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ORIGINAL RESEARCH

IN VITRO EVALUATION OF NOVEL INTRACANAL FORMULATIONS AGAINST ENTEROCOCCUS FAECALIS

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ABSTRACT

Background: *Enterococcus faecalis* is a resilient microorganism commonly associated with endodontic treatment failure. Its resistance to conventional intracanal medicaments necessitates the exploration of more effective alternatives.**Aims and Objectives:** To evaluate and compare the antimicrobial efficacy of various novel and conventional intracanal medicaments against *E. faecalis* using an in vitro model.**Materials and Methods:** Fifty decoronated, instrumented, and sterilized single-rooted human teeth were inoculated with *E. faecalis* (ATCC 29212) and incubated for 21 days. The samples were then randomly assigned to five groups (n=10) based on the medicament used: RC Cal, Doxycycline, Chlorhexidine (2%), Nanochitosan, and Double Antibiotic Paste (DAP). After 7 days of medicament application, dentinal shavings were collected and cultured to determine bacterial viability. Statistical analysis was conducted using ANOVA and Tukey's post hoc test.**Results:** DAP exhibited the highest antimicrobial efficacy with the lowest mean CFU count, followed by Nanochitosan, CHX, Doxycycline, and RC Cal. ANOVA revealed statistically significant differences among groups ($F = 404.65$, $p < 0.0001$). Post hoc analysis confirmed that DAP significantly outperformed other groups ($p < 0.05$).**Conclusion:** DAP and Nanochitosan are effective intracanal medicaments against *E. faecalis*, outperforming traditional agents like RC Cal and Doxycycline. Their use may improve clinical outcomes in endodontic therapy, especially in resistant infections**Keywords:** *Enterococcus faecalis*, intracanal medicament, Double Antibiotic Paste, Nanochitosan, Chlorhexidine, Calcium hydroxide, endodontic infections

INTRODUCTION

Successful endodontic therapy relies heavily on the complete eradication of microbial flora from the root canal system. Among the various microorganisms

implicated in endodontic failures, *Enterococcus faecalis* stands out due to its unique ability to adapt, persist, and survive under harsh environmental conditions commonly encountered within the root canal. This Gram-positive, facultative anaerobic bacterium is frequently isolated

from cases of persistent periapical infections and is a key contributor to post-treatment disease. *E. faecalis* is particularly concerning in retreatment cases due to its intrinsic resistance to conventional root canal disinfectants and its ability to form biofilms, penetrate dentinal tubules, and endure nutrient-deprived environments.¹

Despite rigorous mechanical debridement and the use of irrigating solutions, complete elimination of *E. faecalis* remains a challenge. Intracanal medicaments have therefore been introduced as an adjunctive strategy to enhance antimicrobial efficacy between treatment appointments. Traditional medicaments like calcium hydroxide have shown only limited effectiveness against *E. faecalis*, mainly due to the organism's resistance to high pH environments and its ability to buffer alkaline stress.²

In response to the limitations of conventional agents, recent research has focused on the development and evaluation of novel intracanal formulations with improved antimicrobial properties^{3,4,5}. These include a variety of therapeutic agents such as antibiotic combinations, antimicrobial peptides, herbal extracts, and innovative drug delivery systems involving nanoparticles. These new approaches aim to overcome microbial resistance, enhance penetration into dentinal tubules, and provide sustained antimicrobial action.⁶

Therefore, in vitro evaluation of these novel intracanal formulations is essential to identify more effective alternatives for root canal disinfection. This study aims to compare the antimicrobial efficacy of different novel intracanal medicaments against *Enterococcus faecalis*, with the ultimate goal of improving clinical outcomes in endodontic therapy.

The clinical relevance of this study lies in its evaluation of novel and conventional intracanal medicaments for eradicating *Enterococcus faecalis*, a persistent pathogen in root canal infections that can lead to endodontic treatment failure. By comparing the efficacy of 3% doxycycline gel, 2% chlorhexidine gel, nanochitosan, double antibiotic paste, and calcium hydroxide in reducing *E. faecalis* populations in human mandibular premolars, the study provides evidence to guide clinical decision-making. The findings highlight Double antibiotic paste as highly effective, offering Clinicians reliable options for intracanal medication to improve treatment outcomes. The use of nanochitosan and other formulations also suggests potential for innovative approaches, which could address challenges like antibiotic resistance or tissue irritation. These results can inform endodontic protocols, enhancing the success rate of root canal treatments by

ensuring better microbial control, particularly against resistant strains like *E. faecalis*.

MATERIALS AND METHODS

This was an in vitro, experimental, comparative microbiological study designed to evaluate and compare the antimicrobial efficacy of various novel intracanal formulations against *Enterococcus faecalis*. The study was conducted in the Department of Conservative Dentistry and Endodontics, PMS College of Dental Science & Research, in collaboration with the Department of Microbiology, over a period of 6 months.

A standard strain of *Enterococcus faecalis* (ATCC 29212) was obtained from the microbiology laboratory. The strain was subcultured and maintained on brain heart infusion (BHI) agar and incubated at 37°C for 24 hours to ensure viability and purity.

➤ INTRACANAL MEDICAMENTS:

- 3% Doxycycline gel (DX)
- 2% Chlorhexidine gel (WALDENT CHLORHEX)
- Nanochitosan (Sigma Aldrich)
- Double antibiotic paste
- Calcium hydroxide intracanal medicament (Prime dental RC Cal)

➤ MICROBIOLOGICAL AND CELL VIABILITY TESTING:

- *Enterococcus faecalis strain (ATCC 29212)*
- **MTT Assay** (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)

PROCEDURE

SELECTION AND PREPARATION OF TEETH:

A total of 50 extracted human mandibular premolar teeth with Vertucci's type 1 root canal morphology selected. The crown portion of each tooth was removed 3 mm above the Cemento-Enamel junction (CEJ) using a water cooled diamond saw. The roots were standardized to a length of 15 mm (fig 1). A size 20K file was inserted into each pulp space until it could be seen at the apical foramen.



Figure 1. Root Standardized to a Length of 15mm

ROOT CANAL PREPARATION:

The canals were prepared using rotary files (Protaper Gold, Dentsply Maillefer) upto the F2 size, with irrigation between each file change using 1 ml of 5% Sodium Hypochlorite (NaOCl) for cleaning and disinfection.

After shaping and cleaning, the canals were irrigated with 5 ml of 17% EDTA solution for 3 minutes to remove the smear layer, followed by 1 ml of 5% NaOCl and then saline for final irrigation to ensure complete removal of debris and disinfect the canal.

STERILIZATION OF TEETH:

After cleaning and shaping, the teeth were autoclaved at 121°C, 15 lbs for 15 minutes to ensure sterility.

E. FAECALIS INOCULATION

Once sterility was confirmed, the roots were inoculated with *Enterococcus faecalis*. (ATCC 29212) Bacterial inoculum of *Enterococcus faecalis* were prepared and growth was adjusted to 0.5% McFarland standard. 100mL BHI broth was prepared, sterilized by autoclaving at 121°C 15lbs for 15mins and was dispersed to sterile conical flask (fig 2). 1000µl of inoculum was added and the plate was kept for **incubation at 37°C (14 days for the formation of biofilm)**. After 14 days, the **initial-dentinal shavings were collected** using H files (to assess the bacterial viability).

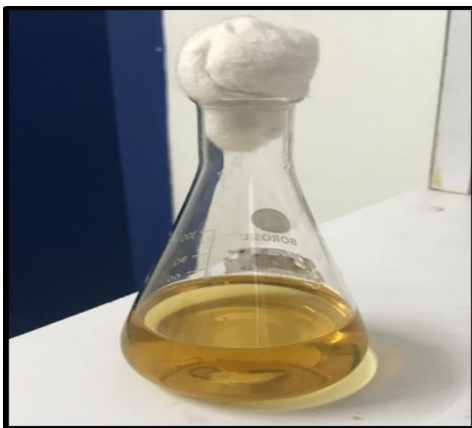


Figure 2. BHI Broth in sterile conical flask

INTRACANALMEDICAMENT PREPARATION

- **3% DOXYCYCLINE GEL**
 - 3.33g Doxycycline hydrochloride
 - 0.1 g Sodium Metabisulfite
 - 50% Propylene glycol
 - Polysorbate 80
 - 0.76 % NaH2PO4
 - 43.37 g 2%Hydroxyethyl cellulose gel
- **CHITOSAN PASTE**

The Chitosan powder was dissolved in 1% acetic acid at 20 mg/ml. The Chitosan pastes were prepared by

mixing 1 ml of chitosan solution (20 mg/ml) with 1 ml of propylene glycol and 3ml of distilled water. The final concentration of chitosan was 4mg/ml in 20% Propylene glycol.

DOUBLE ANTIBIOTIC PASTE

- Metronidazole 400 mg/ml
- Ciprofloxacin 500 mg/ml
- Propylene glycol

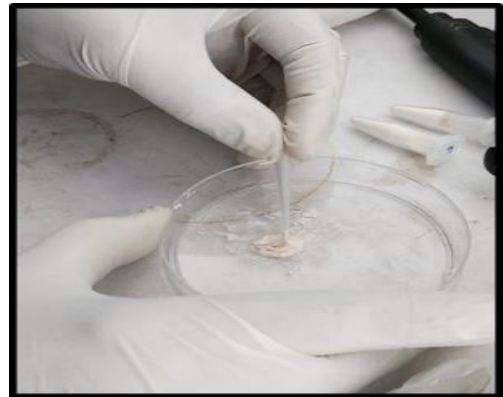


Figure 3. Preparing Double Antibiotic Paste

- **2% CHLORHEXIDINE (CHX) GEL** (Waldent Chlorex)
- **CALCIUM HYDROXIDE INTRACANAL MEDICAMENT**_(Prime dental RC Cal)

INTRACANAL MEDICAMENT APPLICATION

After 14 days of bacterial inoculation, the teeth were fixed in a sterile aluminum apparatus. The root canals were irrigated with sterile saline to remove the loosely bounded bacteria from the pulp space. The teeth were randomly divided into **5 groups (n=10)**. The intracanal medicaments were prepared and applied in the pulp space using **lentulo spirals** (fig 4). After placement, the coronal openings of the canals were sealed with sterile cotton and temporary filling. Then the tooth was stored in artificial saliva to stimulate the oral environment and incubated again for next 7 days. All procedures were carried out in laminar flow chamber to ensure a sterile environment.



Figure 4. Placement of ICM

DENTINAL SHAVING COLLECTION AND BACTERIAL ASSESSMENT

After 7 days of medicament placement, the root canals were irrigated with sterile saline. Dentinal shavings were collected using H files from each root canal. The collected dentinal shavings were then subjected to an **MTT assay** (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) to assess cell viability and to determine the reduction in bacterial cell

RESULTS

TABLE 1. COMPARISON OF % OF VIABILITY

	N	Mean	SD	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
Doxycycline	10	46.5860	1.21774	.54459	45.0740	48.0980	45.56	48.66
RC cal	10	86.1320	3.72006	1.66366	81.5129	90.7511	80.48	89.39
CHX	10	32.4480	3.65234	1.63338	27.9130	36.9830	28.49	37.87
Nano Chitosan	10	28.8840	1.83691	.82149	26.6032	31.1648	25.78	30.64
DAP	10	23.9160	2.71159	1.21266	20.5491	27.2829	20.43	26.88
F Value (One way Anova test)					404.65			
P Value					0.0001*			

viability caused by the intracanal medicaments

STATISTICAL ANALYSIS

- Data analysis- statistical package **SPSS 26.0** (SPSS Inc., Chicago, IL)
- **P<0.05** – level of significance
- **Descriptive statistics**
- Normality of the data - **Shapiro Wilk test**

Inferential statistics -One way Anova test followed by post hoc test.

The order of cell viability from highest to lowest is as follows: **RC cal: > Doxycycline: > CHX: >Nano chitosan>:DAP**

The antimicrobial efficacy of various intracanal formulations against *Enterococcus faecalis* was evaluated in terms of mean colony-forming unit (CFU) counts. A total of five groups (n = 10 per group) were tested: Doxycycline, RC Cal, Chlorhexidine (CHX), Nano Chitosan, and DAP (Double Antibiotic Paste). The observations are summarized as follows: The **RC Cal** group exhibited the **highest mean CFU count** (86.13 ± 3.72), indicating the **least antimicrobial efficacy** among all tested formulations. The **Doxycycline** group showed a **moderate mean CFU count** (46.59 ± 1.22), reflecting a partial antibacterial effect. The **CHX** group had a **lower mean CFU count** (32.45 ± 3.65), suggesting better efficacy than Doxycycline and RC Cal. The **Nano Chitosan** group demonstrated an even **lower mean CFU count** (28.88 ± 1.84), indicating enhanced antibacterial activity. The **DAP** group recorded the **lowest mean CFU count** (23.92 ± 2.71), representing the **most effective antimicrobial action** against *E. faecalis* in this study.

The variability within groups was relatively low, as shown by standard deviations. Confidence intervals for the mean further support the observed trends, with DAP and Nano Chitosan having tightly bound intervals, indicating consistent performance. A **One-Way ANOVA** test revealed a highly significant difference among the groups (**F = 404.65, p = 0.0001**), confirming that the choice of intracanal medicament had a statistically significant impact on the microbial reduction. These results suggest that **DAP and Nano Chitosan formulations** are significantly more effective against *Enterococcus faecalis* compared to traditional medicaments like **RC Cal and Doxycycline**.

TABLE 2. POSTHOC COMPARISON (inter group comparison)- % Viability

Dependent Variable	(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
% VIABILITY	Doxycycline	RC cal	-39.5460	1.77512	.0001*	-45.1437	-33.9483
		CHX	14.13800*	1.77512	.0001*	8.5403	19.7357
		Nano Chitosan	17.70200*	1.77512	.0001*	12.1043	23.2997
		DAP	22.67000*	1.77512	.0001*	17.0723	28.2677
	RC cal	CHX	53.68400*	1.77512	.0001*	48.0863	59.2817
		Nano Chitosan	57.24800*	1.77512	.0001*	51.6503	62.8457
		DAP	62.21600*	1.77512	.0001*	56.6183	67.8137
	CHX	Nano Chitosan	3.56400	1.77512	.584	-2.0337	9.1617
		DAP	8.53200*	1.77512	.001*	2.9343	14.1297
	Nano Chitosan	DAP	4.96800	1.77512	.111	-.6297	10.5657

A post hoc analysis was conducted to compare the percentage viability of *Enterococcus faecalis* among different intracanal medicament groups. The pairwise comparisons revealed the following significant findings:

- **Doxycycline vs. RC Cal:** A statistically significant difference ($p = 0.0001$) was observed, with RC Cal showing **39.55% higher viability** of *E. faecalis*, indicating significantly lower antimicrobial efficacy of RC Cal.
- **Doxycycline vs. CHX, Nano Chitosan, and DAP:** All three groups showed significantly **lower viability** compared to Doxycycline by **14.14%, 17.70%, and 22.67%** respectively ($p = 0.0001$), indicating superior antimicrobial efficacy of these agents.
- **RC Cal vs. CHX, Nano Chitosan, and DAP:** RC Cal had significantly **higher viability** in all comparisons:
 - 53.68% higher than CHX
 - 57.25% higher than Nano Chitosan
 - 62.22% higher than DAP
 These differences were all statistically significant ($p = 0.0001$), confirming RC Cal as the least effective agent.
- **CHX vs. Nano Chitosan:** The difference in viability (3.56%) was **not statistically significant** ($p = 0.584$), indicating comparable efficacy between CHX and Nano Chitosan.
- **CHX vs. DAP:** DAP showed significantly **better efficacy** with 8.53% lower viability ($p = 0.001$).
- **Nano Chitosan vs. DAP:** Although DAP showed a 4.97% lower viability than Nano Chitosan, this difference was **not statistically significant** ($p = 0.111$), suggesting both have similar antimicrobial potential.

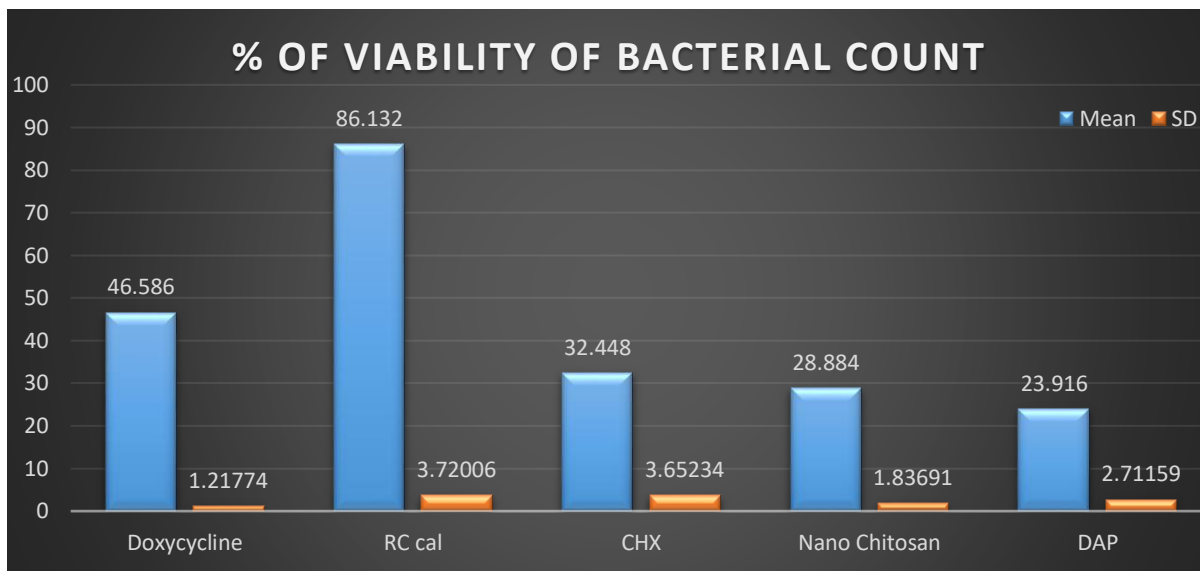


Figure 5- Variability of Bacterial count

Efficacy of intracanal medicament against *E. faecalis* (Reconfirmed with the color change in MTT assay)(Fig 6)

DAP> Nano chitosan> CHX> Doxycycline> RC cal

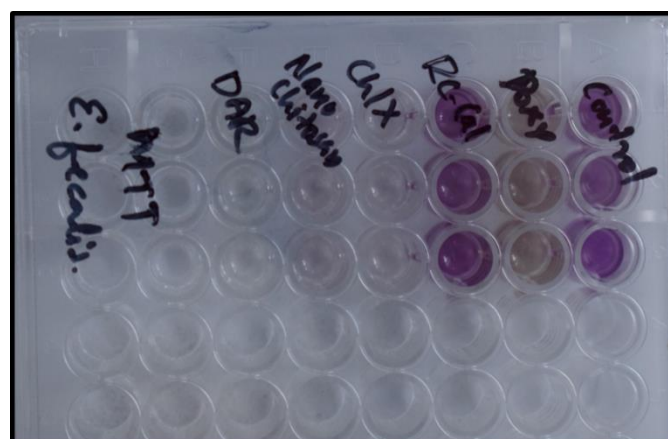
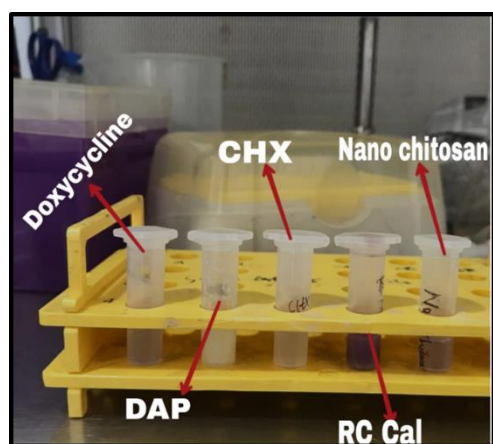


Figure 6. Efficacy of Medicament

DISCUSSION

The eradication of *Enterococcus faecalis* from the root canal system continues to be a major challenge in endodontic therapy. This microorganism possesses a remarkable ability to survive in harsh conditions, penetrate deep into dentinal tubules, form resistant biofilms, and persist even after root canal treatment, making it a frequent cause of persistent and post-treatment apical periodontitis⁶.

In the present study, the Double Antibiotic Paste (DAP), typically a combination of ciprofloxacin and metronidazole, demonstrated the highest antimicrobial efficacy against *E. faecalis*. This finding is in accordance with prior research highlighting the superior performance of antibiotic combinations in eliminating resistant endodontic pathogens⁷. The broad-spectrum nature of ciprofloxacin and metronidazole, combined with their ability to diffuse through dentinal tubules and disrupt bacterial biofilms, contributes to their enhanced antimicrobial effect⁸.

Moreover, studies have shown that DAP, unlike triple antibiotic paste (TAP), causes less dentin discoloration and does not significantly weaken dentinal structure, even with prolonged application⁹.

Nanochitosan, a nanoparticle-based biopolymer known for its intrinsic antimicrobial and biocompatible properties, emerged as another promising medicament in this study. Although slightly less effective than DAP, the difference was not statistically significant. Nanochitosan's efficacy can be attributed to its positive surface charge, which interacts with negatively charged bacterial membranes, leading to disruption and cell death¹⁰. Additionally, nanoparticle drug delivery systems enhance penetration into dentinal tubules and allow for sustained release of active agents¹¹. While chitosan has been previously explored primarily as an irrigant, our findings support its role as an intracanal medicament—a relatively under-investigated application¹².

Chlorhexidine (CHX) 2%, a widely used endodontic irrigant, exhibited substantial antimicrobial activity. Its broad-spectrum efficacy, substantivity, and proven clinical utility are well-documented¹³. CHX acts by disrupting the bacterial cell membrane and precipitating intracellular components¹⁴. However, in our study, CHX was less effective than DAP and Nanochitosan. This reduced efficacy may stem from CHX's limited dentinal penetration and inability to dissolve necrotic tissue or disrupt biofilms as efficiently as nanoparticle systems or antibiotics^{15, 16}.

Doxycycline, a bacteriostatic antibiotic from the tetracycline group, also significantly reduced *E. faecalis* counts, though its performance was inferior to DAP, Nanochitosan, and CHX. Doxycycline inhibits protein synthesis by binding to the 30S ribosomal subunit, impairing bacterial replication¹⁷. Despite its effectiveness, doxycycline's potential for cytotoxicity and antibiotic resistance, particularly when used at higher concentrations, limits its long-term clinical utility^{18, 19}.

RC Cal, a calcium hydroxide-based medicament, showed the least antimicrobial efficacy in this study. This aligns with previous reports suggesting that *E. faecalis* can resist the high pH environment created by calcium hydroxide, possibly due to proton pump mechanisms and efficient buffering capabilities²⁰. Although calcium hydroxide remains a standard intracanal medicament in many clinical scenarios, its limited effectiveness against *E. faecalis*—especially in retreatment cases—raises concerns about its reliability^{21, 22}.

The statistical analysis using one-way ANOVA and post hoc tests confirmed significant differences among the tested groups ($p < 0.0001$), with DAP showing superior efficacy. Notably, the difference between DAP and CHX was statistically significant, whereas the comparison between DAP and Nanochitosan was not, underscoring the potential of nanoparticle-based therapies as alternatives to conventional antibiotic pastes^{23, 24}.

This study contributes to the growing evidence supporting the use of innovative antimicrobial agents in endodontics. Nano-based formulations like Nanochitosan, due to their high efficacy and minimal adverse effects, offer promising alternatives for future treatment protocols²⁵. Moreover, these findings reiterate the need for alternative strategies in the face of rising antibiotic resistance and the limitations of traditional medicaments such as calcium hydroxide^{26, 27}.

Incorporating new intracanal medicaments that exhibit enhanced biofilm disruption and deep dentinal penetration could significantly improve outcomes in endodontic therapy, particularly for refractory cases involving *E. faecalis*^{28, 29}.

CONCLUSION

This in vitro study evaluated the antimicrobial efficacy of various intracanal medicaments against *Enterococcus faecalis*. Among the tested formulations, Double Antibiotic Paste (DAP) demonstrated the most significant antibacterial activity, followed closely by Nanochitosan and Chlorhexidine (CHX). Doxycycline showed moderate effectiveness, while RC Cal, a calcium hydroxide-based medicament, was the least effective. The results highlight the superior performance of novel and antibiotic-based formulations over traditional agents in combating resistant endodontic pathogens. The use of DAP and Nanochitosan as intracanal medicaments presents a promising approach for improving treatment outcomes in persistent endodontic infections, especially those involving *E. faecalis*. Further clinical research is recommended to confirm these findings and assess long-term safety and dentin compatibility.

DECLARATIONS

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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