Original Article

DOI: 10.14235/bas.galenos.2024.81488
Bezmialem Science 2024;12(4):450-5

Inhibitory Efficacy of *Thymus vulgaris* and *Origanum onites* Essential Oils on *Enterococcus faecalis* Biofilm

Thymus vulgaris ve *Origanum onites* Esansiyel Yağlarının *Enterococcus faecalis* Biyofilmi Üzerindeki İnhibitör Etkinliği

Didem SAKARYALI UYAR[1,2], Aylin ÜSKÜDAR GÜÇLÜ[2]

[1]Başkent University Faculty of Dentistry, Department of Pediatric Dentistry, Ankara, Türkiye
[2]Başkent University Faculty of Medicine, Department of Medical Microbiology, Ankara, Türkiye

ABSTRACT

Objective: The crucial aim of irrigation solutions used in root canal treatments is the elimination of biofilm which is considered an important virulence factor of *Enterococcus faecalis*. Essential oils have been investigated to understand their efficacy for biofilm elimination in recent years. This study aimed to evaluate the antimicrobial and antibiofilm effects of *Thymus vulgaris* essential oils (TEO) and *Origanum onites* essential oils (OEO) on *E. faecalis*.

Methods: The antimicrobial effectiveness of TEO and OEO against *E. faecalis* (ATCC 29212) was investigated by broth microdilution method, and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. The effect of TEO and OEO on preventing biofilm formation was evaluated by measuring biofilm biomass using crystal-violet method, and its effect on biofilm viability was evaluated by determining the number of living cells in the biofilm as colony-forming units. Biofilm viability was analyzed with one-sample t-test with statistical significance accepted as $p<0.05$.

Results: The MIC values of TEO and OEO were determined as 0.078 µL/mL, and MBC values were determined as 0.156 µL/mL for OEO and 0.078 µL/mL for TEO. The percentage inhibition of biofilm formation at MIC value for OEO and TEO was determined as 53.9% and 55.6%, respectively. Both essential oils caused a significant reduction in the number of viable cells within the biofilm.

Conclusion: It is concluded that TEO and OEO show high antimicrobial and antibiofilm activity against *E. faecalis* biofilm.

ÖZ

Amaç: Kök kanal tedavilerinde kullanılan irrigasyon solüsyonlarının en önemli amacı *Enterococcus faecalis'*in önemli bir virülans faktörü olan biyofilmi ortadan kaldırmaktır. Sahip oldukları antimikrobiyal aktiviteyle esansiyel yağların çoğu patojenik mikroorganizmaya karşı alternatif olarak kullanılabilecekleri düşünülmektedir. Bu çalışmanın amacı da *Thymus vulgaris* (kekik) esansiyel yağı (TEO) ve *Origanum onites* (sivri kekik) esansiyel yağlarının (OEO) *E. faecalis* üzerindeki *in vitro* antimikrobiyal ve antibiyofilm etkilerini değerlendirmektir.

Yöntemler: TEO ve OEO'nun *E. faecalis*'e antimikrobiyal etkinliği sıvı mikrodilüsyon yöntemiyle araştırılmış, minimum inhibitör konsantrasyonu (MİK) ve minimum bakterisidal konsantrasyonu (MBK) belirlenmiştir. Çalışmalarda *E. faecalis* ATCC 29212 suşu kullanılmıştır. TEO ve OEO'nun biyofilm oluşumunu engelleme etkisi kristal-viyole yöntemiyle biyofilm biyokütlenin ölçülmesi ve biyofilm canlılığına etkisi ise biyofilm içerisindeki canlı hücrelerin sayısının koloni oluşturan birim olarak belirlenmesiyle değerlendirilmiştir. Biyofilm canlılığı one-sample t-testi ile analiz edilmiş ve istatistiksel anlamlılık $p<0,05$ olarak kabul edilmiştir.

Bulgular: TEO ve OEO'nun MİK değerleri 0,078 µL/mL, MBK değerleri ise OEO için 0,156 µL/mL ve TEO için 0,078 µL/mL olarak belirlenmiştir. OEO ve TEO için MİK değerinde biyofilm oluşumu inhibisyonunun yüzdesi sırasıyla %53,9 ve %55,6 olarak belirlenmiştir. Her iki esansiyel yağ da biyofilm içindeki canlı hücre sayısında önemli bir azalmaya neden olmuştur.

Address for Correspondence: Didem Sakaryalı Uyar, Başkent University Faculty of Medicine, Department of Pediatric Dentistry, Ankara, Türkiye
E-mail: dt_didemsakaryali@hotmail.com **ORCID ID:** orcid.org/0000-0001-7850-2375

Received: 15.02.2024
Accepted: 09.07.2024

Cite this article as: Sakaryalı Uyar D, Üsküdar Güçlü A. Inhibitory Efficacy of *Thymus vulgaris* and *Origanum onites* Essential Oils on *Enterococcus Faecalis* Biofilm. Bezmialem Science. 2024;12(4):450-5

ABSTRACT

Therefore, these essential oils can be considered an alternative irrigation solution for eliminating resistant root canal infections.

Keywords: Antibiofilm activity, biofilm, *Enterococcus faecalis*, minimum inhibitory concentration, minimum bactericidal concentration

ÖZ

Sonuç: TEO ve OEO'nun *E. faecalis* biyofilmine karşı yüksek antimikrobiyal ve antibiyofilm aktivite gösterdiği ve bu esansiyel yağların *E. faecalis* biyofilminin ortadan kaldırılmasını sağlayabileceği ve dirençli kök kanal enfeksiyonlarının eliminasyonunda alternatif irrigasyon solüsyonu olarak değerlendirilebileceği düşünülmektedir.

Anahtar Sözcükler: Antibiyofilm aktivitesi, biyofilm, *Enterococcus faecalis*, minimum inhibitör konsantrasyon, minimum bakterisidal konsantrasyon

Introduction

Microorganisms, found in the oral flora, can adhere better to hard and soft tissue surfaces due to their biofilm formation ability. *Enterococcus faecalis* is one of the important biofilm-forming bacteria, which causes resistant periradicular dental infections. *E. faecalis* settles in root canals, mainly the apical part and forms biofilm layer in deeper dentin tubules (1,2). The extracellular matrix, which forms the basic structure of the biofilm, is a protective polymer structure that bacteria constantly form. As it forms, it ensures that the bacteria are more isolated from the buried external environment (2,3). Therefore, the biofilm layer is considered a small community with three-dimensional signal transport, nutrition, and waste channels that microorganisms create as a suitable habitat for themselves. Because of these channels, microorganisms can protect themselves from changes in the external environment and neutralize the effects of agents such as disinfectants and antiseptics used to destroy them (2).

E. faecalis forms a typical biofilm in which the physicochemical properties are modified to dominate according to the environment. In a typical biofilm, living cells are located on the surface of the biofilm, and irregular growth of adjacent cell clusters is observed in aerobic or anaerobic and nutrient deprived-medium (1,3-5). Therefore, resistant periradicular dental infection develops because of the complex structure of the biofilm and the first step to eliminate the infection should be the destruction of the biofilm. At this point, sodium hypochlorite at a concentration of 0.5-6% is routinely applied in dental treatments as it is a cost-effective irrigation solution to eliminate *E. faecalis* biofilm (5). However, the search for alternative irrigation agents continues due to their toxic and caustic nature to the periradicular tissues (5-7).

When considering agents with antibacterial solid activity as well as minimal tissue irritation, herbal-based agents can be thought. Therefore, natural products with herbal ingredients are used in the development of many oral hygiene products because they have rich antimicrobial components (7,8). *Thymus vulgaris* (thyme) essential oils (TEO) and *Origanum onites* essential oils (OEO) are herbal agents with antibacterial activities, but there is insufficient information in the literature regarding the effectiveness of these essential oils (EOs) on *E. faecalis* biofilms. So, this study aimed to determine the antibacterial activity of TEO and OEO on *E. faecalis* biofilm by evaluating minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and antibiofilm activity at different concentrations.

Methods

This microbiological research study was conducted in accordance with the principles of the Declaration of Helsinki. This study was approved by Başkent University Medical and Health Sciences Research Board Institutional Review Board (approval number: KA22/187, date: 28.04.2022). A series of microbiological tests were conducted to calculate MIC, MBC, biofilm formation inhibition percentage, and biofilm viability effect of different concentrations of TEO and OEO to evaluate their effectiveness on *E. faecalis* biofilm within the scope of this study. *E. faecalis* ATCC 29212 was used for all biofilm and antimicrobial activity tests. TEO and OEO were provided by the manufacturer (Caliskan Agriculture, Türkiye). All the EOs were obtained by the hydro-distillation method.

MIC and MBCs for TEO and EOS

For determining MIC values, broth microdilution method was used as recommended by the Clinical and Laboratory Standards Institute (9). The EOs in combination with 1% dimethyl sulfoxide, were diluted twofold in Mueller Hinton Broth (BD Difco™, France). After 24h of incubation at 37 °C, absorbance was measured at 570 nm (BioTek Instruments, ELX 800, USA). All studies were performed in triplicate. The TEO and OEO concentrations ranged from 0.039-10 µL/mL (10). The wells containing only bacterial suspension were used for bacterial growth control, and sterile Mueller Hinton Broth without bacteria or antibacterial agent was used for sterilization control. Plates were incubated at 37 °C for 24h and each experiment was repeated 3 times. MBC was determined by inoculating 10 µL of suspension from each well in Tryptic Soy Agar (Condalab, Spain) after the incubation period of MIC assay (11). MBC was considered the lowest concentration of the test substance in which no microbial growth was observed after the incubation period at 37 °C for 24h.

Biofilm Formation Assay

The biofilm formation assay was performed as described previously (12). Briefly, flat-bottom polystyrene microtiter plates

containing 180 µL of tryptic soy broth (Condalab, Spain) were inoculated with 20 µL of bacterial culture adjusted to $1x10^6$ CFU/mL. After 48h of incubation, biofilm formation was detected by the crystal violet staining method (CVS) (13). The absorbance was measured at 570 nm (11), and biofilm formation ability was evaluated as negative, weak, intermediate and strong for tested strain (14). *Staphylococcus aureus* ATCC 6538 and *S. aureus* ATCC 29213 were used as a positive and negative controls, respectively.

Calculation of Biofilm Formation Inhibition Percentage of EOs

The effect of TEO and OEO against *E. faecalis* biofilm were tested in 96-well plates at MIC and MBC concentrations and evaluated by comparing untreated wells. Briefly, 100 µL of bacterial suspension for each isolate were added to each well containing different concentration of EOs at MIC, MBC concentrations. After 24h of incubation, CVS was applied, and the OD values were quantified as described above. Wells with PBS were used as a control to calculate the percentage of biofilm inhibition (PI) as follows (11):

PI = [(Control OD570 nm-Sample OD570 nm)/Control OD570 nm] x 100

Effects of TEO and OEO on Biofilm Viability

Biofilm formation was carried out without antimicrobial agent as described above for prolonged time. In this study, the medium was refreshed every 48h and biofilm formation was provided in 2 weeks. At the end of the process, the wells containing enterococcal biofilm were washed twice with saline and 200 µL of each agent was added to each well at different concentrations. Then the sonication procedure (Daihan, Korea) for 10 seconds at 30k Hz was performed, and wells were mixed to remove excess biomass after sonication. To calculate viable microorganisms in the biofilm, serially diluted suspensions were cultured and the plates were incubated for 24h at 37 °C. The colonies were counted and the number of viable cells within the biofilm was calculated as CFU/mL. The number of viable microorganisms was compared with those in the control well with PBS (11).

Statistical Analysis

The IBM SPSS for Windows, version 22.0 was used for the statistical analysis (IBM Corp., USA). MIC and MBC were given as average values of repeated tests while calculation of biofilm formation inhibition was given as percentages. Biofilm viability data of TEO and OEO were analyzed by using one-sample t-test and statistical significance was sat at $p<0.05$.

Results

The MIC and MBC values of TEO and OEO were given in Table 1. MIC values of TEO and OEO were same at 0.078 µL/mL; however, TEO had higher MBC (0.156 µL/mL) than OEO (0.078 µL/mL). Biofilm formation inhibition percentages of TEO and OEO were calculated as 53.9% and 47.9% at 0.078 µL/mL concentrations while 55.6% and 44.8% at 0.156 µL/mL concentration for TEO and OEO, respectively. Antibiofilm activity of TEO and OEO on E. faecalis was given in Figure 1 indicating the OD values at 570 nm.

The effects of TEO and OEO on biofilm viability were shown in Table 2 and Figure 2. It was observed that both TEO and OEO has a significant effect on biofilm viability as both EOs caused sharp decrease in the number of viable cell within the biofilm at 0.078 µL/mL concentration. There was not statistical difference between TEO ($p=0.483$; $p\geq0.05$) and OEO ($p=0.169$; $p\geq0.05$) activities on biofilm viability.

Table 1. Antimicrobial efficacy of TEO and OEO

Essential oils	MIC (µL/mL)	MBC (µL/mL)
TEO	0.078	0.156
OEO	0.078	0.078

TEO: Thyme essential oil, OEO: Oregano essential oil, MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration

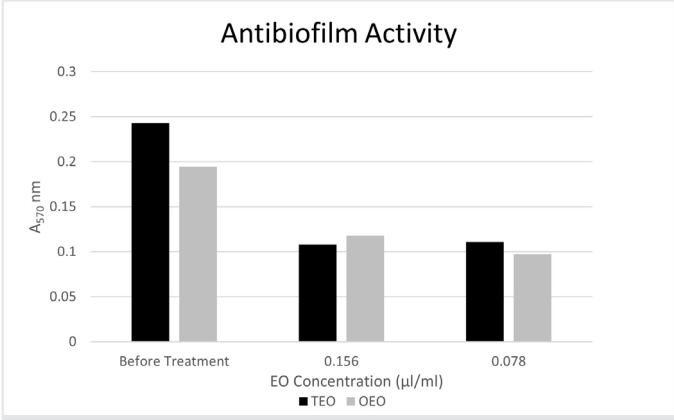

Figure 1. Antibiofilm activity of TEO and OEO on *E. faecalis*
TEO: Thyme essential oil, OEO: Oregano essential oil

Table 2. Effects of TEO and OEO on biofilm viability

EOs	PK	0.019	0.039	0.078	0.156	0.312	0.625	1.25	2.5	5	10	p-value (%95 CI)
TEO	8×10^8	49×10^8	392×10^6	0	0	0	0	0	0	0	0	0.483
OEO	3×10^9	43×10^8	115×10^3	0	0	0	0	0	0	0	0	0.169

TEO: Thyme essential oil, OEO: Oregano essential oil. Concentrations were in CFU/mL units; $p<0.05$ refers significant difference with One Sample t-test

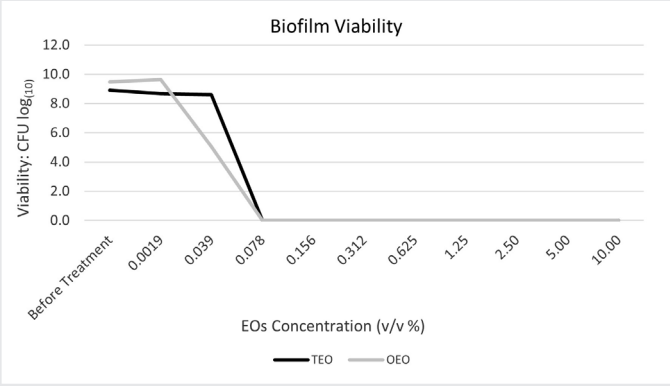

Figure 2. Effects of TEO and OEO on biofilm viability
TEO: Thyme essential oil, OEO: Oregano essential oil

Discussion

Sodium hypochlorite is considered the gold standard for root canal antisepsis and in routine clinical applications, chlorhexidine and sodium hypochlorite are the most frequently used irrigation materials in endodontic treatments to eliminate residual microorganisms (1,8,12). According to the literature on the elimination of endodontic pathogens, chemo-mechanical treatment did not show sufficient success due to the microbial adhesion to the dentin surface because of the specific anatomic structure of the root canal system and the adhesion ability of these endodontic pathogens.

Other than sodium hypochlorite, calcium hydroxide and triple antibiotic paste are the intracanal medicaments that can penetrate deep dentin tubules to eliminate biofilm (6,15). Ethylenediaminetetraacetic acid (EDTA) is one of the most used agents to remove the smear layer from root canals. Since *E. faecalis* biofilm is embedded in deep dentin tubules, EDTA application is assumed to provide an antibacterial effect during the removal of the smear layer. However, its antimicrobial activity against biofilms is still controversial (2). According to the previous *in vitro* studies, irrigation with silver nanoparticles (16), photodynamic therapy with lasers (16,17), or ozone application (18) during the irrigation might increase the antibacterial efficacy of sodium hypochlorite or EDTA.

Tissue-friendly herbal products have been started to be preferred in intracanal irrigation to prevent the exposure of healthy tissues and stem cells around the root from the toxic effects of irrigation solutions or medicaments that overflow from the root apex. Therefore, natural products derived from plants are rich in antimicrobial compounds and are often used to make oral hygiene products. EOs obtained from medicinal plants have been used in the treatment of some diseases due to their antimicrobial effects (18-20). However, there are few studies on their success in root canal treatments (8). According to the improvements in dental materials, the reason for resistant root canal infections seemed to be the presence of some resistant bacteria inside the roots such as *E. faecalis* and its important virulence factor biofilm formation (21,22). Therefore, the aim of this study was to evaluate MIC, MBC, and antibiofilm activity at different concentrations of TEO and OEO to evaluate their efficacy and record them for further laboratory, *in vitro*, and *in vivo* studies.

In the present study, TEO and OEO were preferred to determine antibacterial and antibiofilm efficacies in different concentrations. Thosar et al. (20) reported an *in vitro* study evaluating the antimicrobial activity of TEO and, according to the results zinc oxide cement mixed with TEO showed higher antimicrobial activity than zinc oxide and eugenol group against *Staphylococcus aureus*, *Escherichia coli*, *E. faecalis*, and *Pseudomonas aeruginosa*. Furthermore, Janani et al. (23) reported another *in vitro* study about the antimicrobial efficacy of OEO by evaluating MIC and MBC values amongst *E. faecalis*. According to the results, MIC was found to be 25 µg/mL while MBC was found to be 50 µg/mL. So, OEO was reported to be an effective antimicrobial agent against *E. faecalis*. According to the results of another study (24) comparing the antimicrobial efficacy and removal of the smear layer in dentin tubules, 1% OEO and 5.25% NaOCl had the same antimicrobial activity against *E. faecalis* and 2% or 5% OES had more effective antibacterial action than 5.25% NaOCl. According to these articles, the high antibacterial efficacy of OEO and TEO against *E. faecalis* can be seen. However, within the scope of this study, not only the antimicrobial activity of *E. faecalis* but also the antibiofilm activity of the biofilm it formed was investigated. According to the antibacterial results of these EOs in the present study, TEO and OEO had the same MIC values but, different MBC values. MBC values of TEO were higher than MIC values so, it can be said that TEO might be more unstable and suspicious than OEO according to higher MBC than MIC.

Biofilm formation is considered an important virulence factor of resistant root canal infections. So, in this study, antibiofilm efficacy was evaluated to determine the correct answers for resistant clinical root canal infections. According to the obtained results of biofilm formation inhibition, TEO showed higher inhibition on biofilm formation compared to OEO at both MIC and MBC concentrations. This antibiofilm feature might be a clue for the higher efficacy of TEO rather than OEO at the same MIC value.

According to the results, there were controversial results amongst MBC and biofilm formation inhibition values. TEO had bactericidal efficacy at higher MBC results than OEO. This means suspicious behavior has a toxic effect on tissues while showing higher antibiofilm activity than OEO by biofilm formation inhibition percentages at the MIC level. The results of biofilm viability showed that OEO caused a significant decrease rather than TEO and this result means that it was more effective against the formation of biofilm which is the most important virulence factor of *E. faecalis*. Although, these results showed that both EOs might be used due to higher antimicrobial and antibiofilm activity on *E. faecalis* biofilm even at low concentrations; OEO might be accepted as a more stable EO than TEO due to the same MBC than MIC values and similar antibiofilm activity results before and after the biofilm formation. However, these controversial results should be

evaluated with more *in vitro* studies. A significant cost is spent every year for the treatment of resistant root canal infections. Despite this, patients still need to get the teeth with the high clinical and radiographic success they deserve. Therefore, biocompatible structure and lower costs are important causes besides good antibacterial and antibiofilm effects against *E. faecalis* biofilm to prefer TEO and OEO.

Study Limitation

The strong point of this study was the penetration ability of EOs into dentin tubules. However, to the best of our knowledge, this is the first study comparing the antibacterial and antibiofilm efficacy of TEO and OEO against the *E. faecalis* biofilm. Studying a single bacterial species can be considered as the limitation of the study. The results of the present study should be evaluated for future *in vitro* and clinical studies including *E. faecalis* biofilm.

Conclusion

High antimicrobial effects of different concentrations of TEO and OEO were determined as MIC, MBC, biofilm formation inhibition percentage, and biofilm viability on *E. faecalis* biofilm. Therefore, it can be concluded that TEO and OEO might be used in resistant and recurrent endodontic lesions to destroy and remove the biofilm layer from root canals. However, further microbiological, and clinical studies should be done to evaluate the preliminary results of TEO and OEO and compare them with other irrigation agents such as sodium hypochlorite or chlorhexidine.

Ethics

Ethics Committee Approval: This study was approved by Başkent University Medical and Health Sciences Research Board Institutional Review Board (project no: KA22/187, date: 28.04.2022).

Informed Consent: Informed consent is not required.

Footnotes

Authorship Contributions

Surgical and Medical Practices: D.S.U., A.Ü.G., Concept: D.S.U., A.Ü.G., Design: D.S.U., A.Ü.G., Data Collection or Processing: D.S.U., A.Ü.G., Analysis or Interpretation: D.S.U., A.Ü.G., Literature Search: D.S.U., A.Ü.G., Writing: D.S.U., A.Ü.G.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: This research study was supported by Başkent University Institutional Review Board (project no: DA 22/187).

References

1. Jhajharia K, Parolia A, Shetty KV, Mehta LK. Biofilm in endodontics: A review. J Int Soc Prev Community Dent. 2015;5:1-12.
2. Usha HL, Kaiwar A, Mehta D. Biofilm in endodontics: New understanding to an old problem. J Contemp Dent. 2010;1:44-51.
3. Alghamdi F, Shakir M. The Influence of Enterococcus faecalis as a Dental Root Canal Pathogen on Endodontic Treatment: A Systematic Review. Cureus. 2020;12:e7257.
4. John G, Kumar KP, Gopal SS, Kumari S, Reddy BK. Enterococcus faecalis, a nightmare to endodontist: A systematic review. Afr J Microbiol Res. 2015;9:898-908.
5. Luddin N, Ahmed HM. The antibacterial activity of sodium hypochlorite and chlorhexidine against Enterococcus faecalis: A review on agar diffusion and direct contact methods. J Conserv Dent. 2013;16:9-16.
6. Hecker S, Hiller KA, Galler KM, Erb S, Mader T, Schmalz G. Establishment of an optimized ex vivo system for artificial root canal infection evaluated by use of sodium hypochlorite and the photodynamic therapy. Int Endod J. 2013;46:449-57.
7. Spencer HR, Ike V, Brennan PA. Review: the use of sodium hypochlorite in endodontics--potential complications and their management. Br Dent J. 2007;202:555-9.
8. Zehnder M. Root canal irrigants. J Endod. 2006;32:389-98.
9. Khan A, Miller WR, Axell-House D, Munita JM, Arias CA. Antimicrobial Susceptibility Testing for Enterococci. J Clin Microbiol. 2022;60:e0084321.
10. Juliano C, Magrini GA. Methylglyoxal, the major antibacterial factor in manuka honey: an alternative to preserve natural cosmetics? Cosmetics. 2019;6:1.
11. Üsküdar-Güçlü A, Şimşek D, Ata-Vural I, Ünlü S, Başustaoğlu AC. Antibacterial, antifungal and antibiofilm activity of methylglyoxal: A phytochemical from manuka honey. Mediterr J Infect Microbes Antimicrob. 2021;10:55.
12. Peeters E, Nelis HJ, Coenye T. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J Microbiol Methods. 2008;72:157-65.
13. Ünlü S, Üsküdar Güçlü A, Mirza HC, Altay Koçak A, Başustaoğlu A. In vitro effects of various essential oils on biofilm viability; their antibacterial and antibiofilm activities against clinical Staphylococcus aureus isolates. Turk Hij Den Biyol Derg. 2023;80:491-502.
14. Balaji K, Thenmozhi R, Pandian SK. Effect of subinhibitory concentrations of fluoroquinolones on biofilm production by clinical isolates of Streptococcus pyogenes. Indian J Med Res. 2013;137:963-71.
15. Tennert C, Drews AM, Walther V, Altenburger MJ, Karygianni L, Wrbas KT, et al. Ultrasonic activation and chemical modification of photosensitizers enhances the effects of photodynamic therapy against Enterococcus faecalis root-canal isolates. Photodiagnosis Photodyn Ther. 2015;12:244-51.
16. Afkhami F, Ahmadi P, Chiniforush N, Sooratgar A. Effect of different activations of silver nanoparticle irrigants on the elimination of Enterococcus faecalis. Clin Oral Investig. 2021;25:6893-9.
17. Souza LC, Brito PR, de Oliveira JC, Alves FR, Moreira EJ, Sampaio-Filho HR, et al. Photodynamic therapy with two different photosensitizers as a supplement to instrumentation/irrigation procedures in promoting intracanal reduction of Enterococcus faecalis. J Endod. 2010;36:292-6.
18. Hems RS, Gulabivala K, Ng YL, Ready D, Spratt DA. An in vitro evaluation of the ability of ozone to kill a strain of Enterococcus faecalis. Int Endod J. 2005;38:22-9.

19. Yanling C, Hongyan L, Xi W, Wim C, Dongmei D. Efficacy of relacin combined with sodium hypochlorite against Enterococcus faecalis biofilms. J Appl Oral Sci. 2018;26:e20160608.
20. Thosar NR, Chandak M, Bhat M, Basak S. Evaluation of Antimicrobial Activity of Two Endodontic Sealers: Zinc Oxide with Thyme Oil and Zinc Oxide Eugenol against Root Canal Microorganisms- An *in vitro* Study. Int J Clin Pediatr Dent. 2018;11:79-82.
21. Dioguardi M, Gioia GD, Illuzzi G, Laneve E, Cocco A, Troiano G. Endodontic irrigants: Different methods to improve efficacy and related problems. Eur J Dent. 2018;12:459-66.
22. Sunildath MS, Mathew J, George L, Vineet RV, Thomas P, John D. Canal transportation and centering ability of root canals prepared using rotary and reciprocating systems with and without PathFiles in cone-beam computed tomography-based three-dimensional molar prototypes. J Conserv Dent. 2021;24:246-51.
23. Janani K, Ajitha P, Sandhya R, Teja KV. Chemical constituent, minimal inhibitory concentration, and antimicrobial efficiency of essential oil from oreganum vulgare against *Enterococcus faecalis*: An *in vitro* study. J Conserv Dent. 2019;22:538-43.
24. Ok E, Adanir N, Ozturk T. Antibacterial and smear layer removal capability of oregano extract solution. Eur J Dent. 2015;9:20-4.