RESEARCH PAPER

Antimicrobial Efficacy of Nanoparticle-Coated Gutta-Percha against Enterococcus Faecalis Using a Crystal Violet Assay: An In Vitro Study

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ABSTRACT

Background: Persistent endodontic infections are frequently associated with Enterococcus faecalis, a bacterium known for its resistance to conventional disinfection protocols and its ability to survive within the root canal system. Gutta-percha, although widely used as an obturating material, lacks intrinsic antimicrobial properties**.** Nanoparticle-coated gutta-percha represents a promising strategy to impart antimicrobial functionality to the obturation material itself, thereby targeting residual microorganisms that may persist following chemo-mechanical preparation. Owing to their high surface area-to-volume ratio and unique physicochemical properties, nanoparticles can exert potent antibacterial effects through multiple mechanisms, including disruption of bacterial cell membranes, generation of reactive oxygen species, and interference with microbial metabolic pathways.

Aim: To evaluate the antimicrobial efficacy of silver nanoparticle-coated and zinc–clindamycin nanoparticle-coated gutta-percha against E. faecalis using a crystal violet assay**.**

Materials and Methods: Silver nanoparticles were synthesized by the chemical reduction of silver nitrate using sodium citrate, while clindamycin-incorporated zinc oxide nanoparticles were prepared through a precipitation method using zinc nitrate hexahydrate and sodium hydroxide. The synthesized nanoparticles were conjugated onto gutta-percha cones by continuous stirring at room temperature. Enterococcus faecalis (ATCC 51299) was cultured in Brain Heart Infusion broth and standardized prior to testing. Antimicrobial efficacy was evaluated using a crystal violet assay in sterile 96-well microtiter plates. The samples were divided into three groups: Group I—control (E. faecalis only), Group II—silver nanoparticle-coated gutta-percha, and Group III—zinc oxide–clindamycin nanoparticle-coated gutta-percha. Following incubation, crystal violet staining was performed, and the retained dye was quantified by measuring optical density using a microplate reader. The reduction in optical density values relative to the control group was used to assess the antimicrobial efficacy of the nanoparticle-coated gutta-percha samples against E.faecalis .Statistical analysis was performed using one-way ANOVA and independent t-test.

Results: The control group exhibited the highest optical density values (0.6945 ± 0.1345), indicating greater bacterial retention. Both nanoparticle-coated gutta-percha groups demonstrated significantly lower optical density values, reflecting enhanced antimicrobial activity against Enterococcus faecalis. Silver nanoparticle-coated gutta-percha recorded an optical density of 0.3391 ± 0.1460, whereas zinc oxide–clindamycin nanoparticle-coated gutta-percha showed the lowest optical density value (0.2543 ± 0.1278). The differences among the groups were statistically significant ($p = 0.0001$). Cytotoxicity evaluation revealed no statistically significant difference between the experimental groups ($p = 0.23$).

Conclusion: Nanoparticle modification of gutta-percha significantly enhances its antimicrobial activity against E. faecalis, with zinc–clindamycin nanoparticles demonstrating superior efficacy. This approach may improve the long-term success of root canal treatment.

Keywords: *Enterococcus faecalis, gutta-percha, nanoparticles, Root Canal Therapy.*

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INTRODUCTION

Successful root canal therapy relies on effective elimination of microorganisms and prevention of reinfection within the root canal system. However, persistent endodontic infections remain a major clinical concern, largely due to the survival of resistant species such as Enterococcus faecalis, which is frequently isolated from previously treated teeth with post-treatment disease[1,2,3] . This organism demonstrates remarkable adaptability, including tolerance to high pH, ability to invade dentinal tubules, and capacity to persist in nutrient-limited environments[4,5].

Although advances in chemo-mechanical preparation and irrigant activation have improved disinfection, complete eradication of intracanal pathogens is still unpredictable[6]. The obturation phase therefore plays a critical role in entombing residual microorganisms and preventing recolonization. Gutta-percha remains the material of choice due to its favorable physical and handling properties; however, it is biologically inert and lacks intrinsic antimicrobial activity[7,8]. This limitation has prompted the exploration of bioactive modifications to impart antimicrobial functionality.

In recent years, nanotechnology has emerged as a promising approach in endodontics, enabling the development of materials with enhanced antimicrobial performance[9,10]. Silver nanoparticles have gained attention due to their broad-spectrum bactericidal effects mediated by membrane disruption, protein denaturation, and oxidative stress induction[11,12]. Similarly, zinc oxide nanoparticles exhibit antimicrobial activity through reactive oxygen species generation and ion release, which interfere with bacterial metabolism[13,14]. The incorporation of antibiotics such as clindamycin into nanoparticle systems has been shown to further enhance antimicrobial efficacy through synergistic mechanisms targeting bacterial protein synthesis[15].

Given these advancements, modifying gutta-percha with nanoparticle-based systems represents a novel strategy to improve its biological performance. Therefore, the present study aimed to evaluate the antimicrobial efficacy of silver nanoparticle-coated and zinc–clindamycin nanoparticle-coated gutta-percha against E. faecalis using a crystal violet assay.

METHOD

Preparation of Silver Nanoparticles

Silver nanoparticles (AgNPs) were synthesized by the chemical reduction method. Aqueos 0.01 M aqueous silver nitrate solution (Cynor Laboratories, Surat, Gujarat, India) was heated to boiling, followed by the addition of 1% sodium citrate solution (Vishal Laboratories, Rajkot, Gujarat, India) as a reducing agent. The reaction mixture was continuously stirred at 300 rpm for 4 hours using a magnetic stirrer (Qingdao Antech Scientific Co., Ltd., Qingdao, China). Formation of silver nanoparticles was indicated by a colour change of the solution from colourless to dark green. The synthesized nanoparticles were centrifuged at 6000 rpm for 15 minutes, washed with 1% acetone, and air-dried for further use. Characterization of the synthesized AgNPs was performed by visual observation of the colour change and by UV–Visible spectrophotometry. The UV–Visible absorption spectrum was recorded within the wavelength range of 300–700 nm, and the presence of a characteristic surface plasmon resonance peak confirmed the formation of silver nanoparticles

Preparation of Clindamycin Incorporated Zinc Oxide Nanoparticles

Clindamycin-incorporated zinc oxide nanoparticles (ZnO-CLIN NPs) were synthesized using a chemical precipitation method. Zinc nitrate hexahydrate (Research Lab Fine Chem Industries, Mumbai, Maharashtra, India) was dissolved in 50 mL of distilled water under continuous stirring. Subsequently, 1 mL of clindamycin (Pfizer Products India Pvt. Ltd., Mumbai, Maharashtra, India) was added dropwise to the solution. A 0.1 N sodium hydroxide solution was then added dropwise until the pH reached 12, while maintaining constant stirring at room temperature using a magnetic stirrer (Qingdao Antech Scientific Co., Ltd., Qingdao, China). The reaction mixture was stirred continuously for 2 hours to facilitate nanoparticle formation. Upon completion of the reaction, the resulting white precipitate was collected, thoroughly washed with distilled water to remove unreacted residues, and air-dried for further use. Characterization of the synthesized ZnO–Clindamycin nanoparticles was performed by visual observation of the formation of a white precipitate and by UV–Visible spectrophotometry. The UV–Visible absorption spectrum was recorded with the presence of a characteristic absorption peak at approximately 370 nm confirmed the formation of zinc oxide nanoparticles.

Conjugation of Nanoparticles with Gutta Percha

The synthesized nanoparticles were conjugated with gutta-percha cones (Dia-ProT Gutta Percha, size F1; DiaDent Group International Inc., Chungcheongbuk-do, South Korea). The hydroxyl group present on the surface of the nanoparticles form covalent bonding with gutta-percha, polymer of isoprene unit C_5H_8.

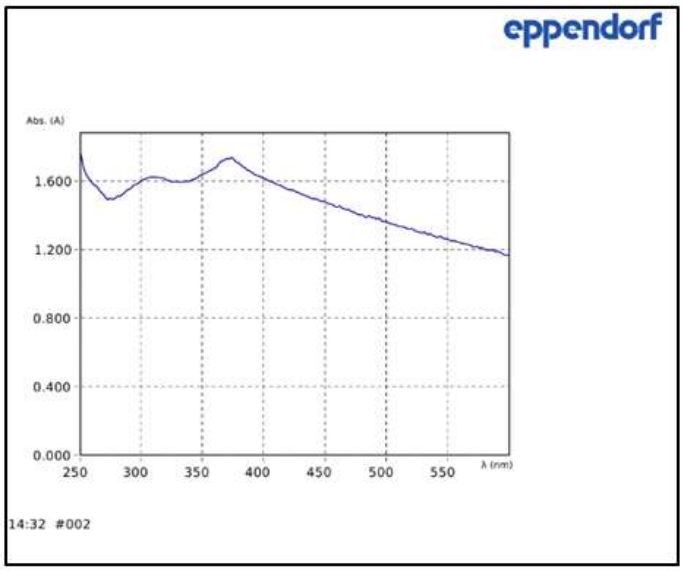


FIG 1- UV Visible spectrum of Zn Nanoparticles. Peak around 370nm indicates the presence of Zn nanoparticles

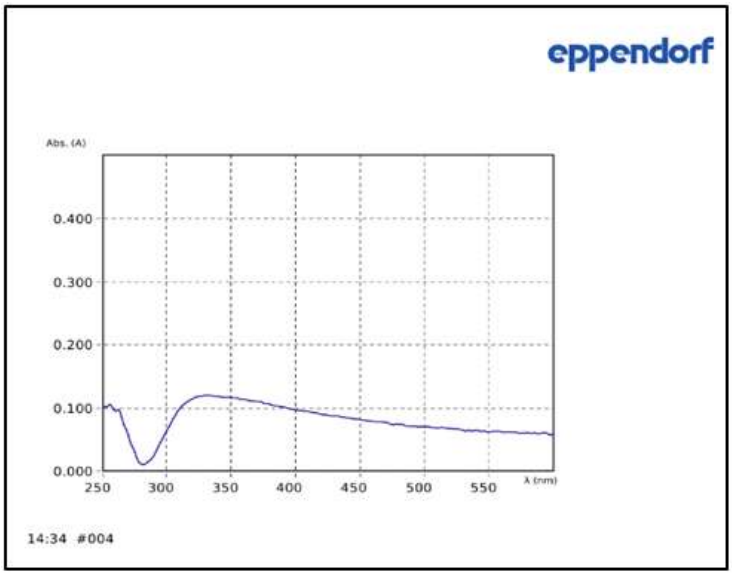


FIG 2- UV Visible spectrum of Ag Nanoparticles. Peak between 300nm to 400 nm indicates the presence of Ag Nanoparticles

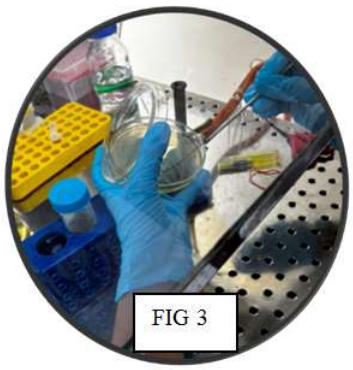
CULTURE PREPARATION

1. Culture Preparation – *E. faecalis* (ATCC 51299)
The (Fig 3) shows handling of a petri dish containing cultured *E. faecalis*. This involves retrieving a bacterial colony from solid media for inoculation.

2. Liquid Culture Development
A single colony of Enterococcus faecalis (ATCC 51299) was aseptically transferred into Brain Heart Infusion (BHI) broth under a laminar airflow cabinet to maintain sterility. The inoculated broth was incubated at 37°C for 24 hours under aerobic conditions in a bacteriological incubator (Kent Scientific Industries, Kochi, Kerala, India) to obtain a fresh bacterial culture. Following incubation, the culture was adjusted to the required bacterial density equivalent to a 0.5 McFarland standard for subsequent antimicrobial testing. The (Fig 4) shows depicts transferring the bacterial colony into Brain Heart Infusion (BHI) broth.

3. Inoculation into 96-Well Plate
A standardized suspension of Enterococcus faecalis was dispensed into sterile 96-well microtiter plates using a micropipette under aseptic conditions. This step ensured uniform bacterial distribution across all wells and provided a standardized platform for evaluating the antimicrobial efficacy of nanoparticle-coated gutta-percha using the crystal violet assay.

The (Fig 5) shows a pipette dispensing the *E. faecalis* suspension into a 96-well microtiter plate.



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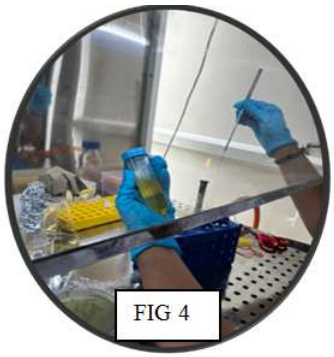

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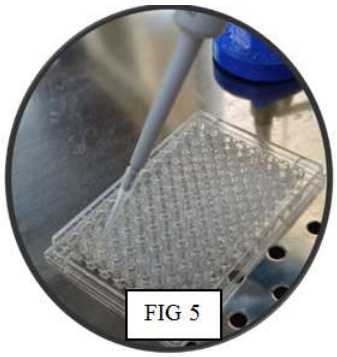


ASSAY

The antimicrobial efficacy of nanoparticle-coated gutta-percha against Enterococcus faecalis was assessed using a crystal violet assay. E. faecalis was cultured overnight in Brain Heart Infusion (BHI) broth and adjusted to a 0.5 McFarland standard. Sterile 96-well microtiter plates were prepared with three experimental groups:

Group I (Control): E. faecalis suspension only

Group II: Silver nanoparticle-coated gutta-percha cones with E. faecalis suspension

Group III: Zinc oxide–clindamycin nanoparticle-coated gutta-percha cones with E. faecalis suspension

Following incubation, the wells were washed with phosphate-buffered saline (PBS) to remove non-adherent bacterial cells. One gutta-percha cone (silver nanoparticle-coated or zinc oxide–clindamycin nanoparticle-coated) was placed in each respective well and incubated at 37°C for an additional 24 hours. After this period, the cones were removed, and the adherent bacterial cells remaining in the wells were stained with 0.1% crystal violet solution for 15 minutes at room temperature. Excess stain was carefully discarded, and the wells were washed three times with distilled water and allowed to air dry. The retained crystal violet was then solubilized by adding 200 µL of absolute ethanol to each well and incubating for 15 minutes. Optical density (OD) was measured at 595 nm using a microplate reader, and the absorbance values obtained were used to quantify the amount of adherent bacterial cells present in each group.

STATISTICAL ANALYSIS

Data was analyzed using the statistical package SPSS 26.0 (SPSS Inc., Chicago, IL) and level of significance was set at $p<0.05$. Descriptive statistics was performed to assess the mean and standard deviation of the respective groups. Inferential statistics to find out the difference between the groups was done using One-way Anova test followed by Bonferroni Posthoc test.

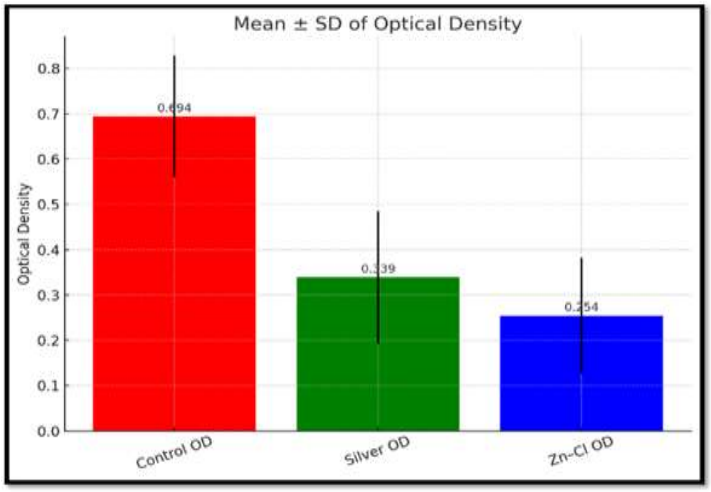
RESULT

The antimicrobial efficacy of nanoparticle-coated gutta-percha was evaluated using the crystal violet assay by measuring optical density (OD), while cytotoxicity was assessed through percentage cell viability. The control group exhibited the highest mean OD value (0.6945 ± 0.1345), indicating the greatest bacterial retention. In contrast, the silver nanoparticle-coated and zinc oxide–clindamycin nanoparticle-coated groups demonstrated significantly lower mean OD values of 0.3391 ± 0.1460 and 0.2543 ± 0.1278, respectively (one-way ANOVA, F = 29.34, p = 0.0001) (Table 1 & Graph 1). Since lower OD values correspond to a reduced number of adherent bacterial cells, the findings indicate enhanced antimicrobial activity of the nanoparticle-coated gutta-percha, with the zinc oxide–clindamycin group exhibiting the greatest antibacterial effect.

Regarding cytotoxicity, the silver nanoparticle-coated group showed a higher mean cell viability (47.99% ± 20.70) than the zinc oxide–clindamycin-coated group (37.07% ± 18.37); however, the difference was not statistically significant (independent t-test, t = 1.24, p = 0.23) (Table 2 & Graph 2). These results suggest that both nanoparticle-coated gutta-percha formulations possess significant antimicrobial activity against Enterococcus faecalis while demonstrating comparable cytotoxicity profiles.

Table 1 - Descriptive Statistics and comparison of Optical density

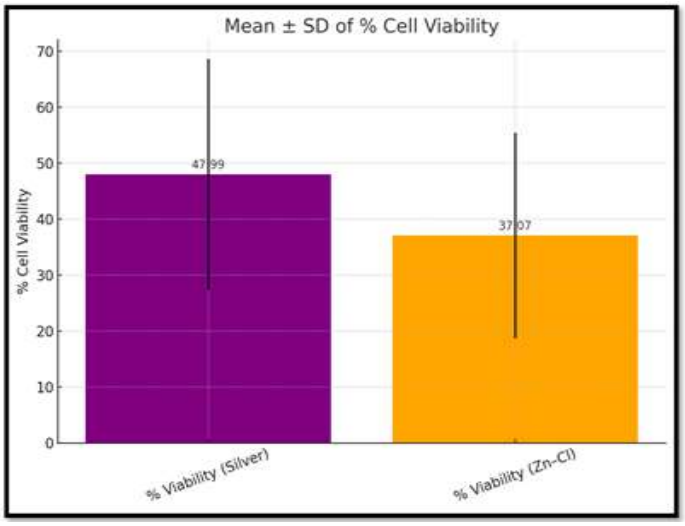
Variable	Mean	SD	Minimum	Maximum
Control OD	0.6945	0.1345	0.4976	0.8650
Silver OD	0.3391	0.1460	0.0753	0.4979
Zn–Cl OD	0.2543	0.1278	0.0774	0.3994
F value		29.34		
P value		0.0001*		



Graph 1: Comparison of Mean Optical Density Values of Enterococcus faecalis Following Exposure to Nanoparticle-Coated Gutta-Percha

Table 2 - Comparison of percentage Viability

Variable	Mean	SD	Minimum	Maximum	T value	P value
% Viability (Silver)	47.99%	20.70	15.10	75.80	1.24	0.23
% Viability (Zn–Cl)	37.07%	18.37	10.70	62.60		



Graph-2: Comparison of Mean Percentage Cell Viability Between Experimental Groups

DISCUSSION

Successful endodontic treatment depends on the effective elimination of microorganisms from the root canal system and prevention of reinfection following obturation. Among the microorganisms implicated in persistent endodontic infections, Enterococcus faecalis has received considerable attention because of its ability to survive under adverse environmental conditions, penetrate dentinal tubules, form organized microbial communities, and resist commonly used intracanal medicaments. Stuart et al , Rocas ,Nair, Love and Evans et al reported that E. faecalis is frequently isolated from root canal-treated teeth associated with post-treatment apical periodontitis[1,2,3,4,5]. Its remarkable ability to withstand nutritional deprivation and antimicrobial challenges makes it an ideal microorganism for evaluating novel antimicrobial strategies in endodontics. Therefore, E. faecalis (ATCC 51299) was selected as the test organism in the present study. Although gutta-percha remains the most widely accepted obturation material due to its favorable physical and biological properties, it does not possess inherent antimicrobial activity[7,8]. Consequently, residual

microorganisms that survive chemomechanical preparation may persist within the root canal system and contribute to treatment failure. To address this limitation, nanoparticle-based modifications have been explored to enhance the antibacterial properties of endodontic materials without compromising their clinical performance[9,10].

In the present study, silver nanoparticle-coated gutta-percha and zinc oxide–clindamycin nanoparticle-coated gutta-percha were evaluated against E. faecalis using a crystal violet assay. The crystal violet assay was selected because it is a simple, reproducible, and widely accepted method for quantifying bacterial adherence through optical density measurements. Crystal violet binds to bacterial cells retained within the wells, and the resulting optical density is directly proportional to the quantity of adherent bacteria. Therefore, lower optical density values indicate fewer retained bacterial cells and consequently greater antimicrobial efficacy.

The results demonstrated a statistically significant difference among the groups (One-way ANOVA; $F = 29.34$, $p = 0.0001$). The control group exhibited the highest mean optical density value (0.6945 ± 0.1345), indicating maximum bacterial retention. In contrast, the silver nanoparticle-coated gutta-percha group showed a significantly lower mean optical density value (0.3391 ± 0.1460), while the zinc oxide–clindamycin nanoparticle-coated gutta-percha group demonstrated the lowest optical density value (0.2543 ± 0.1278). These findings indicate that both nanoparticle-coated formulations effectively inhibited E. faecalis colonization when compared with the control. Furthermore, the lower optical density observed in the zinc oxide–clindamycin group suggests superior antimicrobial efficacy relative to the silver nanoparticle-coated group.

The antibacterial activity observed in the silver nanoparticle group may be attributed to the unique physicochemical properties of silver nanoparticles. Morones et al demonstrated that silver nanoparticles interact with bacterial cell membranes, increase membrane permeability, and interfere with intracellular metabolic processes[11]. Franci et al further reported that silver nanoparticles induce oxidative stress through the generation of reactive oxygen species, leading to bacterial cell damage and death[12]. The significant reduction in optical density observed in the silver nanoparticle group in the present study is consistent with these established mechanisms and supports the potential role of silver nanoparticles as antimicrobial modifiers of obturation materials.

Despite the significant antibacterial activity exhibited by silver nanoparticles, the zinc oxide–clindamycin nanoparticle-coated gutta-percha demonstrated superior antimicrobial performance. This enhanced effect can be attributed to the synergistic action of zinc oxide nanoparticles and clindamycin. Zinc oxide nanoparticles possess intrinsic antibacterial activity through the release of zinc ions and generation of reactive oxygen species, which disrupt bacterial membranes and metabolic pathways[13,14]. In addition, clindamycin inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit, thereby preventing bacterial growth and proliferation[15]. The combined action of these mechanisms likely contributed to the markedly lower optical density values observed in the zinc oxide–clindamycin group.

The findings of the present study are in agreement with those reported by Shrestha and Kishen, who highlighted the ability of nanoparticle-modified endodontic materials to enhance antimicrobial activity against resistant endodontic pathogens. Similarly, Panwar et al demonstrated that nanocurcumin-coated gutta-percha exhibited significantly greater antibacterial activity against E. faecalis than conventional gutta-percha[16]. While both studies support the concept of nanoparticle-enhanced antimicrobial activity, the present study demonstrated a greater reduction in bacterial retention through the incorporation of an antibiotic-loaded nanoparticle system. Unlike nanocurcumin, which relies primarily on its intrinsic antimicrobial activity, the zinc oxide–clindamycin system combines nanoparticle-mediated bacterial disruption with antibiotic-mediated inhibition of protein synthesis, thereby providing a dual antibacterial mechanism. Likewise, Rai et al highlighted the broad-spectrum antimicrobial activity of metallic nanoparticles and suggested that nanoparticle-based systems provide effective alternatives for combating resistant microorganisms[17]. The crystal violet assay employed in the present study is a simple, reproducible, and widely accepted method for quantifying bacterial adherence by optical density measurements, as described by O'Toole[19].

A comparable investigation conducted by Kumar et al evaluated the antibiofilm efficacy of clindamycin-loaded gold nanoparticle-coated gutta-percha against E. faecalis using a crystal violet assay. The authors reported a significant reduction in bacterial biomass compared with conventional gutta-percha, emphasizing the benefits of combining nanoparticles with antimicrobial agents. The findings of the present study corroborate those observations[20,21]. However, while Kumar et al. employed gold nanoparticles primarily as a drug-delivery vehicle, the current study utilized zinc oxide nanoparticles, which themselves possess inherent antimicrobial properties. This additional antibacterial mechanism may explain the superior reduction in optical density observed in the zinc oxide–clindamycin group.

The results of the present investigation also support previous reports evaluating zinc oxide nanoparticles in endodontic applications. Zhang and Chen as well as Raghupathi et al demonstrated significant antibacterial effects of zinc oxide nanoparticles against various bacterial species through oxidative stress-mediated mechanisms[13,14]. The superior antimicrobial efficacy observed in the zinc oxide–clindamycin group in the present study is consistent with these findings and further suggests that incorporation of clindamycin may enhance the antibacterial performance of zinc oxide nanoparticles.

Similarly, Subramanian et al reported that silver nanoparticle-coated gutta-percha exhibited effective antimicrobial activity through oxidative stress-mediated bacterial killing, corroborating the findings obtained for the silver nanoparticle-coated group in the present investigation[23].

Cytotoxicity evaluation revealed mean cell viability values of 47.99 ± 20.70% for the silver nanoparticle-coated group and 37.07 ± 18.37% for the zinc oxide–clindamycin group. Although the silver nanoparticle group exhibited numerically greater cell viability, the difference was not statistically significant (Independent t-test; $t = 1.24$, $p = 0.23$). These findings indicate that the enhanced antimicrobial efficacy achieved by the zinc oxide–clindamycin formulation was not accompanied by a statistically significant increase in cytotoxicity. This observation is clinically relevant because an ideal endodontic material should exhibit potent antibacterial activity while maintaining acceptable biocompatibility.

Within the limitations of this in vitro study, nanoparticle modification significantly improved the antimicrobial efficacy of gutta-percha against E. faecalis. Both silver nanoparticle-coated and zinc oxide–clindamycin nanoparticle-coated gutta-percha demonstrated superior antibacterial activity compared with unmodified controls. However, the zinc oxide–clindamycin formulation exhibited the greatest reduction in bacterial retention, suggesting that combining the intrinsic antimicrobial activity of zinc oxide nanoparticles with the antibacterial action of clindamycin may represent a promising strategy for enhancing the antimicrobial performance of obturation materials.

CONCLUSION

Nanoparticle-coated gutta-percha demonstrated significantly greater antimicrobial efficacy against Enterococcus faecalis than uncoated gutta-percha. Among the tested formulations, zinc oxide–clindamycin nanoparticle-coated gutta-percha exhibited the highest antimicrobial activity, while both nanoparticle coatings showed comparable cytocompatibility. These findings suggest that nanoparticle functionalization, particularly with zinc oxide–clindamycin, is a promising approach for enhancing the antimicrobial properties of obturation materials and may improve the long-term success of root canal treatment.

LIMITATIONS

This study was conducted under in vitro conditions using a single bacterial species (Enterococcus faecalis), which may not fully represent the complex polymicrobial environment of infected root canal systems. The antimicrobial efficacy was evaluated over a short duration using a crystal violet assay, and therefore the long-term antibacterial performance and durability of the nanoparticle coating were not assessed. Further studies involving multispecies microbial models, simulated clinical conditions, and in vivo evaluation are required to validate the clinical applicability of the nanoparticle-coated gutta-percha.

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