eor.istanbul.edu.tr

Official Publication of Istanbul University Faculty of Dentistry

Eur Oral Res 2025; 59(1): 33-39

Original research

The antimicrobial effect of R-limonene and its nano emulsion on *Enterococcus faecalis* - *In vitro* study

Purpose
This study aimed to evaluate the effectiveness of R-limonene and its nano emulsion formulation against *Enterococcus faecalis (E. faecalis)* in infected root canals.

Materials and Methods
A nano emulsion containing 20% R-limonene was prepared using the phase inversion method. *E. faecalis* was cultivated, and MIC/MBC values were determined through the macrodilution method. Standardized *E. faecalis* suspensions were used to infect the root canals ex vivo. Subsequently, R-limonene, nano emulsion containing 20% R-limonene, calcium hydroxide, and sterile saline were applied to the infected root canals for 7 days at the determined MIC/MBC dose. The root canals were sampled, and droplets were plated onto Mueller-Hinton agar plates, and viable bacteria (Colony Forming Units, CFU/mL) were counted. Statistical analyzes were performed based on the data.

Results
Statistically similar efficacy was observed between test materials on the root canal samples. All test materials were significantly more effective than the saline control ($p<0.05$), however, none of the test materials completely eliminated *E. faecalis* from root canals.

Conclusion
Although the effects of R-limonene on *E. faecalis* in infected root canals appeared to be like calcium hydroxide, with its broad antibacterial spectrum, gutta-percha softening and cleansing benefits, it was considered that it could considered among antimicrobials that could be used especially in endodontic retreatments alone or in synergistic combinations.

Keywords: *Antimicrobial effect, calcium hydroxide, Enterococcus faecalis, nano emulsion, R-limonene*

Ilke Doga Seker[1], Tayfun Alacam[2], Gulcin Akca[3], Aysel Yilmaz[4], Sevgi Takka[4]

Introduction

Enterococcus faecalis (*E. faecalis*) is an enteric facultative Gram-positive cocci frequently associated with persistent endodontic infections (1). In addition to several virulence factors displayed by this species, *E. faecalis* isolates expressed varied resistance patterns to some agents used as intracanal medications including calcium hydroxide pastes (2). The proton pump of the *E. faecalis* cell wall, the dentin buffering capacity in the clinical samples, the bacteria's deep penetration into dentin tubules, and the biofilm formation characterize the behavior of this bacterial species in the face of calcium hydroxide in root canal treatment. Therefore, conventional calcium hydroxide cannot penetrate well into dentinal tubules resulting in limited effectiveness of this material on this microorganism. This finding leads researchers to explore new and different forms and combinations of antimicrobial agents and various application methods.

ORCID IDs of the authors: I.D.S. 0000-0001-9531-240X; T.A. 0000-0002-1456-0223; G.A. 0000-0002-8877-4144; A.Y. 0000-0001-8874-1521; S.T. 0000-0001-6451-0497

[1]Department of Endodontics, Faculty of Dentistry, Gazi University, Ankara, Türkiye

[2]Department of Endodontics, Faculty of Dentistry, Lokman Hekim University, Ankara, Türkiye

[3]Department of Medical Microbiology, Faculty of Dentistry, Gazi University, Ankara, Türkiye

[4]Department of Pharmaceutical Technology, Faculty of Pharmacy, Gazi University, Ankara, Türkiye

Corresponding Author: Tayfun Alacam

E-mail: talacam@gazi.edu.tr

Received: 27 March 2024
Revised: 2 April 2023
Accepted: 16 May 2024

DOI: 10.26650/eor.20241459780

How to cite: *Seker ID, Alacam T, Akca G, Yilmaz A, Takka S. The antimicrobial effect of R-limonene and its nano emulsion on Enterococcus faecalis - In vitro study. Eur Oral Res 2025; 59(1): 33-39.* DOI: 10.26650/eor.20241459780

Medicinal plants have been found to have various therapeutic properties such as antimicrobial, anti-inflammatory, sedative/anxiolytic, analgesic, antioxidant, anticoagulant, anti-cariogenic, antiseptic, and antitumor effects (3-5). These properties make them functional in a wide range of endodontic applications, such as irrigation solutions, intracanal drugs, chelating agents, gutta-percha solvents, storage media for traumatic injuries, and repair materials in vital pulp therapy (6). Limonene, a cyclic monoterpene, and the primary component of essential oils in citrus plants has been identified as one of the most important active compounds with antimicrobial effects (7). A previous study showed that *Citrus limonum* essential oil, which has a high content of limonene (73%), was effective against endodontic pathogens, including *E. faecalis* (8). The use of *Citrus limonum* and R-limonene in endodontics is promising because of their antimicrobial, antioxidant, anti-inflammatory, and gutta-percha solvent effects. The antimicrobial mechanism of R-limonene involves disrupting the integrity of the cytoplasmic membrane of microorganisms, causing leakage of cellular components, and inhibiting respiratory enzymes (9, 10). Using R-limonene in the form of a nano emulsion carrier system can further enhance its penetration into microorganism cells and dentinal tubules. Due to its smaller size and its existence as a kinetically stable system, nano emulsions may exhibit improved functional properties compared to conventional emulsions. A nano emulsion carrier system prepared with a mixture of D-limonene and terpene has been shown to significantly increase the antimicrobial activity of the compounds (11).

E. faecalis is a microorganism commonly found in unsuccessful endodontic treatments. If any form of R-limonene will provide a plus benefit as an antimicrobial on this microorganism, the use of R-limonene in retreatment cases will gain a dual benefit along with its organic dissolving property on gutta-percha. This study aimed to investigate the antibacterial effects of R-limonene and its nano emulsion containing 20% R-limonene on root canals infected with *E. faecalis* and to compare these effects against calcium hydroxide. The null hypothesis of this study states there is no difference between pure R-limonene and its nano emulsion formulations and their effects compared to calcium hydroxide on *E.faecalis* infected root canals.

Materials and Methods

Ethical statement

The study protocol was approved by the Gazi University Faculty of Dentistry Clinical Research Ethics Committee with the number 21071282-050.99/06 and dated 12/03/2020.

Chemicals used in the study

(R)-(+)-Limonene was purchased from Alfa Aesar (97%, Stab., Kandel, Germany). Calcium hydroxide, Polysorbate 80 (Tween 80) and 1,2-Propanediol (Propylene Glycol) were purchased from Merck KGaA (Darmstadt, Germany). All other chemicals and reagents were of analytical grade.

Sample size determination

Power analysis was performed according to one-way ANOVA (analysis of variance) for the sample size of the number of viable bacteria after treatment with the test (R-limonene, nano emulsion containing 20% R-limonene) or control materials (calcium hydroxide, saline solution) to be compared. In calculating the sample size, the probability of Type I error (α=0.05) and the power of the test (1-β) were considered as 0.95. When literature studies (12) were examined, it was seen that the average difference of 0.6 points was significant and therefore the effect size was taken as 0.6 points. Using the G Power 3.1.9.2 (Heinrich Heine-Universität, Düsseldorf, Germany) program, it was calculated that the total sample size should be 64 teeth, with 16 teeth in each group [(α=0.05), (1-β=0.95)].

Preparation of R-limonene nano emulsions

R-limonene nano emulsions were prepared using the phase inversion method with some modifications (10, 13, 14). The water phase consisted of sterile distilled water and propylene glycol in a mass ratio of 2:1, while the oil phase included R-limonene and Tween-80. Experiments were performed in triplicate, and nano emulsions were evaluated for their external phase detection, droplet size and zeta potential measurements, microscopic examination, viscosity, flow properties, and stability.

The external phase of the nano emulsions was determined using the dyeing method and it was found that all the prepared nano emulsions formed an oil-in-water emulsion. Droplets smaller than 200 nm were obtained in all formulations, and the mean diameter of the droplets decreased as the surfactant concentration increased. Zeta potential values also decreased with increasing surfactant concentration. A nano emulsion containing 20% (w/w) R-limonene/10% (w/w) Tween-80 formulation was chosen for further testing.

The stability of nano emulsion containing 20% R-limonene was evaluated at 4, 25, and 40 °C for one week, during which time no significant changes in overall physical appearance, droplet size, or zeta potential were observed. Microscopic examination revealed that oil droplets were homogeneously distributed in the water phase. The viscosity of nano emulsion containing 20% R-limonene was determined as 8.128±0.17 mPa.s and exhibited pseudoplastic flow properties.

Determination of the minimum inhibitory and bactericidal concentration (MIC/MBC)

In this study, the standard bacterial strain *E. faecalis* (ATCC 29212) was used. Preliminary experiments indicated the effectiveness of R-limonene in agar diffusion tests (data not shown). Therefore, it was decided to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of both R-limonene and its nano emulsion containing 20% R-limonene. A serial macrodilution method was used to determine MI/MBC, and the relevant data are summarized in Table 1.

Endodontic experiment setup

This study is based on the approach of Turner *et al.* (12). Accordingly, 64 recently extracted human maxillary molar teeth were collected, and the palatal roots were resected and coronally abraded to length of 11 mm. The working length was determined as 1 mm shorter than the length at which the tip of the #10 K-file (Dentsply Sirona, Ballaigues, Switzerland) ap-

peared at the apical foramen and was adjusted to 10 mm (Figure 1). The canals were prepared using ProTaper Gold (PTG; Dentsply Sirona, Ballaigues, Switzerland) nickel-titanium rotary files, and the preparation was completed with PTG F2. During preparation, sodium hypochlorite (Wizard, Rehber Chemistry, Istanbul, Turkey) irrigation (10 mL, 1%) was performed using sterile endodontic needles and a 5 mL syringe. The standardized roots were rinsed in an ultrasonic bath (Eurosonic Micro, Euronda, Vicenza, Italy) first with water for 5 minutes, then with 17% ethylenediaminetetraacetic acid (EDTA; Wizard, Rehber Chemistry, Istanbul, Turkey) for 4 minutes, sodium hypochlorite (1%) for 4 minutes, and finally with water for 5 minutes, respectively. After sterilization in an autoclave (15 min at 121°), three layers of clear nail polish were applied to all outer root surfaces, and the apical regions of the roots were covered with composite resin (Charisma, Kulzer, Hanau, Germany). The roots were then placed in sterile Eppendorf tubes upright.

Infection of root canals

Using brain heart infusion (BHI; Merck KGaA, Darmstadt, Germany) broth, the *E. faecalis* bacterial suspension was adjusted according to the 0.5 McFarland standard ($1.5x10^8$ CFU/mL). Each root canal was infected and incubated for 21 days at 37 °C, sealed in 2 ml of bacterial suspension within separate Eppendorf tubes. The canals were recontaminated with bacterial suspension in a fresh medium every 3 days. The viability (culturing) and purity (gram staining, microscopic examination) of the bacteria were also controlled periodically.

Medication of root canals

After 21 days, the canals were rinsed with 5 mL saline using a sterile endodontic needle and dried with sterile paper points. Roots were distributed into 2 test groups and 2 control groups, each containing 16 roots. In the first group, the determined MIC/MBC value of R-limonene (210,3 mg/mL; dilution in Tween 80) was applied using a 27-gauge dental needle and syringe (Set Inject; Set Medical Instruments, Istanbul, Turkey).

Table 1. *Minimum Inhibitory Concentration and Minimum Bactericidal Concentration (MIC/MBC) values .*

Type of Microorganism	Type of test medicament	Concentration range	MIC/ MBC
E. faecalis	R-limonene	841.2 mg/mL – 1.64 mg/mL	210.3 mg/mL
	Nano emulsion containing 20% R-limonene	195.29 mg/mL – 0.38 mg/mL	48.82 mg/mL

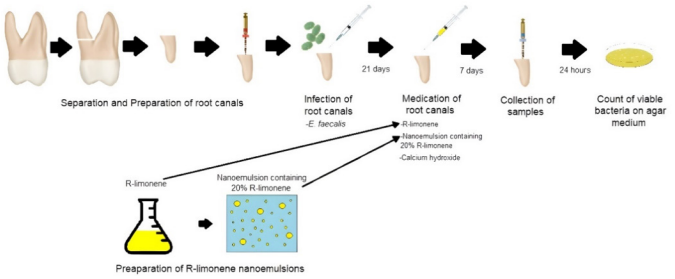

Figure 1. *The diagram depicting the workflow of the experiment.*

In the second group (nano emulsion containing 20% R-limonene), the R-limonene concentration was 195,29 mg/mL. In the third group, a mixture of calcium hydroxide powder (Merck KGaA, Darmstadt, Germany) and distilled water (15) was applied using a sterile lentulo spiral filler (Dentsply Sirona). The fourth group was washed only with sterile saline solution, as the control groups. The canal openings were closed with dental wax, and then all samples were incubated in Eppendorf tubes at 37 °C for 7 days with the closed caps. After seven days, the canals were rinsed with 5 mL of sterile saline solution and dried using sterile paper points (DiaDent, Chongju, Korea). The root canals were sampled using a sterile PTG F3 file, and the dentinal chips were collected into a sterile Eppendorf tube containing 1 mL of BHI broth, followed by vortexing for 30 seconds. Ten-fold serial dilutions were prepared, and inoculations were made on Mueller-Hinton agar (MHA; Merck KGaA, Darmstadt, Germany) plates. The plates were incubated at 37 °C for 24 hours. Colonies that grew at the end of the incubation were counted, and the result for each sample, in terms of viable bacteria count in CFU/mL was recorded.

Statistical analysis

Descriptive statistics for the number of viable bacteria were calculated for R-limonene, nano emulsion containing 20% R-limonene, calcium hydroxide and saline solution. Normality assumptions were examined using the Shapiro-Wilk test. Since the assumption of normal distribution was not provided, the non-parametric Kruskal-Wallis test was used to compare the materials. Pairwise comparisons were made using Dunn's test with Bonferroni correction. All analyzes were performed using SPSS v23 software (IBM Corp. SPSS Statistics for Windows, Armonk, NY, USA) and the upper limit for the significance level was taken as 0.05.

Results

The number of viable bacteria counts, and statistical analysis results are given in Table 2 and Table 3. The Shapiro-Wilk test showed that the viable bacterial count did not follow a normal distribution, and Figure 2 revealed the presence of outliers and/or skewness in the data.

Table 2. *Comparison of materials in terms of the number of viable bacteria.*

Material	Median$^{\Psi}$	IQR	χ^2	P
R-limonene	$3.39x10^6$ a	$6.33x10^6$	37.09	<0.001
Nano emulsion containing 20% R-limonene	$4.16x10^6$ a	$1.25x10^7$		
Calcium hydroxide	$2.08x10^6$ a	$2.32x10^6$		
Saline solution	$1.38x10^8$ b	$4.85x10^7$		

$^{\Psi}$: Within the same column, different letters indicate groups statistically differing from each other based on post hoc comparisons by Dunn's test (P<.05).
IQR: Interquartile Range; χ2 (Kruskal-Wallis Test Statistics Value)

Table 3. *Descriptive statistics on the number of viable bacteria.*

Measurement	Group	M	SD	Min	Max	SW
Number of viable bacteria	R-limonene	$5.56x10^6$	$7.25x10^6$	$1.08x10^5$	$2.64x10^7$	.732*
	Nano emulsion containing 20% R-limonene	$6.31x10^6$	$6.31x10^6$	$2.56x10^3$	$1.61x10^7$	.819*
	Calcium hydroxide	$1.61x10^6$	$1.17x10^6$	$1.80x10^2$	$3.58x10^6$	.878*
	Saline solution	$1.43x10^8$	$3.93x10^7$	$9.20x10^7$	$2.52x10^8$	.905

*$p<0.05$ (The data is not from the Normal Distributions); M: Mean; SD: Standard Deviation; SW: Shapiro-Wilk

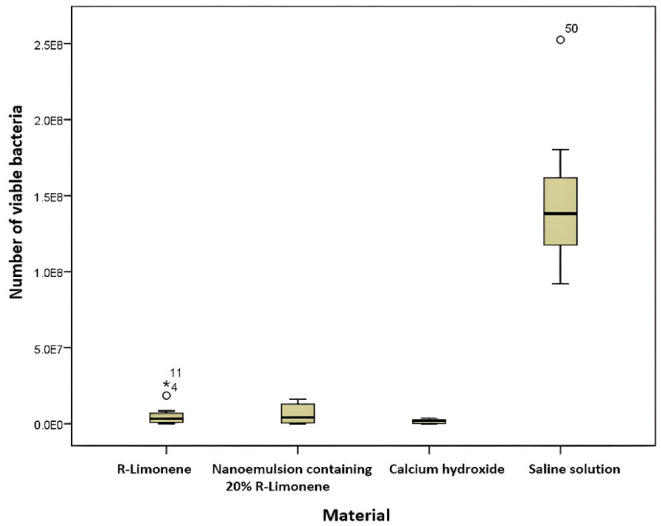

Figure 2. *Distribution graph at the material level in terms of viable bacteria count values.*

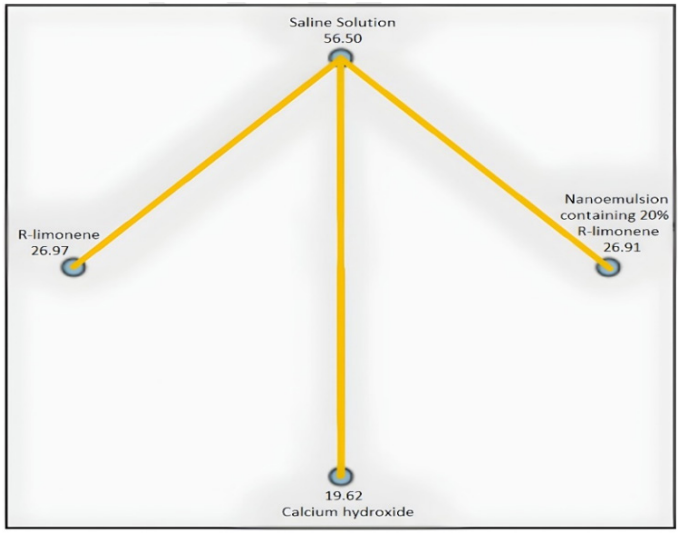

Figure 3. *Graphs of the binary comparison test of the materials over the viable bacteria count values.*

Statistical analysis of the materials according to the number of viable bacteria showed that the measurement values of the saline solution were significantly higher than the other materials ($p<0.05$). On the other hand, there was no statistically significant difference between the nano emulsion containing 20% R-limonene, R-limonene, and calcium hydroxide materials. Pairwise comparisons revealed that each group had significantly lower bacterial count compared to the saline control, and a significant difference was found between each group and the saline solution (Figure 3).

Discussion

There has been increased interest in herbal products recently in dentistry due to their high therapeutic efficacy and fewer adverse effects. Limonene is the main compound contained in all citrus-derived essential oils. It is widely used as a flavoring in commercial applications such as the food and beverage industry, due to its transparency and pleasant citrus fragrance. It is listed in the code of federal regulations as generally considered safe (GRAS) for food preservation (16). In addition, limonene has aroused the interest of many researchers due to its wide spectrum of antimicrobial activities, making it a promising antimicrobial agent (17, 18), as a green solvent for the extraction of natural products (19), and its ability to serve as a substitute solvent for chloroform for softening gutta-percha during endodontic retreatment cases (20).

To minimize the oxidation degradation and hydrophobicity of limonene, nano emulsion technology has been widely used for its encapsulation (11, 21). Nano emulsions are very efficient in terms of improving the stability and decreasing the disruption of encapsulated compounds (22). Furthermore, it is believed that the same encapsulated component displays higher antimicrobial activity compared to the bulk form. This might be due to tiny droplets of the formulation that can easily penetrate bacterial cells and destabilize the lipid envelope of the treated microorganisms (23, 24).

The purpose of this study was to compare the antibacterial properties of R-limonene and its nano emulsion containing 20% R-limonene on *E. faecalis* in infected root canals. It was first planned to develop nano emulsion formulation containing 20% R-limonene using the phase inversion method, known for being cost-effective, time-efficient, and does not require high energy. Initially, it was thought that an emulsion formulation with a droplet size of "nm" would be effective in terms of antibacterial activity, and it was aimed to develop stable and nano-sized emulsions with the phase inversion method. The results of the macrodilution method showed that the nano emulsion containing 20% R-limonene exhibited a lower MIC/MBC against *E. faecalis* compared to R-limonene. This difference can be attributed to the improved transport mechanism of the nano-sized emulsion form of R-limonene to the cell membrane of target microorganisms. Additionally, R-limonene is susceptible to oxidative degradation, which results in direct loss of activity. Therefore, the protection offered by the nano emulsion form of R-limonene against oxidative stress contributed to the increased antimicrobial activity observed in this study. On the other hand, the hydrophobic nature of R-limonene may have prevented

its homogeneous dispersion in the macrodilution method, which could be the reason for the less antimicrobial effect.

MIC/MBC values were determined for R-limonene and nano emulsion containing 20% R-limonene, and these values were then applied to infected canals at the prepared concentration. While using R-limonene and nano emulsion containing 20% R-limonene in the root canals in this study, care was taken to include R-limonene in a similar mass ratio to understand which form of R-limonene would be more effective. The least number of viable bacteria was observed in calcium hydroxide. Subsequently, R-limonene, nano emulsion containing 20% R-limonene, and saline solution were observed, respectively. The number of viable bacteria in the saline group was significantly higher than the other materials in infected root canals.

On the other hand, it was observed that both R-limonene and its nano emulsion containing 20% R-limonene demonstrated similar efficacy against *E. faecalis* on infected root canal dentin as that of calcium hydroxide. According to these results, the fact that there was no difference between calcium hydroxide and pure R-limonene and the nano emulsion form on infected root canals confirmed the null hypothesis. Findings regarding the number of viable bacteria for nano emulsion similar to that of R-limonene were consistent with results reported by Sonu *et al.* (25) who reported that the nano emulsion of limonene and limonene dissolved in dimethyl sulfoxide had an antimicrobial effect on *Bacillus cereus, Escherichia coli, E. faecalis,* and *Salmonella typhi* microorganisms, but there was no difference between them. Although the nano emulsion containing 20% R-limonene was superior in terms of MIC/MBC value, it lost its advantage when exposed to structural materials such as dentin, hydroxyapatite, and collagen found in infected root canals. The preparation method of nano emulsion, the amount of surfactant and active agent concentration, pH, ionic strength, temperature, and sampling technique problems arising from the complexities of root canal anatomy, as well as the penetration factor of microorganisms into dentinal tubules, and subsequent culturing, may be the causes of variation in the number of microorganisms among the groups. An increase in the sample size in future studies would mitigate the effects of variations and may provide more supporting evidence for the benefit of using nano emulsions in infected teeth during the endodontic treatment.

The retreatment requires the complete removal of the filling material from the root canal space, usually made by the association of gutta-percha and some endodontic cement (26). The effectiveness of this procedure is guaranteed by the total removal of the sealer and the gutta-percha from an inadequately shaped and filled root canal system because it is critical to uncover remnants of necrotic tissue or bacteria, and they have to be exposed to a more efficient chemo-mechanical disinfection procedure (27). Organic solvents have to be applied during retreatment to reduce the resistance of filling materials inside the root canal, thus facilitating their removal (28). The orange oil solvent is traditionally used for cleansing and removing various types of cement, pastes, and impression materials from instruments, mixing plates, devices, patient skin, tissues, etc., but it is widely indicated as a solvent during endodontic retreatments. Orange oil was found to be more biocompatible than eucalyptol, xylol, chloroform, and halothane (29). The main ingredient of *Citrus limonum* essential oil is monoterpenoids, especially limonene. Orange oil solvent efficiency was found similar to chloroform, so it is recommended as a suitable alternative to this product. This facilitates chemo-mechanical preparation, and the irrigating solutions can access all ramifications of the entire root canal system during retreatment by decreasing the residual microbial population (30). Thus, citrus oil has discrete effectiveness in the antimicrobial, antioxidant, anti-inflammatory activity, and solvent action on remnants of gutta-percha in endodontic retreatment. Given the association of *E. faecalis* in cases of chronic failure in endodontically treated teeth, a medication especially for this species may be of value (8).

Using normal saline to eliminate calcium hydroxide was one of the limitations of this study. It is not possible to be sure that some remnants of calcium hydroxide were not included in the collected debris, which should be considered in future studies. One of the other limitation of our study is that it was conducted under *in vitro* conditions. The effectiveness of antiseptic substances is affected by factors such as the density of the active substance, incubation time, ambient temperature, root canal condition, pH, pollution level, and the amount of organic matter in the environment. Considerations should include the resistance of microorganisms, the interaction between microorganisms, host factors, inactivation by bacteria in the canal, and the ability of the drug to penetrate the dentin tissue and canal details. Since microorganisms diffuse into the dentin canals, intracanal drugs must also be capable of penetrating the dentin canals or spreading their activity deeply. The wetting properties of antiseptics are also gaining importance. Additionally, biofilms and bacterial aggregation are general mechanisms for the survival of bacteria and are virulence factors that play an important role in development. In addition to the antibacterial effect in irrigation and disinfection of root canals, the prevention of bacterial adhesion should also be considered. The fact that R-limonene, nano emulsion containing 20% R-limonene, and calcium hydroxide showed similar results suggests that R-limonene could serve as an alternative to intracanal medicaments used in root canals, considering its other properties. The organic solvent feature, gutta-percha dissolving capability, and antibacterial effects all indicate that this natural product holds promise for use in endodontics.

Conclusion

Within the limitations of this *in vitro* experiment, it can be concluded that both R-limonene and its nano emulsion containing 20% R-limonene demonstrated similar efficacy against *E. faecalis* on infected root canal dentin as that of calcium hydroxide. Although we have determined that the nano emulsion form does not provide a plus advantage over its pure form in terms of its antibacterial effect on *E. faecalis*, the use of the nano emulsion form may be preferable in retreatment cases, for its gutta-percha dissolving efficacy, existing broad-spectrum antibacterial effects, and protection against oxidative stress. Furthermore, due to its anti-inflammatory, and antioxidant properties, R-limonene can be incorporated into the content of endodontic medicaments, irrigation solutions, and sealers.

Türkçe Özet: *R-Limonen ve nanoemülsiyonunun Enterococcus faecalis üzerindeki antimikrobiyal etkisi- In vitro çalışma. Amaç: Bu çalışma, enfekte kök kanallarında Enterococcus faecalis'e (E. faecalis) karşı R-limonen'in ve onun nanoemülsiyon formülasyonunun etkinliğini değerlendirmeyi amaçlamaktadır. Gereç ve yöntem: %20 R-limonen içeren nanoemülsiyon, faz inversiyon yöntemi kullanılarak hazırlandı. E. faecalis kültüre edildi ve MIC/MBC değerleri makrodilüsyon yöntemi ile belirlendi. Standartlaştırılmış E. faecalis süspansiyonları kök kanallarını enfekte etmek için ex vivo olarak kullanıldı. Daha sonra, R-limonen, %20 R-limonen içeren nanoemülsiyon, kalsiyum hidroksit ve steril salin, belirlenen MIC/MBC dozunda enfekte kök kanallarına 7 gün boyunca uygulandı. Kök kanallarından alınan örnekler, Mueller-Hinton agar plaklarına ekildi ve canlı bakteriler (CFU/mL) sayıldı. Verilere dayalı olarak istatistiksel analizler yapıldı. Bulgular: Test materyalleri arasında istatistiksel olarak benzer etkinlik gözlendi (p>0.05). Tüm test materyalleri, salinden (p<0.05) önemli ölçüde daha etkiliydi, ancak hiçbir test materyali E. faecalis'i kök kanallarından tamamen elimine edemedi. Sonuç: R-limonen'in enfekte kök kanallarında E. faecalis üzerindeki etkileri kalsiyum hidroksite benzer görünse de geniş antibakteriyel spektrumu, guta-perka yumuşatma etkisi ve temizleyici etkisi de göz önüne alınarak endodontik tedavilerde tek başına veya sinerjistik kombinasyonlarla kullanılabilecek antimikrobiyaller arasında bulunabileceği düşünülmüştür. Anahtar kelimeler: Antimikrobiyal etki, kalsiyum hidroksit, Enterococcus faecalis, nanoemülsiyon, R-limonen.*

Ethics Committee Approval: The study protocol was approved by the Gazi University Faculty of Dentistry Clinical Research Ethics Committee with the number 21071282-050.99/06 and dated 12/03/2020.
Informed Consent: Participants provided informed consent.

Peer-review: Externally peer-reviewed.

Author contributions: TA, GA, ST participated in designing the study. IDS, AY participated in generating the data for the study. IDS, AY participated in gathering the data for the study. IDS, AY participated in the analysis of the data. IDS, TA, AY, wrote the majority of the original draft of the paper. IDS, TA, GA, AY, ST participated in writing the paper. IDS, TA, GA, AY, ST has had access to all of the raw data of the study. IDS, TA has reviewed the pertinent raw data on which the results and conclusions of this study are based. IDS, TA, GA, AY, ST have approved the final version of this paper. TA guarantees that all individuals who meet the Journal's authorship criteria are included as authors of this paper.

Conflict of interest: The authors declared that they have no conflict of interest.

Financial disclosure: This study was produced from the doctoral thesis prepared by Seker ID entitled "In Vitro Investigation of the Antibacterial Effects of (R)-limonene Nano emulsions on Root Dentin". This study was supported by Gazi University, BAP Council (Project Number: 03/2020-09). Seker ID and Yilmaz A were supported by the CoHE 100/2000 Ph.D. Scholarship Program. Yilmaz A was supported by TUBİTAK 2211/A Domestic Ph.D. Scholarship Program.

Acknowledgments: The professional language edition was supervised by Gazi University Academic Writing, Application and Research Center. We thank Bulent Altunkaynak, Professor of Statistics at Gazi University for his support in the statistical analysis of our study, and Guven Kayaoglu, Professor of Endodontics at Gazi University for the writing assistance, and proof reading.

References

1. Sunde PT, Olsen I, Debelian GJ, Tronstad L. Microbiota of periapical lesions refractory to endodontic therapy. J Endod 2002;28:304-10. [CrossRef]
2. Byström A, Claesson R, Sundqvist G. The antibacterial effect of camphorated paramonochlorophenol, camphorated phenol and calcium hydroxide in the treatment of infected root canals. Dent Traumatol 1985;1:170-5. [CrossRef]
3. Sinha DJ, Sinha AA. Natural medicaments in dentistry. Ayu 2014;35:113-8. [CrossRef]
4. Tewari R, Kapoor B, Mishra S, Kumar A. Role of herbs in endodontics. Journal of Oral Research and Review 2016;8:95-9. [CrossRef]
5. Vishnuvardhini S, Sivakumar A, Ravi V, Prasad A, Sivakumar J. Herbendodontics–phytotherapy in endodontics: A review. Biomed Pharmacol J 2018;11:1073-82. [CrossRef]
6. Almadi EM, Almohaimede AA. Natural products in endodontics. Saudi Med J 2018;39:124-30. [CrossRef]
7. Jantrawut P, Boonsermsukcharoen K, Thipnan K, Chaiwarit T, Hwang KM, Park ES. Enhancement of antibacterial activity of orange oil in pectin thin film by microemulsion. Nanomaterials (Basel) 2018;8:545. [CrossRef]
8. Seker ID, Akca G, Alacam T. In vitro evaluation of antimicrobial effects of citrus limonum essential oil on some endodontic pathogens. Ann Med Res 2022;29:427-33. [CrossRef]
9. Zahi MR, El Hattab M, Liang H, Yuan Q. Enhancing the antimicrobial activity of d-limonene nano emulsion with the inclusion of ε-polylysine. Food Chem 2017;221:18-23. [CrossRef]
10. Zhang Z, Vriesekoop F, Yuan Q, Liang H. Effects of nisin on the antimicrobial activity of d-limonene and its nano emulsion. Food Chem 2014;150:307-12. [CrossRef]
11. Donsì F, Annunziata M, Sessa M, Ferrari G. Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. LWT - Food Science and Technology 2011;44:1908-14. [CrossRef]
12. Turner S, Love R, Lyons K. An in-vitro investigation of the antibacterial effect of nisin in root canals and canal wall radicular dentine. Int Endod J 2004;37:664-71. [CrossRef]
13. Feng J, Wang R, Chen Z, Zhang S, Yuan S, Cao H, Jafari SM, Yang W. Formulation optimization of d-limonene-loaded nano emulsions as a natural and efficient biopesticide. Colloids Surf A Physicochem Eng Asp 2020;596:124746. [CrossRef]
14. Maté J, Periago PM, Palop A. Combined effect of a nano emulsion of d-limonene and nisin on listeria monocytogenes growth and viability in culture media and foods. Food Sci Technol Int 2016;22:146-52. [CrossRef]
15. Alaçam T, Yoldaş HO, Gülen O. Dentin penetration of 2 calcium hydroxide combinations. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998;86:469-72. [CrossRef]
16. Sun J. D-limonene: Safety and clinical applications. Altern Med Rev 2007;12:259-64.
17. Chikhoune A, Hazzit M, Kerbouche L, Baaliouamer A, Aissat K. Tetraclinis articulata(vahl) masters essential oils: Chemical composition and biological activities. Journal of Essential Oil Research 2013;25:300-7. [CrossRef]
18. Vuuren Sv, Viljoen AM. Antimicrobial activity of limonene enantiomers and 1, 8-cineole alone and in combination. Flavour Fragr J 2007;22:540-4. [CrossRef]
19. Chemat S, Tomao V, Chemat F. Limonene as green solvent for extraction of natural products. In: Mohammad A, editor. Green solvents i: Properties and applications in chemistry. Dordrecht: Springer Netherlands; 2012. p. 175-86. [CrossRef]
20. Uemura M, Hata G, Toda T, Weine FS. Effectiveness of eucalyptol and d-limonene as gutta-percha solvents. J Endod 1997;23:739-41. [CrossRef]
21. Li PH, Chiang BH. Process optimization and stability of d-limonene-in-water nano emulsions prepared by ultrasonic emulsification using response surface methodology. Ultrason Sonochem 2012;19:192-7. [CrossRef]
22. Weiss J, Gaysinsky S, Davidson M, McClements J. Nanostructured encapsulation systems: Food antimicrobials. In: Barbosa-Cánovas G, Mortimer A, Lineback D, Spiess W, Buckle K, Colonna P, editors. Global issues in food science and technology. San Diego: Academic Press; 2009. p. 425-79.
23. Baker J, Hamouda T, Shih A, Myc A, inventors; University of Michigan, assignee. Non-toxic antimicrobial compositions and methods of use. USA patent US-6559189-B2. 2003.

24. Zahi MR, Liang H, Yuan Q. Improving the antimicrobial activity of d-limonene using a novel organogel-based nano emulsion. Food Control 2015;50:554-9. [CrossRef]
25. Sonu KS, Mann B, Sharma R, Kumar R, Singh R. Physico-chemical and antimicrobial properties of d-limonene oil nano emulsion stabilized by whey protein-maltodextrin conjugates. J Food Sci Technol 2018;55:2749-57. [CrossRef]
26. Wennberg A, Ørstavik D. Evaluation of alternatives to chloroform in endodontic practice. Dent Traumatol 1989;5:234-7. [CrossRef]
27. Ørstavik D, Haapasalo M. Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. Dent Traumatol 1990;6:142-9. [CrossRef]
28. Bodrumlu E, Er O, Kayaoğlu G. Solubility of root canal sealers with different organic solvents. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008;106:e67-9. [CrossRef]
29. Rehman K, Khan FR, Aman N. Comparison of orange oil and chloroform as gutta- percha solvents in endodontic retreatment. J Contemp Dent Pract 2013;14:478-82. [CrossRef]
30. Whitworth JM, Boursin EM. Dissolution of root canal sealer cements in volatile solvents. Int Endod J 2000;33:19-24. [CrossRef]