Antibacterial property of chitosan against *E. faecalis* standard strain and clinical isolates

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To investigate applications of chitosan as antibacterial agent for endodontic treatments, we tested its activity against *Enterococcus faecalis* standard strain (ATCC29212) and clinical isolates. We determined the minimum bactericidal concentration (MBC) of 6 types of chitosan against ATCC29212; the most effective types were selected for further tests. Four clinical isolates were cultured from endodontically treated-teeth and identified by biochemical assays and polymerase chain reactions. Bacterial cultures were exposed to 1,700 and 2,100 kDa chitosan at MBC for 1, 3, 5, 10, and 60 min in time-kill assays and plated on brain-heart-infusion (BHI) agar for colony counts. Both types of chitosan showed significantly lower numbers of remaining bacteria (log colony forming units per millimeter, logCFUs/mL) than negative controls (0.1% acetic acid and BHI) at 10 min, and completely eliminated the bacteria at 60 min for all strains. Thus, chitosan could be developed as alternative biocompatible antimicrobial irrigant/medication for endodontic treatments.

**Keywords**: Antibacterial, Chitosan, Clinical isolates, *Enterococcus faecalis*, Time-kill assay

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**INTRODUCTION**

Bacteria and their by-products are main causes of pulp and periapical diseases\(^5\). *Enterococcus faecalis* is one of the most commonly isolated species from endodontically treated teeth\(^4,5\). *E. faecalis* has ability to adapt itself to survive in harsh environments, invade into dentinal tubules and form biofilms\(^6\). It can be found as pure culture or single organism in root-filled teeth with persistent lesions\(^5\). Moreover, *E. faecalis* is resistant to calcium hydroxide due to its ability to live in extreme alkaline pH\(^8,9\). These capacities make *E. faecalis* a challenge in endodontic treatment.

Stuart et al.\(^10\) recommended steps to eliminate *E. faecalis* by using good aseptic technique, increasing apical sizes and using 6% sodium hypochlorite (NaOCl) with combination of 17% ethylenediaminetetraacetic acid (EDTA) and 2% chlorhexidine. NaOCl is effective against *E. faecalis* especially in the higher concentrations and contact time. However, great care is needed when using NaOCl in high concentrations as it can cause many adverse effects, such as damage to soft tissues, oral mucosa or eyes of the patients or operators, extrusion of NaOCl through apical foramen, or bleeding of clothes\(^11,12\). High concentrations of NaOCl can also affect elastic modulus of dentin and may reduce its fracture resistance\(^13\). Therefore, there is a clear need for safer and more biocompatible endodontic irrigants that are effective against *E. faecalis*.

Chitosan has gained interests in medical and pharmaceutical applications as it has many favorable properties, including biocompatibility, nontoxicity, biodegradability, and antimicrobial activity. It is a polysaccharide, consisting of glucosamine and N-acetyl glucosamine, which is derived from deacetylation of chitin found in shells of crustaceans\(^14\). It is naturally abundant and has low production cost\(^15\). Chitosan is used in many medical applications, such as wound dressings, cholesterol lowering agent, hemostatic agent and drug delivery systems\(^16-18\). Chitosan has been approved by the United States Food and Drug Administration (USFDA) as wound dressings, due to its good control of hemorrhage and biocompatibility\(^19\).

Importantly, chitosan has antimicrobial activity against Gram positive and negative bacteria, as well as fungi\(^20,21\). The mechanism of action is proposed to be through the interactions of chitosan’s polycations with the negatively charged surface of microorganisms. These interactions alter the cell permeability and cause leakage of cellular components\(^18\). Chitosan is available in various forms with different molecular weights and different properties. Chitosan has been shown to be effective against several oral microorganisms, such as *Candida albicans*, *Streptococcus mutans*, and *E. faecalis*\(^22,20\). Interestingly, chitosan nanoparticles was shown to be effective in removing smear layer when used as an irrigating solution after the used of NaOCl or NaOCl and EDTA in bovine dentin block models\(^25\).

Due to its antimicrobial and biocompatible properties, chitosan could be a good alternative...
antimicrobial irrigant/medication in endodontic treatments. However, only limited information exists regarding the antibacterial activity of chitosan against *E. faecalis*, especially among clinical isolates from root-filled teeth. Therefore, the aim of this study is to investigate the antibacterial efficacy of chitosan against *E. faecalis* standard strain and clinical isolates.

**MATERIALS AND METHODS**

**Preparation of chitosan solutions**

We prepared stock solutions of 6 types of chitosan as described in Table 1. The chitosan powder was dissolved in 1% acetic acid (Merck, Darmstadt, Germany) to the highest soluble concentration of each type of chitosan. The solutions were sterilized by autoclave and used within the same day.

**Preparation of bacterial culture**

Stock culture of *E. faecalis* American Type Culture Collection (ATCC 29212) was grown in brain heart infusion broth (BHI; HiMedia Laboratories, Mumbai, India) and incubated in aerobic condition in a 37°C incubator. Log-phase culture of *E. faecalis* was prepared and adjusted to approximately 7×10^8 colony forming units per millilitre (CFUs/mL) by adjusting the optical density at 600 nm (OD600) to 0.5.

**Determination of minimum bactericidal concentration (MBC) of chitosan**

Chitosan solutions were prepared by mixing 5 mL of 2×BHI broth with 1 mL of chitosan stock solution and 4 mL of sterile distilled water to obtain 10 mL of chitosan solution in BHI with 0.1% acetic acid. Thus, the final concentration of each type of chitosan was 1:10 dilution of the stock solution. Since the solubility of the different types of chitosan varies, the concentrations of the solutions used for MBC determinations were different depending on the maximum soluble concentrations of the chitosan as shown in Table 1. Then, 2-fold serial dilutions of the chitosan solutions were prepared for the determination of MBC. Approximately 5×10^6 CFUs/mL of bacterial suspension was mixed with the 2-fold serial dilutions of chitosan. After 1 day of incubation in aerobic condition at 37°C, 10 µL of the solution was spread onto BHI agar (HiMedia Laboratories) plates by using glass beads. The agar plates were incubated at 37°C for 1 day and bacterial growth was observed. The lowest concentration of chitosan that could kill all of *E. faecalis* cells was determined as the MBC. The experiment was repeated 3 times. In this experiment, 2.5% NaOCl (2.5% Chlorinated soda solution, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand) was used as a positive control, and 0.1% acetic acid, and BHI alone were used as negative controls.

**Time-kill assay**

To determine the time required for chitosan to kill *E. faecalis*, a time-kill assay was performed where the bacteria were exposed to chitosan solutions for various time periods and plated to observe the number of remaining viable cells. Log-phase culture of *E. faecalis* was prepared and approximately 10^6 CFUs/mL were exposed to 1,700 and 2,100 kDa chitosan solution at MBC in 100 µL reactions. After 1, 3, 5, 10 and 60 min, 1 mL of BHI was added to stop the reaction, and the solution was removed after centrifugation at 13,000 rpm for 1 min. Then, the bacteria was resuspended in 1 mL of BHI broth, further diluted to 10- and 100-fold, and 100 µL of the dilutions was plated on BHI agar. The plates were incubated at 37°C for 1 day and colony-forming units on each plate were recorded. The experiment was repeated 3 times. Mean logCFUs/mL of remaining viable cells was used to compare the antibacterial activity. In this experiment, 2.5% NaOCl was used as a positive control, and 0.1% acetic acid, and BHI alone were used as negative controls.

**Clinical sample collection**

Clinical samples were collected from root-filled teeth.
of 15 adult patients who attended the Department of endodontics, Faculty of dentistry, Chulalongkorn University for non-surgical endodontic retreatment. The study protocol was approved by the ethics committee of the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand (ref. 061/2017). All participants gave informed consent before sample collection. Inclusion criteria were previously root-filled teeth with failed treatment as determined on the basis of clinical and radiographic examinations, including presence of apical radiolucent lesion, and persistent symptoms, such as pain on palpation, or sinus tract openings. Exclusion criteria were teeth with periodontitis or have probing depth>5 mm, and cases with extreme loss of tooth structure that precluded rubber dam isolation.

The sampling procedures was modified from Möller. Aseptic techniques were used throughout the endodontic procedures. For each case, the tooth was isolated by rubber dam application and disinfected with 30% hydrogen peroxide followed by 10% tincture iodine. Access cavity was prepared using sterile burs with manual irrigation using sterile normal saline solution. Root canal fillings were removed by using gate- glazed burs and H-files (Dentsply Maillefer, Ballaigues, Switzerland) without any chemical solvent. Working length was determined using radiographs and an apex locator (Dentaport Root ZX, J Morita, Irvine, CA, USA). Then, the root canal was filed with K-file files (Dentsply Maillefer) at working length to file size 25. A sterile paper point was introduced to the working length and left for 60 s. The samples were transferred into BHI broth and sent for microbiological analysis.

Isolation and identification of E. faecalis
The samples were vortexed and the bacterial suspension was plated on Mitis Salivarius Agar (MSA; Difco Mitis Salivarius Agar, Becton, Dickinson and Difco, Chicago, IL, USA) and incubated at 37°C for 1 day. Dark blue colonies with smooth surfaces on MSA (Fig. 1A) were selected for Gram staining and isolated for biochemical tests. Gram’s staining was performed using crystal violet (20 s), Gram’s iodine (1 min), ethanol (10 s), and safranin (1 min) (reagents from AppliChem, Darmstadt, Germany, and SDFCL, Mumbai, India). The isolated pure cultures were characterized by testing for sorbitol fermentation and Streptococcus faecalis (SF) broth (reagents from HiMedia Laboratories). Isolates with positive tests were confirmed to be E. faecalis by polymerase chain reaction (PCR)

Identification of the Enterococcus genus was performed using amplification of 16s rRNA gene sequences. Primers E1 (5'-TCA ACC GGG GAG GGT-3') and E2 (5'-ATT ACT AGC GAT TCC GG-3'), which bind to positions 632–646 and 1353–1369 of the 16s rRNA gene, respectively, produced a PCR amplicon of 738 bp. For E. faecalis, the species-specific primers EFLF (5'-GTT TAT GCC GCA TGG CAT-3' GenBank accession no. Y18293) and EFLR (5'-CGG TCA GGG GAC GTT CAG-3' GenBank accession no. Y18293), which located at positions 165–187 and 457–474 of the E. faecalis 16S rDNA, respectively, were used to produce a PCR amplicon of 310 bp. The PCR reaction mixture contained 1 µL of each specific primer, 2.5 µL of 10x PCR buffer, 2 µL of 25 mM of MgCl₂, 0.25 µL of Taq DNA polymerase, and 0.2 mM of each deoxyribonucleoside triphosphates (reagents from Thermo Fisher Scientific, WA, Massachusetts, USA). PCR amplification was performed in a DNA thermocycler (T100™ Thermal Cycler, Bio-Rad Laboratories, Foster city, CA, USA). Cycling parameters for PCR reactions using E. faecalis-specific primers consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, and a final step of 72°C for 10 min. Gel electrophoresis was used to separate PCR amplicons along with ExcelBand 100 bp DNA ladder (Smobio, Hsinchu City, Taiwan, Republic of China) using 1% agarose gel and visualized on an UV transilluminator after staining with ethidium bromide. Clinical isolates with positive PCR results were stored at −80°C and prepared and tested in the time-kill assay as described for the standard strain.

Statistical analysis
The antibacterial activity of chitosan at various time points was measured as log colony forming units per milliliter (logCFUs/mL) of remaining E. faecalis.
Two-way ANOVA was used to analyze the effects of two variables (agent and time) on the logCFUs/mL of remaining *E. faecalis*. Kruskal-Wallis *H* test and Mann-Whitney *U* test were used to compare the antibacterial activity of each type of chitosan in different bacterial strains and at 5 incubation times (1, 3, 5, 10 and 60 min). Data were analyzed using IBM SPSS Statistics for Windows, Version 22.0 (IBM, Armonk, New York, USA). A *p*-value of <0.05 was considered statistically significant.

**RESULTS**

**Antibacterial activity of chitosan against *E. faecalis* standard strain**

We first examined the antibacterial activity of 6 types of chitosan with different molecular weights by determining the MBC of chitosan against an *E. faecalis* standard strain (ATCC 29212). As shown in Table 2, the 1,700 and 2,100 kDa chitosan have the lowest MBC at 2 mg/mL, suggesting that they have the highest antibacterial activity. Therefore, these were selected for further studies.

Since our goal is to use chitosan in endodontic treatments, especially as an antimicrobial irrigant, we needed to determine the time required for chitosan to kill *E. faecalis*. A time-kill assay was performed by exposing *E. faecalis* to chitosan at MBC for various amounts of time (1, 3, 5, 10, and 60 min). The numbers of colony forming units (CFUs) of remaining viable *E. faecalis* cells at each time points were counted and compared to the negative controls (0.1% acetic acid and BHI media alone). The results are shown in Fig. 2A. Mean logCFUs/mL of *E. faecalis* cells was significantly lower than the negative controls at 5 and 10 min after exposure to 1,700 kDa (*p*=0.046 and 0.037, respectively) and 2,100 kDa chitosan (*p*=0.05 and 0.037, respectively). Importantly, both types of chitosan at MBC could reduce the number of *E. faecalis* by 3 orders of magnitude (99.9%) in 10 min and could eliminate all *E. faecalis* at 60 min of exposure. The results suggest that chitosan may be a useful antimicrobial agent against *E. faecalis*, but it requires a contact time of longer than 10 min. To further test if chitosan would also be effective against clinical strains of *E. faecalis*, we next isolated *E. faecalis* from patients that require endodontic retreatment.

**Isolation of *E. faecalis* from root-filled teeth requiring retreatment**

From 15 patients with root-filled teeth requiring endodontic retreatment, we identified 5 samples that carried Gram-positive cocci that grew as dark blue colonies with smooth surface on MSA media (Fig. 1A). These colonies were isolated and tested for fermentation of sorbitol and in SF broth. Isolates with positive results were further identified by PCR assay using species-specific primers. Among the 5 clinical isolates, 4 isolates were confirmed as *E. faecalis* by PCR (named clinical isolates A, B, C, and D, Fig. 1B). The remaining isolate (E), which was negative to *E. faecalis* PCR assay, was tested with *Enterococcus* genus PCR primers and gave negative results; this isolate was excluded from further study. Thus, we obtained 4 clinical isolates of *E. faecalis* from root canals (A–D) for further testing with chitosan.

**Antibacterial activity of chitosan at different contact times against *E. faecalis* clinical isolates**

Figures 2 B–E show the results of the time-kill assays of 1,700 and 2,100 kDa chitosan at MBC against clinical isolates A–D, respectively. In comparison to the negative controls (0.1% acetic acid and BHI alone), chitosan significantly reduced the mean logCFUs/mL of remaining *E. faecalis* at 10 min (*p*=0.037) and completely eliminated the bacteria after 60 min in all isolates. Interestingly, isolates A and D were completely killed by both types of chitosan at 10 min, while isolate B were eliminated by 2,100 kDa chitosan and isolate C by 1,700 kDa chitosan at 10 min.

When the results of the time-kill assay of standard strain and all clinical isolates were combined (Fig. 2F), analysis by two-way ANOVA showed that there is a statistically significant interaction between the effects of agents and time of exposure on the number of remaining *E. faecalis* (*p*<0.001). This suggests that chitosan has increasing antibacterial activity over time on *E. faecalis*.

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### Table 2 Minimum bactericidal concentration (MBC) of chitosan against *E. faecalis* ATCC29212

| Chitosan type      | Mean (SD) MBC (mg/mL) |
|--------------------|-----------------------|
| 30 kDa             | 2.9 (1.0)             |
| 890 kDa            | 3 (0)                 |
| 1,700 kDa          | 2 (0)                 |
| 2,100 kDa          | 2 (0)                 |
| Oligomer (7–9 kDa) | 2.5 (0.9)             |
| Polymer (900–100 kDa) | 3 (0)              |
In this study, we showed that 1,700 and 2,100 kDa chitosan has antibacterial activity against *E. faecalis* standard strain and clinical isolates. At MBC (2 mg/mL), both types of chitosan could eliminate 10⁶ cells/mL of *E. faecalis* in 60 min and could significantly reduce the number of *E. faecalis* after 10 min of contact time. This suggests that both types of chitosan are good candidates to be developed into a useful antibacterial agent in endodontic treatments.

*E. faecalis* is the most prevalent species of microorganisms found in root canal-treated teeth, especially in poorly filled canals.⁴,⁷ It can invade into dentinal tubules and adheres to dentin collagen in the presence of human serum, which helps it to survive in root-filled teeth.⁵ Moreover, *E. faecalis* can live in extreme alkaline environment due to its proton pump activity, which makes it resistant to calcium hydroxide medication. Distel *et al.* showed that *E. faecalis* can form biofilm in medicated root canals in vivo. Thus, elimination of *E. faecalis* in the root canal is an important step towards the success of endodontic treatment. As currently available antimicrobial irrigants...
have adverse effects on host tissues, this study aimed to test for antimicrobial property of chitosan, a highly biocompatible natural product, on *E. faecalis* in order to improve treatment protocols in retreatment cases.

From our results, we found that chitosan with molecular weights of 1,700 and of 2,100 kDa have the lowest MBC against *E. faecalis*. The antimicrobial mechanism of chitosan has been proposed to mediate by the interactions of the positively charged chitosan molecules with the negatively charged bacterial cell membranes\(^{30}\). The antimicrobial activity of various chitosan derivatives is influenced by their degree of deacetylation, molecular weight, source of chitin, and other modifications\(^{31-33}\). Previous studies suggest that chitosan with higher degree of deacetylation and lower molecular weight tend to have higher antimicrobial activity\(^{31}\). Higher degree of deacetylation of chitosan enhances the release of free amino groups which react to bacterial membrane and increase antibacterial efficacy\(^{32}\).

The degree of deacetylation of all chitosan used in our studies was at least 90%; this may be a contributing factor to its antimicrobial activity. In addition, it has been suggested that low molecular weight chitosan may bind more effectively to bacterial membrane, due to its mobility, attraction and ionic interactions between positively charged chitosan and the negatively charged bacterial cell membranes\(^{31}\). Nevertheless, No et al.\(^{20}\) found that, although the antibacterial efficacy of chitosan oligomer against Gram negative bacteria increased with decreasing molecular weight, Gram positive bacteria was inhibited by chitosan with various molecular weights. Thus, *E. faecalis*, a Gram positive bacteria, may be inhibited by various molecular weight of chitosan. In our study, the 1,700 and 2,100 kDa chitosan were the most effective against *E. faecalis*.

Initially, we tested the activity of chitosan against a standard strain of *E. faecalis*. However, many studies found that clinical isolates may have different antibiotics susceptibility\(^{33,34}\). Because bacteria in root-filled teeth experience limited nutrients and survive in harsh environment, they usually develop stress responses that may affect antibiotic resistance. Thus, to ensure that chitosan would be effective in clinical settings, we also tested the activity of selected chitosan on clinical isolates. We collected *E. faecalis* from root canal-treated teeth because previous study suggested that *E. faecalis* is more commonly found in root canal retreatment cases than in primary treatment cases\(^{36}\). We identified *E. faecalis* by both biochemical and species-specific PCR\(^{35}\). The use of culture-based methods combined with molecular techniques gave a more reliable and accurate result for the identification of *E. faecalis* from clinical samples. In our study, from 15 patients, only 4 clinical isolates (26.7%) of *E. faecalis* were obtained and tested with chitosan. This is a limitation of this study and more clinical isolates should be tested to ensure the efficacy of chitosan before clinical use.

Time of exposure required for antimicrobial activity is an important factor determining the types of clinical applications chitosan could be used. We performed time-kill assays to evaluate the activity of chitosan against *E. faecalis* at 1, 3, 5, 10 and 60 min. To be practical for clinical use as an endodontic irrigant, the contact time required should not exceed 10 min. The commonly used irrigant, NaOCl, also requires a contact time of 10 min to kill *E. faecalis* (ATCC 29212)\(^{39}\). Indeed, chitosan can reduce the number of viable bacteria by 99.9% in standard strain and completely eliminate certain clinical isolates (A and D) after 10 min of exposure. However, some clinical isolates (B and C) requires a longer exposure time. Nevertheless, all tested strains were completely killed after 60 min of exposure. This demonstrates the different sensitivity among different bacterial strains and emphasizes the needs to test the activity of antimicrobial agents not only against standard strain, but also clinical isolates. Our results suggest that 1,700 and 2,100 kDa chitosan at MBC may be used as an irrigant to reduce the number of *E. faecalis* in root canals, but it may not be able to completely eliminate the bacteria in all cases at 10 min. The contact time required may be reduced if a higher concentration of chitosan is used. This warrants further investigations. Additionally, these types of chitosan may be more effective in other applications with longer contact time, such as in medication or sealer.

Previous studies on applications of chitosan in endodontic treatments were varied in chitosan concentrations, chitosan types and methods of study. Suzuki\(^{37}\) reported that antibacterial effect against *E. faecalis* of a chitosan-citrate solution was obtained at 5 min. They used chitosan oligomer in 10% citrate buffer solution and observed 87.9, 87.8, 99.7 and 99.8% reduction of bacterial viability at 5, 15, 30 and 60 min, respectively. In comparison to our results, the chitosan we tested showed higher antibacterial activity by reducing 99.9% (3 orders of magnitude) of *E. faecalis* in 10 min. In another study, chitosan nanoparticles required 8 h of contact time to completely kill *E. faecalis* (10^6 cells/mL)\(^{38}\), even though chitosan nanoparticles are proposed to have higher surface area and charge density that allow more interaction with negatively charged surface of bacterial cell membranes\(^{39}\). Interestingly, this study also reported that, when dentin was subsequently treated with nanoparticles after using NaOCl, EDTA or chlorhexidine as irrigants, *E. faecalis* adherence to dentin was significantly reduced\(^{38}\). Another study showed that chitosan nanoparticles can eliminate planktonic *E. faecalis* cells (ATCC 29212 and OG1RF) in 8–12 h of application\(^{40}\). However, the antibacterial activity was reduced in 7-days biofilm as some bacteria in biofilm still survived after 72 h of treatment, especially in deep biofilm layer\(^{40}\). Therefore, biofilm model should be used to test efficacy of chitosan in future studies. In addition, a previous study showed that 5-min applications of 0.1, 0.2 and 0.37% chitosan in 1% acetic acid resulted in deterioration of dentin surface and intratubular dentin\(^{41}\). The author recommended the application of 0.2% chitosan for 3 min, which efficiently removed smear layer with no destruction of peritubular dentin. Because the chitosan solutions tested in our study were in 0.1%
acetic acid, we do not expect the dentin structure to be affected, but this should be evaluated in future studies. Since chitosan may require a long contact time to completely eliminate *E. faecalis*, applications of chitosan in endodontic medication and sealers are of interest. Elsaka and Elnaghy^43^ found that calcium hydroxide combined with chitosan solutions showed better antimicrobial efficacy against *E. faecalis* infected root canal dentin than calcium hydroxide mixed with saline. Furthermore, it did not significantly affect the bond strength of RealSeal sealer to dentin. The addition of chitosan to RealSeal self-etching primer increased antibacterial activity against *E. faecalis* compared with the original primer without significant effects on push-out bond strength^44^.

Moreover, the authors reported that the antibacterial properties of 7-day aged modified self-etching primer was not significantly different from freshly prepared one^45^. A study by Shrestha *et al*. showed that bacterial inhibition of chitosan nanoparticles aged in saliva to simulate coronal leakage into root canal system was observed at 30 days, but was significantly reduced at 90 days^46^.

These studies suggest that chitosan could sustain its antibacterial activity for at least 7–30 days, which can be beneficial as medication or sealer in endodontic treatment. Our study showed antibacterial property of chitosan against *E. faecalis* standard strain and clinical isolates. However, since bacteria may exist in the root canals as multi-species biofilm, biofilm model would be needed to confirm antibacterial property of chitosan in future studies. In addition, further development of chitosan for endodontic applications will also require investigations of the effect of chitosan on physical properties and interaction with other substances used in root canal treatments.

**CONCLUSION**

Chitosan with molecular weight of 1,700 and 2,100 kDa are effective against *E. faecalis* standard strain and clinical isolates. It can significantly reduce the number of bacteria after 10 min of contact time and completely eliminated the bacteria in 60 min. Thus, chitosan is a promising candidate for further development into alternative biocompatible antimicrobial agent for endodontic treatments.

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