The antimicrobial effect of R-limonene and its nanoemulsion on Enterococcus faecalis - In vitro study

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Research Article

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Abstract

Objectives: This study aimed to investigate the antimicrobial effects of pure R-limonene and its nanoemulsion formulation on Enterococcus faecalis-infected root canals.

Materials and Methods: First, a nanoemulsion formulation of R-limonene was prepared with the phase inversion method. E. faecalis was cultivated and MIC/MBC values were determined using the macrodilution method. Then, standardized suspensions of E. faecalis were used for infecting the root canals of extracted teeth. Finally, pure R-limonene at the determined MIC/MBC dose, a nanoemulsion containing 20% R-limonene, calcium hydroxide (Ca(OH)$_2$), and sterile saline solution were applied to the infected root canals for 7 days. The root canals were then sampled, droplets were plated onto Mueller-Hinton agar plates, and viable bacteria (CFU/mL) were counted. Kruskal-Wallis test and Dunn's test with Bonferroni correction were used for the statistical analyses. The statistical significance was set to p<0.05.

Results: The root canal samplings revealed no statistically significant differences among the test materials (p>0.05); all were significantly lower than the saline control values (p<0.05). None of the tested materials eliminated E. faecalis from the root canals.

Conclusions: The antimicrobial effects of R-limonene, its 20% nanoemulsion, and Ca(OH)$_2$ were similar in E. faecalis-infected root canals.

Clinical Relevance: Nanoemulsions with their enhanced physicochemical properties have led research toward new prospects of treating and preventing intracanal infection. This study aimed to determine whether R-limonene nanoemulsion or natural form would be effective at eradicating E. faecalis within the root canal system.

Introduction

Medicinal plants have antimicrobial, anti-inflammatory, sedative/anxiolytic, analgesic, antioxidant, anticoagulant, anti-cariogenic, antiseptic, and antitumor effects [1–3]. Thanks to these effects, in endodontics, medicinal plants have a wide range of use as irrigation solutions, intracanal medicament, chelation agents, gutta-percha solvent, a storage medium that can be used in traumatic injuries, and repair material in vital pulp treatments [4].

The limited effectiveness of Ca(OH)$_2$, which is frequently used in root canal disinfection, against endodontic pathogens such as E. faecalis has led researchers to search for new antimicrobials and more effective application methods. The antimicrobial activity of orange oil was also evaluated and showed significant inhibitory effects against many microorganisms including E. faecalis. Limonene is the most important active ingredient in terms of antimicrobial effect [5]. Limonene is an important cyclic monoterpene and is the parent compound of essential oils of citrus plants. In a previous study, Citrus limonum essential oil, which contains high content of limonene (73%), was found to be effective against endodontic pathogens, including E. faecalis [6]. C. limonum and limonene have the potential to be used in
endodontics with their antimicrobial, antioxidant, anti-inflammatory, and gutta-percha solvent effects. The antimicrobial mechanism of action of R-limonene has been explained as disrupting the cytoplasmic membrane integrity of microorganisms, causing leakage of some cellular components such as ions and proteins by changing membrane permeability, and inhibition of respiratory enzymes [7, 8].

The use of R-limonene as a nanoemulsion instead of its pure use can better penetrate the microorganism cells due to its nano size. A nanoemulsion carrier system prepared with a mixture of D-limonene and terpene has significantly increased the antimicrobial activity of encapsulated compounds compared to unencapsulated ones [9].

This study aimed to investigate the antibacterial effects of pure R-limonene and nanoemulsion formulations containing different ratios of R-limonene and compare their effects against Ca(OH)₂ on E. faecalis-infected root canals.

**Materials And Methods**

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

Power analysis was performed according to one-way ANOVA for the sample size of the materials to be compared. Using the G Power 3.1.9.2 (Henrick 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)].

The flowchart of the methodology is shown in Fig. 1.

**Preparation of R-limonene nanoemulsions**

R-limonene [(R)-(+) -Limonene, 97%, Stab., Alfa Aesar, Kandel, Germany] nanoemulsions were prepared with the phase inversion method with some modifications [8, 10, 11]. Sterile distilled water and propylene glycol at a mass ratio of 2:1 was used as the water phase in all nanoemulsion formulations, and R-limonene and Tween-80 (Merck, Darmstadt, Germany) were used as the oil phase. The experiments were repeated in three parallels. Nanoemulsions were evaluated in terms of macroscopic examination and external phase detection, droplet size and zeta potential measurement, microscopic examination, viscosity, flow properties, and stability of nanoemulsions were also evaluated. The external phase of the nanoemulsions was determined using the dyeing method and it was observed that all the prepared nanoemulsions formed an oil/water emulsion for the purpose. Nano-sized droplets (< 200 nm) were obtained in all formulations and the average diameter of the droplets decreased as the surfactant concentration increased. It was observed that the zeta potential values decreased as the surfactant increased. Nanoemulsion formulation containing 20% (w/w) R-limonene, 10% (w/w) Tween-80 (NE20) was chosen. This formulation was subjected to a one-week stability test at 4, 25, and 40°C. During the one-week stability follow-up, there were no significant changes in overall physical appearance, droplet
size, and zeta potential for this formulation. As a result of microscopic examination, it was observed that
the oil droplets were homogeneously dispersed in the water phase. The viscosity of NE20 was found to
be $8.128 \pm 0.17$ mPa.s. NE20 was found to have pseudoplastic flow property.

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

*E. faecalis* (ATCC 29212) standard bacterial strain was used. In pilot experiments, R-limonene was
effective in agar diffusion tests (data not shown). So, it was decided to find MIC/MBC by serial
macrodilution method. MIC/MBC values are given in Table 1.

<table>
<thead>
<tr>
<th>Type of Microorganism</th>
<th>Type of test medicament</th>
<th>Concentration range</th>
<th>MIC/MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em></td>
<td>R-limonene</td>
<td>841,2 mg/mL – 1,64 mg/mL</td>
<td>210,3 mg/mL</td>
</tr>
<tr>
<td></td>
<td>Nanoemulsion containing 20% R-limonene</td>
<td>195,29 mg/mL – 0,38 mg/mL</td>
<td>48,82 mg/mL</td>
</tr>
</tbody>
</table>

**Endodontic Experiment Setup**

The method applied by Turner, Love and Lyons [12] was taken as a reference. 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 adjusted to 10 mm. The canals were prepared using
ProTaper Gold (PTG; Dentsply Sirona, Ballaigues, Switzerland) nickel-titanium rotary files, and the
preparation was completed with PTG F2 using sodium hypochlorite (10 mL, 1%) irrigation. The roots were
rinsed in an ultrasonic bath (Eurosonic Micro, Euronda, Vicenza, Italy) first with water for 5 minutes, then
with EDTA (17%) for 4 minutes, sodium hypochlorite (1%) for 4 minutes, and finally with water for 5
minutes, respectively. After sterilization in an autoclave, 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. The roots were
then placed in sterile Eppendorf tubes upright.

**Infection of root canals**

Using BHI broth, the *E. faecalis* bacterial suspension was adjusted according to the 0.5 McFarland
standard ($1.5 \times 10^8$ CFU/mL). Each root canal was infected and incubated for 21 days at 37°C, sealed in 2
ml of bacterial suspension in individual Eppendorf tubes. The canals were recontaminated with bacterial
suspension in a fresh medium every 3 days. The viability (culturing) and the purity (gram staining,
microscopic examination) of the bacteria were also controlled periodically.
Medication of root canals

After 21 days, the canal was rinsed with 5 mL saline using a sterile endodontic needle and dried with sterile paper points.

Roots were distributed to 2 experimental groups and 2 control groups with 16 roots in each. In the first group, the determined MIC/MBC value (210.3 mg/mL; dilution in Tween 80) of R-limonene was applied using a 27-G needle. In the second group (NE20), the R-limonene concentration was 195.29 mg/mL. In the third and fourth groups, Ca(OH)$_2$-distilled water mixture [13] and saline, respectively, were applied as the controls. The canal openings were closed with dental wax and all samples were incubated in Eppendorf tubes at 37°C for 7 days with the caps closed. After seven days, the canals were rinsed with 5 mL of sterile saline solution, and dried with sterile paper points. 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, and vortexed for 30 seconds.

Ten-fold serial dilutions were prepared, and inoculations were made on MHA plates. The plates were incubated at 37°C for 24 hours, and viable bacteria counts were recorded in CFU/mL.

Statistical analysis

Descriptive statistics for the number of viable bacteria were made for each material. 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. P < 0.05 was accepted as the statistical significance limit.

Results

The number of viable bacteria counts and statistical analysis results are given in Tables 2 and 3.

<table>
<thead>
<tr>
<th>Material</th>
<th>Median $^\Psi$</th>
<th>IQR</th>
<th>$\chi^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-limonene</td>
<td>3.39x10$^6$ $^a$</td>
<td>6.33x10$^6$</td>
<td>37.09</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Nanoemulsion containing 20% R-limonene</td>
<td>4.16x10$^6$ $^a$</td>
<td>1.25x10$^7$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium hydroxide</td>
<td>2.08x10$^6$ $^a$</td>
<td>2.32x10$^6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline solution</td>
<td>1.38x10$^8$ $^b$</td>
<td>4.85x10$^7$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^\Psi$: Within the same column, different letters indicate groups statistically differing from each other based on post hoc comparisons by Dunn's test (P < .05).
Table 3
Descriptive statistics on the number of viable bacteria

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Group</th>
<th>M</th>
<th>SD</th>
<th>Min</th>
<th>Max</th>
<th>SW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of viable bacteria</td>
<td>R-limonene</td>
<td>5.56x10^6</td>
<td>7.25x10^6</td>
<td>1.08x10^5</td>
<td>2.64x10^7</td>
<td>.732*</td>
</tr>
<tr>
<td></td>
<td>Nanoemulsion containing 20% R-limonene</td>
<td>6.31x10^6</td>
<td>6.31x10^6</td>
<td>2.56x10^3</td>
<td>1.61x10^7</td>
<td>.819*</td>
</tr>
<tr>
<td></td>
<td>Calcium hydroxide</td>
<td>1.61x10^6</td>
<td>1.17x10^6</td>
<td>1.80x10^2</td>
<td>3.58x10^6</td>
<td>.878*</td>
</tr>
<tr>
<td></td>
<td>Saline solution</td>
<td>1.43x10^8</td>
<td>3.93x10^7</td>
<td>9.20x10^7</td>
<td>2.52x10^8</td>
<td>.905</td>
</tr>
</tbody>
</table>

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

The number of viable bacteria did not provide the assumption of normal distribution. As seen in Fig. 2, there were outliers and/or skewness in the data.

When the materials were compared in terms of the number of viable bacteria, the measurement values of the saline solution were found to be statistically significantly higher than the other materials (p < 0.05). There was no statistically significant difference between NE20, R-limonene, and Ca(OH)_2 materials. A significant difference was found between each group and saline solution (Fig. 3).

Discussion

In this study, it was first planned to develop nanoemulsion formulations for R-limonene (NE20) using the phase inversion method, which is low cost, not time-consuming, 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.

In this study, it was thought that the nanoemulsion form of R-limonene increased antimicrobial activity in the macrodilution method. The fact that the NE20 had a lower MIC/MBC value on *E. faecalis* compared to pure R-limonene can be explained by the increase in the transport mechanism to the cell membrane of the target microorganisms with the nano-sized emulsion form of R-limonene. Additionally, R-limonene is susceptible to oxidative degradation, which results in direct loss of activity. Therefore, the protection of the nanoemulsion form of R-limonene compared to pure R-limonene against oxidative stress may also have caused an increase in antimicrobial activity. In addition, due to the hydrophobic nature of R-limonene, it may not be homogeneously dispersed in the macrodilution method and may have shown a less antimicrobial effect.
While using in the root canals pure R-limonene and NE20, care was taken to include R-limonene in a similar mass ratio to understand which form of R-limonene would be more effective. Thus, it is aimed to compare the nanoemulsion and natural form of R-limonene. For this reason, pure R-limonene was used in MIC/MBC value, and NE20 was applied at the concentration it was prepared. The least number of viable bacteria was observed in Ca(OH)$_2$. Then, R-limonene, NE20, and saline solution come respectively. However, there was statistically no significant difference between Ca(OH)$_2$, R-limonene, and NE20. The fact that there was no significant difference between R-limonene and Ca(OH)$_2$ showed that R-limonene could be an alternative to intracanal medicaments used in root canals, considering other properties. Both its organic solvent feature, gutta-percha dissolving feature, and antibacterial effect give positive signs that this natural product can be used in endodontics.

In terms of viable bacteria count in infected root canals, nanoemulsion showed similar results to pure R-limonene. Sonu, Mann, Sharma, Kumar and Singh [14] reported that similar to this study, the nanoemulsion of limonene oil and limonene oil 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.

While the NE20 was superior in terms of MIC/MBC value, it lost this superiority when it encountered structural materials such as dentin, hydroxyapatite, and collagen in infected root canals. However, the close results with Ca(OH)$_2$ suggest that the nanoemulsion formulation could be promising.

There are also some difficulties in working with R-limonene. R-limonene is unstable, and volatile. It should be well protected from external factors such as temperature, oxygen, and light. Especially changes in temperature and oxidative degradation can cause changes in its activity. The bacterial membrane is thought to be more sensitive to R-limonene at low temperatures; this is attributed to R-limonene being more volatile at high temperatures [15, 16]. However, the nanoemulsion form of R-limonene is more stable for oxidative degradation.

One of the weaknesses of our study is that it was conducted under *in vitro* conditions. Effective substance density, incubation time, ambient temperature, condition of the root canal, pH and pollution level, and amount of organic matter in the environment are important in the effectiveness of antiseptic substances. 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 should be considered. Since microorganisms diffuse into the dentin canals, intracanal drugs must also be able to penetrate the dentin canals or spread their activity deeply. The wetting properties of antiseptics are also gaining importance. Additionally, biofilms and bacterial aggregation are a general mechanism for the survival of bacteria and are virulence factor that plays 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.

**Conclusion**
When R-limonene and nanoemulsion formulation containing 20% R-limonene (NE20) were compared with Ca(OH)$_2$, in terms of the number of viable *E. faecalis* bacteria on infected dentine, no statistically significant difference was found between them. Considering the use of R-limonene in root canals due to its versatile positive effects, although we have determined that the nanoemulsion form does not provide an advantage when compared with its pure form in terms of its antibacterial effect on *E. faecalis*, the use of the nanoemulsion form may be a reason for preference considering the hydrophobic properties of natural oils. Considering its existing broad-spectrum antibacterial effect and comparable effect with Ca(OH)$_2$ on *E. faecalis*, R-limonene can be considered as an alternative agent that can be added to the content of endodontic medicament, irrigation solutions, and sealers.

**Declarations**

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**Author Contributions:**

Ilke Doga SEKER: data collection and analysis

Tayfun ALACAM: conceived the idea and led the writing

Gulcin AKCA: review of manuscript, data collection

Aysel YILMAZ: data collection and analysis

Sevgi TAKKA: review of manuscript, data collection

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

**Informed consent:** For this study, formal consent is not required.

**Conflict of Interest:** The authors declare that they have no conflict of interest.

**References**


**Figures**
**Figure 1**

Flowchart of the experimental stages
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.