ANTIMICROBIAL EFFICACY OF 2-HYDROXYISOCAPROIC ACID (HICA) TOWARDS *ENTEROCOCCUS FAECALIS* AS ALTERNATIVE ENDODONTIC INTRACANAL MEDICAMENT

Jasmiad N[1], Mohamed Zohdi N[2], Mohamad Zain N[3], and Abu Bakar N[1].
[1]Centre of Paediatric Dentistry and Orthodontics Studies, Faculty of Dentistry, Universiti Teknologi MARA, Sungai Buloh Campus, Sungai Buloh, Selangor, Malaysia.
[2]Centre of Comprehensive Care Studies, Faculty of Dentistry, Universiti Teknologi MARA, Sungai Buloh Campus, Sungai Buloh, Selangor, Malaysia.
[3]Centre of Preclinical Science Studies, Faculty of Dentistry, Universiti Teknologi MARA, Sungai Buloh Campus, Sungai Buloh, Selangor, Malaysia.

Correspondence:
Norashikin Abu Bakar,
Centre of Paediatric Dentistry and Orthodontics Studies,
Faculty of Dentistry,
Universiti Teknologi MARA,
Sungai Buloh Campus, Sungai Buloh, Selangor, Malaysia.
Email: norashikin8234@uitm.edu.my

Abstract
Introduction: Antibiotics are used as intracanal medicaments in endodontic therapy. However, increasing antimicrobial resistance has led to research on alternative medications. 2-hydroxyisocaproic acid (HICA) is a derivative of leucine, a normal component of human plasma that shows potential as an intracanal medicament. **Objective:** To determine the antimicrobial efficacy of HICA against *Enterococcus faecalis (E. faecalis)*. **Methods:** The antimicrobial activity was screened by measuring the Zone of Inhibition (ZOI) using the disk diffusion method, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC). The positive control was a Triple Antibiotic Paste (TAP) containing minocycline, metronidazole, and ciprofloxacin. Sixty-six teeth were prepared, and the canals were filled with *E. faecalis* suspension before incubation for 21 days. Later, HICA at a concentration of 16 mg/mL, TAP at 1 mg/mL, and saline (negative control) were introduced and further incubated for seven days under anaerobic conditions at 37°C. Viable bacterial counts in the canals were used as indicators of bacterial growth. **Results:** At 256 mg/mL concentration, HICA exhibited a ZOI of 15.72 ± 1.60 mm, and MIC and MBC were 8 mg/mL and 16 mg/mL, respectively. TAP, at a concentration of 4 mg/mL, exhibited a ZOI of 30.74 ± 0.71 mm, and its MIC was equal to MBC at 0.25 mg/mL. The viable bacterial counts extracted from the canal were $4.33 \pm 0.23 \log_{10}$ for saline, $4.03 \pm 0.21 \log_{10}$ for HICA and $3.97 \pm 0.34 \log_{10}$ for TAP. Compared with saline, both TAP and HICA exhibited a significant difference in bacterial counts ($p < 0.01$). Interestingly, the efficacies of TAP and HICA were similar, with p-values of 0.79. **Discussion:** The ZOI indicated that HICA exhibited an antimicrobial effect. *E. faecalis* growth was repressed at 8 mg/mL, and cell death was observed at 16 mg/mL. At 16 mg/mL, HICA showed antimicrobial efficacy comparable to 1 mg/mL of TAP. **Conclusion:** HICA shows potential as an alternative intracanal medicament to eradicate *E. faecalis* in endodontic treatment.

***Keywords*:** 2-hydroxyisocaproic acid (HICA), *E. faecalis,* Triple Antibiotic Paste

Introduction

Polymicrobial infections, consisting of aerobic and anaerobic bacteria, are usually identified in endodontic infections (1-3). Chugal et al. (4) stated that Gram-negative anaerobic rods are predominant in primary endodontic infection, while fewer species are identified in secondary endodontic infections. A study by Ercan et al. (5) reported that anaerobic Gram-positive cocci predominated the microbiota. Commonly used antibiotics as intracanal medicaments in regenerative endodontics are double antibiotic paste (DAP) containing metronidazole and ciprofloxacin, and triple antibiotic paste (TAP) containing metronidazole, ciprofloxacin and minocycline (6, 7). Calcium hydroxide ($Ca(OH)^2$) is widely used as an intracanal medicament in

endodontics. It has been reported to have a favourable outcome in revascularising an infected immature permanent tooth (8, 9). However, Maniglia-Ferreira et al. (10) and Arruda et al. (11) reported in their studies that $Ca(OH)^2$ is insufficient to fully eradicate endodontic pathogens, especially *Enterococcus faecalis (E. faecalis)* and *Candida albicans (C. albicans)*.

The increasing threat of antimicrobial resistance is alarming, and overprescribing antibiotics in the dental field contributes to the problem. A study by Loffler and Bohmer (12) stated that unnecessary antibiotics are prescribed by 30%–50% of dentists. Root canal infection is a common dental infection treated with systemic and topical antibiotics. Over prescription and abuse of antibiotics increase microorganism resistance, making it more challenging to manage infections. To address this issue, alternative agents should be explored for their potential use in managing endodontic infections.

Sakko et al. (13, 14) studied the antimicrobial and antifungal effects of 2-hydroxyisocaproic acid (HICA). HICA is a derivative of leucine, a normal component of human plasma and a product of *Lactobacillus plantarum* during animal protein fermentation. Other studies by Nieminen et al. (15) and Selis et al. (16) reported that the antimicrobial effect and cytotoxicity of HICA showed its potential as an alternative intracanal medicament, not only for root canal treatment but also for regenerative endodontics, as it has a lower cytotoxic effect than calcium hydroxide at 1 mg/mL and is not cytotoxic at < 10 mg/mL (16).

The antimicrobial activity of 2-hydroxyisocaproic acid (HICA) has yet to be fully established in dentistry. Therefore, this study aimed to investigate the antimicrobial efficacy of HICA against *E. faecalis* and compare its effectiveness with that of triple antibiotic paste (TAP) as a potential alternative endodontic intracanal medicament.

Materials and Methods

Bacterial suspension and intracanal medicament preparation

Strict aseptic conditions were ensured by performing the procedure in a sterile biological safety cabinet. *E. faecalis* strain (ATCC 4082) was grown anaerobically at $37^{O}C$ for 24 hours in brain heart infusion (BHI) agar and later subcultured in BHI broth (17). HICA (Sigma-Aldrich, Germany) was mixed with sterile distilled water to a concentration of 256 mg/ml. TAP, consisting of ciprofloxacin, metronidazole, and minocycline, was mixed at a ratio of 1:1:1 with sterile water (18, 19) to produce a stock solution of 4 mg/mL concentration.

Experimental design

Figure 1 shows the experimental design used in this study. This study focused on assessing the antibacterial activity of HICA against *E. faecalis* using multiple methodologies, including the disk diffusion method, determination of minimum inhibitory concentrations, determination of minimum bactericidal concentration, and evaluation as an intracanal medicament in a human tooth model.

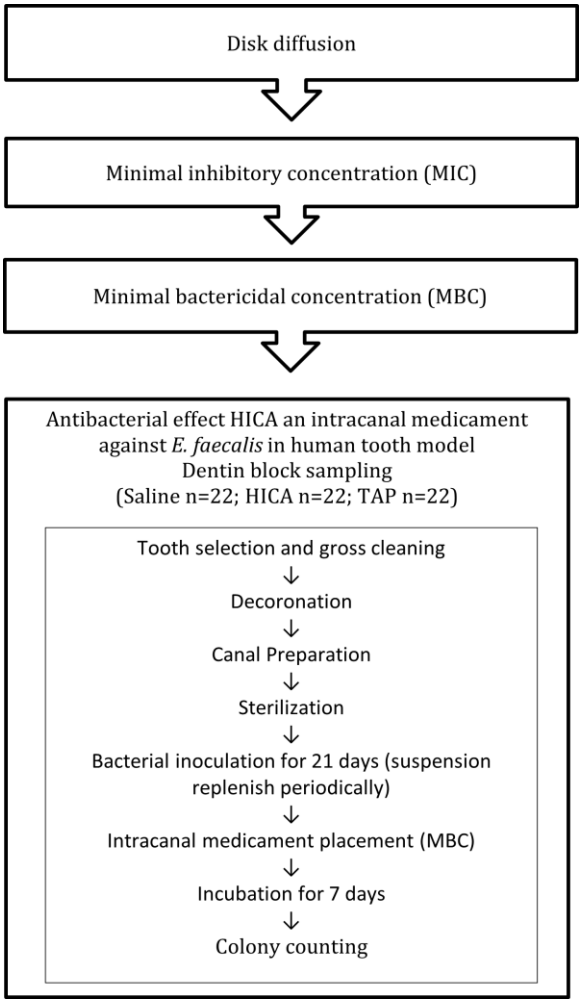

Figure 1: Flow chart of the experimental design of this study.

Antimicrobial susceptibility testing

The disk diffusion method was used to investigate the antimicrobial activities of HICA, TAP (positive control), and saline (negative control). BHI agar plates were prepared and cultured with bacterial suspensions adjusted to 0.5 McFarland standard (20). Filter disks loaded with 256 mg/mL HICA, 4 mg/mL TAP, and saline were placed on agar and incubated for 24 hours at 37°C under anaerobic conditions. The diameters (mm) of the zones of inhibition (ZOI) surrounding the disks (Figure 2) were measured (21, 22) using a digital calliper.

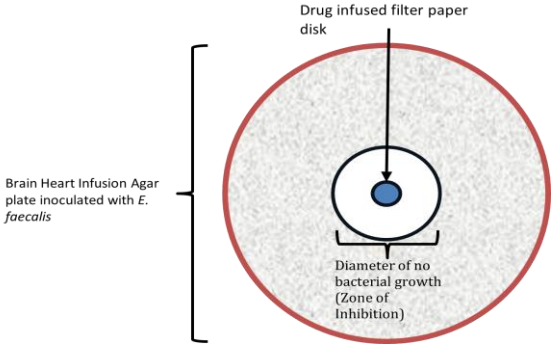

Figure 2: Disk diffusion method for antimicrobial susceptibility testing of *E. faecalis.*

Minimum inhibitory concentration and minimum bactericidal concentration

The MICs for HICA and TAP were determined using the broth microdilution method with BHI broth in 96-well flat-bottom microtiter plates in accordance with CLSI guidelines (23). In a 96-well plate, 100 µL BHI broth was dispensed into each well. Subsequently, 100 µL of a 256 mg/mL HICA solution was added to the initial well. Serial dilutions were then carried out, resulting in final HICA concentrations of 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 mg/mL. The same procedure was followed for 100 µL of 4 mg/mL TAP, serving as a positive control, with final TAP concentrations of 0.125, 0.25, 0.5, 1, and 2 mg/mL. Several colonies from the overnight agar cultures were suspended in sterile BHI broth. The bacterial density of the suspension was spectrophotometrically adjusted (OD600 nm) to achieve an inoculum equivalent to 0.5 McFarland, approximately 1.5×10^8 CFU/mL (23).

Finally, 10 µL of 0.5 McFarland standard solution of *E. faecalis* bacterial suspension, diluted to 1/20, was added to each well at a final concentration of approximately 5×10^5 CFU/mL (24). A blank control group containing only BHI was also included. The microplates were incubated at 37°C for 24h under anaerobic conditions and later evaluated using a microplate reader (Tecan's Infinite® 200 PRO) at 600 nm to obtain optical density (OD) (25). The MIC is the lowest concentration that inhibits bacterial growth. Then, 10 µL of each suspension with concentrations greater than the MIC was plated onto BHI agar plates and cultured at 37°C for another 24h. MBC was established at the lowest concentration, with no evident bacterial growth on the cultivated BHI agar. All processes were performed in triplicate and repeated five times (21).

Dentin block sampling

A dentin block was created to model the intracanal environment to promote bacterial growth before applying the medication. A total of 66 extracted permanent teeth with a single canal were used in this study. The sample size was calculated using G-power analysis with a power of 80% ($\beta = 0.2$), α level of 0.05, and effect size of 0.4. Teeth with lateral canals were excluded, as were those with extensive internal or external root resorption, root caries, or calcified canals. Collected teeth were stored in 0.1% thymol solution, and after removal of calculus and soft tissues, samples were stored in saline (26-29). Radiographs were obtained for each tooth to ensure the samples met the inclusion and exclusion criteria.

Decoronation was performed and the canal was prepared to size F5 (Protaper Next, Dentsply Maillefer, Ballaigues, Switzerland) at a working length of 12 mm. Using 30 gauges side-vented needle, the canal was irrigated with 2 ml of 5.25% sodium hypochlorite to dissolve any remaining intracanal tissue. Then, 2 ml 17% ethylenediaminetetraacetic acid (EDTA) is flushed into the canal to remove the smear layer (30). Ten millilitres of saline were used for the final irrigation. Self-cured glass ionomer cement (Riva, SDI) was placed at the apical foramen to prevent leakage of the bacterial suspension. The blocks were then suspended in acrylic resin before autoclaving at 121°C for 30 min (31, 32).

To ensure successful sterilisation process, two randomly selected sample cultures were used. After sterilisation, root canals were inoculated with *E. faecalis* suspension (1.5×10^8 CFU/mL) and then incubated at 37°C for 21 days under anaerobic conditions (20). The intracanal bacterial culture within the root canal was replenished every 48 hours to maintain biofilm (6). After incubation, the infected roots were randomly divided into three groups for

intracanal medicament application (saline, 16 mg/mL HICA, and 1 mg/mL TAP). The canals were irrigated with 10 mL of sterile saline and dried using sterile paper points. Intracanal medicament was delivered into the root canals using a 30-gauge needle until the working length and further incubated for seven days (29). After seven days, the canals were irrigated with 10 mL of sterile saline to ensure complete removal of the medicament. Sterile paper points were subsequently placed into the canal for 60 seconds, manipulated around the dentinal walls, and then transferred into a microcentrifuge tube containing 1.0 ml sterile saline. The microcentrifuge tube was vortexed, and 10-fold serial dilutions were performed. From the 10-fold serial dilutions, 100 µl suspension was transferred onto agar plates (triplicates) and incubated for 24h at 37°C under anaerobic conditions. Colony forming units (CFU)/mL from the plates were calculated using a colony counter. CFU number was calculated using the following formula (28, 33). The results are presented in $\log^{10}$.

$$\textbf{Total CFU/ml} = \frac{\text{Number of colonies}}{\text{Volume plated (ml)} \times \text{Dilution factor}}$$

Data analysis

Statistical analyses were performed using the IBM SPSS Statistics Version 27. To evaluate the normality of the data, a normality test was conducted using the Shapiro–Wilk test and one-way ANOVA for normal distribution data. A post-hoc Tukey test was performed to compare the efficacy of the intracanal medicaments.

Results

The zones of inhibition, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) of HICA and TAP are shown in Table 1.

Table 1 shows that the mean value for the zone of inhibition (ZOI) of TAP is significantly larger than that of HICA despite the use of a higher concentration of HICA. The MIC and MBC of HICA are also notably higher in concentration to produce a similar effect of 0.25 mg/mL of TAP.

Each dentin-block group consisted of 22 samples (n = 66). When plated on agar during colony counting for teeth treated with TAP, the viable bacterial count was lower than that of HICA and negative control groups (Figure 3).

Table 1: Zone of Inhibition, Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of HICA and TAP towards *E. faecalis*

Microorganism	Zone of Inhibition (diameter in mm)		HICA (concentration in mg/mL)		TAP (concentration in mg/mL)	
	HICA (256 mg/mL)	TAP (4 mg/mL)	MIC	MBC	MIC	MBC
E. faecalis	15.72 ± 1.60	30.74 ± 0.71	8	16	0.25	0.25

HICA: 2-Hydroxyisocaproic acid
TAP: Triple Antibiotic Paste
AST: Antimicrobial Susceptibility Testing
MIC: Minimum Inhibitory Concentration
MBC: Minimum Bactericidal Concentration

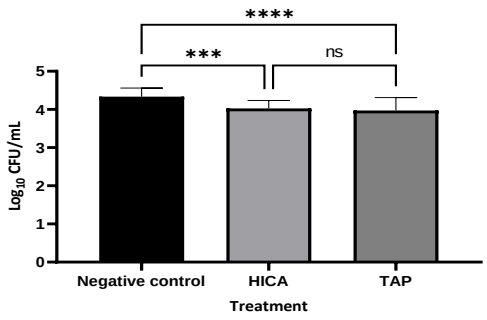

Figure 3: The bar graph represents the means ± SD values of bacterial count ($\log_{10}$ CFU/mL) for different treatment groups (n=22). Statistical analysis was conducted using one-way analysis of variance (ANOVA) to assess the overall differences between groups. Post hoc Tukey tests were performed for pairwise comparisons. Results are reported as follows: ns indicates no significant difference, *** denotes P<0.001, and **** indicates P<0.0001.

A post-hoc Tukey test was performed, and the results are depicted in Figure 3, comparing the antimicrobial

efficacy between the groups. TAP and HICA were significantly superior to the control ($p < 0.01$). The antimicrobial efficacies of HICA and TAP were not significantly different ($p > 0.05$).

Discussion

Antibiotic susceptibility testing (AST), using the disk diffusion test, is one of the most basic methods for determining the susceptibility of microorganisms to chemical agents or drugs. Bacteria were cultured on sterile agar before placing sterile filter paper disks impregnated with an antimicrobial agent at the desired concentration. The absence or presence of bacterial growth around the disks indicated the ability of the agent to inhibit microorganisms (22). Minimum inhibitory concentration is the lowest concentration achieved by antibacterial agents in a controlled in vitro experiment that prevents visible bacterial growth of the organism tested (34). The minimum bactericidal concentration (MBC) is the lowest concentration that reduces 99.9% of the number of bacteria, where no bacterial growth was observed on the inoculated agar within the stipulated time (34, 35).

HICA is a byproduct of the leucine-acetyl-CoA pathway. HICA formation involves the transamination of leucine to 2-ketoisocaproic acid (KICA). KICA is then reduced to HICA as the end product (36). To eliminate HICA, it must be converted back to KICA by an oxidation reaction catalysed by hydroxyisocaproic acid dehydrogenase (HicDH). The mechanism of the antimicrobial activity of HICA was investigated, and bacterial cell membrane penetration was reported. Changes in the cell membrane cause depolarisation, permeabilisation, and membrane rupture, leading to the leakage of cellular contents and, eventually death (37).

E. faecalis was selected for this study because it is commonly found in failed root canal treatments or persistent infections (38, 39). It has also been reported that *E. faecalis* is present in 38% of root canals that are unsuccessfully treated (40). A systematic review by Alghamdi and Shakir (41) concluded that *E. faecalis* was the primary pathogen in root canal treatment failure. Disk diffusion tests were performed to evaluate the antimicrobial activity of the medicaments in vitro. Antibiotics loaded in the sterile filter disks placed on the inoculated agar diffused through the agar, creating an area of bacterial growth inhibition that was later measured. HICA has been shown to have an antimicrobial effect against *E. faecalis*. TAP showed a larger ZOI, even though the concentration was lower than that of HICA at 4 mg/mL compared to 256 mg/mL. MIC and MBC were determined to evaluate the medicaments' efficacy before dentin block treatment. The values differ from previous studies, which investigated the efficacy of HICA at a lower dose.

The MIC of HICA was shown to be much higher at a concentration of 8 mg/mL compared to 0.25 mg/mL of TAP, indicating that *E. faecalis* is more susceptible to TAP. Based on the findings of a study by Sakko et al. (14), the MIC that inhibits 90% growth of *E. faecalis* was 9.0 mg/mL, which is similar to the outcome of this study. However, the MBC for HICA was much lower in this study at 18 mg/mL compared to 36 mg/mL. The difference in efficacy might be due to variations in the *E. faecalis* strains (ATCC 29212 and T-75359) used in their study (14). Triple antibiotic pastes have been introduced by Hoshino et al. (42) and Sato et al. (43). Based on Hoshino's research, 25 µg/mL was sufficient to eradicate root canal bacteria using TAP. Alireza et al. reported that the MIC for TAP against *E. faecalis* was 77.5 µg/mL (44). This study's findings showed that a higher concentration of TAP at 0.25 mg/mL is required to eradicate *E. faecalis* fully (ATCC 4082).

These results showed that TAP was bactericidal at a concentration of 0.25 mg/mL. However, 1 mg/mL was used in this study in line with the recommended dosage for regenerative endodontic procedures recommended by the American Association of Endodontists (AAE). AAE clinical considerations for a Regenerative Procedure Revised in 2021 suggested concentrations of 1 – 5 mg/mL TAP by mixing equal parts of antibiotics (45).

Dentin block showed a lower viable bacterial count for TAP compared to negative control and HICA. Both HICA and TAP showed significant antimicrobial effects compared to saline. At a 16 mg/mL concentration, HICA was as effective as 1 mg/mL TAP in inhibiting *E. faecalis* growth. To the best of our knowledge, no study has compared the efficacies of HICA and TAP. Sakko et al. (29) compared the effects of 40% HICA paste, 2% chlorhexidine solution, 40% calcium hydroxide paste, and 0.9% saline ex vivo, and reported more than 90% bacterial growth inhibition by HICA compared to saline. The same study reported that HICA has an antimicrobial effect comparable to chlorhexidine and is not substantially affected by potential inhibitors, such as hydroxyapatite and dentin powder, compared to $Ca(OH)^2$ (29).

Ideally, intracanal medicaments should possess broad-spectrum antimicrobial effects while preserving stem cell viability, especially in regenerative endodontics. Selis et al. studied the biocompatibility of HICA (16) and compared the cytotoxic effects of HICA and $Ca(OH)^2$ on primary human periodontal ligament (hPDL) fibroblasts. The findings showed that HICA was neither cytotoxic nor genotoxic at concentrations < 10 mg/mL. At 1 mg/mL, HICA is reported to be less cytotoxic than calcium hydroxide, demonstrating the potential of HICA as an intracanal medicament in regenerative endodontic procedures (16).

This study has several limitations; in vitro and ex vivo experiments might not be able to represent intraoral conditions fully. Multiple factors, such as polymicrobial infections, may affect the efficacy of medication. The concentration of intracanal medicaments may also vary, depending on the microorganisms present.

Conclusion

HICA shows potential as an alternative intracanal medicament to eradicate *E. faecalis* in endodontic treatment. Further research regarding its effect on multiple microorganisms, adverse effects on tooth structure, and cytotoxicity on dental pulp stem cells needs to be explored.

Acknowledgement

The authors would like to express their gratitude to the Research Laboratory support staff of the Faculty of Dentistry, UiTM Sungai Buloh, for their assistance and support during this research.

Competing interests

The authors declare that they have no conflicts of interest.

Ethical Clearance

Ethical approval for this study was obtained from the Research Ethics Committee of UiTM. Patient data were not collected or used during the study period. The Ethic Committee reference number: REC/07/2021(EX/119).

Financial support

We gratefully acknowledge the financial support provided for this study by the Special Research Grant (600-FPG (PT.5/2)(06/2021)) Faculty of Dentistry UiTM. This funding has been instrumental in enabling the research efforts and advancements made within the scope of this project. We extend our sincere appreciation to the Faculty of Dentistry for their commitment to fostering academic pursuits and facilitating our research endeavours.

References

1. Pereira RS, Rodrigues VAA, Furtado WT, Gueiros S, Pereira GS, Avila-Campos MJ. Microbial analysis of root canal and periradicular lesion associated to teeth with endodontic failure. Anaerobe. 2017; 48:12-18.
2. Bronzato JD, Davidian MES, de Castro M, de-Jesus-Soares A, Ferraz CCR, Almeida JFA, *et al.* Bacteria and virulence factors in periapical lesions associated with teeth following primary and secondary root canal treatment. Int. Endod. J..2021; 54(5):660-671.
3. Mussano F, Ferrocino I, Gavrilova N, Genova T, Dell'Acqua A, Cocolin L, *et al.* Apical periodontitis: preliminary assessment of microbiota by 16S rRNA high throughput amplicon target sequencing. BMC Oral Health. 2018; 18(1):1-8.
4. Chugal N, Wang JK, Wang R, He X, Kang M, Li J, *et al.* Molecular characterization of the microbial flora residing at the apical portion of infected root canals of human teeth. J Endod. 2011; 37(10):1359-1364.
5. Ercan E, Dalli M, Yavuz İ, Özekinci T. Investigation of Microorganisms in Infected Dental Root Canals. Biotechnology & Biotechnological Equipment. 2014; 20(2):166-172.
6. Porciuncula de Almeida M, Angelo da Cunha Neto M, Paula Pinto K, Rivera Fidel S, Joao Nogueira Leal Silva E, Moura Sassone L. Antibacterial efficacy and discolouration potential of antibiotic pastes with macrogol for regenerative endodontic therapy. Aust. Endod. J. 2021; 47(2):157-162.
7. Makandar SD, Noorani TY. Triple antibiotic paste - Challenging intracanal medicament: A systematic review. J Int Oral Health. 2020; 12(3):189-196.
8. Cehreli ZC, Isbitiren B, Sara S, Erbas G. Regenerative endodontic treatment (revascularization) of immature necrotic molars medicated with calcium hydroxide: a case series. J Endod. 2011; 37(9):1327-1330.
9. Shaik J, Garlapati R, Nagesh B, Sujana V, Jayaprakash T, Naidu S. Comparative evaluation of antimicrobial efficacy of triple antibiotic paste and calcium hydroxide using chitosan as carrier against *Candida albicans* and *Enterococcus*

faecalis: An in vitro study. J Conserv Dent. 2014; 17(4):335-339.
10. Maniglia-Ferreira C, de Almeida-Gomes F, Pinto MMN, de Sousa Barbosa FT, de Farias Filho DM, Albuquerque NLG. In vitro evaluation of the antimicrobial effects of different intracanal medications in necrotic immature teeth. Eur Arch Paediatr Dent. 2016; 17(4):251-255.
11. Arruda MEF, Neves MAS, Diogenes A, Mdala I, Guilherme BPS, Siqueira JF, Jr., *et al*. Infection Control in Teeth with Apical Periodontitis Using a Triple Antibiotic Solution or Calcium Hydroxide with Chlorhexidine: A Randomized Clinical Trial. J Endod. 2018; 44(10):1474-1479.
12. Loffler C, Bohmer F. The effect of interventions aiming to optimise the prescription of antibiotics in dental care - A systematic review. PLoS One. 2017; 12(11):1-23.
13. Sakko M, Moore C, Novak-Frazer L, Rautemaa V, Sorsa T, Hietala P, *et al*. 2-hydroxyisocaproic acid is fungicidal for *Candida and Aspergillus* species. Mycoses. 2014; 57(4):214-221.
14. Sakko M, Tjäderhane L, Sorsa T, Hietala P, Järvinen A, Bowyer P, *et al*. 2-Hydroxyisocaproic acid (HICA): a new potential topical antibacterial agent. Int J Antimicrob Agents. 2012; 39(6):539-540.
15. Nieminen MT, Novak-Frazer L, Rautemaa V, Rajendran R, Sorsa T, Ramage G, *et al*. A novel antifungal is active against *Candida albicans* biofilms and inhibits mutagenic acetaldehyde production in vitro. PLoS One. 2014; 9(5):1-10.
16. Selis D, Pande Y, Smoczer C, Wheater M, Alhabeil J, Paurazas S, *et al.* Cytotoxicity and Genotoxicity of a New Intracanal Medicament, 2-hydroxyisocaproic Acid-An In Vitro Study. J Endod. 2019; 45(5):578-583.
17. AlSaeed T, Nosrat A, Melo MA, Wang P, Romberg E, Xu H, *et al.* Antibacterial Efficacy and Discoloration Potential of Endodontic Topical Antibiotics. J Endod. 2018; 44(7):1110-1114.
18. Shokouhinejad N, Khoshkhounejad M, Alikhasi M, Bagheri P, Camilleri J. Prevention of coronal discoloration induced by regenerative endodontic treatment in an ex vivo model. Clin Oral Investig. 2018; 22(4):1725-1731.
19. Devaraj S, Jagannathan N, Neelakantan P. Antibiofilm efficacy of photoactivated curcumin, triple and double antibiotic paste, 2% chlorhexidine and calcium hydroxide against *Enterococcus fecalis* in vitro. Sci Rep. 2016; 6:1-6.
20. Sakko M, Tjaderhane L, Sorsa T, Hietala P, Rautemaa R. 2-Hydroxyisocaproic acid is bactericidal in human dental root canals ex vivo. Int Endod J. 2017; 50(5):455-463.
21. Al-Madi EM, Almohaimede AA, Al-Obaida MI, Awaad AS. Comparison of the Antibacterial Efficacy of *Commiphora molmol* and Sodium Hypochlorite as Root Canal Irrigants against *Enterococcus faecalis* and *Fusobacterium nucleatum*. Evid Based Complement Alternat Med. 2019; 1-7
22. Hudzicki J. Kirby-Bauer disk diffusion susceptibility test protocol. American Society for Microbiology. 2009; 1-23.
23. Clinical and Laboratory Standards Institute (CLSI). *Performance standards for antimicrobial susceptibility testing;* 30th ed. CLSI document M100; 2020. Available at: https://www.nih.org.pk/wp-content/uploads/2021/02/CLSI-2020.pdf. Accessed 18 October 2022.
24. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clinical Microbiology and Infection. 2003; 9(8):ix-xv.
25. Nagata H, Inagaki Y, Yamamoto Y, Maeda K, Kataoka K, Osawa K, *et al.* Inhibitory effects of macrocarpals on the biological activity of *Porphyromonas gingivalis* and other periodontopathic bacteria. Oral Microbiol Immunol. 2006; 21(3):159-163.
26. Jacobs JC, Troxel A, Ehrlich Y, Spolnik K, Bringas JS, Gregory RL, *et al.* Antibacterial Effects of Antimicrobials Used in Regenerative Endodontics against Biofilm Bacteria Obtained from Mature and Immature Teeth with Necrotic Pulps. J Endod. 2017; 43(4):575-579.
27. Baranwal R, Duggi V, Avinash A, Dubey A, Pagaria S, Munot H. Propolis: A Smart Supplement for an Intracanal Medicament. Int J Clin Pediatr Dent. 2017; 10(4):324-329.
28. Chua EG, Parolia A, Ahlawat P, Pau A, Amalraj FD. Antifungal effectiveness of various intracanal medicaments against *Candida albicans*: an ex-vivo study. BMC Oral Health. 2014; 14:1-8.
29. Sakko M, Tjaderhane L, Sorsa T, Hietala P, Rautemaa R. Antimicrobial 2-hydroxyisocaproic acid and chlorhexidine resist inactivation by dentine. Int Endod J. 2016; 49(4):352-360.
30. Hwang D, Fong H, Johnson JD, Paranjpe A. Efficacy of different carriers for the triple antibiotic powder during regenerative endodontic procedures. Aust Endod J. 2018; 44(3):208-214.

31. Afkhami F, Pourhashemi SJ, Sadegh M, Salehi Y, Fard MJ. Antibiofilm efficacy of silver nanoparticles as a vehicle for calcium hydroxide medicament against *Enterococcus faecalis*. J Dent. 2015; 43(12):1573-1579.
32. Moradi Eslami L, Vatanpour M, Aminzadeh N, Mehrvarzfar P, Taheri S. The comparison of intracanal medicaments, diode laser and photodynamic therapy on removing the biofilm of *Enterococcus faecalis* and *Candida albicans* in the root canal system (ex-vivo study). Photodiagn Photodyn Ther. 2019; 26:157-161.
33. Shafiei Z, Haji Abdul Rahim Z, Philip K, Thurairajah N. Antibacterial and anti-adherence effects of a plant extract mixture (PEM) and its individual constituent extracts (*Psidium sp., Mangifera sp*., and *Mentha sp*.) on single- and dual-species biofilms. PeerJ. 2016; 4:1-19.
34. Testing ECoAS. Methods for the determination of susceptibility of bacteria to antimicrobial agents. Terminology Eucast Defin Doc. 1998; 4:291-6.
35. Parvekar P, Palaskar J, Metgud S, Maria R, Dutta S. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of silver nanoparticles against *Staphylococcus aureus.* Biomater Investig Dent. 2020; 7(1):105-9.
36. Smit BA, Engels WJ, Wouters JT, Smit G. Diversity of L-leucine catabolism in various microorganisms involved in dairy fermentations, and identification of the rate-controlling step in the formation of the potent flavour component 3-methylbutanal. Appl Microbiol Biotechnol. 2004; 64(3):396-402.
37. Pahalagedara A, Flint S, Palmer J, Brightwell G, Gupta TB. Antibacterial efficacy and possible mechanism of action of 2-hydroxyisocaproic acid (HICA). PLoS One. 2022; 17(4):1-20.
38. Baumgartner JC, Siqueira JF, Jr., Xia T, Rocas IN. Geographical differences in bacteria detected in endodontic infections using polymerase chain reaction. J Endod. 2004; 30(3):141-144.
39. Baras BH, Sun J, Melo MAS, Tay FR, Oates TW, Zhang K, *et al.* Novel root canal sealer with dimethylaminohexadecyl methacrylate, nano-silver and nano-calcium phosphate to kill bacteria inside root dentin and increase dentin hardness. Dent Mater. 2019; 35(10):1479-1489.
40. Sundqvist G. Taxonomy, ecology, and pathogenicity of the root canal flora. Oral Surg Oral Med Oral Pathol. 1994; 78(4):522-530.
41. Alghamdi F, Shakir M. The Influence of *Enterococcus faecalis* as a Dental Root Canal Pathogen on Endodontic Treatment: A Systematic Review. Cureus. 2020; 12(3):1-7.
42. Hoshino E, Kurihara-Ando N, Sato I, Uematsu H, Sato M, Kota K, *et al.* In-vitro antibacterial susceptibility of bacteria taken from infected root dentine to a mixture of ciprofloxacin, metronidazole and minocycline. Int Endod J. 1996; 29(2):125-130.
43. Sato I, Ando-Kurihara N, Kota K, Iwaku M, Hoshino E. Sterilization of infected root-canal dentine by topical application of a mixture of ciprofloxacin, metronidazole and minocycline in situ. Int Endod J. 1996; 29(2):118-124.
44. Alireza Adl, Nooshin SS, Mohamad M. A Comparison between the Antimicrobial Effects of Triple Antibiotic Paste and Calcium Hydroxide against *Entrococcus Faecalis*. Iran Endod J. 2012; 7(3):149-155.
45. American Association of Endodontist Clinical Considerations for a Regenerative Procedure. 2021. Available at: https://www.aae.org/specialty/wp-content/uploads/sites/2/2021/08/ClinicalConsiderationsApprovedByREC062921.pdf. Accessed 18 October 2022.