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In vitro antibacterial effect of a nano-zinc oxide eugenol sealer alone and in combination with chitosan, propolis, and nanosilver on *Enterococcus faecalis*

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ABSTRACT

Background: This study aimed to assess the antibacterial effect of a nano-zinc oxide eugenol (nZOE) sealer alone and in combination with chitosan, propolis, and nanosilver on *Enterococcus faecalis*.
Materials and Methods: In this *in vitro*, experimental study, nanosilver, chitosan, and propolis with 10wt%, 20wt%, and 60wt% concentrations, respectively, were added to nZOE sealer, and their antibacterial activity against *E. faecalis* was evaluated by agar diffusion and broth microdilution tests. The diameter of the growth inhibition zones was measured, and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were calculated for all materials. Data were analyzed by *t*-test (alpha = 0.05).
Results: The addition of nanosilver, chitosan, and propolis to nZOE did not change the diameter of growth inhibition zone in agar diffusion test. Propolis and eugenol alone showed the lowest MIC and MBC. Chitosan alone showed the highest MIC and MBC. Furthermore, nZOE showed lower MBC than micro-ZOE ($P = 0.000$). All groups containing nZOE showed the lowest MIC and MBC values.
Conclusion: The addition of propolis to nZOE can enhance its antibacterial activity against *E. faecalis in vitro*.

Key Words: Anti-bacterial agents, chitosan, *Enterococcus faecalis*, eugenol, propolis, silver, zinc oxide

INTRODUCTION

Bacteria and their byproducts are responsible for pulpal and periapical diseases. Thus, their elimination from the root canal system is a major goal in endodontic treatment. Physical instrumentation can significantly decrease the bacterial load in the root canal; however, due to anatomical complexities of the root canal system, especially in primary teeth, complete elimination of microorganisms is not feasible.[1-5] Chemical irrigants and intracanal medicaments with antibacterial properties are used as an adjunct to mechanical instrumentation to further minimize the microbial load.[4] The success of root canal treatment depends on complete elimination of necrotic pulp residues and microorganisms from the root canal system.[2,6] Evidence shows that the

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presence of bacteria in the canal during obturation decreases the long-term success of treatment.[7]

Enterococcus faecalis has been isolated from endodontically failed teeth with persistent periapical lesions. It is also commonly found in cases of periodontitis, gingivitis, and failed root canal treatment.[3,5,7] It can invade the dentinal tubules, resist hard ecological conditions, cope with unfavorable intracanal conditions, and form biofilm along with other bacteria. Thus, it is known as a resistant endodontic pathogen.[5,8] It is among the main microorganisms comprising the microbial flora of the root canal system and is often associated with refractory periapical lesions after endodontic treatment.[1,5,6] It is also responsible for endodontic treatment failure of primary teeth.[1,6] A previous study showed the involvement of *E. faecalis* in 80% of the cases of endodontic treatment failure.[6]

Achieving a high success rate in endodontic treatment depends on adequate cleaning and shaping and optimal obturation of the root canal system.[9-11] Thus, to prevent the growth and proliferation of residual bacteria in the root canal, root-filling materials and endodontic sealers should preferably have antibacterial activity and must be able to preserve this property over time.[10] Evidence shows that eugenol-based sealers have higher antibacterial activity compared with resin-based or calcium hydroxide-based sealers against *E. faecalis*.[1,8,12] Zinc oxide eugenol (ZOE) sealer has long been used as a suitable endodontic sealer. Although its extension beyond the apex can cause periapical irritation, it has advantages such as optimal dimensional stability, solubility, and high antibacterial activity, making it a suitable sealer for pulpectomy of primary teeth.[1,2]

Nanotechnology is currently used to produce many dental materials such as composite resins, dental bonding agents, impression materials, ceramics, dental implant coatings, and fluoride mouthwashes.[11] Nanoparticles can penetrate deeper into dentinal tubules, have higher antibacterial properties, and can decrease microleakage. Due to such excellent properties, nanoparticles are also used in production of endodontic sealers.[9,11,13] Nano-ZOE (nZOE) sealer is synthesized by mixing zinc oxide nanoparticles with eugenol solution and has superior properties compared with the conventional ZOE sealer.[13] A previous study showed significantly lower adhesion of *E. faecalis* to dentinal walls treated with zinc oxide nanoparticles.[9] Furthermore, nanoparticles have higher antibacterial activity against bacterial biofilm.[14]

The efficacy of endodontic sealers may be improved by the addition of nanoparticles or any other chemical agent that can help eliminate the residual bacteria from the root canal system. Accordingly, the prognosis and success of treatment would improve.[11,14-16] Chitosan, propolis, and nanosilver are three important materials in biomedicine due to their optimal physical, chemical, and biological properties.[4,9,17,18] Chitosan is a naturally abundant polysaccharide, which is obtained from chitin. It has optimal biocompatibility, low cytotoxicity, favorable biodegradability, and suitable antibacterial, antifungal, and hemostatic properties. It has anti-inflammatory effects and enhances wound healing.[14,16,19,20]

Propolis is a resinous material rich in flavonoids, which is produced by the honeybees, and has favorable antibacterial and antifungal properties, low cytotoxicity, and healing properties.[20,21] The antibacterial properties of nanosilver particles have been previously documented, and evidence shows that decreasing the size of silver particles to nanoscale increases their antibacterial activity, biocompatibility, and efficient contact area.[17,18]

To the best of the authors' knowledge, the antibacterial effect of nZOE sealer alone and in combination with chitosan, propolis, and nanosilver has not been previously evaluated. Thus, this study aimed to assess the antibacterial effect of nZOE sealer alone and in combination with chitosan, propolis, and nanosilver on *E. faecalis*.

MATERIALS AND METHODS

This *in vitro*, experimental study evaluated the antibacterial effect of nZOE sealer alone and in combination with chitosan, propolis, and nanosilver on *E. faecalis* by two methods of agar diffusion and broth microdilution. The study was approved by the Ethics Committee of Zanjan University of Medical Sciences (IR.ZUMS.REC.1399.384).

This study was conducted on 11 groups, including different combinations of powder and liquid. The liquid included eugenol (Morvabon, Tehran, Iran), distilled water, and dimethyl sulfoxide (Merck, Germany). The powders included nano-zinc oxide powder with 20–25 nm particle size (Arminano,

Tehran, Iran), nanosilver powder with a particle size of 10–60 nm (Arminano, Tehran, Iran), chitosan powder with 90% degree of distillation (Nano Pooyesh Yekta, Tehran, Iran), propolis (Rodin Mehr Arad, Ardabil, Iran), and micro-zinc oxide powder (Morvabon, Tehran, Iran). All materials were weighed using a scale (Sartorius, Gottingen, Germany) with 0.0001 accuracy in milligrams.

The study groups were as follows:

Group 1: micro-zinc oxide + distilled water, Group 2: nano-zinc oxide + distilled water, Group 3: nanosilver + distilled water, Group 4: chitosan + distilled water, Group 5: propolis + distilled water, Group 6: eugenol, Group 7: nano-zinc oxide + eugenol, Group 8: micro-zinc oxide + eugenol, Group 9: nano-zinc oxide + 10wt% nanosilver + eugenol, Group 10: nano-zinc oxide + 20wt% chitosan + eugenol, and Group 11: nano-zinc oxide + 60wt% propolis + eugenol.

Bacterial culture

Standard-strain *E. faecalis* (ATCC 33186) was obtained in lyophilized form from the Industrial Scientific and Research Center of Iran. A microbial suspension was prepared with 0.5 McFarland standard concentration containing 1.5×10^8 colony-forming units (CFUs)/mL. For the microdilution test, microbial suspension was diluted with sterile saline by 1:100 to obtain 1.5×10^6 CFUs/mL.[22]

Agar diffusion test

For agar diffusion test, the powders in all groups (except Group 6) were mixed with their respective solution (eugenol/distilled water) in a 1:4 ratio.[23] In Group 6, eugenol was used alone. Bacterial suspension was inoculated on brain heart infusion agar plates by sterile swabs. Next, a Pasteur pipette was used to create 5 holes with 5 mm diameter on each plate. The test materials were transferred into the holes, and the plates were incubated at 37° for 24 h. A caliper was then used to measure the diameter of the growth inhibition zones.[22]

Broth microdilution test

For the microdilution test, in all groups except for Group 6 (eugenol), 0.5 g of powder was dissolved in 1 mL of solvent (eugenol or distilled water) along with 1 mL of dimethyl sulfoxide in a microtube to reach 2 mL volume. In Group 6, 1 mL of eugenol was mixed with 1 mL of dimethyl sulfoxide in a microtube. A 96-well plate was used for this test. A total of 150 μL of brain heart infusion broth was added to all wells of a row except for the 10th well. Each row was used for one material. Next, 150 μL of the mixture in each group was added to the first well of the respective row and mixed with the culture medium by up-and-down movement of the sampler. Next, 150 μL of the first well was collected and transferred to the second well and mixed; 150 μL of the second well was collected and transferred to the third well, and this process was continued until the 9th well. Finally, 150 μL of the 9th well was collected and discarded. Finally, each well had a content volume of 150 μL. Accordingly, the concentration of products in each group was diluted from ½ to 1/512. Afterward, 15 μL of the bacterial suspension with 1.5×10^6 CFUs/mL was added to all wells containing the culture medium to reach a final bacterial concentration of 1.5×10^5 CFUs/mL. The 10th and 11th wells served as the negative and positive control groups, respectively. The negative control well received no additive, while the positive control well-received 15 μL bacterial suspension. Each plate was then incubated at 37°C for 24 h. Next, the contents of each well were cultured on brain heart infusion agar plates and incubated at 37°C for 24 h. The culture was performed by a swab, and the swab was heated on a flame for 5 s after each culture to ensure no contamination.[22]

The minimum inhibitory concentration (MIC) of each group was calculated visually by noticing bacterial growth on solid culture plate. In cases where visual assessment was not possible due to discoloration, MIC along with minimum bactericidal concentration (MBC) was calculated. To calculate the MBC, samples were transferred from wells without bacterial growth to brain heart infusion agar plates by a swab, and the plates that showed no bacterial growth indicated the MBC value of the respective product.

Statistical analysis

Data were analyzed by SPSS version 22 (SPSS Inc., IL, USA). The measures of central dispersion were reported for the diameter of growth inhibition zones in agar diffusion test. For the broth microdilution test, the MIC and MBC values of the groups were compared pairwise by *t*-test at 0.05 level of significance.

RESULTS

Agar diffusion test results

Table 1 presents the mean diameter of growth inhibition zones in agar diffusion test. As shown,

Groups 1–4 had no antibacterial activity, but the remaining groups had antibacterial activity against *E. faecalis*.

Microdilution test results

Table 2 presents the MIC and MBC of the first six groups (materials dissolved in distilled water and eugenol alone). According to the results, the eugenol group showed the lowest MIC and MBC and inhibited the growth and proliferation of *E. faecalis* in all wells. After eugenol, propolis showed the lowest MIC and MBC. Both eugenol and propolis groups showed lower MIC and MBC than other groups. Furthermore, chitosan showed the highest MIC and MBC and had lower antibacterial activity against *E. faecalis* at certain concentrations compared with other groups. Nano-zinc oxide had similar MIC but significantly lower MBC than micro-zinc oxide ($P = 0.000$).

The micro-zinc oxide group showed lower MIC ($P = 0.000$) but similar MBC compared with the nanosilver group. The micro-zinc oxide group showed significantly lower MIC ($P = 0.029$) and MBC ($P = 0.000$) than the chitosan group. The micro-zinc oxide group demonstrated significantly higher MIC ($P = 0.000$) and MBC ($P = 0.000$) than the propolis group.

The nano-zinc oxide group showed significantly lower MIC ($P = 0.000$) and MBC ($P = 0.000$) than the nanosilver group. The chitosan group demonstrated significantly higher MIC ($P = 0.000$) and MBC ($P = 0.000$) than the propolis group.

The results of microdilution test for the materials dissolved in eugenol revealed that all materials inhibited the growth and proliferation of *E. faecalis* in all wells, and thus, their minimum concentration, i.e., 0.9 μg/mL, was considered their MIC and MBC.

DISCUSSION

This study assessed the antibacterial effect of nZOE sealer alone and in combination with chitosan, propolis, and nanosilver on *E. faecalis*. To the best of the authors' knowledge, this study is the first to compare the antibacterial properties of the abovementioned materials alone and in combination with nZOE sealer. The results showed that the addition of nanosilver, chitosan, and propolis to nZOE did not change the diameter of growth inhibition zone in agar disc diffusion test. Propolis and eugenol alone showed the lowest MIC and MBC. Chitosan alone showed the highest MIC and MBC. Furthermore, nZOE had lower MBC than micro-ZOE ($P = 0.000$). All groups

Table 1: Mean diameter of the growth inhibition zones (mm) in the agar diffusion test

Groups	Mean±SD	Minimum	Maximum
Micro-zinc oxide + distilled water	-	Resistant	Resistant
Nano-zinc oxide + distilled water	-	Resistant	Resistant
Nanosilver + distilled water	-	Resistant	Resistant
Chitosan + distilled water	-	Resistant	Resistant
Propolis + distilled water	2.12±13.5	12	15
Eugenol	0.70±12.5	12	13
Nano-zinc oxide + eugenol	0.70±13.5	13	14
Micro-zinc oxide + eugenol	13	13	13
Nano-zinc oxide + 10wt% nanosilver + eugenol	0.70±12.5	12	13
Nano-zinc oxide + 20wt% chitosan + eugenol	0.70±12.5	12	13
Nano-zinc oxide + 60wt% propolis + eugenol	1.41±13	12	14

SD: Standard deviation

Table 2: Minimum inhibitory concentration and minimum bactericidal concentration of the first six groups (materials dissolved in distilled water and eugenol alone)

Groups	MIC (μg/mL)	MBC (μg/mL)
Micro-zinc oxide + distilled water + dimethyl sulfoxide	31.2	62.5
Nano-zinc oxide + distilled water + dimethyl sulfoxide	31.2	31.2
Nanosilver + distilled water + dimethyl sulfoxide	62.5	62.5
Chitosan + distilled water + dimethyl sulfoxide	125	125
Propolis + distilled water + dimethyl sulfoxide	0.9	1.9
Eugenol	1.9	1.9

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration

containing nZOE showed the lowest MIC and MBC values.

Evidence shows that nZOE sealer is superior to micro-ZOE since it optimally inhibits the growth of bacterial biofilm in dentinal tubules, has lower cytotoxicity, better dimensional stability, and superior sealing properties and antibacterial activity compared with micro-ZOE, and is therefore preferred to it.[9,17] Sirelkhatim *et al*.,[24] in their review study, discussed that nZOE particles, due to their small size, can easily penetrate through the bacterial membrane. Furthermore, smaller particles have higher antibacterial activity.[24] Moradpoor *et al*.[25] reported that zinc oxide nanoparticles have selective toxicity against bacteria, superior antibacterial activity, and minimum effect on human cells compared with micro-zinc oxide.

In the present study, Groups 1–4 (micro-zinc oxide, nano-zinc oxide, nanosilver, and chitosan in distilled water) did not show any antibacterial activity against *E. faecalis* in agar diffusion test, which was in agreement with the results of some previous studies.[26-28] Inadequate dissolution of materials and their poor dispersion in the culture medium may be responsible for this finding. However, this finding was in contrast to the results of some others,[29-31] which may be due to the use of a different type of solvent or different technique of synthesis of materials.

In the present study, Groups 1 (nano-zinc oxide + eugenol) and 11 (nano-zinc oxide + 60wt% propolis) showed maximum growth inhibition zone (14 mm), and Groups 9 (nano-zinc oxide + 10wt% nanosilver + eugenol), 10 (nano-zinc oxide + 20wt% chitosan + eugenol), and 11 (nano-zinc oxide + 60wt% propolis + eugenol) showed the smallest growth inhibition zones (12 mm diameter). This result was in line with the findings of Haghgoo *et al*.,[15] Hala *et al*.,[9] and Beshr and Abdelrahim.[19] No antibacterial activity of nanosilver and chitosan in agar diffusion test appears to be due to aqueous structure of the culture medium and insolubility of nanosilver and chitosan in water. Nonetheless, these results were different from those of Pecarski *et al*.,[32] and Shayani Rad *et al*.,[17] which may be due to the use of higher concentrations of materials in their study and the use of different bacterial species.[17,32] It should be noted that the agar diffusion test is suitable for assessment of antibacterial activity of water-soluble (hydrophilic) materials.[10,33] Thus, the microdilution test was also used in this study in addition to the agar diffusion test to more accurately assess the antibacterial effects of the materials. The present study revealed that propolis + distilled water and eugenol showed the lowest MBC, and propolis + distilled water showed the lowest MIC among Groups 1–6. Propolis + distilled water had significantly higher antibacterial activity than other groups with distilled water solvent. Several studies have demonstrated the optimal efficacy of propolis in various fields such as prevention of biofilm formation, endodontic purposes, bone regeneration, formation of hard tissue barrier in pulpotomy, and as a pulp capping agent.[20,34] The MIC and MBC values of propolis in the present study were 0.9 and 1.9 µg/µL, respectively, indicating that it was 60 times stronger than the propolis used in the study by Kousedghi *et al*.[35] They collected propolis from Azerbaijan, Iran, and reported MIC and MBC values of 64 and 128 µg/µL, respectively. In the present study, propolis was obtained from Ardabil, Iran. The composition of propolis reportedly depends on its origin, which can affect its antibacterial activity as well.[36] Kartal *et al*.[37] reported that propolis collected from Kayseri, Turkey, had MIC and MBC values of 0.3 and 0.6 µg/µL, respectively. Variations in the reported results can be attributed to different compositions of propolis and different methodologies of studies.

Consistent with the present results, Elsheshtawy *et al*.[38] and Mattigatti *et al*.[39] reported that the addition of propolis to endodontic sealers significantly increased their antibacterial activity compared with zinc oxide. Elsheshtawy *et al*.[38] indicated that the addition of propolis to Metapex and ZOE sealers improved their antibacterial activity in primary necrotic teeth. Furthermore, Mattigatti *et al*.[39] discussed that propolis had high antibacterial activity against *E. faecalis in vitro* and can be used as an intracanal medicament. Divya *et al*.[40] suggested that propolis can be used in combination with Endoflas sealer for pulpectomy of primary teeth with severe involvement of the pulp and periapical tissue due to its optimal disinfecting property and enhancement of tissue healing. Such results can be due to synergistic antibacterial effects of propolis and zinc oxide.

The mean diameter of the growth inhibition zone for nano-zinc oxide and micro-zinc oxide with eugenol was 13.5 and 13 mm, respectively, in the present study. Furthermore, nano-zinc oxide with distilled water showed significantly lower MBC in the

microdilution test. In line with the present results, Ghaderian *et al*.[41] used 5 and 100 nm nanoparticles and obtained MIC and MBC values of 100 and 25 μg/μL, respectively. In the present study, 20–25 nm particles were used, yielding MIC and MBC values of 31.5 and 62.5 μg/μL, respectively, which was within the range reported by Ghaderian *et al*.[41] It has been confirmed that by a reduction in particle size to 100 nm or smaller, the contact area and charge of the particles increase, which enhance reactions and contact between positively charged nanoparticles and bacteria with a negatively charged cell wall, resulting in higher antibacterial activity.[25,42] Hala *et al*.[9] reported that nZOE had higher antibacterial activity than micro-ZOE sealer after 3 weeks and inhibited *E. faecalis* by 99.5% (vs. 98.6% by micro-ZOE). Similarly, Shayani Rad *et al*.[17] demonstrated that increasing the size of nano-zinc oxide particles from 63 to 87 nm decreased their inhibitory effect on *E. faecalis*. The results of the abovementioned studies are in line with the present findings. However, Emami-Karvani and Chehrazi[30] failed to show optimal antibacterial activity of zinc oxide nanoparticles against *Staphylococcus aureus* and *Escherichia coli*, which may be due to different techniques of synthesis of nanoparticles, variations in their size, and use of different microorganisms.

In the present study, eugenol had the lowest MBC and the second lowest MIC (1.9 μg/μL). Poggio *et al*.[10] demonstrated that eugenol-containing sealers had higher antimicrobial activity than those without eugenol. Due to its phenolic hydrophobic structure, eugenol can affect the lipid membrane, cell wall, and bacterial mitochondria and cause severe leaking through the bacterial membrane and subsequent bacterial death.[23] Due to hydrophobicity of eugenol, it could not show any superiority to other groups in the agar diffusion test. In line with the present results, Thosar *et al*.[43] showed that eugenol had the lowest MIC and MBC (1 μg/μL) in comparison with tea tree, lavender, thyme, and mint oils. Dragland *et al*.[44] reported a MIC of 1.2 μg/μL for eugenol against *E. faecalis*. Different techniques of synthesis of eugenol can affect its antibacterial properties and explain the variations in the results reported in the literature.[45]

In the present study, the MIC and MBC of nano-zinc oxide were lower than those of nanosilver. The MIC and MBC of nanosilver particles with 60 nm size were both 62.5 μg/μL against *E. faecalis*. Furthermore, the nano-zinc oxide + nanosilver group had no significant difference with other groups in the agar diffusion test. The present results were in agreement with the findings of Haghgoo *et al*.[15] and Kangarlou *et al*.[46] Haghgoo *et al*.[15] showed that the addition of nanosilver by 5wt% to ZOE sealer did not improve its antibacterial activity in the agar diffusion test. Kangarlou *et al*.[46] demonstrated that the addition of 10wt% nanosilver to AH26 and AH Plus sealers did not change the diameter of the growth inhibition zone of *E. faecalis*. This finding can be due to insolubility of nanosilver in the culture medium. In contrast to the present study, Halkai *et al*.[47] reported MIC and MBC of 5 μg/μL for silver nanoparticles, which may be due to the different shape, size, and synthesis technique of silver nanoparticles in their study since they used the colloidal form, and particles were 45 nm in size. The use of colloidal form of nanosilver leads to complete homogenous dissolution of nanosilver in the solvent and enhances the dispersion of silver ions in the culture medium.

In the present study, chitosan had the highest MIC and MBC values. Similarly, Wang *et al*.[48] reported that chitin and chitosan had no antibacterial activity in the agar diffusion test. They used distilled water as the solvent similar to the present study. Future studies are recommended to use acetic acid solvent to better benefit from the antimicrobial activity of chitosan. Unlike the present study, Loyola-Rodríguez *et al*.[16] reported that the addition of chitosan to endodontic sealers enhanced their antimicrobial activity against *E. faecalis* in the agar diffusion test.[16] The antibacterial properties of chitosan derivatives can change depending on physical properties such as distillation, molecular weight, and type of solvent.[49,50] Loyola-Rodríguez *et al*.[16] used acetic acid solvent with high degree of distillation and low-molecular-weight chitosan, which may explain the difference between their results and the present findings. They revealed that nanosilver had higher antibacterial activity than chitosan in the agar diffusion test, which was in line with the results of the microdilution test in the present study. In contrast to the present results, El-Sharif and Hussain[31] reported MIC and MBC of chitosan against *E. faecalis* to be 0.75 and 1.2 μg/μL, respectively. Yadav *et al*.[51] reported a MIC of 4.5 μg/μL for chitosan against *E. faecalis*.[51] This difference can be due to different solvents used in the two studies since we used distilled water.

Among the tested materials in the present study, propolis had high antibacterial activity. It can be used in combination with root canal filling materials due to its optimal biological properties such as enhancement of wound healing and high antimicrobial activity in low concentrations. Nano-zinc oxide showed significantly higher antibacterial activity than micro-zinc oxide.

This study had some limitations. The *in vitro* design of the study may make it difficult to generalize the results to the clinical setting. Furthermore, the agar diffusion test has some limitations such as lack of standardization of inoculum density, agar viscosity, plate storage conditions, size and number of specimens per plate, time and temperature of incubation, and dependence on the solubility and diffusion characteristics of both the test material and media.[52] Further studies are recommended to assess other properties such as biocompatibility, cytotoxicity, and resorption rate of these sealers prior to their clinical use. Furthermore, future studies are required on the effect of different synthesis techniques on antimicrobial properties of propolis, nanosilver, and chitosan. Moreover, the antibacterial activity of tested materials should be assessed against other pathogenic microorganisms. Furthermore, materials that showed the lowest MIC and MBC should be tested in lower concentrations to find their minimum concentration with optimal antibacterial activity. In addition, materials with high MIC and MBC should be tested in terms of safety for human cells. Physical properties of sealers after the addition of propolis should also be investigated.

CONCLUSION

The addition of propolis to nZOE can enhance its antibacterial activity against *E. faecalis in vitro*.

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Conflicts of interest
The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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