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Investigation of Cajuput and Lemongrass Essential Oils Supplemented in Alcohol-Free Mouthwash: Anti-Inflammation on Human Gingival Fibroblast Cells *In Vitro*

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Abstract

Alcohol-free mouthwash containing essential oils is a helpful adjunct to self-performed oral hygiene of patients with periodontal disease due to its antimicrobial and anti-inflammatory properties. This study aimed to investigate the anti-inflammatory activity of cajuput and lemongrass essential oils supplemented in alcohol-free mouthwash on human gingival fibroblast cells and RAW 264.7 macrophages, which were incubated in a complete medium. In the current *in vitro* study, 3 types of mouthwash, including 12 % (v/v) alcohol-free mouthwash, 0.8 % (v/v) cajuput essential oil and 0.4 % (v/v) lemongrass essential oil supplemented in alcohol-free mouthwash, and 0.12 % chlorhexidine mouthwash, were evaluated. All 3 types were determined for cytotoxicity by MTT assay. The sub-IC_{50} concentration of each mouthwash was calculated to investigate anti-inflammatory activities via the inhibition of lipopolysaccharide-activated nitric oxide production, cyclooxygenases-2, interleukin-1β and interleukin-6 gene expression. The wound healing rate was determined by scratch assay. The results demonstrated that the sub-IC_{50} concentrations of essential oils in 0.8 % (v/v) cajuput essential oil and 0.4 % (v/v) lemongrass essential oil supplemented in alcohol-free mouthwash significantly inhibited nitric oxide production in lipopolysaccharide-activated RAW 264.7 macrophages and reduced the gene expression of cyclooxygenases-2, interleukin-1β and interleukin-6 in lipopolysaccharide-activated human gingival fibroblast cells, while stimulating the migration of human gingival fibroblast cells, similar to the sub-IC_{50} concentration of 0.12 % chlorhexidine mouthwash. These findings suggested that essential oils contained in this mouthwash formula exhibited an anti-inflammatory effect and promoted oral wound healing, which might be used as an alternative agent for patients with periodontal disease.

Keywords: Anti-inflammation, Cajuput essential oil, Lemongrass essential oil, Human gingival fibroblast cells, Alcohol-free mouthwash

Introduction

Inflammation is a response of the host immune system to various harmful stimuli, including infection or injury. It is an important defense mechanism to eliminate injurious stimuli and promote the healing process. However, if inflammatory stimuli are not resolved, acute inflammation may develop into chronic inflammation, leading to progressive damage or chronic disease [1].

The microorganisms and the host immune response are associated with the pathogenesis of chronic oral inflammation, especially periodontal disease [2]. Periodontal disease is the most prevalent disease in the oral cavity, including gingivitis and periodontitis. It is characterized by the breakdown of gingival and periodontal tissue and the alveolar bone destruction resulting from the imbalance between the microbial biofilm and immuno-inflammatory response. Lipopolysaccharide (LPS), the outer membrane of Gram-negative bacteria, is the main virulence factor that causes inflammation in the tissue. During the inflammatory process, LPS initiates the cell-mediated immune response. Multiple cell types, including activated macrophages, lymphocytes and gingival fibroblasts, secrete high inflammatory mediators; for example, tumor necrosis factor- α (TNF-α), interleukin-(IL-)1β, IL-6, nitric oxide and cyclooxygenase-2

(COX-2). These mediators are critical in chronic gingival inflammation and periodontal tissue destruction [3,4]. Therefore, inhibiting the production of inflammatory mediators is a crucial therapeutic approach to reducing gingival and periodontal inflammation.

Chlorhexidine mouthwash (CHX) at concentrations of 0.2 and 0.12 % is commonly used as an adjunctive agent for the treatment of oral diseases due to its antiseptic, antibacterial, antifungal and anti-inflammatory properties. Nevertheless, the adverse effects of CHX, including altered taste sensations, tooth staining, dry mouth (xerostomia) and exacerbated pain, have been explained after its use [5]. Thus, CHX should not be used in the long-term in dental treatment.

Currently, herbal mouthwash can be used as an alternative to CHX as its compounds exhibit antimicrobial, antioxidant and anti-inflammatory properties. A recent systematic review and meta-analysis by Mathur *et al.* [6], demonstrated that green tea-based herbal mouthwash was not significantly different compared with standard CHX in reducing plaque and gingival inflammation. Charles *et al.* [7], reported that the use of mouthwash supplemented with polyherbal essential oils was as effective as 0.12 % CHX in reducing plaque and gingivitis. The enhancement of essential oils contained in mouthwash that presents anti-inflammatory activity is of interest in this study. Lemongrass essential oil extracted from *Cymbopogon citratus* and cajuput essential oil extracted from *Melaleuca cajuputi Powell* have provided evidence supporting their antimicrobial and anti-inflammatory properties [8-10]. Several studies have investigated the anti-inflammatory activity of lemongrass essential oil and its main chemical component (citral), demonstrating the inhibition of pro-inflammatory cytokines (IL-1β and IL-6) in LPS-activated macrophages of BALB/c mice [11], and reducing the nitric oxide production through suppression of inducible nitric oxide synthase (iNOS) and NF-κB expression in LPS-activated RAW 264.7 cells [12]. The anti-inflammatory activity of cajuput essential oil has been little studied. Raungsawat *et al.* [13], reported that cajuput essential oil (8 µL/mL) and lemongrass essential oil (4 µL/mL) formulated in alcohol-free mouthwash showed a strong potential for anticandidal activity. A recent cytotoxicity study in gingival fibroblast cells by Thanabhinunt *et al.* [14], found that alcohol-free mouthwash supplemented with both cajuput essential oil (8 µL/mL) and lemongrass essential oil (4 µL/mL) was less cytotoxic than alcohol-free mouthwash with cajuput essential oil (8 µL/mL), and 0.12 % CHX. The combination of cajuput and lemongrass essential oils exhibits a better anticandidal effect [13], and affects cytotoxicity less [14]. However, to our knowledge, the anti-inflammatory property of cajuput essential oil and lemongrass essential oil contained in alcohol-free mouthwash has not been examined. Thus, the objective of this study was to investigate the anti-inflammatory effect of cajuput and lemongrass essential oils supplemented in alcohol-free mouthwash on human gingival fibroblast cells.

Materials and methods

Preparation of cajuput and lemongrass essential oils

Fresh lemongrass leaves were collected from Chiang Mai, Thailand, and cajuput leaves were collected from Southern Thailand. The preparation of cajuput essential oil and lemongrass essential oil was performed using the standard protocol of the Faculty of Pharmacy, Chiang Mai University, Thailand. Briefly, 5 kg of fresh lemongrass leaves and cajuput leaves were mixed with distilled water and extracted in a Soxhlet extractor for 8 h. Both essential oils were stored in a hermetically sealed glass bottle, covered with aluminum foil to protect the substances from light, and kept under refrigeration.

Preparation of mouthwashes

Three types of mouthwash were used in this study. Type 1: Alcohol-free mouthwash (AM) was prepared by mixing the basic ingredients, which consisted of 12 % (v/v) propylene glycol, 5 % (v/v) of sorbitol 70 % solution, 0.4 % (w/v) menthol, 1 % (w/v) Sodium benzoate, and distilled water. Type 2: Alcohol-free mouthwash supplemented with 0.8 % (v/v) cajuput essential oil and 0.4 % (v/v) lemongrass essential oil supplemented in alcohol-free mouthwash (CLAM) was developed by mixing 0.8 % (v/v) cajuput essential oil and 0.4 % (v/v) lemongrass essential oil into AM [12]. Type 3: 0.12 % CHX was obtained from the Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand.

Cell cultures

Human gingival tissues were obtained from healthy donors following third wisdom tooth extraction or crown lengthening surgery with an informed consent form at the dental clinic of Chiang Mai University, Thailand. They were obtained for human gingival fibroblasts (HGFs) isolation. All protocols for this study were approved by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University, Thailand, project number 38/2021. The gingival tissues were washed 3 times with Dulbecco's modified

Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 2 % penicillin (10,000 U/mL)-streptomycin (10 mg/mL) (Gibco, Grand Island, NY, USA). They were cut into 1 - 3 mm^3 pieces, placed in 35-mm culture dishes filled with a complete medium (DMEM supplemented with 10 % fetal bovine serum [FBS; Gibco, Grand Island, NY, USA] and 1 % penicillin-streptomycin), and incubated at 37 °C in a humidified atmosphere containing 5 % CO_2. The complete medium was replaced with a fresh medium every 2 - 3 days until cells reached 80 % confluence. Cells were collected with 0.25 % trypsin-EDTA solution (Gibco, Grand Island, NY, USA). We selected HGFs between passages 3 and 6 for all experiments.

Determination of cytotoxicity by MTT assay

The cytotoxicity was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay. HGFs were seeded into 96-well plates at a density of 1×10^4 cells/well with a complete medium and incubated for 24 h at 37 °C in 5 % CO_2 to facilitate cell attachment. After incubation, cells were treated with various concentrations of 3 types of mouthwash using 2-fold serial dilution from 1:2 to 1:256 for 24 h. Cells treated with a complete medium were used as the control. Then, 5 mg/mL MTT (Sigma-Aldrich, MO, USA) was dissolved in phosphate buffer saline pH 7.4 (PBS; Sigma-Aldrich, MO, USA), and 50 µL of the dissolved MTT solution was added into each well for 3 h. At the end of this period, all solutions were removed, and 100 µL of Dimethyl sulfoxide (DMSO; Sigma-Aldrich, MO, USA) in ethanol (1:1 v/v) was added to each well for 15 min. The absorbance at 540 nm was measured by an ELISA microplate reader (TECAN Sunrise, Switzerland). The percentages of cell viability were calculated using the following equation:

$$\% \text{ Cell viability} = \frac{\text{Absorbance (sample)}}{\text{Absorbance (control)}} \times 100$$

IC_{50} (the concentration of mouthwash that caused 50 % cell viability) was used to determine the cytotoxic concentrations of 3 types of mouthwash, and the sub-IC_{50} concentration ($IC_{50}/2$) of each mouthwash was selected for further experimentation.

Measurement of nitric oxide production

HGFs and RAW 264.7 cells were used to investigate the effect of mouthwashes on nitric oxide production using Griess assay. HGFs and RAW 264.7 cells (1×10^5 cells/well) were seeded into 24-well plates with DMEM without phenol red supplemented with 10 % FBS and incubated for 24 h at 37 °C in 5 % CO_2. Subsequently, 2 types of cells were treated with 3 types of mouthwash at the sub-IC_{50} concentrations in the presence or absence of LPS (10 µg/mL) (LPS from *Escherichia coli* serotype 0127: B8, Sigma-Aldrich, MO, USA) for 24 h. As an indicator of nitric oxide production, the nitrite accumulation was measured in the culture supernatants by Griess reagent kit (G-7921, Invitrogen, USA) according to the manufacturer's instructions. The culture supernatants (150 µL) were transferred to the wells of the 96-well plate and mixed with 20 µL of Griess reagent and 130 µL of distilled water for 30 min at room temperature. Cells treated with a medium were used as the negative control. Cells treated with a medium and LPS (10 µg/mL) were used as the positive control. The absorbance at 540 nm was measured by an ELISA microplate reader (TECAN Sunrise, Switzerland), and the amount of nitrite was determined from the standard curve generated with sodium nitrite (0 - 1,000 µM).

Measurement of pro-inflammatory mediators' gene expression by real-time quantitative polymerase chain reaction (RT-qPCR)

HGFs were plated at a density of 1×10^5 cells/well into 6-well plates with DMEM without phenol red supplemented with 10 % FBS and incubated for 24 h at 37 °C in 5 % CO_2 and then treated with 3 types of mouthwash at the sub-IC_{50} concentrations in the presence or absence of LPS (10 µg/mL). Cells treated with a medium were used as the negative control. Cells treated with a medium and LPS (10 µg/mL) were used as the positive control. After treatment, total RNA was extracted by Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Piscataway, USA) according to the manufacturer's instructions. Total RNA concentrations and purity were verified at 260/280 nm using NanoDrop 2000 spectrophotometry (Thermo Fisher Scientific, MA, USA). Then, total RNA was reversed to cDNA using ReverTra Ace qPCR RT Master Mix with gDNA remover Kit (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. The gene expressions of COX-2, IL-1β, IL-6 (target genes) and GAPDH (housekeeping gene) were evaluated using the LightCycler 480 RT-qPCR system (Roche Applied Science, Penzberg, Germany).

The oligonucleotide primers were obtained from Bio Basic Inc (Toronto, Canada). The primer sequences of each gene are shown in **Table 1**. The protocols of PCR reaction consisted of preincubation at 95 °C for 5 min, according to 40 cycles of amplification. The amplification process consisted of denaturation at 95 °C for 5 s, annealing at 60 °C for 10 s, and extension at 72 °C for 20 s. The melting curve from 60 to 95 °C was utilized to confirm the specific products. The levels of the target genes were normalized by GAPDH expression and determined using the $2^{-\Delta\Delta Ct}$ method [15].

Table 1 Primer sequences used for RT-qPCR.

Primer name	Direction	Nucleotide Sequence (5´to 3´)
COX-2	Forward	CCC TTG GGT GTC AAA GGT AA
	Reverse	GCC CTC GCT TAT GAT CTG TC
IL-1β	Forward	GCA CGA TGC ACC TGT ACG AT
	Reverse	CAC CAA GCT TTT TTG CTG TGA GT
IL-6	Forward	GGT ACA TCC TCG ACG GCA TCT
	Reverse	GCC TCT TTG CTG CTT TCA C
GAPDH	Forward	AAA TCC CAT CAC CAT CTT CCA GGA GC
	Reverse	CAT GGT TCA CAA CCA TGA CGA ACA

COX-2 = Cyclooxygenase-2, IL-1β = Interleukin-1 beta, IL-6 = Interleukin-6, GAPDH = Glyceraldgehyde-3-phosphate dehydrogenase

Determination of cell migration by scratch assay

HGFs (2×10^4 cells/well, 24-well plates) were plated with a complete medium and incubated at 37 °C in 5 % CO_2 for 24 h until the cell monolayer reached 80 % confluence. The cell monolayer was scratched manually with a 200 μL sterile pipette tip to produce the wound area. Then, cells were washed 3 times with DMEM to remove detached cells and treated with mouthwash types at the sub-IC_{50} concentrations. Cells treated with a completed medium were used as the control. An inverted microscope (Leica DFC3000 G equipped with a CCD camera) and LAS X software (Leica Microsystems, Wetzlar, Germany) were used to record the images of cell migration (wound closure) every 12 for 36 h. The images were measured using ImageJ software (ImageJ bundled Java 1.8.0_172) and the percentages of wound closure were calculated using the following formula [16]:

$$\% \text{ Wound closure} = \frac{(\text{The wound area of 0 h} - \text{the wound area of x h})}{\text{The wound area of 0 h}} \times 100$$

Statistical analysis

The data were shown as mean ± standard deviation (SD). The statistical analysis of data was performed by IBM SPSS statistics software version 26.0 for Windows using one-way analysis of variance (ANOVA) followed by Tukey's HSD and Dunnett's test and applied for multiple comparisons at the $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ levels.

Results and discussion

Cytotoxicity of mouthwashes on HGFs

The cell viability of HGFs treated with various concentrations of 3 types of mouthwash using 2-fold serial dilution was measured by MTT assay, and IC_{50} was used to determine the cytotoxicity. The results showed that the IC_{50} of AM and CHX were 6 % (v/v) and 0.00375 %, respectively, while the IC_{50} of essential oils in CLAM were 0.05 % (v/v) cajuput essential oil and 0.025 % (v/v) lemongrass essential oil supplemented in alcohol-free mouthwash (**Figure 1**). The sub-IC_{50} concentrations of AM and CHX were 3 % (v/v) and 0.001875 %, respectively, whereas the sub-IC_{50} concentrations of essential oils in CLAM were 0.025 % (v/v) cajuput essential oil and 0.0125 % (v/v) lemongrass essential oil supplemented in alcohol-free mouthwash, which did not significantly affect the viability of HGFs as compared with the control

(**Figure 1**). Therefore, 3 types of mouthwash at the sub-IC_{50} concentrations were selected for further experiments.

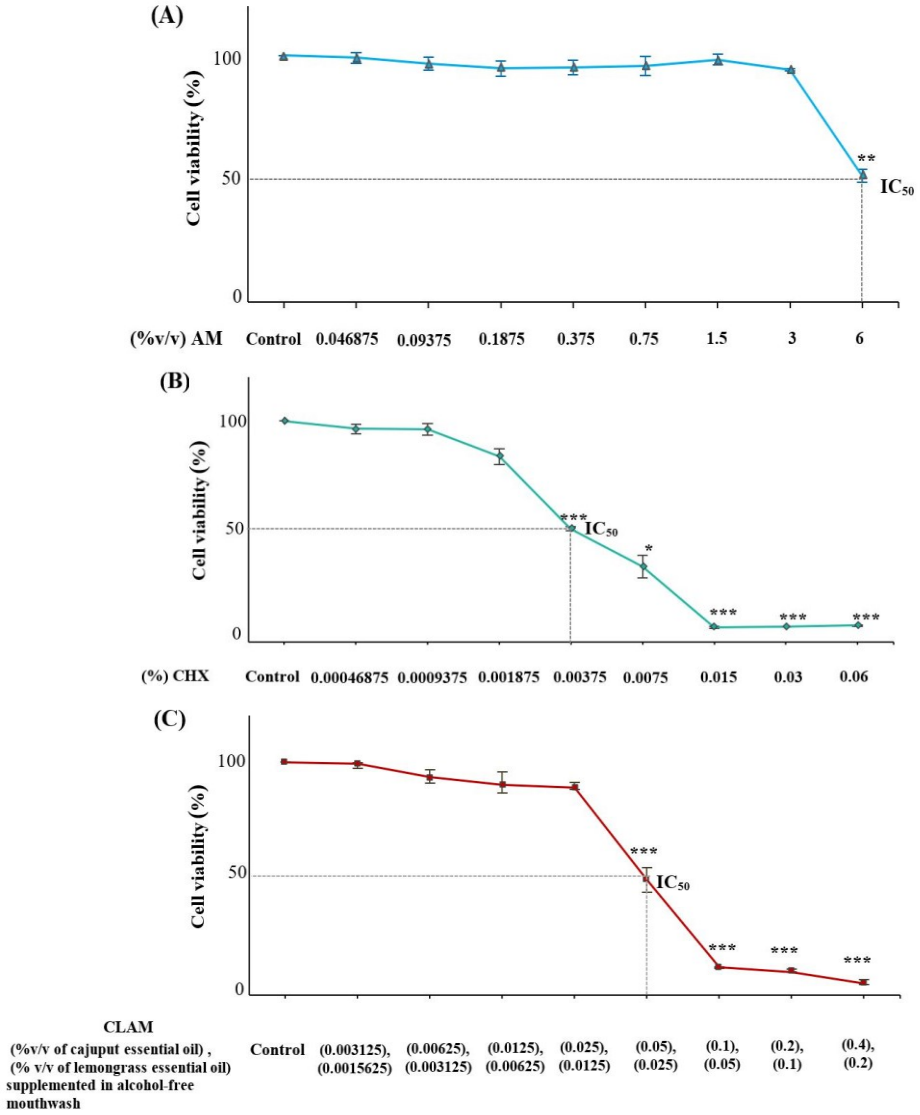

Figure 1 Cytotoxic effect of 3 types of mouthwash: A) AM; B) CLAM; and C) CHX at various concentrations using 2-fold dilution on HGFs, determined by MTT. IC_{50} was estimated from curves by plotting cell viability (%) versus the concentration of mouthwashes (% v/v). All data were expressed as the mean ± SD of 3 independent experiments. *, **, ***, significant difference at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively, after comparison with control group.

Effect of mouthwashes on LPS-activated nitric oxide production in HGFs and RAW 264.7 cells

The nitric oxide production in LPS-activated HGFs and RAW 264.7 cells treated with 3 types of mouthwash at the sub-IC_{50} concentrations was determined as nitrite production using the Griess assay. The results showed that LPS-activated RAW 264.7 cells (the positive control) released significantly higher nitric oxide levels compared with non-LPS-activated RAW 264.7 cells (the negative control) (**Figure 2(A)**). On the other hand, the nitric oxide production in LPS-activated HGFs was not significantly different from non-LPS-activated HGFs (**Figure 2(B)**). LPS-activated HGFs might not have a significant role in nitric oxide synthesis. Macrophages are well known to be the primary cells in the host inflammatory response, which can generate nitric oxide. This cellular response is induced by LPS, the outer membrane of Gram-negative bacteria, through toll-like receptor 4 (TLR4) on the cell membrane of macrophages [17]. Additionally, RAW 264.7 cells treated with LPS or LPS/IFN-γ can produce nitric oxide through iNOS [18], and are widely used as a model primary macrophage [19]. Therefore, we chose LPS-activated RAW 264.7

cells as a study model to investigate the anti-inflammatory activity of 3 types of mouthwash at the sub-IC_{50} concentrations by inhibiting nitric oxide production. As shown in **Figure 2(A)**, the sub-IC_{50} concentrations of CHX and essential oils in CLAM significantly inhibited LPS-activated nitric oxide production by 52.2 ± 4.99 % and 56.1 ± 4.06 %, respectively, compared with the positive control. Meanwhile, treatment with the sub-IC_{50} concentration of AM did not reduce LPS-activated nitric oxide production significantly, resulting in 90.9 ± 2.4 % compared with the positive control.

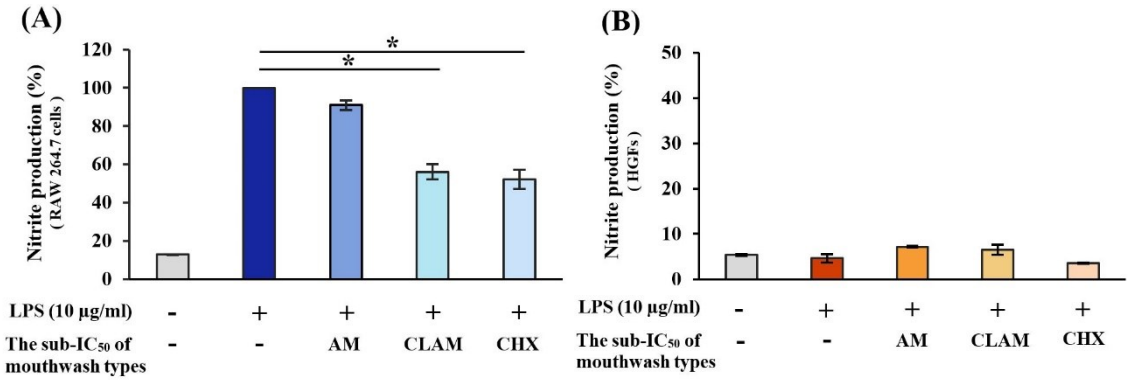

Figure 2 Effect of 3 types of mouthwash groups at the sub-IC_{50} concentrations on LPS-activated nitric oxide production in (A) RAW 264.7 cells and (B) HGFs. All data were expressed as the mean ± SD of 3 independent experiments. *, significant difference at $p \leq 0.05$ after comparison with the positive control.

Among the various pro-inflammatory mediators, nitric oxide has a vital role in inflammatory processes. Excessive amounts of nitric oxide synthesized by iNOS in macrophages are potentially detrimental as nitric oxide can stimulate the pro-inflammatory signaling to generate oxidative stress, causing cytotoxicity, tissue breakdown and chronic inflammation [20]. Subsequently, suppressing nitric oxide production from macrophages may be an important approach to preventing inflammatory disease.

Wisidsri *et al.* [21], determined that lemongrass essential oil has an anti-inflammatory effect in LPS-stimulated macrophages. Hong *et al.* [22], reported that lemongrass essential oil showed a potential inhibitory effect on nitric oxide production in LPS-activated macrophages. Consistent with these existing studies, the current findings presented that cajuput and lemongrass essential oils in CLAM inhibited the nitric oxide production in LPS-activated RAW 264.7 cells. Moreover, the nitric oxide inhibitory effect of these essential oils in CLAM was greater than that of AM. It showed that cajuput essential oil and lemongrass essential oil contained in CLAM predominantly expressed an anti-inflammatory property. Furthermore, a literature review described that nitric oxide had been linked with inflammatory processes. Our findings indicated the essential oils in CLAM effectively reduced LPS-activated inflammatory response in RAW 264.7 cells by inhibiting nitric oxide production, which may have a beneficial effect in preventing periodontal disease and other diseases associated with inflammation.

Effect of mouthwashes on pro-inflammatory mediators' gene expression in HGFs

Pro-inflammatory mediators, including IL-6, IL-1β and COX-2, are known to be regulated through the NF-κB signaling pathway [23,24]. These mediators are released from HGFs stimulated by LPS and cause damage in the surrounding gingival and periodontal tissues [25]. Consequently, we investigated how 3 types of mouthwash at the sub-IC_{50} concentrations affected the induction of pro-inflammatory mediators, including COX-2, IL-6 and IL-1β, in non-LPS and LPS-stimulated HGFs. To investigate the effects of 3 types of mouthwash at the sub-IC_{50} concentrations on COX-2, IL-6 and IL-1β, gene expression was determined by RT-qPCR. As shown in **Figure 4**, LPS stimulation at 10 µg/mL (the positive control) significantly elevated the gene expression of COX-2, IL-6 and IL-1β, compared with the control group without LPS (the negative control). In non-LPS-activated groups, the treatment with 3 types of mouthwash at the sub-IC_{50} concentrations did not significantly affect the gene expression of COX-2, IL-6 and IL-1β as compared with the negative control. In LPS-activated groups, the treatment with the sub-IC_{50} concentrations of CHX and essential oils in CLAM significantly inhibited the gene expression of COX-2, IL-6 and IL-1β, while the treatment with the sub-IC_{50} concentration of AM did not significantly reduce the gene expression of COX-2, IL-6 and IL-1β as compared with the positive control.

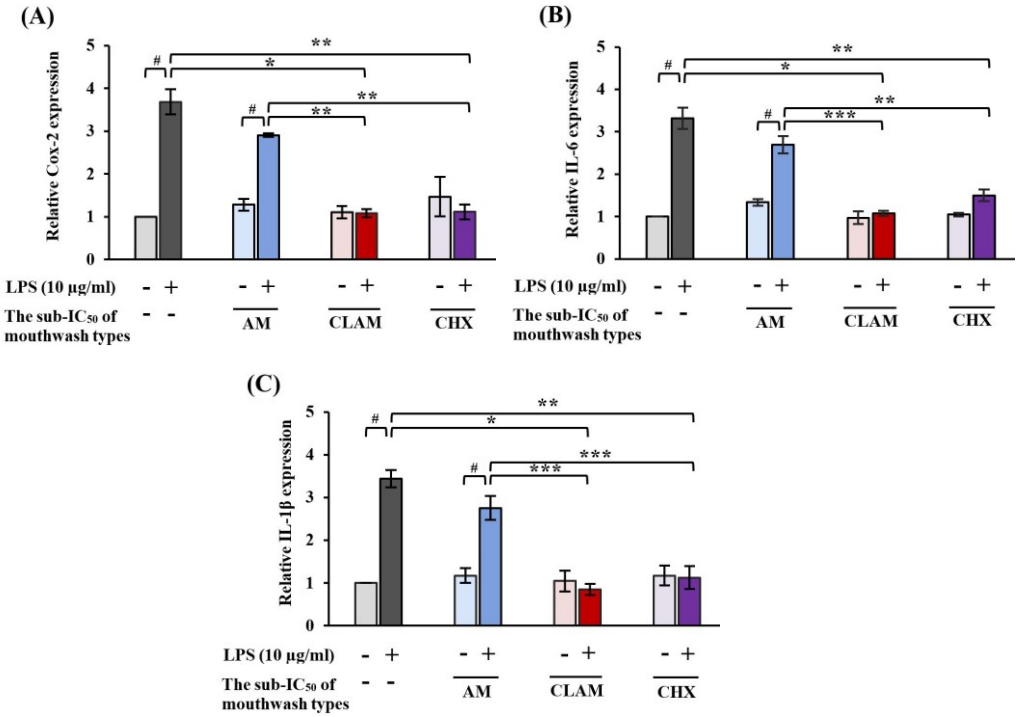

Figure 4 The effect of 3 types of mouthwash at the sub-IC_{50} concentrations on (A) COX-2, (B) IL-6, and (C) IL-1β gene expression in HGFs treated with and without LPS. All data were expressed as the mean ± SD of 3 independent experiments. $^{\#}$, significant difference at $p < 0.05$ after comparison with non-LPS-treated groups. *, **, ***, significant difference at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively, after comparison with LPS-treated groups.

Overall, these data demonstrated that the anti-inflammatory activity of the sub-IC_{50} concentrations of essential oils in CLAM on COX-2, IL-6 and IL-1β gene expression in LPS-activated HGFs was similar to that of the sub-IC_{50} concentration of CHX. It was more significant than the anti-inflammatory activity of the sub-IC_{50} concentration of AM. It revealed that the sub-IC_{50} concentrations of essential oils contained in CLAM were 0.025 % (v/v) cajuput essential oil and 0.0125 % (v/v) lemongrass essential oil, which expressed the greatest anti-inflammatory activity. The exact mechanism of the anti-inflammatory effect of cajuput and lemongrass essential oils is unclear. However, the anti-inflammatory properties of lemongrass essential oil and cajuput essential oil can be attributed to the presence of their main components, such as citral (neral and geranial) and terpinen-4-ol, respectively. Mitoshi *et al.* [26], reported that lemongrass essential oil elicited significant *in vivo* anti-allergic and anti-inflammatory effects. The finding of Boukhatem *et al.* [27], indicated that oral administration of lemongrass essential oil significantly reduced skin inflammation in a mouse model. Both reports investigated lemongrass essential oil with its main chemical composition, similar to that of Han *et al.* [28]. The report of Nogueira *et al.* [29], explored the essential oil compounds of *Melaleuca alternifolia*, an herb of the same family as cajuput, which possess an *in vitro* anti-inflammatory property, parallel to the report of Hart *et al.* [30]. In the present study, we expect that the chemical compositions of cajuput and lemongrass essential oils in CLAM are similar to those of the existing studies.

Analyzing the gene expression of COX-2, IL-6 and IL-1β, we can observe a significant reduction in the 3 genes when LPS-activated HGFs were treated with cajuput and lemongrass essential oils in CLAM. The anti-inflammatory properties of these essential oils were described, demonstrating that lemongrass essential oil inhibited the production of cytokines (IL-1β and IL-6) in LPS-activated macrophages of BALB/c mice [11], and *Melaleuca alternifolia*, an herb of the same family as cajuput, having the potential of reducing the production of *in vitro* IL-1β, IL-6 and IL-10 in LPS-activated monocytes [27], as confirmed by the results of the present study in relation to IL-6 and IL-1β. Consequently, it was estimated that cajuput essential oil and lemongrass essential oil in CLAM exerted anti-inflammatory abilities since these essential oils attenuated the expression of COX-2, IL-6 and IL-1β as an important target for preventing the inflammation conditions induced by bacterial pathogens.

The combination of herb-herb extract or drug-herb extract can lead to synergistic, additive and antagonistic effects. Satthanakul *et al.* [31], reported that the combination of lemongrass essential oil with CHX demonstrated a synergistic effect against *Candida alblicans*. The report of Ishijima *et al.* [32], found that the combination of 2 essential oils (lemongrass oil-perilla oil) and their chemical compositions (citral-perilladehyde) had synergistic antimicrobial activity. Some researchers suggested that the potent antimicrobial effect of lemongrass essential oil might result from the synergism among the monoterpenes (citral) and other components present in the essential oil [33]. Ortiz *et al.* [34], indicated that the interaction of naproxen and citral provided a significant synergistic effect on the anti-inflammatory property. It has been proposed that a synergistic interaction can be obtained from 2 essential oils or components with different but related complementary mechanisms. In the current study, our findings revealed that cajuput and lemongrass essential oils in CLAM had an anti-inflammatory effect. The combination of these essential oils in CLAM probably had synergistic anti-inflammatory activity. However, the exact mechanism of the anti-inflammatory property of this combination in CLAM should be further investigated.

Effect of mouthwashes on HGFs migration

Wound healing is a process of repairing soft tissues and skin after infection or injury, consisting of inflammatory, proliferative and remodeling phases [35]. The cell migration is one of the key steps during the proliferative phase that is responsible for wound healing [36]. The effects of the sub-IC_{50} concentrations of CHX and essential oils in CLAM on cell migration were carried out by scratch assay for 3 different time periods (12, 24 and 36 h). The results showed that the sub-IC_{50} concentrations of CHX and essential oils in CLAM increased the migration of HGFs, a significantly different effect from the non-treated cells in all periods. The completed wound closures were observed after 36 h of treatment with the sub-IC_{50} concentrations of CHX and essential oils in CLAM, while in the non-treated cells, they were not completed (**Figure 5**). It was revealed that wound closure could be induced after treatment with the sub-IC_{50} concentrations of CHX and essential oils in CLAM.

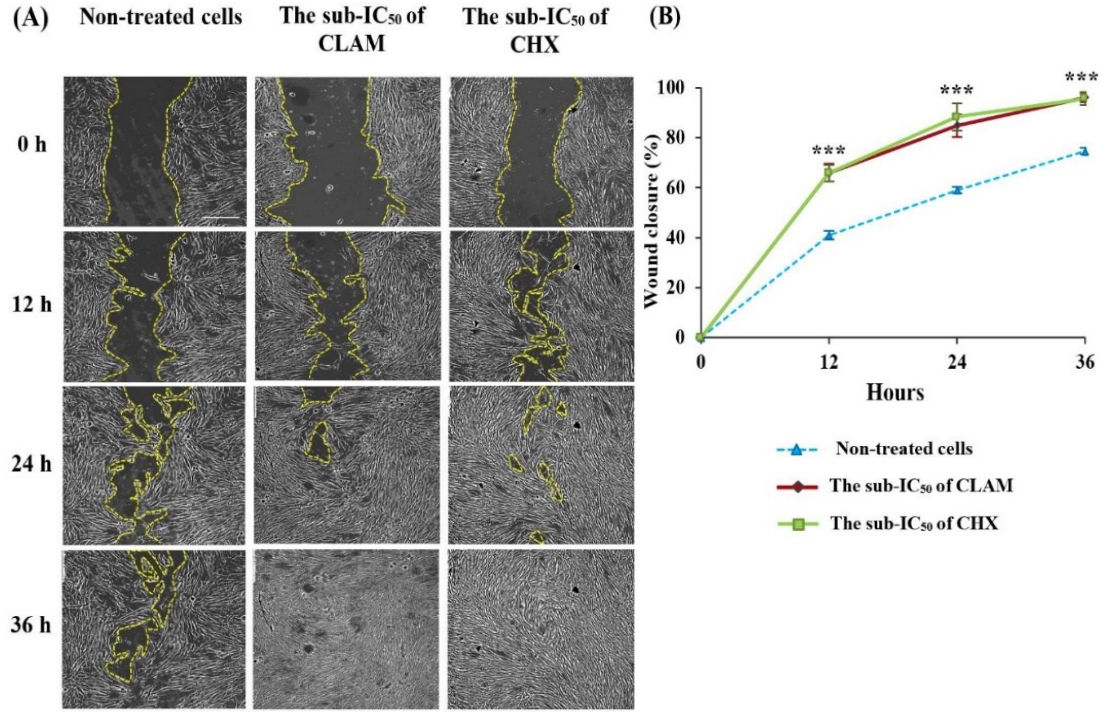

Figure 5 The effect of mouthwash types at the sub-IC_{50} concentrations on the migration of HGFs during 0, 12, 24 and 36 h was determined by scratch assay. A) Representative images of wound closure indicate cell migration in each period. Scale bar = 553.8 µm. B) % wound closure was calculated by the remaining cell-free area in each period, expressed as a percentage of the initial migrated area at 0 h. All data were expressed as the mean ± SD of 3 independent experiments. ***, significant difference at $p \leq 0.001$, compared with non-treated cells.

Oral tissue repair involves a complex reaction. Gingival fibroblasts, the most abundant cells in the connective tissue of the gingiva, contribute to gingival tissue hemostasis through their ability to generate new cells and remodel tissues. In the wound healing process, the inflammatory phase initiates the process, where neutrophils, macrophages and fibroblasts migrate into the wound. Then, the proliferative phase reveals an increase in fibroblasts. The remodeling phase is the final phase of the healing process, where the fibroblasts support the reconstruction of the extracellular matrix and deposit collagen, constituting newly formed granulation tissue [35,37]. Hence, fibroblasts play a crucial role in the healing of damaged tissue. In this scratch assay, it was observed that cajuput and lemongrass essential oils in CLAM significantly improved cell migration ability compared with the non-treated cells. Additionally, the promotion of cell migration ability of cajuput and lemongrass essential oils in CLAM was equal to that of CHX, suggesting that these essential oils in CLAM have a positive effect on wound healing.

The inflammatory reaction is implicated in wound healing, in which resident macrophages and fibroblasts produce the inflammatory mediators to prevent infection. However, prolonged inflammatory response contributes to tissue damage and impairs wound healing. Thus, anti-inflammation is essential in recruiting fibroblasts, which migrate to the lesion site and initiate the proliferative phase for accelerating wound healing [38].

The wound healing properties of cajuput and lemongrass essential oils can be best illustrated by their antimicrobial and anti-inflammatory activities. Several investigators suggested that essential oil extracted from lemongrass suppressed the production of inflammatory mediators, including IL-6, IL-1β [11], and nitric oxide [21,22], downregulating the LPS-induced COX-2 expression [39]. The previous study described that both cajuput and lemongrass essential oils formulated in alcohol-free mouthwash had a strong inhibitory effect against *Candida albicans* [13]. The results of this study showed that the cajuput and lemongrass essential oils in CLAM inhibited the production of nitric oxide and suppressed the gene expression of COX-2, IL-6 and IL-1β. It is also possible that cajuput and lemongrass essential oils in CLAM improved healing by downregulating the expression of inflammatory mediators, which are mentioned to be crucial to cell migration and proliferation [40].

Additionally, keratinocytes are the predominant cells of re-epithelization in the wound-healing process, where they proliferate and migrate to cover the wound [41]. Interestingly, our further study should point to the effect of cajuput and lemongrass essential oils in CLAM on the migration and proliferation of keratinocytes and their wound healing *in vivo*.

Conclusions

Based on the findings in this study, we conclude that 0.025 % (v/v) cajuput essential oil and 0.0125 % (v/v) lemongrass essential oil supplemented in alcohol-free mouthwash exhibited anti-inflammatory effects by inhibiting the nitric oxide production in LPS-activated RAW 264.7 cells and reducing COX-2, IL-6 and IL-1β expression in LPS-activated HGFs, as well as promoting oral wound healing. It is acknowledged that adding anti-inflammatory and antioxidant molecules in natural herb extracts to oral hygiene products can relieve the progression of periodontal disease [42]. Thus, this mouthwash formula may provide useful options for preventing periodontal disease. Nevertheless, this anti-inflammatory effect should be further researched *in vitro*, including inhibiting NF-κB expression, and *in vivo* studies to understand the precise mechanisms of the anti-inflammatory effect.

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