Research Article

Nanoemulsion-based mouthwash with *Litsea cubeba* essential oil and *Piper betle* extract for inflammatory dental condition

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Abstract

A nanoemulsion-based mouthwash incorporating *Litsea cubeba* essential oil and *Piper betle* extract was developed for inflammatory dental conditions. The optimized formulation, identified using pseudo-ternary phase diagrams, contained 0.9% essential oil and 1% dry extract, yielding droplet sizes of 22.36 nm, a near-neutral zeta potential (−0.50 mV), and a pH of 7.2. The formulation demonstrated physicochemical stability over a three-month period. Anti-inflammatory activity, evaluated through nitric oxide inhibition in lipopolysaccharide-stimulated RAW 264.7 macrophages, surpassed that of 0.12% chlorhexidine. In antibacterial assays, the formulation achieved a 99.74% reduction of *Streptococcus mutans* in a time-kill test. Molecular docking analysis further supported the antimicrobial potential of citral and eugenol via interaction with *Streptococcus mutans* glucosyltransferase B. These findings suggest that the developed nanoemulsion-based mouthwash may offer a promising natural approach for managing oral inflammatory conditions.

Keywords

Introduction

Oral health is an integral part of general health, with conditions such as dental caries and periodontal diseases being among the most common global health issues (Booth et al. 2024). These diseases are predominantly caused by bacterial colonization, especially by *Streptococcus mutans (S. mutans)*, and are often exacerbated by inflammation of the gingival tissues (Hamman et al. 2024; Kodgi et al. 2024). The standard treatment includes the use of chemical-based mouthwashes, such as chlorhexidine digluconate (CHX) 0.12%, which has demonstrated high antibacterial efficacy (Motallaei et al. 2021; Zuttion et al. 2024). However, prolonged use of CHX is associated with undesirable side effects, including tooth discoloration, taste alteration, and mucosal irritation, prompting the search

for safer and more sustainable alternatives (Drugs and Lactation Database 2024; Simmons et al. 2024).

Natural plant-based bioactives have gained increasing interest for their potential in human health care due to their multifaceted biological activities and favorable safety profiles (Shinkai et al. 2024). The increasing demand for natural oral care products has driven extensive research into herbal and plant-based mouthwashes (Rajendiran et al. 2021; Tidke et al. 2022; Wang et al. 2023). Several studies have demonstrated the potential of natural bioactives in oral care (Cai et al. 2020; Chatzopoulos et al. 2022). Herbal mouthwashes containing essential oils or plant extracts, such as *Fructus mume*, *Salvadora persica*, *Zingiber officinale*, *Azadirachta indica*, and *Matricaria chamomilla*, have demonstrated efficacy in reducing gingival inflammation compared to placebos, though their effects on plaque reduction remain inconsistent. While some formulations, like *Persica* and *Azadirachta indica*, significantly reduced *S. mutans* colony counts, others, such as *Zingiber officinale*, showed only short-term effects. Leiva-Cala et al. demonstrated a 41.4% reduction in inflammation with *Aloe vera* gel, outperforming chlorhexidine, while Kamath et al. observed a greater decrease in Gingival Index (0.64 vs. 0.54) and Bleeding on Probing (23.7 vs. 29.2) in the *Aloe vera* group (Cai et al. 2020). Goes et al. reported a 25.6% reduction in Visible Plaque Index and a 29.9% decrease in Gingival Bleeding Index with *Matricaria chamomilla* mouthwash, compared to 39.9% and 32.0%, respectively, with chlorhexidine (Talpos Niculescu et al. 2024). Yeturu et al. noted a plaque reduction of 20.38% with *Aloe vera* and 31.59% with chlorhexidine, while gingival scores improved by 9.88% and 16.30%, respectively (Goes at al. 2016). Atwa et al. highlighted honey's strong antibacterial effect, reducing *S. mutans* counts from 255.6 CFU to 104.4 CFU, alongside pH modulation from 6.85 to 5.86 before recovery to 6.84 (Cai et al. 2020). Lastly, Golshah et al. observed a reduction in Gingival Index from 1.00 to 0.77 over 8 weeks with a 2% resveratrol emulgel, demonstrating its efficacy in managing gingivitis (Cai et al. 2020). Sung-Ho Lee et al. (2021) evaluated the antibacterial and anti-inflammatory effects of natural extracts and mixtures using 11 pathogenic oral bacteria, 2 nonpathogenic bacteria, and RAW 264.7 macrophages. The natural mixtures demonstrated superior antibacterial and anti-inflammatory effects compared to individual extracts or chemical mouthwashes, likely due to the synergistic combination of components. However, the study lacked evidence linking specific extracts to their effects within the mixtures. Further research is needed to elucidate the composition-effect relationship and mechanisms underlying microbial suppression (Yeturu et al. 2016; Lee et al. 2021).

Among the many promising botanicals, **Litsea cubeba** essential oil and *Piper betle* leaf extract have attracted research interest for their potent antimicrobial, anti-inflammatory, and antioxidant properties (Kamle et al. 2019; Nayaka et al. 2021; Wang et al. 2022). Essential oil

content in *Litsea cubeba* fruits varies depending on geographic origin and variety, ranging from 1.79% to 4.79% in samples from China (Si et al. 2012; Fan et al. 2023). Across multiple studies, citral-comprising geranial (E-citral) and neral (Z-citral)-was consistently identified as the dominant component, accounting for 51–72% of total oil content. Other major constituents include D-limonene (10–18.8%), citronellal (~14%), linalool, and 4-methyl-3-pentenal (Yang et al. 2014; Wang et al. 2018; Hung et al. 2023). The chemical profile of *Litsea cubeba* essential oil is primarily composed of monoterpenes and sesquiterpenes, and demonstrates significant regional variation (Gao et al. 2016). These findings suggest that citral-rich *Litsea cubeba* essential oil holds strong potential for applications in pharmaceuticals and aromatherapy due to its antimicrobial and anti-inflammatory properties. Meanwhile, *Piper betle* leaves have been widely investigated for their rich phytochemical profile and associated pharmacological potentials. Numerous studies have confirmed the presence of various bioactive compounds including phenolics, flavonoids, terpenes, alkaloids, saponins, tannins, steroids, and organic acids. Among these, hydroxychavicol is consistently reported as the predominant constituent, with relative abundances ranging from 66.55% to 69.46%, followed by eugenol (4.86%-11.92%–20.37%), 4-chromanol (24.0%–27.81%), isoeugenol (~2.90%), and chavicol (~3.2%). Minor compounds such as 4-allyl-1,2-diacetoxybenzene (0.76%–3.21%), neophytadiene, elemicin, and propionic acid have also been identified, particularly in ethanolic and aqueous extracts (Deshpande and Kadam 2013; Venkadeswaran et al. 2016). To sum up, *Litsea cubeba* essential oil, rich in citral, has demonstrated remarkable antimicrobial activity (Si et al. 2012; Fan et al. 2023; Liu et al. 2024) while *P. betle* extract, abundant in phenolic compounds, is well-documented for its antibacterial, anti-inflammatory, and antioxidant effects (Alam et al. 2013; Ali et al. 2018). Both have shown efficacy against *S. mutans* and in reducing gingival inflammation in preclinical studies (Rahim et al. 2011; Okonogi et al. 2021; Songsang et al. 2022).

However, the clinical utility of these plant bioactives is often limited by their hydrophobic nature, volatility, poor water solubility, and instability in conventional formulations, which reduce their bioavailability and efficacy (Yeturu et al. 2016). Moreover, challenges such as unpleasant taste, odor, and limited shelf-life can affect patient compliance. Addressing these drawbacks necessitates formulating strategies that enhance stability, solubility, and targeted delivery of natural compounds in the oral cavity.

Nanoemulsion-based delivery systems offer a promising solution by encapsulating lipophilic bioactives within nanoscale droplets (<200 nm), thereby improving solubilization, chemical stability, and bioavailability (Tenjarla 1999; Çağlar et al. 2023). The reduced droplet size increases surface area and facilitates better interaction with microbial membranes, enhancing antimicrobial efficacy (Zarenezhad et al. 2021). The choice of excipients is

critical; surfactants like Cremophor RH40 and cosurfactants such as PEG 400 are selected for their ability to form kinetically stable nanoemulsions with low polydispersity, minimal mucosal irritation, and favorable biocompatibility suitable for oral applications (Lakyat et al. 2023). These components improve formulation clarity, viscosity, and mucoadhesion, which promote prolonged retention and uniform distribution in the oral cavity, essential for effective mouthwash performance.

Therefore, this study was designed to develop and characterize a nanoemulsion-based herbal mouthwash incorporating *Litsea cubeba* essential oil and *Piper betle* dry extract, aiming to optimize their antimicrobial and anti-inflammatory efficacy. The study also evaluated the antibacterial activity and anti-inflammatory potential of the formulation, comparing it to 0.12% chlorhexidine digluconate mouthwash, a standard in oral care. For the first time, molecular docking analysis of key bioactive compounds, including citral and eugenol, is performed to explore their interaction with *S. mutans*. To this end, by addressing the solubility, stability, and efficacy limitations of natural products, this research aims to provide a scientifically validated, patient-friendly alternative to conventional chemical mouthwashes, contributing to the development of safer and more effective oral health solutions.

Materials and methods

Materials

Fresh *Piper betle* leaves, harvested at twelve months of maturity, were collected from a tropical region in March 2024. The botanical identity was authenticated through morphological and DNA analysis. After washing, the leaves were dried at 50 °C to a final moisture content of 5.9%, finely milled, and stored at room temperature until further analysis. DNA sequencing was performed by an independent laboratory to confirm species identity. *Litsea cubeba* essential oil, characterized by a high citral content, was obtained from a certified supplier specializing in medicinal plant extracts.

Standard citral (geranial and neral) and eugenol (purity > 99%) were procured from Sigma-Aldrich (Singapore). Excipients, including polyethylene glycol (PEG) 400 from B.L. Hua & Co. Ltd. (Thailand), and Cremophor RH 40 (PEG-40 hydrogenated castor oil) from BASF (Germany), were used in the formulation. Isopropyl myristat was obtained from 3C Cosmetic (Vietnam). Levofloxacin and other reagents of analytical grade or higher were supplied by Sigma-Aldrich Chemical Co. (United States of America, USA). All materials and chemicals were carefully selected to ensure quality and reproducibility in the study.

A 0.12% chlorhexidine gluconate mouthwash (Kin Gingival, Kin Laboratories, Barcelona, Spain) was used as the reference formulation for anti-inflammatory and antibacterial assessments in this study.

Herbal preparation and characterization

Piper betle leaf extract was obtained by extracting *Piper betle* leaf powder (300 g per batch, n = 3) with 1.8 L of 80% ethanol (v/v) at 70 °C for 6 hours. The extracts were then cooled at 5 °C for 24 hours, filtered, and concentrated using a water bath (PharmaTest, Germany) at 60 °C. To further remove residual solvent, the concentrated extracts were dried in a vacuum cabinet at 60 °C before storage at 18 °C for subsequent experiments (Fig. 1). The percentage of extraction yield was calculated by equation (1).

$$\%Yield = \frac{\text{The mass of dry extract} \times (1\text{-}\%\text{Humidity of dry extract})}{\text{The mass of dry plant material} \times (1\text{-}\%\text{Humidity of dry plant})} \times 100$$

Litsea cubeba essential oil was obtained from Viet Nam Medicine Joint Stock Company. Prior to its incorporation into the mouthwash formulation, the essential oil was analyzed to verify that the citral content met the minimum requirement of 40%, ensuring its suitability for inclusion in the formulation.

The major constituents of *Piper betle* dry extract and *Litsea cubeba* essential oil were identified and quantified using gas chromatography–mass spectrometry (GC-MS) on a QP2010 system (Shimadzu, Japan) (Madhumita et al. 2019). A DB-5MS UI column (30 m × 0.25 mm) was utilized, with helium as the carrier gas at a flow rate of 0.76 mL/min. The injection temperature was set at 280 °C, and the mass spectrometer operated at an ionization temperature of 220 °C. The temperature program included an initial hold at 70 °C for 2 minutes, followed by a ramp to 180 °C at 10 °C/min (held for 0.5 minutes), and a final increase to 270 °C at 30 °C/min. The mass spectrometer recorded signals in the 15–500 m/z range. The identification of the essential oil was performed by Catech Center using validated analytical method to ensure the quality and authenticity of the material.

Quantitative analysis confirmed eugenol as the principal component of *Piper betle* dry extract and citral (geranial and neral) as the predominant compounds in *Litsea cubeba* essential oil, verifying their chemical integrity and suitability for formulation development (Liu et al. 2023).

Determination of minimum inhibitory concentration (MIC)

The antimicrobial activity of *Piper betle* dry extract and *Litsea cubeba* essential oil was evaluated using the agar dilution method. Serial dilutions of the *Piper betle* dry extract (5.00, 2.50, 1.25, 0.63, 0.32, 0.16, and 0.08 mg/mL) were prepared in dimethyl sulfoxide (DMSO). Similarly, serial dilutions of *L. cubeba* essential oil (2.00%, 1.00%, 0.50%, 0.25%, 0.125%, 0.063%, and 0.032% v/v) were prepared in DMSO supplemented with 0.05% (v/v) Tween 20 to enhance solubility. Each dilution was incorporated into molten Mueller-Hinton agar cooled to 50 °C before plating. Standardized bacterial
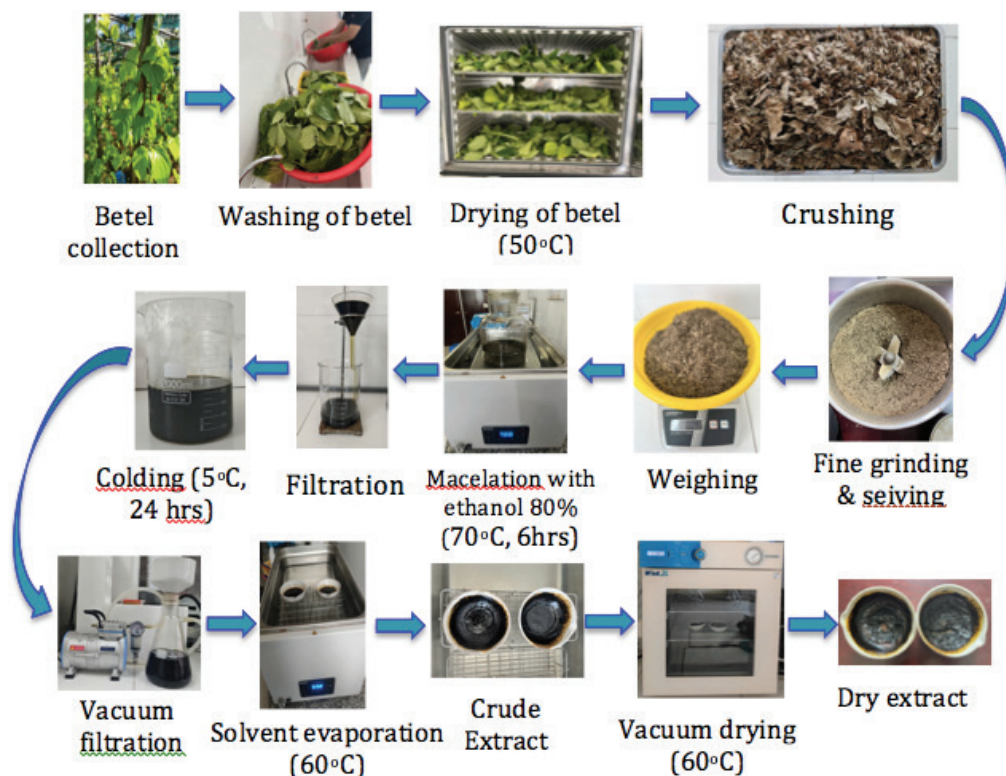
Figure 1. *Piper betle* extract preparation and extraction process.

suspensions (McFarland 0.5) were inoculated onto the agar plates (1–2 μL per spot), followed by drying at room temperature for 15 minutes and incubation at 37 °C for 18 hours. Negative control plates containing uninoculated medium were included to confirm the absence of contamination, while positive control plates containing untreated agar were used to confirm bacterial viability.

The MIC was defined as the lowest concentration at which no visible bacterial growth was observed. This method ensured high reliability and reproducibility in assessing the antimicrobial efficacy of the tested compounds (Michael et al. 2020).

Nanoemulsion-based mouthwash preparation

Phase diagram construction

Based on prior research, isopropyl myristate (IPM), Cremophor RH 40, and PEG 400 were selected as the oil phase, surfactant, and co-surfactant, respectively (Nguyen et al. 2024). Pseudo-ternary phase diagrams were constructed using Chemix School 7.0 software and the water titration method under controlled stirring at room temperature. Surfactant and co-surfactant mixture (Smix) and IPM were prepared at weight ratios ranging from 9:1 to 1:9, followed by incremental water addition. The resulting dispersions were classified as nanoemulsions, emulsions, or gels based on optical clarity and homogeneity. Stable nanoemulsion regions

were identified and mapped, facilitating the selection of formulations with optimal solubility, stability, and potential bioavailability for *Litsea cubeba* essential oil (Nguyen et al. 2024).

Nanoemulsion-based mouthwash formulation

Nanoemulsion formulations incorporating *Litsea cubeba* essential oil and *Piper betle* extract were developed based on phase diagram analysis. *Litsea cubeba* essential oil (0.6%, 1.2%, 1.8%, or 2.4% w/v) was dissolved in the oil phase, followed by the addition of Smix and water in appropriate proportions. The mixture of *Piper betle* dry extract (2% or 4% w/v) in Cremophor RH 40 was then incorporated under continuous magnetic stirring for 15 minutes to form a stable nanoemulsion.

The final mouthwash formulation was prepared by blending the nanoemulsion with the solution of other excipients (Fig. 2), including xylitol (20% w/v), aspartame (2% w/v), glycerin (10% w/v), sodium bicarbonate (1% w/v), strawberry flavor (2% w/v), and distilled water (q.s. to 100 mL). This mixture was combined with 100 mL of the prepared nanoemulsion, yielding final concentrations of *Litsea cubeba* essential oil (0.3%, 0.6%, 0.9%, or 1.2% w/v) and *Piper betle* dry extract (1% or 2% w/v). The compositions of formulations F1–F8, calculated for the preparation of 100 mL of mouthwash, are presented in Table 1.

Eight formulations were evaluated for physicochemical properties, including pH, transmittance, precipitation, and stability. The irritation potential was assessed using
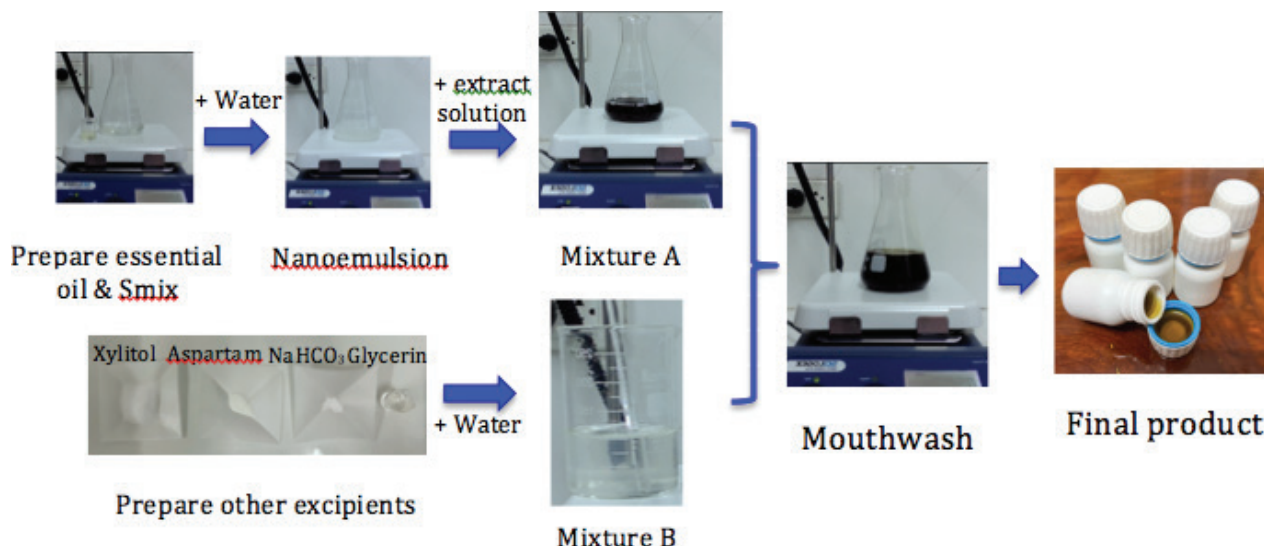
Figure 2. Mouthwash preparation process.

Table 1. The compositions of formulations F1–F8.

Composition	F1	F2	F3	F4	F5	F6	F7	F8
Litsea cubeba essential oil (g)	0.3	0.3	0.6	0.6	0.9	0.9	1.2	1.2
Piper betle dry extract (g)	1	2	1	2	1	2	1	2
IPM (g)	2	2	2	2	2	2	2	2
Smix (Cremophor RH40: PEG 400) (g)	20	20	20	20	20	20	20	20
Cremophor RH40 (g)	4	4	4	4	4	4	4	4
Xylitol (g)	10	10	10	10	10	10	10	10
Aspartame (g)	1	1	1	1	1	1	1	1
Glycerin (g)	5	5	5	5	5	5	5	5
Sodium bicarbonate (g)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Strawberry flavor (g)	1	1	1	1	1	1	1	1
Distilled water	q.s. to 100 mL							

the Hen's Egg Test—Chorioallantoic Membrane (HET-CAM) assay (Gilleron et al. 1996), following the standard protocol [40] and described in the next section. The optimal formulation was selected based on achieving a balance between the concentration of active ingredients and the potential for mucosal irritation.

Characterization of nanoemulsion-based mouthwash

The final formulation was evaluated for visual appearance, pH, droplet size, polydispersity index (PI), zeta potential, thermodynamic stability, and physicochemical stability.

Visual appearance and pH

Color and transparency were classified into four levels: (+) opaque, (++) translucent, (+++) transparent, and (++++) water-clear. pH was measured using a calibrated pH meter with standard buffer solutions (pH 4.0, 7.0, and 10.0) (Peeran et al. 2024).

Droplet size, PI, and Zeta Potential

Dynamic light scattering analysis (Zetasizer Nano ZS, Malvern, UK) determined droplet size and PI based on the Stokes-Einstein equation. Zeta potential was measured via phase-analysis light scattering to assess colloidal stability (Yeturu et al. 2016). Measurements were conducted in deionized water under standard conditions.

Thermodynamic stability

Stability was evaluated via centrifugation (3000 rpm, 15 min) and six heating-cooling cycles (4 °C to 45 °C, 48 h per cycle), followed by visual inspection for phase separation (Peeran et al. 2024).

Physicochemical stability

The final nanoemulsion-based mouthwash formulation was stored at room temperature for three months, with evaluations at 0, 1, and 3 months. Appearance and bioactive compound content (eugenol and citral) were analyzed using GC–MS. A standard mixture containing 100 ppm citral and 10 ppm eugenol was used for calibration. Deviations greater than 10% in compound content were considered indicative of instability (Yeturu et al. 2016).

Irritation assessment

Mucosal compatibility was evaluated via the HET-CAM assay. Nine fertilized chicken eggs were incubated at 37 ± 0.5 °C and 65 ± 1% RH for 8 days. Samples (300 μL) were applied to the exposed chorioallantoic membrane, with vascular responses (coagulation, lysis, hemorrhage) recorded at 0 s, 30 s, 120 s, and 300 s. The sample irritation scores were determined based on Table 2, and the irritation scores were classified as none (<1), slight (<5), moderate (<9), or severe (9–21) (Gilleron et al. 1996).

Table 2. Scoring system for evaluating irritation potential in the hen's egg chorioallantoic membrane test.

Effect	Score		
	30 s (0.5 min)	120 s (2 min)	300 s (5 min)
Vascular lysis	5	3	1
Hemorrhage	7	5	3
Coagulation	9	7	5

Cytotoxicity assessment

The cytotoxicity of the nanoemulsion-based mouthwash was evaluated using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay in RAW 264.7 macrophages (ATCC-TIB-71) (Johan van Meerloo et al. 2011). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotics under standard conditions (37 °C, 5% CO_2).

Cells (30,000/well) were seeded in 96-well plates and incubated for 24 hours before exposure to serial dilutions of the mouthwash (16–256 µg/mL) for 4 hours. After treatment, cells were washed with phosphate-buffered saline, incubated with MTT (5 mg/mL, 20 µL/well) for 4 hours, and the resulting formazan was dissolved in DMSO (100 µL/well). Absorbance was measured at 540 nm using a microplate reader (Varioskan, Thermo Fisher, USA). Viability was calculated relative to untreated controls, ensuring biocompatibility for further anti-inflammatory testing.

In vitro *anti-inflammatory evaluation*

The anti-inflammatory potential was assessed by measuring nitric oxide (NO) inhibition in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Three samples were included in the study: a blank formulation prepared following the composition and process described in Table 1 but excluding the dry extract and essential oil; a nanoemulsion-based formulation containing *Piper betle* extract and *Litsea cubeba* essential oil; and a commercial 0.12% chlorhexidine mouthwash used as a reference control.

Cells (20,000/well) were seeded in 96-well plates, serum-starved for 3 hours, and treated with test formulations for 2 hours before LPS (1 µg/mL) stimulation for 24 hours. Supernatants were collected and reacted with Griess reagent, and absorbance was measured at 540 nm. NO inhibition was quantified using a sodium nitrite standard curve, and IC_{50} values were determined via nonlinear regression. L-NG-Monomethylarginine, Acetate Salt (L-NMMA) was used as a reference inhibitor (Johan van Meerloo et al. 2011). This approach provided a comparative evaluation of the formulation's anti-inflammatory efficacy.

Antibacterial activity assessment

Bactericidal activity

Antimicrobial testing was conducted in accordance with CLSI guidelines (M100, 31st edition). Bacterial suspensions (10^8 CFU/mL) were prepared in Mueller-Hinton broth for *E. coli* and *S. aureus*, and brain heart infusion broth for *S. mutans*. Each suspension (20 µL) was mixed with 2 mL of nanoemulsion-based mouthwash or 0.12% chlorhexidine mouthwash and incubated for 30 seconds at room temperature. Residual bacterial counts were determined by plating onto Mueller-Hinton agar or brain heart infusion agar. A negative control (0.9% NaCl) was included. Experiments were performed in triplicate.

Molecular docking of citral and eugenol

Molecular docking simulations were conducted using MOE 2019 to evaluate interactions between key mouthwash components (geranial, neral, eugenol, chlorhexidine) and *S. mutans* gtfB (PDB: 8FKL) (Karnjana et al. 2023). The triangle-based docking algorithm was used, generating up to 500 solutions per iteration. Docking scores, root-mean-square deviation (RMSD), and interaction types were analyzed to assess binding affinity and antibacterial potential.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical comparisons were performed using the Student's t-test for two-group analyses and one-way ANOVA for multiple comparisons. A p-value < 0.05 was considered statistically significant.

Results and discussion

Plant extraction and chemical quantification

Piper betle leaves were extracted using 80% ethanol at 70 °C for 6 hours, yielding a dark brown extract with a characteristic aroma. The extraction yield was 6.15 ± 0.25% (w/w). Quantitative analysis revealed that the eugenol content in the dry extract was 104 ± 2 mg/g, consistent with previous reports indicating the effectiveness of ethanol in extracting phenolic compounds from plant materials (Deshpande and Kadam 2013; Singh et al. 2016; Venkadeswaran et al. 2016; Purba et al. 2019).

Litsea cubeba essential oil, obtained from Viet Nam Medicine Joint Stock Company, was analyzed using gas chromatography–mass spectrometry (GC-MS) prior to its incorporation into the mouthwash formulation. The primary constituents of *Litsea cubeba* essential oil were identified as citral (73.00%), D-limonene (4.62%), (R)-(+)-citronellal (3.72%), β-linalool (3.26%), and 6-methyl-5-hepten-2-one (3.30%). Detailed composition data are presented in Table 3 and Suppl. material 1. The high citral content is consistent with previous studies that have highlighted its notable antimicrobial and anti-inflammatory properties (Dalimunthe et al. 2021; Fan et al. 2023; Liu et al. 2023). Quantitative analysis using a calibration curve (citral standard at 100 ppm) confirmed a total citral content of 49.6% (Suppl. material 1), ensuring consistency in both formulation and expected biological activity.

Table 3. GC-MS analysis of the *Litsea cubeba* essential oil.

Peak No.	Retention time (min)	Area	Area (%)	Compound name
1	5.126	618,912	1.01	sec-Butyl Acetoacetate
2	5.593	158,619	0.26	β-Phellandrene
3	5.700	2,015,925	3.30	6-Methyl-5-hepten-2-one
4	5.885	78,676	0.13	2,3-Dehydro-1,8-cineole
5	6.551	2,820,580	4.62	D-Limonene
6	6.633	49,252	0.08	Eucalyptol
7	7.255	1,003,388	1.64	trans-Linalool Oxide
8	7.515	73,760	0.12	cis-Linalool Oxide
9	7.696	1,992,877	3.26	β-Linalool
10	8.582	2,272,169	3.72	(R)-(+)-Citronellal
11	8.725	171,019	0.28	cis-Verbenol
12	9.033	285,273	0.47	Carane, 4,5-epoxy-, trans-
13	9.161	251,808	0.41	5-Isopropyl-2-methylbicyclo[3.1.0]hexan-2-ol
14	9.383	159,997	0.26	α-Terpineol
15	9.780	904,205	1.48	β-Citronellol
16	10.024	18,434,314	30.16	β-Citral
17	10.163	1,068,202	1.75	trans-Geraniol
18	10.353	153,906	0.25	1-Carvomenthenone
19	10.480	25,281,187	42.84	α-Citral
20	10.704	563,181	0.92	Epoxy-linalool oxide
21	10.900	147,931	0.25	3,7-Dimethyl-2,6-octadien-1-ol
22	11.005	198,590	0.32	Citronellic acid
23	11.182	156,664	0.26	Neric acid
24	11.501	384,600	0.63	2,7-Dimethyl-2,7-octanediol
25	11.656	664,299	1.09	Geranic acid
26	12.028	749,324	1.22	2,3-Dimethylcyclohexanol
Total		**61,112,561**	**100.00**	

These extracts were selected for nanoemulsion formulation, with eugenol and citral serving as key quantitative markers for quality control.

Minimum inhibitory concentration determination

The MICs of *Piper betle* dry extract and *Litsea cubeba* essential oil against *S. aureus*, *E.coli*, and *S. mutans* were assessed following CLSI guideline (M07-ed11) using the agar dilution method (Clinical and Laboratory Standards Institute 2018). MIC results revealed that the essential oil exhibited strong inhibitory effects, particularly against *E. coli* (MIC = 0.125% w/v) and *S. aureus* (MIC = 0.25% w/v), while showing moderate activity against *S. mutans* (MIC = 0.5% w/v). In contrast, the *P. betle* extract demonstrated a more selective profile, with potent inhibition of *E. coli* (MIC = 0.32 mg/mL), modest activity against *S. aureus* (MIC = 5 mg/mL), and negligible effect on *S. mutans* (MIC > 5 mg/mL) (Fig. 3, Table 4). Images showing the determination of the MICs of the essential oil and the dry extract against the tested bacterial strains are presented in Suppl. material 2.

The findings suggest that *Litsea cubeba* essential oil exhibits broad-spectrum antimicrobial activity, which can be attributed to its high content of bioactive monoterpenes such as citral and limonene. Previous studies have demonstrated that *Litsea cubeba* essential oil disrupts bacterial cell membranes and interferes with energy metabolism, which contributes to its low minimum inhibitory concentration values against both Gram-positive and Gram-negative bacteria (Lv et al. 2011). Its relatively lower efficacy against *Streptococcus mutans* may be due to the species' ability to form thick biofilms and its aciduric nature, which may reduce sensitivity to lipophilic compounds. In contrast, *Piper betle* dry extract displayed significant antimicrobial activity against *Escherichia coli*, consistent with earlier reports linking its antimicrobial properties to phenolic compounds such as chavicol, eugenol, and hydroxychavicol (Chung et al. 2013). Additionally, *Piper betle* dry extract contains bioactive compounds like eugenol, which are known for their anti-inflammatory and soothing properties. The combination of these two natural agents in a mouthwash formulation may enhance its overall efficacy by addressing both bacterial inhibition and inflammation.

Nanoemulsion-based mouthwash preparation

Phase diagram construction

Mixtures of the selected components were prepared at various Smix:oil (IPM) weight ratios of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9 (w/w). Pseudo-ternary phase diagrams were subsequently constructed using the water titration method. Among the evaluated surfactant/co-surfactant mixtures, the combination of Cremophor RH 40 and PEG 400 at a 3:1 (w/w) ratio demonstrated the ability to form clear, homogeneous solutions with IPM over a wide range
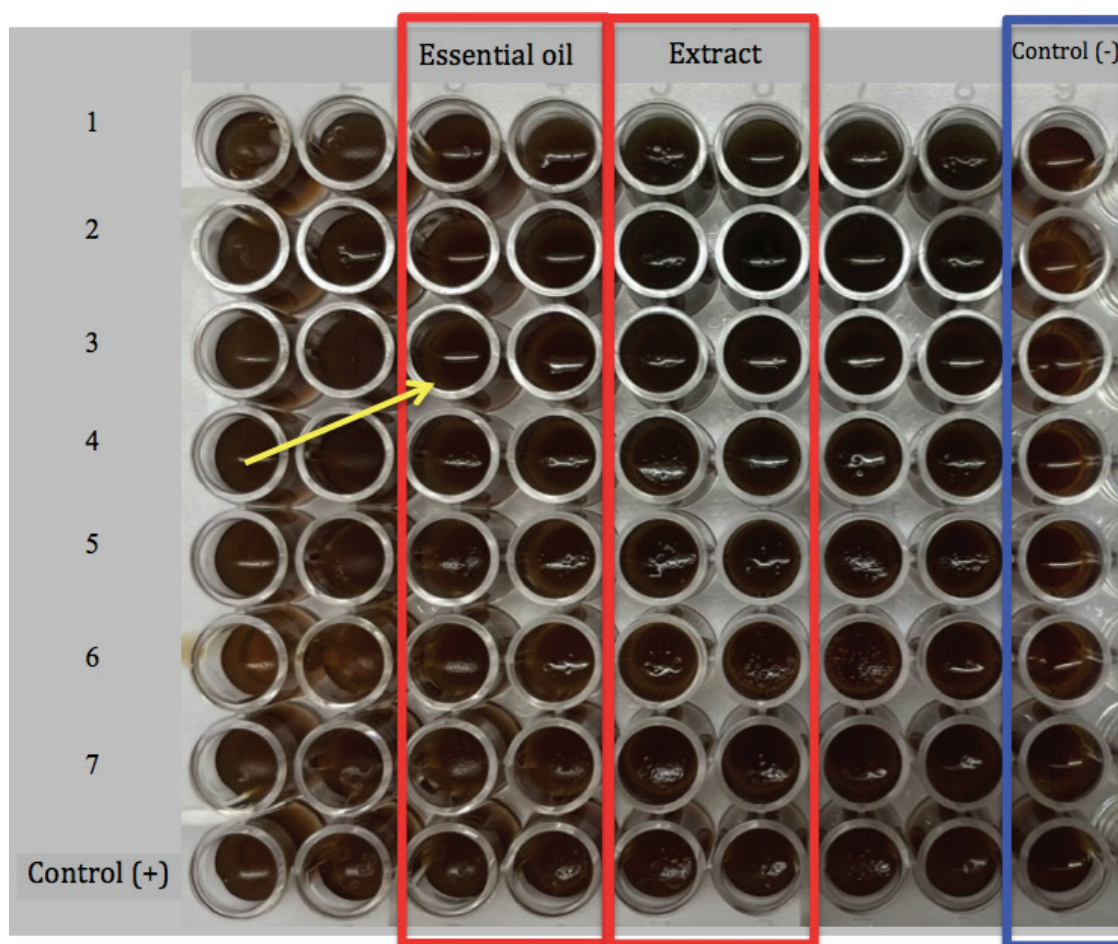
Figure 3. Determination of the Minimum Inhibitory Concentration of the test substance against *Streptococcus mutans*.

Table 4. Minimum inhibitory concentration (MIC) values against selected bacterial strains.

Bacterial strains	MIC value		
	Staphylococcus aureus ATCC 29213	*Escherichia coli* ATCC 25922	*Streptococcus mutans* ATCC 25715
Litsea cubeba essential oil (% w/v)	0.25	0,125	0,5
Piper betle dry extract (mg/mL)	5	0,32	>5

of Smix:oil ratios (from 9:1 to 3:7). This system exhibited a broad nanoemulsion region, indicating its capacity to form stable self-emulsifying nanoemulsions. Accordingly, the Smix formulation comprising Cremophor RH 40 and PEG 400 at a 3:1 (w/w) ratio was selected for further development in combination with IPM and Litsea cubeba essential oil to formulate the nanoemulsion system. The corresponding pseudo-ternary phase diagram, with the nanoemulsion region highlighted in dark gold, is presented in Fig. 4 (constructed using Chemix School software).

Nanoemulsion-based mouthwash formulation

Nanoemulsion-based mouthwashes were formulated with varying concentrations of *Litsea cubeba* essential oil and *Piper betle* dry extract. All formulations (F1–F8) exhibited homogeneity and transparency (+++ on the transparency scale), indicating successful nanoemulsion formation.
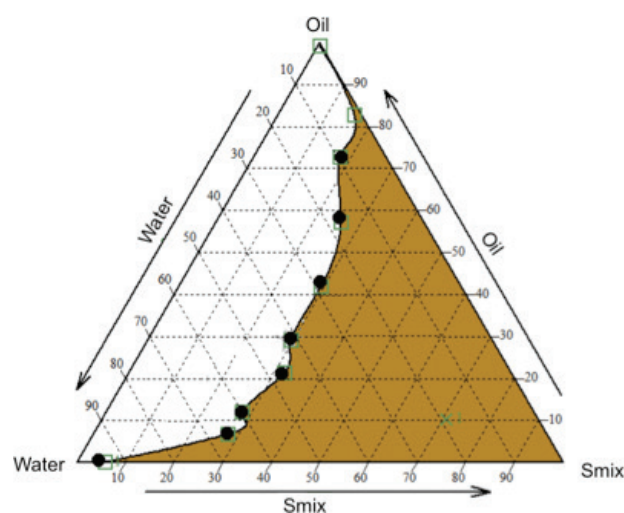

Figure 4. The pseudoternary phase diagram with the nanoemulsion region highlighted in dark gold of Smix (Cremophor RH 40 and PEG 400 ratio of 3:1 w/w) and IPM system.

The pH values ranged from 6.5 to 7.5, aligning with the natural pH of the oral cavity, which is approximately 6.5–7.0. Maintaining an appropriate pH is crucial to prevent mucosal irritation and ensure patient comfort during use. These findings are consistent with previous studies on nanoemulsion-based mouthwashes, which reported similar pH ranges and transparency levels (Had et al. 2023).

Irritability testing showed in Fig. 5 that formulations F1–F5, containing lower concentrations of *Litsea cubeba* essential oil (≤0.9%) and *Piper betle* dry extract (≤1%), were non-irritant (score = 0). Conversely, formulations F6–F8, with higher concentrations of *Litsea cubeba* essential oil (≥1.2%) and/or *Piper betle* dry extract (≥2%), exhibited irritant potential. This suggests that increasing the concentrations of active ingredients may enhance antimicrobial efficacy but also raises the risk of mucosal irritation. Therefore, optimization of ingredient concentrations is essential to balance efficacy and safety.

Formulation F5, containing 0.9% *Litsea cubeba* essential oil and 1% *Piper betle* dry extract, demonstrated optimal transparency, stability, and tolerability. This formulation was selected for further development due to its balanced composition, which ensures both antimicrobial efficacy and patient safety. The selection of appropriate concentrations is supported by studies indicating that formulations with moderate concentrations of active ingredients tend to offer effective antimicrobial activity without compromising safety (Had et al. 2023).
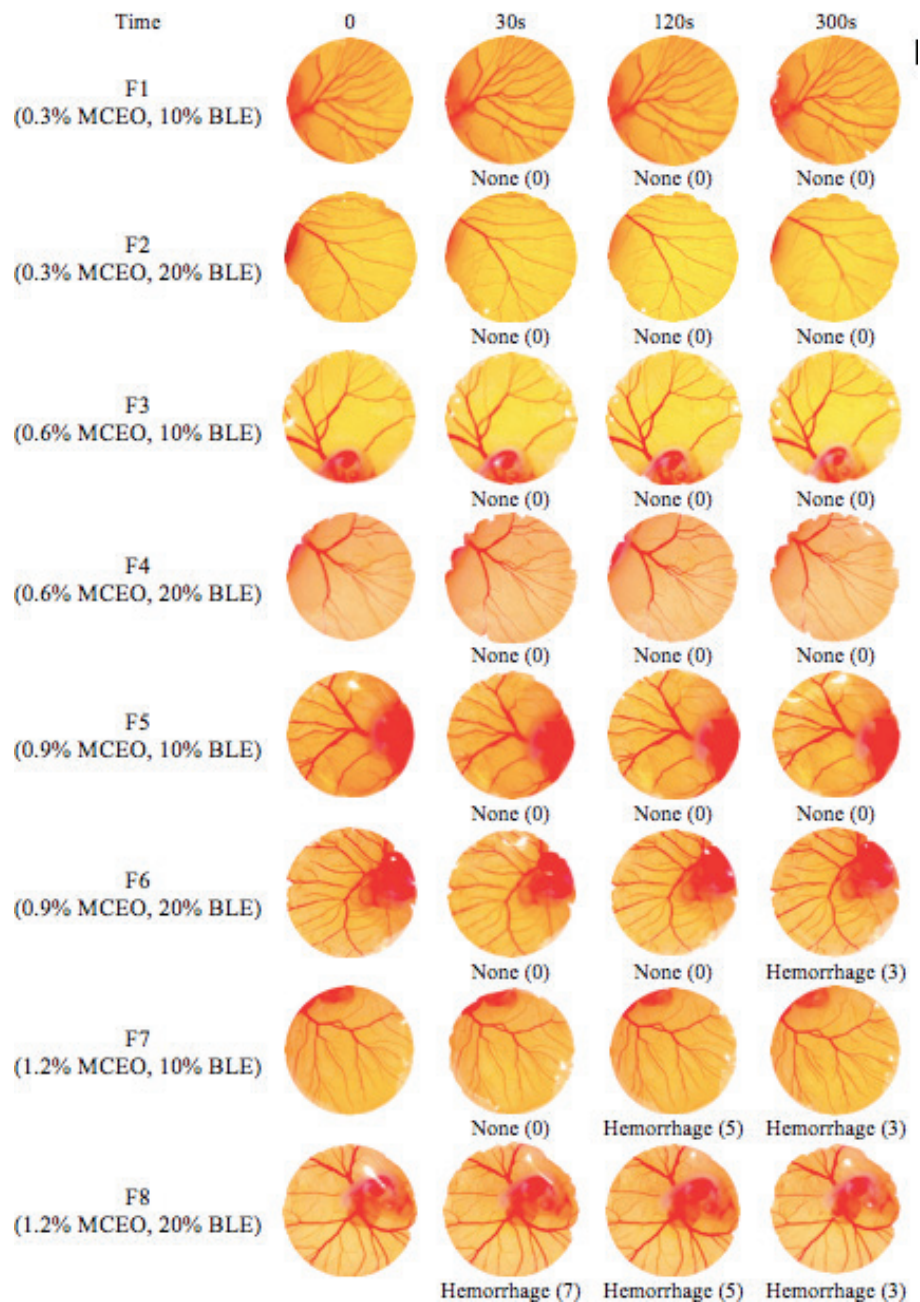

Figure 5. HET-CAM test results of formulations: positive control (1% NaOH, score 17), negative control (0.9% NaCl, score 0), and nanoemulsion formulas (F1–F5: score 0; F6: score 3; F7: score 8; F8: score 15).

Characterization of nanoemulsion-based mouthwash

The optimized nanoemulsion-based mouthwash (0.9% *Litsea cubeba* essential oil, 1% *Piper betle* dry extract) exhibited an average droplet size of 22.36 ± 0.05 nm, a narrow size distribution (PI = 0.099 ± 0.063), and a near-neutral zeta potential (-0.50 ± 0.00 mV) (Fig. 6). The near-neutral zeta potential (−0.50 ± 0.00 mV) observed in the optimized nanoemulsion can be attributed to the use of nonionic excipients, particularly PEG 400 and Cremophor RH 40, which provide steric rather than electrostatic stabilization. These surfactants adsorb onto droplet surfaces, forming a hydrophilic barrier that prevents aggregation through steric hindrance (Tran et al. 2021). Such stabilization is effective even when zeta potential is low, especially in systems with low oil phase content, as in our formulation (Tran et al. 2021). This mechanism explains the colloidal stability observed during the heating-cooling cycles despite the lack of strong surface charge.

The quantification results showed that the concentrations of citral and eugenol in the formulated mouthwash were 0.44% and 0.09%, respectively (Fig. 7). The formulation remained physicochemically stable for at least three months, maintaining its transparency (+++), dark gold appearance, and active ingredient retention, with citral and eugenol levels at 94.8 ± 0.9% and 98.9 ± 2.5% of their initial concentrations, respectively (Table 5).

Table 5. Stability of nanoemulsion-based mouthwash containing *Piper betle* dry extract (eugenol biomarker) and *Litsea cubeba* essential oil (citral biomarker) after 3 months at room temperature.

Parameter	Time		
	0 month (Initial)	1 month	3 months
Appearance	Homogeneous, clear, transparent (+++), and dark gold color		
Citral amount	440 µg (100%)	426 ± 8 µg (96.9 ± 2.3%)	417 ± 4 µg (94.8 ± 0.9%)
Eugenol content	90 µg (100%)	90 ± 2 µg (100.3 ± 2.3%)	89 ± 2 µg (98.9 ± 2.5%)

The Hen's Egg Test-Chorioallantoic Membrane assay confirmed that the nanoemulsion-based mouthwash was non-irritating, with an irritation score of 0, comparable to the negative control (Fig. 8). This suggests that the formulation is safe for mucosal application, supporting its potential use in oral care products. The HET-CAM assay is a well-established method for assessing ocular and mucosal irritation, providing reliable results for the safety evaluation of topical formulations (Patricia et al. 2019).

Cytotoxicity assessment & in-vitro anti-inflammatory activity

The *in-vitro* anti-inflammatory effects of the nanoemulsion-based mouthwash were evaluated using an LPS-induced RAW 264.7 NO inhibition assay. Cytotoxicity was first assessed via the MTT assay, confirming that the mouthwash, reference control L-NMMA, and 0.12% chlorhexidine mouthwash were non-toxic at concentrations up to 256 µg/mL, with cell viabilities of 82.8 ± 2.4%, 91.8 ± 1.5%, and 85.1 ± 2.5%, respectively. According to ISO 10993–5:2009, cell viability > 80% indicates biocompatibility, supporting its safety for biomedical applications. Despite the high Cremophor RH 40 (PEG-40 hydrogenated castor oil) content, the formulation exhibited no cytotoxicity, consistent with prior studies on nanoemulsions containing approximately 20% PEG-40 hydrogenated castor oil across multiple cell lines (Rachmawati et al. 2017).

Fig. 9 presents the nitric oxide (NO) inhibitory activity of a nanoemulsion-based mouthwash containing *L. cubeba* essential oil and *P. betle* extract in comparison to 0.12% chlorhexidine mouthwash. The nanoemulsion formulation exhibited a significant inhibitory effect, with an IC50 of 173.71 ± 8.52 µg/mL, suggesting a strong potential for modulating inflammatory responses. Conversely, 0.12% chlorhexidine mouthwash demonstrated limited NO inhibition, reaching only 22% inhibition at 256 µg/mL, precluding the determination of an IC50 within the tested concentration range. Given that chlorhexidine is primarily recognized for its antimicrobial properties rather than direct anti-inflammatory activity (Yeturu et al. 2016), these findings underscore its limited impact on NO-mediated inflammatory pathways. The superior NO inhibitory effect of the nanoemulsion-based formulation suggests that *L. cubeba* essential oil and *P. betle* extract may exert anti-inflammatory effects through the modulation of NO production. These results support the potential of nanoemulsion technology in enhancing the bioactivity and therapeutic efficacy of phytochemical-based oral care formulations, warranting further mechanistic and clinical investigations.

Antibacterial activity assessment

Bactericidal activity

The present study compared the bactericidal efficacy of a nanoemulsion-based mouthwash with that of a 0.12% chlorhexidine mouthwash against three clinically relevant bacterial strains: *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, and *Streptococcus mutans* ATCC 25157. As shown in Table 6, both mouthwashes significantly reduced bacterial counts compared to the control group (P < 0.05), as demonstrated by the results of the time-kill assay.

Specifically, the nanoemulsion mouthwash achieved bactericidal rates of 98.00% against *S. aureus*, 98.06% against *E. coli*, and 99.74% against *S. mutans*. In comparison, the chlorhexidine formulation displayed slightly higher efficacy against *S. aureus* (99.50%) and *S. mutans* (>99.99%), but was less effective against *E. coli* (97.79%). These findings align with previous reports suggesting that nanoemulsion systems enhance antimicrobial delivery by disrupting bacterial membranes through surfactant-induced destabilization and increased surface area contact (Li et al. 2015).
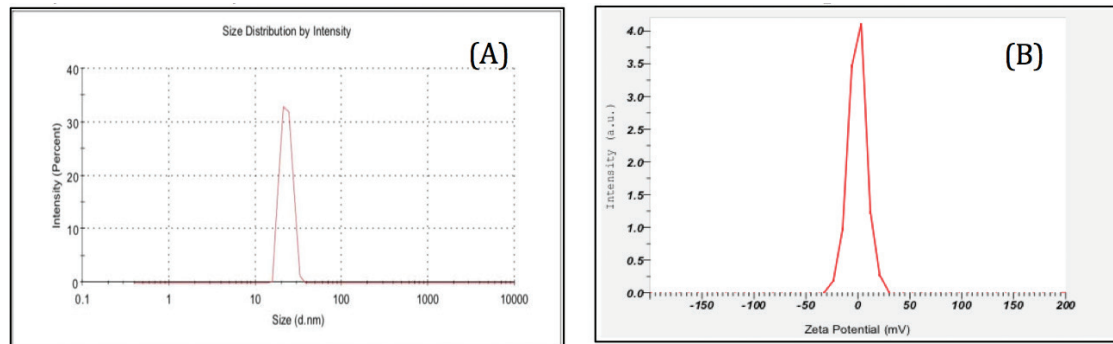
Figure 6. Droplet size, distribution (**A**), and zeta potential (**B**) of the nanoemulsion containing may change essential oil and betel leaf extract.
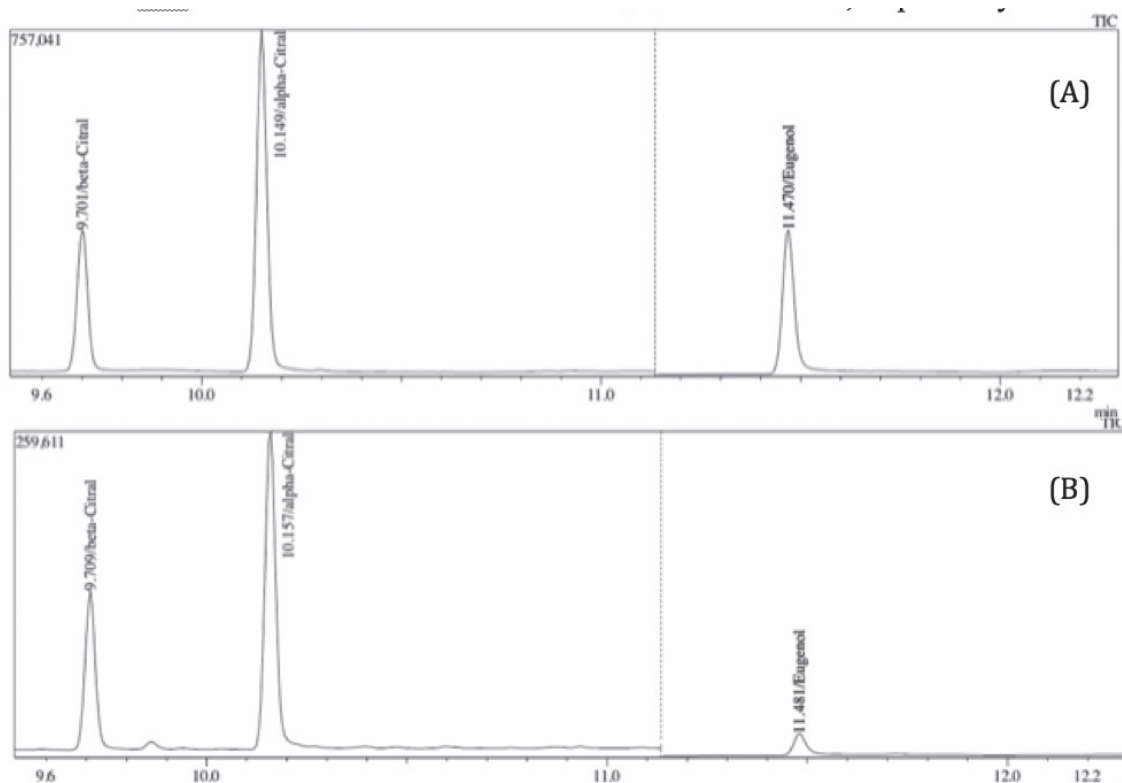

Figure 7. GC–MS chromatograms of (**A**) standard mixture and (**B**) nanoemulsion-based mouthwash.

Table 6. The time-kill assay.

Test strain	Bacterial count in control sample (CFU/ml)	Nanoemulsion-based mouthwash		0.12% chlorhexidine mouthwash	
		Bacterial count in sample (CFU/ml)	Bacterricidal rate %	Bacterial count in sample (CFU/ml)	Bacterricidal rate %
Staphylococcus aureus ATCC 29213	$8,10 \times 10^6$	$1,62 \times 10^5$	98,00%	$4,00 \times 10^4$	99,50%
Escherichia coli ATCC 25922	$6,40 \times 10^6$	$1,24 \times 10^5$	98,06%	$1,41 \times 10^5$	97,79%
Streptococcus mutans ATCC 25157	$1,20 \times 10^6$	$3,10 \times 10^3$	99,74%	$1,04 \times 10^3$	>99,99%

The high bactericidal activity of the nanoemulsion mouthwash against *S. mutans* (99.74%) is of particular relevance in the context of dental caries prevention. As *S. mutans* is the primary pathogen implicated in cariogenesis due to its acidogenicity and ability to form robust biofilms, targeting this organism effec-

tively is crucial. Nanoemulsion systems, owing to their submicron droplet size and physicochemical properties, are known to enhance antimicrobial penetration and retention at mucosal surfaces. Prior studies have reported that the use of surfactants in nanoemulsions promotes membrane destabilization and increased
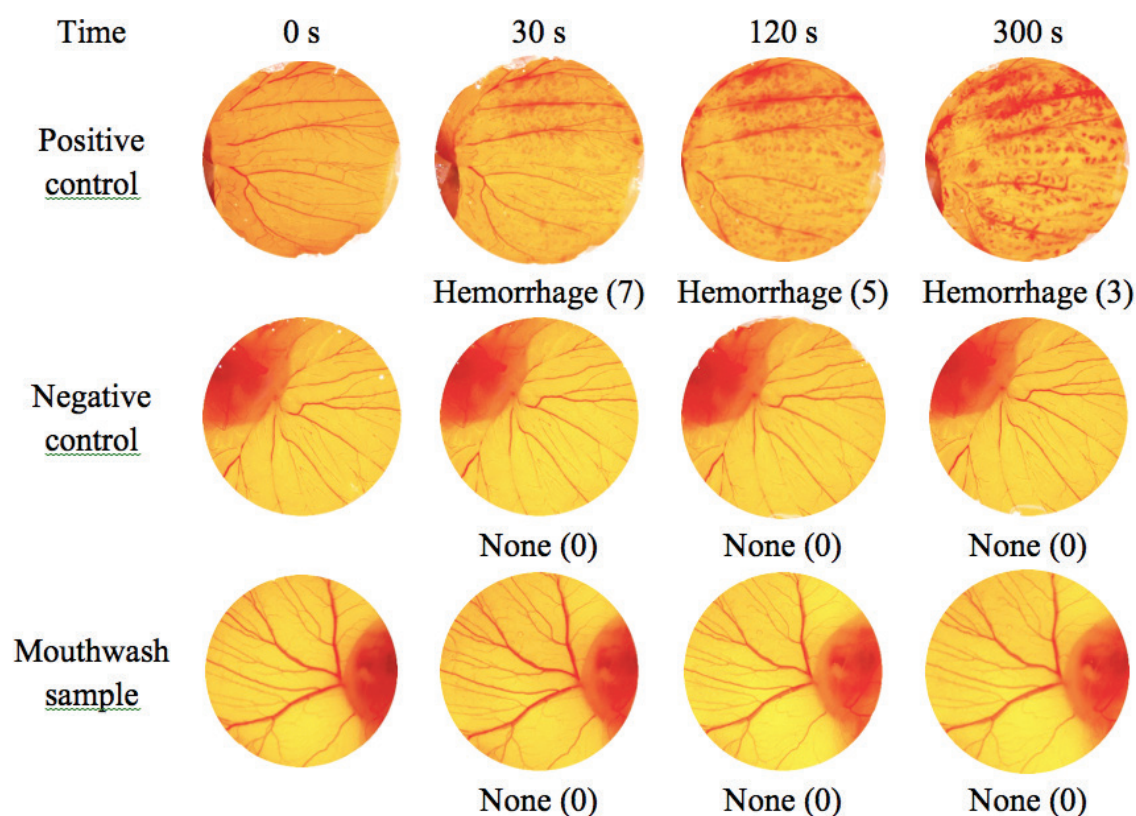
Figure 8. HET-CAM test results: positive control (1% NaOH, score 15), negative control (0.9% NaCl, score 0), and nanoemulsion-based mouthwash containing *Litsea cubeba* essential oil and *Piper betle* dry extract (score 0).

permeability in bacterial cells, leading to cell lysis and death (Al-Adham et al. 2013). These mechanisms are likely contributors to the strong antimicrobial action observed in this study.

Interestingly, while chlorhexidine exhibited the highest efficacy against *S. mutans* (>99.99%) and *S. aureus* (99.50%), it was marginally less effective against *E. coli* (97.79%) compared to the nanoemulsion (98.06%). This may be attributed to the outer membrane of Gram-negative bacteria such as *E. coli*, which acts as a permeability barrier against cationic agents like chlorhexidine. The nanoemulsion droplets, by contrast, may circumvent this limitation due to their ability to fuse with lipid membranes and deliver active compounds more efficiently, as demonstrated in prior lipid-based delivery studies (Wang et al. 2020).

Molecular docking of citral and eugenol

Molecular docking simulations were performed using MOE 2019 software to evaluate the binding interactions between citral, eugenol, and *Streptococcus mutans* virulence-associated protein (PDB ID: 8FKL). Glucosyltransferase B (GtfB), a key virulence factor of *S. mutans*, was selected as the target protein due to its critical role in biofilm formation and cariogenicity (Schormann et al. 2023).

The molecular docking results are summarized in Fig. 10 and Table 7. The monoterpenes geranial and neral exhibited binding affinities of -5.49 and -5.32 kcal/mol, respectively, interacting with Lys715 of the 8FKL protein as hydrogen bond acceptors (Fig. 7). In contrast, eugenol, characterized by the presence of a hydroxyl functional group, acted as a hydrogen bond donor, forming an

Table 7. Docking simulation results with docking score energy (DS, kcal/mol), root-mean-square deviation (RMSD, Å) and types of interaction.

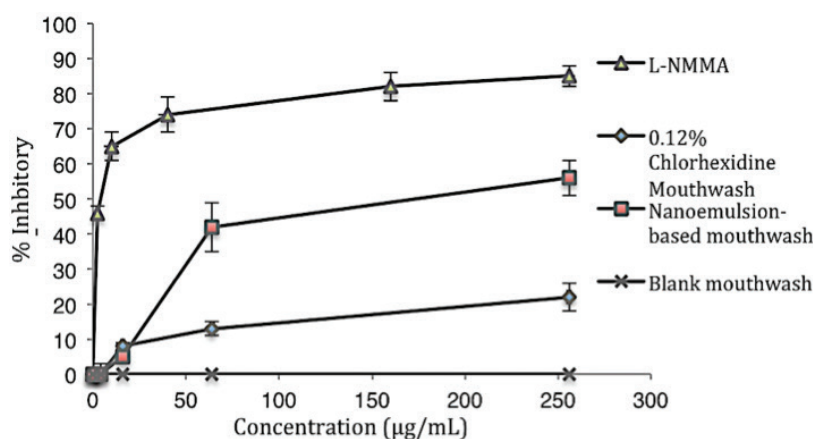| Ligand	DS	RMSD	Hydrogen bond				
			Ligand	Protein	Type	Distance	Energy
Citral alpha	-5.49	1.34	O	N (Lys715)	H-acceptor	2.81	-2.4
Citral beta	-5.32	1.37	O	N (Lys715)	H-acceptor	2.90	-2.5
Eugenol	-5.48	1.74	O	O (Ser690)	H-donor	3.06	-1.0
Chlorhexidine	-7.48	2.56	N	O (Ser690)	H-donor	2.91	-0.9
			N	O (Asp688)	H-donor	2.74	-3.9
			N	O (Asn646)	H-donor	2.76	-3.4
			N	C (Gly689)	H-acceptor	3.27	-0.5
			C	Six-ring (Phe691)	H-p	4.37	-0.5

Figure 9. Inhibition percentages of NO production in LPS-induced RAW 264.7 cells by blank mouthwash, mouthwash with *Litsea cubeba* essential oil and *Piper betle* dry extract, and 0.12% chlorhexidine mouthwash (n = 3).
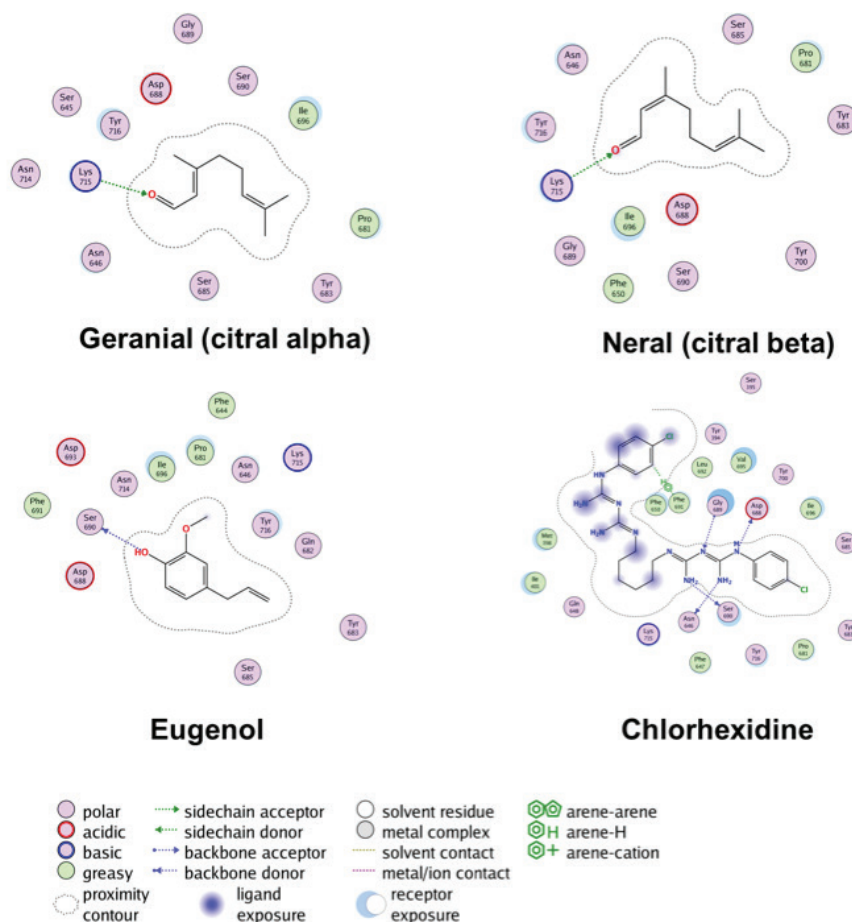

Figure 10. Protein PBD8FKL docked with cital alpha, citral beta, eugenol and chlorhexidine.

interaction with Ser690 of 8FKL. The reference compound chlorhexidine displayed the strongest binding affinity (-7.48 kcal/mol), likely attributed to its higher molecular weight and the formation of multiple hydrogen bond interactions with key residues of 8FKL, including Ser690, Asp688, Asn646, Gly689, and Phe691. These findings suggest that the tested compounds, particularly eugenol and the monoterpenes, have the potential to inhibit the 8FKL protein of *S. mutans*, highlighting their prospective role in antimicrobial strategies. While eugenol's antibacterial

effects are well-documented (Adil et al. 2014), this study is the first to report citral's interaction with *S. mutans*, warranting further investigation into its therapeutic potential for oral health applications (Adil et al. 2019).

While the present study provides promising physico-chemical and biological evidence supporting the potential of this nanoemulsion-based mouthwash, certain limitations should be acknowledged. The results are primarily derived from *in vitro* experiments, which may not fully replicate the complex biological interactions within the

oral environment. Furthermore, although both *Litsea cubeba* essential oil and *Piper betle* extract were demonstrated to possess individual antimicrobial properties, their potential synergistic effects when combined were not specifically evaluated in the current study. This aspect warrants further investigation and is being considered for future research. In addition, a control sample of the blank mouthwash base (without active components) was not included in the antibacterial assays, which may have limited the ability to fully isolate the contributions of the excipients. Lastly, while molecular docking revealed plausible mechanisms of antimicrobial action for citral and eugenol, their actual bioavailability and interactions within the nanoemulsion matrix remain to be further elucidated. These limitations highlight the need for subsequent *in vivo* and clinical studies to validate the formulation's long-term safety, efficacy, and clinical applicability.

Conclusion

This study successfully developed and characterized a nanoemulsion-based mouthwash incorporating *Piper betle* dry extract and *Litsea cubeba* essential oil for potential application in dental disease management. The optimized formulation encapsulated 0.9% *Litsea cubeba* essential oil and 1% *Piper betle* dry extract, forming nanosized spherical droplets (~22.36 nm) with a zeta potential of -0.50 mV. The final mouthwash exhibited physiologically compatible pH (6.5–7.5), stable citral (~0.5%) and eugenol (~0.1%) content, and maintained physicochemical stability for over three months. Biological evaluations demonstrated that the formulation was non-irritating (HET-CAM assay) and exhibited significant anti-inflammatory activity in an LPS-stimulated RAW 264.7 macrophage model, surpassing 0.12% chlorhexidine mouthwash. Furthermore, antibacterial studies revealed substantial activity against *Streptococcus mutans*, with 99.74% bacterial reduction in a time-kill assay. Molecular docking studies further supported the antimicrobial potential of citral and eugenol through interactions with *S. mutans* glucosyltransferase B (GtfB). These findings highlight the potential of this nanoemulsion-based mouthwash as a novel natural alternative for oral healthcare, offering both anti-inflammatory and antibacterial benefits with improved tolerability. Further clinical investigations are warranted to validate its therapeutic efficacy and long-term safety in dental applications.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statements

The authors declared that no clinical trials were used in the present study.

     The authors declared that no experiments on humans or human tissues were performed for the present study.

     The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.

     The authors declared that no experiments on animals were performed for the present study.

     The authors declared that no commercially available immortalised human and animal cell lines were used in the present study..

Author contributions

All authors contributed to the study conception and design. All authors read and approved the final manuscript. Ngoc Nha Thao Nguyen: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Resources, Validation, Visualization, Supervision, Writing-Original draft preparation, Writing-Reviewing and Editing. Thi Trang Dai Nguyen: Methodology, Investigation, Data curation. Thanh Si Nguyen: Methodology, Investigation, Data curation, Formal analysis, Writing-Original draft preparation. Dang Tuyet Minh Than: Methodology, Investigation, Data curation. Thi Thanh Yen Le: Investigation, Data curation. Huu Nhan Nguyen: Investigation, Data curation.

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Data availability

All of the data that support the findings of this study are available in the main text.

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Supplementary material 1

Quantification of essential oil and extract

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Supplementary material 2

MIC determination

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