J Dent Mater Tech 2025; 14 (2): 64-71
https:// 10.22038/jdmt.2025.84628.1759

J Dent Mater Tech

Original Article Open Access

Antimicrobial properties of novel dental bioceramic sealers and conventional sealers against *Enterococcus faecalis*

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Abstract

Objective: This study aimed to evaluate and compare the antimicrobial properties of two newly formulated tricalcium silicate-based (TCS) bioceramic sealers with those of two commercially available sealers against *Enterococcus faecalis*.

Methods: The antibacterial properties of four sealers were tested: TCS sealer, TCS-Amo (containing amoxicillin), Pulpdent and Endoseal MTA. Antibacterial activity was assessed using the agar diffusion test (ADT) and direct contact test (DCT) at four different time intervals: immediately after setting (T0), 24 hours (T1), 72 hours (T3) and 168 hours (T7). In the ADT, antibacterial activity was determined by measuring the diameter (mm) of the zone of inhibition (ZOI). In the DCT, results were expressed as mean bacterial viability (CFU/mL). Statistical analysis was conducted using two-way ANOVA ($P<0.05$).

Results: According to the ADT results, ZOI diameters in the TCS and TCS-Amo groups increased significantly over time ($P<0.001$). These two groups also demonstrated significantly larger ZOI values at T0, T3, and T7 compared to the other groups ($P<0.05$). In the DCT, bacterial viability in the TCS and TCS-Amo groups increased significantly over time ($P<0.001$). The experimental sealers showed significantly lower bacterial viability than Endoseal MTA at the early time points (T0 and T1), but higher viability at the later stages (T3 and T7) ($P<0.05$).

Conclusion: The TCS and TCS-Amo sealers exhibited strong antibacterial activity in the ADT, with TCS-Amo showing the highest efficacy on day 7. However, their effectiveness declined over time in the DCT, indicating limited long-term antimicrobial activity against *E.faecalis.*

Keywords: Agar diffusion test, Antibacterial agents, Bioceramics, *Enterococcus faecalis*, Root canal sealants, Silicates

Introduction

Pathogenic bacteria are the main cause of pulp and periapical diseases. The presence of microorganisms in the root canal system can lead to pathological changes in the periradicular tissues, the development of periapical lesions, and bone loss around the affected tooth. Therefore, the main goal of root canal treatment is to sufficiently clean the root canal and eliminate the microorganisms from the root canal system (1-3).

Root canal cleaning is performed using both chemical and mechanical methods. However, evidence shows that despite thorough chemical and mechanical cleaning, it is still impossible to completely eradicate all microorganisms from the root canal system. Therefore, root canal filling materials must possess antimicrobial properties to remove residual bacteria and biofilm and prevent the recurrence of periapical diseases (2).

Among the various commercially available filling materials, gutta-percha is currently regarded as the best filling material (4). However, gutta-percha has a poor sealing ability and insufficient antimicrobial properties. It does not bond to the root canal walls on its own, thus an additional substance is required to fill the space between gutta-percha and dentin to achieve an optimal obturation (5, 6). For this reason, sealers are used along with gutta-percha as filling materials. Sealers must adhere to both gutta-percha and dentin to create and maintain an effective seal. The optimal characteristics of sealers include preventing leakage, strengthening the root structure, and showing biocompatibility and radiopacity. Furthermore, canal fillers must have antimicrobial properties to prevent the growth and proliferation of microorganisms (7, 8).

Endodontic sealers are classified into various groups based on their chemical composition and formulation:

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Accepted: 30 May 2025. Submitted: 14 December 2024.

zinc oxide eugenol-based, salicylate-based, fatty acid-based, glass ionomer-based, silicone-based, epoxy resin-based, methacrylate-based, and tricalcium silicate (TCS)-based sealers (9). In recent years, TCS-based sealers have gained popularity due to their favorable properties such as biocompatibility, flowability and antimicrobial effects (10, 11).

The favorable characteristics of TCS-based sealers are attributed to the calcium hydroxide by-products formed during their initial setting reaction with water (12). These sealers demonstrate alkaline behavior (13). When the surrounding environment becomes saturated with calcium ions, the pH can exceed 11, establishing conditions that are unfavorable for bacterial growth (14).

Enterococcus, Streptococcus, Peptostreptococcus, and Actinomyces species are particularly implicated in the failure of endodontic treatment and the recurrence of pulpal and periapical lesions (15). Enterococci are Gram-positive and facultative anaerobic bacteria capable of surviving in both aerobic and anaerobic environments. Although enterococci represent a minor component of the normal oral flora, they play a significant role in pulpal and periapical infections. *Enterococcus faecalis* is the most frequently identified species in periodontal disease, root canal infections, and periapical abscesses (16-18).

In this study, two experimental TCS-based bioceramic sealers were developed, each consisting of TCS powder and a water-based paste containing a thickening agent. The formulations were designated as TCS and TCS-Amo, with the latter additionally incorporating the antibiotic amoxicillin.

Given the documented antimicrobial activity of TCS-based bioceramic sealers in previous studies (19, 20), the present study aimed to evaluate the antimicrobial efficacy of two newly formulated tricalcium silicate-based sealers, with and without amoxicillin, against *Enterococcus faecalis* and to compare their performance with Endoseal MTA (a TCS-based sealer) and Pulpdent (a zinc oxide eugenol-based sealer).

Materials and methods

This study was approved by the Ethics Committee of Mashhad University of Medical Sciences (Ethics Code: IR.MUMS.DENTISTRY.REC.1403.024).

TCS synthesis

The characteristics of the materials used for TCS synthesis are listed in Table 1. The Pechini method was utilized in the production of TCS powder (21). Nano-silica ($nSiO_2$) was used as the silicon source, and calcium nitrate tetrahydrate (CaNT) served as the calcium source. Each component was separately dissolved in distilled water to achieve a calcium-to-silicon molar ratio of 3:1. Citric acid, previously dissolved in water, was added to the combined solution at a 1:1 molar ratio concerning the total cation content. The mixture was stirred until a clear solution was obtained.

Next, ethylene glycol (EG) was added to the citric acid mixture at a 2:1 molar ratio. The resulting solution was heated in a fume cupboard at 80–100 °C with continuous stirring to evaporate excess water, forming a foamy gel. This gel was dried overnight at 150 °C. The dried material (xerogel) was then ground into a powder and calcined in a furnace at 1300 °C for three hours. The calcined material was subsequently milled using a mixer mill to obtain a fine TCS powder.

Preparation of bioceramic cements

In this study, a novel double-paste injectable tricalcium silicate (TCS) cement was developed. Paste A

Table 1. Characteristics of materials used for the synthesis of tricalcium silicate-based sealers

Material	Chemical/Physical Properties	Manufacturer
Nano-Silica ($nSiO_2$)	Purity: 99.95%, Particle size: 20–30 nm	SPIC (Chennai, India)
Nano-Hydroxyapatite (nHA)	Molar mass: 502.31 g/mol, Particle size: 10–30 nm	ARMINANO (Tehran, Iran)
Ethylene Glycol (EG)	Density: 1.11 g/cm^3, Purity: >99%	Drm-chem (Saveh, Iran)
Amoxicillin (AMX)	Broad-spectrum β-lactam antibiotic	Daanapharma (East Azarbaijan, Iran)
Calcium Nitrate Tetrahydrate (CaNT)	$Ca(NO_3)_2{\cdot}4H_2O$, Purity: >98.5%	Sigma-Aldrich (Missouri, USA)
Polyethylene Glycol (PEG)	Average molecular weight: 400	Sigma-Aldrich (Missouri, USA)
Citric Acid (CA)	Purity: >99%	Merck Group (Darmstadt, Germany)
Polyvinyl Alcohol (PVA)	Average molecular weight: 89,000–98,000	Merck Group (Darmstadt, Germany)
Calcium Chloride ($CaCl_2$)	Purity: >98%, Molar mass: 110.99 g/mol	Merck Group (Darmstadt, Germany)
Tungsten Oxide (WO_3)	Purity: 99.95%, Particle size: 20 nm	US-Nano (Huston, United States)

was formulated by mixing TCS powder with PEG 400. Paste B was a water-based formulation containing 2 wt% polyvinyl alcohol (PVA), 10 wt% nano-silica ($nSiO_2$), 2 wt% calcium chloride ($CaCl_2$), 5 wt% tungsten trioxide (WO_3), and 5 wt% nano-hydroxyapatite (nHAp) in distilled water. The antibiotic-containing cement (TCS-Amo) was prepared by incorporating 30 mM of amoxicillin (AMX) into Paste B.

To prepare the TCS and TCS-Amo cements, Paste A and Paste B were mixed at a 1:1 volume ratio. The components were thoroughly combined to form a uniform, injectable paste for subsequent testing.

Grouping

The antimicrobial properties of two newly developed bioceramic sealers (TCS and TCS-Amo) were compared with those of Pulpdent (Watertown, Massachusetts, USA) and Endoseal MTA (Maruchi, Wonju, Gangwon-do, Korea). Therefore, four groups of specimens were tested: TCS, TCS-Amo, Pulpdent, and Endoseal MTA.

Pulpdent and Endoseal MTA were prepared according to the manufacturer's instructions.

For each group, separate samples were prepared and tested at different time points: immediately after setting (T0), 24 hours (1 day, T1), 72 hours (3 days, T3), and 168 hours (7 days, T7). This allowed for a time-dependent comparison of antimicrobial activity across all sealers.

Antibacterial properties

Two methods were used to compare the antibacterial properties of sealers against *Enterococcus faecalis*: the agar diffusion test (ADT) and the direct contact test (DCT).

The *Enterococcus faecalis* strain was obtained from the Microbiology Laboratory of the Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran. To prepare the bacterial suspension for testing, three colonies were inoculated into 10 mL of Brain Heart Infusion (BHI) broth and incubated at 37°C for 24 hours in a shaker incubator. The resulting turbid culture, indicating bacterial growth, was then measured using a spectrophotometer and adjusted to a concentration of 10^8 CFU/mL, corresponding to a 0.5 McFarland standard.

To ensure sterility and rule out any contamination, several control tests were performed alongside the main experiments. First, BHI broth was added to each sealer sample without bacterial inoculation to verify the sterility of the sealers. Second, BHI broth alone was incubated to confirm that the liquid medium was uncontaminated. Additionally, plain BHI agar plates were incubated without bacterial inoculation to assess the sterility of the solid medium. These controls confirmed that any bacterial growth observed in the test samples was due to the deliberate introduction of *Enterococcus faecalis*, and not from contamination by materials or the environment."

Agar diffusion test (ADT)

After mixing, each sealer was placed into circular molds measuring 6 mm in diameter and 1 mm in depth. The samples were incubated at 37 °C with 97% humidity to prevent drying and simulate the moisture conditions of dentin.

BHI agar plates were inoculated with 150 µL of bacterial suspension that was spread uniformly with a sterile swab. Wells measuring 6 mm in diameter and 1 mm in depth were created in the agar at regular intervals, and sealer discs were placed into these wells (Figure 1). Vancomycin discs were used as positive controls, while empty wells served as negative controls. The plates were incubated at 37 °C for 24 hours, and the

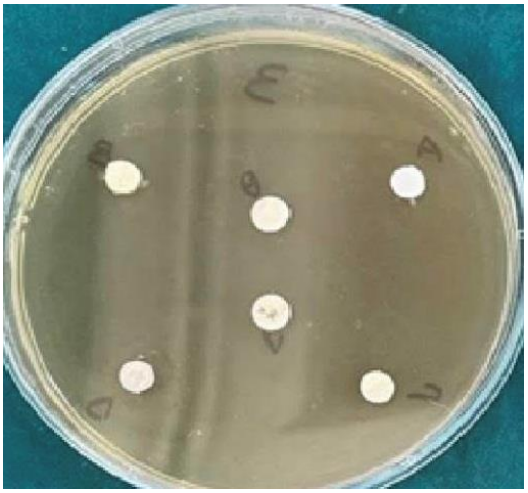

Figure 1. Agar diffusion test (ADT) performed on a Brain Heart Infusion (BHI) agar plate with wells containing sealer discs

Figure 2. Microtubes used for direct contact testing (DCT) to assess the antimicrobial properties of sealers

diameter of the zone of inhibition (ZOI) around each sealer was measured at each time point (T0, T1, T3, T7). All experiments were performed in triplicate.

Direct contact test (DCT)

After mixing, each sealer was weighed, and 0.2 to 0.4 g of the material was placed at the bottom of the sterile microtubes (Figure 2). The microtubes were incubated at 37 °C in a humidified chamber (95–100% relative humidity) to prevent drying. Antimicrobial testing was conducted after incubation periods of 1, 3, and 7 days.

On the test day, 50 µL of microbial suspension was diluted in 15 mL of BHI broth. Then, 250 µL of this solution was added to the surface of each sealer and incubated for three hours.

Subsequently, 10 µL from each microtube was serially diluted in BHI broth, and 10 µL of the final dilution was spread onto BHI agar plates.

After 24 hours of incubation at 37°C, colony counting was performed using a colony counter, and results were reported as colony-forming units per milliliter (CFU/mL). Each experiment was conducted in triplicate. Antibacterial activity was calculated using the following formula:

$$Antibacterial\ activity\ percentage\ (\%) = [\frac{(CFU/mL(control) - CFU/mL(sample))}{CFU/mL(control)}] \times 100$$

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics version 23 (IBM Corp., Armonk, NY, USA). The data were analyzed using Two-way ANOVA to evaluate differences among the groups over time. A significance level of P<0.05 was considered statistically significant.

Results

Agar diffusion test (ADT) results

Table 2 presents the mean ZOI diameter values of four sealer groups across different time points. Two-way ANOVA revealed a significant interaction between sealer type and time point (P<0.001), indicating that the changes in antimicrobial activity over time differed significantly among the tested sealers. Therefore, one-way ANOVA was used for analysis followed by Tukey's post hoc test for pairwise comparisons.

Within-group comparisons showed that both experimental sealers exhibited statistically significant increases in antimicrobial activity over time (P<0.001 for both). TCS maintained stable values at T0 and T1, followed by a significant rise at T3 and T7 (P<0.05). TCS-Amo showed a significant decrease at T1, followed by a significant increase at T3 and peaking at T7 (P<0.05). Pulpdent showed no significant changes across time points (P=0.31). Endoseal MTA demonstrated significantly larger ZOI diameters at T1 and T7 compared to T0 and T3 (P<0.05; Table 2).

Between-group analysis revealed significant differences at all time points (P<0.05; Table 2). Immediately after setting (T0), experimental sealers exhibited significantly greater ZOI diameter values compared to the conventional sealers (P<0.05). At T1, TCS and Endoseal MTA groups showed significantly greater antibacterial activity compared to TCS-Amo and Pulpdent groups (P<0.05). When measured 72 hours after setting (T3), both experimental sealers demonstrated significantly larger ZOI values than the conventional sealers (P<0.05). Moreover, the ZOI value of the TCS group was significantly greater than that of the TCS-Amo group (P<0.05). At T7, TCS-Amo exhibited the highest ZOI diameter, which was significantly higher than the other groups (P<0.05). The ZOI diameter of the TCS group was also significantly greater than that of the conventional sealers (P<0.05; Table 2).

Table 2. Comparison of the zone of inhibition (ZOI) diameter (mm) in the study groups across different time points

Groups	T0 Mean ± SD	T1 Mean ± SD	T3 Mean ± SD	T7 Mean ± SD	P-value
TCS	15.17 ± 0.51^{aA}	15.19 ± 0.44^{aA}	38.95 ± 3.87^{aB}	38.25 ± 0.56^{bB}	<0.001
TCS-Amo	16.80 ± 0.57^{aB}	11.15 ± 1.10^{bA}	29.44 ± 2.11^{bC}	41.68 ± 0.38^{aD}	<0.001
Pulpdent	12.10 ± 0.38^{b}	12.00 ± 0.83^{b}	11.82 ± 0.12^{c}	11.29 ± 0.53^{c}	0.31
Endoseal MTA	11.29 ± 1.10^{bA}	14.70 ± 1.10^{aB}	11.38 ± 1.01^{cA}	12.75 ± 0.32^{cB}	0.007
P-value	<0.001	0.002	<0.001	<0.001	

Different uppercase superscript letters indicate a statistically significant difference within the same group between different time points.
Different lower case superscript letters indicate a statistically significant difference between groups at the same time point.
SD: standard deviation

Direct contact test (DCT) results

Table 3 compares the mean bacterial viability (CFU/mL) values of all four sealer groups across different time points. Two-way ANOVA revealed a significant interaction between sealer type and time (P<0.001). Therefore, one-way ANOVA and Tukey's post hoc test were used for analysis.

Within-group comparisons showed statistically significant changes in bacterial viability in TCS, TCS-Amo and Endiseal MTA groups (P<0.001). In both experimental sealers, bacterial viability was comparable between T0 and T1, then significantly increased at T3 and T7 (P<0.05). Pulpdent showed insignificant fluctuations in antibacterial activity over time (P=0.12). The bacterial viability of Endoseal MTA was relatively stable at T0 and T1, and significantly reduced at T3 and T7 (P<0.05).

At each time point, significant differences were found between the groups (P<0.001). Immediately after setting (T0) and 24 hours later (T1), Endoseal MTA demonstrated significantly greater bacterial viability compared to the other groups (P<0.05). At T3 and T4, the experimental sealers showed significantly greater bacterial viability compared to the conventional sealers (P<0.05).

Discussion

This study evaluated the antimicrobial properties of two newly developed bioceramic sealers, TCS and TCS-Amo, compared to two commercially available endodontic sealers, Endoseal MTA (a tricalcium silicate-based sealer) and Pulpdent (a zinc oxide eugenol-based sealer). The assessment was performed using both agar diffusion (ADT) and direct contact (DCT) methods at different time points. E. faecalis was chosen as the test organism due to its known resistance to endodontic treatment and frequent association with persistent periapical infections and root canal failure.

When interpreting the results of antibacterial tests, it is essential to recognize that while a lower bacterial viability (log CFU/ml) in the direct contact test (DCT) indicates greater antimicrobial activity, a larger ZOI in the agar diffusion test (ADT) similarly denotes enhanced antibacterial performance.

According to the ADT results, both experimental sealers, TCS and TCS-Amo, demonstrated a clear time-dependent increase in antibacterial activity, as reflected by their increasing ZOI values. TCS reached its peak antimicrobial performance at 72 hours (T3), while TCS-Amo exhibited the largest ZOI at 168 hours (T7), indicating a prolonged release of its active components. This sustained activity may be attributed to the solubility of the materials and the gradual release of antimicrobial ions, such as calcium, which increase the local pH and create an alkaline environment unfavorable for bacterial growth (22).

TCS-Amo initially showed a decrease in ZOI diameter at 24 hours but significantly improved at 72 and 168 hours. This trend could be attributed to the gradual release of amoxicillin over time, allowing it to maintain and even enhance its efficacy at later stages. The inclusion of amoxicillin in the sealer likely enhanced its antimicrobial properties. Previous studies by Baer et al. (23) and Dornelles et al. (24) reported that sealers incorporating amoxicillin-loaded microspheres were more effective against E. faecalis, while the addition of amoxicillin had no impact on the physical properties of the sealers.".

The commercial sealers, however, behaved differently compared to the TCS and TCS-Amo sealers. Pulpdent displayed relatively consistent but moderate ZOI values across all time points, suggesting a limited time-dependent change in its antibacterial performance. Endoseal MTA, on the other hand, showed slight fluctuations, with a significant peak at 24 hours, followed by a return to near-baseline value at T3 and a small significant increase at T7.

Table 3. Comparison of mean bacterial viability in different study groups across multiple time intervals (log(CFU/mL))

Group	T0 Mean ± SD	T1 Mean ± SD	T3 Mean ± SD	T7 Mean ± SD	P-value
TCS	6.96 ± 0.01^{aA}	6.88 ± 0.02^{aA}	7.11 ± 0.03^{aB}	7.35 ± 0.09^{aB}	<0.001
TCS-Amo	6.96 ± 0.07^{aA}	6.79 ± 0.03^{aA}	7.26 ± 0.008^{aB}	7.15 ± 0.02^{aB}	<0.001
Pulpdent	6.74 ± 0.02^{a}	6.66 ± 0.03^{a}	6.82 ± 0.10^{b}	6.67 ± 0.01^{b}	0.12
Endoseal MTA	7.25 ± 0.01^{bA}	7.19 ± 0.02^{bA}	6.76 ± 0.05^{bB}	6.59 ± 0.05^{bB}	<0.001
P-value	<0.001	<0.001	<0.001	<0.001	

Different uppercase superscript letters indicate a statistically significant difference within the same group between different time points.
Different lower case superscript letters indicate a statistically significant difference between different groups at the same time point.
SD: standard deviation

Between-group comparisons revealed that TCS and TCS-Amo exhibited significantly greater antibacterial activity against *E. faecalis* than conventional sealers at almost all time points except T1 when Endoseal MTA showed a comparable efficacy to TCS.

Although ADT is commonly used for evaluating antimicrobial properties, it has some limitations. The results obtained through ADT can be influenced by the material's solubility in the culture medium, and thus it may yield inaccurate readings for materials that do not diffuse well through the agar gel. Additionally, factors such as the buffering capacity of the agar can affect the size of the ZOI. Furthermore, ADT cannot differentiate between bactericidal and bacteriostatic properties, making it insufficient for determining the exact mechanism of antimicrobial action (5, 25). Given these limitations, direct contact testing (DCT) was also used in this study, as it provides a more accurate reflection of the direct contact between the sealers and bacteria, minimizing potential confounding factors.

The antimicrobial trends revealed through the DCT differed from those observed in the ADT. Unlike the ADT in which TCS and TCS-Amo showed increasing antimicrobial activity over time, the DCT results indicated a progressive increase in bacterial viability for both experimental sealers, suggesting a decline in antibacterial activity over the seven days.

For both TCS and TCS-Amo sealers, bacterial viability remained stable between T0 and T1 but significantly increased at T3 and T7. This may be due to a gradual reduction in calcium ion release and a corresponding drop in pH. TCS-Amo showed a significant decrease in bacterial viability at T1, possibly due to the initial release of amoxicillin. This was followed by an increase in bacterial viability at T3 and T7, suggesting a decreased release rate of amoxicillin over time (24, 26). In contrast, Pulpdent maintained stable antibacterial activity across all time points, implying that its antimicrobial efficacy is not significantly affected by aging. This consistent performance is likely due to the rapid setting properties of Pulpdent and its stable release of antimicrobial agents, particularly eugenol, a phenolic compound that effectively inhibits bacterial growth (25). Endoseal MTA exhibited a gradual and significant increase in antibacterial activity at T3 and T7. This is likely due to continuous hydration and sustained ion release, which creates an alkaline environment that enhances antimicrobial properties over time (27).

Overall, the DCT findings indicate that TCS and TCS-Amo offer strong short-term antibacterial effects, especially during the first day after setting. However, their effectiveness diminishes over time, which contrasts with the more sustained or delayed activity of commercial sealers, particularly Endoseal MTA. In clinical terms, the initial high efficacy of the experimental sealers may be beneficial during the early post-obturation phase, when microbial threats are greatest. However, their decreasing antibacterial activity over time, suggests a need for enhanced sustained-release formulations. On the other hand, materials like Endoseal MTA, which maintain or improve their performance over time, may offer longer-lasting protection against reinfection. These findings highlight the need for enhanced long-term release mechanisms in novel sealers to maintain their antimicrobial potential throughout time.

This study was conducted under in vitro conditions, which may not fully replicate the complex environment of the root canal system. The antibacterial assessment was limited to *E. faecalis*, while clinical infections often involve polymicrobial communities. Future studies should focus on evaluating the long-term antibacterial effects of these sealers in clinical studies, assessing their performance against multispecies biofilms, and investigating key physical and biological properties, such as biocompatibility, sealing ability, and resistance to degradation and bacterial infection over time.

Conclusions

According to the obtained results, the following statements can be concluded:

- In the agar diffusion test, the novel TCS-based sealers demonstrated significantly greater antibacterial activity compared to commercial sealers at almost all time intervals.
- TCS and TCS-Amo sealers demonstrated strong initial antibacterial effects in the direct contact test, particularly at 24 hours.
- Based on the direct contact testing, both experimental sealers showed a decline in effectiveness over time, suggesting a limited long-term antimicrobial effect.
- Commercial sealers offered more sustained antibacterial performance in direct contact testing compared to the experimental sealers.

Acknowledgment

The authors express their gratitude to the Vice Chancellor for Research of Mashhad University of Medical Sciences. Special thanks go to the Dental

Materials Laboratory for providing essential resources and facilities, and to Mr. Zahabi for his technical assistance during the study.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

S.M. contributed to the conceptualization and methodology; H.B. contributed to the visualization, methodology, data curation, writing, reviewing, and editing the manuscript; M.J.B. contributed to the visualization, data curation, and writing of the initial manuscript; M.J. contributed to the methodology and data curation; A.S. contributed to the supervision, investigation, data curation, writing and editing the final manuscript. All authors approved the final manuscript.

Ethical approval

This study was approved by the ethics committee of Mashhad University of Medical Sciences (Ethics Code: IR.MUMS.DENTISTRY.REC.1403.024).

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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