Article published online: 2024-06-26

Antibacterial Activity of Calcium Hydroxide and Zinc Oxide Combined with Several Solutions against *Enterococcus faecalis* Growth

Syatirah-Najmi Abdullah[1] Wan Nur Faizatulakmal Wan Ahmad Zaki[1] Syarifah Musyirah Qistina Sayed Mansor[1] Aws Hashim Ali Al-Kadhim[2] Siti Aisyah Abd Ghafar[1] Rohazila Mohamad Hanafiah[1]

[1] Department of Basic Science, Faculty of Dentistry, Universiti Sains Islam Malaysia, Menara B Persiaran MPAJ, Jalan Pandan Utama, Ampang Kuala Lumpur, Malaysia
[2] Department of Prosthodontics and Conservative, Faculty of Dentistry, Universiti Sains Islam Malaysia, Menara B Persiaran MPAJ, Jalan Pandan Utama, Ampang Kuala Lumpur, Malaysia

Address for correspondence Rohazila Mohamad Hanafiah, BSc, MSc, PhD, Department of Basic Science, Faculty of Dentistry, Universiti Sains Islam Malaysia, Menara B Persiaran MPAJ, Jalan Pandan Utama, 55100, Ampang Kuala Lumpur, Malaysia (e-mail: rohazila@usim.edu.my).

Eur J Gen Dent

Abstract

Objective This study aims to compare the antimicrobial activity of calcium hydroxide (CaOH) and zinc oxide (ZnO) when incorporated with other solutions such as 2% chlorhexidine (CHX), 2.5% sodium hypochlorite (NaOCl), 1% povidone-iodine (PVP-I), and sterilized distilled water (ddH_2O) against *Enterococcus faecalis*.

Materials and Methods The materials were prepared by mixing CaOH and ZnO with other solutions (CHX, PVP-I, NaOCl, and ddH_2O) separately. The antibacterial activity of CaOH and ZnO mixtures against *E. faecalis* was done by using disk diffusion assay (DDA). Twofold serial dilutions of the mixtures were used against *E. faecalis* to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values. Biofilm inhibition of *E. faecalis* had been measured by using crystal violet assay.

Statistical Analysis The quantitative data of this study had been analyzed by using two-way analysis of variance with software SPSS version 27. The result is considered as significant if the value of analysis is *p*-value less than 0.05.

Results From the DDA results, the lowest zone of inhibition toward *E. faecalis* was CaOH-PVP-I (6.00 ± 0.00 mm), while the highest zone of inhibition toward *E. faecalis* was CaOH-CHX (22.73 ± 0.02 mm). Besides that, ZnO-PVP-I showed the lowest zone of inhibition (16.50 ± 0.06 mm), while ZnO-CHX showed the highest zone of inhibition (18.30 ± 0.08 mm) against *E. faecalis*. The MIC and MBC values of CaOH-CHX and ZnO-CHX were 0.78 and 6.25 mg/mL, respectively. In biofilm assay, CaOH-CHX and ZnO-CHX were reduced biofilm formation of *E. Faecalis*.

Conclusion Both CaOH-CHX and ZnO-CHX showed the highest antimicrobial activities toward *E. faecalis*. CaOH and ZnO alone showed no antimicrobial activities against *E. faecalis*.

Keywords
- calcium hydroxide
- zinc oxide
- *Enterococcus faecalis*

DOI https://doi.org/10.1055/s-0044-1786547.
ISSN 2320-4753.

Thieme Medical and Scientific Publishers Pvt. Ltd., A-12, 2nd Floor, Sector 2, Noida-201301 UP, India

Introduction

Failure in root canal treatment can be due to various factors. One of the prime factors is the persistence of bacteria inside the root canal. A previous study reported that the most isolated pathogenic bacteria present in the root canal system responsible for endodontic treatment failure is *Enterococcus faecalis*.[1,2] *E. faecalis* is a coccus-shaped, Gram-positive bacteria and facultative anaerobe. *E. faecalis* can resist the harsh environment inside the root canal and adapt to it.[3] Zhang et al reported that *E. faecalis* has a higher prevalence in persistent endodontic infection compared with untreated intra articular infection.[4]

In dental clinic settings, calcium hydroxide (CaOH) is widely used as a medicament during root canal treatment. Ba-Hattab et al mentioned that CaOH is the most popular choice for endodontic therapy due to its ideal properties toward the root canal.[5] CaOH has high pH that contributes to the alkaline environment, inducing the formation of reparative dentine and lowering the bacterial loads for the affected dentin.[6]

OdontoPASTE is a commercially available zinc oxide (ZnO)-based paste. Its usage is to temporarily obturate root canals and is extremely successful in reducing bacterial contamination during endodontic therapy.[7] Pain after endodontic operations is frequently caused by inflammation of the pulp and periapical tissues. OdontoPASTE that has anti-inflammatory properties with clindamycin hydrochloride (a broad-spectrum antibiotic) and triamcinolone acetonide (a steroid-based anti-inflammatory medication) that act through direct contact with the inflamed tissue and help to alleviate this discomfort.

However, studies stated that CaOH and ZnO are unable to totally eradicate bacteria inside the root canal such as *E. faecalis* and only exhibited a minor antimicrobial property.[8,9] According to Djearamane et al ZnO was resistant to *E. faecalis* growth.[10] Adding to that, *E. faecalis* colonies in dentinal tubules that have size up to 300 µm cause it to be hard to eliminate by the intracanal medicaments. Adl et al reported that CaOH does not remove the bacteria in the dentinal tubules effectively as it is unable to penetrate it.[11] This finding was supported by Dewi et al reported *E. faecalis* colonized in dentinal tubules was not affected and remained after being treated with CaOH.[12] Hence, even with the use of medicaments that possess antibacterial properties, it is hard to eradicate *E. faecalis* from the root canal. *E. faecalis* also has resistance toward CaOH by the formation of biofilm alongside surviving in alkaline conditions up to a pH of 11.5 for more than 10 days.[13] To overcome the problem, the combination of CaOH and ZnO with other solutions had been studied.

Hence, this study aims to evaluate the antibacterial activity of CaOH and ZnO combined with other solutions such as 2% CHX, 2.5% sodium hypochlorite NaOCL, and PVP-I toward *E. faecalis*.

Materials and Methods

Methodology

This experimental study was conducted to demonstrate the antimicrobial activity of materials used in endodontic treatment; CaOH paste and ZnO paste mixed with other solutions toward *E. faecalis*. ►**Fig. 1** is the workflow of the study.

Preparation of Materials

Calcicur that is CaOH paste (Voco dental, Indian Land, United States) and OdontoPASTE that is ZnO paste (Voco dental, Indian Land, United States) were prepared in two different concentrations (50 and 100 mg/mL) and mixed with four different solutions; 0.12% chlorhexidine (CHX) (Oradex, Damansara, Malaysia), 3% sodium hypochlorite (NaOCl), (Coltene, Switzerland) 1% povidone-iodine (PVP-I) (Betadine, Stamford, United States), and sterilized distilled water (negative control).

Microorganisms and Culture Conditions

Enterococcus faecalis (ATCC 29212) bacteria was obtained from the laboratory of microbiology at the Faculty of Dentistry Universiti Sains Islam Malaysia. The bacteria were cultured in Tryptic Soy Broth (Oxoid, Thermo Fisher Scientific, United Kingdom, England) and incubated at 37°C for 24 hours under aerobic conditions.[14]

Preparation of Media Plates for the Antimicrobial Sensitivity Testing

Mueller Hinton agar (MHA) (Oxoid, Thermo Fisher Scientific, United Kingdom, England) and Mueller Hinton broth (MHB) (Oxoid, Thermo Fisher Scientific, United Kingdom, England) were used in antimicrobial sensitivity testing. All media were prepared according to the manufacturer's instructions and sterilized by autoclaving at 121°C for 3 hours. All plates are labeled according to the treatment for each section.

Disk Diffusion Agar Test

Antibacterial activity was determined by using the disk diffusion method with modification.[15] The modification of this method was on concentration of the samples and type of bacteria. Overnight cultures of *E. faecalis* (ATCC 29212) were adjusted to 0.5 McFarland standards (1.5×10^8 colony forming unit [CFU]/mL; Sigma-Aldrich, St. Lous, United States). Then, the bacteria were cultured onto MHA using sterile cotton swabs. Paper disks were impregnated with 50 and 100 mg/mL of CaOH-CHX, CaOH-NaOCl, CaOH-PVP-I, CaOH-ddH_2O, ZnO-CHX, ZnO-NaOCl, ZnO-PVP-I, and ZnO-ddH_2O, respectively. Zones of inhibition were measured from the diameter of the disks to the circumference of the inhibition zone after incubating at 37°C for 24 hours, aerobically.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

E. faecalis was subcultured in the MHB and incubated at 37°C overnight. Gram staining was employed to ensure no contamination in the colony. The MIC was performed using the twofold serial dilution method in a 96-well plate (Sigma-Aldrich, St. Lous, United States). Approximately 100 µL of *E. faecalis* (1.5×10^8 CFU/mL) were added into 100 µL of various

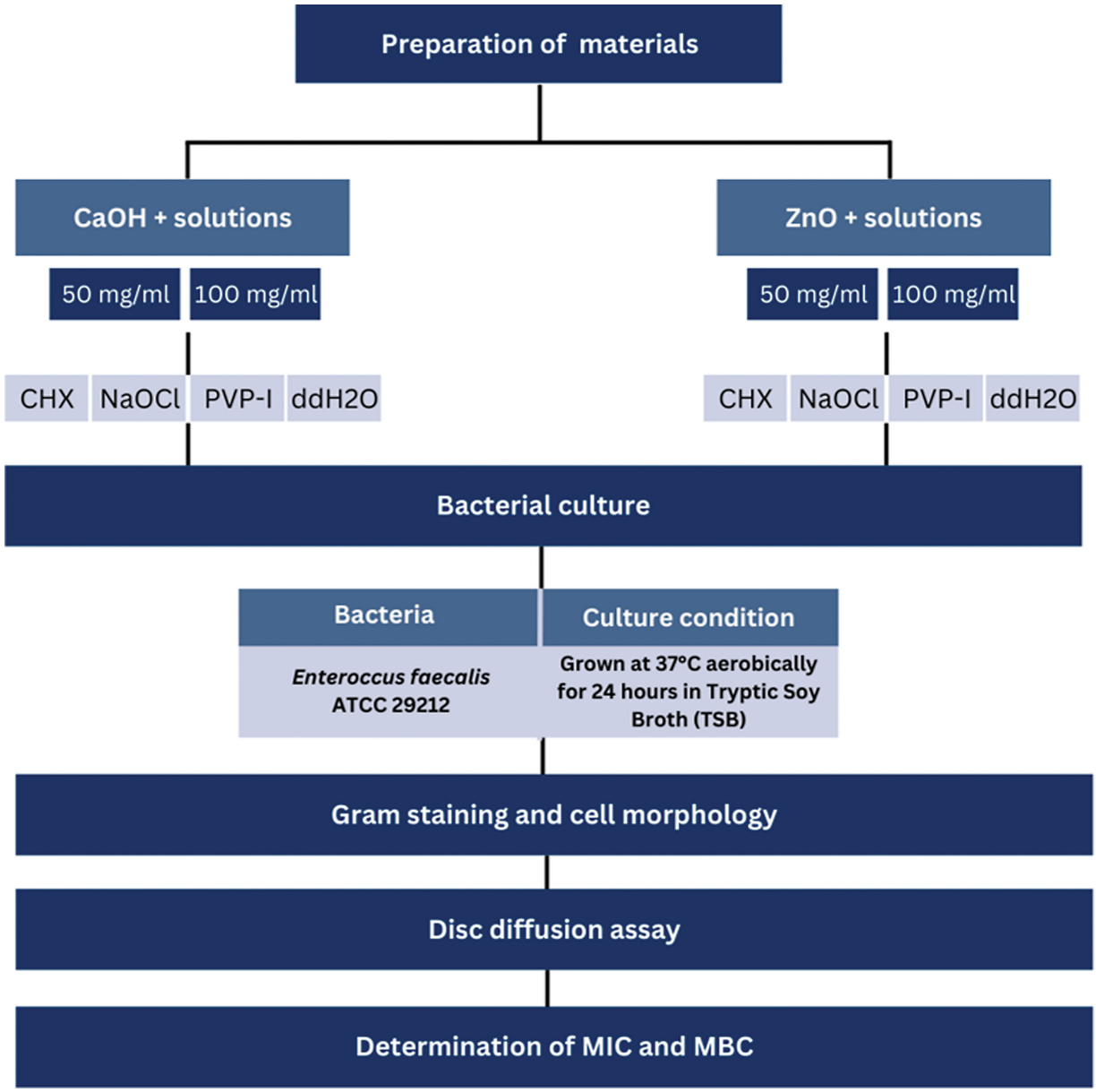

Fig. 1 Workflow of this study to determine antibacterial activities of CaOH and ZnOH combined with several solutions against *Enterococcus faecalis*. CaOH, calcium hydroxide; CHX, chlorhexidine; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; NaOCL, 2.5% sodium hypochlorite; PVP-I, 1% povidone-iodine; ZnOH, zinc hydroxide.

combination solutions in 96-well. The details of the combination solutions were shown below:

a) CaOH
Column A: Sterilized MHB only
Column B: CaOH-dd$H_2$0 (50–0.39 mg/mL)
Column C: CaOH-CHX (50–0.39 mg/mL)
Column D: CaOH -PVP-I (50–0.39 mg/mL)
Column E: CaOH-NaOCl (50–0.39 mg/mL)
Column F: *E. faecalis* only

b) ZnO
Column A: Sterilized MHB only
Column B: ZnO-ddH_2O (50–0.39 mg/mL)
Column C: ZnO-CHX (50–0.39 mg/mL)
Column D: ZnO-PVP-I (50–0.39 mg/mL)
Column E: ZnO-NaOCl (50–0.39 mg/mL)
Column F: *E. faecalis* only

After 24 hours of incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich, St. Lous, United States) was added to all wells (10 µL per well). The color changes were observed after incubation at 37°C for 1 to 4 hours. The MBC was determined by directly plating 10 µL of an aliquot from wells that displayed no growth in MIC wells onto MHA. After incubating overnight at 37°C, the MBC value was identified. MBC of different combination solutions was defined as the lowest concentration that kills *E. faecalis.*

Anti-biofilm Assay

Anti-biofilm assay was done by following Hanafiah et al.[15] Briefly, 100 µL of mixtures at MIC was added into 100 µL of *E. faecalis* (1.5×10^8 CFU/mL). *E. faecalis* treated with CaOH-ddH_20 and ZnO-ddH_2O acted as negative control, respectively. After inoculation, all the samples were incubated in an incubator at 37°C for 24 hours. Biofilm formation was determined by crystal violet assay. The culture medium was then decanted, and the plates were gently washed twice with 200 µL of sterile distilled water to remove the planktonic and loosely bound cells. The adherent bacteria were stained with 50 µL of 0.1% crystal violet for 15 minutes. After rinsing twice with 200 µL of water, the bound dye was extracted from the stained cells using 200 µL of 99% ethanol. Biofilm formation was then quantified by measuring the absorbance of the solution at 600 nm in a microplate reader.

Results

Disk Diffusion Assay of CaOH and ZnO Combined with Solutions against *E. faecalis*

The inhibition zone of CaOH combined with several solutions against *E. faecalis* was shown in ►**Fig. 2**. The mean of the diameter inhibition zone for *E. faecalis* after treated with CaOH-CHX and CaOH-NaOCl was found to be significant when compared with control (CaOH-ddh2O) ($p < 0.05$), while there was no significant difference between PVP-I and control. CaOH-CHX shows the highest zone of inhibition compared with other groups in concentrations 50 and 100 mg/mL that were 22.26 ± 0.03 and 22.73 ± 0.02mm, respectively.

The inhibition zone of ZnO combined with several solutions against *E. faecalis* was shown in ►**Fig. 3**. The mean of the diameter inhibition zone for *E. faecalis* after being treated with ZnO-CHX, ZnO-NaOCl, and ZnO-PVP-I were found to be significant, ($p < 0.05$) when compared with control. ZnO-CHX shows the highest zone of inhibition compared with other groups in concentrations 50 and 100 mg/mL that were 17.40 ± 0.02 and 18.30 ± 0.08 mm, respectively.

MIC and MBC of CaOH and ZnO Combined with Solutions against *E. faecalis*

The MIC and MBC values of CaOH and ZnO combined with solutions against *E. faecalis* were shown in ►**Table 1**. The MIC and MBC values of CaOH-CHX and ZnO-CHX against *E. faecalis* were 0.78 and 6.25 mg/mL, respectively. However, the MIC and MBC values of CaOH-PVP-I and CaOH-ddH_2O were more than 50 mg/mL, respectively. Meanwhile, the MIC and MBC values of ZnO-PVP-I and ZnO-NaOCL were 6.25 mg/mL and more than 50 mg/mL, respectively. The MIC and MBC values of ZnO-ddH_2O were 3.13 and more than 50 mg/mL, respectively. From the analysis, CHX solutions enhanced the antibacterial activity of CaOH and ZnO against *E. faecalis.*

Anti-Biofilm Activity

►**Fig. 4** showed significant ($p < 0.05$) anti-biofilm activity of mixtures at MIC concentrations against *E. faecalis.* Biofilm formation of *E. faecalis* reduced significantly, when it was treated with CaOH-CHX and ZnO-CHX compared with control, respectively. Treatment with CaOH-CHX and ZnO-CHX inhibited 70 and 75% biofilm formation of *E. faecalis,* respectively. CaOH-PVP and CaOH-NaOCL did not inhibit biofilm of *E. faecalis* at MIC concentration. Meanwhile, ZnO-PVP and ZnO-NaOCL inhibited 20 to 25% of biofilm formation of *E. faecalis* when compared with control, respectively.

Discussions

E. faecalis is a significant pathogen in the oral mucosa.[16,17] It is thought to have a key part in the etiology of post-treatment apical periodontitis because it is more prevalent in secondary endodontic infections than original infections. *E. faecalis* may

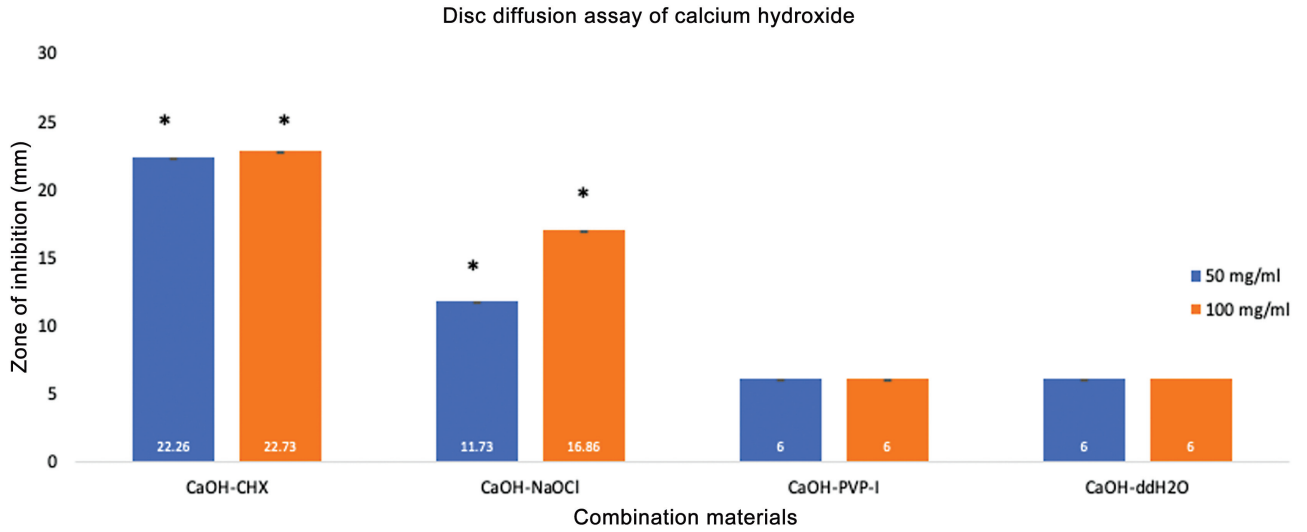

Fig. 2 The inhibition zone of CaOH combined with several solutions against *Enterococcus faecalis.* Values are represented as mean ± standard deviation. Values on the same group followed by asterisk differ significantly ($p < 0.05$). CaOH, calcium hydroxide; CHX, chlorhexidine; NaOCL, 2.5% sodium hypochlorite; PVP-I, 1% povidone-iodine; ZnO, zinc oxide.

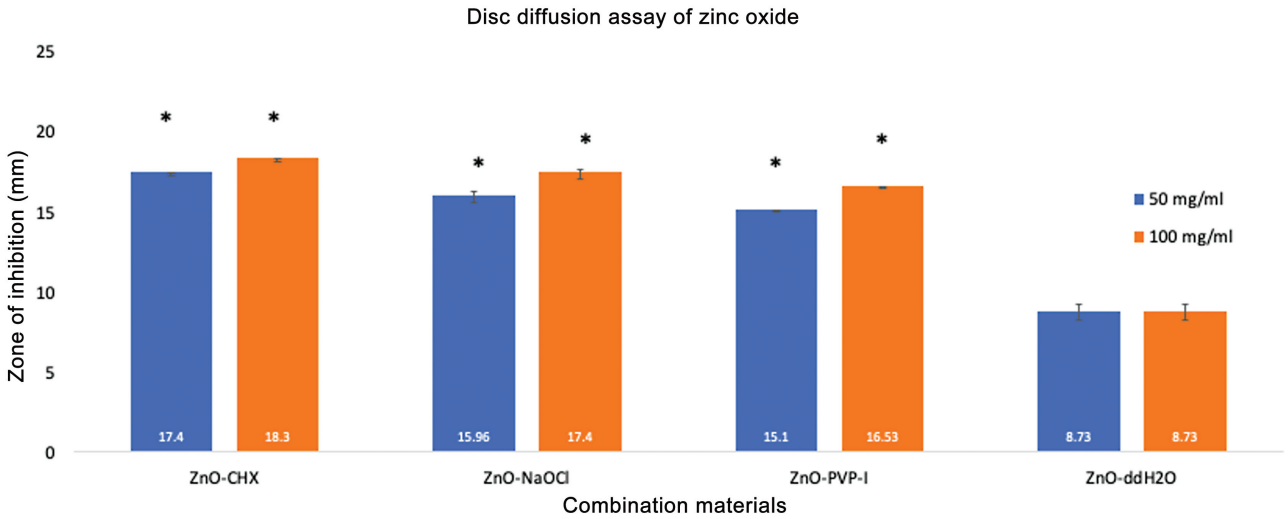

Fig. 3 The inhibition zone of ZnO combined with several solutions against *Enterococcus faecalis*. Values are represented as mean ± standard deviation. Values on the same group followed by asterisk differ significantly ($p < 0.05$). CaOH, calcium hydroxide; CHX, chlorhexidine; NaOCL, 2.5% sodium hypochlorite; PVP-I, 1% povidone-iodine; ZnO, zinc oxide.

Table 1 The MIC and MBC values of CaOH and ZnO combined with solutions against *Enterococcus faecalis*

Samples	MIC (mg/mL)	MBC (mg/mL)
CaOH-CHX	0.78	6.25
CaOH-NaOCL	3.13	6.25
CaOH-PVP-I	>50	>50
CaOH-ddH_2O	>50	>50
ZnO-CHX	0.78	6.25
ZnO-PVP-I	6.25	>50
ZnO-NaOCL	6.25	>50
ZnO-ddH_2O	3.13	>50

Abbreviations: CaOH, calcium hydroxide; CHX, chlorhexidine; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; NaOCL, 2.5% sodium hypochlorite; PVP-I, 1% povidone-iodine; ZnO, zinc oxide.
CHX was presented as an effective solution that can enhance the antimicrobial activity of CaOH and ZnO toward *E. faecalis*.

enter the root-filled channel through coronal leakage during or after root-canal therapy as secondary invaders.[18] *E. faecalis* is resistant to several antibiotics and antimicrobial compounds, such as chloramphenicol, tetracyclines, macrolides, and clindamycin,[17,19] making it difficult to eliminate. Furthermore, several irrigants used in root canal therapy showed little or no bactericidal action against *E. faecalis.*

The study on antibacterial activity of CaOH and ZnO combined with several solutions has proven that CaOH-CHX showed the most effective combination as antibacterial agent against *E. faecalis* when compared with other mixtures. Meanwhile, a combination of CaOH-PVP-I displayed the lowest antibacterial agents against *E. faecalis*. CHX is a synthetic antimicrobial agent that is widely used in antiplaque and antigingivitis formulations for the treatment of periodontitis due to its wide-spectrum action against both Gram-negative and Gram-positive bacteria, as well as in other applications against yeasts, fungi, and some viruses.[20] Some studies have failed to show any benefits in incorporating CHX that is in contrast to our findings. The reasons are the methods and materials used, microbiological assessments, time periods of experiments, strains and concentrations of *E. faecalis,* methods of bacterial inoculation, and depths of sampling.[21]

According to Nozari et al, the combination of CaOH and ZnO, especially when associated with CHX, provides a powerful antimicrobial effect against *E.* faecalis during root canal treatments.[22] Evans et al discovered that *E. faecalis* becomes susceptible to the combination of CaOH and chlorhexidine when examining the mechanism of resistance to CaOH in bovine dentin. Because of its method of action, CHX takes just a short time to exert an antibacterial effect on *E. faecalis*.[23] Its cationic molecule interacts with the negative charge of phosphate groups in the bacterial cell wall, causing metabolic damage and, as a result, bacterial death. This is also supported by another study that has the same result as our result where they observed that the combination of CaOH-CHX performed better than CaOH alone.[24]

OdontoPaste (ZnO) contains clindamycin hydrochloride that causes cell lysis of bacterial DNA by inhibiting the formation of peptide bonds.[25] Clindamycin hydrochloride is effective toward *E. faecalis* with 50,000 µg/mL concentration in OdontoPaste. In our study, the ZnO was effective against *E. faecalis* alone. However, when we incorporated CHX and ZnO together the results showed an increase of antibacterial activity against *E. faecalis* ($p < 0.05$). A study suggested that CHX is functional as part of the final irrigation solution in root canal infected with E. *faecalis* prior to obturating the canal.[26]

Povidone-iodine is often used as an alternative agent because of its increased antibacterial activity. Iodine has a strong oxidizing property resulting in disulfide linkages and effectively inhibits many root canal bacteria growth. However,

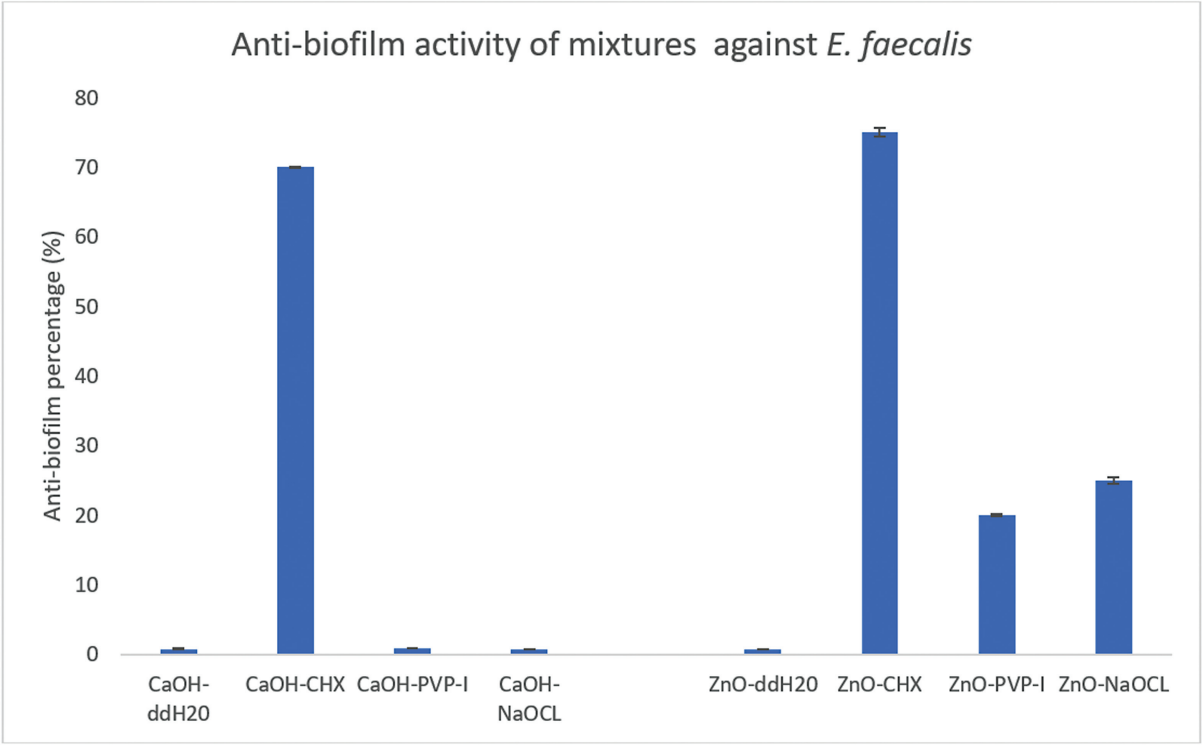

Fig. 4 Anti-biofilm activity of calcium hydroxide (CaOH) and zinc oxide (ZnO) combined with other solution against *Enterococcus faecalis*. In this analysis, CaOH-CHX, ZnO-CHX, ZnO-PVP-I, and ZnO-NaOCL were significantly reduced biofilm of *E. faecalis* at minimum inhibitory concentration ($p < 0.05$). CHX, chlorhexidine; NaOCL, 2.5% sodium hypochlorite; PVP-I, 1% povidone-iodine.

in this study, CaOH-PVP-I mixture did not give any effect on *E. faecalis*. This may be due to basic chemical knowledge indicating that povidone-iodine is neutral when compared with CaOH.[27] Interestingly, the combination of ZnO-PVP-I has mild antibacterial activity against *E. faecalis*. There were no studies found that investigated the antibacterial effect of ZnO when mixed with PVP-I toward *E. faecalis*. Another study also investigated the combination of CaOH-PVP-I against *E. faecalis*. In the investigation, the combination of CaOH-PVP-I did not result in a significant reduction in *E. faecalis* when compared with CaOH-CHX which is equal to our result.[27]

When CaOH and ZnO combined with NaOCl, there is significant zone reduction. Besides that, the antimicrobial activity of CaOH and ZnO incorporated with NaOCl was not as good or better than other samples. Previous studies also reported that NaOCl was resistant to *E. faecalis*.[28] Several studies have established NaOCl's usefulness as an irrigant solution. It is widely utilized in endodontic therapy, and the concentration of 2.5% NaOCl was chosen since it is commonly used. It can preserve enough chlorine to eradicate a considerable proportion of bacteria.[24] Based on a study, their findings confirmed that when CaOH is combined with NaOCl, the antibacterial efficacy of the mixture is larger than CaOH alone.[24] This conclusion is comparable to the results of Farhad et al, who examined the antibacterial efficiency of CaOH in conjunction with H_2O, CHX, or NaOCl against *E. faecalis*. Their findings revealed that the antibacterial potency of CaOH can be increased by preparing it with antibacterial irrigants such as NaOCl.[29] However, we could not find any other studies that investigate the combination of ZnO with NaOCl against *E. faecalis*.

ZnO and CaOH alone showed antibacterial activity against *E. faecalis*; however, synergetic effects were shown after the materials combined with CHX. Both combinations strongly inhibit *E. faecalis*. The results of this study revealed that the mixtures of CaOH-CHX and ZnO-CHX have antibacterial properties. This showed that they promoted greater antibacterial activity against *E. faecalis* by the association of CHX. Although based on our knowledge, both CaOH and ZnO can promote antibacterial effects, the activity against *E. faecalis* is favored by association with CHX. Through this study, we found that research on the combination between ZnO and other intracanal medicament materials is rarely done and as we have already investigated this study by using combination of ZnO with CHX, NaOCl, and PVP-I, we acknowledged that this is the significances of our study and we recommend for others to try investigating combination of ZnO with other medicaments too so that we can do comparison of our results in the future.

Conclusion

In conclusion, this study showed that CaOH-CHX and ZnO-CHX possessed the highest effect of antibacterial activity against *E. faecalis*. This finding provides an insight on the use of these combination agents in endodontic treatment. These combination agents could be useful in the formulation of disinfectants and the development of intracanal medicaments for root canal treatment.

Funding
This research was sponsored by the University Grant Scheme from Universiti Sains Islam Malaysia (PPPI/USIM-RACER_0120/FPG/051000/12420).

Conflicts of Interest
None declared.

References

1 Alghamdi F, Shakir M. The influence of *Enterococcus faecalis* as a dental root canal pathogen on endodontic treatment: a systematic review. Cureus 2020;12(03):e7257
2 Barbosa-Ribeiro M, Arruda-Vasconcelos R, Louzada LM, Dos Santos DG, Andreote FD, Gomes BPFA. Microbiological analysis of endodontically treated teeth with apical periodontitis before and after endodontic retreatment. Clin Oral Investig 2021;25(04): 2017–2027
3 John G, Kumar KP, Gopal SS, Kumari S, Reddy BK. *Enterococcus faecalis*, a nightmare to endodontists: a systematic review. Afr J Microbiol Res 2015;9(13):898–908
4 Zhang C, Du J, Peng Z. Correlation between *Enterococcus faecalis* and persistent intraradicular infection compared with primary intraradicular infection: a systematic review. J Endod 2015;41 (08):1207–1213
5 Ba-Hattab R, Al-Jamie M, Aldreib H, Alessa L, Alonazi M. Calcium hydroxide in endodontics: an overview. Open J Stomatol 2016;06 (12):274–289
6 Song M, Yu B, Kim S, et al. Clinical and molecular perspectives of reparative dentin formation: lessons learned from pulp-capping materials and the emerging roles of calcium. Dent Clin North Am 2017;61(01):93–110
7 Hegde VR, Jain A, Patekar SB. Comparative evaluation of calcium hydroxide and other intracanal medicaments on postoperative pain in patients undergoing endodontic treatment: a systematic review and meta-analysis. J Conserv Dent 2023;26(02):134–142
8 Kranz S, Guellmar A, Braeutigam F, et al. Antibacterial effect of endodontic disinfections on *Enterococcus faecalis* in dental root canals-an in-vitro model study. Materials (Basel) 2021;14(09):2427
9 Eftekhar B, Moghimipour E, Pourakbar Jahandideh P, Jalali S, Mahmoudian M. Analgesic effect of odontopaste and a compound intracanal medicament between root canal therapy appointments. Jundishapur J Nat Pharm Prod 2013;8(04):169–174
10 Djearamane S, Loh ZC, Lee JJ, et al. Remedial aspect of zinc oxide nanoparticles against *Serratia marcescens* and *Enterococcus faecalis*. Front Pharmacol 2022;13:891304
11 Adl A, Hamedi S, Sedigh Shams M, Motamedifar M, Sobhnamayan F. The ability of triple antibiotic paste and calcium hydroxide in disinfection of dentinal tubules. Iran Endod J 2014;9(02):123–126
12 Dewi A, Upara C, Krongbaramee T, Louwakul P, Srisuwan T, Khemaleelakul S. Optimal antimicrobial concentration of mixed antibiotic pastes in eliminating *Enterococcus faecalis* from root dentin. Aust Endod J 2021;47(02):273–280
13 Jhajharia K, Parolia A, Shetty KV, Mehta LK. Biofilm in endodontics: a review. J Int Soc Prev Community Dent 2015;5(01):1–12
14 Sharma P, Pandey D, Rizvi AF, Gupta AK. Antimicrobial activity of *Cassia alata* from Raipur region against clinical and MTCC isolates. Int J Curr Microbiol Appl Sci 2015;4(01):330–339
15 Hanafiah RM, Kamaruddin K, Saikin N, Yakop MF, Lim V, Ghafar S. Antibacterial properties of *Clinacanthus nutans* extracts against *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*: an in-vitro study. J Int Dent Medical Res. 2019;12(02):401–404
16 Łukomska-Szyma'nska M, Zarzycka B, Grzegorczyk J, Półtorak K, Sokołowski J, Łapi'nska B. *Streptococcus mutans and Enterococcus faecalis* as crucial pathogens of oral cavity. Dent Forum 2016; 44:47–52
17 Pinheiro ET, Mayer MPA. *Enterococcus faecalis* in oral infections. J Interdiscipl Med Dent Sci 2015;3:160
18 Chávez de Paz LE, Bergenholtz G, Dahlén G, Svensäter G. Response to alkaline stress by root canal bacteria in biofilms. Int Endod J 2007;40(05):344–355
19 Hollenbeck BL, Rice LB. Intrinsic and acquired resistance mechanisms in enterococcus. Virulence 2012;3(05):421–433
20 Al-Obaidy SSM, Greenway GM, Paunov VN. Enhanced antimicrobial action of chlorhexidine loaded in shellac nanoparticles with cationic surface functionality. Pharmaceutics 2021;13(09):1389
21 Saatchi M, Shokraneh A, Navaei H, Maracy MR, Shojaei H. Antibacterial effect of calcium hydroxide combined with chlorhexidine on Enterococcus faecalis: a systematic review and meta-analysis. J Appl Oral Sci 2014;22(05):356–365
22 Nozari A, Karimkhani A, Motamedifar M, Arasteh P. The antimicrobial effects of zinc oxide-calcium hydroxide mixture fillers: determining the ideal mixture ratio. Iran J Microbiol 2019;11(03):239–245
23 Evans M, Davies JK, Sundqvist G, Figdor D. Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. Int Endod J 2002;35(03):221–228
24 Mahdi Al-F. Antibacterial activity of calcium hydroxide combined with chlorhexidine or sodium hypochlorite against gram positive and gram negative bacteria. J Natural Sciences Research 2014; 4:55–61
25 Govindaraju L, Jenarthanan S, Subramanyam D, Ajitha P. Antibacterial activity of various intracanal medicament against *Enterococcus faecalis, Streptococcus mutans* and *Staphylococcus aureus*: an in vitro study. J Pharm Bioallied Sci 2021;13(Suppl 1):S157–S161
26 Kim HS, Woo Chang S, Baek SH, et al. Antimicrobial effect of alexidine and chlorhexidine against *Enterococcus faecalis* infection. Int J Oral Sci 2013;5(01):26–31
27 Punathil S, Moyin S, Bhat SS, Hedge S, Pai A, James J. Comparison of antibacterial effect of calcium hydroxide combined with chlorhexidine and povidone-iodine against *Enterococcus faecalis* in dentinal tubules of human incisors: an in vitro comparative study. J Pharm Bioallied Sci 2020;12(Suppl 1):S448–S452
28 Radcliffe CE, Potouridou L, Qureshi R, et al. Antimicrobial activity of varying concentrations of sodium hypochlorite on the endodontic microorganisms *Actinomyces israelii, A. naeslundii, Candida albicans* and *Enterococcus faecalis*. Int Endod J 2004;37(07): 438–446
29 Farhad AR, Barekatain B, Allameh M, Narimani T. Evaluation of the antibacterial effect of calcium hydroxide in combination with three different vehicles: an in vitro study. Dent Res J (Isfahan) 2012;9(02):167–172