Original Article

Antimicrobial Activity of Phytic Acid, Citric Acid, and EDTA with and without Propolis against Enterococcus Faecalis and Candida Albicans

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Abstract

Introduction: This study aimed to investigate the antimicrobial efficacy of chelation agents on Enterococcus faecalis (E. faecalis) and Candida albicans (C. albicans) when used alone or in combination with propolis. **Methods:** One hundred fifty mandibular premolar teeth were selected. Each canal was prepared with Reciproc R25. The roots were then divided into two parts along their long axis (n=300). For *E. faecalis* and *C. albicans*, the samples were divided into 16 groups (14 experimental and 2 control) as follows: Group 1A-1B [17% Ethylenediaminetetraacetic acid (EDTA)], Group 2A-2B [10% Citric Acid (CA)], Group 3A-3B [1% phytic acid/inositol hexaphosphate (IP6)], Group 4A-4B (17% EDTA+8 mg/mL propolis), Group 5A-5B (10% CA+8 mg/mL propolis), Group 6A-6B (1% IP6+8mg/mL propolis), Group 7A-7B (8 mg/mL propolis), Control A-B (Dimethyl Sulfoxide). Each tooth was randomly irrigated with 2 mL of one of the group solutions or dispersions for 5 min, and the solutions were examined for the bactericidal effect. **Results:** For *C. albicans*, all groups showed less optical density (OD) than the control group ($P<0.05$). The propolis group and the IP6 group had higher OD values than the CA group ($P<0.05$). For *E. faecalis*, on the other hand, significantly lower OD values were observed in the propolis+ CA group, compared to the CA and propolis groups ($P<0.05$). There was no significant difference between microbial growth among IP6, EDTA, propolis+ CA, propolis+IP6, and propolis+ EDTA groups ($P>0.05$). **Conclusion:** CA and IP6 showed promising results in eliminating *E. faecalis*, one of the collective organisms responsible for failed root canals.

Keywords: Antimicrobial efficacy, Citric acid, EDTA, Endodontic microbiology, Phytic acid

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Introduction

Endodontic treatment aims to completely remove vital or necrotic tissues, microorganisms, and their products from the root canal system (1). The complex structure of the root canal system as well as the resistance of bacterial biofilms, sometimes preclude sufficient and satisfactory root canal preparation (2). *Enterococcus faecalis* (*E. faecalis*) is a bacterial species isolated from the root canals of teeth with permanent periapical periodontitis, capable of adapting to even adverse environmental conditions and forming a biofilm. Similarly, *Candida albicans* (*C. albicans*) is the most frequently isolated species in persistent root canal infections (3). It is critical to eliminate these microorganisms, which are the primary causes of unsuccessful root canal treatment.

The main technique for reducing the bacterial population of root canals is mechanical preparation (3). However, despite mechanical preparation, inaccessible areas and a smear layer consisting of inorganic/organic components usually persist on the root canal walls (4, 5). Therefore, irrigation solutions are integral to conventional root canal therapy (6).

Ethylenediaminetetraacetic acid (EDTA) is the most widely used chelation agent for removing the inorganic portion of the smear layer. In addition, using different concentrations of sodium hypochlorite (NaOCl) in conjunction with EDTA, has been advocated for complete smear layer removal (7). Additionally, citric acid (CA) is a weak acidic root canal irrigant used for removing the smear layer (8). Studies have evaluated CA concentrations between 1%-50% in removing the smear layer and have also investigated its antimicrobial activity (9, 10).

It has been reported that EDTA and CA have little or minimal antimicrobial properties (11, 12); however, a previous study revealed that EDTA could remove 83% of *Streptococcus gordonii* biofilms (13). Recently, phytic acid (inositol hexaphosphate-IP6) has been recognized as an alternative agent with the potential to replace EDTA. It has been established that IP6 is a biocompatible chelating agent on osteoblasts that can remove the smear layer (14). There are, however, few studies in the literature concerning the antimicrobial activity of IP6, which has recently been introduced to endodontic practice (15-17).

Propolis is a suitable agent for eradicating *E. faecalis* and *C. albicans*, which can be used as an alternative canal irrigant (18). Studies have shown that the antibacterial properties of NaOCl and propolis are similar (18, 19). Compared to chlorhexidine (CHX), they have no superiority over each other in destroying bacteria; however, propolis has been shown to reduce the number of bacteria significantly (20). In addition, there are studies in which propolis was used to transport calcium hydroxide material that is an intracanal medicament to root canals (21, 22).

Based on our literature review, no study has yet investigated the impact of using adjunct propolis with chelating agents, such as EDTA, CA, and IP6; on their antimicrobial properties.

In the present study, the authors aimed to investigate the antimicrobial efficacy of chelating agents on *E. faecalis* and *C. albicans* when used alone or in combination with propolis.

Material and Methods

Chemicals

Propolis was commercially supplied from Istanbul Technical University Arı Teknokent Company (BEEO, Istanbul, Turkey), EDTA and CA from Promida Dental (Promida Ltd., Eskişehir, Turkey), and IP6 from Sigma Aldrich (Sigma Aldrich, Saint Louis, USA).

Microorganisms

In this study, test microorganisms included *C. albicans* (ATCC 10231) obtained from Refik Saydam Hygiene Center (Ankara, Turkey) and *E. faecalis* (ATCC 29212) obtained from Dicle University Clinical Microbiology Laboratory (Diyarbakir, Turkey).

Sample Selection and Preparation

The present study was conducted on 150 sound mandibular premolar teeth with mature apices and Vertucci class 1 root canal configuration (23). The specimens were cleaned of surface debris, calculus, as well as tissue residues, and then, stored in normal saline. Teeth were sectioned with a diamond disc under the cementoenamel junction to achieve a typical tooth length of 10 mm. Canal working length was determined with a #15K file (Dentsply Maillefer, Ballaigues, Switzerland), 1 mm short of the apex. According to the manufacturer's instructions, each canal was prepared with Reciproc R25 instruments (VDW, Munich, Germany). The canals were irrigated with 5 mL of 2.5% NaOCl during instrumentation.

The final irrigation used 5 mL of 2.5% NaOCl, 5 mL of 17% EDTA, and 10 mL of distilled water. The antimicrobial testing stage followed a procedure similar to that of Jaiswal et al. (24). The roots were then divided into two parts along their long axis (n=300). Afterward, the samples were sterilized in a steam autoclave at 120˚C for 15 min. The teeth were randomly divided into two main experimental groups (n=140) and two control groups (n=10). Teeth in each group, respectively, are shown in Table I.

Table I. Groups in this study (for experimental groups, n=20 and control groups, n=10).

Enterococcus faecalis	*Candida albicans*
Group 1A: 2 mL 17% EDTA	Group 1B: 2 mL 17% EDTA
Group 2A: 2 mL 10% CA	Group 2B: 2 mL 10% CA
Group 3A: 2 mL 1% IP6	Group 3B: 2 mL 1% IP6
Group 4A: 1 mL 17% EDTA+8 mg/mL propolis	Group 4B: 1 mL 17% EDTA+8 mg/mL propolis
Group 5A: 1 mL 10% CA+8 mg/mL propolis	Group 5B: 1 mL 10% CA+8 mg/mL propolis
Group 6A: 1 mL 1% IP6+8mg/mL propolis	Group 6B: 1 mL 1% IP6+8mg/mL propolis
Group 7A: 2 mL 8mg/mL propolis	Group 7B: 2 mL 8mg/mL propolis
Control A: 2 mL Dimethyl Sulfoxide	Control B: 2 mL Dimethyl Sulfoxide

EDTA: Ethylenediaminetetraacetic acid, IP6: Phytic acid/Inositol hexaphosphate, CA: Citric acid

Every tube was sterilized in an autoclave (Hirayama HV-85L Otoklav, Saitama, Japan) for 20 min at 120 lb. Each tube received 2 mL of Nutrient Broth (NB) and Mueller Hinton Broth (MHB). It was then incubated for 24 h at 37˚C to test the efficacy of the sterilizing process. The MHB was used for *E. faecalis* and NB for *C. albicans*. The teeth were then transferred to a laminar flow hood (BIOASE Class II, Biosafety Cabinet, Shandong, China) for inoculation with 50 µL of *E. faecalis* and *C. albicans* dispersion at 10^8 CFU/mL.

Half of the samples (n=150) were placed in Eppendorf tubes containing 2 mL of medium inoculated with *E. faecalis* and the other half (n=150) with *C. albicans*. The teeth were incubated for three days at 37˚C. Each tube in which the teeth were kept was labeled with a letter (1A-7A and Control A for *E. faecalis* and 1B-7B and Control B for *C. albicans*). Afterward, the teeth were placed in a single collective recipient to allow unbiased irrigation during the assignment. Each tooth was randomly assigned to one of the groups and irrigated with 2 mL of one of the following solutions or dispersions and shaken in a vortex mixture for 5 min: (1A and 1B) 20 teeth were irrigated with EDTA (17%); (2A and 2B) 20 teeth were irrigated with CA (10%); (3A and 3B) 20 teeth were irrigated with IP6 (1%); (4A and 4B) 20 teeth were irrigated with EDTA (17%)+propolis (8 mg/mL); (5A and 5B) 20 teeth were irrigated with CA (10%)+propolis (8 mg/mL); (6A and 6B) 20 teeth were irrigated with IP6 (1%)+propolis (8 mg/mL); (7A and 7B) 20 teeth were irrigated with propolis (8 mg/mL); (Control A and Control B) 10 teeth were irrigated with a dimethyl sulfoxide (DMSO) solution (control). To examine the bactericidal effect of the irrigant, 200 µL of each group's solution samples were filled into a 96-well polystyrene microplate. Turbidity was then measured using a microplate absorbance reader (Thermo Fisher Scientific, Massachusetts, USA) at a wavelength of 585 nm without interference with the testing solution. After the antimicrobial efficacy evaluation, apical, middle, and coronal section images were taken from one sample of each group using a scanning electron microscope in order to observe the smear layer (Figures1-8).

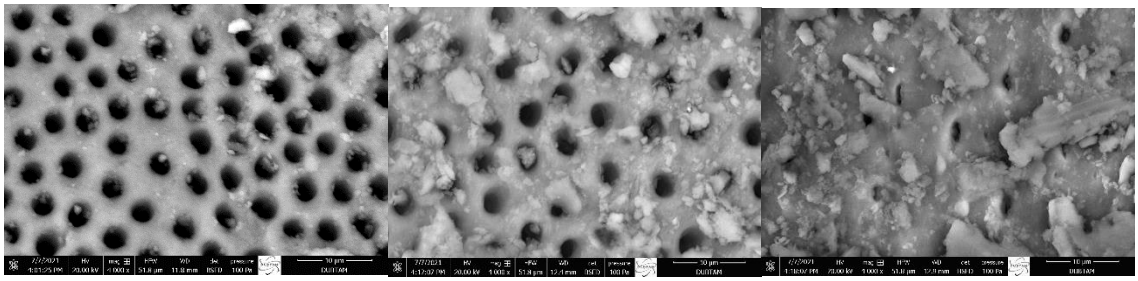


Figure 1. Coronal, middle, and apical section images (×4,000) in the Ethylenediaminetetraacetic acid group (left to right order)

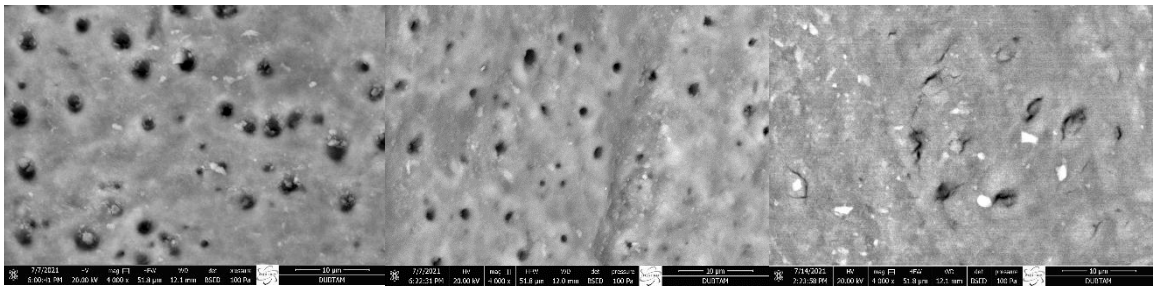


Figure 2. Coronal, middle, and apical section images (×4,000) in the Ethylenediaminetetraacetic acid+ propolis group (left to right order)

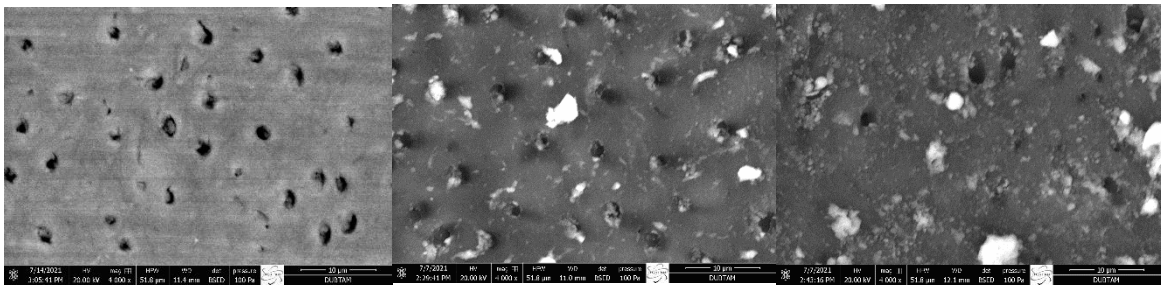


Figure 3. Coronal, middle, and apical section images (×4,000) in the Inositol hexaphosphate group (left to right order)

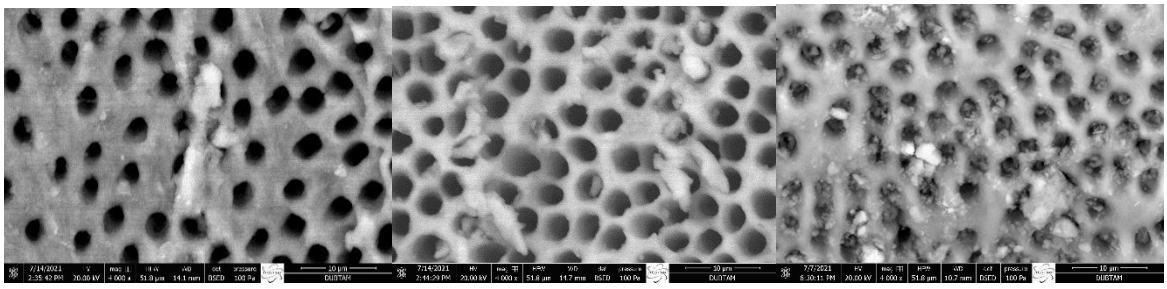


Figure 4. Coronal, middle, and apical section images (×4,000) in the Inositol hexaphosphate+ propolis group (left to right order)

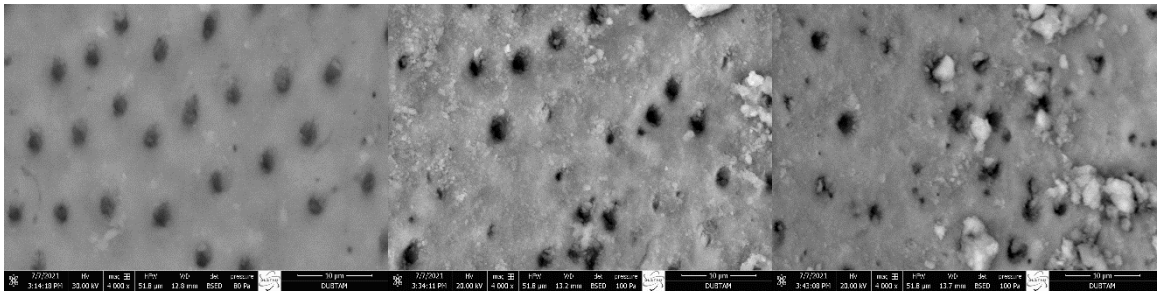


Figure 5. Coronal, middle, and apical section images (×4,000) in the Citric acid group (left to right order)

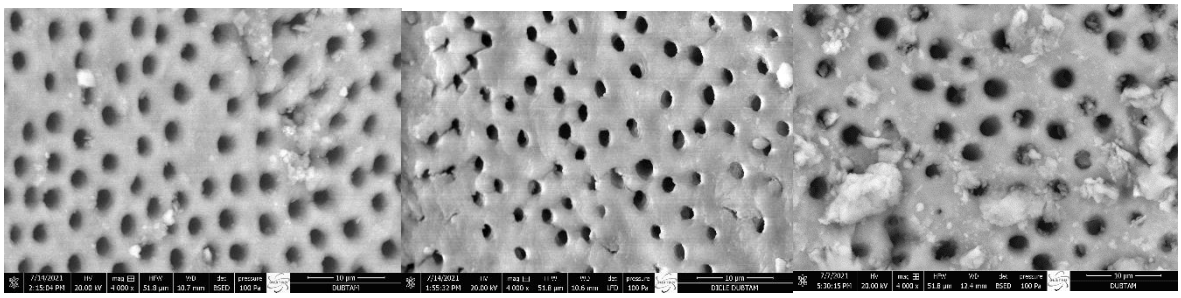


Figure 6. Coronal, middle, and apical section images (×4,000) in the Citric acid+propolis group (left to right order)

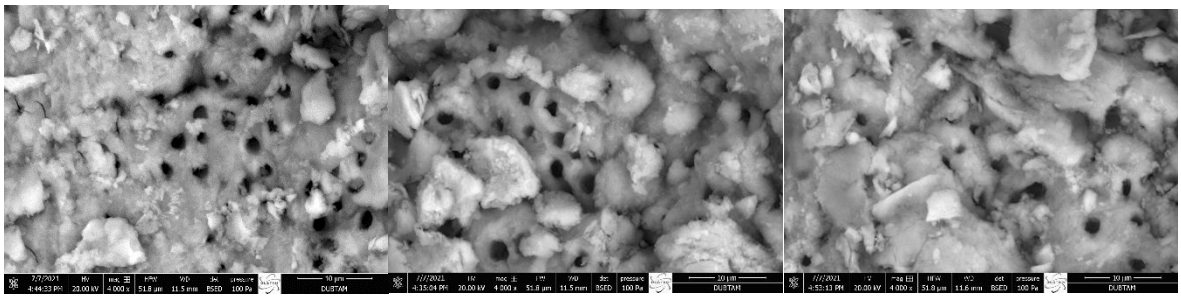


Figure 7. Coronal, middle, and apical section images (×4,000) in the control group (left to right order)

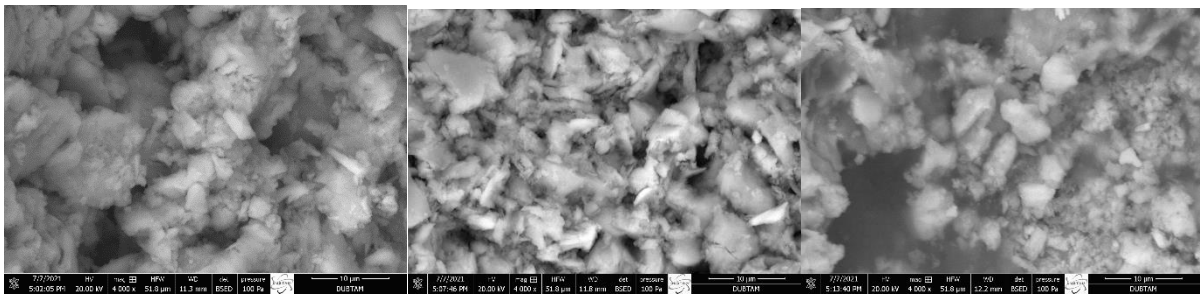


Figure 8. Coronal, middle, and apical section images (×4,000) in the propolis group (left to right order)

Statistical Analysis

Statistical analysis was performed using the SPSS software (Version 22, IBM Corp, Armonk, New York, USA). Since the data sets did not show normal distribution, based on the Shapiro-Wilk normality test, the groups were compared using the Kruskal Wallis H test. Median, minimum, and maximum values were calculated for each group, and the threshold for statistical significance was set at P<0.05.

Ethics committee approval

The study protocol was approved by the Local Ethics Committee of the Dicle University, Faculty of Dentistry (Meeting Number: 2021-24).

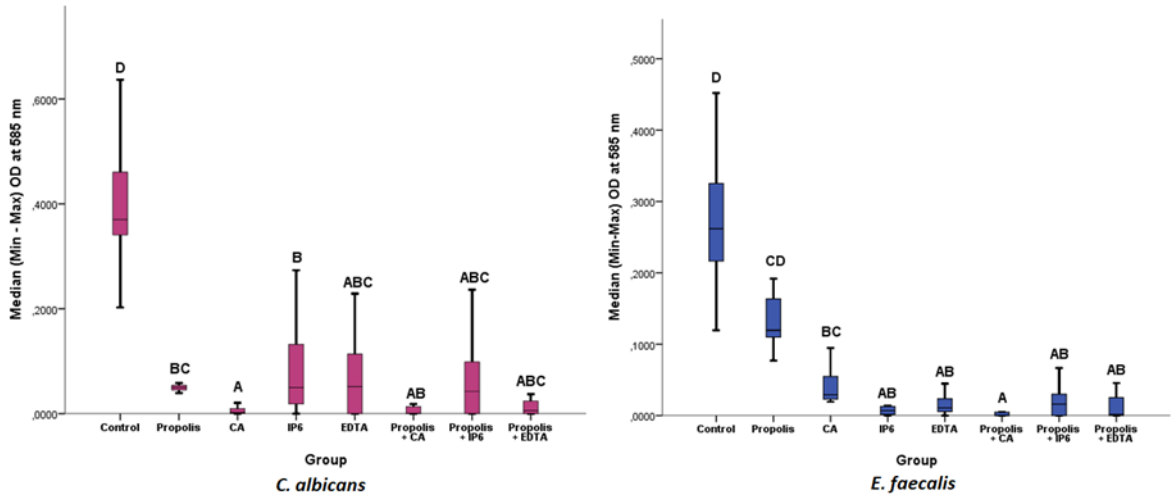
Results

Graph 1 graphically displays the optical density (OD) values observed for samples from all groups. Table II shows the median (minimum-maximum) OD values from all groups. For the *C. albicans*, all groups showed less OD than the control group (P<0.05). The OD values in the propolis group were significantly higher than those in the CA group (P<0.05). The IP6 group had significantly higher OD values than the CA group (p<0.05). The bacterial growth did not differ significantly in EDTA, propolis+IP6, and propolis+EDTA groups (P>0.05). Regarding *E. faecalis*, a significant difference was found between the control group and other groups, except for propolis (P<0.05). The bacterial growth did not differ significantly between the control and propolis groups (P>0.05). On the other hand, significantly lower bacterial growth was observed in the propolis+CA group, compared to the CA and propolis groups (P<0.05). This shows that the combination of propolis+CA has a higher antibacterial effect. The bacterial growth, however, did not differ significantly among IP6, EDTA, propolis+CA, propolis+IP6, and propolis+EDTA groups (P>0.05).

Table II. The median, minimum, and maximum optical density values in the experimental and control groups for Candida albicans and Enterococcus faecalis

Group	*Candida albicans*	*Enterococcus faecalis*
Propolis+EDTA	0.01 (0-0.09)^{ABC}	0 (0-0.07)^{AB}
Propolis+IP6	0.04 (0-0.39)^{ABC}	0.02 (0-0.08)^{AB}
Propolis+CA	0 (0-0.20)^{AB}	0 (0-0.04)^{A}
EDTA	0.05 (0-0.33)^{ABC}	0.01 (0-0.11)^{AB}
IP6	0.05 (0-0.27)^{B}	0.01 (0-0.19)^{AB}
CA	0 (0-0.05)^{A}	0.03 (0.02-0.09)^{BC}
Propolis	0.05 (0.04-0.20)^{BC}	0.12 (0.08-0.30)^{CD}
Control	0.37 (0.20-0.64)^{D}	0.26 (0.12-0.45)^{D}

Data followed by superscript capital letters in columns are statistically different (P<0.05), EDTA: Ethylenediaminetetraacetic acid, IP6: Phytic acid/Inositol hexaphosphate, CA: Citric acid



Graph 1. The median, minimum, and maximum optical density values in the experimental and control groups for Candida albicans and Enterococcus Faecalis. The bars marked with the same letter did not show statistically significant differences from each other in the statistical analysis (P<0.05).

In the groups with the chelating agent, fewer dentin tubules were open and more debris were accumulated from the coronal section to the apical section (Figure 1-6). In addition, the dentinal tubules were not open, as expected, since propolis and DMSO groups did not affect the smear layer (Figure 7-8).

Discussion

The removal of microorganisms from the root canal is an essential requirement for a successful endodontic procedure. Few, if any, studies have investigated the use of endodontic chelators combined with propolis against *C. albicans* and *E. faecalis* microorganisms, which are considered resistant to endodontic treatment. The present study aimed to compare the antibacterial effect of IP6, CA, EDTA, and their combinations with propolis, on *E. faecalis* and *C. albicans*.

A study using different percentages of CA, alone and modified with CHX, showed that 10% CA was not effective against *C. albicans* (25). Smith and Wayman indicated that CA did not show any antimicrobial effect against *C. albicans* and *E. faecalis*. They concluded that the higher the concentration of CA solution, the higher the antimicrobial capacity (26). Another study evaluating

the minimum bactericidal concentration of phosphoric acid, CA, and EDTA solutions for *E. faecalis* determined the contact time required for 10% and 25% CA and 17% EDTA to exert bactericidal activity. It was reported that the EDTA solution did not show any bactericidal effect even after 60 min of application, while 10% and 25% CA solutions inhibited the growth of *E. faecalis* within 3 min of contact (27). Contrary to these findings, data has also been obtained in the literature on the antibacterial effect of EDTA solution. In a study, Şen et al. compared 2.5% and 5% NaOCl, CHX, EDTA, as well as routine antifungal medications, and found the highest antifungal effect in EDTA (28). In another study, Dagna et al. compared 5% NaOCl, Cloreximide, 3% hydrogen peroxide, and 17% EDTA regarding antimicrobial efficacy against *E. faecalis* and *Streptococcus mutans*. They stated that EDTA had a good antimicrobial effect on these microorganisms and attributed this to its ability to separate the biofilm from the root canal walls (29). The present study revealed that EDTA has an antimicrobial activity similar to IP6 and CA on both *E. faecalis* and *C. albicans*. Its combination with propolis, however, did not induce a significant difference in this activity.

In this study, the use of 10% CA, alone or combined with propolis, significantly reduced bacterial growth. The reason for this difference may be the longer incubation period (5 min) with chelator agents and the chelator's increased percentage in this study. In a study comparing the antibacterial activity of irrigants on Gram-negative and facultative anaerobic bacteria, the substances with the highest antimicrobial activity were listed in descending order: 4% NaOCl; 2.5% NaOCl; 2% CHX; 0.2% CHX, EDTA, and CA; as well as 0.5% NaOCl (30). In a recent study, Sowjanya et al. found that IP6 showed a greater diameter of zone inhibition than NaOCl against *E. faecalis* (17). In another study conducted by Puvvada et al., in which they compared the antimicrobial effects of IP6, CHX, NaOCl, IP6+NaOCl, and IP6+CHX solutions, it was reported that IP6 alone showed greater inhibition zones, and its antimicrobial activity was higher, compared to other irrigants. The study also reported that IP6 enhanced the antimicrobial effect of NaOCl and CHX against *E. faecalis* quite well (15). The present study found that in addition to IP6, the combination of IP6+propolis also had antimicrobial effects on *E. faecalis*. Furthermore, it was found that IP6 showed relatively less antimicrobial activity against *C. albicans* than the other groups, while it exhibited acceptable antimicrobial activity against *E. faecalis*. Therefore, IP6 may be an alternative antimicrobial chelator to EDTA in retreatment of failed root canal treatments.

NaOCl continues to be the gold standard for canal irrigation. Some studies, comparing propolis and NaOCl, have shown that the antibacterial properties of propolis and NaOCl are similar (24, 31). Therefore, this study attempted to improve the antimicrobial effect of chelators with propolis. When tested on *C. albicans*, there was no significant difference between groups combined with propolis and groups with a solitary chelator. Regarding. In contrast, combination of CA with propolis significantly increased its antimicrobial activity on *E. faecalis*, compared to the use of solitary CA.

In the present study, when chelators were used against *C. albicans* in combination with propolis or alone, the combination of propolis relatively increased the antibacterial effect of the chelators. However, this difference was not statistically significant. For *E. faecalis*, only the combination of CA with propolis increased the antibacterial effect, compared to the use of this chelator alone. Additionally, all chelators showed significantly greater antimicrobial activity than the control group.

This study attempted to develop a novel final root canal irrigant to eliminate the smear layer and disinfect the root canal. However, at present, no single irrigant is capable of eliminating both the inorganic structure and the organic structure of the smear layer. Therefore, the current irrigation protocol recommends using NaOCl to debride the organic component of the smear layer, followed by 17% EDTA to demineralize the inorganic component of the smear layer. Finally, rinsing with a final irrigant with an antiseptic, such as NaOCl or CHX, is recommended to kill any remaining bacteria in the root canal. Combinations of CA with and without propolis can be an alternative to the final irrigant used in root canal preparation.

The cell toxicity and biofilm disinfection efficacy of the combined irrigant should also be studied. The stability of the combined irrigant is unknown. Further studies are needed to verify whether solutions with added propolis interfere with the chelation mechanism of IP6, CA, and EDTA, or whether propolis would modify the toxicity of this acid solution.

Conclusion

CA and IP6 showed promising results in eliminating *E. faecalis*, one of the collective organisms responsible for failed root canal therapies. This study demonstrates that the combination of propolis and root canal chelating agents ameliorates the disinfection of root canals. However, further research is necessary to evaluate its biocompatibility and ability to remove the smear layer.

Conflict of interest

The authors declare no conflict of interest.

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