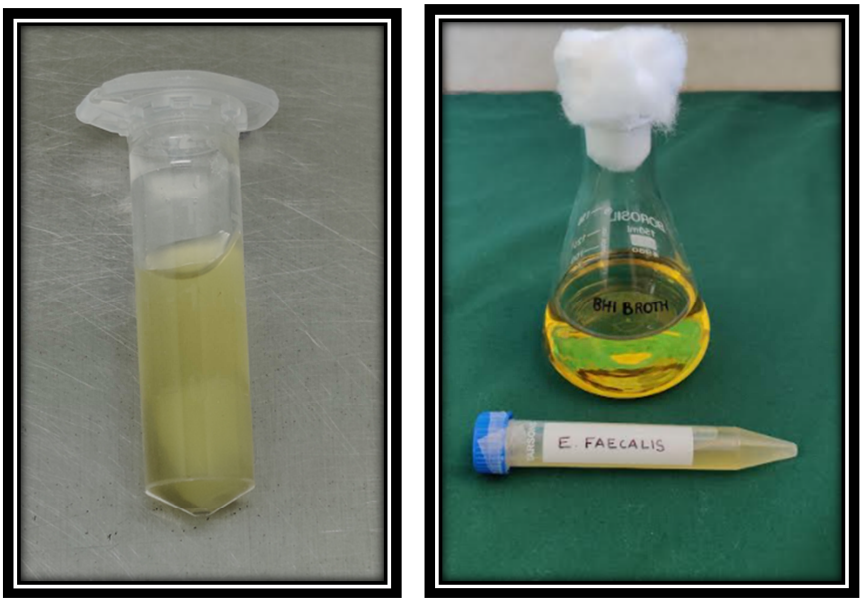


Fig 10: Test samples were inoculated with *E. faecalis*

Fig 11: Samples were kept in incubator

Fig 12: Preparation of TAP

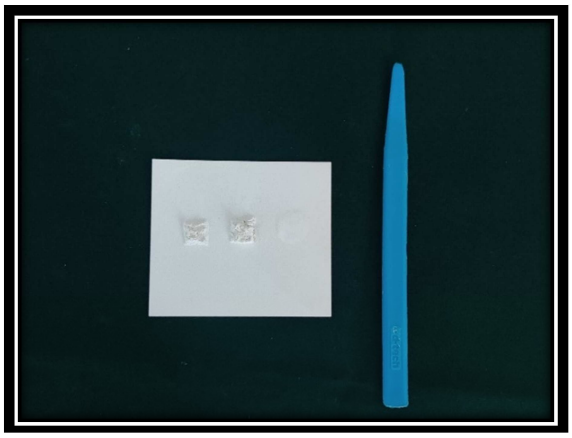


Fig 13: Preparation of NAC AND LEV

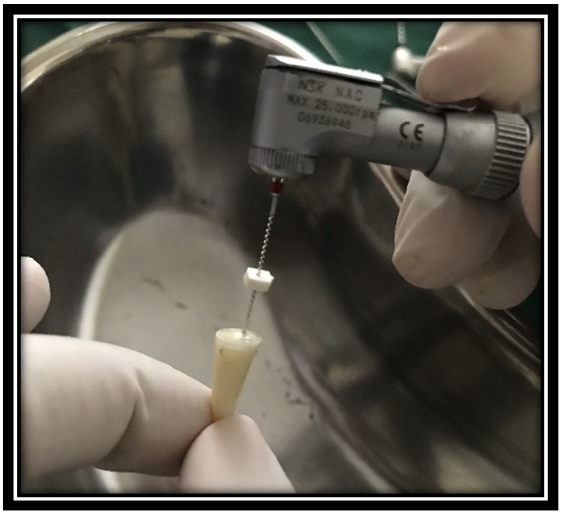


Fig 14: Placement of the medicament inside the canals using lentulospiral

Fig 15: Colony forming unit of Group 1 (TAP)

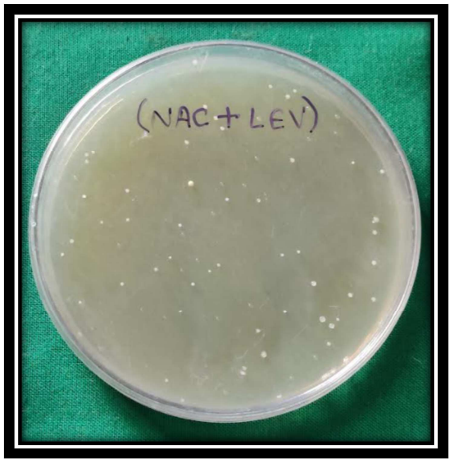


Fig 16: Colony forming unit of Group 2 (NAC+LEV)

Fig 17: Colony forming unit of Group 3 (SALINE)

RESULTS

Table 1. Colony forming units of medicaments (cfu) ($x10^3$)

Sr no. (Sample)	Triple antibiotic paste (group 1)	N-acetylcysteine and levofloxacin (group 2)	Saline – positive control (group 3)
1	100×10^3	45×10^3	345×10^3
2	200×10^3	78×10^3	400×10^3
3	156×10^3	43×10^3	298×10^3
4	325×10^3	67×10^3	250×10^3
5	184×10^3	48×10^3	378×10^3
6	234×10^3	76×10^3	222×10^3
7	167×10^3	29×10^3	218×10^3
8	190×10^3	34×10^3	290×10^3
9	201×10^3	56×10^3	350×10^3
10	127×10^3	28×10^3	367×10^3
11	167×10^3	45×10^3	399×10^3
12	135×10^3	26×10^3	369×10^3
13	178×10^3	37×10^3	267×10^3
14	145×10^3	50×10^3	227×10^3
15	209×10^3	57×10^3	198×10^3
16	235×10^3	35×10^3	498×10^3
17	350×10^3	53×10^3	555×10^3
18	265×10^3	56×10^3	543×10^3
19	287×10^3	66×10^3	567×10^3
20	123×10^3	71×10^3	543×10^3
21	112×10^3	59×10^3	444×10^3

Table 2: Summary of Colony forming units (CFU) ($x10^3$) in Group 1, Group 2 and Group 3

Groups	Min	Max	Mean	SD	SE	95% CI for mean	
						Lower Bound	Upper Bound
Group 1	100.00	350.00	194.76	68.20	14.88	163.72	225.80
Group 2	26.00	78.00	50.43	15.66	3.42	43.30	57.56
Group 3	198.00	567.00	368.00	120.24	26.24	313.27	422.73

Table 3: Comparison of Group 1, Group 2 and Group 3 with Colony forming units (CFU) ($x10^3$) by one way ANOVA

Sources	Sum of Squares	df	Mean Square	F-value	p-value
Between groups	1061866.13	2	530933.06	82.3020	0.0001*
Within groups	387060.95	60	6451.02		
Total	1448927.08	62			

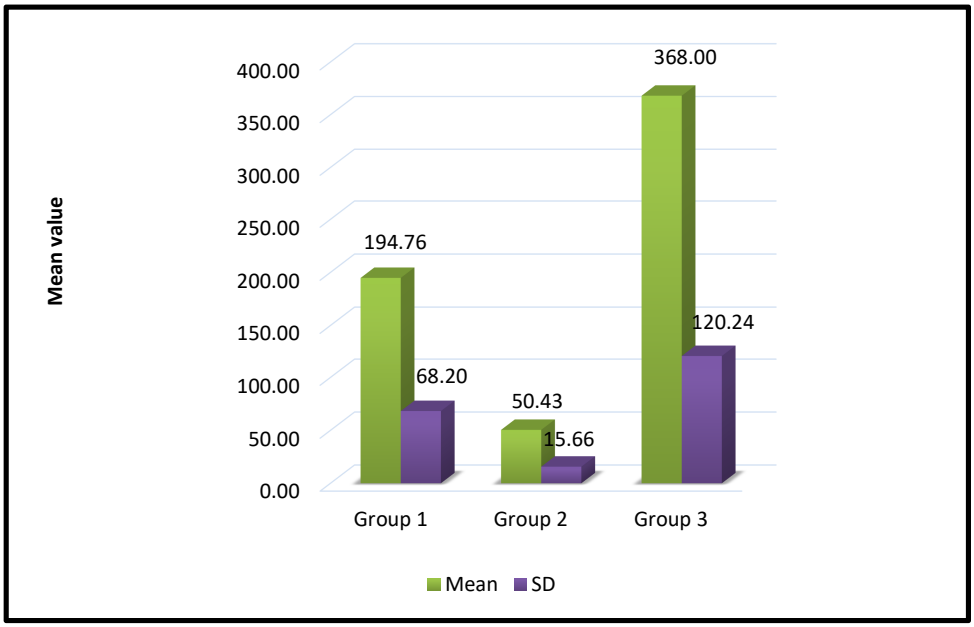
*$p<0.05$

Table 4: Pair wise comparison of Group 1, Group 2 and Group 3 with Colony forming units (CFU) ($x10^3$) by Tukeys multiple posthoc procedures

Groups	Mean diff.	Std. Error	p-value
Group 1 vs Group 2	144.3333	24.7867	0.0001*
Group 1 vs Group 3	-173.2381	24.7867	0.0001*
Group 2 vs Group 3	-317.5714	24.7867	0.0001*

*$p<0.05$

Graph 1: Comparison of Group 1, Group 2 and Group 3 with Colony forming units (CFU) ($x10^3$)



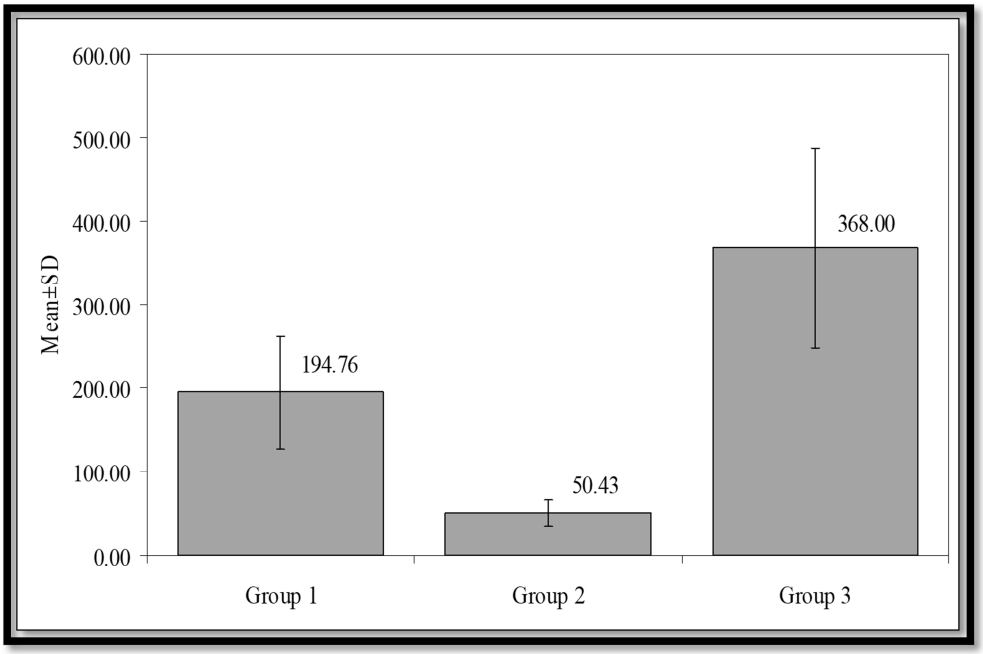
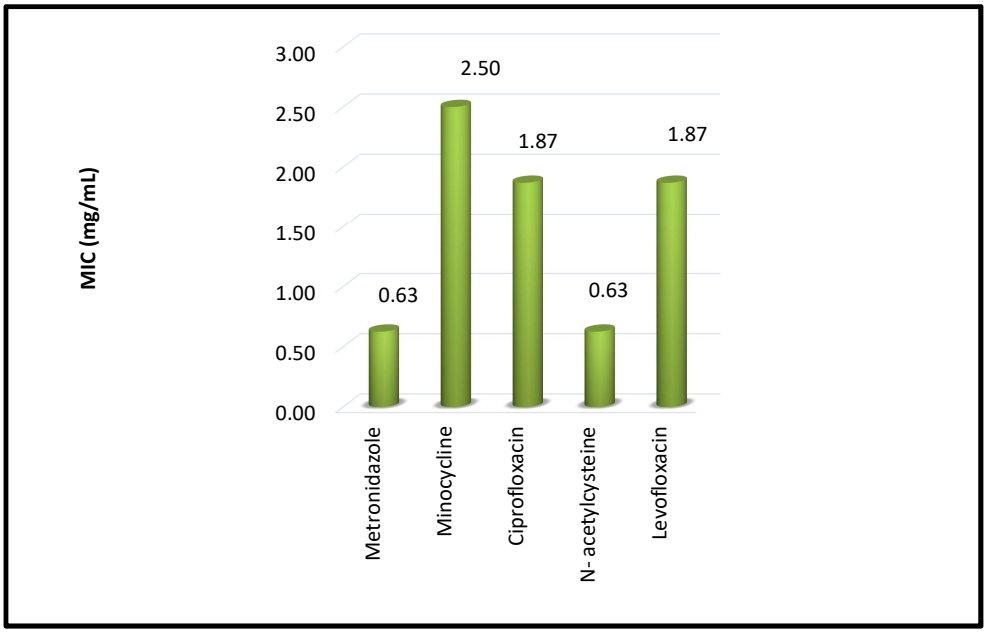


Table 5: Minimum inhibitory concentration MIC (mg/mL)

Compound	MIC (mg/mL)
Metronidazole	0.625
Minocycline	2.5
Ciprofloxacin	1.87
N- acetylcysteine	0.625
Levofloxacin	1.87

Graph 2 : Minimum inhibitory concentration MIC (mg/mL)



Results:

The following hypothesis was taken for this study-

H0: The null hypothesis taken was that there is no difference in the antimicrobial efficacy of a combination of N-acetyl cysteine and Levofloxacin with Triple antibiotic paste against E. faecalis biofilm.

H1: There is a difference in the antimicrobial efficacy of a combination of N-acetyl cysteine and Levofloxacin with Triple antibiotic paste against E. faecalis biofilm.

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 done using the CFU (Table 2 and Table 3) 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 test (Table 4, Table 5) has shown that Group 2 is the most efficient compared to Group 1 and Group 3 in terms of MIC,and CFU counts.

Tukeys multiple posthoc test 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 showed the lowest mean CFU count (50.43) compared to group 1 and group 3 (68.20 and 368.00 respectively).

Therefore, in this study, **Group 2** i.e. N-acetylcysteine in combination with Levofloxacin showed a statistically significant reduction in biofilm compared to other two groups and proved to be an effective antimicrobial when used as an intracanal medicament.

DISCUSSION

Pulpal involvement as a result of trauma or caries in immature permanent teeth can cause the loss of pulpal vitality as well as directly affect root development, resulting in short roots with very thin walls, increasing the risk of fracture and obstructing conventional root canal treatment[2].

The treatment for these pulpal pathologies is to remove the pulp tissue and disinfect the root canal system using the various irrigant and intracanal medicament options available today[2]. Apexification is a procedure of induction of a calcified apical barrier in the apical zone of an incompletely formed root, in which the pulp is diagnosed as non-vital, and is one of the possible treatments to perform in an immature permanent tooth with short roots that requires endodontic therapy[3].

Apexification with calcium hydroxide was once the standard therapy for an infected, non-vital, immature tooth[3]. Calcium hydroxide is commonly used as an intracanal medicament and to generate an apical barrier[3]. However, until the root canal treatment is finished, many visits to the dentist for long periods are required. Mineral trioxide aggregate (MTA) is utilized instead of calcium hydroxide to decrease the treatment time. Even after apexification with MTA, the root remains thin and feeble[4].

Science and technology advancements have had enormous positive effects on today's globe. It has made a significant contribution to all aspects of our life. Treatment approaches that were once thought to be impossible are now considered feasible. Regenerative treatment is one such breakthrough. Biological ways to repair

teeth are among the many clinical dental benefits that regenerative treatment promises[5].

A study by Zaheer Shaik et al, as well as the vast majority of other studies evaluating the antimicrobial efficacy of intra canal medicaments, used single-rooted teeth to eliminate any anatomical complexities that could lead to bias in the study. As a result, single-rooted incisors and canines with a single canal were included in the current study [30].

0.1% Thymol was utilized as a storage solution in this study because it renders antifungal action.[30]

The tooth specimens were sectioned to obtain 6 mm of the middle third of the root. Since apical dentine is mostly sclerotic, mid root dentin blocks were used in the study.[30]

Following the model proposed by Haapasalo and Ørstavik 1976, the root canals were enlarged with no. 3 Gates-Glidden burs (ISO size 090) to standardize the internal diameter of the root.[30]

The irrigants used in this study were 3% sodium hypochlorite (NaOCl), 17% ethylene diamine tetra acetic acid (EDTA), and 0.9 percent normal saline. Even at low concentrations, sodium hypochlorite has strong antimicrobial activity and kills bacteria quickly. It is effective against the majority of bacteria found in the root canal. Bystrom and Sundquist 1983 discovered that concentrations above 2% have the dissolving characteristics required for the removal of vital and necrotic tissue. As a result, 3 percent sodium hypochlorite was used because it is a powerful antimicrobial that dissolves pulp remnants.[34]

A review article published in 2010 by Violich and Chandler concluded that using a 17 percent EDTA solution as an irrigant chelates the inorganic tissue and aids in the removal of debris that becomes compacted into the dentinal tubules when the root canal is instrumented.[7] This debris, which includes soft tissue and dentine, is referred to as the smear layer which prevents the root canal system from being thoroughly irrigated. As a final flush, 0.9 percent sterile normal saline was used to neutralize the various chemicals of the irrigant through its flushing action.[35]

7 days old biofilm was chosen to evaluate the antibacterial efficacy because the incubation period was sufficient for the formation of clumps of bacteria bounded by carbohydrate matrix, which was a feature normally found in biofilm. [29,37,39]

Microdilution and agar dilution are both quantitative approaches for determining MIC values (Kim et al. 2007). Although the results obtained with the agar dilution method show a good correlation with the microdilution method done by Amsler et al. 2010, the agar dilution method is laborious and time-consuming, and more importantly, the factors that negatively affect the disc diffusion method may also contribute to the lack of accuracy with the agar dilution method, particularly when dealing with sparingly soluble biosurfactants[36]. Microdilution , on the other hand, is a standardized, accurate, low-cost, and simple approach (Jorgensen and Ferraro 2009)[36].

The new microdilution which is the resazurin-based 96-well plate microdilution method was used to test the study's MIC results. This approach has been utilized in several investigations.[37]The use of resazurin dye as a redox indicator to the modified microdilution method used in this study has an action on active bacterial cells. It converts the non-fluorescent resazurin (blue) to the fluorescent

resorufin (pink), which may then be further reduced to hydroresorufin (red) (O'Brien et al. 2000), providing a direct assessment of bacterial metabolic activity.[37]

In the present study, Propylene Glycol was selected as the vehicle of choice to prepare the paste because it exhibits better diffusion of medicament deep within dentinal tubules which enhances antimicrobial action.[38,39]

The drugs were used for seven days to check for any antibacterial properties. Sjogren et al. discovered that a 7-day application of a TAP medication was enough to reduce canal bacteria to a level that resulted in a negative culture[20].

Thales Galvao et al in 2017 studied the efficacy of three methods for inserting calcium hydroxide-based paste in root canals that is conventional technique using a hand instrument (MAN), rotary Lentulo spiral (LEN) technique, and a combined technique combining conventional hand files with sonic activation through the EndoActivator device (EA) and concluded that ,a combined approach utilizing hand files with sonic activation showed no enhancements over the LEN or MAN techniques on the quality of intracanal placement of calcium hydroxide paste.[22]

The efficacy of three methods - McSpadden compactors, Lentulo drills, and files for inserting calcium hydroxide-based paste in root canals was studied, and it was concluded that using $Ca(OH)_2$ paste with lentulospiral produced a more homogeneous filling than manual technique as it has an action of propelling the medicament centrifugally which helps in a better placement[40].Following this, a lentulospiral was used in this study to place small amounts of intra canal medicaments one at a time[40].

However in a study by Jussaro et al 2020, the capacity of different methods (Lentulospiral, Ultrasonic agitation, Endoactivator sonic agitation) for insertion of

calcium hydroxide paste in the filling of lateral canals was evaluated and it was concluded that methods which use agitation device such as ultrasonic, promoted higher filling of calcium hydroxide in simulated lateral canals.[45]

After 7 days the specimens were prepared with GG drill till no.5 and the shavings of which were transferred to agar plates inoculated with BHI broth .The colonies on agar plates were counted by a blinded microbiologist to eliminate bias.[36]
The colonies were counted using the formulae used by many studies for evaluating colonies of E. faecalis after using an antimicrobial medicament.[37,44,56]

Endodontics can be on the cutting edge of regenerative therapy, which is the future of dentistry[6]. Regenerative endodontics offers the possibility of restoring vitality to a non-vital tooth. It aims to replace injured and diseased pulp tissue with functional pulp tissue and reparative dentin, which is impossible to achieve through apexification.[3]

The presence of a root canal infection is an important factor that can influence the outcome of treating necrotic immature tooth. As a result, the most important step in endodontic regeneration is the effective eradication of pathogenic microflora that causes infection.[6]

The microbiota of root canal infections has been studied. In his work, Gajan EB found that necrotic pulp tissue in initial root canal infections indicated a polymicrobial flora with gram-negative species of several kinds of obligate anaerobic bacteria, accounting for 90% of all bacteria[42].In a study published in 2004, Orstavik D found that Enterococcus faecalis was the most common bacterium found in samples of secondary root canal infections as well as cases of reinfection.

Many in vitro antimicrobial experiments have demonstrated that E. faecalis is capable of growing into dentinal tubules (Orstavik & Haapasalo 1990, Siqueira & Uzeda 1996, Gomes et al. 2001).[29,43,44]In a study published in 1985, Byström et al. determined that root canal infection is caused by a combination of bacteria, the most common of which is E.faecalis, coupled with polymicrobial anaerobic species.[46] E. faecalis' ability to survive or adapt to adverse environmental circumstances may provide it an edge over other species. It could explain its ability to survive in root canal infections, where nutrients are sparse and root canal medicaments are difficult to reach.[45]Hence E.faecalis was chosen as the study's test organism.

To achieve success, certain medicaments are essential to create a microbial-free environment in the root canal system. [47]

Intracanal calcium hydroxide is a regularly used medicament .Calcium hydroxide's antibacterial activity is due to the release of hydroxyl ions in an aqueous environment. Damage to the bacterial cytoplasmic membrane, protein denaturation, and DNA damage are likely to be responsible for their deadly effects on bacterial cells[48].

Calcium hydroxide's antibacterial effectiveness is due to its high pH (12.5), which has a damaging effect on bacterial cell membranes and protein structure. $Ca(OH)_2$ cannot eliminate E.faecalis from the surface layers of dentinal tubules, according to Haapasalo and Orstavik.[20] In their research, Siqueira and de Uzeda came to the same conclusion.[49]After relatively long periods of $Ca(OH)_2$/saline combination treatment, Safavi et al. found that E. faecalis remained alive in dentinal tubules.[29]

With all the above mentioned drawbacks of calcium hydroxide newer medicaments like antibiotics were tried.[36]

Infections in the root canal system are polymicrobial, containing both aerobic and anaerobic bacteria, according to Mohammadi Z's research.[51]As a result, due to the canal's complexity, a single antibiotic therapy may not be adequate to treat the infection. To combat the varied microorganisms present, a mix of antibiotics is required as it may also reduce the likelihood of resistant bacteria strains developing.[51]

With the advent of non-instrumentation endodontic treatment ,lesion sterilization and tissue repair, local application of antibiotics has been investigated Triple antibiotic paste was the most practical combination introduced in 2006 in order to achieve a satisfactory result. Because of its antibacterial effects in endodontic regenerative operations, TAP including metronidazole, ciprofloxacin, and minocycline has been proposed as a root canal medicament.[52]

Pai and colleagues investigated the effects of Ca(OH)2 and TAP. Inter-appointment flare-ups occurred in three of twenty individuals with 15 % Ca(OH)2. In the case of the TAP, however, none of them experienced inter-appointment flare-ups.[53]In another investigation, the TAP was found to have superior disinfection characteristics when compared to $Ca(OH)_2$[20]Another study by Cheng et al evaluated the antibacterial efficacy of a combination of TAP and $Ca(OH)_2$ with 2% chlorhexidine(CHX) on E. faecalis, and found that TAP could eradicate bacteria to a depth of 400 µm, whereas Ca(OH)2 with CHX could only destroy bacteria to a depth of 200 µm of dentin.[20]

TAP can influence gram-negative, gram-positive, and anaerobic bacteria, and this combination is effective against odontogenic bacteria. However, there are some drawbacks to this paste :

1. Teeth treated with TAP have demonstrated slight crown darkening. The presence of minocycline in the paste is most likely to blame. As a result, extreme caution and care should be exercised in aesthetic zones.[54]
2. Another unfavorable side effect of TAP conditioning of the radicular dentin appears to be an indirect negative impact on the survival of the stem cells of the apical papilla (SCAP).[55]
3. According to some researchers, the TAP and the concentration employed in the regeneration and revascularization protocol can result in significant dentine loss and an increase in roughness.[52]
4. In addition to the significant rise in the organic phase, such an increase can result in reduced dentine wettability and a significant drop in the inorganic phase of the treated dentine. TAP can demineralize the dentine surface due to its acidic nature (pH = 2.9). Furthermore, studies have demonstrated that 1 g/mL TAP causes a significant reduction in root micro-hardness and dentine demineralization.[52]
5. Another hazard to be aware of while using TAP in the root canal space is the difficult removal of the paste. Because TAP penetrates and binds to the dentinal structure, current irrigation procedures are unable to adequately remove it.[13]

One of the main goals of modern clinical microbiology is to try to develop new tactics and incorporate more promising antibiotics that can reduce the prevalence of biofilm.

N-acetylcysteine (NAC) has been offered as a viable root canal medicament alternative (Karapinar et al. 2016). N-acetylcysteine (NAC) has been shown to have an antibacterial effect on endodontic pathogenic microorganisms in prior investigations.[32] NAC is an antioxidant with thiol groups that efficiently reduces extracellular polysaccharide formation (EPS), eliminating mature biofilms in the same way that a mucolytic agent does while also lowering bacterial adhesion[32]. According to Silveira L et al, the presence of extracellular polysaccharide (EPS) is one mechanism that could explain why bacteria in biofilms are less susceptible to antimicrobial treatments than bacteria in planktonic forms[56]. This outcome could be explained by NAC's major mode of action, it's ability to cause EPS disruption in biofilms.

NAC was recently discovered to be effective against E. faecalis biofilms. Its combination with other antimicrobial compounds, as a mucolytic agent, could result in increased efficacy against biofilms.[57]

Because antimicrobial susceptibility in bacteria associated with broken biofilm is increased (El-Azizi et al., 2005), it's possible that an antibiofilm/antimicrobial agent combination might be synergistic (Olofsson et al., 2003).[57]N-acetylcysteine aids penetration of penicillins, polymyxins, and fluoroquinolones into the biofilm's deepest layers, overcoming the antibiotic resistance of causative bacterial agents.[27]

Levofloxacin (LEV) has been shown to efficiently permeate into bacterial biofilms when compared to other fluoroquinolones. Levofloxacin belongs to a novel class of fluoroquinolones, and its antibacterial and antiviral action against Streptococcus pneumoniae and other respiratory infections has been extensively researched and documented (Marchetti & Viale, 2003)[27]

Inhibition of new glycocalyx formation, release, or activation of exopolysaccharide breakdown enzymes, and electrostatic interference with bacterial adherence are all proposed modes of action for levofloxacin on biofilms (Eng et al., 1991). As a result, levofloxacin activity on early adhered cells may help to avoid the formation of biofilms.[58]

EPS acts as a barrier, preventing the agent from penetrating. In this way, combining an antimicrobial drug-like levofloxacin with a proven mucolytic agent or a possible EPS disruptor like NAC might improve antimicrobial efficacy against biofilms.[14]

The inhibition produced by different brands of a medicament against a particular organism depends upon various extrinsic and intrinsic factors. As per Jinlun Feng et al, the effective drug dose (MIC) of N – acetylcysteine, and Levofloxacin against *E. faecalis* is 1.56mg/mL and 2.5mg/mL. In this study, the mean MIC [Tab.2] against *E.faecalis* for N-acetylcysteine was 0.625 mg/mL and levofloxacin was 1.87mg/mL , whereas Metronidazole , Minocycline and Ciprofloxacin had MIC of 0.625mg/mL, 2.5mg/mL, 1.87mg/mL respectively.

In the present study, the first sample (S1) reading of the combination (N-Acetylcysteine and Levofloxacin) was 45×10^3 and that of Triple antibiotic paste was

$100 \times 10^{3.}$ Comparing the medicaments with the control group saline (Group-3), triple antibiotic paste (Group-1) and NAC+ LEV (Group-2) showed very strong evidence against the null hypothesis (P = 0.0001) [Figure 1] [Table 1].

Besides when combination of NAC+LEV (Group 2) was compared with TAP (Group1) , NAC+LEV showed strong evidence against the null hypothesis [$p<0.05$]. Thus, the null hypothesis was rejected.

Fewer studies have documented the antibacterial activity of NAC+LEV but the exact mechanism remains uncertain.Quah et al.,[14] considered the first one to ever study the antibacterial efficiency of N-acetylcysteine and Levofloxacin, by utilizing the agar diffusion test against *E. faecalis* and concluded that this combination is an effective intracanal medicament.

Compared to this combination (NAC + LEV) , TAP exhibited a significantly lesser antibacterial action and the reason for such finding could be due to the dentin buffering action. According to Portenier et al.[14] dentin inhibits the antibacterial action of TAP, However, Quah et al. discovered that the antibacterial properties of NAC are unaffected by the presence of dentin, which is a benefit of NAC over TAP.[14]

In a study on the effectiveness of N-acetyl cysteine, 2% chlorhexidine, and their combination as intracanal medicaments on Enterococcus faecalis biofilm, it was found that NAC has nearly equal antimicrobial property as 2% CHX, whereas their combination demonstrated a synergistic action, demonstrating the agent's compatibility and efficacy with an antimicrobial agent[59].

The presence of EPS is one mechanism that could explain why bacteria in biofilm are less sensitive to antimicrobial agents than bacteria in planktonic forms.

EPS serves as a barrier, preventing the agent from penetrating.[56]In this regard, combining an antimicrobial substance – LEV with a proven mucolytic agent or a potential EPS disruptor like NAC should improve antimicrobial efficacy against biofilms.

Numerous studies on the efficacy of NAC and LEV on biofilms of various organisms have been conducted. In a study, Yamur AKDA et [al 60]evaluated the effect of levofloxacin and a mucolytic (NAC) against biofilms by Staphylococcus aureus and Pseudomonas aeruginosa for the treatment of cystic fibrosis. The most significant issue in the treatment of cystic fibrosis-related infections is the difficulty of antibiotic penetration into the lung's dehydrated and viscous mucus layer. As a result, N-acetylcysteine (NAC) was chosen as a mucolytic agent to improve levofloxacin penetration. And it was discovered that when the combination was used, there was a significant reduction in biofilm.[14]This result demonstrated the ability of NAC to improve the penetration of LEV against a biofilm, demonstrating a synergistic combination against biofilms. This could also explain the combination's increased antimicrobial efficacy against TAP.

Another study by Carlos et al[28] showed low number of colonies remaining in the NAC at pH 11 group compared to the 2 percent CHX group suggests that the antibacterial potential of NAC was greater than that of 2 percent CHX, which supports Quah et al research's[30] that due to NAC's ability to directly damage the biofilm's three-dimensional structure, the antibacterial can more easily penetrate the biofilm and damage the intermolecular and intramolecular bacterial wall proteins.

However, Prather et al.[54] evaluated the effect of TAP on dentin's chemical structure and mechanical properties. In his research, he discovered that the TAP and

Modified TAP currently used in regenerative endodontics caused a significant reduction in dentin microhardness, which he attributed to the antibiotic mixture's demineralizing effect on dentin[52]. TAP use has resulted in etching of dentin and mild eroding of dentinal structure, resulting in a decrease in mechanical properties of dentin, resulting in increased tooth brittleness and fracture.[13] However, no such effects on dentin by NAC + LEV have been reported in literature till now.

The results obtained indicated that there is significant difference between all the three groups (p value <0.05). Among all the three groups, Group 2(NAC+LEV) performed followed by Group 1(TAP) and Group 3(Saline).

The present study shows that the combination of NAC+LEV is effective in the complete eradication of root canal bacteria in 7 days, and thus minimizes any damage to the root fracture resistance of the treated tooth. Therefore NAC+LEV with both Anti-oxidant and antibacterial activity can be a potential intracanal medicament and replacement for Triple antibiotic paste with further research.

In this study, the time period for which medicaments were introduced in the canals was 7 days. However, as per the literature, several studies document the use of TAP for over 3 months.[13] In conclusion of these studies, though dentin demineralization was on a hike, still there was also an upsurge in the antimicrobial efficacy at the same time. Hence, there could be a possible situation wherein if in this study, the medicament would have been placed for a longer duration, better results would have been yielded.

Thus the limitation of the present study is the time limit for placement of intracanal medicament.

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

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.

CONCLUSION

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

1) Minimum Inhibitory concentration(MIC) of N-Acetylcysteine and Metronidazole was least compared to other antibiotics.
2) Combination of N-Acetylcysteine plus Levofloxacin (group 2) was significantly better than Triple antibiotic paste (group 1) against E. Faecalis biofilm.
3) Combination of N-Acetylcysteine plus Levofloxacin (group 2) was significantly better than saline (group 3) against E. Faecalis biofilm.
4) Triple antibiotic paste (group1) was significantly better than saline (group 3) against E. Faecalis biofilm.

SUMMARY

The study was conducted in the "Department of Conservative Dentistry and Endodontics, Vishwanath Katti Institute of Dental Sciences, KAHER Belagavi" with the aim - "Comparative evaluation of antimicrobial efficacy of a combination of N-acetyl cysteine and Levofloxacin with Triple antibiotic paste against *Enterococcus faecalis* biofilm : An In-vitro study"

Microorganisms play a major role in development of pulpal and periapical diseases. Elimination of these microorganisms during root canal therapy helps to obtain endodontic success. Thus, for complete disinfection of the root canal system an intracanal medicament along with biomechanical preparation is necessary. CH is most commonly used intracanal medicament, but certain studies have shown it to be resistant against E.*faecalis* and prolonged exposure to radicular dentin resulted in reduced fracture resistance. This led to the development of TAP which is most widely used in endodontic revascularization and healing of larger periapical lesions.

Triple antibiotic paste containing ciprofloxacin, metronidazole and minocycline is an effective medicament for disinfection of infected necrotic immature teeth. However this paste has shown some disadvantages such as crown discoloration, demineralization effect on root dentin, cytotoxic effects on cells and has shown less potency against biofilm.

Recently, a combination of N-acetyl cysteine (NAC) and levofloxacin (LEV) which has a higher disruptive effect on biofilm and less cytotoxic effects on the cells has been introduced and has shown successful results.

The positive effects of NAC treatment have primarily been attributed to its mucus-dissolving properties by disrupting disulfide bonds in the mucus and its ability to decrease biofilm formation, resulting in significant reductions in bacterial infections.

Fluoroquinolones such as levofloxacin (LEV) could readily equilibrate across the biofilm and therefore, they seem to be effective in stopping the growth of biofilms.

Also, studies have shown that N-acetylcysteine can increase the therapeutic activity of fluoroquinolones.

Hence, the aim of the current study is to evaluate the efficacy of biofilm removal using combination of N-acetyl cysteine and Levofloxacin, a potential intracanal medicament compared with the gold standard Triple antibiotic paste.

Sixty eight extracted human single -rooted, single- canal teeth were chosen which fulfilled the inclusion and exclusion criteria. A diamond disc was used to section 6mm of middle 3rd of root. After sectioning, a sterile Gates Glidden drill no. 3 was used to standardize the internal diameter of the root canal.

Following which irrigation protocols were followed where the specimens were placed in an ultrasonic bath of 17% EDTA for five minutes followed by 3% NaOCl for five minutes in order to remove the organic and inorganic debris. Apex of the specimens were sealed with paraffin wax.

Then for the contamination of the Specimens (63 specimens): E. faecalis (ATCC 439) was be grown in BHI agar for 24 hours. The culture was be suspended in 5 mL of BHI broth and incubated for 4 hours at 37°C and its turbidity was be

adjusted at 0.5 Mcfarland standard (1×10^8 CFUmL−1) .The dentin specimens were transferred into fresh broth containing *E.faecalis*. Contamination of the dentin specimens was carried out for a period of 21 days.

For antimicrobial assessment, at the end of 21 days, the specimens were irrigated with 5 mL of sterile saline to remove the incubation broth. Then they were divided into 3 groups (n-21).

GROUP 1: Triple Antibiotic paste

GROUP 2: N-Acetylcysteine plus Levofloxacin

GROUP 3: Positive Control - Saline

Both the combinations that is, Triple antibiotic paste , N-acetylcysteine and Levofloxacin were prepared according to the values obtained after evaluating minimum inhibitory concentration with 1mL of propylene glycol .The respective medicaments were placed in root canal for seven days.

Microbial cells assessment was carried out at the end of seven days of incubation. Harvesting of dentin was carried out at a depth of (400 μm) by preparing the root canal circumferentially using sterile Gates Glidden drills no.5 in a slow speed handpiece.

The fine dentin shavings were collected in a test tube containing 1 mL of sterile BHI broth and incubated in an anaerobic environment at 37°C for 24 hours. After 24 hours the contents were serially diluted 100 μL of broth in 100 μL of sterile saline five times.

About 50 µL of the dilution was then plated on BHI agar plates and incubated for 24 hours at 37°C. The colonies on the agar plates were counted and recorded by a blinded microbiologist, represented in colony-forming units (CFU) per mL and readings was tabulated. Formulae used for evaluating the colony forming unit:

$$\text{Colony forming unit/mL} = \frac{\text{number of colonies formed}}{\text{Volume plated (mL)} \times \text{Total Dilution used(mL)}}$$

The results obtained indicated that there is significant difference between all the three groups (p value <0.05). Among all the three groups, Group 2(NAC+LEV) performed best, due to better antimicrobial and antioxidant property of NAC and added advantage of antibiotic LEV.

Therefore, 'null hypothesis' that there is no difference in the antimicrobial efficacy of a combination of N-acetyl cysteine and Levofloxacin with Triple antibiotic paste against E. Faecalis biofilm was rejected.

REFERENCES

1. Johnson WT, Noblett WC. Cleaning and Shaping in: Endodontics: Principles and Practice. 4th ed. Saunders, Philadelphia, PA, 2006.
2. Harlamb SC. Management of incompletely developed teeth requiring root canal treatment. Australian dental journal. 2016 Mar;61:95-100.
3. Aamir Rashid Purra , Fayaz Ahmed Ahangar, Sachin chadgal and Riyaz Farooq. Mineral Trioxide aggregate apexification: A novel approach. J Conserv Dent.2016 Jul-Aug,19(4):377-380.
4. Thibodeau B, Trope M. Pulp revascularization of a necrotic infected immature permanent tooth: case report and review of the literature. Pediatric dentistry. 2007 Jan 1;29(1):47-50
5. Jung C, Kim S, Sun T, Cho YB, Song M. Pulp-dentin regeneration: current approaches and challenges. *J Tissue Eng*. 2019;10:2041731418819263. Published 2019 Jan 29.
6. Diogenes A, Henry MA, Teixeira FB, Hargreaves KM. An update on clinical regenerative endodontics. Endodontic Topics. 2013 Mar;28(1):2-3.
7. Meshal G. Al- Shammari .Regenerative Endodontic Procedure in immature permanent teeth.2021 Jun 14.DOI:10.5772/intechnopen.96986.
8. 8.Faisal Alghamdi and Marwa Shakir.The Influence of Enterococcus faecalis as a dental root canal pathogen on endodontic treatment :A systematic review.2020 Mar 13.DOI:10.7759/cureus.7257.
9. Nakashima M, Akamine A. The application of tissue engineering to regeneration of pulp and dentin in endodontics. Journal of endodontics. 2005 Oct 1;31(10):711-8.

10. Frough Reyhani M, Rahimi S, Fathi Z, et al. Evaluation of Antimicrobial Effects of Different Concentrations of Triple Antibiotic Paste on Mature Biofilm of Enterococcus faecalis. *J Dent Res Dent Clin Dent Prospects*. 2015;9(3):138-143.
11. 11.Horsley H, Malone-Lee J, Holland D, et al. Enterococcus faecalis subverts and invades the host urothelium in patients with chronic urinary tract infection. *PLoS One*. 2013;8(12):e83637. Published 2013 Dec 10. doi:10.1371/journal.pone.0083637
12. Windley W 3rd, Teixeira F, Levin L, Sigurdsson A, Trope M. Disinfection of immature teeth with a triple antibiotic paste. *J Endod*. 2005;31(6):439-443.
13. Parhizkar A, Nojehdehian H, Asgary S. Triple antibiotic paste: momentous roles and applications in endodontics: a review. *Restor Dent Endod*. 2018;43(3):e28. Published 2018 Jun 20.
14. Khosravi MR, Khonsha M, Ramazanzadeh R. Combined Effect of Levofloxacin and N-Acetylcysteine against Enterococcus faecalis Biofilm for Regenerative Endodontics: An in Vitro Study. Iranian Endodontic Journal. 2018;14(1):40-6.
15. Moon JH, Choi YS, Lee HW, Heo JS, Chang SW, Lee JY. Antibacterial effects of N-acetylcysteine against endodontic pathogens. Journal of Microbiology. 2016 Apr 1;54(4):
16. Lewis K. Riddle of biofilm resistance. Antimicrobial agents and chemotherapy. 2001 Apr 1;45(4):999-1007.
17. Shigeta M, Tanaka G, Komatsuzawa H, Sugai M, Suginaka H, Usui T. Permeation of antimicrobial agents through Pseudomonas aeruginosa biofilms: a simple method. Chemotherapy. 1997;43(5):340-5.

18. Pham TDM, Ziora ZM, Blaskovich MAT. Quinolone antibiotics. *Medchemcomm.* 2019;10(10):1719-1739. Published 2019 Jun 28.

19. Lee BN, Moon JW, Chang HS, Hwang IN, Oh WM, Hwang YC. A review of the regenerative endodontic treatment procedure. *Restor Dent Endod.* 2015;40(3):179-187. doi:10.5395/rde.2015.40.3.179

20. Ghabraei S, Bolhari B, Sabbagh MM, Afshar MS. Comparison of Antimicrobial Effects of Triple Antibiotic Paste and Calcium Hydroxide Mixed with 2% Chlorhexidine as Intra canal Medicaments Against Enterococcus faecalis Biofilm. *J Dent (Tehran).* 2018;15(3):151-160.

21. Suzuki T, Yamamoto T, Torikai T, Ohashi Y. Combination Effect of Cefuroxime and Levofloxacin Against Bacteria Isolated from the Healthy Conjunctival Sac and Endophthalmitis Cases Using a Fractional Inhibitory Concentration Index. J Ocul Pharmacol Ther. 2017 Jan/Feb;33(1):19-23. doi: 10.1089/jop.2016.0073. Epub 2016 Dec 1. PMID: 27906556.

22. HOSSEINI MM, ZARE JM, Fesharaki M, OSTAD SM. Cytotoxicity of triple antibiotic paste and calcium hydroxide against cultured human dental pulp fibroblasts.

23. Yamada M, Kojima N, Paranjpe A, Att W, Aita H, Jewett A, Ogawa T. N-acetyl cysteine (NAC)-assisted detoxification of PMMA resin. Journal of Dental Research. 2008 Apr;87(4):372-7.

24. Moon JH, Jang EY, Shim KS, Lee JY. In vitro effects of N-acetyl cysteine alone and in combination with antibiotics on Prevotella intermedia. Journal of Microbiology. 2015 May 1;53(5):321-9.

25. Ghabraei S, Marvi M, Bolhari B, Bagheri P. Minimum intra canal dressing time of triple antibiotic paste to eliminate Enterococcus faecalis (ATCC 29212) and determination of minimum inhibitory concentration and minimum bactericidal concentration: an ex vivo study. Journal of Dentistry (Tehran, Iran). 2018 Jan;15(1):1.

26. Kaur M, Kendre S, Gupta P, Singh N, Sethi H, Gupta N, Acharya R. Comparative Evaluation of Anti Microbial effects of Triple Antibiotic Paste and Amox and its derivatives against E. Faecalis: An in vitro study. Journal of Clinical and Experimental Dentistry. 2017 Jun;9(6):e799.

27. Pei Y, Liu H, Yang Y, Yang Y, Jiao Y, Tay FR, Chen J. Biological activities and potential oral applications of N-acetylcysteine: progress and prospects. Oxidative medicine and cellular longevity. 2018 Apr 22;2018.

28. Manoharan A, Das T, Whiteley GS, Glasbey T, Kriel FH, Manos J. The effect of N- acetylcysteine in a combined anti biofilm treatment against antibiotic-resistant Staphylococcus aureus. Journal of Antimicrobial Chemotherapy. 2020 May 3

29. Haapasalo M, Ørstavik D. In vitro infection and of dentinal tubules. Journal of dental research. 1987 Aug;66(8):1375-9.1

30. Kim D, Kim E. Antimicrobial effect of calcium hydroxide as an intracanal medicament in root canal treatment: a literature review - Part I. In vitro studies. *Restor Dent Endod*. 2014;39 (4):241-252.

31. Ridhalaksani, R., Nazar, K., Djauharie, N. K., Meidyawati, R., & Npa, D. A. (2018). The antibacterial potential of n-acetylcysteine as an endodontic irrigant on the clinical isolates of the enterococcus faecalis biofilm. *International Journal of Applied Pharmaceutics*, *9*, 17–19. https://doi.org/10.22159/ijap.2017.v9s2.05

32. Quah SY, Wu S, Lui JN, Sum CP, Tan KS. N-acetylcysteine inhibits growth and eradicates biofilm of Enterococcus faecalis. *J Endod*. 2012;38(1):81-85. doi:10.1016/j.joen.2011.10.004

33. Jain P, Yeluri R, Garg N, Mayall S, Rallan M, Gupta S, Pathivada L. A Comparative Evaluation of the Effectiveness of Three Different Irrigating Solution on Microorganisms in the Root Canal: An Invivo Study. J Clin Diagn Res. 2015 Dec;9(12):ZC39-42. doi: 10.7860/JCDR/2015/13133.6991. Epub 2015 Dec 1. PMID: 26816990; PMCID: PMC4717765.

34. Bystrom A, Sundqvist G. The antibacterial action of sodium hypochlorite and EDTA in 60 cases of endodontic therapy. *Int Endod J*. 1985;18(1):35-40. doi:10.1111/j.1365-2591.1985.tb00416.

35. Mohammadi Z, Shalavi S, Jafarzadeh H. Ethylene diamine tetra acetic acid in endodontics. Eur J Dent. 2013 Sep;7(Suppl 1):S135-S142. doi: 10.4103/1305-7456.119091. PMID: 24966721; PMCID: PMC4054072.

36. Luber P, Bartelt E, Genschow E, Wagner J, Hahn H. Comparison of broth microdilution, E Test, and agar dilution methods for antibiotic susceptibility testing of Campylobacter jejuni and Campylobacter coli. J Clin Microbiol. 2003 Mar;41(3):1062-8. doi: 10.1128/JCM.41.3.1062-1068.2003. PMID: 12624030; PMCID: PMC150256

37. Elshikh M, Ahmed S, Funston S, Dunlop P, McGaw M, Marchant R, Banat IM. Resazurin-based 96-well plate microdilution method for the determination of minimum inhibitory concentration of biosurfactants. Biotechnol Lett. 2016 Jun;38(6):1015-9. doi: 10.1007/s10529-016-2079-2. Epub 2016 Mar 11. PMID: 26969604; PMCID: PMC4853446.

38. Cruz, E.V. & Kota, K & Huque, J & Iwaku, M & Hoshino, E. (2002). Penetration of propylene glycol into dentine. International endodontic journal. 35. 330-6. 10.1046/j.1365-2591.2002.00482.x.

39. Srinivas, S. & Jibhkate, N. & Baranwal, R. & Avinash, Alok & Tandil, Y. & Rathi, S.. (2016). Propylene Glycol: A New Alternative for an Intra canal Medicament. Journal of International Oral Health. 8. 611-614. 10.2047/jioh-08-05-16.

40. Gilson Sydney, Carlos estrela, Antonio bastisto .Root canal filling with calcium hydroxide paste using lentulospiral at different speeds .Dental press Endod.2011 apr-jun ;1(1);58-63

41. Bansal R, Jain A, Mittal S. Current overview on challenges in regenerative endodontics. J Conserv Dent 2015;18:1-6

42. Gajan EB, Aghazadeh M, Abashov R, Salem Milani A, Moosavi Z. Microbial Flora of Root Canals of Pulpally-infected Teeth: Enterococcus faecalis a Prevalent Species. *J Dent Res Dent Clin Dent Prospects.* 2009;3(1):24-27.

43. Peters LB, Wesselink PR, Buijs JF, van Winkelhoff AJ. Viable bacteria in root dentinal tubules of teeth with apical periodontitis. *J Endod*. 2001;27(2):76-81. doi:10.1097/00004770-200102000-00002

44. Siqueira JF, De Uzeda M, Fonseca ME. A scanning electron microscopic evaluation of in vitro dentinal tubules penetration by selected anaerobic bacteria. Journal of endodontics. 1996 Jun 1;22(6):308-10.

45. Wang QQ, Zhang CF, Chu CH, Zhu XF. Prevalence of Enterococcus faecalis in saliva and filled root canals of teeth associated with apical periodontitis. *Int J Oral Sci*. 2012;4(1):19-23. doi:10.1038/ijos.2012.17

46. Gaston JR, Andersen MJ, Johnson AO, Bair KL, Sullivan CM, Guterman LB, White AN, Brauer AL, Learman BS, Flores-Mireles AL, Armbruster CE. *Enterococcus faecalis* Polymicrobial Interactions Facilitate Biofilm Formation, Antibiotic Recalcitrance, and Persistent Colonization of the Catheterized Urinary Tract. Pathogens. 2020 Oct 13;9(10):835. doi: 10.3390/pathogens9100835. PMID: 33066191; PMCID: PMC7602121.

47. Neelakantan P, Romero M, Vera J, Daood U, Khan AU, Yan A, Cheung GSP. Biofilms in Endodontics-Current Status and Future Directions. Int J Mol Sci. 2017 Aug 11;18(8):1748. doi: 10.3390/ijms18081748. PMID: 28800075; PMCID: PMC5578138.

48. Estrela C, Pesce HF. Chemical analysis of the liberation of calcium and hydroxyl ions of calcium hydroxcide pastes in the presence of connective tissue of the dog.

49. Siqueira Jr JF, Lopes HP. Mechanisms of antimicrobial activity of calcium hydroxide: a critical review. International endodontic journal. 1999 Sep;32(5):361-9.

50. Bhandari S, S AT, Patil CR.An in Vitro Evaluation of Antimicrobial Efficacy of 2% Chlorhexidine Gel, Propolis and Calcium Hydroxide Against Enterococcus faecalis in Human Root Dentin. J Clin of Diagn Res.2014; 8(11):ZC060-ZC063. 2014 | November

51. Mohammadi Z, Jafarzadeh H, Shalavi S, Yaripour S, Sharifi F, Kinoshita JI. A Review on Triple Antibiotic Paste as a Suitable Material Used in Regenerative Endodontics. *Iran Endod J*. 2018;13(1):1-6.

52. Nerness A. *The effect of triple antibiotic paste and EDTA on the surface loss and surface roughness of radicular dentin.*2014

53. Pai S, Vivekananda Pai AR, Thomas MS, Bhat V. Effect of calcium hydroxide and triple antibiotic paste as intracanal medicaments on the incidence of inter-appointment flare-up in diabetic patients: An in vivo study. *J Conserv Dent.* 2014;17(3):208-211.

54. Thomas MS. Crown discoloration due to the use of triple antibiotic paste as an endodontic intra-canal medicament. Saudi Endodontic Journal. 2014 Jan 1;4(1):32.

55. Althumairy RI, Teixeira FB, Diogenes A. Effect of dentin conditioning with intracanal medicaments on survival of stem cells of apical papilla. Journal of endodontics. 2014 Apr 1;40(4):521-5.

56. Silveira LF, Baca P, Arias-Moliz MT, Rodríguez-Archilla A, Ferrer-Luque CM. Antimicrobial activity of alexidine alone and associated with N-acetylcysteine against Enterococcus faecalis biofilm. International journal of oral science. 2013 Sep;5(3):146-9.

57. Abu Hasna, A., Khoury, R. D., Toia, C. C., Gonçalves, G. B., de Andrade, F. B., Carvalho, C. A. T., Camargo, C. H. R., & Valera, M. C. (2020). In vitro Evaluation of the Antimicrobial Effect of N-acetylcysteine and Photodynamic Therapy on Root Canals Infected with Enterococcus faecalis. *Iranian Endodontic Journal*, *15*(4), 236-245. https://doi.org/10.22037/iej.v15i4.26865

58. Ishida H, Ishida Y, Kurosaka Y, Otani T, Sato K, Kobayashi H. In vitro and in vivo activities of levofloxacin against biofilm-producing Pseudomonas aeruginosa. Antimicrob Agents Chemother. 1998 Jul;42(7):1641-5. doi: 10.1128/AAC.42.7.1641. PMID: 9660997; PMCID: PMC105659.

59. Palaniswamy U, Lakkam SR, Arya S, Aravelli S. Effectiveness of N-acetyl cysteine, 2% chlorhexidine, and their combination as intracanal medicaments on Enterococcus faecalis biofilm. J Conserv Dent. 2016 Jan-Feb;19(1):17-20. doi: 10.4103/0972-0707.173186. PMID: 26957787; PMCID: PMC4760006.

60. Duijvestijn YC, Brand PL. Systematic review of N-acetylcysteine in cystic fibrosis. Acta Paediatr. 1999 Jan;88(1):38-41. doi: 10.1080/ 08035259950170574. PMID: 10090545

ANNEXURE – I

ETHICAL CLEARANCE CERTIFICATE

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CERTIFICATE

This is to Certify that the synopsis titled

COMPARATIVE EVALUATION OF ANTIMICROBIAL EFFICACY OF A COMBINATION OF N- ACETYLCYSTEINE AND LEVOFLOXACIN WITH TRIPLE ANTIBIOTIC PASTE AGAINST ENTEROCOCCUS FAECALIS BIOFILM: IN VITRO STUDY

Submitted by

Dr. MAHIMA GUPTA *P. G. Student / Staff, Guided by* DR PREETI DODDWAD *from Department of* CONSERVATIVE DENTISTRY & ENDODONTICS *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date :

Member Secretary
Research and Ethical Committee
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ANNEXURE – II

BIOSTATISTIC CLEARANCE CERTIFICATE

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Biostatistics Clearance Certificate

This is to certify that the Biostatistics aspect of the Dissertation / Research work of Dr. Malvika Gupta entitled "Comparative Evaluation of antimicrobial efficacy of a combination of N-Acetyl Cysteine and Levofloxacin Vs Triple Antibiotic paste against E. faecalis biofilm. An – In-vitro study" has been done under my guidance and considered satisfactory.

Place : Belagavi

Date : 01/12/21

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

PLAGIARISM CHECK CERTIFICATE

Scientific Correspondence and Review Committee

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PLAGIARISM CHECK REPORT

Name of the Applicant : DR. MAHIMA GUPTA
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Batch & Year : 2019-22
Department : CONSERVATIVE DENTISTRY AND ENDODONTICS

The soft copy of Research Work / Manuscript by DR. MAHIMA GUPTA ..... entitled "COMPARATIVE EVALUATION OF ANTIMICROBIAL EFFICACY OF A COMBINATION OF N-ACETYLCYSTEINE AND XEVOFLOXACIN WITH TRIPLE ANTIBIOTIC PASTE AGAINST E-FAECALIS BIOFILM : AN INVITRO STUDY ....."

under the guidance of DR. PREETI. K. DODWAD .....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 .....7.....%, which is **within / not within** the acceptable limits of 10% as per the UGC guidelines.

Member Secretary
Scientific Correspondence and Review Committee
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