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Journal of Infection and Chemotherapy xxx (xxxx) xxx

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: www.elsevier.com/locate/jic

Original Article

In vitro evaluation of the antimicrobial properties of terpinen-4-ol on apical periodontitis-associated bacteria

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ARTICLE INFO

Keywords:
Antibacterial
essential oils
Terpinen-4-ol
Apical periodontitis
Enterococcus faecalis

ABSTRACT

Manuka oil and tea tree oil are essential oils with known antibacterial properties that are believed to be caused by one main component: terpinen-4-ol. Terpinen-4-ol has potent antibacterial activity against caries-related microorganisms. However, few studies have investigated the antimicrobial effects of terpinen-4-ol on bacteria in apical periodontitis. Thus, the objective of the present study was to evaluate the antibacterial and antibiofilm potential of terpinen-4-ol against *Enterococcus faecalis*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum*, which have all been detected in apical periodontitis. The minimum inhibitory and minimum bactericidal concentrations of terpinen-4-ol were determined to assess its activity against biofilms. The minimum inhibitory concentration of terpinen-4-ol was 0.25% against *E. faecalis* and *F. nucleatum*, 0.05% against *P. gingivalis*, and 0.1% against *P. intermedia*. The minimum bactericidal concentration of terpinen-4-ol was 1.0% against *E. faecalis*, 0.2% against *P. gingivalis* and *P. intermedia*, and 0.5% against *F. nucleatum*. In the biofilm evaluations, all terpinen-4-ol-treated bacteria had significant reductions in biofilm viability compared with controls in experiments assessing attachment inhibitory activity. Furthermore, structural alterations and decreased bacterial cell clumping were observed under scanning electron microscopy, and significantly decreased cell survival was noted using fluorescence microscopy. Together, these results suggest that terpinen-4-ol is a potential antibacterial agent with bactericidal properties, and can also act on established biofilms.

1. Introduction

Oral cavity infections are widely observed in patients with periodontitis and dental caries, and in those undergoing endodontic treatment. Apical periodontitis is one of the most common chronic diseases and involves infection by endodontic bacteria. Various bacteria, including *Enterococcus faecalis*, have been detected and confirmed in periapical lesions [1,2].

E. faecalis is one of the most commonly isolated bacteria from teeth with persistent periapical disease requiring re-root canal treatment; however, it is rarely present in primary endodontic infections [3–5]. It has been reported that when this bacterium is isolated during failed endodontic treatment or at the time of root filling, the failure rate of retreatment is relatively high [5]. Furthermore, the inhibition of *E. faecalis* biofilms requires very high concentrations of antibiotics such as ampicillin, vancomycin, and linezolid [6]. In addition, many species of anaerobic gram-negative bacteria, such as *Porphyromonas gingivalis, Prevotella intermedia*, and *Fusobacterium nucleatum*, have been isolated and identified from root canals of teeth with periapical lesions. Some of these bacteria may also be closely associated with the development of periapical lesions [7]. Many bacterial cell components and products have various effects on periodontal tissue, including the induction of inflammation.

In the clinic, medications that have traditionally been used to treat root canal infections include iodine glycerin, guaiacol, and parachlorophenol; calcium hydroxide is currently the medication of choice.

Abbreviations: ATCC, American Type Culture Collection; BHI, brain heart infusion; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; MO, manuka oil; PBS, phosphate-buffered saline; TTO, tea tree oil; SEM, scanning electron microscope.

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https://doi.org/10.1016/j.jiac.2023.10.021
Received 18 August 2023; Received in revised form 12 October 2023; Accepted 29 October 2023
Available online 3 November 2023

To treat root canal infections, each reagent has advantages and disadvantages in terms of bactericidal effects, duration, method of removal, and toxic effects on cells. Calcium hydroxide, which has strong alkaline properties, is presently widely used as an intracanal medication in clinical practice. However, it has poor solubility and diffusibility, and its effects are limited to areas of contact. Moreover, calcium hydroxide is difficult to remove from the concave root canal wall, and *E. faecalis* appears highly resistant to its antibacterial effects [8–10]. There is therefore a need for an antimicrobial agent that produces relatively low irritability in tissues, is effective for complicated root canal morphologies, and is easy to use.

Of the naturally occurring bioactive agents with promising antimicrobial activity, essential oils have attracted attention [11,12]. Essential oils are volatile, natural, and complex compounds that are characterized by a strong odour. They are formed by aromatic plants as secondary metabolites [13], and include manuka oil (MO) and tea tree oil (TTO). MO is an essential oil that is extracted from *Leptospermum scoparium*, which is native to New Zealand [14]. TTO is a valuable essential oil that is extracted by steam distillation from the leaves of *Melaleuca alternifolia* [15]. Both oils have antibacterial, anti-inflammatory, and antiviral properties; in the field of medicine, they are reportedly effective against tinea pedis, atopic dermatitis, and candidiasis [16–20]. It has also been reported that MO and TTO have strong antibacterial activity against periodontopathic and cariogenic bacteria in the dental field [21].

The diverse chemical structures of essential oils containing MO and TTO include two groups with different biosynthetic origins [22]—terpenes and terpenoids—and another group of aliphatic and aromatic compounds, all of which are characterized by low molecular weights [13]. Terpinen-4-ol is classified as a monoterpene, and is a main component of both MO and TTO [13]. This compound has antibacterial and antifungal activities, and has been reported to show potent antibacterial activity against caries-related microorganisms [12, 23]. However, there are few studies on the antimicrobial effects of terpinen-4-ol on bacteria in apical periodontitis.

In the present study, we investigated the antibacterial activities of essential oils, and their main component terpinen-4-ol, against the apical periodontitis-related bacteria *E. faecalis*, *P. gingivalis*, *P. intermedia*, and *F. nucleatum*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

In the present study, *E. faecalis* (American Type Culture Collection [ATCC] 29212) was obtained from the RIKEN BioResource Center (Saitama, Japan). *E. faecalis* strains were cultured on supplemented trypticase soy agar (3% trypticase soy broth, 0.3% yeast extract, 5% heat-inactivated horse serum, and 1.5% agar; Difco Laboratories, Detroit, MI, USA) or in brain heart infusion (BHI; 37 g/L; Difco Laboratories) broth supplemented with menadione (1 μg/mL; Sigma, St. Louis, MO, USA) and hemin (5 μg/mL; Sigma) at 37 °C in 5% CO_2 [24]. *P. gingivalis* (ATCC 33277), *P. intermedia* (ATCC 25611), and *F. nucleatum* (ATCC 25586) were purchased from ATCC (Manassas, VA, USA) and grown on sheep blood agar plates (Becton Dickinson, Franklin Lakes, NJ, USA) or in BHI broth supplemented with menadione (1 μg/mL; Sigma) and hemin (5 μg/mL; Sigma) under anaerobic conditions. All strains were grown to the late logarithmic phase (3×10^7 to 5×10^7 colony-forming units (CFUs)/mL).

2.2. Preparation of reagents

MO was obtained from TCN Co., Ltd. (Nagoya, Japan), TTO was obtained from Tea Tree Therapy (Ventura, CA, USA), and terpinen-4-ol was obtained from Tokyo Chemical Industry (Tokyo, Japan). Jojoba oil was purchased from FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan). The MO and TTO were diluted in jojoba oil to have final concentrations of 0.05%–0.48% (v/v) and 0.48%–4.76% (v/v), respectively. The terpinen-4-ol was diluted to a final concentration of 0.01%–0.25% (v/v) in dimethyl sulfoxide (Sigma) and phosphate-buffered saline (PBS).

2.3. Determination of minimum inhibitory and bactericidal concentrations

MO and TTO, terpinen-4-ol were adjusted to final concentrations of 0.05%–0.48% (v/v) and 0.48%–4.76% (v/v), 0.01%–0.25% (v/v) and added to 96-well plates. All strains were grown to the late logarithmic phase (3×10^7 to 5×10^7 CFUs/mL). A bacterial suspension without MO and TTO, terpinen-4-ol served as the positive control. After incubation for 48 h at 37 °C under anaerobic conditions, levels of microbial growth over time were measured using an Infinite F50 microplate reader (Tecan, Männedorf, Switzerland) at 595 nm. The MIC of MO and TTO were defined as the lowest MO and TTO concentration without bacterial growth. Jojoba oil was used as the positive control. The MIC of terpinene-4-ol was defined as the lowest terpinen-4-ol concentration without bacterial growth. The dimethyl sulfoxide used in the dilution solvent was adjusted to a final concentration of 1.0%, and PBS was used as the positive control. After incubation under anaerobic conditions at 37 °C for 48 h, the levels of microbial growth over time were measured using a microplate reader at 595 nm. The MBC values were determined after the MIC reading by plating 10 μL from each well with no apparent growth onto a trypticase soy agar plate for *E. faecalis* and 5% sheep blood agar plate for *P. gingivalis*, *P. intermedia*, and *F. nucleatum* and incubating at 37 °C under anaerobic conditions; viability was assessed after 72 h. MBC was defined as the lowest concentration capable of causing complete inhibition of bacterial growth.

2.4. Biofilm detachment assay

The effects of terpinen-4-ol against preformed biofilms of *E. faecalis, P. gingivalis*, *P. intermedia*, and *F. nucleatum* were evaluated using a modified version of the protocol established by Cordeiro et al. [25]. Briefly, All strains were grown to the late logarithmic phase (3×10^7 to 5×10^7 CFUs/mL) in 96-well plates. The plates were incubated with BHI broth for 24 h at 37 °C under static conditions to allow biofilms to form in the wells. The broth was then removed, and PBS solutions containing different concentrations of terpinen-4-ol were added. After 1 h of incubation at 37 °C, the contents of each well were discarded, and the plates were then gently washed with sterile distilled water to remove planktonic cells before being dried at room temperature. After drying, a 1% crystal violet solution was added and left for 40 min. The dye was then discarded, and any excess on the well walls was removed by washing with distilled water. After drying, absolute ethanol was added to the wells, and the dye was eluted for 30 min. Plates were then read on a microplate reader at 595 nm.

2.5. Scanning electron microscopy analysis (SEM)

In order to analyse the potential effect of terpinen-4-ol against preformed biofilms, SEM analysis was performed basically according to the methods described by Bose et al. and Jafri et al. [26,27]. The plates were incubated with BHI broth and inoculum to form biofilms as described in the “2.4 Biofilm detachment assay” part. The contents of each well were then removed, and PBS solutions containing MIC concentrations of terpinen-4-ol were added. PBS without terpinen-4-ol was added to the control well. After incubation for 1 h at 37 °C, the supernatant or broth was discarded, and the plates were gently rinsed twice with 1 mL of PBS. Biofilms on the plates were then fixed with 5% glutaraldehyde in graded concentrations of ethanol (50%, 60%, 70%, 80%, 90%, 95%, and 100%) and dehydrated in room temperature air. After the fixed plates were gold-sprayed, the biofilms were observed under an S-3400 N scanning electron microscope (SEM; Hitachi Co. Ltd., Tokyo, Japan) equipped

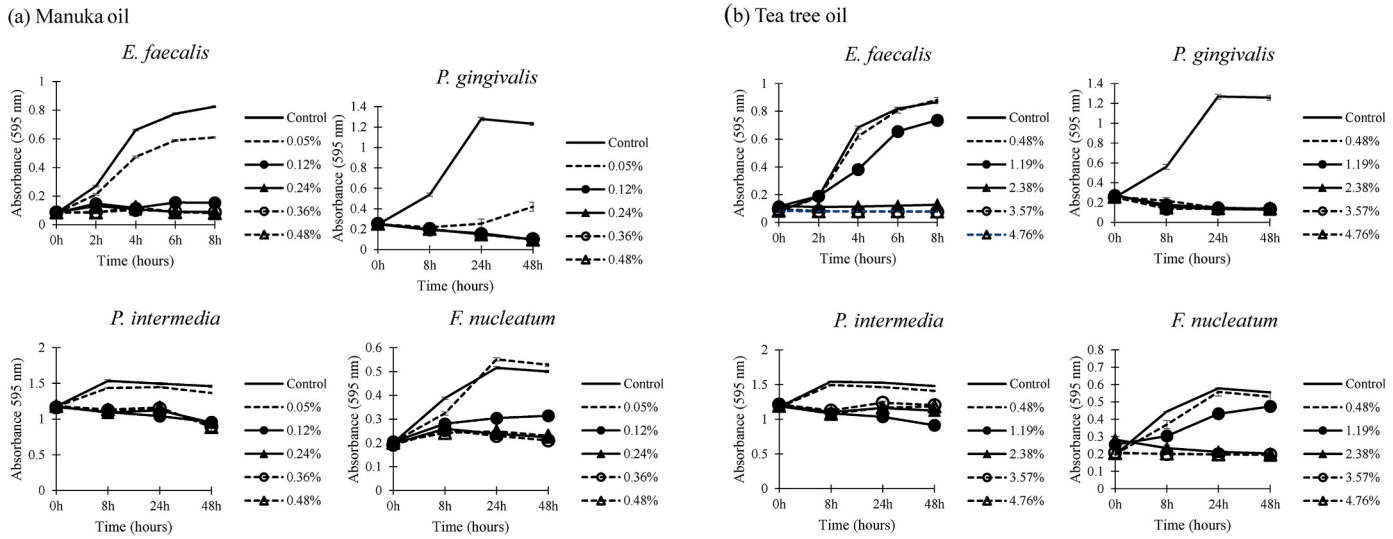

Fig. 1. Determination of the minimum inhibitory concentrations of manuka oil and tea tree oil against *Enterococcus faecalis*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum.* (a) Manuka oil (adjusted to a final concentration of 0.05%–0.48%) and (b) tea tree oil (adjusted to a final concentration of 0.48%–4.76%) were added to 96-well plates. A bacterial suspension without manuka or tea tree oil served as the positive control. After incubation at 37 °C for 48 h under anaerobic conditions, levels of microbial growth over time were measured using a microplate reader. Jojoba oil was used as the positive control. The minimum inhibitory concentrations of both Manuka oil and tea tree oil inhibited bacterial growth at the lowest concentrations in *P. gingivalis* compared with the other bacteria.

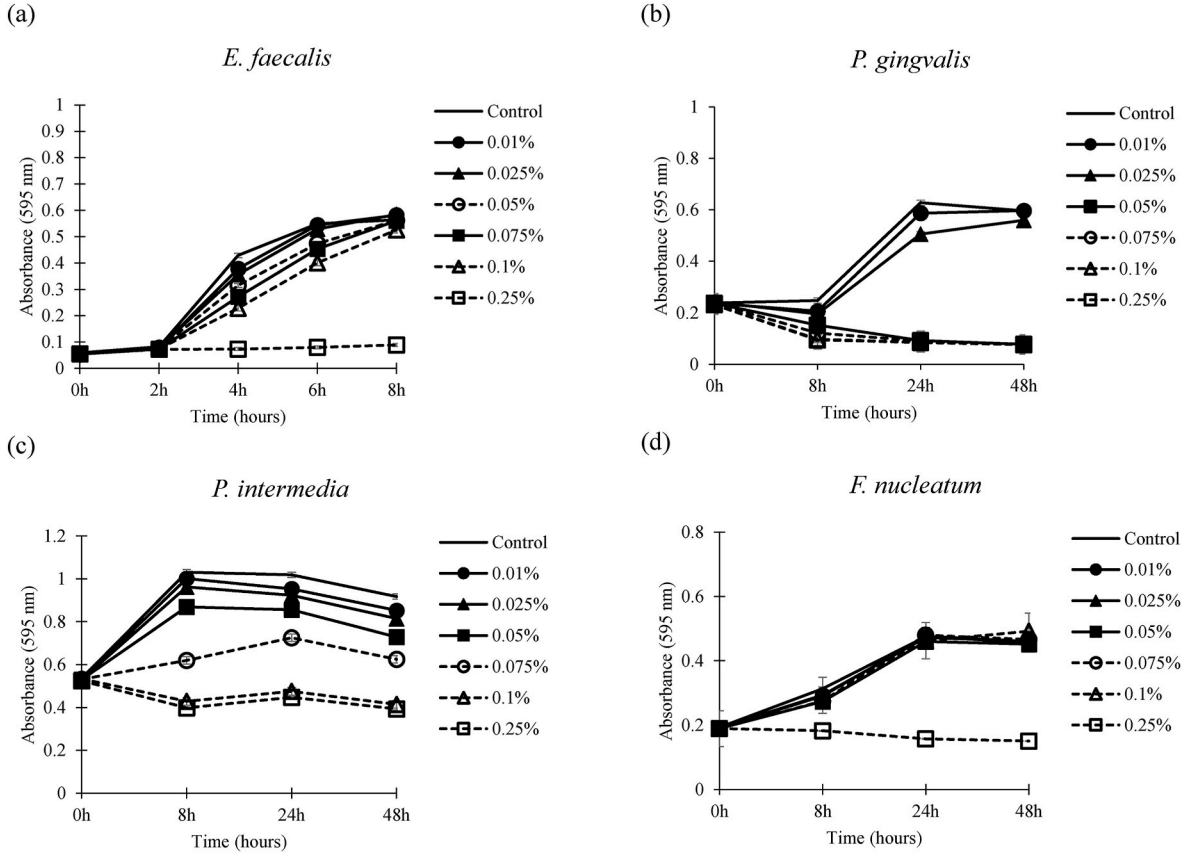

Fig. 2. Determination of the minimum inhibitory concentrations of terpinen-4-ol against *Enterococcus faecalis*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum*. Terpinen-4-ol was adjusted to a final concentration of 0.01%–0.25% and placed into 96-well plates. A bacterial suspension without terpinen-4-ol (phosphate-buffered saline only) served as the positive control. After incubation under anaerobic conditions at 37 °C for 48 h, the levels of microbial growth over time were measured using a microplate reader at 595 nm. The minimum inhibitory concentration of terpinen-4-ol inhibited bacterial growth at the lowest concentrations in *P. gingivalis* compared with the other bacteria.

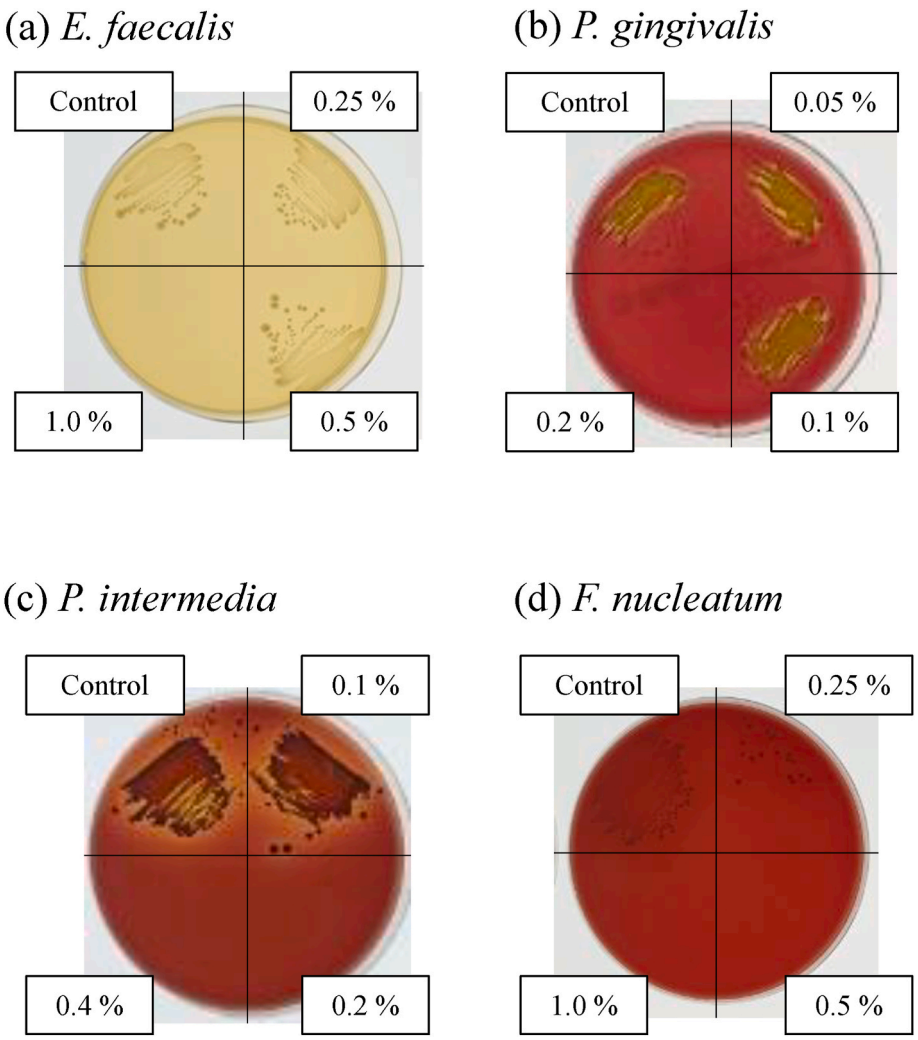

Fig. 3. Determination of the minimum bactericidal concentrations (MBCs) of terpinen-4-ol against *Enterococcus faecalis*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum*. The MBCs were obtained after plating 10 μL aliquots from each well onto trypticase soy agar (*E. faecalis*) or 5% sheep blood agar (*P. gingivalis*, *P. intermedia*, and *F. nucleatum*) and were defined as the lowest concentrations of terpinen-4-ol at which 99.9% of the bacteria were killed. The plates were incubated at 37 °C for 72 h under anaerobic conditions. The minimum bactericidal concentrations of terpinen-4-ol were the lowest against *P. gingivalis* and *P. intermedia* compared with the other bacteria.

with a Genesis APEX2 energy-dispersive X-ray analyser (EDAX Inc., Mahwah, NJ, USA) at 10-kV accelerating voltage.

2.6. Fluorescence microscopy

In order to analyse the potential of terpinen-4-ol against preformed biofilms, 96-well plates were incubated with BHI broth and inoculum to form biofilms as described in the "2.4 Biofilm detachment assay" part. The contents of each well were then removed, and PBS solutions containing MIC or MBC of terpinen-4-ol were added. After 1 h of incubation at 37 °C, the contents of each well were discarded and plates were gently washed with sterile distilled water to remove the planktonic biofilm. Bacteria were then labelled with 3.34 mM of SYTO9 and 20 mM of propidium iodide using a LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, Carlsbad, CA, USA) for 15 min according to the manufacturer's instructions. The excitation/emission wavelengths were as follows: 480/500 nm for SYTO9 and 490/635 nm for propidium iodide. The fluorescence from the stained cells was observed using a fluorescent microscope (BZ-X800; Keyence, Osaka, Japan; × 400 magnification) [28]. Live bacteria were stained green and dead bacteria were stained red, and the areas selected for biofilm analysis were randomly defined. The following formula was used to evaluate the cell viability (% of control) of biofilms of *E. faecalis*, *P. gingivalis*, *P. intermedia*, and *F. nucleatum* after treatment with MIC and MBC of terpinen-4-ol: cell viability (%) = live cells (μm^2)/(live cells [μm^2] + dead cells [μm^2]).

2.7. Statistical analysis

Results are expressed as mean values with standard deviations. One-way analysis of variance followed by Tukey's *post hoc* test was used for the statistical analysis of data from all assays. We considered $p < 0.05$ to be significant. JMP 16.0 (SAS Institute, Cary, NC, USA) was used for all statistical calculations.

3. Results

3.1. Determination of the MIC of MO, TTO, and terpinen-4-ol against *E. faecalis, P. gingivalis, P. intermedia, and F. nucleatum*

The antibacterial activities of MO, TTO, and terpinen-4-ol against *E. faecalis*, *P. gingivalis, P. intermedia*, and *F. nucleatum* were evaluated by determining the respective MICs. The MIC of MO was 0.24% against *E. faecalis* and *F. nucleatum*, and 0.12% against *P. gingivalis* and *P. intermedia* (Fig. 1a). The MIC of TTO was 3.57% against *E. faecalis*,

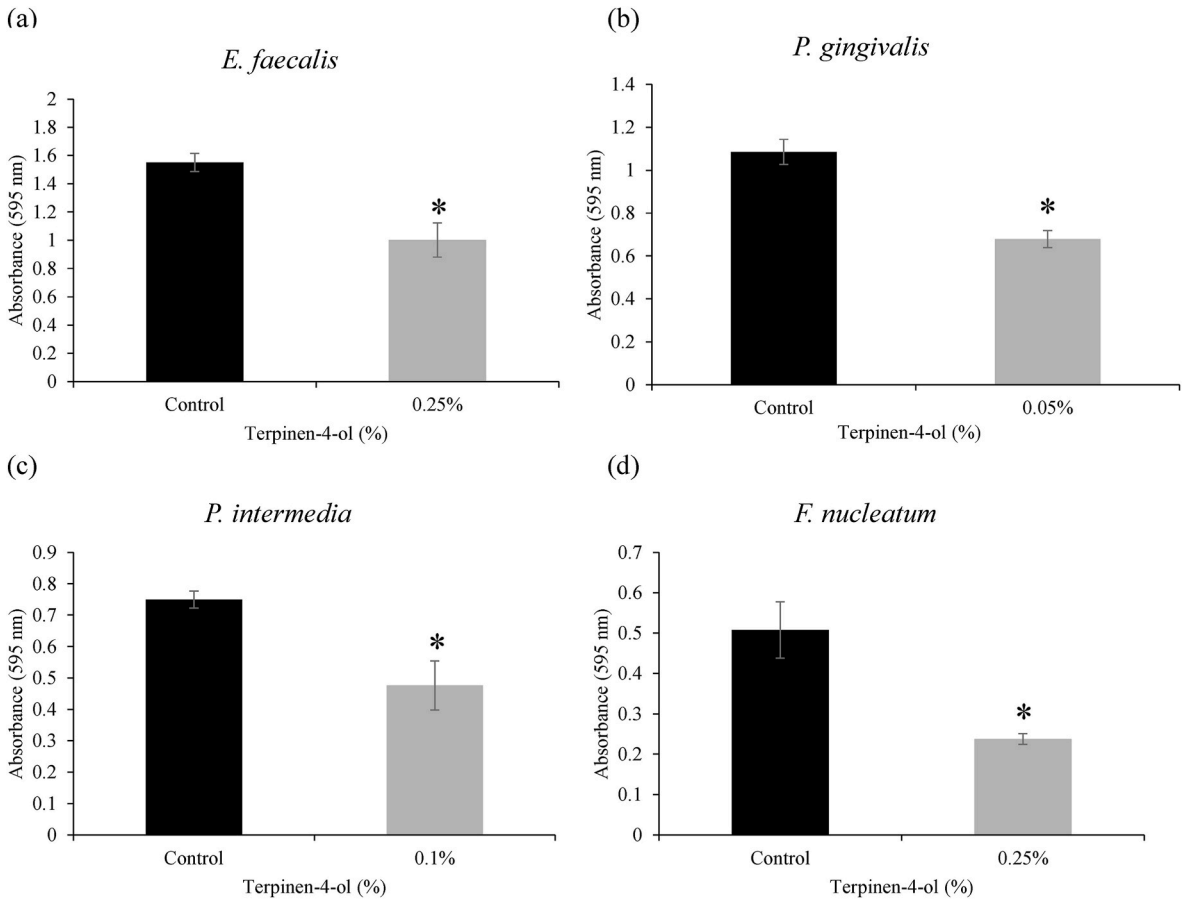

Fig. 4. Effects of terpinen-4-ol on biofilm viability. The effects of terpinen-4-ol against biofilms of (a) *Enterococcus faecalis*, (b) *Porphyromonas gingivalis*, (c) *Prevotella intermedia*, and (d) *Fusobacterium nucleatum* (on plastic surfaces with a 1-h incubation period) were compared with the control (phosphate-buffered saline only). Statistical analyses were performed using Student's *t*-test (*$p < 0.05$). Terpinen-4-ol significantly reduced the biofilms of all four bacteria compared with the untreated biofilms ($p < 0.05$).

0.48% against *P. gingivalis*, 1.19% against *P. intermedia*, and 2.38% against *F. nucleatum* (Fig. 1b). The MIC of terpinen-4-ol was 0.25% against *E. faecalis* and *F. nucleatum*, 0.05% against *P. gingivalis*, and 0.1% against *P. intermedia* (Fig. 2). These results suggest that MO, TTO, and terpinen-4-ol are all able to inhibit the growth of these bacteria, although there were differences in their effective concentrations.

3.2. Determination of the MBC of terpinen-4-ol against E. faecalis, P. gingivalis, P. intermedia, and F. nucleatum

The MBC of terpinen-4-ol was 1.0% against *E. faecalis*, 0.2% against *P. gingivalis* and *P. intermedia*, and 0.5% against *F. nucleatum* (Fig. 3); the respective MIC/MBC ratios against these bacteria were 1:4, 1:4, 1:2, and 1:2. These findings indicate that terpinen-4-ol has bactericidal properties against all strains analysed in the present study.

3.3. Effects of terpinen-4-ol on biofilm viability

We next determined the attachment inhibitory activities of terpinen-4-ol against preformed biofilms of *E. faecalis*, *P. gingivalis*, *P. intermedia*, and *F. nucleatum* on plastic surfaces (Fig. 4). Terpinen-4-ol (0.25%) significantly reduced *E. faecalis* and *F. nucleatum* viability compared with untreated *E. faecalis* and *F. nucleatum* biofilms ($p < 0.05$) (Fig. 4a, d). Moreover, *P. gingivalis* and *P. intermedia* viability was significantly reduced by both 0.05% and 0.1% terpinen-4-ol compared with untreated *P. gingivalis* and *P. intermedia* biofilms ($p < 0.05$) (Fig. 4b and c). These results suggest that terpinen-4-ol has a role in the detachment of biofilms of these bacteria.

3.4. SEM analysis

After biofilms of *E. faecalis*, *P. gingivalis*, *P. intermedia*, and *F. nucleatum* were treated with terpinen-4-ol at their respective MIC values for 1 h, changes or alterations in bacterial cell morphology were evaluated under SEM (Fig. 5). Compared with controls, terpinen-4-ol treatment decreased bacterial cell aggregation and slightly altered bacterial cell morphology (becoming rough and irregular) in all bacterial types. Destruction of bacterial cell structure compared with controls was also observed in all bacterial types.

3.5. Fluorescence microscopy

Live cells in biofilms emitted a green fluorescent signal, while dead cells produced red fluorescence (Fig. 6). In all biofilms, the untreated control emitted strong green fluorescence. Compared with controls, terpinen-4-ol (at MIC and MBC) did not completely kill all bacterial cells within the biofilms; however, cell viability was significantly reduced in all biofilms (Figs. 6 and 7).

4. Discussion

The present study demonstrated the antibacterial effects of terpinen-4-ol against four bacteria that can cause apical periodontitis: *E. faecalis*, *P. gingivalis*, *P. intermedia*, and *F. nucleatum*.

In the current study, the MIC of MO was between 0.12% and 0.24% against the bacteria tested, while the MIC of TTO was between 0.48% and 3.57% (Fig. 1). Several previous studies have also reported the

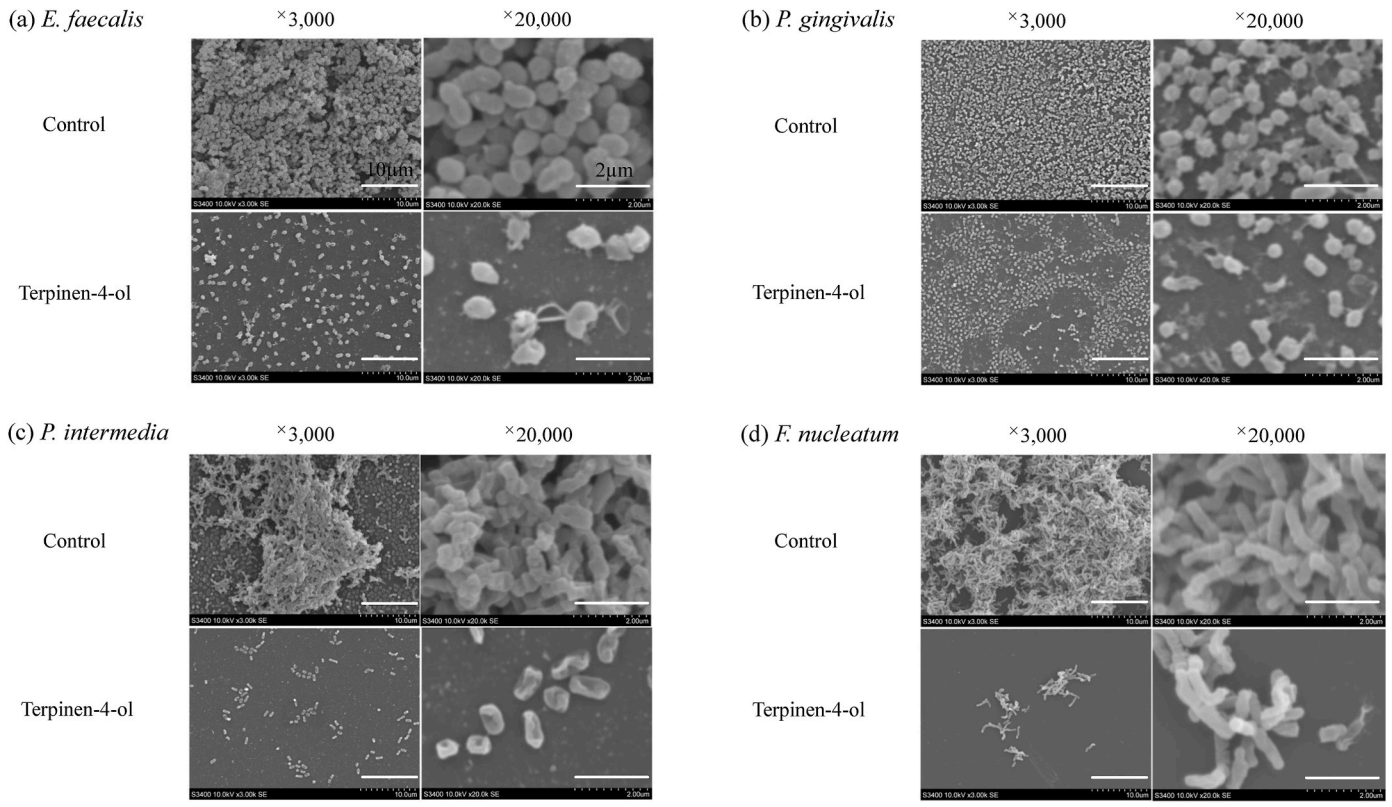

Fig. 5. Scanning electron microscopy analysis. Biofilms of *Enterococcus faecalis*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum* were treated for 1 h with terpinen-4-ol at their respective minimum inhibitory concentration (MIC) values. (a) *E. faecalis* (MIC: 0.25%), (b) *P. gingivalis* (MIC: 0.05%), (c) *P. intermedia* (MIC: 0.1%), and (d) *F. nucleatum* (MIC: 0.25%). Magnification: ×3000 and × 20,000, scale bars: 10 μm and 2 μm. Compared with controls, terpinen-4-ol treatment decreased bacterial cell aggregation and slightly altered bacterial cell morphology (becoming rough and irregular) in all bacterial types. Destruction of bacterial cell structure compared with controls was also observed in all bacterial types.

antibacterial activity of MO and TTO against bacteria. For example, Takarada et al. investigated the antibacterial effects of essential oils on oral bacteria and reported that MO and TTO were the most effective against *P. gingivalis* [21]. In addition, Hammer et al. evaluated the antibacterial activity of TTO on 161 isolates of oral bacteria from 15 genera and reported that isolates of *Porphyromonas*, *Prevotella*, and *Veillonella* had the lowest MIC and MBC, whereas isolates of *Streptococcus*, *Fusobacterium*, and *Lactobacillus* had the highest MIC and MBC [29]. Furthermore, Thosar et al. investigated the antibacterial activities of five essential oils against oral pathogens and found that TTO, thyme oil, and peppermint oil were effective against oral pathogens, with *E. faecalis* being the least susceptible among the investigated oral pathogens [30]. Similar to the results of other studies examining MICs against bacteria, our results indicate that both MO and TTO inhibit bacterial growth and development. Although there are many differences between the investigations—such as the types of MO and TTO used and the experimental conditions—both MO and TTO exhibited antibacterial properties in the current study, which is consistent with other reports on the antibacterial properties of MO and TTO.

Terpinen-4-ol, a major component of MO and TTO, is classified as a monoterpene; these compounds have known antibacterial and antifungal activities. In the current study, the MIC of terpinen-4-ol was between 0.05% and 0.25% against the included bacteria, while the MBC was between 0.2% and 1.0%. The MIC/MBC ratios of *E. faecalis*, *P. gingivalis*, *P. intermedia*, and *F. nucleatum* were 1:4, 1:4, 1:2, and 1:2, respectively. A bactericidal substance is capable of killing bacterial cells, whereas bacteriostatic substances inhibit or slow bacterial growth without causing death. A drug is generally considered to have bactericidal activity when the MIC/MBC ratio is < 4 [25,31]. Our results therefore suggest that terpinen-4-ol has bactericidal properties against all four analysed bacterial strains.

Based on the MIC and MBC results of terpinen-4-ol, we investigated its bactericidal activity against biofilms using SEM and fluorescence microscopy. In experiments evaluating the effects of terpinen-4-ol on biofilm detachment, a significant reduction in biofilms was observed in all bacterial biofilms compared with controls (Fig. 4). Biofilms are organised, structured microbial communities embedded in polymeric matrices that release planktonic cells to colonise surfaces [32]. Several previous studies have evaluated the antibacterial and antibiofilm activities of terpinen-4-ol against bacteria. For example, Bordini et al. reported that terpinen-4-ol inhibits biofilm formation in *Streptococcus mutans* and *L. acidophilus* at concentrations of 0.24% and 0.95%, respectively [33]. Cordeiro et al. reported that terpinen-4-ol has antibacterial and antibiofilm activity against *Staphylococcus aureus*, with concentrations of 0.25% for MIC, 0.5% for MBC, and 0.25% for antibiofilm activity [25]. In addition, Maquera-Huacho et al. reported the antibacterial and antibiofilm activity of terpinen-4-ol against *P. gingivalis* and *F. nucleatum*, with concentrations of 0.06% for both MICs, 0.06% for both MBCs, and 0.06% and 0.24%, respectively, for antibiofilm activity [27]. Although there were many methodological differences among these studies, such as the type of catalyst used and the culture time, the results of the present investigation are generally similar to those of previously reported studies.

In the current study, the SEM observation of terpinen-4-ol-treated biofilms revealed decreased bacterial aggregation and altered cell structure in all bacterial biofilms. Terpinen-4-ol induces membrane loss to disrupt the integrity and physiology of microbial cells [33]. Thus, because terpinen-4-ol is lipophilic and targets the structure, function, and integrity of microbial membranes, it appears unlikely that true bacterial resistance will develop in response to treatment with this

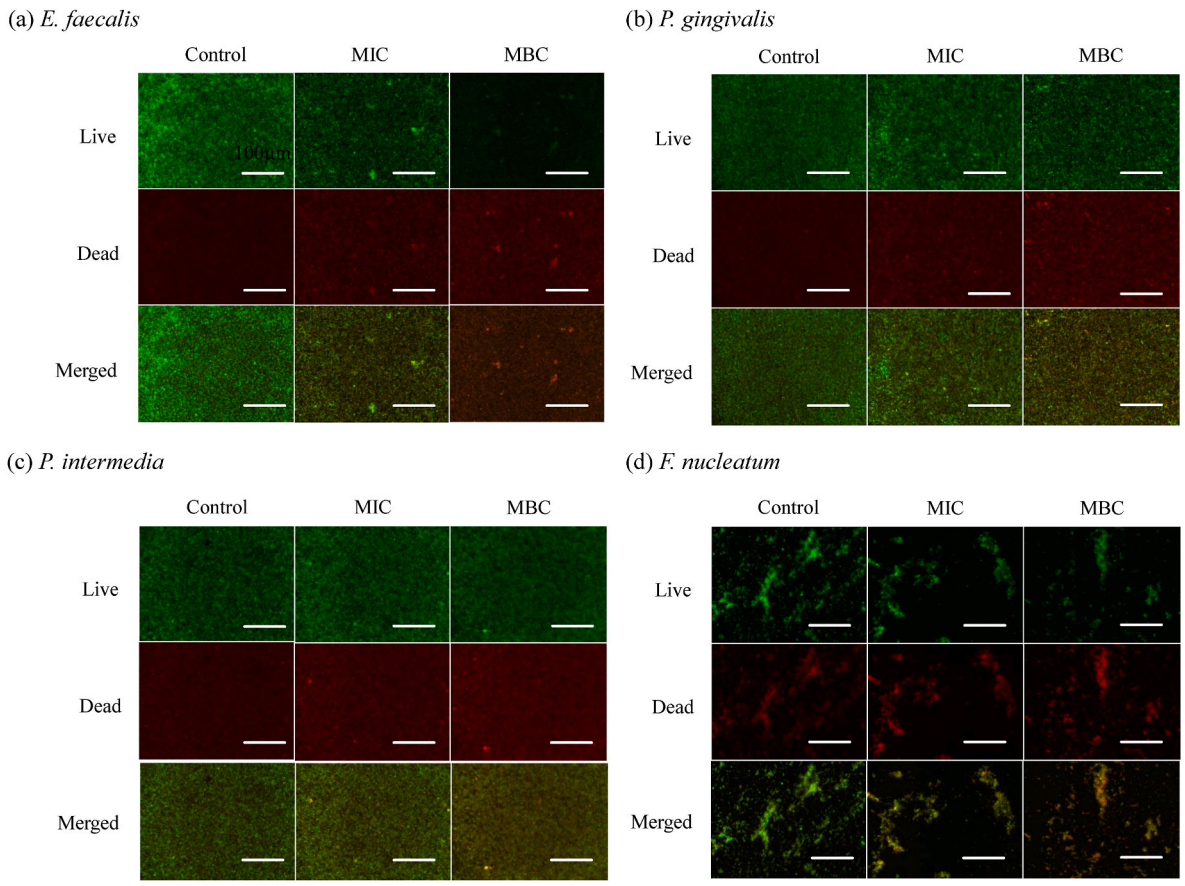

Fig. 6. Live/dead staining images of biofilms of (a) *Enterococcus faecalis*, (b) *Porphyromonas gingivalis*, (c) *Prevotella intermedia*, and (d) *Fusobacterium nucleatum* that were treated for 1 h with terpinen-4-ol (concentration: minimum inhibitory concentration [MIC] or minimum bactericidal concentration [MBC]). Live bacteria are stained green and dead bacteria are stained red. Magnification: × 400, scale bars: 100 μm. Compared with controls, terpinen-4-ol (at both the MIC and MBC) did not completely kill all bacterial cells within the biofilms.

compound. In addition, monoterpenes disrupt the synthesis of genetic material, induce the coagulation of cytoplasmic constituents, form toxic compounds, disrupt normal cell communication (e.g., quorum sensing), and lower intracellular pH [34]. In experiments evaluating the viability of biofilms treated with terpinen-4-ol (at MIC or MBC), we observed a significant reduction in cell viability in all bacterial biofilms compared with controls in the current study. However, terpinen-4-ol treatment using the MBC did not result in the complete destruction of bacterial biofilms. This may be because the biofilms were already mature in the present study. Nonetheless, our results indicate that terpinen-4-ol is effective in breaking/eliminating biofilms that have already formed. This finding is consistent with other reports of the antibiofilm properties of terpinen-4-ol [25,27,31,35].

The possible toxic effects of terpinen-4-ol on cells should be investigated to further evaluate its potential clinical applications. A few studies suggest that terpinen-4-ol has cytotoxic effects on fibroblasts; however, there are also reports indicating that it has low cytotoxicity. It has been reported that low concentrations of terpinen-4-ol do not have toxic effects on fibroblasts or epithelial cells, thus allowing for its topical application with relatively few side effects [27,35]. Cytotoxic doses of terpinen-4-ol against tumour cells are also significantly less efficacious against non-tumour fibroblast cells [36]. Moreover, one study reported that terpinen-4-ol (0.24%) was less cytotoxic than chlorhexidine to mouse L929 mouse fibroblast cells [27]; in another study, terpinen-4-ol (0.125%–1.0%, 24 h) did not show cytotoxicity to human fibroblasts [37].

For the clinical application of terpinen-4-ol, further studies are needed. A limitation of the present study was the use of only four oral pathogenic species, and the development of single-species biofilms. In the oral cavity, biofilms are formed by various bacteria, including apical periodontitis bacteria; these bacteria are intricately related to each other. The present investigation was a preliminary study only, and further studies using multi-species oral biofilms are needed to mimic the complex microbiology of apical periodontitis.

5. Conclusions

The present results indicate that terpinen-4-ol is a potential antimicrobial agent that has bactericidal properties against bacterial cells, in addition to its effects on established biofilms. Our findings suggest that terpinen-4-ol is a potential alternative to intracanal medications for root canal treatment. However, further studies of terpinen-4-ol are needed to evaluate its activity against biofilm formation by other bacterial species, and thus elucidate its usefulness.

Declaration of interest

None.

Funding

This study was financially supported by JSPS KAKENHI (Grant Number JP22K10002) from the Japan Society for the Promotion of Science.

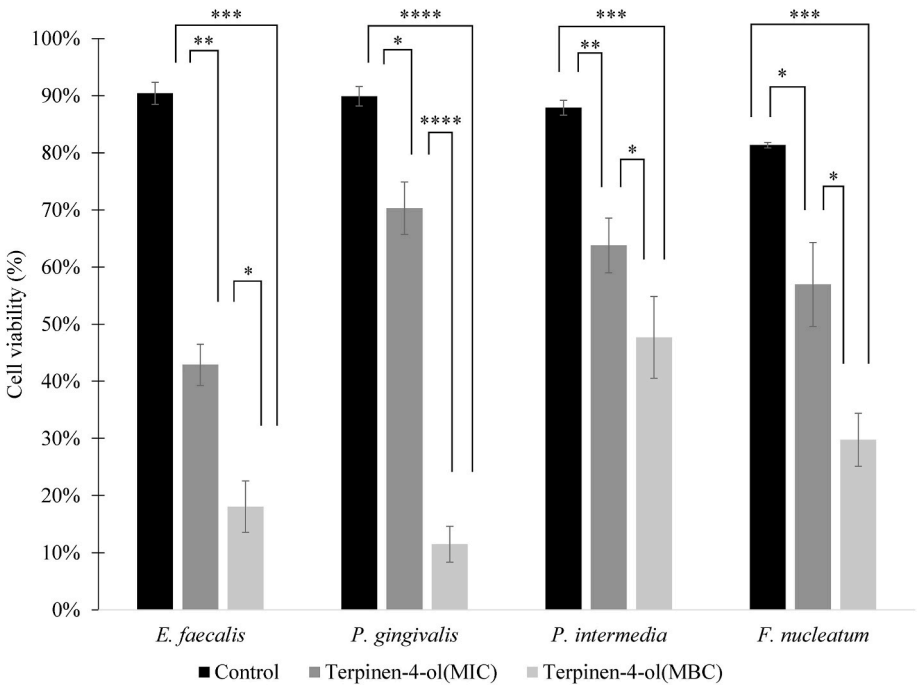

Fig. 7. Cell viability (% of control) of biofilms of *Enterococcus faecalis*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum* following treatment with terpinen-4-ol at the minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC). Cell viability (%) = live cells (μm^2)/(live cells [μm^2] + dead cells [μm^2]). Statistical analysis was performed using analysis of variance and Tukey's tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$). Cell viability was significantly reduced in all biofilms.

Author contributions

All authors have approved the final article.

HK, AH, and NW designed the study and collected data, HK, AH, HM, AY, HW, WS, RZ and SW collected data and conducted data analysis. HK, AH, and NW wrote the manuscript.

Acknowledgements

We thank Bronwen Gardner, PhD, from Edanz (https://jp.edanz.com/ac) for editing the English text of a draft of this manuscript.

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