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Original Article

In vitro effects of fibrin hydrogel incorporated with *Litsea cubeba* essential oil on viability of periodontal pathogens and human gingival fibroblasts

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Periodontitis;
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Abstract *Background/purpose:* The adjunctive use of antibiotics in periodontal therapy may lead to resistance and undesired effects, which can be mitigated by alternative agent such as *Litsea cubeba*. Thus, this study aimed to formulate fibrin hydrogel incorporated with *L. cubeba* essential oil (LC-EO) and test its antimicrobial property against key periodontal pathogens and cytotoxicity on human gingival fibroblasts (HGFs).
Materials and methods: Fibrin hydrogels were incorporated with various concentrations of LC-EO and the releasing of LC-EO was determined by measuring the wavelength absorbance. Their antibacterial activity was tested against *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* by the direct contact method. The cytotoxicity to HGFs was determined by MTT assay.
Results: The absorbance wavelength of LC-EO at 230 nm was the optimal wavelength using for the standard curve. The release of 1.0% (v/v) LC-EO from fibrin hydrogel in PBS was 0.03% (v/v) in the first hour and lasted for 24 h. The gradually release of LC-EO was observed until day 30 with the concentration of 0.008% (v/v). The minimum bactericidal concentration against *A.*

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actinomycetemcomitans and *P. gingivalis* was 1.0 % (v/v) and 0.25% (v/v), respectively. The lowest cytotoxicity to HGF cells was observed at 0.0625% (v/v), yielding the cell viability of 73.65 ± 8.24 % at 24 h.

Conclusion: Fibrin hydrogel with LC-EO effectively inhibited bacterial growth in a dose-dependent manner. Cytotoxicity assays also revealed concentration-dependent effects on HGF. Our results suggest the potential use of fibrin hydrogel with LC-EO as a novel adjunctive treatment in periodontal diseases.

Introduction

Periodontitis is a prevalent chronic inflammatory condition affecting tooth-supporting structures, where disease progression depends on host factors responding to subgingival biofilm accumulation. The release of proinflammatory cytokines leads to tissue destruction and osteoclastogenesis, resulting in severe alveolar bone loss in affected patients.[1] While scaling and root planing are effective for most cases, some patients harbor specific microorganisms, notably *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*, associated with treatment-resistant cases.[2–4] Hence, adjunctive antimicrobial therapies like systemic antibiotics, local antimicrobials, and mouthrinses have been introduced.

Systemic antibiotic failure may stem from inadequate concentrations in periodontal pockets, prompting exploration of local antimicrobial treatments.[5,6] However, their efficacy can be compromised by saliva and gingival crevicular fluid flow. To address this, various drug delivery systems like polymethylmethacrylate cement, hydroxyapatite tricalcium phosphate, collagen fibers, and fibrin hydrogel have been employed to prolong drug release.[7,8] Due to concerns about antibiotic resistance and oral flora disruption,[9–11] alternative antimicrobials are being explored.

As a comprehensive review of drug delivery and medicinal plants with antimicrobial activity have been performed, fibrin hydrogel and essential oil from *Litsea cubeba* attracts our interest as a possible novel alternative for adjunct periodontal treatment. Fibrin hydrogel is a fibrin network that mimics the natural blood clotting mechanism. It forms a three-dimensional network that widely used as a scaffold or drug carrier to promotes hemostasis.[12] Its osteoinductive and biodegradable properties also increase therapeutic satisfaction. Using fibrin hydrogel in guided tissue regeneration (GTR) for intrabony defect treatment was reported no tissue loss or harmful effects.[13,14]

L. cubeba (Lour.) *Pers*, indigenous to China, Japan, and Southeastern Asia. It's essential oils are a complex mixture of monoterpenes, phenols, and sesquiterpenes.[15] *L. cubeba* essential oil (LC-EOs) demonstrated antioxidant, antibacterial, and antifungal activities.[16] These properties have made LC-EOs a remedy in traditional Chinese medicine for inflammation, headache, and intoxication.[17] Recent studies highlight its potential in reducing proinflammatory cytokines and inhibiting osteoclastogenesis.[18–21] The application of *L. cubeba* in dentistry remains limited. Previous studies indicate its effectiveness in suppressing proinflammatory cytokine production in periodontal ligament fibroblasts and reducing bone loss in periodontitis models.[22,23] The antimicrobial effects have been shown when it was incorporated in dental materials, i.e., denture soft liners.[24]

In this context, we investigated the antimicrobial properties against some key periodontal pathogens and its cytotoxicity to evaluate the potential of fibrin hydrogel incorporated with LC-EOs as a novel adjunctive periodontal treatment.

Materials and methods

Fibrin hydrogel incorporated with *L. cubeba* essential oil

LC-EO (Thai-China Flavours and Fragrances Industry Co., Ltd., Nonthaburi, Thailand) from fresh fruits was extracted by steam distillation. Fibrin hydrogel was prepared from 1:1 mixture of fibrinogen 8 mg/mL in 2.5 mmol/L calcium ion solution and 2 NIH Unit/mL thrombin (Baxter Healthcare, Bangkok, Thailand) in tris-buffered saline solution (TBS) which consisted of TrisHCl 100 mM (Sigma–Aldrich, St. Louis, MO, USA) and NaCl 150 mM (Sigma–Aldrich). LC-EO was diluted with TBS and added into the thrombin solution before mixing with fibrinogen solution to yield the final concentration of LC-EO ranged from 0.0625% (v/v) to 2.5% (v/v).

Bacterial cultivation

A. actinomycetemcomitans ATCC 29523 and *P. gingivalis* W50 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and preserved in our microbiology laboratory. *A. actinomycetemcomitans* was maintained in Brain heart infusion agar (BHI; Difco Laboratories, Detroit, MI, USA), incubated for 48 h at 37 °C in 5% CO_2. *P. gingivalis* was maintained in anaerobic basal agar (Oxoid, Hampshire, UK) supplemented with 5% human blood and incubated under anaerobic condition, using anaerobic jar with a gas pack (Anaeropack anaero, MGC trading, Bangkok, Thailand), at 37 °C for 72 h.

Human gingival fibroblasts and culture conditions

Human gingival fibroblasts (HGF) cell line (ATCC CRL-2014) were used in the cytotoxicity test. The cells were grown in Dulbecco's modified Eagle's medium high glucose (DMEM) (Hyclone, South logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1% penicillin-streptomycin solution (Hyclone) in a humidified atmosphere at 37 °C and 5% CO_2. Cells were detached from tissue culture plastic by using 0.25% EDTA—Trypsin (Gibco, Langley, OK, USA) prior to reaching confluency.

In vitro release of *L. cubeba* essential oil from the fibrin hydrogel

The accuracy and precision of the measurement method to determine the LC-EO released from the fibrin hydrogel had been tested according to The United State Pharmacopoeia. Firstly, the absorbance of LC-EO was screened in the range of 200 and 300 nm using a microplate spectrophotometer (EPOCH, Bio-Tek instruments Inc, Winooski, VT, USA) to identify λ_{max}. Then the standard curve was created using five standard solutions of LC-EO (v/v) prepared in ethanol, i.e., 0.006%, 0.003%, 0.0015%, 0.00075%, 0.000375%. Three optical density readings of each standard concentration were performed. The relative standard deviation of the measurement was calculated for inter-day and inter-person precision, while the percentage of relative error or percent bias (% bias) was used for proving the accuracy. The limit of detection (LOD) and limit of quantification (LOQ) were measured from the absorbance of the blank solution.

To measure the release of LC-EO from fibrin hydrogel, the specimens were separately immersed in 400 μL phosphate-buffered saline (PBS), pH 7.2 and incubated at 37 °C in the dark. The PBS solution was collected from triplicate samples at 0, 1, 12, 24, 72, 168 (7 d), 336 (14 d) and 720 (30 d) h and stored at −20 °C until observing the absorbance. The solutions of 1% (v/v) of LC-EO in PBS were measured in parallel.

Antimicrobial test

The antimicrobial effectiveness of LC-EO in fibrin hydrogel was determined by the direct contact method. Prior to the experiment, *A. actinomycetemcomitans* was cultured in brain heart infusion broth (Difco), incubated for 48 h at 37 °C in 5% CO_2. *P. gingivalis* was cultured in anaerobic basal broth (Oxoid) and incubated under anaerobic condition, at 37 °C for 48 h. The bacterial inoculum was adjusted the turbidity to 0.5 McFarland standards (1×10^7 colony-forming unit, CFU/mL) using McFarland densitometer (Biosan Medical-Biological Research & Technologies, Riga, Latvia).

Fibrin hydrogel incorporated with LC-EO at various concentrations (v/v), i.e., 0.0625%, 0.125%, 0.25%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, were prepared. Then, a 40-μL of each concentration was added into a 1.5-mL microcentrifuge tube. After few-second setting time, 400 μL 0.5 McFarland standard cultures of *A. actinomycetemcomitans* or *P. gingivalis*, was added and incubated at 37 °C in anaerobic condition. After 24-h incubation, the viable bacteria, expressed as CFU, were determined by plate counting. Percent bacterial reduction was calculated by comparing with the positive controls (the bacterial cultures with fibrin hydrogel alone). The lowest concentration of LC-EO in fibrin gel that showed 99.9% growth inhibition was considered as the minimum bactericidal concentration.

Cytotoxicity test

The cytotoxicity test of fibrin hydrogel containing various concentrations of LC-EO on HGFs was adapted from ISO 10993-5. HGFs were grown in DMEM supplemented with 10% (v/v) FBS and 1% penicillin-streptomycin solution. HGFs (1×10^4 cells/well) were seeded in the lower compartment of the 24-well Transwell (Corning Inc., Corning, NY, USA) and incubated at 37 °C in 5% CO_2. After 24 h, the media with non-adherent cells was replaced with 400 μL of the fresh medium and 40 μL each of fibrin hydrogel alone, with LC-EO at 0.0625%, 0.125%, 0.25%, 0.5%, 1%, 1.5% (v/v), were loaded into the upper compartments. The plates were then incubated for 24 h. Chlorhexidine gluconate (CHX) 0.12% (M-Dent, Bangkok, Thailand) was used as the positive control. After 24-h incubation, the upper compartments were removed and the cells were washed with 500 μL of PBS. The cell viability was assessed using the MTT assay (Sigma—Aldrich). The absorbance was read at 570 nm using a microplate spectrophotometer (Bio-Tek). The percentage of cell viability was calculated by comparing with the controls (fibrin hydrogel alone).

Statistical analyses

Data analysis was performed using PASW statistics software (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0, Armonk, NY, USA). Data shown were mean ± standard deviation of triplicates unless otherwise indicated. Statistical significance was calculated using Kruskal—Wallis, one-way analysis of variance test, followed by the Mann—Whitney U Test. All experiments were performed at least three times. Statistically significance difference was defined as $P < 0.05$.

Results

In vitro release of *L. cubeba* essential oil from the fibrin hydrogel

The absorbance of LC-EO ranging from 200 to 300 nm was shown in Fig. 1A, which indicated the maximum absorbance of 3.973 at the wavelength of 230 nm (λ_{max}). This wavelength was further used to create the standard curve for LC-EO concentrations, as shown in Fig. 1B. The linear regression (R^2) calculated from LC-EO at five concentrations (v/v), i.e., 0.006, 0.003, 0.015, 0.0075, 0.00375, was 0.9997. The LOD and LOQ was 0.208 and 0.230, respectively. The precision was determined by repeating the method three times and computing the percentage of relative standard deviation. Using this standard curve, the release of LC-EO from the fibrin hydrogel incorporated with various concentrations was determined. Fig. 2A showed the

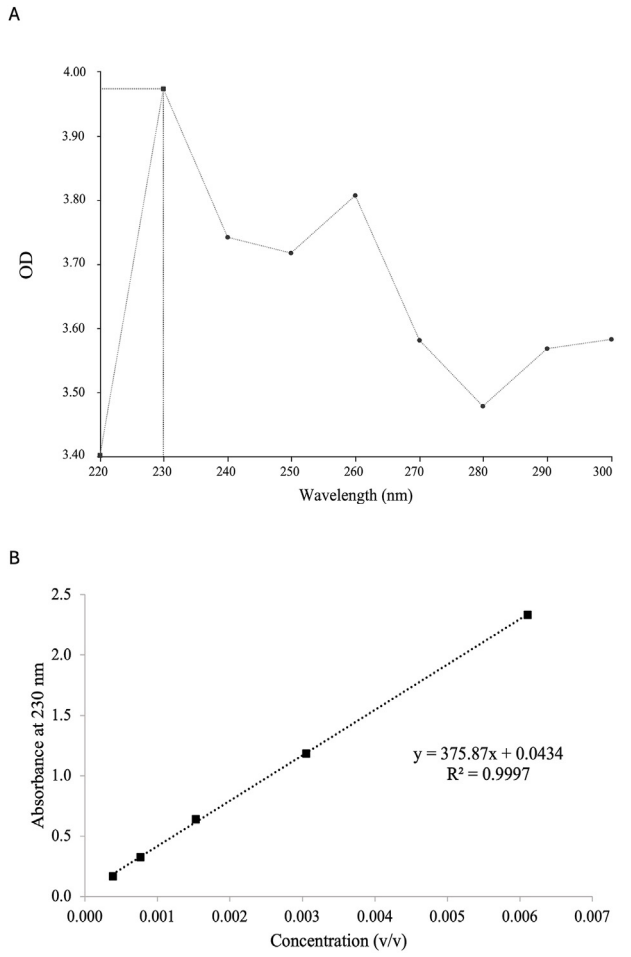

Figure 1 Determination of *Litsea cubeba* essential oil (LC-EO) concentration using optical density (OD). (A) Spectra absorption of LC-EO at wavelength between 220 and 300 nm showed the maximum absorbance at 230 nm, which should be used for further analysis. (B) The standard curve for LC-EO concentration (v/v) using OD at 230 nm which presented the least square analysis and equation.

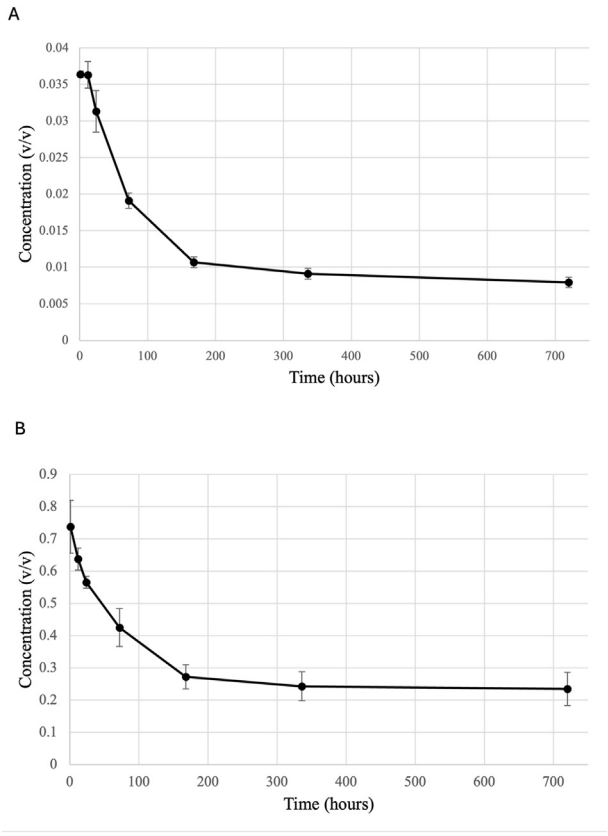

Figure 2 The release of *Litsea cubeba* essential oil (LC-EO) from fibrin hydrogel with 1.0% (v/v) LC-EO at various time points. (A) The concentration of LC-EO released from fibrin hydrogel with 1.0% LC-EO. (B) The control 1.0% LC-EO detected in phosphate-buffered saline (PBS). The measured time points were 1, 12, 24, 72, 168, 336 and 720 h.

release of LC-EO in PBS from the fibrin hydrogel with 1.0% (v/v) LC-EO. The concentration of LC-EO in the initial release during the first hour to 24 h was approximately 0.03% (v/v). Then the release of LC-EO was gradually reduced and reach the concentration approximately 0.01% by day 7 (168 h). At the end of the experimental period of 30 days (720 h), 0.008% of LC-EO was detected. When compared to the stability of 1.0% LC-EO in PBS, 0.74% of LO-EO remained after 1 h and continuously declined in a similar pattern, as shown in Fig. 2B. Approximately 0.25% was reach at day 7 and still remained until day 30.

Antimicrobial activity of fibrin hydrogel incorporated with *L. cubeba* essential oil

The antimicrobial effectiveness of fibrin hydrogel with LC-EO was shown in Fig. 3, the bacterial growth of *A. actinomycetemcomitans* and *P. gingivalis* after 24-h exposure to fibrin hydrogel with LC-EO was inhibited in a dose-dependent manner. The difference in the antimicrobial susceptibility of *A. actinomycetemcomitans* and *P. gingivalis* indicated as the minimal bactericidal concentration of 1.0% and 0.25% (v/v) LC-EO, respectively.

Effect of fibrin hydrogel incorporated with *L. cubeba* essential oil on human gingival fibroblasts

The percent HGF cell viability at 24-h exposure to fibrin hydrogel with various concentrations of LC-EO was showed in Fig. 4. The cell viability decreased in a dose-dependent manner. It was observed that fibrin hydrogel with 0.0625% (v/v) of LC-EO had the lowest cytotoxicity with 73.65 $\pm$ 8.24 % cell viability at 24 h. At concentration of LC-EO in fibrin hydrogel $\geq$0.5% (v/v), cell viability less than 2.28%, which was comparable to 0.12% chlorhexidine gluconate.

Discussion

This study aimed to explore the possible use of LC-EO in periodontitis treatment by incorporating in the drug delivery vehicle, fibrin hydrogel. We first investigated the release of LC-EO from fibrin hydrogel, then determined the

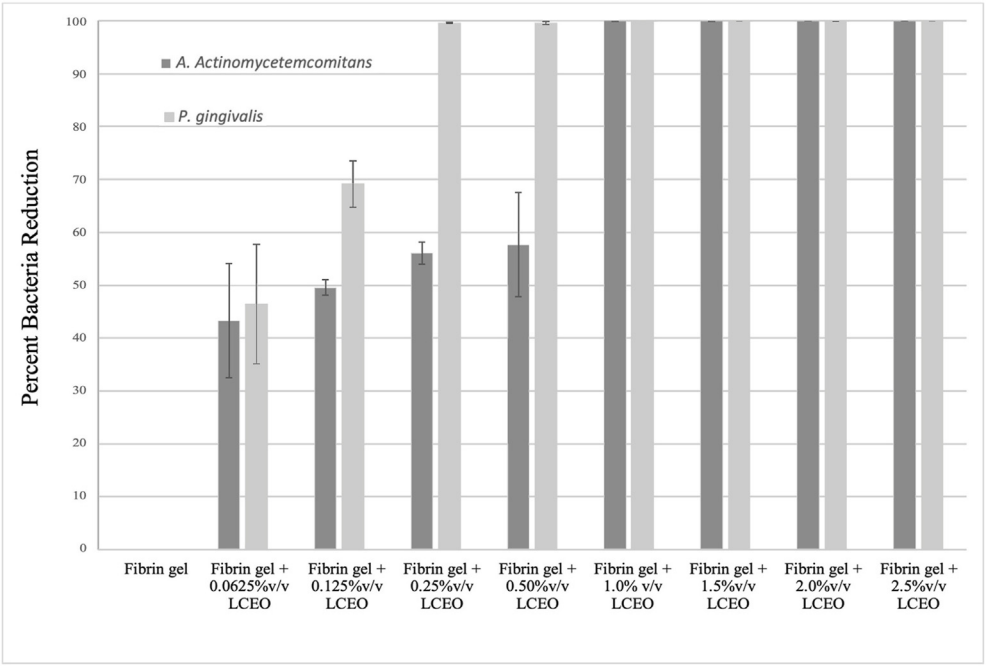

Figure 3 Percent reduction presented as mean ± standard deviation (SD) of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* after 24 h exposure to fibrin hydrogel with various concentrations of *Litsea cubeba* essential oil (LC-EO).

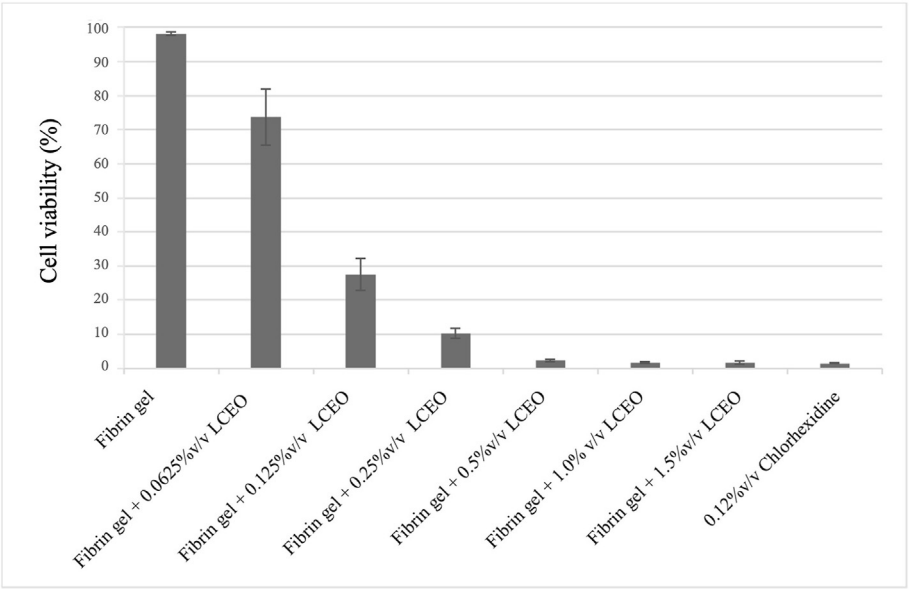

Figure 4 Percent cell viability presented as mean ± standard deviation (SD) of human gingival fibroblasts (HGFs) exposed to fibrin hydrogel with various concentrations of *Litsea cubeba* essential oil (LC-EO) for 24 h using 3-(4,5-demethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay.

antimicrobial effects on periodontal pathogens, i.e., *A. actinomycetemcomitans* and *P. gingivalis*, and toxicity on human gingival fibroblasts.

LC-EO contains a complex mixture of compounds, with aldehydes (citral, citral isomer, citronellal, and citronellal isomer) accounting for approximately 70%.[25] The composition and yield of LC-EO can vary based on several factors, such as cultivation methods, growing areas and season, vegetative stage, and isolation methods. Gas chromatography/mass spectrometry (GC/MS) and high-performance liquid chromatography (HPLC) are commonly used to analyze LC-EO's main constituents.[20,25–27] However, these methods are expensive and require sophisticated equipment. In this study, we determined the concentration of LC-EO by measuring the absorbance of the essential oil. This simpler and cost-effective method has been previously

used to assess the concentration of LC-EO released from the scaffold.[28,29] Spectral characteristics of LC-EO showed the maximum absorption and less interference at 230 nm. To precisely determine the LC-EO, we used linear regression and LOQ to adjust the LC-EO concentration being in the range where the percentage of relative standard deviation were acceptable.

To control the LC-EO release, fibrin hydrogel is our choice due to its biocompatibility and biodegradability. The release of LC-EO in PBS from fibrin hydrogel showed an initial burst, followed by a gradual decrease, which is commonly observed in the controlled release system.[30] In addition, the solubility of LC-EO from the fibrin hydrogel might be lower than that of the other hydrophilic drugs or substances.[31] We found the rapid release with low concentration of LC-EO within the first 12—24 h, gradually decrease and stable from day 7 until day 30. Regarding the stability, in the control group approximately 0.25% (v/v) still be detected in PBS. This slow release of LC-EO from fibrin hydrogel is advantageous for sustained antimicrobial effects in periodontal pockets.

As an adjunct treatment in periodontitis patients, we tested its antimicrobial activity against key pathogens, *A. actinomycetemcomitans* and *P. gingivalis*. We found that the fibrin hydrogel with 1.0% LC-EO effectively inhibited the growth of both species. The MBC for *P. gingivalis* (0.25%) was four times lower than that for *A. actinomycetemcomitans* (1.0%). For clinical application, the concentration of LC-EO released from fibrin hydrogel should be high enough for exerting antimicrobial effect. According to our results, the highest amount of LC-EO found within 24 h was approximately 0.03% (v/v), which could inhibit approximately 50% of bacterial growth, indicating a potent effect even at the low concentration. Nevertheless, we expected that at disease sites greater LC-EO concentration could be achieved in the initial burst-release of loaded fibrin hydrogel in the periodontal pocket which is due to the dissolution of hydrogel by the fibrinolytic mechanism as well as the protease activity from anaerobic bacteria in subgingival biofilms.[32—35] Accordingly, we expected that fibrin hydrogel ensures the controlled release of LC-EO and the concentration should be more than these *in vitro* results which was conducted in PBS.

To ensure clinical safety, we evaluated the cytotoxicity of fibrin hydrogel containing LC-EO. At a concentration of 0.065% (v/v) LC-EO, the viability of HGFs was between 70 and 80% after 24 h. This suggests that cytotoxicity may be lower at reduced concentrations in periodontal pockets. Although higher concentrations exhibited some toxicity, they were less toxic compared to chlorhexidine gluconate.[36] Acute and genetic toxicity studies also indicated only slight toxicity.[37] Additionally, LC-EO was classified as a weak sensitizer for dermal use.[38] However, since our results are based on a single human fibroblast cell line, they may not be generalizable to other cell lines and could complicate replication and comparison with studies using different cell types.[39] To confirm these findings and improve their clinical relevance, further cytotoxicity evaluations should be conducted using a variety of cell types, including primary cells and multiple cell lines.

Regarding the mechanism of action on antimicrobial and cytotoxicity, previous studies have been reported the damage of cell membrane, and the change of cell metabolism.[26,40] Multiple components in LC-EO, including citral, aldehydes, hydrocarbons, and alcohols, may contribute synergistically to these effects.[16,26]

Taken together, our study highlights the controlled release of LC-EO from fibrin hydrogel and its antimicrobial efficacy against periodontal pathogens. The observed cytotoxicity suggests the need for careful consideration of LC-EO concentrations in clinical applications. Future research should focus on clinical evaluations and wound healing assessments. In summary, fibrin hydrogel with LC-EO presents a promising adjunctive treatment option for periodontal diseases, warranting further investigation in clinical settings.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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