Odontology (2024) 112:444–452
https://doi.org/10.1007/s10266-023-00857-2

ORIGINAL ARTICLE

Rational design of EDTA-incorporated nanoflowers as novel and effective endodontic disinfection against biofilms

Tugrul Aslan[1] · **Şeyma Dadi**[2] · **Ozgur Kafdag**[1] · **Nimet Temur**[3] · **Nilay Ildiz**[4] · **Ismail Ocsoy**[3] · **Yakup Ustun**[1]

Received: 27 June 2023 / Accepted: 12 September 2023 / Published online: 3 October 2023

Abstract
The ethylenediaminetetradiacetic acid (EDTA) is one of the most commonly used irrigation solutions. Although EDTA has a very low antimicrobial property, it is used to remove inorganic part of smear layer in areas of root canal system. Herein, we developed EDTA-incorporated nanoflowers (EDTA NFs), for the first time, as novel and effective irrigation solution with quite high antimicrobial property to provide complete disinfection in root canal system. We both systematically elucidated the formation of the EDTA NFs with various techniques, and their catalytic and antimicrobial activities in the presence of hydrogen peroxide (H_2O_2) were documented through intrinsic EDTA property and peroxidase-like activities.

Keywords EDTA nanoflowers · Catalytic activity · Endodontic disinfection · Biofilms

Introduction

Microbial infection of the root canal system is one of the proven main etiologic factors in pulpal and periapical disease [1]. Free-floating microorganisms could be present in the root canal system and these microorganisms have tended to adhere each other to grow a biofilm in the root canal space [2]. When the biofilm is matured, bacteria become more resistant to antimicrobial agents and eventually persistent endodontic infections (PEIs) occur [3]. *Enterococcus faecalis* (*E faecalis*) has been detected in 77% of PEIs and listed as one of the main actors of PEIs [4]. Not only bacteria, but also fungal pathogens have been detected in PEIs. For instance, *Candida albicans (C albicans)* is the most common fungus in the root canal of infected teeth.

To treat the root canal infection, elimination of microbial biofilm is mandatory. Mechanical operation can provide removal of biofilms, and tissue remnants from infected dentin [5]. However, most of the root canal system remains uninstrumented after mechanical operation [6, 7]. Therefore, chemical irrigation solutions have been actively used for eradication of biofilms in uninstrumented areas of root canal system. The most commonly used irrigation solutions are sodium hypochlorite (NaOCl), ethylenediaminetetradiacetic acid (EDTA), and chlorhexidine gluconate (CHX). NaOCl is a proteolytic solution and has potent antibiofilm activity and the ability to dissolve organic tissues [7]. However, it could be harmful to the host, has high toxicity, and can cause allergic reactions owing to cellular destruction effect [8]. CHX is effective on both Gram-positive and Gram-negative bacteria and it has substantively antibacterial action in dentine and has lower tissue toxicity than NaOCl [9], but it is unable to eradicate biofilm or dissolve organic tissue [10]. Alexidine (ALX) is a similar solution (introduced first as mouth-rinse) to CHX that composed from bisbiguanides. ALX has stronger effect than CHX against major virulence factors such as bacterial lipopolysaccharide and lipoteichoic acid, and also it has a longer substantivity than CHX [11, 12]. EDTA is an also another frequently used irrigation solution in endodontic. EDTA is especially used for removing inorganic part of smear layer but it has a very low antibacterial property [10].

✉ Yakup Ustun
yakupustun33@hotmail.com

[1] Department of Endodontics, Faculty of Dentistry, Erciyes University, Kayseri, Turkey

[2] Department of Nanotechnology Engineering, Abdullah Gül University, 38080 Kayseri, Turkey

[3] Department of Analytical Chemistry, Faculty of Pharmacy, Erciyes University, Kayseri 38039, Turkey

[4] Medical Imaging Department, Bandırma Onyedi Eylul University, Vocational School of Health Services, 12 10200 Bandirma, Turkey

There is still need for alternative irrigation solutions for complete and effective disinfection of root canals. In endodontic, new and promising products may produce opportunities to kill bacteria, disruption of biofilm, and control dentinal tubule infection by the advances in nanotechnology [8, 9]. A wide range of nanoparticles (NPs) with a potentially effective antimicrobial activity for endodontic disinfection have been developed. However, these NPs require a prolonged contact time to kill the bacteria, induce some toxicity and tooth coloration, all of which can be considered as significant drawbacks [8, 13]. Recently, discovery of organic–inorganic hybrid nanomaterials (NMs) called "Nanoflowers (NFs)" provided a new class of hybrid NMs with greatly enhanced catalytic activities and stabilities [14]. The main principle of NF formation is to incorporate proteins/enzymes and metal ions especially copper II ions (Cu^{2+}) as organic and inorganic components, respectively, in phosphate-buffered saline (PBS). Benefiting from both different enzymes and metal ions allowed researchers to develop and fabricate NFs with various scientific and industrial applications [15–18]. The recent studies reported that amino acids, standard plant molecules, and whole plant extracts can be alternative organic component to enzymes to produce novel NFs [19–31].

Herein, we reported synthesis of EDTA nanoflowers (EDTA NFs) and exhibited their enzyme like catalytic and antimicrobial activities in the presence of hydrogen peroxidase (H_2O_2) toward guaiacol and model pathogens, respectively, through Fenton reaction. The potential mechanism of Fenton-like reaction relies on transition metal ions. Basically, transition metal (M^{2+}) ions give interaction with H_2O_2 to produce M^{1+}ions in the first step. These M^{1+}ions also reacted with H_2O_2 and highly reactive hydroxyl radicals were generated. Finally, these free hydroxyl radicals attack to model substrates and oxidized them. We also aimed to rationally use EDAT NFs as effective endodontic disinfection agents against biofilms formed by *E faecalis* and *C albicans* microorganisms.

Materials and methods

Chemicals and materials

Ethylenediaminetetraacetic acid (EDTA), copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), salt precursors for preparation of phosphate-buffered saline (PBS) including NaCl, KCl, Na_2HPO_4, KH_2PO_4, $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$ were purchased from Sigma-Aldrich. All chemicals were used as received without purification. Ultrapure water (18.2MΩ; Millipore Co., USA) was used in all experiments.

Synthesis of EDTA NFs

The synthesis of EDTA NFs was carried out using a modified method [24]. Briefly, freshly prepared Cu^{2+} and EDTA solutions were mixed in 10 mM PBS solution (pH 7.4) (final concentrations of Cu^{2+} and EDTA were fixed to 0.08 mM and 0.02 mg/ml, respectively) and the resulting mixture was incubated at room temperature (RT: 25°C) for 3 days without disturbing. After incubation, blue precipitate at the bottom of reaction tube was obtained and considered as indication of EDTA NF formation. The EDTA hold two nitrogen atoms and four carboxyl groups and all may react with Cu^{2+} ions based on experimental conditions to form EDTA NFs.

We also used 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), and N-hydroxysulfosuccinimide (Sulfo-NHS) called EDC/NHS or conventional protein labeling chemistry to actives carboxyl groups of EDTA and provide six bonds between EDTA and Cu^{2+} in the formation of EDTA NF.

Sample preparation

The ethical board of the research foundation of Erciyes University of Medical Sciences in Kayseri, Turkey approved this investigation (Ethics Approval Number: 2018-151). Forty extracted human single-rooted teeth were collected. Roots with previous endodontic treatment, anatomic irregularities or curvatures, fracture lines were excluded. The samples were prepared with some modifications from study of Wu et al [32]. The crown and the apical portion of the tooth were sectioned off with a diamond disk, and the middle third was sectioned with a low-speed sectioning saw (IsoMet 2000 Precision Saw; IsoMet, Buehler, IL) under water cooling. One hundred 6 × 8 × 0.5 mm (width × length × thickness) dentin disks were prepared. The dentin disks were treated with 17% EDTA in an ultrasonic bath for 1 minute to eliminate the smear layer and then rinsed in sterile saline for 1 minute. All dentin disks were sterilized by autoclave at 135˚C for 15 minutes (min). Two dentin disks randomly selected from each group were incubated in brain–heart infusion (BHI, Merck) broth for 24 hours at 37 ˚C to ensure that there was no bacterial contamination.

Treatment of infected specimens

The dentin disks were randomly divided into 6 groups ($n = 20$/group) according to the irrigation solutions (Table 1).

Bacterial strain and biofilm generation

E faecalis (ATCC 29212) was plated in the BHI broth and incubated anaerobically at 37˚C for 24 hr. Also, the fungal

Table 1 Classification of samples

Group No	Irrigation solutions	Number of samples	Microorganism
1	5.25% NaOCl solution	20	*E faecalis* (*n*:10) and *C albicans* (*n*:10)
2	17% EDTA solution	20	*E faecalis* (*n*:10) and *C albicans* (*n*:10)
3	EDTA–Cu^{2+} NF solution	20	*E faecalis* (*n*:10) and *C albicans* (*n*:10)
4	2% CHX solution	20	*E faecalis* (*n*:10) and *C albicans* (*n*:10)
5	1% Alexidine solution	20	*E faecalis* (*n*:10) and *C albicans* (*n*:10)
6	1.5% H_2O_2 solution	20	*E faecalis* (*n*:10) and *C albicans* (*n*:10)

Each experimental group has 2 subgroups according to the microorganism (*E faecalis* (*n*:10) or *C albicans* (*n*:10) used for biofilm formation. Each specimen was immersed in 2 mL of the tested solution for 10 minutes. The samples were gently washed for 1 min with 5 mL distilled water.

suspension of *C albicans* (ATCC 90028) was plated on Sabouraud Dextrose Agar (SDA, Becton, Dickinson and Company), and stored in an incubator aerobically at 37 ˚C for overnight to grow. The colonies of bacteria and fungi were diluted in fresh BHI and Sabouraud dextrose broth medium (SDB,Becton, Dickinson and Company), respectively. After incubated to match the turbidity equivalent to a 0.5 McFarland standard, corresponding to an optical density of 0.08–0.1 absorbance at 600 nm in a spectrophotometer. Sterilized dentin disks (*n*:40) were placed in sterilized 24-well tissue culture plates (Nunc; Thermo Scientific, Darmstadt, Denmark) and inoculated with 3.0 mL *E faecalis* (*n*:20) and *C albicans* (n:20) suspension (1×10^8 colony-forming unit (cfu)/mL for bacteria and 10^7cfu/ml for fungal density) under appropriate conditions for 21 days at 37 ˚C. The inoculation was repeated every 72 h using a 24-h prepared culture to remove dead cells and ensure bacterial viability. After incubation, the specimens were aseptically removed from the wells and gently rinsed with sterile PBS for 1 min to remove loosely attached planktonic bacteria.

Results

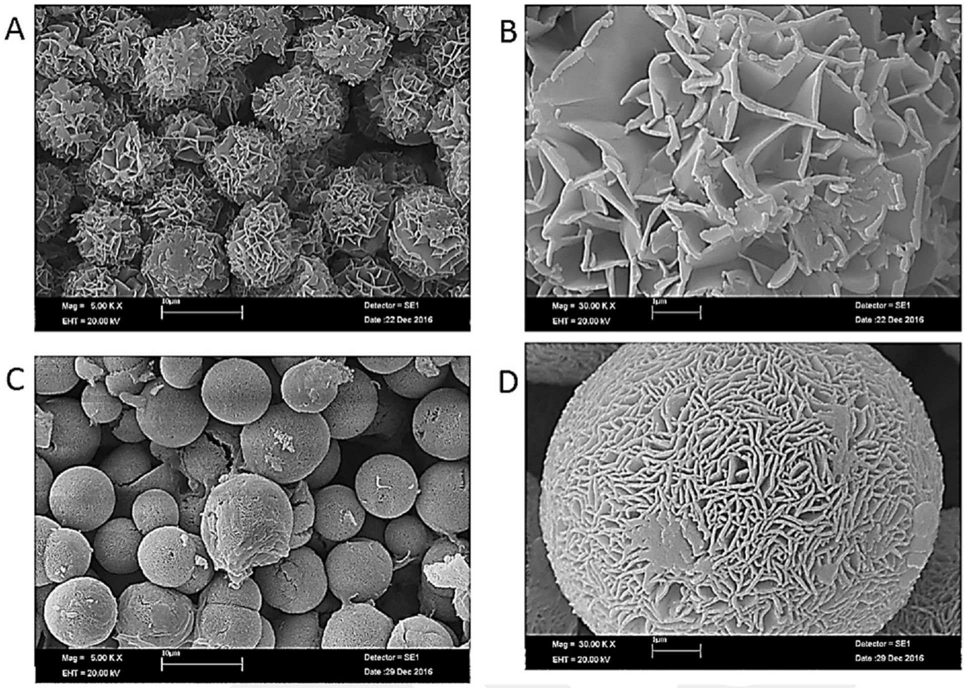
Preparation and Characterization of EDTA NFs

In the synthesis of EDTA NFs, reported strategy was followed with some modification [24]. EDTA was, for the first time, used as organic component and Cu^{2+} ions acted as inorganic component to form EDTA NFs in PBS solution. EDTA contains two nitrogen atoms and four carboxyl groups and gives complexation reaction with various metal ions through four or six binding mechanism. The formation and antibiofilm activity of EDTA NFs are illustrated in scheme. Typically, Cu^{2+} reacts with phosphate ions (PO_4^{3-}) to form $Cu_3(PO_4^{3-})_2$ primary nanocrystals in nucleation step. Then, EDTA molecules give coordination reaction with Cu^{2+} of primary nanocrystals through nitrogen atoms and carboxyl groups to create EDTA–$Cu_3(PO_4^{3-})_2$ petal-like structures in growth step. The petals combine each other to both form large petals and EDTA NFs prior to end of the growth process. In the final step called completion of NF formation, anisotropic growth of EDTA NFs was saturated and eventual EDTA NFs formation was kinetically completed in terms of shape and size. The structures of produced EDTA NFs were evaluated using various characterization techniques including SEM, EDX, FT-IR, and XRD. While the peroxidase-like activity of the EDTA NFs was tested using UV–Vis spectrometry, their antibiofilm or antimicrobial activities were elucidated with SEM and Confocal images.

The EDTA NFs were produced with and without EDC/NHS chemistry to track effect of activated carboxyl groups of morphology of the EDTA NFs. For instance, Fig. 1A, B showed that the EDTA NFs are spherical, monodispersed, uniform and have porous structure. The boundaries of EDTA–$Cu_3(PO_4^{3-})_2$ petals in the EDTA NFs were clearly seen the magnified image of EDTA NFs (Fig. 1). In addition to that, treatment of EDTA molecules with EDC/NHS prior to their addition into Cu^{2+}-containing PBS solution in the EDTA NFs synthesis resulted in more compact structure as shown in Fig. 1C, D. The magnified image in Fig. 1D showed that the EDTA NFs have small pore sizes and blooming structure compared to the EDTA NFs formed without EDC/NHS chemistry.

While Cu^{2+} is considered as corner stone, $Cu_3(PO_4^{3-})_2$ nanocrystals play a role as skeleton in the EDTA NFs formation. The presence of Cu metal in the EDTA NFs was revealed with EDX analysis (Fig. 2A), the diffraction peaks of $Cu_3(PO_4^{3-})_2$ nanocrystals mostly matched with those of JCPDS card (00-022-0548) were characterized in XRD patterns (Fig. 2B). The structures of the EDTA NFs were also analyzed with the FT-IR spectrums (Fig. 2C). The bonds of vibrations in PO_4^{3-} molecule gives the peaks at around 1042 cm^{-1} and 557 cm^{-1}, all of which are sings of PO_4^{3-} incorporation to the EDTA NFs. The bending and stretching peaks of nitrogen atoms can be observed at around to 1621 cm^{-1}. The weak peak of carboxyl group is assigned to ~1500 cm^{-1}. The peaks of CH_2 and OH vibrations were recorded at around 3385 cm^{-1} and 2987 cm^{-1}, respectively.

Fig. 1 **A**,**B** SEM images of EDTA NFs without EDC/NHS, **C**,**D** EDTA NFs with EDC/NHS.



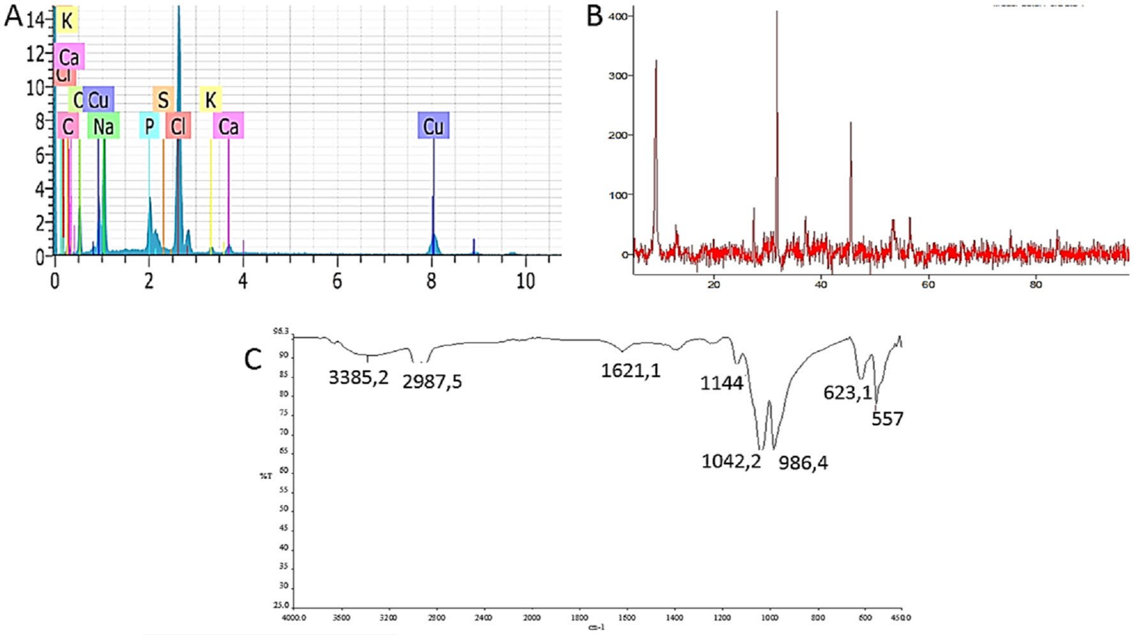


Fig. 2 **A** EDX analysis of the EDTA NFs, **B** XRD pattern of the EDTA NFs and **C** FT-IR spectrum of the EDTA NFs.

Enzyme-like catalytic activity of EDTA NFs

The peroxidase mimic activity of the EDTA NFs was measured toward guaiacol used as a model substrate showed as in Fig. 3A. Typically, the certain amount of EDTA NFs (1mg/mL) was added to 0.1 M, 1 mL of KH_2PO_4 (pH 6.8) solution containing 24 mM hydrogen peroxide (H_2O_2) and 45 mM guaiacol. During incubation of the resulting solution, EDTA NF acted as Fenton agent and oxide the guaiacol to 3,3-dimethoxy-4,4-diphenoquinone through Fenton reaction as presented in Fig. 3B. The absorbance of the product occurred based on oxidation of guaiacol was measured at 470 nm at 25 ˚C via UV–vis spectrophotometer.

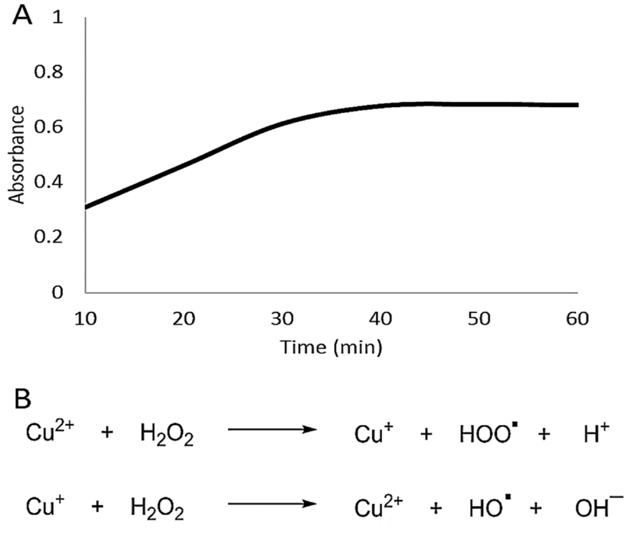


Fig. 3 Enzyme mimic activity of EDTA NFs acted as **A** Fenton agent and **B** Potential Fenton reaction mechanism.

In typical Fenton reaction mechanism, it is well documented that copper-related compounds show peroxidase-like activity in the presence of the H_2O_2. In our system, Cu^{2+} ions in the EDTA NFs react with H_2O_2 and generate Cu^{1+} ions. Those Cu^{1+} ions also give reaction with H_2O_2 and quite reactive hydroxyl radicals were formed. The free hydroxyl radicals initiate the oxidation of model substrate and cascade-like reaction was continued till completion of substrate oxidation. The peroxidase-like catalytic activity of the EDTA NFs is increased dependent upon concentrations of H_2O_2 and the EDTA NFs.

To verify the viability of *E faecalis* and *C albicans* biofilms, two additional randomly selected dentin disks from each group were examined using a confocal laser scanning microscope (Nikon Ti Eclipse Confocal Microscope-Fluorescence Imaging, Amsterdam, Netherlands). The bacterial and fungal biofilm formations on root canal walls and into dentinal tubules were monitored with SEM images in Fig. 4. The images of the biofilms formed by *E faecalis* and *C albicans* are given in Fig. 4A–C and D–F, respectively.

The percentages of bacteria that survived after irrigation on *C albicans* biofilm were 45.84% in 17% EDTA; 19.11% in EDTA CuNC, 4.86 in 1% ALX, 4.18 in 2% CHX, and 4.84 in 1.5% H_2O_2; 12.59 in 5.25% NaOCl were observed (See Table 2).

When the pair wise comparisons were evaluated, it was observed that the antimicrobial efficacy of EDTA NFs solutions increased significantly compared to 17% EDTA($p <$ 0.05), and it was observed that EDTA NFs solution is as effective as ALX, CHX, NaOCl, and H_2O_2 solutions against tested microorganisms and there was no significant difference among them in means viability of *E faecalis* and *C albicans* biofilms after application of solutions ($p > 0.05$).

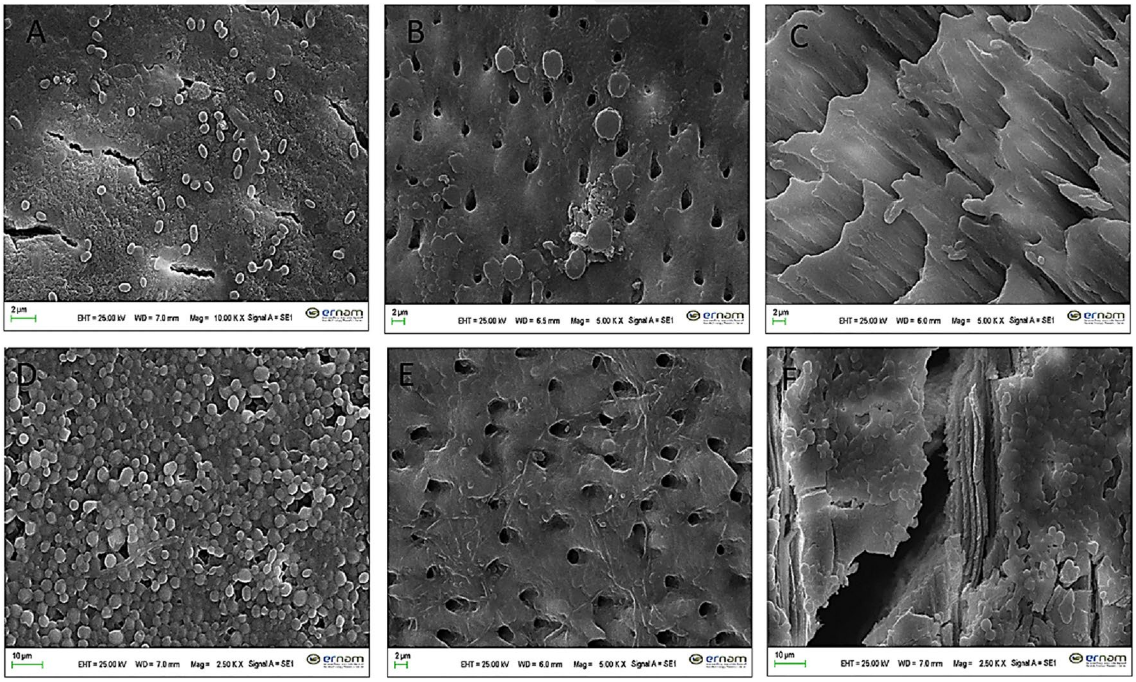


Fig. 4 SEM images of 3-week-old/21-day bacterial biofilms formed on dentin surfaces. **A**,**B** SEM micrographs of *E faecalis* colonies on root canal walls and **C** *E faecalis* colonies into dentinal tubules. **D**,**E** SEM micrographs of *C albicans* colonies on root canal walls and **F** *C albicans* colonies into dentinal tubules.

Table 2 Viability percentage of *E faecalis* and *C albicans* biofilms after treatment with tested irrigation solutions.

Microorganism	Tested Chemicals	Mean (live cells)	95% confidence interval	% viability
Enterococcus faecalis	17% EDTA	70.69	47.78–92.31	36.2
	EDTA NF	18.64	11.72–26.73	10.7
	2% CHX	10.97	14.64–36.58	10.91
	5.25% NaOCl	6.62	− 24.40–37.60	5.56
	1% ALX	12.08	5.98–30.13	11.1
	1.5% H_2O_2	6.64	− 58.35–72.65	6.07
Candida albicans	17% EDTA	173.82	126–228.92	45.84
	EDTA NF	43.87	36.69–49.79	19.11
	2% CHX	7.66	− 23.35–35.57	4.18
	5.25 % NaOCl	17.06	4.38–27.45	12.59
	1% ALX	9.53	− 1.29–22.26	4.86
	1.5% H_2O_2	6.61	− 18.21–31.42	4.84

There was no statistically significant difference between the other groups ($p>0.05$).

The viability of *E faecalis* and *C albicans* after application of chemical test solutions are given in Table 2. The percentages of bacteria that survived after irrigation on the *E faecalis* biofilm were 36.2 in 17% EDTA; 10% in EDTA NFs; 11.1 in 1% ALX; 10.91 in 2% CHX; 6.07 in 1.5% H_2O_2; and 5.56 in 5.25% NaOCl were observed, respectively. (See Table 2).

Discussion

Our results show that EDTA NFs are unique materials for irritation solutions with their catalytic and antimicrobial activities. According to surveys from around the world [33, 34] Sodium hypochlorite was reported as the most common irrigation solution used in endodontics. According to results of this study, NaOCl is the most effective solution over viability of *E faecalis* biofilm but there was no significant difference among the experimental groups. Although NaOCl solutions have a wide range of effect on intracanal microorganisms, unfortunately it has some limitations such as being toxic and non-substantive, ineffective in smear layer removal and corrosive [35, 36], and also when NaOCl is used as a final irrigant, the dentin may be altered and it may affect the bonding of the sealer [37]. Therefore, there is still need for an alternative disinfectant.

Biofilm formations are composed of an extracellular polysaccharide matrix and this matrix makes microorganisms more resistant for different environmental situations [38]. Biofilms developed in vitro for short periods of time may not have the same resistance as a mature biofilm. In this study, a 21-day-old *E faecalis* biofilm was formed based on a previous study that observed mature *E faecalis* biofilm formation after this period of time [39]

In endodontic studies, EDTA solution is used to remove inorganic part of smear layer or any debris during the operation, and it is used in regeneration protocols as last irrigation solution to release growth factors from dentin [40]. On the other hand, it has a very low antibacterial effect [41] only on direct exposure for extended time, 17% EDTA solution extracts bacterial surface proteins by combining with metal ions from the cell envelope, and that causes microorganism death [42].

However, in the present study, 17% EDTA did not effectively inactivate microorganism and/or destruct biofilms (Fig. 5A and B). In contrast to that, benefiting from Fenton reaction property of EDTA NFs in the presence of H_2O_2 makes them quite effective antimicrobial agents. For instance, although *E faecalis* and *C albicans* cells preserve their rigid and intact membrane even after treatment with EDTA, the EDTA NFs led to inactivation of *E faecalis* and *C albicans* with membrane deformation and loss cell structure owing to attraction of generated highly reactive free hydroxyl radicals (Fig. 5C and D). The novel form of EDTA with NFs structures caused destruction of the biofilms which can be observed in SEM images (Fig. 5C and D).

In the present study, EDTA NF solution has nearly a three times more antimicrobial and antifungal activity than 17% EDTA solution. H_2O_2 is a widely used biocide for disinfection and sterilization and it also have use in endodontics as an irrigant.H_2O_2 is active against viruses, bacteria, yeasts, even bacterial spores. It has greater activity against Gram-positive than Gram-negative bacteria. Production of catalase or superoxide dismutase by several bacteria can afford those species some protection against H_2O_2. H_2O_2 produces hydroxyl free radicals (–OH), which attack several cell components such as proteins and DNA [43]. In the present study, H_2O_2 solution was added to start Fenton reaction in EDTA NF solution but we added only H_2O_2 solution group to clarify its own antimicrobial effect on target microorganisms.

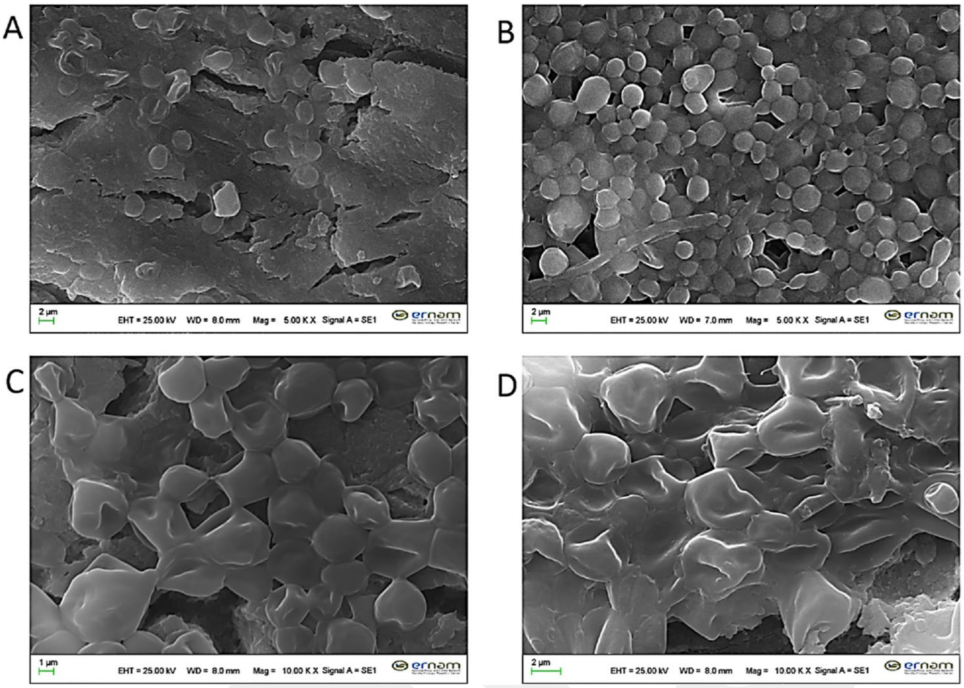


Fig. 5 SEM images of **A** *E faecalis* biofilm and **B** *C albicans* biofilm after 5-min free EDTA treatment. SEM images of **C** *E faecalis* biofilm and **D** *C albicans* biofilm after 5-min EDTA NFs treatment.

H_2O_2 solution was found to be effective on both *E faecalis (*6.07% viability) and on *C albicans* (4.84% viability). By this way, it is understood that both Fenton reaction and H_2O_2 have own antimicrobial–antifungal effect and additive effect on EDTA NF solution.

Kim et al [44] reported in their study that CHX and ALX have similar antibacterial efficiency on *E faecalis* infection. In the present study, CHX and ALX solutions showed similar activity against *E faecalis* and *C albicans* biofilms. Additionally, the efficiency of NaOCl on the against of *E faecalis* and *C albicans* biofilms have been reported in previous studies [45, 46]. In this study, the cell viabilities in the presence of 5.25% NaOCl is lower compared to EDTA NFs solution. However, there is no significant difference among the EDTA NF and considering the toxic nature of NaOCl, EDTA NFs can be used as irrigation solution.

Further investigations with EDTA NFs solution are needed about smear layer removing efficiency and effect on viability on SCAPs.

For further evaluation, confocal microscopy studies were carried out to show inactivation of *E faecalis* and *C albicans* cells (Fig. 6). For LIVE/DEAD staining, the mixture of 1.67 mM SYTO 9 and 18.3 mM propidium iodide (PI) was utilized. While SYTO 9 dye reacts with all microorganism to stain their membranes green, the PI dye only stain dead cells or damaged membranes to red.

Figure 6 A1 and A2 shows how 17% EDTA solution affected cell viability of *E. faecalis* and *C. albicans* cells, respectively. After treatment, these cells with EDTA NFs, cell viability of *E faecalis,* and *C albicans* cells are exhibited in Fig. 6 BA and B2 We interpret that EDTA NFs much effectively killed *E faecalis* and *C albicans* cells 17% EDTA solution. As control %5.25 NaOCl, 1.5 % H_2O_2, 1% ALX, and 2% CHX shows antimicrobial activities for *E faecalis* and *C albicans* cells as presented in Fig. 6C1, C2, D1, D2, E1, E2 and F1,F2, respectively. We concluded with these results that the EDTA NFs displayed highly enhanced antimicrobial activities against both *E faecalis* and *C albicans* cells compared to all irrigation solutions currently used in clinics.

Conclusion

In conclusion, we have synthesized EDTA NFs with their catalytic and antimicrobial activities via Fenton-like reaction in the presence of H_2O_2. We have used EDTA NFs solution as an irrigation solution to remove biofilms caused by *E faecalis* and *C albicans* in the root canal of infected teeth. The structure of biofilms was investigated with SEM and confocal microscopy before and after EDTA NFs treatment.

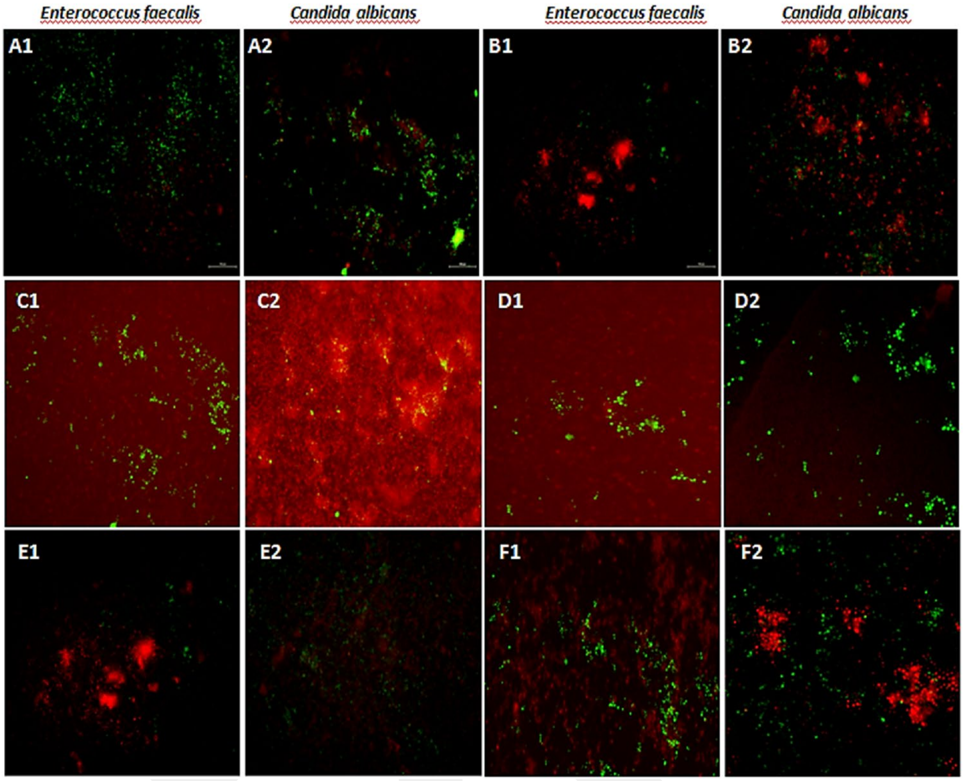


Fig. 6 **A**1 Antimicrobial efficiency of %17 EDTA on *E. faecalis* biofilm, **A**2) Antimicrobial efficiency of %17 EDTA on *C albicans* biofilm, **B**1 Antimicrobial efficiency of EDTA NFs on *E faecalis* biofilm, **B**2 Antimicrobial efficiency of EDTA NFs on *C albicans* biofilm. **C**1 Antimicrobial efficiency of %5.25 NaOCl on *E faecalis* biofilm, **C**2 Antimicrobial efficiency of %5.25 NaOCl on *C albicans* biofilm. **D**1 Antimicrobial efficiency of 1.5 % H_2O_2 on *E faecalis* biofilm, **D**2 Antimicrobial efficiency of %1.5 H_2O_2 on *C albicans* biofilm. **E**1 Antimicrobial efficiency of 1% ALX on *E faecalis* biofilm, **E**2 Antimicrobial efficiency of 1% ALX on *C albicans* biofilm. **F**1 Antimicrobial efficiency of 2% CHX on *E faecalis* biofilm, **F**2 Antimicrobial efficiency of 2% CHX on *C albicans* biofilm.

Acknowledgment This work was supported by grants from the Erciyes University Scientific Research Office (TSA-2018-8023).

Data availability The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing financial interest.

References

1. Kakehashi S, Stanley HR, Fitzgerald RJ. The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. Oral Surg Oral Med Oral Pathol. 1965;20(3):340–9.
2. Nair PR. Light and electron microscopic studies of root canal flora and periapical lesions. J Endod. 1987;13(1):29–39.
3. Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. The calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol. 1999;37(6):1771–6.
4. Fouad AF, Zerella J, Barry J, Spångberg LS. Molecular detection of enterococcus species in root canals of therapy-resistant endodontic infections. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2005;99(1):112–8.
5. Haapasalo M, Shen YA. Current therapeutic options for endodontic biofilms. Endod Top. 2010;22(1):79–98.
6. Peters OA, Schönenberger K, Laib A. Effects of four Ni–Ti preparation techniques on root canal geometry assessed by micro computed tomography. Int Endod J. 2001;34(3):221–30.
7. Paqué F, Ganahl D, Peters OA. Effects of root canal preparation on apical geometry assessed by micro-computed tomography. J Endod. 2009;35(7):1056–9.
8. Shrestha A, Kishen A. Antibacterial nanoparticles in endodontics: a review. J Endod. 2016;42(10):1417–26.
9. Samiei M, Farjami A, Dizaj SM, Lotfipour F. Nanoparticles for antimicrobial purposes in endodontics: a systematic review of in vitro studies. Mater Sci Eng C. 2016;58:1269–78.
10. Heling I, Chandler NP. Antimicrobial effect of irrigant combinations within dentinal tubules. Int Endod J. 1998;31(1):8–14.
11. Zorko M, Jerala R. Alexidine and chlorhexidine bind to lipopolysaccharide and lipoteichoic acid and prevent cell activation by antibiotics. J Antimicrob Chemother. 2008;62(4):730–7.
12. Barrios R, Ferrer-Luque CM, Arias-Moliz MT, Ruiz-Linares M, Bravo M, Baca P. Antimicrobial substantivity of alexidine and chlorhexidine in dentin. J Endod. 2013;39(11):1413–5.
13. Moazami F, Sahebi S, Ahzan S. Tooth discoloration induced by imidazolium based silver nanoparticles as an intracanal irrigant. J Dent. 2018;19(4):280–6.
14. Ge J, Lei J, Zare RN. Protein–inorganic hybrid nanoflowers. Nat Nanotechnol. 2012;7(7):428–32.
15. Ocsoy I, Dogru E, Usta S. A new generation of flowerlike horseradish peroxides as a nanobiocatalyst for superior enzymatic activity. Enzyme Microb Technol. 2015;75:25–9.
16. Sun J, Ge J, Liu W, Lan M, Zhang H, Wang P, Wang Y, Niu Z. Multi-enzyme co-embedded organic–inorganic hybrid nanoflowers: synthesis and application as a colorimetric sensor. Nanoscale. 2014;6(1):255–62.
17. Wang LB, Wang YC, He R, Zhuang A, Wang X, Zeng J, Hou JG. A new nanobiocatalytic system based on allosteric effect with

dramatically enhanced enzymatic performance. J Am Chem Soc. 2013;135(4):1272–5.

18. Celik C, Tasdemir D, Demirbas A, Kati A, Gul OT, Cimen B, Ocsoy I. Formation of functional nanobiocatalysts with a novel and encouraging immobilization approach and their versatile bioanalytical applications. Rsc Adv. 2018;8(45):25298–303.
19. Baldemir A, Kose NB, Ildiz N, Ilgun S, Yusufbeyoglu S, Yilmaz V, Ocsoy I. Synthesis and characterization of green tea (Camellia sinensis (L.) Kuntze) extract and its major components-based nanoflowers: a new strategy to enhance antimicrobial activity. Rsc Adv. 2017;7(70):44303–8.
20. Ildiz N, Baldemir A, Altinkaynak A, Ozdemir N, Yilmaz V, Ocsoy I. Self assembled snowball-like hybrid nanostructures comprising *Viburnum opulus* L. extract and metal ions for antimicrobial and catalytic applications. Enzyme Microb Technol. 2017;102:60–6.
21. Wu ZF, Wang Z, Zhang Y, Ma YL, He CY, Li H, Chen L, Huo QS, Wang L, Li ZQ. Amino acids-incorporated nanoflowers with an intrinsic peroxidase-like activity. Sci Rep. 2016;6(1):22412.
22. Celik C, Ildiz N, Ocsoy I. Building block and rapid synthesis of catecholamines-inorganic nanoflowers with their peroxidase-mimicking and antimicrobial activities. Sci Rep. 2020;10:2903.
23. Koca FD, Yilmaz DD, Onmaz NE, Yilmaz E, Ocsoy I. Green synthesis of allicin based hybrid nanoflowers with evaluation of their catalytic and antimicrobial activities. Biotechnol Lett. 2020;42:1683–90.
24. Dadi S, Celik C, Ocsoy I. Gallic acid nanoflower immobilized membrane with peroxidase-like activity for m-cresol detection. Sci Rep. 2020;10:16765.
25. Koca FD, Yilmaz DD, Onmaz NE, Ocsoy I. Peroxidase-like activity and antimicrobial properties of curcumin-inorganic hybrid nanostructure. Saudi J Biol Sci. 2020;27(10):2574–9.
26. Dadi S, Celik C, Mandal AK, Ocsoy I. Dopamine and norepinephrine assistant-synthesized nanoflowers immobilized membrane with peroxidase mimic activity for efficient detection of model substrates. Sci Rep. 2021;11:117–25.
27. Gul OT, Ocsoy I. Preparation of magnetic horseradish peroxidase-laccase nanoflower for rapid and efficient dye degradation with dual mechanism and cyclic use. Mater Lett. 2021;303: 130501.
28. Gul OT, Ocsoy I. Co-Enzymes based nanoflowers incorporated-magnetic carbon nanotubes: a new generation nanocatalyst for superior removal of cationic and anionic dyes with great repeated use. Environ Technol Innov. 2021;24: 101992.
29. Yilmaz SG, Demirbas A, Karaagac Z, Dadi S, Celik C, Yusufbeyoglu S, Ildiz N, Mandal AK, Cimen B, Ocsoy I. Synthesis of taurine-$Cu_3(PO_4)_2$ hybrid nanoflower and their peroxidase-mimic and antimicrobial properties. J Biotechnol. 2022;343:96–101.
30. Dadi S, Temur N, Gul OT, Yilmaz V, Ocsoy I. In situ synthesis of horseradish peroxidase nanoflower@carbon nanotube hybrid nanobiocatalysts with greatly enhanced catalytic activity. Langmuir. 2023;39(13):4819–28.
31. Dadi S, Cardoso MH, Mandal AK, Franco OL, Ildiz N, Ocsoy Ismail. Natural molecule-incorporated magnetic organic-inorganic nanoflower: investigation of its dual fenton reaction-dependent enzyme-like catalytic activities with cyclic use. ChemistrySelect. 2023;8(13):e202300404.
32. Wu D, Fan W, Kishen A, Gutmann JL, Fan B. Evaluation of the antibacterial efficacy of silver nanoparticles against *Enterococcus faecalis* biofilm. J Endod. 2014;40(2):285–90.
33. Dutner J, Mines P, Anderson A. Irrigation trends among American association of endodontists members: a web-based survey. J Endod. 2012;38(1):37–40.
34. Gopikrishna V, Pare S, Pradeep Kumar A, Lakshmi Narayanan L. Irrigation protocol among endodontic faculty and post-graduate students in dental colleges of India: A survey. J Conserv Dent. 2013;16(5):394–8.
35. Karuvilla JR, Kamath MP. Antimicrobial activity of 2.5% sodium hypochlorite and 0.2% chlorhexidine gluconate separately and combined, as endodontic irrigants. J Endod. 1998;24(7):472–6.
36. Hülsmann MRT, Nordmeyer S. Complications during root canal irrigation. Endod Top. 2007;16(1):27–63.
37. Rocha AW, de Andrade CD, Leitune VC, Collares FM, Samuel SM, Grecca FS, et al. Influence of endodontic irrigants on resin sealer bond strength to radicular dentin. Bull Tokyo Dent Coll. 2012;53(1):1–7.
38. Mohammadi Z, Abbot PV. The properties and applications of chlorhexidine in endodontics. Int Endod J. 2009;42:288–302.
39. Guerreiro-Tanomaru JM, de Faria-Júnior NB, Duarte MAH, Ordinola-ZapatabR Graeff MSZ, Tanomaru-Filho M. Comparative Analysis of *Enterococcus faecalis* biofilm formation on different substrates. J Endod. 2013;39(3):346–50.
40. Hancerliogullari D, Erdemir A, Kisa U. The effect of different irrigation solutions and activation techniques on the expression of growth factors from dentine of extracted premolar teeth. Int Endod J. 2021;54(10):1915–24.
41. Wang D, Shen Y, Ma J, Hancock REW, Haapasalo M. Antibiofilm effect of D-enantiomeric peptide alone and combined with EDTA in vitro. J Endod. 2017;43(11):1862–7.
42. Haapasalom EU, Zandi H, Coil JM. Eradication of endodontic infection by instrumentation and irrigation solutions. Endod Top. 2005;10:77–102.
43. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. Clin Microbiol Rev. 1999;12(1):147–79.
44. Kim HS, Woo Chang S, Baek SH, Han SH, Lee Y, Zhu Q, Kum KY. Antimicrobial effect of alexidine and chlorhexidine against *Enterococcus faecalis* infection. Int J Oral Sci. 2013;5(1):26–31.
45. Tong Z, Zhang Y, Wei X. The effect of human serum and dentin powder alone or in combination on the antibacterial activity of sodium hypochlorite against *Enterococcus faecalis*. Arch Oral Biol. 2019;97:72–6.
46. Oncu A, Celikten B, Aydın B, Amasya G, Açık L, Sevimay FS. Comparative evaluation of the antifungal efficacy of sodium hypochlorite, chlorhexidine, and silver nanoparticles against *Candida albicans*. Microsc Res Tech. 2022;85(12):3755–60.