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Evaluation of the Efficacy of Methylglyoxal and Thyme Oil in Elimination of *Enterococcus faecalis* Biofilm: An in vitro Study

Enterococcus faecalis Biyofilminin Elimine Edilmesinde Metilglioksal ve Kekik Yağının Etkinliğinin Değerlendirilmesi: Bir in vitro Çalışma

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ABSTRACT Objective: The antimicrobial activity of methylglyoxal and thyme essential oil (TEO) against *Enterococcus faecalis*, a pathogen responsible for resistant root canal infections due to its biofilm-forming ability, has not been sufficiently documented. This study aimed to evaluate the antibacterial and antibiofilm activities of the methylglyoxal and TEO for removing *E. faecalis* biofilm on root canal using microbiological culture and confocal laser scanning microscopy (CLSM). **Material and Methods:** A total of 128 root samples were incubated with *E. faecalis* for three weeks to form mature biofilm. The infected samples were then, randomly distributed into 4 groups and irrigated with 5.25% sodium hypochlorite, methylglyoxal (40% in water), TEO (100% pure), and 0.9% isotonic sodium chloride (negative control). After the irrigation procedure, samples were assessed through microbiological culture and CLSM. The statistical significance level was set at $p<0.05$. **Results:** Culture results indicated no statistically significant difference between sodium hypochlorite and methylglyoxal ($p=0.250$; $p\geq0.05$), sodium hypochlorite and TEO ($p=0.274$; $p\geq0.05$) and methylglyoxal and TEO ($p=0.500$; $p\geq0.05$). In addition, CLSM images showed a higher population/prevalence of dead cells in samples treated with methylglyoxal and sodium hypochlorite compared to isotonic sodium chloride and TEO. **Conclusion:** Methylglyoxal and TEO demonstrated similar efficacy as sodium hypochlorite in removing *E. faecalis* biofilm, suggesting these two tested agents' potential as alternate irrigation solutions to sodium hypochlorite in root canal treatment.

Keywords: Antibiofilm activity; *Enterococcus faecalis*; Manuka honey; methylglyoxal; thyme essential oil

ÖZET Amaç: Metilglioksal ve kekik esansiyel yağının [thyme essential oil (TEO)], biyofilm oluşturma yeteneği nedeniyle dirençli kök kanalı enfeksiyonlarından sorumlu bir patojen olan E*nterococcus faecalis*'e karşı antimikrobiyal aktivitesini gösteren yeterli araştırma bulunmamaktadır. Bu çalışmada, *E. faecalis* biyofilmini uzaklaştırmak için metilglioksal ve TEO'nun, antibakteriyel ve antibiyofilm aktivitesini mikrobiyolojik kültür ve lazer taramalı konfokal mikroskopla değerlendirilmiştir. **Gereç ve Yöntemler:** Bu çalışma kapsamında kök örnekleri (n=128) *E. faecalis* ile 3 hafta süreyle inkübe edilerek olgun biyofilm oluşturuldu. Daha sonra enfekte örnekler rastgele 4 farklı gruba dağıtıldı ve %5,25 sodyum hipoklorit, %0,9 izotonik sodyum klorür, metilglioksal (suda %40) ve TEO (saf %100) ile irrigasyon yapıldı. İrrigasyon işleminin ardından örnekler mikrobiyolojik kültür yöntemi ve konfokal lazer tarama mikroskobu ile değerlendirildi. İstatistiksel anlamlılık düzeyi $p<0,05$ olarak kabul edildi. **Bulgular:** Kültür sonuçlarına göre sodyum hipoklorit ile metilglioksal ($p=0,250$; $p\geq0,05$), sodyum hipoklorit ile TEO ($p=0,274$; $p\geq0,05$), metilglioksal ile TEO ($p=0,500$; $p\geq0,05$) arasında istatistiksel olarak anlamlı fark saptanmadı. Ayrıca lazer taramalı konfokal mikroskopla alınan görüntülerde, irrigasyon sonrası metilglioksal ve sodyum hipoklorit gruplarında izotonik sodyum klorür ve TEO gruplarına göre daha fazla ölü hücre görüntülendi. **Sonuç:** Metilglioksal ve TEO, *E. faecalis* biyofilminin uzaklaştırılmasında sodyum hipoklorit ile benzer etkinlik göstermiştir ki test edilen bu iki ajanın, kök kanal tedavisinde sodyum hipoklorite alternatif irrigasyon solüsyonları olarak potansiyelini ortaya koymuştur.

Anahtar Kelimeler: Antibiyofilm aktivite; *Enterococcus faecalis*; Manuka balı; metilglioksal; kekik esansiyel yağı

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Enterococcus faecalis, one of the most resistant bacteria known in dentistry, is a pathogen capable of causing significant diseases within the oral cavity.[1] In addition to causing resistant infections in soft and hard tissues surrounding the teeth, such as gingiva and bone, *E. faecalis* has also been implicated as a contributing factor in the development of endodontic lesions.[2] These lesions, which result from an infectious process affecting the dental pulp tissue represent a potential complication in endodontic treatments. The most crucial objective of these treatments is to effectively reduce the number of bacteria present within the root canals. This is a prerequisite for the success of the procedure.[3,4]

Despite the application of various root canal cleaning tools, irrigation solutions and antimicrobial drugs, the removal of resistant microorganisms such as *E. faecalis* from root canals poses a significant challenge in ensuring clinical success.[5,6] These bacteria are the most frequently isolated bacteria from root canals and can play an active role even alone in resistant or recurrent endodontic infections. *E. faecalis* is typically the primary cause of unsuccessful root canal treatments, accounting for 22-77% of cases.[7,8]

Despite the considerable challenges posed by the intricate anatomy of the root canal and the existence of lateral canals, the complete elimination of pathogens from root canals remains a feasible objective. The combination of instrumentation, irrigation, and intracanal medicaments may prove effective in eliminating the majority of pathogens.[9,10] The current standard of care employs a combination of mechanical and chemical cleaning methods for the disinfection of root canals. For this purpose, irrigation solutions such as 5.25% sodium hypochlorite (NaOCl), 3% hydrogen peroxide and 2% chlorhexidine are employed, which have been demonstrated to markedly diminish the bacterial load within root canals. Among these solutions, NaOCl is the most commonly preferred agent due to its cost-effectiveness and accessibility.[3,11]

Essential oils are primarily composed of oxygenated monoterpenes and monoterpene hydrocarbons, with a predominant component known as carvacrol. They are recognized for their potent antioxidant properties. Thyme essential oil (TEO) is composed of fundamental constituents, including carvacrol and thymol. Thymol has been demonstrated to prevent the growth of oral pathogens, while carvacrol has been shown to possess antibacterial activity against a number of bacteria present in the oral microbiota and root canals of teeth.[12-16] Thosar et al. published an article examining the efficacy of essential oils against a range of oral pathogens, including *Staphylococcus aureus, E. faecalis, Escherichia coli,* and *Candida albicans*.[12] Their findings suggest that TEO may serve as an effective intracanal antiseptic solution against these pathogens. Furthermore, methylglyoxal has been demonstrated to possess a non-peroxide antibacterial effect and is present in high concentrations in Manuka honey. The antimicrobial efficiency of methylglyoxal has been evidenced in the context of numerous microorganisms, including *E. coli, E. faecalis, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Streptococcus mutans, S. aureus, Streptococcus pyogenes, Pseudomonas aeruginosa,* and *Lactobacillus*.[17,18]

To the best of our knowledge, there are studies investigating the antibacterial activity of various phytochemicals such as methylglyoxal and TEO, on a variety of bacterial pathogens.[12-18] However, there is no study investigating their effectiveness on *E. faecalis* biofilm formed in the root canals of extracted teeth. The aim of this study was to evaluate the antibacterial and antibiofilm activity of methylglyoxal and TEO as alternate irrigation solutions to NaOCl for removing *E. faecalis* biofilm formed in the root canals of extracted teeth using microbiological culture and laser scanning confocal microscopy (CLSM). The null hypothesis of this study was that there was no difference between methylglyoxal, TEO, NaOCl and physiological saline regarding the antimicrobial and antibiofilm efficacy.

MATERIAL AND METHODS

PRIORI POWER ANALYSIS AND ETHICAL CONSIDERATION

The power analysis indicated that a total of 64 tooth samples were required to test the statistical signifi-

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cance of the results obtained at 90% power, 5% error level, and 0.5 effect size. This entailed including 16 samples in each of the groups. This study was approved by Başkent University Institutional Review Board and Ethics Committee (date: July 14, 2021, no: K94603339-604.01.02.47988) and conducted in accordance with the principles of the Declaration of Helsinki.

INCLUSION AND EXCLUSION CRITERIA OF THE TEETH

Teeth with completed root formation, which was confirmed to have single canal and which did not exhibit any anatomical structural anomalies such as internal resorption, calcification and root dilation in radiographic examination. Additionally, the teeth had not undergone root canal treatment prior to the study. Teeth exhibiting signs of hypoplasia or hypomineralisation, as well as those displaying incomplete root formation with multiple root canals, were excluded from the study.

PREPARATION OF THE TOOTH SAMPLES

The soft tissue residues on the teeth included in the study were cleaned with the assistance of a scaler and stored in saline solution to prevent dehydration until the commencement of the experiment. To obtain tooth samples with standard root length, the root length was measured to be 8 mm, and the crown part was removed under water cooling with a diamond disc from under the enamel-cementum junction. The working length of the root canals was determined by employing the number 15 K-file, with preparation extending to the number 30 file. Subsequently, the apical third of all root canals was expanded up to the F3 size using the rotary system (Pro-Taper Universal, Dentsply Maillefer, Switzerland) to simulate the formation of an open apex.

The preparation of root canals was performed using sterile saline solution (10 mL) as the irrigation solution. For the final irrigation, 2 mL of 3% NaOCl and 17% Ethylenediaminetetraacetic acid solutions were used for 2 minutes to remove the smear layer. Following the preparation and irrigation of all root canals, all roots were bisected vertically along their midlines under water cooling with a diamond disc (2n: 128). In addition, the external surfaces of the tooth root, which had been bisected at the midpoint, were flattened to guarantee that the root fragment was fully embedded in the tissue culture plates.[19-21] As the concluding step in the sample preparation, all preparated root samples were sterilized in an autoclave at 121 °C for 15 minutes.

INFECTION OF ROOT CANALS WITH E. FAECALIS

Following the preparation of root samples, they were infected with *E. faecalis* ATCC 29212. The dentin block model method was employed to form an *E. faecalis* biofilm. A pure culture of *E. faecalis* was cultivated on 5% Sheep Blood Agar medium and incubated overnight at 37 °C. The bacterial suspension was prepared by adjusting at the optical density to 0.5 McFarland standard in 5 mL of Tryptone Soy Broth (TSB) medium. The bacterial suspension was diluted 1/100 to obtain ~ 1.5×10^6 Colony Forming Unit per millimeter (CFU/mL) with TSB for inoculation of the root samples. The root samples were placed into the wells of flat-bottomed 24-well culture plates and covered with 1 mL of the prepared bacterial suspension, and then incubated at 37 °C. The suspension was replenished every two days, following a three-week period of washing with phosphate buffered saline. At the end of the 21-day period, the bacterial suspension was removed, and the root samples were gently washed with phosphate-buffered saline to remove any unadherent planktonic bacteria.

APPLICATION OF IRRIGATION SOLUTIONS

Following a three-week incubation period, the samples (2n=128) infected with *E. faecalis* and exhibiting biofilm formation were randomly distributed to the groups for the application of irrigation solutions. Following the distribution of the root samples into four groups according to the irrigation solutions, each infected root sample was maintained in a culture plate containing 3 mL of each irrigation solution for 10 minutes in contact with teeth samples. Then, the samples were transferred to 3 mL of sterile physiological saline solution in order to terminate the antibacterial effect. The Methylglyoxal solution (40% in water, Sigma Aldrich, U.S.A.) and TEO (100% pure, Caliskan Agriculture, Türkiye) were utilized as irrigation solutions.

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Group 1: 5.25% Sodium Hypochlorite (Positive Control)

Group 2: 0.9% Isotonic Sodium Chloride Solution (Physiological Saline) (Negative Control)

Group 3: 40% Concentration Methylglyoxal Solution (Test Group)

Group 4: 100% Concentration TEO (Test Group)

EVALUATION OF SAMPLES BY QUANTITATIVE CULTURE METHOD

Following the irrigation procedure, the samples were rinsed with 3 mL of sterile saline solution in order to remove the neutralization solution. The number of viable cells in the biofilm on root samples after the irrigation process was counted and compared between the groups. The root samples from each group were covered with 1 mL of Tryptone Soy Broth and sonicated (Daihan, Korea) at an amplitude of 30% for a duration of 10 seconds, with the objective of detaching the bacterial cells present in the biofilm. Following sonication, the broth from each well was serially diluted, with 100 µl of each tube subsequently inoculated into the Tryptone Soy Agar. The petri dishes were incubated at 37 °C for 24 hours. Following the incubation period, the number of viable cells on each dilution was counted and calculated as CFU/mL.

EVALUATION OF SAMPLES BY A CONFOCAL LASER SCANNING MICROSCOPE

In order to visualize the change in the density of the *E. faecalis* biofilm before and after the irrigation process, two randomly selected root samples from each group were imaged with a confocal laser scanning microscope (Zeiss LSM 510, Germany). The manufacturer's instructions were followed for the use of SYTO 9 and propidium iodide (Thermo Fisher Scientific, U.S.A.), which allow the visualization of the dead and living cells within the biofilm on the sample. Subsequently, the samples were washed with sterile saline solution and stained with a 20 µL solution of SYTO-propidium iodide for 20 minutes in the dark. After that, the stained samples were rinsed with sterile distilled water for one minute, after which the stained root halves were examined with CLSM. Excitation and emission wavelengths were 500/530 nm for SYTO 9 and 552/617 nm for propidium iodide, respectively. Simultaneous dual-channel imaging was employed to visualize green fluorescence (indicative of living cells) and red fluorescence (indicative of dead cells). The CLSM images of samples with *E. faecalis* biofilm following the irrigation procedure with methylglyoxal, TEO, sodium hypochlorite, and isotonic sodium chloride were presented in Figure 1, Figure 2, Figure 3, and Figure 4, respectively. The live and dead cells observed in green and red are designated as A and B respectively, in Figure 1, Figure 2, Figure 3, and Figure 4.

STATISTICAL ANALYSIS

The data were analyzed using the SPSS V. 22 (IBM) package program. The mean±standard deviation for each irrigation group was provided in Table 1 for the CFU/mL count. A pairwise evaluation of each group according to the CFU/mL counts of *E. faecalis* after the irrigation process was presented in Table 2. This analysis was conducted using a t-test at a 95% confidence interval of difference. The statistical signifi-

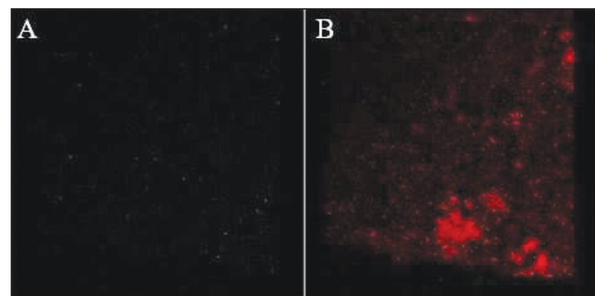

FIGURE 1: Images of **(A)** live (green) and **(B)** dead (red) cells in *Enterococcus faecalis* biofilm taken with confocal laser scanning microscope after the irrigation procedure with methylglyoxal.

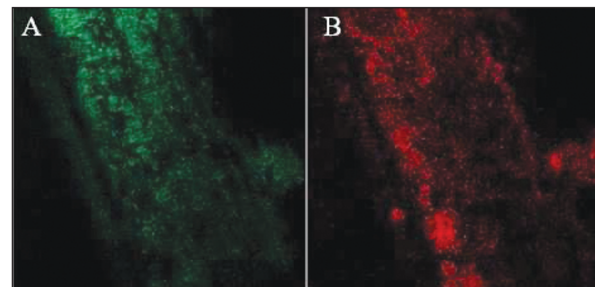

FIGURE 2: Images of **(A)** live (green) and **(B)** dead (red) cells in *Enterococcus faecalis* biofilm taken with confocal laser scanning microscope after the irrigation procedure with thyme oil.

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cance level was set at α=0.05, with a significance level of $p<0.05$.

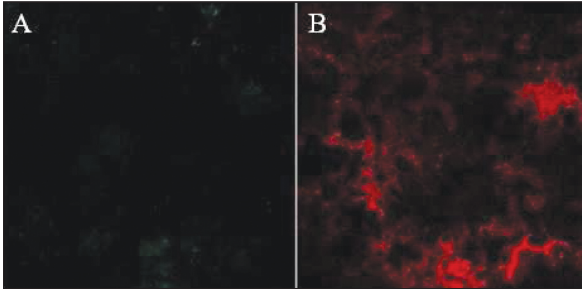

FIGURE 3: Images of **(A)** live (green) and **(B)** dead (red) cells in *Enterococcus faecalis* biofilm taken with confocal laser scanning microscope after the irrigation procedure with sodium hypochlorite.

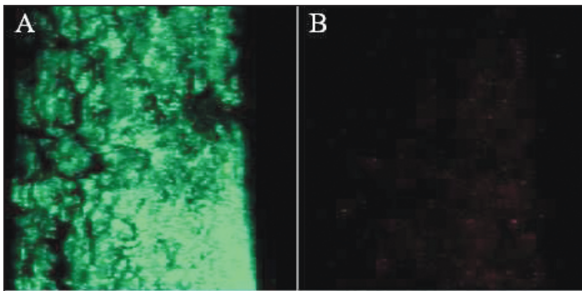

FIGURE 4: Images of **(A)** live (green) and **(B)** dead (red) cells in *Enterococcus faecalis* biofilm taken with confocal laser scanning microscope after the irrigation procedure with isotonic sodium chloride.

TABLE 1: Counts of *Enterococcus faecalis* bacteria after irrigation process amongst each irrigation solution and statistical significance results of before and after the irrigation process.

Groups/Irrigation solutions	$\bar{X}$±SD	p-value
G1: 5.25% sodium hypochlorite	3.19±0.27	0.074
G2: Isotonic sodium chloride solution	7.20±0.28	
G3: Methylglyoxal	2±0.43	
G4: Thyme essential oil	1.9±0.57	

SD: Standard deviation; Comparison of groups was performed with one-way analysis of variance with a statistical significance level of $p<0.05$.

TABLE 2: Pairwise evaluation of each group according to counts of *Enterococcus faecalis* after irrigation process.

Groups	$\bar{X}$±SD	p-value (95% CI)
G1-G2	-4.004±0.006	0.001*
G1-G3	1.195±0.700	0.250
G1-G4	1.292±0.837	0.274
G2-G3	5.199±0.706	0.061
G2-G4	5.296±0.843	0.071
G3-G4	0.097±0.138	0.500

*Refers statistical significance; SD: Standard deviation; Statistical analysis was performed with t-test as paired samples with a statistical significance level of $p<0.05$.

RESULTS

As demonstrated by the statistical analysis of the obtained data, the mean and standard deviation of the CFU counts were presented in Table 1. Prior to the irrigation process, the baseline CFU of *E. faecalis* was established at a level of 1.5×10^8 CFU/mL for each group. Following the irrigation process, a statistically significant reduction in the CFU counts of *E. faecalis* was observed. The statistical differences amongst the baseline CFU are presented in Table 1. The statistical analysis revealed a statistically significant decrease in the *E. faecalis* counts for each group, as illustrated in Table 1.

The pairwise evaluation revealed a statistically significant difference between paired groups, allowing for the determination of the interchange of CFU counts of *E. faecalis* following the irrigation process. As illustrated in Table 2, the discrepancy between the NaOCl and isotonic sodium chloride solutions was statistically significant ($p=0.001$; $p<0.05$). However, the statistical analysis between remaining paired groups did not yield statistically significant results ($p\geq0.05$), indicating that there was no statistically significant difference between the NaOCl and experimental groups as methylglyoxal ($p=0.250$; $p\geq0.05$) and TEO ($p=0.274$; $p\geq0.05$). Additionally, no statistically significant difference was observed between the experimental groups; methylglyoxal and TEO ($p=0.500$; $p\geq0.05$). Figure 1, Figure 2, Figure 3 and Figure 4 illustrate the images of *E. faecalis* biofilm taken with a confocal laser scanning microscope following irrigation with methylglyoxal, TEO, NaOCl, and isotonic sodium chloride solutions, respectively. The images featuring green color (Figure 1A, Figure 2A, Figure 3A and Figure 4A) illustrate the presence of living cells, while the images with red color (Figure 1B, Figure 2B, Figure 3B and Figure 4B) indicate the presence of dead cells within the *E. faecalis* biofilm. Therefore, it can be observed that a greater number of living cells are present in the isotonic sodium chloride irrigation solution in comparison to the methylglyoxal, TEO and NaOCl solutions.

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DISCUSSION

In periradicular infections, the most resistant bacteria in infected root canals is *E. faecalis*, which is a gram-positive, facultatively anaerobic bacteria.[1,2] Its resistance to high pH and high salt concentration, as well as its ability to form biofilm, are important virulence factors of *E. faecalis*.[22] The eradication of *E. faecalis* is a significant challenge due to its capacity to withstand antimicrobial agents and to form a biofilm, which serves as a protective barrier against the surrounding environment.[23] In addition, the presence of deep dentin tubules located in the dentin tissue provides an ideal environment for *E. faecalis* biofilm formation.[24-26] Therefore, eliminating *E. faecalis* from the root canal system is a crucial and challenging aspect of successful endodontic treatments.

The ability of *E. faecalis* to form biofilms represents a crucial virulence factor in the context of resistant or recurrent endodontic lesions. It exhibits a high binding ability to the dentin surface and the ability to grow in biofilm due to the presence of dentin tubules. Consequently, dentin discs were employed as a substrate for biofilm formation in previous studies. Accordingly, in the present study, the roots of extracted teeth were prepared mechanically to create a deeper dentin tubule structure, thereby facilitating enhanced penetration of *E. faecalis*.[24]

The *E. faecalis* biofilm is more resistant to disinfecting solutions after three weeks of formation and is referred to as a mature biofilm.[24-26] However, within this period, bacteria remain active and in the exponential growth phase, and the structural development of the biofilm or extracellular polymeric matrix production is not yet complete after colonization.[24,25] A homogenous and dense form of *E. faecalis* biofilm was observed with a CLSM in all samples assessed to confirm the biofilm formation on dentin surfaces. The CLSM images confirmed the presence of a mature biofilm.[24] Therefore, in this study, a three-week incubation of *E. faecalis* was applied before the irrigation procedure to ensure the biofilm maturation. The primary objective of this study was to assess the efficacy of two phytochemichal irrigation solutions in eradicating mature biofilms of *E. faecalis*.

The current cleaning and shaping techniques are insufficient to ensure a bacteria-free root canal environment.[3,24] Therefore, a chemical irrigation solution is necessary to reduce the number of bacteria and their toxicity near a mechanical root canal treatment. In routine clinical applications, the most commonly used irrigation solutions are NaOCl and chlorhexidine, which are employed to eliminate residual microorganisms. Sodium hypochlorite has the ability to dissolve organic tissues and exhibits potent antibiofilm activity. It can be prepared in different concentrations between 0.5-5.25% and provides low-cost, antiseptic lubrication.[24,27] However, NaOCl has high toxicity for periradicular tissue and might also cause allergic reactions.[27,28] A previous study have demonstrated that dead cells were significantly higher in the 5.25% NaOCl group than in the experimental groups, indicating almost complete removal and dissolution of *E. faecalis* biofilms.[29] These findings are in accordance with the results of previous studies which demonstrated the pronounced antibiofilm effect of 5.25% NaOCl, attributable to its ability to dissolve organic tissue and target the extracellular matrix of the biofilm.[24,26]

It was previously assumed that the use of high concentration NaOCl was more efficient in removing bacteria from the root canal system. However, the use of NaOCl in high concentrations may cause complications due to overflow from the root tip during endodontic treatments.[30,31] Therefore, this study was designed to assess the efficacy of two distinct phytochemical, methylglyoxal and TEO in comparison with NaOCl, a commonly utilized irrigation solution as positive control, and isotonic sodium chloride solution.

In determining the optimal irrigation solution, it is essential to consider the concentration and irrigation time, as these factors significantly influence the efficacy of endodontic treatment. In a study conducted by Ma et al., the efficiency of five different antimicrobial solutions was evaluated against the survival of *E. faecalis*.[32] The solutions included 5.25% NaOCl, 1% NaOCl, 2% chlorhexidine, 0.2% chlorhexidine, and 0.9% NaOCl. The results of the study indicated that 1% NaOCl was the most effective in reducing the number of dead *E. faecalis* cells

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in the biofilm. Bukhary and Balto evaluated the efficiency of 5.25% NaOCl with other irrigation solutions for a 10 minute period against a 3-week-old biofilm model of *E. faecalis*.[24] The results demonstrated that a 10 minute irrigation with 5.25% NaOCl resulted in a significantly higher rate of cell death than other experimental groups. In the present study, the optimal time and concentration were identified as those that yielded the highest success rate for NaOCl. Therefore, 5.25% NaOCl was selected with a 10 minute irrigation time to facilitate a comparative analysis of the maximum effect of NaOCl with methylglyoxal and TEO in this study.

Phytochemicals were employed to eliminate the adverse effects associated with NaOCl. The antibacterial features of honey have been attributed to various factors in recent times.[33] Manuka, the common name for Leptospermum scoparium, a tea tree native to New Zealand, has been shown to possess antibacterial activity. This non-peroxide antibacterial effect is attributed to methylglyoxal, which is present in high concentrations in Manuka honey.[33-35] A number of studies have demonstrated the antibacterial effect of Manuka honey on a range of gram positive and negative bacterial pathogens, including *E. coli, E. faecalis, A. actinomycetemcomitans, P. gingivalis, S. mutans and Lactobacillus*.[33-35] Methylglyoxal is particularly effective in inhibiting bacterial growth by disrupting cellular divisions, arresting bacterial proliferation, and inducing degradation of bacterial DNA, even at relatively low concentration. The efficacy of honey as an antibacterial agent was found to increase in proportion to the concentration of methylglyoxal present in the honey.[17] Consequently, methylglyoxal was investigated as an experimental group on tooth sample surfaces with mature biofilm formation in the present study. Because, its efficiency against *E. faecalis* was demonstrated to be effective even at low concentrations.

Thyme is a member of the *Lamiaceae* family and is native to Europe, specially Mediterranian region. It is a hardy, perennial herbaceous plant or shrub that belongs to more than 300 species. It has been recognized as one of the Hippocrates 400 simple solutions. The essential oil of thyme (*Thymus spp, T. citriodorits, T. vulgaris*) is derived from the leaves, and its primary components are 20-40% of the oil. Thymol, the active constituent of thyme, has been demonstrated to impede the proliferation of oral pathogens within the oral cavity. Furthermore, its combination with other essential oils has been shown to diminish the incidence of dental caries. Additionally, carvacrol has been demonstrated to possess antibacterial activity against a range of bacterial strains.[12-14,18] Consequently, a number of studies have indicated that TEO displays antibacterial activity and is a valuable addition to dental practice, particularly in the management of endodontic lesions.[12-16] However, to the best of our knowledge, there were no studies evaluating its efficiency over the mature form of *E. faecalis* biofilm on root canal.[12,15] These previous studies demonstrated a high antibacterial and antibiofilm effect of TEO on *E. faecalis*.[12,15] Additionally, Marinković et al. emphasized that TEO exhibited similar antibiofilm activity with NaOCl.[16] Therefore, the objective of this study was to evaluate its efficacy over the mature *E. faecalis* biofilm and compare it with another phytochemical, methylglyoxal.

The findings of the present study indicate that all irrigation solutions demonstrated a statistically significant reduction in *E. faecalis* counts (CFU) in biofilm structure. However, no statistically significant difference was observed between methylglyoxal and NaOCl as well as TEO and NaOCl, indicating that these irigation solutions exhibited promising antibacterial and antibiofilm efficacy. In light of the insignificant difference between the alternate agents and NaOCl, these solutions might be recomended during endodontic treatments to ensure clinically successful outcomes. Additionally, Figure 1, Figure 2, Figure 3 and Figure 4 illustrate the presence of both dead and living cells subsequent to the irrigation procedure. The obtained counts (CFU) corroborate this observation, as the lowest number of dead cells were evident in the isotonic sodium chloride solution, with comparable levels of dead cells evident in the methylglyoxal, TEO, and NaOCl images. Therefore, the hypothesis of this study was rejected due to the statistical difference between the isotonic sodium chloride solution and the other irrigation solutions used in the study, namely methylglyoxal, TEO and NaOCl.

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A limitation of this study was the assessment of a single sample from each root surface, rather than from each region, such as the cervical, middle and apical thirds of the root surface. Therefore, the results were not distributed across the root region, which might be different due to the features of the dentin tubules. However, there was no statistical difference between methylglyoxal and TEO, so further in-situ, in-vitro and in-vivo studies should be conducted to obtain more detailed results. The results showed that there was a statistical difference between NaOCl and isotonic sodium chloride solution. Thus, isotonic sodium chloride solution showed the lowest efficiency on *E. faecalis* biofilm of all irrigation solutions. Therefore, still cannot be recommended during the treatment of resistant or recurrent endodontic lesions.

CONCLUSION

The results of this study stated that the highest removal of *E. faecalis* biofilm was obtained in the NaOCl group. In addition, both methylglyoxal and TEO showed the similar efficacy with NaOCl for *E. faecalis* removal. The images of dead and live cells in *E. faecalis* biofilm taken with CLSM showed the similar efficacy of these two tested phytochemicals compared to NaOCl. Therefore, it could be concluded that methylglyoxal and TEO could be used as an alternative to NaOCl as an irrigation solution. However, further microbiological and cytotoxicity studies should be performed to demonstrate lower toxicity levels compared to NaOCl prior to in vivo studies.

Source of Finance

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

***Idea/Concept:** Didem Sakaryalı Uyar, Aylin Üsküdar Güçlü; **Design:** Didem Sakaryalı Uyar, Aylin Üsküdar Güçlü; **Control/Supervision:** Didem Sakaryalı Uyar, Aylin Üsküdar Güçlü; **Data Collection and/or Processing:** Didem Sakaryalı Uyar, Aylin Üsküdar Güçlü; **Analysis and/or Interpretation:** Didem Sakaryalı Uyar, Aylin Üsküdar Güçlü; **Literature Review:** Didem Sakaryalı Uyar, Aylin Üsküdar Güçlü; **Writing the Article:** Didem Sakaryalı Uyar; **Critical Review:** Didem Sakaryalı Uyar, Aylin Üsküdar Güçlü; **References and Fundings:** Didem Sakaryalı Uyar; **Materials:** Didem Sakaryalı Uyar, Aylin Üsküdar Güçlü.*

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REFERENCES

1. Siqueira JF Jr, Rôças IN. Clinical implications and microbiology of bacterial persistence after treatment procedures. J Endod. 2008;34(11):1291-301.e3. PMID: 18928835.
2. Zhu X, Wang Q, Zhang C, Cheung GS, Shen Y. Prevalence, phenotype, and genotype of Enterococcus faecalis isolated from saliva and root canals in patients with persistent apical periodontitis. J Endod. 2010;36(12):1950-5. PMID: 21092811.
3. Arneiro RA, Nakano RD, Antunes LA, Ferreira GB, Fontes K, Antunes LS. Efficacy of antimicrobial photodynamic therapy for root canals infected with Enterococcus faecalis. J Oral Sci. 2014;56(4):277-85. PMID: 25500925.
4. Xhevdet A, Stubljar D, Kriznar I, Jukic T, Skvarc M, Veranic P, et al. The disinfecting efficacy of root canals with laser photodynamic therapy. J Lasers Med Sci. 2014;5(1):19-26. PMID: 25606335; PMCID: PMC4290519.
5. Akbari Aghdam M, Soroush Barhaghi MH, Aghazadeh M, Jafari F, Beomide Hagh M, Haghdoost M, et al. Virulence genes in biofilm producer Enterococcus faecalis isolates from root canal infections. Cell Mol Biol (Noisy-le-grand). 2017;63(5):55-9. PMID: 28719346.
6. Zenuz AT, Eslami H, Kafil HS, Safari E, Ghanizadeh M, Mohammadi A. The application of antimicrobial photodynamic therapy on Pseudomonas aeuroginosa and Enterococcus fecalis using hypericin and methylene blue photosensitizers. Biomed Pharmacol J. 2016;9(2):443-50. http://dx.doi.org/10.13005/bpj/957
7. Janani M, Jafari F, Samiei M, Lotfipour F, Nakhlband A, Ghasemi N, et al. Evaluation of antibacterial efficacy of photodynamic therapy vs. 2.5% NaOCl against E. faecalis-infected root canals using real-time PCR technique. J Clin Exp Dent. 2017;9(4):e539-e44. PMID: 28469819; PMCID: PMC5410674.
8. Gholizadeh P, Eslami H, Yousefi M, Asgharzadeh M, Aghazadeh M, Kafil HS. Role of oral microbiome on oral cancers, a review. Biomed Pharmacother. 2016;84:552-8. PMID: 27693964.
9. Singh S, Nagpal R, Manuja N, Tyagi SP. Photodynamic therapy: an adjunct to conventional root canal disinfection strategies. Aust Endod J. 2015;41(2):54-71. PMID: 25404404.

10. Tennert C, Feldmann K, Haamann E, Al-Ahmad A, Follo M, Wrbas KT, et al. Effect of photodynamic therapy (PDT) on Enterococcus faecalis biofilm in experimental primary and secondary endodontic infections. BMC Oral Health. 2014;14:132. PMID: 25366394; PMCID: PMC4236465.
11. Hasheminia S, Farhad AR, Saatchi M, Rajabzadeh M. Synergistic antibacterial activity of chlorhexidine and hydrogen peroxide against Enterococcus faecalis. J Oral Sci. 2013;55(4):275-80. PMID: 24351914.
12. Thosar N, Basak S, Bahadure RN, Rajurkar M. Antimicrobial efficacy of five essential oils against oral pathogens: an in vitro study. Eur J Dent. 2013;7(Suppl 1):S071-S07. PMID: 24966732; PMCID: PMC4054083.
13. Shapiro S, Guggenheim B. The action of thymol on oral bacteria. Oral Microbiol Immunol. 1995;10(4):241-6. PMID: 8602337.
14. Yu D, Pearson SK, Bowen WH, Luo D, Kohut BE, Harper DS. Caries inhibition efficacy of an antiplaque/antigingivitis dentifrice. Am J Dent. 2000;13(Spec No):14C-17C. PMID: 11763908.
15. Galovičová L, Borotová P, Valková V, Vukovic NL, Vukic M, Štefániková J, et al. Thymus vulgaris essential oil and its biological activity. Plants (Basel). 2021;10(9):1959. PMID: 34579491; PMCID: PMC8467294.
16. Marinković J, Ćulafić DM, Nikolić B, Đukanović S, Marković T, Tasić G, et al. Antimicrobial potential of irrigants based on essential oils of Cymbopogon martinii and Thymus zygis towards in vitro multispecies biofilm cultured in ex vivo root canals. Arch Oral Biol. 2020;117:104842. PMID: 32707220.
17. Johnston M, McBride M, Dahiya D, Owusu-Apenten R, Nigam PS. Antibacterial activity of Manuka honey and its components: an overview. AIMS Microbiol. 2018;4(4):655-64. PMID: 31294240; PMCID: PMC6613335.
18. Liu F, Jin P, Gong H, Sun Z, Du L, Wang D. Antibacterial and antibiofilm activities of thyme oil against foodborne multiple antibiotics-resistant Enterococcus faecalis. Poult Sci. 2020;99(10):5127-36. PMID: 32988551; PMCID: PMC7598324.
19. Ganesh A, Nagendrababu V, John A, Deivanayagam K. The effect of addition of an eps degrading enzyme with and without detergent to 2% chlorhexidine on disruption of enterococcus faecalis biofilm: a confocal laser scanning microscopic study. J Clin Diagn Res. 2015;9(11):ZC61-5. PMID: 26675655; PMCID: PMC4668526.
20. Zeng C, Willison J, Meghil MM, Bergeron BE, Cutler CW, Tay FR, et al. Antibacterial efficacy of an endodontic sonic-powered irrigation system: an in vitro study. J Dent. 2018;75:105-12. PMID: 29908290.
21. Aktaş N, Öztaş N, Özçelik B, Oyardı Ö. Evaluation of the antimicrobial efficacy of herbal alternatives and ozone in root canal disinfection. IJAR. 2023;13(2):70-3. doi: 10.36106/ijar
22. Kafil HS, Mobarez AM, Moghadam MF. Adhesion and virulence factor properties of Enterococci isolated from clinical samples in Iran. Indian J Pathol Microbiol. 2013;56(3):238-42. PMID: 24152500.
23. Zand V, Milani AS, Amini M, Barhaghi MH, Lotfi M, Rikhtegaran S, Sohrabi A. Antimicrobial efficacy of photodynamic therapy and sodium hypochlorite on monoculture biofilms of Enterococcus faecalis at different stages of development. Photomed Laser Surg. 2014;32(5):245-51. PMID: 24308707.
24. Bukhary S, Balto H. Antibacterial efficacy of octenisept, alexidine, chlorhexidine, and sodium hypochlorite against enterococcus faecalis biofilms. J Endod. 2017;43(4):643-7. PMID: 28258812.
25. Du T, Wang Z, Shen Y, Ma J, Cao Y, Haapasalo M. Effect of long-term exposure to endodontic disinfecting solutions on young and old Enterococcus faecalis biofilms in dentin canals. J Endod. 2014;40(4):509-14. PMID: 24666901.
26. Wang Z, Shen Y, Haapasalo M. Effectiveness of endodontic disinfecting solutions against young and old Enterococcus faecalis biofilms in dentin canals. J Endod. 2012;38(10):1376-9. PMID: 22980181.
27. Alghamdi F, Shakir M. The influence of enterococcus faecalis as a dental root canal pathogen on endodontic treatment: a systematic review. Cureus. 2020;12(3):e7257. PMID: 32292671; PMCID: PMC7152576.
28. 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(13):898-908. doi: 10.5897/AJMR2014.7122
29. Guida A. Mechanism of action of sodium hypochlorite and its effects on dentin. Minerva Stomatol. 2006;55(9):471-82. English, Italian. PMID: 17146426.
30. Jhajharia K, Parolia A, Shetty KV, Mehta LK. Biofilm in endodontics: a review. J Int Soc Prev Community Dent. 2015;5(1):1-12. PMID: 25767760; PMCID: PMC4355843.
31. Radcliffe CE, Potouridou L, Qureshi R, Habahbeh N, Qualtrough A, Worthington H, et al. Antimicrobial activity of varying concentrations of sodium hypochlorite on the endodontic microorganisms Actinomyces israelii, A. naeslundii, Candida albicans and Enterococcus faecalis. Int Endod J. 2004;37(7):438-46. PMID: 15189432.
32. Ma J, Tong Z, Ling J, Liu H, Wei X. The effects of sodium hypochlorite and chlorhexidine irrigants on the antibacterial activities of alkaline media against Enterococcus faecalis. Arch Oral Biol. 2015;60(7):1075-81. PMID: 25956995.
33. Kumar ND, Kalluru RS, Ahmed S, Abhilashini A, Jayaprakash T, Garlapati R, et al. Comparison of the antibacterial efficacy of Manuka honey against e.faecalis and E.coli - an in vitro study. J Clin Diagn Res. 2014;8(8):ZC29-31. PMID: 25302263; PMCID: PMC4190789.
34. Üsküdar-Güçlü A, Şimşek D, Ata-Vural I, Ünlü S, Başustaoğlu A. Antibacterial, antifungal and antibiofilm activity of methylglyoxal: a phytochemical from Manuka honey. Mediterr J Infect Microbes Antimicrob. 2021;10:55. doi: 10.4274/mjima.galenos.2021.2021.55
35. Beena JP, Sahoo P, Konde S, Raj NS, Kumar NC, Agarwal M. Manuka honey: a potent cariostatic agent- an in vitro study. Int J Clin Pediatr Dent. 2018;11(2):105-9. PMID: 29991862; PMCID: PMC6034044.

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