

## Antibacterial effect of chlorhexidine-cetrimide combination, *Salvia officinalis* plant extract and octenidine in comparison with conventional endodontic irrigants

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The aim of the present study was to compare the antimicrobial effect of sodium hypochlorite (NaOCl), 2% chlorhexidine (CHX), a CHX/cetrimide solution (CHX+CTR), octenidine hydrochloride (OCT) and *Salvia officinalis* plant extract against *Enterococcus faecalis*. Seventy decoronated single-rooted human teeth were infected and divided into 6 test ( $n=10$ ) and 2 control groups ( $n=5$ ) (negative, sterile samples and positive, infected samples). Following irrigants were then applied to test groups: 2.5% NaOCl, 5.25% NaOCl, CHX, CHX+CTR, *S. officinalis* extract and OCT. The dentin chips were obtained from inner root canal walls and analyzed by counting the number of colony forming units (CFU). The 2.5% NaOCl, 5.25% NaOCl, CHX and OCT groups presented no bacterial growth (CFU=0). *S. officinalis* and CHX+CTR groups reduced the number of *E. faecalis* cells but could not eliminate all. OCT may have potential as an endodontic irrigant in treatment of infected root canals.

**Keywords:** Cetrimide, Chlorhexidine, *Enterococcus faecalis*, Octenidine hydrochloride, *Salvia officinalis*

### INTRODUCTION

Bacteria are the primary cause of the development of necrotic pulps, periapical pathosis and post-treatment disease following root canal treatment<sup>1</sup>. Hence, the eradication of microorganisms and their by-products from the root canal system is compulsory for the success of the endodontic treatment<sup>2</sup>. While mechanical instrumentation is one of the most important steps in the control of root canal infection, it cannot achieve total elimination of bacteria itself. Due to the complexity of root canals, as they contain many cul-de-sacs, fins and lateral canals, almost half of the root canal walls are left unprepared with only instrumentation<sup>3</sup>. Therefore, the use of antimicrobial irrigation solutions has been advised as an adjunct to mechanical instrumentation<sup>4</sup>.

*Enterococcus faecalis*, a facultative anaerobic gram-positive bacterium, is responsible for endodontic treatment failures and asymptomatic persistent infections<sup>5</sup>. Once a root canal is invaded by *E. faecalis*, it becomes harder to disinfect the root canals<sup>6</sup>. Difficulty in eliminating *E. faecalis* from the root canals may be related to the resistance of *E. faecalis* to antimicrobial agents as well as its ability to penetrate deep into dentinal tubules and to form biofilm<sup>7</sup>. In order to promote success in endodontic therapy, it is crucial to eliminate the bacteria responsible for root canal failures as part of study designs. If irrigants can be proven effective against *E. faecalis*, it is likely they will decrease the rate of endodontic treatment failures.

Sodium hypochlorite (NaOCl) is a household bleaching material that has been used in concentrations

ranging from 0.5 to 5.25%. It is the most popular root canal irrigant with its antibacterial activity and dissolving effects on the necrotic tissues. NaOCl ionizes in water into sodium and hypochlorite ions, establishing equilibrium with hypochlorous acid. The excellent antibacterial activity of NaOCl is derived from this acid. It disrupts several vital functions of the bacteria, resulting in bacterial death<sup>8</sup>. However, NaOCl is highly toxic and can cause damage when in contact with the periradicular tissues, especially at high concentrations<sup>9</sup>.

Chlorhexidine gluconate (CHX) has been recommended as an alternative to NaOCl due to its relative absence of toxicity and high antimicrobial efficacy<sup>9,10</sup>. Antibacterial activity of CHX is also effective for up to 12 weeks. However, CHX does not have any capacity to dissolve pulp tissue and remove debris from the root canal walls<sup>11</sup>. Cetrimide (CTR) is a cationic surfactant that reduces the surface tension of solutions, thereby improving their contact with surfaces of difficult access<sup>12</sup>. It was reported that incorporation of CTR into CHX solution improves the antibacterial effects of CHX<sup>13,14</sup>.

Octenisept<sup>®</sup> is a new antiseptic used in the treatment of traumatic, acute, chronic, surgical or burn wounds, and for mucose membrane disinfection and mouth rinses consisting of octenidine hydrochloride (OCT) and phenoxyethanol. OCT possesses broad spectrum antimicrobial effects against both gram-positive and gram-negative bacteria, fungi, yeasts and some virus species<sup>15-20</sup>. Previous studies have demonstrated that OCT compared favourably with CHX against dental plaque-forming bacteria<sup>17,21</sup>.

Most of the irrigation solutions commonly used in

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endodontic treatment still have a number of issues, which means that an alternative solution with good antimicrobial activity, less toxicity and better tissue dissolving properties is still being researched. The role of natural extracts for endodontic purpose has been researched for plants such as *Arctium lappa*, *Morinda citrifolia*, *Triphala*, Green Tea Polyphenols, *Liquorice*, *Ocimum sanctum*, *Cinnamomum zeylanicum* and *Syzygium aromaticum* due to their antimicrobial efficacy against *E. faecalis*<sup>22-26</sup>. *Salvia officinalis*, commonly known as sage, belongs to the Lamiaceae (Labiatae) family. It has been generally recognized as safe (GRAS) for its intended use by U.S. Food and Drug Administration (FDA) as per 21 CFR section 182.20 and 21CFR977029-66-5. *S. officinalis* is used both in culinary and medicinal preparations<sup>27</sup>. Therapeutic effects including antioxidant, antispasmodic, antimicrobial, anti-inflammatory, carminative and mucolytic agent; as well as a hormonal regulator and to control mild to moderate states of Alzheimer's disease, reducing patients agitation have already been reported<sup>27-34</sup>. Although data from the previous studies is not strictly relevant to endodontics, the safe use of *S. officinalis* extract for the treatment of several human ailments generally refers to its use will most likely be safe for endodontic purposes as well. To our knowledge, this is the first study evaluating the antimicrobial effectiveness of *S. officinalis* against *E. faecalis* infection in root canals.

The main aim of the present study was to assess the antimicrobial activity of the methanol extract of *S. officinalis*, CHX+CTR and OCT as irrigation solutions against *E. faecalis* in comparison with the conventional irrigants (NaOCl and CHX) currently used in root canal treatment by using a dentin block model.

## MATERIALS AND METHODS

### *Preparation of root samples*

This research was conducted according to strict compliances outlined by World Medical Association Declaration of Helsinki. The teeth used in this study were extracted for medical reasons, and all patients gave informed consent before sample collection. Seventy freshly extracted single straight-rooted teeth were used. The study included teeth with complete root formation and root length equal to or longer than 8 mm. Exclusion criteria included teeth with cracks or fractures and teeth with obliterated and/or calcified canals. The teeth were stored in a 0.01% NaOCl (Caglayan Kimya Konya, Turkey) until use. The root surfaces were carefully cleaned of any calculus or soft tissue debris. The crowns were removed with a diamond bur (MANI, Tochigi, Japan) under continuous water cooling, leaving roots 8 mm in length. The working length of each tooth was then determined visually by subtracting 1 mm from the length of an inserted #15 K-file (Dentsply Maillefer, Ballaigues, Switzerland) with its tip visualized at the apical foramen. The canals of the roots were then prepared with ProTaper series (Dentsply Maillefer) NiTi rotary files in a crown-down manner. The ProTaper files

were used as Sx-F3 according to the manufacturer's instructions. Between each file, the canals were irrigated with 1 mL of 1% NaOCl (Caglayan Kimya). Subsequently, the root samples were rinsed with distilled water, placed into vials containing phosphate-buffered saline (PBS) solution and sterilized in a steam autoclave (HMC Hirayama, Saitama, Japan) for 20 min at 121°C. Finally, the root canals were dried using sterile paper points (Gapadent, Hamburg, Germany).

### *Preparation of bacterial cell suspension*

*E. faecalis* (A197A) was adapted and maintained on tryptone soy broth (TSB; BioMerieux, Charbonnières-Bains, France) with 2 mg mL<sup>-1</sup> streptomycin, was used as a test microorganism. Streptomycin sulphate (AppliChem, Darmstadt, Germany) at a concentration of 2 mg mL<sup>-1</sup> was included in all growth media during the study to avoid possible contamination of the experimental setup. A loopful of *E. faecalis* was incubated in 5 mL TSB at 37°C overnight. After 12-h incubation, the suspension was spectrophotometrically adjusted to an optical density of 500 at 600 nm to obtain a standard bacterial solution.

### *Infection of root samples with E. faecalis*

The root samples were randomly divided into 6 experimental ( $n=10$ ), a positive control group ( $n=5$ ) and a negative control group ( $n=5$ ) and placed into separate tubes. Sterile root canals were used as a negative control group to show reliability of test procedures. Experimental and positive control groups were infected with *E. faecalis* suspension for 21 days at 37°C, and the media was changed every second day with a fresh 5 mL suspension having same optical density to achieve sufficient bacterial growth. The canals were dried with sterile paper points before applying the test irrigation solutions to the root canals.

### *Test irrigation solutions*

Experimental groups tested were:

- Group 1: 2.5% NaOCl (Caglayan Kimya)
- Group 2: 5.25% NaOCl (Caglayan Kimya)
- Group 3: 2% CHX (Klorhex<sup>®</sup>, Drogosan, Turkey)
- Group 4: CHX+CTR (Savlex<sup>®</sup>, Drogosan)
- Group 5: Methanol extract of *S. officinalis* (The fresh-cultivated extract was obtained from Selcuk University, Faculty of Agriculture)
- Group 6: OCT (Octenisept<sup>®</sup>, Schülke & Mayr, Germany)

Infected samples with no irrigant application served as the positive control group.

Following application of 5 mL of each test solution via a syringe with a 25-gauge needle for 3 min, the canals were rinsed with 1 mL of distilled water, dried with sterile paper points and stored in a freezer for 1 h at -27°C.

### *Microbiological analysis*

After 1 h of freezing at -27°C, a 3-mm apical portion of the root samples was resected. The dentin powder was

removed from inner root canal lumen with the sequential use of sterile gates-glidden burs (#3, 4 and 5) (MANI) at low speed. Dentin samples were obtained directly over separated test vials containing 2 mL of phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA) and glass beads. For each sample, new sterile gates-glidden burs were used. The dentin shavings collected in the vials were then vigorously shaken on a Vortex mixer (VWR, Bedfordshire, UK) for 30 s. PBS with resuspended *E. faecalis* cells was serially diluted (1:10, 1:100, 1:1,000, 1:10,000) by transferring 100  $\mu$ L into a test tube containing PBS, and two droplets of 25  $\mu$ L from each of the four parallel dilutions were inoculated on trypticase soy agar (TSA; BioMerieux) plates and incubated at 37°C for 48 h. All steps of this experiment were performed under strict asepsis in a laminar flow hood (Labconco, MO, USA) to avoid contamination from outside microorganisms. A CFU counting technique was used for recovery of viable *E. faecalis*.

#### Scanning electron microscopy

Scanning electron microscopy (EVO LS10, Zeiss, Oberkochen, Germany) was used to visualise the establishment of bacterial colonisation on dentinal walls in the positive control group and to confirm no bacterial growth in the negative control group. Scanning electron microscopy (SEM) microphotographs were obtained in representative areas of the samples.

#### Statistical analysis

The CFU values were transformed to  $\log_{10}$  values. Mean  $\log_{10}$  CFU values with standard deviations were

calculated. Levene's test was performed to analyze the homogeneity of the variances. Statistical analysis was performed using the SPSS 17.0 (SPSS, Chicago, IL, USA). CFU values were subjected to the Kruskal-Wallis test for significant differences, because the data were non-parametric. Finally, the Mann-Whitney U-test was used for group comparisons. The level of statistical significance was set at 0.05 for all analyses.

## RESULTS

The results are summarized in Fig 1. The results of the negative control group showed no growth after all procedures (CFU=0), whereas the positive control

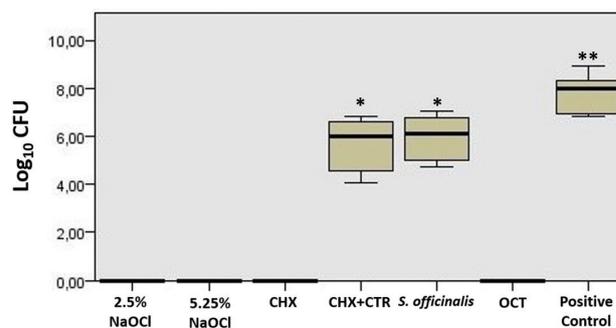


Fig. 1 Box plot graph including recovered mean CFU counts (as  $\log_{10}$  values) of 2.5% NaOCl, 5.25% NaOCl, CHX, CHX+CTR, *S. officinalis* and OCT groups.

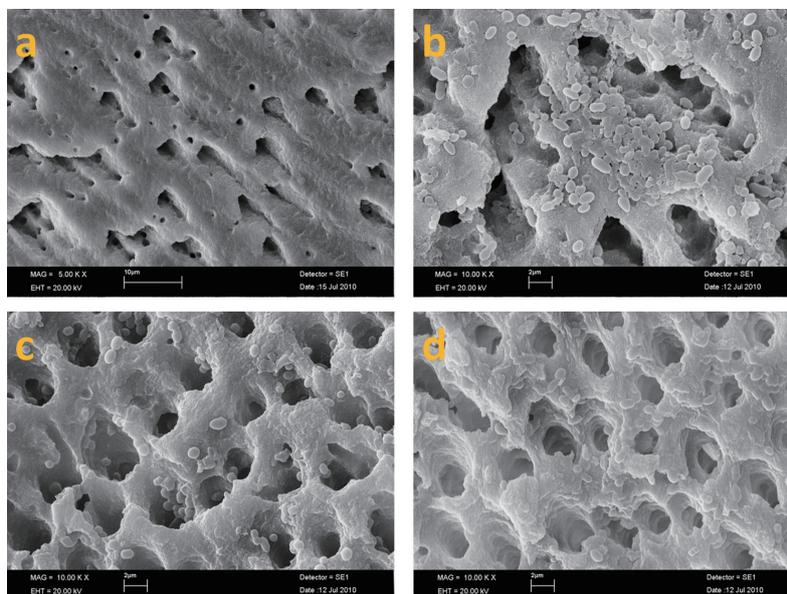


Fig. 2 Scanning electron microscopic micrographs of root canal dentin. (Original magnification  $\times 10,000$ ). a) Negative control group shows evidence of no bacterial growth. b) Positive control group shows sufficient *Enterococcus faecalis* (A197A) growth on root canal walls after 21-day incubation. c) the CHX+CTR group. d) the methanol extract of *S. officinalis* group.

group yielded vigorous growth, confirming the bacterial infection ( $\log_{10}$  CFU=7.94±0.02). In addition, SEM image of negative control group showed the evidence of no bacterial growth (Fig. 2a) and SEM image of positive control group confirmed the biofilm formation inside the root canal system and (Fig. 2b). All tested groups showed mean  $\log_{10}$  values significantly lower than the positive control group ( $p<0.05$ ). The NaOCl (2.5 and 5.25%) groups, the CHX group and the OCT group could eliminate all *E. faecalis* cells (CFU=0), and there was no difference between the negative control group (CFU=0). The CHX+CTR group ( $\log_{10}$  CFU=5.65±1.06) and the methanol extract of *S. officinalis* group ( $\log_{10}$  CFU=5.90±0.92) were reduced *E. faecalis* cells but they could not achieve total elimination (Figs. 2c and d). The differences between two groups were not significant statistically ( $p>0.05$ ).

## DISCUSSION

The dentin powder model developed by Haapasalo and Ørstavik<sup>35</sup> with some modifications was preferred because of the reported good correlation between histology and the culturing of dentin dust in their study. That study was also later confirmed by Peters *et al.*<sup>36</sup>, who showed that the grinding and culturing of dentin gave better quantitative information about the extent of the infection. Consequently, we used gates-glidden burs at low speed to remove dentin from the root canal walls and dentinal tubules, allowing more predictable sampling. All samples were stored in a freezer for 1 h at -27°C before obtaining the dentin powder in order to avoid killing bacterial cells due to the excessive heat created by the friction from using gates-glidden burs on the dentin. In the present study, the apical 3-mm sections of roots were resected to eliminate differences due to the apical delta and apical lateral canals<sup>37</sup>. In addition, this process may be required to prevent contamination with external surfaces of the root samples because the diameter of the apical end of root was smaller than the diameter of the gates-glidden burs used to get the dentin powder<sup>38</sup>.

*Enterococcus faecalis*, which is a commonly-used organism for dentin disinfection studies due to its resistance against root canal disinfectants was chosen for this study. It is the most frequently isolated microorganism in persistent root canal infections, and also, it is easy to maintain and cultivate under laboratory conditions<sup>5,9,12,14,20,39</sup>. Its capability to invade dentinal tubules was also confirmed previously *ex vivo*<sup>35</sup>.

Some studies assessing the antimicrobial efficacy of the irrigants used neutralizing agents to prevent any residual activity<sup>10,39,40</sup> whereas some studies did not<sup>14,17,41</sup>. No deactivating agent was used in this study to reduce the carry-over effect of the disinfectant solutions because the neutralizing agents could have various antimicrobial effects which might also deteriorate the results<sup>14</sup>. Moreover, there is a lack of any “universal” neutraliser agent appropriate for OCT and methanol extract of *S. officinalis*. Instead using any neutraliser

agents for only NaOCl and CHX groups, distilled water was used to neutralize the effect of all tested irrigants. Therefore, all groups were tested under same conditions in the present study.

The results of the present study showed that CHX has strong bactericidal activity on *E. faecalis* as 2.5 and 5.25% NaOCl solutions. This finding is consistent with the results of other investigators, who showed no differences in bactericidal efficacy between NaOCl and CHX<sup>10,39-42</sup>.

CTR is a cationic detergent that has an antibacterial activity against many gram-positive and gram-negative bacteria<sup>41</sup>. Furthermore, CHX can easily and thoroughly penetrate into the root canals and dentinal tubules when used in combination with CTR<sup>9</sup>. Contrary to our expectations, Savlex® (a CHX+CTR commercial product) was significantly less effective than 2% CHX and NaOCl solutions against *E. faecalis*. In comparison, Onçağ *et al.*<sup>9</sup> reported that CHX+CTR gluconate combination was more effective in killing *E. faecalis* than 5.25% NaOCl solution. In another *in vitro* study, no difference was found in the bactericidal effect of NaOCl and CHX+CTR solution when tested in various concentrations<sup>41</sup>. Portenier *et al.*<sup>13</sup> claimed that the combination of CHX and CTR killed *E. faecalis* more rapidly than CHX alone, and at lower concentrations.

It is possible that the differences of concentrations of CHX and CTR compounds in tested solutions could explain the differences in our data compared to other studies. Moreover, Arias-Moliz *et al.*<sup>14</sup> demonstrated that application of the CTR alone and right after the irrigation with CHX was able to act more directly on *E. faecalis* rather than the combined application of CTR and CHX in irrigation solutions.

To overcome drawbacks associated with currently used irrigants, use of natural plant extracts as endodontic irrigants might be of interest as part of a growing trend to research natural remedies in dental treatment. Methanol and ethyl acetate are known to be the most suitable solvents for extraction of antioxidants from plant materials<sup>34</sup>. Methanol extract of *S. officinalis* was found to be more effective on *E. faecalis* than its ethanol form in our pilot studies in which we used the filter paper disc method (data not shown). In addition, when methanol is applied as a single agent against *E. faecalis*, it could not kill any bacteria in our pilot experiments (data not shown). Therefore, the antibacterial activity of methanol extract of *S. officinalis* was not associated with the effect of methanol itself.

*S. officinalis* as a source of natural antioxidants used in health care products has been shown to be non-toxic to rat hepatocytes with diluted concentrations of the extract<sup>43</sup>. In addition, Horiuchi *et al.*<sup>33</sup> isolated the effective compounds from *S. officinalis* extract and identified them as carnosol and carnosic acid. They also claimed that carnosol and carnosic acid increase the gram-positive bacteria cell membrane permeability, thereby improving the diffusion of antimicrobial agents into the cells.

The antimicrobial efficacy of *S. officinalis* against

*E. faecalis* has been investigated using the agar diffusion method<sup>33,34</sup>. However, the studies had observed the bacterial activity of the extracts against planktonic forms only. Considering the clinical relevance of *E. faecalis* biofilms in endodontics rather than free bacterial forms, we evaluated the antimicrobial characteristics of *Salvia officinalis* extract by using root dentin samples with a biofilm model. This was a preliminary study to evaluate the antibacterial characteristics of *S. officinalis* extract against *E. faecalis*.

However, in contrast to our expectations, methanol extract of *S. officinalis* failed to completely disinfect the root canals, whereas the total elimination of *E. faecalis* cells was observed in our previous filter paper disc studies (data not shown). In fact, the observed reduction of this bactericidal activity may be attributed to the differences in the experimental models and probably are a result of the inhibitory effect of the substances present in dentin<sup>45</sup>. Thus, it becomes apparent that *S. officinalis* extract has a much lower antimicrobial efficacy when compared with NaOCl and CHX. The results of this study indicate that it can have a potential for use in endodontics for control of root canal flora. Whilst these extracts may not be useful as endodontic irrigants due to the weaker antimicrobial action on biofilms in comparison with conventional endodontic irrigants used in the present study, it may have potential to be used as intracanal medicaments due to its reported anti-inflammatory characteristics. Future studies are required to confirm its antimicrobial and anti-inflammatory activities as an endodontic material for other endodontic applications.

The results obtained from our study demonstrated that OCT is as effective as well characterized endodontic irrigants including NaOCl solutions and CHX on *E. faecalis*. OCT acts as a cation active substance on the cell membrane; thus, it destroys bacterial cells by interacting with their cell walls and intracellular components. The susceptibility of bacteria to OCT has been reported to be comparable with CHX<sup>15,16,19</sup>. Moreover, Kocak *et al.*<sup>21</sup> have reported that OCT had a more persistent antimicrobial effect on *Streptococcus mutans* in plaque than CHX. In addition, the result of a previous study<sup>18</sup> demonstrated that the antimicrobial efficacy of various concentrations of different concentrations of OCT was more effective than 5.25% NaOCl against selected endodontic microorganisms. With the experimental procedure described in this study that depends on a dentin powder model, it may be assumed that OCT could retain its high antimicrobial properties due to its resistance against organic substances contained in the dentin structure.

## CONCLUSION

Based on the results of this *in vitro* study, CHX+CTR and methanol extract of *S. officinalis* showed bacterial reduction; but could not achieve total bacterial elimination. A wound disinfectant OCT was as effective as NaOCl and CHX in removing from *E. faecalis* infected root canals and hence OCT may have potential

as an endodontic irrigant.

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