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Antimicrobial efficacy of intracanal medicaments targeting *E. faecalis* biofilm: An *in vitro* study

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Abstract:
Enteroacoccus faecalis biofilms are a major cause of persistent endodontic infections and treatment failure. Therefore, it is of interest to evaluate and compare the antimicrobial efficacy of triple antibiotic paste, simvastatin, propolis and chitosan against *E. faecalis* biofilms in extracted human teeth. Antimicrobial activity was assessed by colony-forming unit counts at the apical and middle thirds following medicament application. Chitosan showed the lowest bacterial counts and significantly greater antimicrobial efficacy than the other tested intracanal medicaments. Thus, data show chitosan as a promising biocompatible anti-biofilm agent for intracanal disinfection.

Keywords: Antibiofilm activity, chitosan, *enterococcus faecalis*, intracanal medicaments, propolis, simvastatin, triple antibiotic paste (TAP)

Background:
Effective elimination of microorganisms from the root canal system is fundamental to the success of endodontic therapy **[1]**. Despite advances in instrumentation and irrigation, complete disinfection remains elusive because of the intricate root canal anatomy, which provides protected niches for microbial survival **[2]**. Residual infection within these areas continues to be a leading cause of post-treatment apical periodontitis and endodontic failure **[3]**. Among the microorganisms associated with persistent endodontic infections, *Enterococcus faecalis* occupies a central role **[4]**. It is frequently isolated from teeth with failed root canal treatment in about 24-77% of cases and is particularly difficult to eradicate due to its ability to withstand nutrient deprivation, tolerate high pH environments and penetrate deep into dentinal tubules **[5]**. Its propensity to form biofilms further enhances its survival, as biofilm-associated bacteria exhibit significantly greater resistance to antimicrobial agents than planktonic forms **[6].** The biofilm mode of growth represents a major therapeutic challenge in endodontics **[7]**. The protective extracellular matrix, altered metabolic activity and coordinated bacterial behavior within biofilms markedly reduce the effectiveness of conventional chemo-mechanical preparation and irrigation **[8].** Consequently, intracanal medicaments are indispensable adjuncts to root canal therapy, serving to reduce residual microbial load, disinfect inaccessible areas and prevent bacterial regrowth between appointments **[9]**. Calcium hydroxide has long been regarded as the intracanal medicament of choice owing to its high alkalinity and broad antimicrobial activity **[10]**. However, increasing evidence suggests that its effectiveness against *E. faecalis* is limited, particularly when the bacteria are organized in biofilms **[11]**. The buffering capacity of dentin can reduce its pH, diminishing its antimicrobial action, while its restricted penetration into dentinal tubules and inconsistent disruption of mature biofilms further compromise its efficacy **[12]**. These shortcomings have driven the search for alternative medicaments capable of targeting resistant, biofilm-associated microorganisms **[13]**. In this context, several agents with distinct mechanisms of action have gained attention. Triple antibiotic paste (TAP), comprising ciprofloxacin, metronidazole and minocycline, offers broad-spectrum antimicrobial activity and has demonstrated effectiveness against endodontic pathogens, including *E. faecalis*; however, concerns related to tooth discoloration, cytotoxicity and antibiotic resistance remain **[14, 15]**. Simvastatin, beyond its lipid-lowering effects, has shown antimicrobial and antibiofilm activity along with anti-inflammatory and osteogenic potential, making it a promising adjunct in endodontic disinfection **[16]**. Propolis, a natural resinous product of honeybees, exhibits antimicrobial, anti-inflammatory and antioxidant properties and has shown favorable biocompatibility in endodontic applications **[17]**. Chitosan, a naturally derived biopolymer, possesses inherent antimicrobial activity through disruption of bacterial cell membranes and has showed potential in inhibiting biofilm formation and enhancing penetration into dentinal tubules **[18]**. Given the growing emphasis on managing biofilm-mediated endodontic infections and the limitations of conventional intracanal medicaments, a comparative evaluation of these alternative agents is warranted **[19]**. *In vitro* biofilm models provide a standardized and reproducible platform to assess their antimicrobial efficacy **[20].** Therefore, it is of interest to evaluate and compare the antimicrobial efficacy of four intracanal medicaments—triple antibiotic paste, simvastatin, propolis and chitosan against *Enterococcus faecalis* biofilm using an *in vitro* model.

Methodology:
The study involved the collection and preparation of 85 extracted human teeth. The inclusion criteria required the selection of fully developed single-rooted premolars, non-restored teeth and teeth with a straight root and single canal. The exclusion criteria included grossly decayed teeth, teeth with developmental anomalies and teeth with fractures or craze lines. All selected teeth were cleaned, radiographically evaluated, autoclaved and decoronated to standardize the root length to 12 mm. The working length was established using a #15 K-file and cleaning and shaping were performed using Pro-Taper Gold rotary files up to F3 (0.3, 9%), with irrigation using 5 ml of 5.25% sodium hypochlorite. Smear layer removal was achieved by using 5 ml of 17% EDTA for 3 minutes, followed by saline irrigation and drying. For biofilm formation, *Enterococcus faecalis* (ATCC 29212) was cultured in Blood Agar and a suspension was prepared by dissolving the colonies in BHI broth to obtain a concentration of 0.5 McFarland (1.5×10^8 CFU/ml). Each canal was inoculated with 1 ml of the bacterial suspension and

incubated for four weeks. Fresh medium was added daily until the fourth week to allow for mature biofilm formation. The preparation of intracanal medicaments involved several steps. For Triple Antibiotic Paste (TAP), equal amounts of ciprofloxacin, metronidazole and doxycycline were mixed to obtain a concentration of 10 mg/ml, dissolved in 1 ml of sterile distilled water and heated for 2 hours for complete dissolution. It was then combined with 4% methylcellulose to achieve a paste consistency. Simvastatin was dissolved in 1 ml sterile distilled water to obtain a 10 mg/ml solution and 4% methylcellulose was added to form a paste. Chitosan was dissolved in a 1% acidic solution. One milliliter of this solution was mixed with 1 ml propylene glycol and 3 ml distilled water to obtain a final concentration of 4 mg/ml. For propolis, 5 g of crude propolis was mixed with 80% ethyl alcohol in a 1:15 ratio and evaporated at 40–45 °C. The mixture was filtered and 1 g of 1% sodium alginate was added to achieve the desired consistency. After biofilm formation, the samples were divided into five groups, with 17 samples per group based on the intracanal medicament used: Group A (Control Group, No Medicaments), Group B (Triple Antibiotic Paste, 10 mg/ml), Group C (Simvastatin, 10 mg/ml), Group D (Propolis) and Group E (Chitosan, 4 mg/ml). The medicaments were placed using a no. 40 lentulospiral and coronal sealing was performed with Resin-Modified Glass Ionomer Cement (RMGIC). The samples were then incubated for 7 days at 98% humidity. Following the incubation period, the medicaments were removed and transverse sections (1 mm) from the middle and apical thirds were obtained. The samples were centrifuged, cultured on BHI agar and incubated for 24 hours. Colony-forming units (CFU) were counted using the conventional bacterial counting method. Statistical analysis was performed using SPSS software (Windows version 22.0) and the power of the study was estimated to be 0.95. One-way ANOVA and Tukey's Post-hoc test were applied to determine the significance of mean values and for intergroup comparison.

Results:

At the apical third, a statistically highly significant difference in mean CFU counts was observed among the five experimental groups ($F = 52.97$, $p < 0.001$), indicating that the type of intracanal medicament significantly influenced the reduction of *E. faecalis* **(Table 1, Figure 1)**. The control group (Group A), which received no medicament, demonstrated the highest bacterial count ($8.65 \pm 0.27 \times 10^4$ CFU), confirming persistent bacterial survival in the absence of antimicrobial intervention. Among the medicament groups, Group E (Chitosan) exhibited the lowest mean CFU count ($2.82 \pm 0.35 \times 10^4$ CFU), reflecting the greatest antibacterial activity. This was followed by Group D (Propolis) with $4.12 \pm 0.27 \times 10^4$ CFU, Group B (Triple Antibiotic Paste) with $4.24 \pm 0.26 \times 10^4$ CFU, and Group C (Simvastatin) with $4.53 \pm 0.35 \times 10^4$ CFU. Tukey's multiple comparison test showed that all medicament groups (Groups B, C, D, and E) produced significantly lower CFU counts than the untreated control (Group A), with all comparisons showing $p < 0.001$ **(Table 2, Figure 2)**. Pairwise comparisons among Triple Antibiotic Paste, Simvastatin, and Propolis (Groups B, C, and D) revealed no statistically significant differences ($p > 0.05$), indicating comparable antimicrobial efficacy among these three agents. However, Chitosan (Group E) demonstrated significantly lower bacterial counts than Triple Antibiotic Paste ($p < 0.05$), Simvastatin ($p < 0.01$), and Propolis ($p < 0.05$), confirming its superior antimicrobial performance. When compared with the untreated control, Chitosan reduced the bacterial load by approximately 67.3%, whereas additional reductions of 33.3%, 37.7%, and 31.4% were observed relative to Triple Antibiotic Paste, Simvastatin, and Propolis, respectively. These findings indicate that Chitosan was the most effective intracanal medicament for eliminating *E. faecalis* in the apical region. A similar pattern of antimicrobial activity was observed in the middle third of the root canal. One-way ANOVA demonstrated a statistically highly significant difference among the five groups ($F = 56.6$, $p < 0.001$), confirming that the tested medicaments differed significantly in their antibacterial efficacy **(Table 3, Figure 3)**. The untreated control (Group A) again exhibited the highest bacterial count ($8.71 \pm 0.28 \times 10^3$ CFU), whereas Chitosan (Group E) showed the lowest mean CFU count ($2.06 \pm 0.40 \times 10^3$ CFU). The remaining groups demonstrated intermediate values, with Propolis (Group D) recording $3.47 \pm 0.33 \times 10^3$ CFU, Triple Antibiotic Paste (Group B) $3.59 \pm 0.36 \times 10^3$ CFU, and Simvastatin (Group C) $3.82 \pm 0.30 \times 10^3$ CFU. Post-hoc analysis confirmed that each medicament group exhibited significantly lower CFU counts than the untreated control (all $p < 0.001$) **(Table 4, Figure 4)**. Similar to the apical third, there were no statistically significant differences among Triple Antibiotic Paste, Simvastatin, and Propolis ($p > 0.05$), suggesting equivalent antibacterial efficacy among these medicaments. In contrast, Chitosan showed significantly lower bacterial counts than Triple Antibiotic Paste ($p < 0.05$), Simvastatin ($p < 0.01$), and Propolis ($p < 0.05$), further confirming its superior antimicrobial action in the middle third. Relative to the untreated control, Chitosan achieved an approximate 76.4% reduction in bacterial count at the middle third. Compared with Triple Antibiotic Paste, Simvastatin, and Propolis, Chitosan further reduced bacterial counts by 42.6%, 46.2%, and 40.7%, respectively, highlighting its enhanced antimicrobial efficacy throughout the root canal. Overall, the findings consistently showed that Chitosan (Group E) exhibited the greatest antibacterial activity against *Enterococcus faecalis* in both the apical and middle thirds of the root canal. Although Triple Antibiotic Paste, Simvastatin, and Propolis all significantly reduced bacterial counts compared with the untreated control, no significant differences were observed among these three medicaments. Chitosan, however, produced significantly greater bacterial reduction than each of the other tested intracanal medicaments, indicating its superior potential as an intracanal antimicrobial agent. Representative microbiological findings are presented in **Figure 5**, which illustrates the growth of *E. faecalis* on Brain Heart Infusion (BHI) agar. **Figure 6** shows the intracanal medicaments evaluated in the study, namely Triple Antibiotic Paste, Simvastatin, Propolis, and Chitosan. Representative agar plates showing bacterial colony growth following treatment with each medicament and

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the untreated control are presented in **Figure 7**, visually supporting the quantitative CFU findings.

Table 1: Colony forming unit (CFU) of *E. faecalis* in apical third

Group	*E. faecalis* CFU count (x10⁴) (Mean ± SE) (n=17)	F value	*P* Value
Group A	8.65 ± 0.27	52.97	<0.001
Group B	4.24 ± 0.26		
Group C	4.53 ± 0.35		
Group D	4.12 ± 0.27		
Group E	2.82 ± 0.35		

Table 2: Comparison (P value) of difference in mean *E. faecalis* CFU count (x 10⁴) between groups by tukey test at apical third

Comparison	Mean diff.	q value	*P* value	95% CI of diff
Group A vs. Group B	4.41	14.53	P < 0.001	3.210 to 5.613
Group A vs. Group C	4.12	13.56	P < 0.001	2.916 to 5.319
Group A vs. Group D	4.53	14.92	P < 0.001	3.328 to 5.731
Group A vs. Group E	5.82	19.18	P < 0.001	4.622 to 7.025
Group B vs. Group C	-0.29	0.97	P > 0.05	-1.496 to 0.908
Group B vs. Group D	0.12	0.39	P > 0.05	-1.084 to 1.319
Group B vs. Group E	1.41	4.65	P < 0.05	0.210 to 2.613
Group C vs. Group D	0.41	1.36	P > 0.05	-0.790 to 1.613
Group C vs. Group E	1.71	5.62	P < 0.01	0.504 to 2.907
Group D vs. Group E	1.29	4.26	P < 0.05	0.093 to 2.496

Table 3: *E. faecalis* CFU count (x 10³) of five groups at middle third

Group	*E. faecalis* CFU count (x10³) (Mean ± SE) (n=17)	F value	*P* value
Group A	8.71 ± 0.28	56.6	< 0.001
Group B	3.59 ± 0.36		
Group C	3.82 ± 0.30		
Group D	3.47 ± 0.33		
Group E	2.06 ± 0.40		

E. faecalis CFU count of five groups at middle third were summarized in Mean ± SE and compared by ANOVA.

Table 4: Comparison (P value) of difference in mean *E. faecalis* CFU count (x 10³) between groups by Tukey test at middle third

Comparison	Mean diff.	q value	*P* value	95% CI of diff
Group A vs. Group B	5.12	15.14	P < 0.001	3.781 to 6.455
Group A vs. Group C	4.88	14.45	P < 0.001	3.545 to 6.219
Group A vs. Group D	5.24	15.49	P < 0.001	3.898 to 6.572
Group A vs. Group E	6.65	19.67	P < 0.001	5.310 to 7.984
Group B vs. Group C	-0.24	0.7	P > 0.05	-1.572 to 1.102
Group B vs. Group D	0.12	0.35	P > 0.05	-1.219 to 1.455
Group B vs. Group E	1.53	4.53	P < 0.05	0.1923 to 2.867
Group C vs. Group D	0.35	1.04	P > 0.05	-0.984 to 1.690
Group C vs. Group E	1.77	5.22	P < 0.01	0.428 to 3.102
Group D vs. Group E	1.41	4.18	P < 0.05	0.075 to 2.749

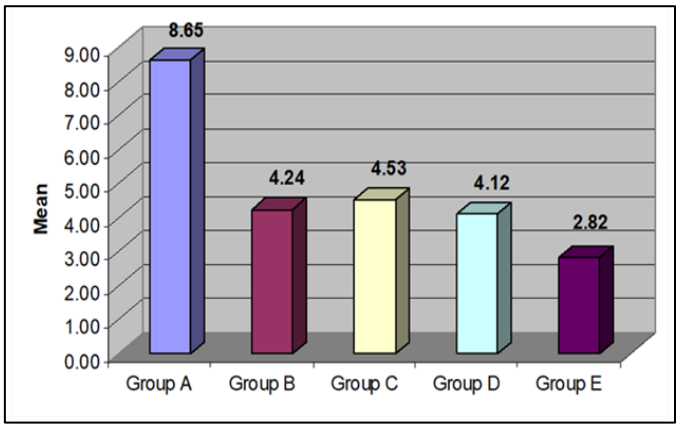

Figure 1: Mean *E. faecalis* CFU count of five groups at apical third

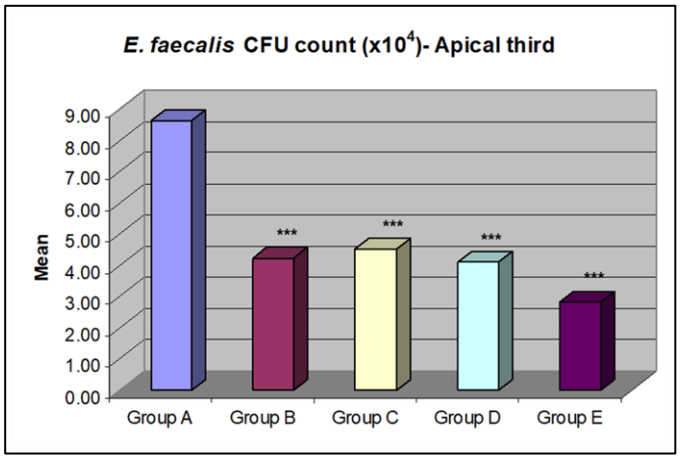

Figure 2: Comparisons of difference in mean *E. faecalis* CFU count between five groups at apical third

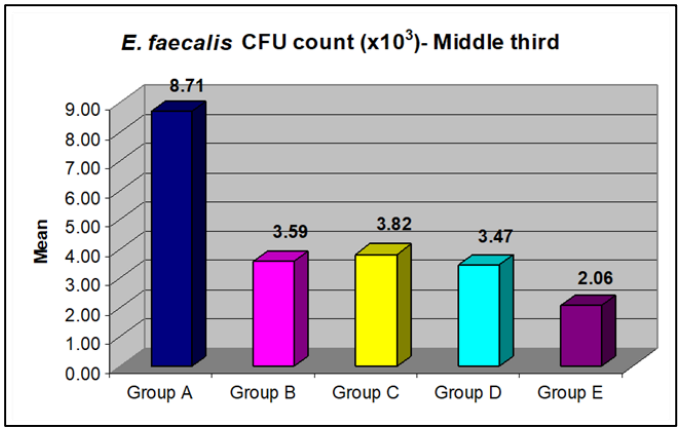

Figure 3: Mean *E. faecalis* CFU count of five groups at middle third

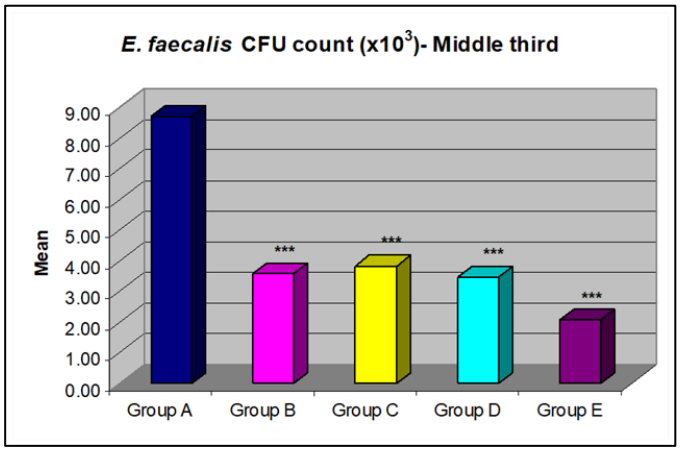

Figure 4: Comparisons of difference in mean *E. faecalis* CFU count between five groups at middle third

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Figure 5: *E. faecalis* grown on BHI Agar

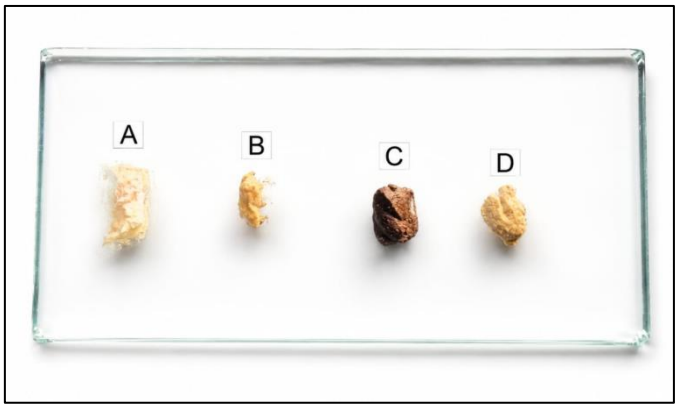

Figure 6: Intracanal Medicaments A) TAP B) Simvastatin C) Propolis D) Chitosan

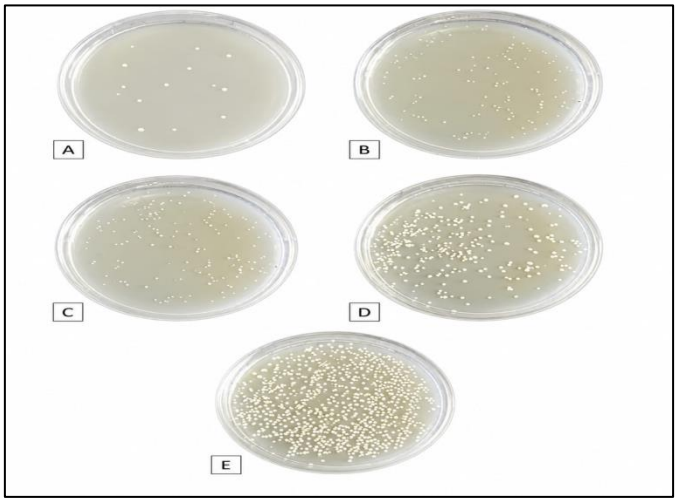

Figure 7: Colonies on agar plates A) Chitosan treated B) Propolis treated C) TAP treated D) Simvastatin treated E) Control group

Discussion:

Effective intracanal disinfection is determined not solely by the intrinsic antimicrobial strength of a medicament, but by its ability to adequately penetrate dentinal tubules, interact with the extracellular biofilm matrix and maintain sustained activity within the complex root canal system. Variations in molecular weight, structural configuration, diffusion capacity and mechanism of microbial interaction significantly influence therapeutic outcomes **[21]**. In this context, the present study comparatively evaluated the antibiofilm efficacy of four intracanal medicaments against *Enterococcus faecalis* to identify the agent demonstrating superior penetration ability and bactericidal performance. The results revealed that chitosan exhibited the highest antimicrobial efficacy against *E. faecalis* biofilm. This enhanced effectiveness can be attributed to its distinctive physicochemical and biological characteristics. The low molecular weight and reduced particle size of chitosan facilitate superior diffusion through the extracellular polymeric substance of the biofilm and allow deeper penetration into dentinal tubules. Improved depth of penetration ensures direct contact with microorganisms residing in anatomically inaccessible areas **[22]**. Furthermore, chitosan's polycationic nature, due to the presence of protonated amino groups, enables strong electrostatic interaction with negatively charged bacterial cell membranes. This interaction disrupts membrane integrity, increases permeability, induces leakage of intracellular components and ultimately results in bacterial cell death **[23]**. Thus, the combined effect of biofilm matrix disruption and direct bactericidal action likely accounts for the statistically superior antimicrobial outcome observed in this group. Following chitosan, propolis showed the second-highest antimicrobial activity. Its antibacterial properties are primarily attributed to bioactive constituents such as flavonoids and phenolic compounds, which interfere with enzymatic systems and inhibit protein synthesis within bacterial cells **[24]**. However, unlike chitosan, propolis did not exhibit a pronounced direct bactericidal effect against mature biofilm structures. Its comparatively larger molecular configuration and resinous consistency may restrict its diffusion into deeper dentinal tubules and the inner layers of the biofilm matrix. Furthermore, the volatility of its active components may reduce its sustained antimicrobial activity over time **[25]**. Consequently, although propolis displayed considerable antimicrobial potential, its relatively limited penetration capability may explain its lower efficacy when compared to chitosan. In the present study Triple antibiotic paste showed better antimicrobial activity than simvastatin, likely due to the synergistic effect of metronidazole, ciprofloxacin and minocycline. Metronidazole acts against anaerobic bacteria by generating reactive nitro radicals that damage bacterial DNA, leading to cell death. Ciprofloxacin inhibits DNA gyrase and topoisomerase IV, thereby preventing DNA replication and repair and is effective against *E. faecalis* due to its ability to partially penetrate biofilms and dentinal tubules. Minocycline suppresses bacterial protein synthesis, reducing both bacterial growth and biofilm formation. The combined inhibition of DNA integrity, replication and protein synthesis contributes to enhanced antimicrobial property of Triple Antibiotic Paste (TAP) **[26]**. However the components of Triple Antibiotic Paste have relatively large molecular sizes, which limit their penetration into deeper dentinal tubules. Inadequate

dentinal penetration may result in sub-inhibitory concentrations in deeper dentin layers, creating favorable conditions for bacterial survival and resistance **[27]**. These factors may account for the comparatively lower antimicrobial efficacy of TAP when compared with chitosan and propolis. Thasanakit *et al.* (2023) **[28]** stated that unlike Chitosan, TAP mainly acts only direct antimicrobial killing rather than by disrupting the structural integrity of the biofilm, which may reduce its effectiveness against mature and structurally complex biofilms. Furthermore, Saravanan and Ravindran (2025) **[29]** reported that, unlike biocompatible agents such as chitosan and propolis, TAP exhibits cytotoxic effects on stem cells, particularly stem cells of the apical papilla (SCAP) and its extrusion may cause apical tissue necrosis. Additionally, minocycline has a high affinity for calcium, leading to the formation of insoluble complexes within dentinal tubules that undergo oxidation over time and result in tooth discoloration. In contrast Simvastatin exhibited least antimicrobial efficacy among all the tested intracanal medicaments. Although widely recognized as a lipid-lowering agent that inhibits bacterial HMG-CoA reductase, blocking isoprenoid biosynthesis. This depletes lipid precursors, disrupts undecaprenyl phosphate production and impairs peptidoglycan transport. As a result, cell wall synthesis and membrane integrity are compromised. Recently simvastatin has been increasingly explored for its pleiotropic effects, including anti-inflammatory and antibacterial properties **[30].** In this study it has shown the least antimicrobial activity, this might be due to the fact that simvastatin is lipophilic (hydrophobic), which may limit its ability to traverse the hydrophilic extracellular polymeric substance (EPS) of *E. faecalis* biofilms. This may further limit its access to bacterial cells. The antimicrobial action of simvastatin appeared predominantly bacteriostatic at lower concentrations, likely through interference with essential metabolic pathways and inhibition of bacterial proliferation rather than direct membrane disruption. While higher concentrations may enhance bactericidal action, such increases raise concerns regarding cytotoxicity to periapical tissues **[31]**. This concentration-dependent balance between antimicrobial efficacy and biocompatibility may account for its intermediate performance among the tested medicaments. Taken together, the findings of the present investigation emphasize that antimicrobial success within the root canal system is profoundly influenced by molecular characteristics, penetration depth and mechanism of bacterial interaction. Medicaments capable of effectively permeating the biofilm matrix while exerting direct bactericidal effects show superior therapeutic potential. Among the materials evaluated, chitosan consistently showed the most promising antibiofilm performance **[32]**. However, the *in vitro* design of this study constitutes a limitation, as clinical conditions involve polymicrobial infections, host immune responses, tissue remnants and complex canal anatomy that may influence antimicrobial behavior. Therefore, further *in vivo* and clinical studies are warranted to validate these findings and to determine the optimal concentration and formulation for predictable and safe clinical application.

Conclusion:

Chitosan exhibited the high antimicrobial activity against mature *Enterococcus faecalis* biofilm, followed by propolis, triple antibiotic paste and simvastatin. These results highlight the potential of biocompatible, naturally derived medicaments as effective alternatives to antibiotics. Further studies with larger sample sizes and *in vivo* validation are needed to confirm these findings for clinical practice.

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