



In vitro evaluation of antimicrobial effects of *Citrus limonum* essential oil on some endodontic pathogens

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Abstract

Aim: To investigate the antimicrobial effects of *Citrus limonum* and calcium hydroxide comparatively and to evaluate MIC, MBC, and MFC levels for some microorganismal strains of the root canal microbial flora.

Materials and Methods: *Enterococcus faecalis*, *Candida albicans*, *Staphylococcus aureus*, *Streptococcus mutans*, and *Escherichia coli* standard strains were used. Microorganisms were cultivated and used for the evaluation of the antimicrobial effect of the different concentrations of the *C.limonum* and calcium hydroxide using disc diffusion and agar well diffusion methods. Then MIC, MBC, and MFC values were recorded.

Results: Wider inhibition zones were obtained for *C.limonum* essential oil compared to calcium hydroxide. In the agar well diffusion method, while the widest inhibition zone was obtained for *C.albicans*, the narrowest inhibition zone was observed for *E.faecalis* for *C.limonum*. For calcium hydroxide, the widest inhibition zone was in *C.albicans* cultures, however, the narrowest inhibition zone was observed for *S.mutans*. In the agar disc diffusion method, the widest inhibition zone was observed for *C.albicans*, and the narrowest inhibition zone was spotted in *S.aureus* for *Citrus limonum*. For calcium hydroxide, while the widest inhibition zone again reached for *C.albicans*, the narrowest inhibition zone was found for *E.faecalis*. The lowest MIC and MBC value for *Citrus limonum* was observed in *S.mutans*. For calcium hydroxide, the lowest MIC and MBC values were recorded for *S.aureus*, *E.coli*, and *S.mutans*.

Conclusion: Data in experimental conditions showed that *C.limonum* essential oil may be an alternative candidate, and superior to calcium hydroxide in terms of antimicrobial activity for all chosen members of intracanal flora.



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Introduction

In the treatment of root canals, mechanical preparation, antimicrobial irrigation, and intra-canal medication are used in the fight against intra-canal infection. However, in cultures taken at the stage of filling in primary treatments and in recurrent cases after unsuccessful root canal treatments, it is seen that some species can still maintain their viability.

Traditionally used calcium hydroxide, some other chemicals, and antibiotics are not completely effective for all root canal microorganisms, due to the presence of resistant species and the possible side effects of the agents used; thus, the search continues for alternative materials and methods in antimicrobial therapy. The resistance of

Enterococcus faecalis to calcium hydroxide, which is a traditionally used intracanal antimicrobial agent is one of the important reasons why this search continues [1].

Medicinal plants have antimicrobial, anti-inflammatory, sedative/anxiolytic, analgesic, antioxidant, anticoagulant, anti-cariogenic, antiseptic, and antitumor effects [2]. Thanks to these effects, they have wide use in endodontics as irrigation solution, intra-canal medicament, chelation agent, gutta-percha solvent, a storage medium that can be used for traumatic injuries and repair material in vital pulp treatments [3].

Citrus is one of the most important taxonomic subunits of the family *Rutaceae*. One of the best-known and most widely used species of the *Citrus* genus is lemon-*Citrus limonum*. The main ingredients of *Citrus limonum* essential oil are monoterpenoids, especially D-limonene. Its secondary metabolites include flavonoids, as well as other

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compounds such as phenolic acids, coumarins, carboxylic acids, amino acids, and vitamins. *Citrus limonum* essential oil is classified as safe by the American Food and Drug Administration (FDA) [4]. Anticancer, antioxidant, anti-inflammatory, antibacterial, antifungal, antiviral, antiallergic, hepatoregenerative, antidiabetic, and antiobesity effects have been reported for essential oils obtained from a lemon. It is also known to have effects on the cardiovascular, respiratory, nervous, and skeletal systems. In particular, its anti-inflammatory, antibacterial, antiviral, and antifungal effects are associated with the main compound of D-limonene [5].

Citrus limonum has a wide variety of uses. It is being researched as a natural preservative, especially in the food industry, where it is important to combat microorganisms. Studies on various microorganisms have obtained results that support *Citrus limonum* as a natural antimicrobial agent [6, 7].

The retreatment requires the complete removal from root canal space of the filling material, usually made by the association of gutta-percha and some endodontic cement [8]. The effectiveness of this procedure is guaranteed by the removal of the total amount of the sealer and the gutta-percha from an inadequately shaped and filled root canal system because it is critical for uncovering remnants of necrotic tissue or bacteria, and they have to be exposed to a more efficient chemo-mechanical disinfection procedure [1]. Organic solvents have to be applied during retreatment to reduce the resistance of filling materials inside the root canal, thus facilitating their removal [9]. Different chemical solvents are available on the market, and they can dissolve obturation materials in different ways. The success of non-surgical endodontic retreatment is related to the complete removal of filling materials from the root canal system because the presence of residual filling material in a root canal may result in retreatment failure. Many techniques, instruments, and substances have been employed aiming to remove gutta-percha. The use of hand instruments either without or with solvents is emphasized because the latter decreases the risks of damaging the tooth structure during the gutta-percha removal [10]. Among the chemical solvents, xylene, eucalyptol, orange oil, and chloroform have been some of the options more commonly employed [8]. The orange oil solvent is traditionally used for the cleansing and removal of types of cement, pastes, impression materials from instruments, mixing plates, devices, patient skin, and 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 [11]. 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 of the residual microbial population [12]. Thus, citrus oil has discrete effectiveness of antimicrobial, antioxidant, anti-inflammatory activity, and solvent action on remnants of gutta-percha in endodontic retreatment. Given the association of *Enterococcus faecalis* in cases of chronic failure in endodontically treated teeth, a medica-

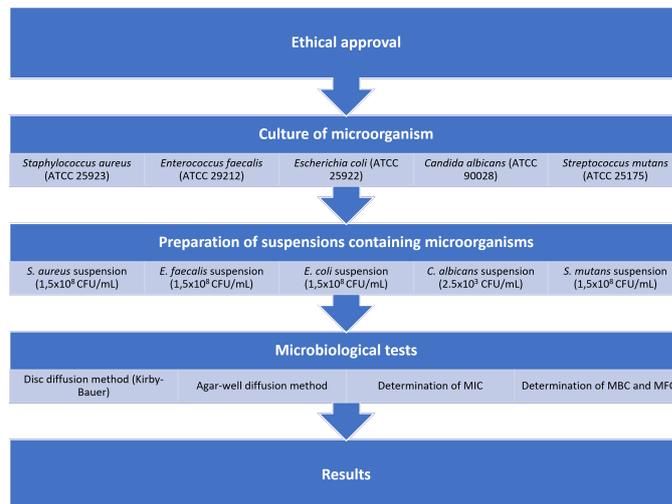


Figure 1. The flowchart of the methodology

tion specifically for this species may be of value.

This study aimed to compare the antimicrobial effect of *Citrus limonum* essential oil with calcium hydroxide against some endodontic pathogens including *Enterococcus faecalis* determining the minimum inhibitory and minimum bactericidal/fungicidal concentrations for effective use. Our study hypothesizes that Citrus limonum, a biocompatible natural product, can be an alternative to intracanal medicaments thanks to its both solvent and antimicrobial effects.

Material and Methods

Ethics committee approval was obtained from the Gazi University Faculty of Dentistry Clinical Research Ethics Committee with decision number 21071282-050.99/06, dated 12/03/2020, for this study.

The flowchart of the methodology is shown in Figure 1.

Citrus limonum essential oil (Art de Huile /Arin deep Ltd. Co, Istanbul, Turkey; Table 1, 2), calcium hydroxide powder (Merck KGaA, Darmstadt, Germany), distilled water, and sterile saline were used.

Calcium hydroxide was prepared by mixing 0.0536 g of calcium hydroxide powder with 0.0744 mL of distilled water [13].

Microbiological procedures

Test microorganisms and growth conditions

As test microorganisms, *Staphylococcus aureus* (ATCC 25923, *S. aureus*), *Enterococcus faecalis* (ATCC 29212, *E. faecalis*), *Escherichia coli* (ATCC 25922, *E. coli*), *Candida albicans* (ATCC 90028, *C. albicans*), and *Streptococcus mutans* (ATCC 25175, *S. mutans*) strains were used in this study. *S. aureus* was cultured on 5% sheep blood agar (Orbak, Ankara, Turkey); *E. faecalis* was cultured on trypticase soy agar (TSA, Merck, KGaA, Darmstadt, Germany); *E. coli* was grown on brain heart infusion agar (BHIA, Merck, Germany), and *C. albicans* were cultured on Sabourraud dextrose agar (SDA, Merck, KGaA, Darmstadt, Germany) at 37°C for 24–48 h aerobically. *S. mutans* were grown at trypticase soy agar (TSA, Merck,

Table 1. Citrus limonum essential oil properties

Product	
Description	Small tree from 3 to 5 meters high belonging to the Rutaceae family, the lemon tree is an evergreen with white flowers. Native from the north of India it is cultivated in Australia, south of America, Oceania, and around the Mediterranean.
Botanical name	<i>Citrus limonum</i>
Country of manufacturing	Italy
Certifications	EEC ORGANIC certified by FR-BIO-01
Product Analytical Data	Raw material from Organic Farming
Organoleptic parameters	
Colour	Light yellow to dark green
Odor	Lemon characteristic
Appearance	Clear liquid, maybe cloudy with lowering temperatures
Physicochemical parameters	
Density	0.850 - 0.858 (at 20 °C (D20/20))
Refractive Index	1.473 - 1.476 (at 20 °C (ND20))
Optical rotation	57 ° to 66°
Screening 200 pesticides	Conforms
Flavour Inventories	
N°COE	139
N°FDA	182.20
N°FEMA	2625
BelFrit	Yes
Composition	
INCI CosIng	CITRUS LIMON PEEL OIL
INCI PCPC	CITRUS LIMON (LEMON) PEEL OIL
N°CAS EINECS	84929-31-7
N°CAS TSCA	8008-56-8
N°CAS EINECS	284-515-8
Geographic Origin	Italia
Part of the plant	Zest

KGaA, Darmstadt, Germany), supplemented with 5% sucrose at 37 °C for 24–48 h under 5% CO₂ microaerophilic conditions. Then freshly grown bacterial and fungal suspensions were prepared in the sterilized test tubes containing brain heart infusion broth for *S. aureus*, *E. faecalis*, *E. coli*, *S. mutans*, and Sabourraud dextrose broth (SDB, Merck, Germany) for *C. albicans*. The final concentrations of the bacterial strains were adjusted to 1.5x10⁸ CFU/mL, and the final concentration of fungal suspension was adjusted to 2.5x10³ CFU/mL according to the turbidity of 0.5 McFarland test standard. Additionally, the inoculums were adjusted according to their Optical density (OD_{450nm}: 0,600) spectrophotometrically. For *S. aureus* and *E. coli*, Mueller Hinton agar (MHA, Merck, Germany), for *C. albicans* SDA, *S. mutans*, and *E. faecalis* trypticase soy agar was prepared, sterilized, and poured on the sterilized Petri plates at the equal amount of 20 mL.

Table 2. Citrus limonum essential oil components

Component	%
Limonene	73.273
α-Thujone / Sabinene	12.733
γ-Terpinene	7.595
α-Pinene	1.957
β-Myrcene	1.194
Neral	0.949
Cymene	0.675
Geranial	0.56
Nerly acetate	0.466
Geranyl Acetate	0.325
α-Terpinol	0.085
α-Terpinene	0.06
Ocimene	0.048
Caryophyllene	0.023
Camphene	0.022
Fenchone	0.018
β-Bisabolene	0.018

* It is the analysis report of the Scientific and Technology Application and Research Center, Burdur Mehmet Akif Ersoy University. (Report date is 03.04.2018)

Disc diffusion method (Kirby-Bauer)

One hundred µL amount of each bacterial and fungal suspension was spread onto the Petri dishes containing MHA for *S. aureus* and *E. coli*, SDA for *C. albicans*, and TSA for *S. mutans* and *E. faecalis* with a sterile cotton swab. Then sterile blank discs with 6 mm diameter were placed on the media plates, and 25 µL amount of 100% *Citrus limonum* essential oil sterile saline solution was put onto the discs. As a control, 0.0536 g amount of Ca(OH)₂ powder was weighed and suspended in 0.0744 mL distilled water. Then the same amount of Ca(OH)₂ was put onto the discs, too. They were put into the incubators aerobically for 24 hours and 5% CO₂ containing incubator microaerophilic conditions for 24-48 hours. After the incubation, bacterial and fungal growth inhibition around discs were examined, and the diameter of the inhibition zones was measured with a digital caliper (Mitutoyo, SP, Brazil) by an independent observer. Tests were performed in duplicate for each test strain, and the arithmetic mean numbers of the inhibition zone diameters were calculated for the graphical aspects. Data were recorded in millimeters.

Agar-well diffusion method

Same bacterial and fungal suspensions were used for the agar-well diffusion test method. For each material, two holes with a diameter of 6 mm per material were prepared on the agar plates aseptically. Each of the strains was spread on their specific agar plates as mentioned before. Then 100% *Citrus limonum* essential oil, calcium hydroxide, and sterile saline were pipetted into wells with a diameter of 6 mm formed on the inoculated agar surface to be 50 µL and tested on the specific media above for each strain type described previously. Then bacterial and fungal strains were incubated at 37 °C for 24-48h under aerobic and microaerophilic conditions. At the end of each incubation time, the diameter of inhibition zones

Table 3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration/Minimum Fungicidal Concentration (MBC/MFC) values

Type of Microorganism	Type of test medicament	Concentration range	MIC/MBC
Staphylococcus aureus	Citrus limonum essential oil (%)	100 % - 0.19 %	25 %
	Calcium hydroxide (mg/mL)	720.43 mg/mL-1.40 mg/mL	11.25 mg/mL
Escherichia coli	Citrus limonum essential oil (%)	100 % - 0.19 %	12.5 %
	Calcium hydroxide (mg/mL)	720.43 mg/mL-1.40 mg/mL	11.25 mg/mL
Enterococcus faecalis	Citrus limonum essential oil (%)	100 % - 0.19 %	25 %
	Calcium hydroxide (mg/mL)	720.43 mg/mL-1.40 mg/mL	22.52 mg/mL
Streptococcus mutans	Citrus limonum essential oil (%)	100 % - 0.19 %	6.25 %
	Calcium hydroxide (mg/mL)	720.43 mg/mL-1.40 mg/mL	11.25 mg/mL

Type of Microorganism	Type of test medicament	Concentration range	MIC/MFC
Candida albicans	Citrus limonum essential oil (%)	100 % - 0.19 %	12.5 %
	Calcium hydroxide (mg/mL)	720.43 mg/mL-1.40 mg/mL	22.52 mg/mL

was measured with a digital caliper (Mitutoyo, SP, Brazil) by a blinded, independent observer. Tests were performed duplicate for each test strain, and the arithmetic mean numbers of the inhibition zone diameters were calculated for the graphical aspects.

Determination of minimum inhibitory concentration (MIC)

To obtain the MIC values of the agents in this study, the serial dilution method was used in 96-well microplates with U-shaped bottom.

Enterococcus faecalis, *Escherichia coli*, *Staphylococcus aureus*, Mueller-Hinton Agar (Merck KGaA, Darmstadt, Germany), *Candida albicans* Sabouraud dextrose medium (Merck KGaA, Darmstadt, Germany), and *Streptococcus mutans* tryptic soy medium (Merck KGaA, Darmstadt, Germany) in a dilution were performed.

First, 100 µL media was placed in all wells except the first well. Then starting from the first well, the dilution process was continued by placing 100 µL of active substance in each well. Finally, the serial dilution process was completed by adding 100 µL of microorganisms to other wells, except for the negative control well.

The concentration range of *Citrus limonum* essential oil in this series dilution process was from 100% to 0.19%. The concentration range of calcium hydroxide was 720.43 mg/mL to 1.40 mg/mL. The entire dilution process was repeated three times.

Determination of minimum bactericidal concentration (MBC) and Minimum fungicidal concentration (MFC)

MBC and MFC were determined by taking 20 µL samples from each well and inoculating microorganisms into their own special agar media plates. Petri plates were incubated in the same traditional microbiological conditions and incubation periods as previously described. The minimum concentration at which no overgrown colonies were seen on the agar plates was set at MBC/MFC. The results were expressed in both MIC and MBC/MFC values. All the tests were performed three times. After incubation, the grown colonies were counted and calculated as CFU/mL.

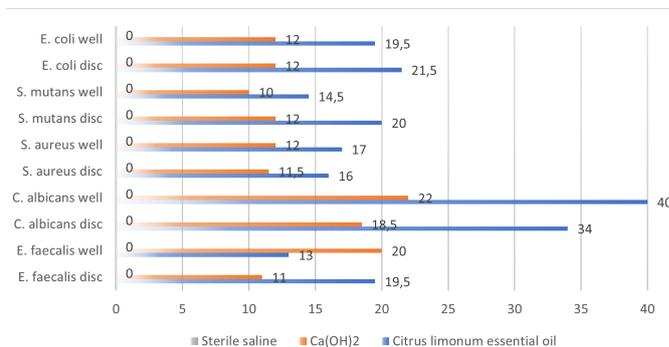


Figure 2. Agar diffusion method results

Results

The results of the agar diffusion method were shown in Figure 2.

For *Citrus limonum* essential oil in the agar well diffusion method, *C. albicans* reached the widest inhibition zone, while the narrowest inhibition zone was observed for *E. faecalis*. For calcium hydroxide, *C. albicans* again reached the widest inhibition zone, and the narrowest inhibition zone was observed to have *S. mutans*. Sterile saline did not form any zones.

For *Citrus limonum* essential oil in the agar disc diffusion method, *C. albicans* reached the widest inhibition zone, while the narrowest inhibition zone was spotted in *S. aureus*. For calcium hydroxide, *C. albicans* again reached the widest inhibition zone, and the narrowest zone of inhibition was found for *E. faecalis*. Sterile saline did not form any zones.

The MIC and MBC/MFC values are shown in Table 3.

The lowest MIC and MBC value for *Citrus limonum* essential oil was observed in *S. mutans*. This value was 6.25%.

For calcium hydroxide, the lowest MIC and MBC values were recorded for *S. aureus*, *E. coli*, and *S. mutans*. This value was 11.25 mg/mL.

Discussion

Today, in both dentistry and endodontics, the number of studies is increasing that provide evidence for the uses of

medicinal plants. This, in turn, has created a widespread interest in the production of medicament, which is alternatively derived from more natural, sustainable, and natural products [3]. The constant increase in species resistant to synthetic drugs, antibiotics, and side effects have led researchers to look for herbal alternatives in endodontics, as well [14]. In endodontics, due to the cytotoxic reactions of most commercial intracanal drugs used and their inability to destroy bacteria in the dentin tubules, the idea of using biological drugs derived from natural plants in medicine is supported as an alternative.

Microorganisms inhibitions of essential oils show different action mechanisms and may have been partially based on microorganism hydrophobicity. As a result, fats affect the bilayer lipid structure of the cell membrane, interfere with the respiratory chain, and cause leakage of vital cell contents [15]. Disruption of bacterial enzyme systems is another potential action. Many components of essential oils permeabilize the cell membrane and can increase the penetration of antibiotics. The influence of bacterial enzyme systems may be another mechanism of action [16].

Citric and acidic fruits contain healthy and nutritive contents. The peels of *Citrus* fruits are rich in flavonoids, especially many polymethoxylated flavones, which are very rare in other plants. Chemical analysis of *Citrus limonum* essential oil shows that the highest concentration of the ingredient is limonene. The ratio of limonene indicated in the analysis report obtained from the manufacturer for our experimental material was determined as 73.27%. Antimicrobial, anti-inflammatory, antioxidant effects are especially thought to be caused by this component. It is believed that the antimicrobial action of terpenes such as limonene is the result of affecting the cytoplasmic membrane of microorganisms. It is suggested that possible mechanisms of action are loss of membrane integrity by accumulation in the cell membrane, inhibition of respiratory enzymes, and loss of proton motive force [17]. Its anti-inflammatory effect is believed to be caused by inhibition of TNF- α induced NF-KB translocation in fibroblasts and reduction of IL-6 levels [5].

The use of new antibacterial substances primarily requires antimicrobial sensitivity tests. For this purpose, our work was carried out in two stages. The effects of *C. limonum* on microorganisms that are often found in root canals were compared with the agar diffusion test with calcium hydroxide, traditionally applied. In our study, *Citrus limonum* essential oil was found to be more effective than calcium hydroxide on the tried microorganisms. This finding is in parallel with the findings of similar studies [7, 18-22].

A wide variety of microorganisms such as Gram +, Gram -, anaerobic, aerobic and fungal can cause root canal infection. *Enterococcus faecalis* (*E. faecalis*), *Candida albicans* (*C. albicans*), *Staphylococcus aureus* (*S. aureus*), *Streptococcus mutans* (*S. mutans*), and *Escherichia coli* (*E. coli*) are microorganisms in which the effectiveness of antiseptics used in our study was evaluated. Any antiseptic that acts on them will likely affect other more sensitive microorganisms, as well.

Studies have shown that *Enterococcus faecalis* is the most commonly isolated species in stubborn infections that occur in root canal-treated teeth [23-25]. *Enterococcus fae-*

calis is a gram + facultative anaerobic bacterium commonly found in root canals [26]. Although untreated necrotic pulp teeth make up only a very small part of the initial flora, *enterococci* and especially *E. faecalis* has been isolated in 24% to 77% of positive cultures of filled root canals that give signs of chronic apical periodontitis [27]. Located in the dentin tubules *E. faecalis* is resistant to the use of calcium hydroxide as an intracanal medicament [1, 28]. It can adhere to the dentin and form invasive biofilm into the dentin tubules, gaining the property of adaptation and cross-protection in the stress environment. This condition is effective in demonstrating resistance to intracellular antimicrobial processes [29]. Calcium hydroxide, traditionally used in endodontic therapy, is often found, especially in treatment relapses. Its inability to counter *E. faecalis* led researchers to look for different medicaments and try different activating methods. Laird *et al.* [20] evaporated a mixture of *citrus* essential oil (Citri-V™ Orange: Bergamot, 1:1 v/v) on stainless steel by applying them to *E. faecalis* biofilms, and they ensured their complete elimination. Previous studies have also shown that *citrus* vapors disrupt cell membrane integrity, increase membrane permeability, cause loss of membrane potential, and reduce intracellular ATP [30]. In experimental conditions in our study, *Citrus limonum* essential oil is effective on *E. faecalis* and forms wider inhibition zones in the agar than calcium hydroxide. This result may be important in the antimicrobial material choice for endodontic retreatment cases together with the consideration of its solvent action on gutta-percha filling.

Streptococcus mutans is the main pathogenic agent most involved in dental plaque and responsible for dental caries. *S. mutans* is a facultative anaerobe, gram-positive coccus [31]. Liu *et al.* [21] determined that *Citrus limonum* essential oil effectively prevented the development of *S. mutans* from adhering to glass and saliva-coated enamel surfaces and stopped the transcription and enzyme activity of glycosyltransferase. Sun *et al.* [32] found that lemon (*Citrus limonum*) essential oil suppressed the expression of the *luxS* and *srtA* gene associated with biofilm formation and acid tolerance. They reported that *S. mutans* can reduce acid tolerance and biofilm formation.

Extraradicular biofilm of *Staphylococcus aureus* has been associated with refractory periapical disease in tissues and biomaterial surfaces [33]. It is a Gram-positive and facultative anaerobic microorganism. *Staphylococci* are pyogenic microorganisms; they are involved in the formation of abscesses and pus. They produce a large number of toxins and enzymes. Oliveira *et al.* [7] reported that *Citrus limonum* and *Citrus aurantium* essential oils are effective in biofilms containing multiple species and that the reduction in germ load obtained with them does not only remain similar to sodium hypochlorite, but also more effective than chlorhexidine. Ali *et al.* [18] by investigating the effects of *Citrus limonum* extracts on *S. aureus*, *E. coli*, and *C. albicans* through the agar diffusion test, determined the positive antimicrobial activity of methanolic extracts compared to the standards. Kumar *et al.* [19] tested the solvent extracts from *C. sinensis* and *C. limonum* on planktonic cultures of *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumonia*,

and *Salmonella typhi*, and found them as potent as methicillin and penicillin.

Escherichia coli is a temporary colony in the oral cavity and can often be seen in long-term antibiotic treatments, immunodeficiency, and hospitalized patients. Endotoxin produced by this bacterium may play a role in the development of early periapical inflammatory lesions and bone resorption. In the study by Soković *et al.* [22] in the evaluation of certain essential oils, including *C. limonum*, it was found that its development was prevented in disc diffusion and microdilution tests in planktonic cultures of *E. coli*. Oliveira *et al.* [7] found similar results.

Candida albicans is part of the normal microbiota and is associated with failed endodontic treatments and is seen as a dentinophilic microorganism [34]. Lamine *et al.* [35] tested the essential oils of different citrus species, such as *C. limonum*, *C. aurantium*, *C. reticulata*, and *C. sinensis* on *Candida albicans*, *Aspergillus flavus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella paratyphi B*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Bacillus subtilis*. *C. limonum* has shown the highest antibacterial activity among other citrus essential oils.

Similarly, *C. limonum* essential oil was found to be effective on all these microorganisms in the agar test in our study. In the second phase of the study, MIC, MBC, and MFC tests were performed. The microdilution method is a safe test for determining microbial resistance to antimicrobial material. Minimal inhibition concentration indicates the lowest concentration required to prevent the noticeable reproduction of microorganisms. The biological balance between the toxicity and antimicrobial effects of drugs to be achieved at low densities should be the basic philosophy of root canal disinfection. It has been reported that moderate compounds will be sufficient to purify root canals from microorganisms, and their high concentrations will increase periapical inflammation. Studies on this subject show that canal disinfectants should be used at the lowest concentration where they can be effective. In our study, it was determined that *Citrus limonum* essential oil affected all microorganisms tried in dilution of 25% by determining minimum inhibition and minimum bactericidal/fungicidal concentrations on selected microorganisms. For *S. mutans*, this value was found to be 6.25%. For calcium hydroxide in our control group, this value was 11.25 mg/mL suspension in distilled water.

MIC value for *C. limonum* on different microorganisms was found to be between 25% and 6.25%. The work of Man *et al.* [36] have tested micellar and aqueous extracts of lemon (*Citrus limonum*), frankincense (resin obtained from *Boswellia sacra*), myrtle (*Myrtus communis*), garden thyme (*Thymus vulgaris*), oregano (*Origanum vulgare*), and lavender (*Lavandula angustifolia*) essential oils on *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. MIC value of micellar extract of *C. limonum* was between 12.5% and 6.3%; MBC value was between 6.3% and 50%; MIC, and MBC value of aqueous extract was >50% and 25%. Lamine *et al.* [35] found MIC values for *C. limonum* in the range of 1-4%. Ben Hsouna *et al.* [6] evaluated the antimicrobial effects of *C. limonum* on Gram-positive (*B. cereus*, *E. faecalis*, *P. aureus*, *P.*

epidermis, *B. subtilis*, *L. Monocytogenes*, and *M. luteus*) and Gram-negative (*P. aeruginosa*, *E. coli*, *P. Enteritidis*, and *K. pneumoniae*) with inhibition zone and MIC values. They found *C. limonum* is as effective as a natural antimicrobial agent. Gucwa *et al.* [37] studied MIC and MFC values of essential oils of *Thymus vulgaris*, *Citrus limonum*, *Pelargonium graveolens*, *Cinnamomum cassia*, *Ocimum basilicum*, and *Eugenia caryophyllus* on *C. albicans* and *C. glabrata* isolates. They found *C. limonum* MIC and MFC values for *C. albicans* isolates range from 0.005% to 2.5%, and for *C. glabrata* isolates, a MIC value between 0.005% and 0.625%, and an MFC value between 0.005% and 1.25%.

Within the limits of the study, *Citrus limonum* has provided some encouraging results in eradicating major endodontic pathogens; *S. aureus*, *E. coli*, *C. albicans*, *S. mutans*, and *E. faecalis* in pure culture and was comparable with calcium hydroxide. This may be especially pertinent in cases of conventional endodontic retreatment where *E. faecalis* is the most commonly recovered bacterial species [25].

The weakness of our study is that it is being done in vitro conditions. In vivo conditions differ from the tube environment. *E. faecalis* withstands the effects of calcium hydroxide in the root canal; however, in our experimental conditions, calcium hydroxide is affected even at a suspension of 45.02 mg/mL. Drug resistance of microorganisms in the oral environment, the resistance of bacteria, adaptation to limited food sources, the interaction between microorganisms, host factors, inactivation by bacteria in the canal, anatomy of a root canal, and the ability of the drug to penetrate the dentin tissue and canal details should be taken into account. Effective substance density, incubation period, the temperature of the environment, pH and pollution level, amount of organic matter in the environment are important in the effectiveness of antiseptic substances. As microorganisms spread into the dentin tubules, intracanal drugs should also be able to penetrate the dentin tubules. The wetting properties of antiseptics are also gaining importance. In addition, biofilms and bacterial interaction are a general mechanism for bacteria to survive and are a virulence factor that plays an important role in development. In addition to the antibacterial effect, the prevention of bacterial adhesion should also be taken into account when irrigating and disinfecting root canals.

Another weakness of the study is the unstable, fragile and volatile structural properties of limonene, the main component of the oil we use. Limonene is sensitive to oxidative degradation resulting in a direct loss of activity [38]. Oxygen can easily deteriorate if it is not well protected from external factors such as light and temperature. In this sense, any temperature change can cause changes in its activity. In particular, the bacterial cell wall is thought to be more susceptible to limonene at low temperatures; this is because limonene is more volatile in increased heat [39, 40]. Furthermore, due to the hydrophobic nature of limonene and the inability to achieve a homogeneous distribution in water, it needs to be used in high concentrations to achieve antimicrobial yields.

These limitations indicate that it may be useful to resort to encapsulation techniques for more effective and durable

use of *Citrus limonum*.

Conclusions

Citrus limonum essential oil was found to be more effective on selected microorganisms in vitro than calcium hydroxide. Laboratory tests are the first step for evaluating the antimicrobial effects of antiseptics. Agar diffusion and microdilution tests of irrigation solutions do not define clinical conditions. After evaluation with cytotoxicity tests by the MIC values obtained in this study, and after conducting the antimicrobial evaluation of *C. limonum* in clinical conditions, it may be a candidate for the list of endodontic antimicrobials.

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