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ORIGINAL ARTICLE

Medicine Science International Medical Journal

Medicine Science 2026;15(1):165-71

Comparative evaluation of the antimicrobial activity of different essential oils and chlorhexidine gluconate mouthwash: An *in vitro* study

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Received 16 July 2025; Accepted 08 September 2025
Available online 22 February 2026 with doi: 10.5455/medscience.2025.07.191

Abstract

The increase of opportunistic pathogens in the oral biofilm triggers the development of periodontal diseases. In order to prevent these diseases, a wide variety of chemical plaque removal oral care products are used. In this study, it was aimed to investigate the in vitro antimicrobial activities of 7 different commercially available essential oils and Chlorhexidine gluconate (CHX) mouthwash against four different standard bacterial strains and to compare the antimicrobial effects of essential oils with CHX solution. The antimicrobial activity of different commercially available essential oils and CHX solution against the most common oral bacteria *Staphylococcus aureus (S. aureus)*, *Streptococcus mutans (S. mutans)*, *Enterococcus faecalis (E. faecalis)* and *Bacillus subtilis (B. subtilis)* strains were analyzed comparatively. Antimicrobial activity was investigated by disc diffusion method. Thyme oil showed the highest antimicrobial effect on *S. aureus* and *B. subtilis* against the other test products (p=0.006 and p=0.004, respectively), while CHX showed the highest effect against *S. mutans* and *E. faecalis* species (p=0.006 and p=0.006, respectively). Thyme, peppermint and rosemary oils showed no significant difference in antimicrobial activity compared to CHX against *S. aureus* (p=0.479, p=0.164 and p=0.164, respectively), *S. mutans* (p=0.301, p=0.072 and p=0.072, respectively) and *B. subtilis* (p=0.072, p=0.072 and p=0.813, respectively) strains. Due to their effects on oral pathogens, essential oils such as thyme oil, peppermint oil, and rosemary oil have been considered as potential alternatives to CHX solution as oral antiseptic solutions.

Keywords: Antimicrobial activity, essential oils, chlorhexidine, mouthwash

Introduction

Since the oral microbiome is the gateway of the oral cavity to the body, it has an important role in protecting systemic health as well as oral health. The oral cavity is regularly inoculated and colonized with microorganisms from the first feeding within 8-16 hours following birth, and the process of acquiring an established oral microbiome begins [1]. The oral mucosa and the soft and hard tissues of the teeth are the two primary sites colonized by bacteria. In addition to the core microbiome common to all individuals, a variable microbiome is unique to each individual, depending on physiological changes and lifestyle [2].

The oral microbiome is constantly changing in a lifestyle-dependent manner, including personal oral hygiene practices, medications, dietary habits, and habits such as smoking and alcohol [3]. The commensal oral microbiome is crucial in maintaining oral health as it provides resistance to colonization by pathogens [4]. Periodontal diseases are triggered by dysbiosis of subgingival microbial communities [5]. Important microbial species in the oral biofilm, particularly *Streptococcus mutans*, are considered to be etiologic agents in the development of periodontal disease [6]. Among these species, stable plaque, which may include opportunistic pathogens, can cause long-term inflammation leading to periodontal disease [7].

Antimicrobial mouthwashes are chemical methods that remove oral microbial biofilm and are recommended to be used in addition to, or in some limited cases instead of, mechanical removal of microbial biofilm [8]. Since periodontal diseases

CITATION
Akbayirli U, Gul G, Ozturk I, Kaya S. Comparative evaluation of the antimicrobial activity of different essential oils and chlorhexidine gluconate mouthwash: An *in vitro* study. Med Science. 2026;15(1):165-71.

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DOI: 10.5455/medscience.2025.07.191

have a chronic progression, the use of mouthwashes to support periodontal treatment between treatment periods is recommended [9]. Routine daily use of mouthwashes has also been reported to reduce the development of periodontal disease and the rate of disease progression [10]. In addition, mouthwashes and antiseptics can be used prophylactically to prevent secondary infections due to oral mucositis after chemotherapy and radiotherapy and to prevent infections that may develop in the wound area after dental operations [11]. Chlorhexidine gluconate, benzidamine hydrochloride, hyaluranic acid and alcohol-containing mouthwashes and antiseptics are frequently used for this purpose [12]. However, although these chemicals have therapeutic and prophylactic effects, they have been reported to cause a wide range of functional and morphological side effects locally [13,14]. Considering the existing side effects of these chemotherapeutic agents, there is an increasing interest in the search for herbal preparations that may show similar efficacy.

In the literature, there are a lot of studies on the use of essential oils extracted from plants due to their antimicrobial effects according to the type of active substance they have [15,16]. Some of these studies focus on the use of these natural products in periodontology [17,18]. In this *in vitro* study, it was aimed to evaluate the antimicrobial activities of seven different commercially available essential vegetable oils against four different standard bacterial strains and to determine their effects on the oral microbiome by comparing them with Chlorhexidine gluconate (CHX) found in mouthwashes. Unlike the studies in the literature where the antimicrobial effects of essential oils were examined, the comparison of the effects of different essential oils and CHX in our study shows the unique value of the study.

Material and Methods

Project outline

In this *in vitro* study, the standard disc diffusion test of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) was used to test the antimicrobial activity of essential oils [19]. This study does not require Ethics Committee approval. All research procedures complied with the principles of Declaration of Helsinki.

Essential oils

The essential oils were selected from those used in traditional and alternative medicine methods through a literature review and obtained commercially. As a result, a total of 7 essential oils were included in this analysis (Table 1). There are studies in the literature investigating the antimicrobial activity of essential oils with reference to the test recommendations of EUCAST [20,21].

Microbial strains

In this study, bacteria selected from the "American Type Culture Collection" (ATCC) standard strains recommended for use by EUCAST and with known susceptibility characteristics were used (Table 2). The standard bacterial strains used for this purpose were obtained commercially in lyophilized form and were inoculated into plate medium before starting the study. The plates were placed in an incubator and incubated at 37 °C for 24 hours to revitalize the bacterial strains.

Inoculum preparation

Bacterial suspensions to be used in the study were prepared from fresh colonies of strains grown on plate media using sterile swabs. The swab was immersed in tubes containing 2-3 mL sterile saline (0.9% NaCl) and the colonies were mixed thoroughly by stirring. Microorganism suspensions in the tubes were prepared to McFarland turbidity standard of 0.5 (1.5 x 10^8 CFU/mL). The turbidity of the bacterial suspensions was standardized by measuring with a densitometer. The prepared inoculum suspensions were spread homogeneously and evenly over the entire surface of Mueller-Hinton Agar (MHA)/Mueller-Hinton Fastidious (MH-F) medium in three directions.

Antimicrobial discs

Sterile blank antimicrobial discs stored at +4 ºC before the study were kept at room temperature before starting the study. The essential oils and CHX were absorbed in a sterile petri dish at the rate of 15 µL per 6 mm diameter sterile blank antibiotic discs. The petri dishes were kept at +4 ºC for 1 hour with the lids closed to ensure adequate absorption of the oils and CHX into the discs.

Disc diffusion test

The impregnated discs removed from the oven were placed on the inoculated MHA/MH-F media at such a distance that the zones on the surface of the media did not touch each other, with 6 discs per plate medium. Ciprofloxacin (5 µg/disc) disc was placed in the same media as positive control based on EUCAST Internal Quality Control standards and sterile blank antibiotic disc was placed as negative control. All inoculated MHA/MH-F media were incubated at 37 ºC for 18-24 hours. Each test was repeated three times in parallel.

Evaluation of results

Inhibition zone diameters formed at the end of incubation were evaluated from the reverse side of the plate on a dark background illuminated with reflected light using a digital caliper. Inhibition zones were measured and recorded in millimeters (mm). A disc diameter of 6 mm was included in the measurement and positive control discs were used as the base for measurement. Triplicate analyses of the test results for each oil, CHX and Ciprofloxacin were averaged.

Statistical Analysis

Statistical analyses were performedusing SPSS Version 26 (Armonk, NY: IBM Corp). In the statistical evaluation of the results, Kruskal-Wallis test was used for multiple comparisons and Mann-Whitney U test was used to analyze the differences between two groups. The findings were evaluated at 95% confidence interval (95% CI) and 5% significance level. A p-value <0.05 was considered statistically signifcant in all analyses.

DOI: 10.5455/medscience.2025.07.191

Result

The *in vitro* antibacterial activities of the tested essential oils and CHX positive control Ciprofloxacin discs on *Staphylococcus aureus* (*S. aureus*), *Streptococcus mutans* (*S. mutans*), *Enterococcus faecalis* (*E. faecalis*), and *Bacillus subtilis* (*B. subtilis*) strains were measured using the disc diffusion test.

The Figure shows the distribution of the inhibition zone diameters in millimeters formed by the test products on these four strains. The key observations indicating the antimicrobial efficacy of the test products on the four strains based on the inhibition zone diameters are as follows:

- *S. aureus*: Thyme oil > CHX > Peppermint oil ≈ Rosemary oil > Grapefruit oil
- *S. mutans*: CHX > Thyme oil > Peppermint oil > Rosemary oil
- *E. faecalis*: CHX > Thyme oil > Rosemary oil > Peppermint oil
- *B. subtilis*: Thyme oil > Peppermint oil > Rosemary oil ≈ Grapefruit oil > CHX

Inhibition zone diameters detected in essential oils (mean ± SD); For peppermint oil; *S. aureus* 15±1 mm, *S. mutans* 14.6±0.57 mm, *E. faecalis* 9±0 mm, *B. subtilis* 15.6±0.57 mm; For rosemary oil; *S. aureus* 15±1 mm, *S. mutans* 13.6±0.57 mm, E. feacalis 9.3±0.57 mm, *B. subtilis* 13±1 mm; For thyme oil; *S. aureus* 17±1 mm, *S. mutans* 16.6±0.57 mm, *E. faecalis* 10±0 mm, *B. subtilis* 17.6±0.57 mm; For grapefruit oil; *S. aureus* 14.3±0.57 mm, *S. mutans* 0±0 mm, *E. faecalis* 0±0 mm, *B. subtilis* 13±1 mm were detected. No antimicrobial effect was detected in ginger, sage, and coconut oils. The inhibition zone diameters (mean ± SD) detected in the CHX product compared to essential oils were as follows; *S. aureus* 16.3±0.57 mm, *S. mutans* 17.3±0.57 mm, *E. feacalis* 17±0 mm, *B. subtilis* 12.6±0.57 mm.

Among the 7 essential oils investigated for antimicrobial activity in this study, Thymus vulgaris (Thyme), Mentha piperita (Peppermint) and Rosmarinus officinalis (Rosemary) oils were found to have antimicrobial activity against all of the microorganisms studied. Citrus paradisi (Grapefruit) oil was found to have antimicrobial activity only against *S. aureus* and *B. subtilis*. Zingiber officinale (Ginger), Salvia officinalis (Sage) and Cocos nucifera (Coconut) oils had no activity against *S. aureus*, *S. mutans*, *E. faecalis* and *B. subtilis*. The positive control, Ciprofloxacin disc, showed the highest activity in all four bacterial strains, while the negative control, sterile blank disc, showed no activity. Based on the inhibition zone diameters, it was concluded that Thyme oil showed the highest antimicrobial effect among all oils. Thyme oil showed the highest antimicrobial effect on *S. aureus* and *B. subtilis* against the other test products (p=0.006 and p=0.004, respectively), while CHX showed the highest effect against *S. mutans* and *E. faecalis* species (p=0.006 and p=0.006, respectively) (Table 3).

In the binary analysis between CHX, which has proven efficacy in mouthwashes, and thyme oil, it was observed that the antimicrobial activity of both groups was similar for *S. aureus* (p=0.479), *S. mutans* (p=0.301) and *B. subtilis* (p=0.072). When both groups were analyzed in terms of *E. faecalis*, CHX was significantly more effective than thyme oil (p=0.046). In the binary analysis between CHX and peppermint oil, it was observed that the antimicrobial activity of both groups was similar for *S. aureus* (p=0.164), *S. mutans* (p=0.072) and *B. subtilis* (p=0.072). When both groups were analyzed in terms of *E. faecalis*, CHX was significantly more effective than peppermint oil (p=0.046). In the binary analysis between CHX and rosemary oil, there was no significant difference in antimicrobial activity between both groups for *S. aureus* (p=0.164), *S. mutans* (p=0.072), *E. faecalis* (p=0.059) and *B. subtilis* (p=0.813) (Table 4).

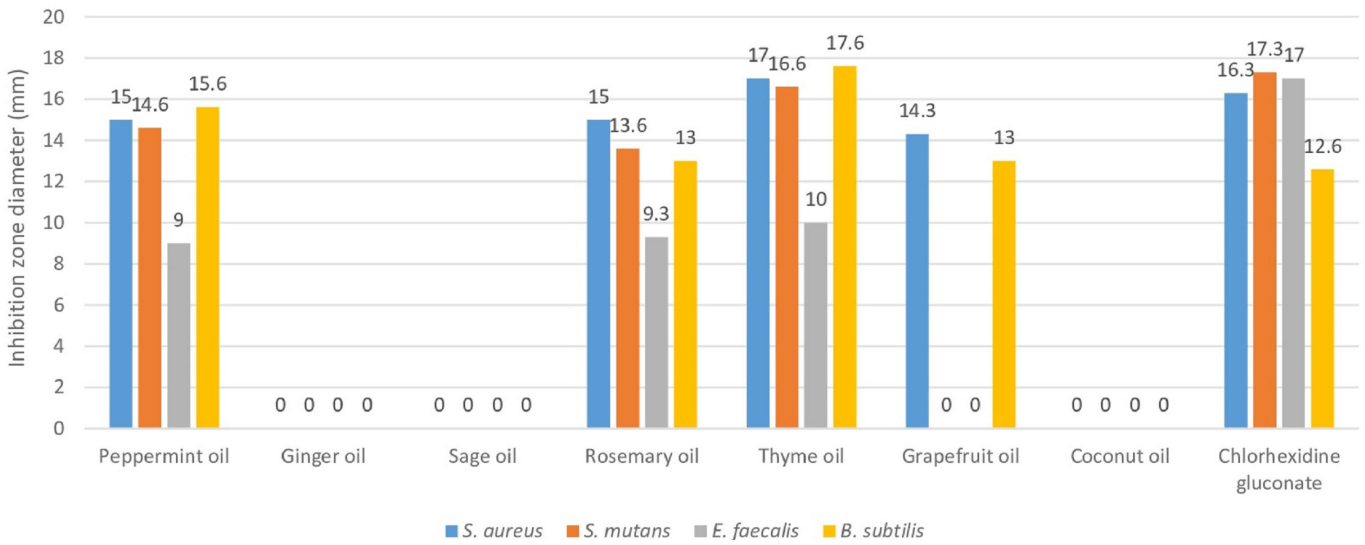

Figure 1. Antimicrobial activities of essential oils and chlorhexidine gluconate according to inhibition zone diameter (median values) measurements in four bacterial strains

DOI: 10.5455/medscience.2025.07.191

Table 1. Essential oils used in the study

Plant species from which the essential oil is extracted	Essential oil
Mentha piperita	Peppermint oil
Zingiber officinale	Ginger oil
Salvia officinalis	Sage oil
Rosmarinus officinalis	Rosemary oil
Thymus vulgaris	Thyme oil
Citrus paradisi	Grapefruit oil
Cocos nucifera	Coconut oil

Table 2. Standard lyophilized bacterial strains used in the study

Name of bacterial strains	ATCC code of the bacterial strains
Staphylococcus aureus	25923
Streptococcus mutans	25175
Enterococcus faecalis	29212
Bacillus subtilis	196659

ATCC: American type culture collection

Table 3. Inhibition zone diameter means (mm) and standard deviations (SD) of analysis products

Variables	*S. aureus*	*S. mutans*	*E. faecalis*	*B. subtilis*
Peppermint oil	15±1	14.6±0.57	9±0	15.6±0.57
Ginger oil	0±0	0±0	0±0	0±0
Sage oil	0±0	0±0	0±0	0±0
Rosemary oil	15±1	13.6±0.57	9.3±0.57	13±1
Thyme oil	17±1	16.6±0.57	10±0	17.6±0.57
Grapefruit oil	14.3±0.57	0±0	0±0	13±1
Coconut oil	0±0	0±0	0±0	0±0
CHX	16.3±0.57	17.3±0.57	17±0	12.6±0.57
Ciprofloxacin (positive control)	32.3±1.52	21.6±0.57	21.6±0.57	36.3±0.57
Sterile blank disc (negative control)	0±0	0±0	0±0	0±0
p*	0.006	0.006	0.006	0.004

*: Kruskal–Wallis test, CHX: Clorhexidine gluconate

Table 4. Comparison of antimicrobial effects of chlorhexidine gluconate and three different essential oils

Bacterial strains	p*		
	Comparison between the thyme oil and the CHX	Comparison between the peppermint oil and the CHX	Comparison between the rosemary oil and the CHX
S. aureus	0.479	0.164	0.164
S. mutans	0.301	0.072	0.072
E. faecalis	0.046	0.046	0.059
B. subtilis	0.072	0.072	0.813

*: Mann-Whitney U test, CHX: Clorhexidine gluconate

DOI: 10.5455/medscience.2025.07.191

Discussion

Currently, oral diseases are known to be a major health problem worldwide. Among a large number of oral diseases, periodontal disease is one of the major oral health conditions. Chronic inflammatory responses, such as alveolar bone destruction and connective tissue loss, resulting from the triggering of the host immune response by pathogenic bacteria, form the basis of periodontal diseases [22].

Mechanical plaque cleaning is the most effective way known for oral hygiene. The aim of this practice is to mechanically remove plaque on the tooth surface and thus eliminate pathogenic microorganisms in the oral environment [23]. Although mechanical cleaning is the way to prevent microorganism colonization in the mouth, conditions such as patient compliance, malpractice, and the presence of complicated prostheses and/or appliances in the mouth prevent effective cleaning. Therefore, the use of chemotherapeutics for chemical plaque cleaning to support oral hygiene and provide antimicrobial efficacy is among oral care recommendations. A wide variety of antimicrobial mouthwashes are used for this purpose [24].

CHX is considered the "gold standard" among anti-plaque agents due to its potent bactericidal and bacteriostatic effects [25]. CHX creates a slow and long-lasting antibacterial effect by binding to the oral mucosa [26]. Since it is the most preferred agent in mouthwashes, it is widely used in dentistry. However, long-term use of CHX has been associated with local adverse effects such as transient changes in the sense of taste and tooth pigmentation [27]. Due to these negative effects, recent studies have focused more on natural and herbal ingredients. Essential oils, which can be used in various medical applications, also have the potential to be natural antimicrobial agents as a result of the widespread antimicrobial resistance [28]. There are many studies in the literature examining the antimicrobial effects of the essential oils in our study [29,30].

In this study, it was aimed to evaluate the *in vitro* antibacterial effects of CHX, which is commonly used in mouthwashes, and seven different essential oils on 4 critical bacterial strains in the oral microbiome. The four bacterial strains tested in our study are among the agents frequently mentioned in the literature on oral microbiome studies and are well known for their pathogenic properties in dysbiosis conditions [31,32]. In our study, CHX mouthwash showed the highest antimicrobial activity against *S. aureus*, *S. mutans*, *E. faecalis* and *B. subtilis*. There are many studies in the literature examining the effects of CHX mouthwashes on the oral microbiome. CHX has been shown to inhibit plaque formation and reduce gingivitis in addition to reducing oral bacteria in in vivo and *in vitro* studies.

In a study conducted by de Albuquerque RF et al. on 60 patients [33], saliva was collected before and after CHX-containing mouthwashes and microbiological analysis was performed. The number of microorganisms was calculated according to colony forming units (CFU) and a more than 99% reduction in CFU levels was found in *S. aureus* and *S. mutans* strains after rinsing with CHX. In the *in vitro* study of Aghazadeh et al. [34] microbiologic culture inoculation was performed for colony counting by mixing bacterial species with a tube containing CHX. The researchers concluded that CHX had a significantly greater effect on the *S. aureus* strain. Neglia et al. [35] investigated the bactericidal activity of a strain of *E. faecalis*, a component of the normal oral microbiota that causes failure of root canal treatments, with an endodontic irrigation solution. The results of the study showed that the bacterial load in teeth washed with this solution gradually decreased so that no bacteria could be detected a few days after washing. In the study of Portenier et al. [36] the effect of CHX solution against cells in three different phases of *E. faecalis* (starvation, stationary, growth) was examined and it was observed that cells in the growth phase were most sensitive to the bactericidal effect. In this study, the data showing the antibacterial effect of CHX mouthwash on the oral microbiome agree with the results of studies in the literature.

In our study, inhibition zone diameters were taken as a basis among the essential oils we examined and the most effective oil was found to be thyme. Thyme oil, which is used in the treatment of halitosis and toothache in dentistry, is known to be one of the 10 most commercially used essential oils worldwide due to its antioxidant, antibacterial and antifungal effects [37].

Thyme oil obtained from Thymus vulgaris plant and commercially available was used in our study. It is known that the main compounds responsible for the antimicrobial effects of this essential oil are thymol and carvacrol [38]. Khan et al. [39] found that thymol was most effective against the caries-causing bacterium *S. mutans* and reduced the bacterial population by 93% at a concentration of 200 µg/mL, while carvacrol compound reduced the *S. mutans* population by 91% at the same concentration. The results of the study showed that thymol and carvacrol compounds have strong antimicrobial and antibiofilm effects against *S. mutans*. Therefore, it was concluded that the use of essential oils containing these compounds in toothpaste or mouthwashes is important in reducing oral bacteria. In the study by Perez et al. investigating the antibiofilm activity of Thymus vulgaris essential oil against Methicillin-resistant *S. aureus* (MRSA) strain [40], it was observed that it reduced the biomass of pre-formed biofilms of *S. aureus* ATCC 25923 strain and 3 out of 4 clinical MRSA isolates by 4 mg/mL. The researchers thought that thyme oil could be used as an alternative to antibiotics used in the treatment of chronic bacterial infections due to bacterial biofilms. In this study, thyme oil showed a strong antimicrobial effect against all four bacterial strains tested. The effect of thyme oil on *S. aureus*, *S. mutans* and *B. subtilis* was similar to CHX, while CHX was more effective against *E. faecalis* species.

Among the essential oils examined in our study, peppermint and rosemary oils were found to have strong antimicrobial effects against all bacterial strains tested. It is well known that peppermint oil extracted from Mentha piperita plant has strong antimicrobial, antioxidant and anti-inflammatory effects. These effects have been shown to be mainly due to the menthol, menthone, neomenthol and iso-menthone components contained in the oil [41]. Rosemary oil

DOI: 10.5455/medscience.2025.07.191

obtained from Rosmarinus officinalis plant has anti-inflammatory and antimicrobial effects mainly because it contains carnosol and carnosic acid [42].

In the study by Valková et al. investigating the *in vitro* antimicrobial activity of lavender, peppermint and rosemary essential oils [43], disc diffusion method was applied as in our study. Inhibition zone diameters were measured against different bacterial strains and found to be 5.3±0.6 mm for peppermint oil and 10.3±0.6 mm for rosemary oil in *S. aureus* strain. In our study, inhibition zone diameters against *S. aureus* bacteria were measured as 15±1 mm for both oils. In another study [44], in which the *in vitro* antibacterial effects of peppermint essential oil were analyzed by both liquid microdilution test and disc diffusion test, *S. aureus* and *B. subtilis* strains, which were also included in our study, were used. In the study, MIC and inhibition zone diameter were measured and the values were found to be 8 µL/mL and 8 mm for *S. aureus* and 2.5 µL/mL and 18 mm for *B. subtilis*, respectively. According to the results of the study, it was reported that peppermint essential oil has the potential to be applied as an antibacterial agent. In a study investigating the antimicrobial effect of rosemary extracts [45], commercially used rosemary extract was dissolved in ethanol (100 mg/mL) and tested against various microorganisms. The MIC values of the ethanol solution were found to be 0.5% for *S. aureus* and 0.13% for *S. mutans*, two strains also included in our study. In this study, peppermint oil and rosemary oil showed a strong antimicrobial effect against all four bacterial strains tested. The effect of peppermint oil on *S. aureus*, *S. mutans* and *B. subtilis* was similar to CHX, while CHX was more effective against *E. faecalis* species. The antimicrobial effect of rosemary oil was similar to CHX on all four bacterial strains tested.

Our study found that ginger, sage, and coconut oils did not exhibit antimicrobial activity against the tested bacterial strains. This outcome may be attributed to various factors, including the chemical composition and concentration of the oils used in the study, their limited duration of effect due to high volatility, natural resistance mechanisms observable in the tested bacterial strains, or the diffusion rate in the test method. As a result, it is known that the antimicrobial activity of essential oils is not pronounced for all strains [46].

This study has some limitations. The antimicrobial effect of the essential oils and CHX agent used in this study was evaluated using the disc diffusion test; the MIC/MBC test was not performed. However, since the disc diffusion test alone cannot demonstrate therapeutic applicability, the change in the effect of these agents at different concentrations over different periods of time on bacteria could not be fully clarified. Furthermore, the absence of cytotoxicity or biocompatibility tests for the essential oils used has also led to limited interpretation of the antimicrobial effects. Therefore, the antimicrobial effects of essential oils can be better understood through prospective studies that evaluate the dose/duration efficacy of these agents and determine MIC/MBC.

Conclusion

According to current literature, uterine incisional defects There are studies in the literature showing that essential oils, which are used in toothpaste, gel and mouthwash formulations in dentistry due to their antimicrobial and anti-inflammatory properties, have significant therapeutic efficacy in dental and gum diseases. Today, *in vitro* and in vivo studies continue to be conducted to better understand the therapeutic effects of essential oils on periodontal diseases and to develop new essential oil-based formulations. In this study, it was concluded that, in addition to traditional CHX mouthwash use, essential oils such as thyme oil, peppermint oil, and rosemary oil may have potential as oral antiseptics against oral pathogens due to their antibacterial effects, pending further research.

Conflict of Interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
The authors declare that they have received no financial support for the study.

Ethical Approval
Ethical committee approval was unnecessary for this study.

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