



Development of a nanoemulsion containing essential oil from *Eugenia caryophyllus* and evaluation in vitro and in silico of its antibacterial and antibiofilm activity against *Streptococcus mutans*

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Abstract

This study developed a *Eugenia caryophyllus* nanoemulsion and evaluated its performance antimicrobial and toxicity. Nanoemulsion was produced with the high agitation method, using essential oil and a surfactant in the oily phase, and the aqueous phase consisted of a surfactant and ultrapure water. Nanoemulsion characterization included mean droplet diameter, zeta potential, and polydispersity index. Antimicrobial activity evaluation minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) and the nanoemulsion's ability to destroy and inhibit biofilm in vitro and ex vivo on bovine incisor specimens. In silico toxicity predictions were determined using Molinspiration Cheminformatics, pkCSM, ProTox-II, and OSIRIS Property Explorer software. Molecular docking was conducted using AutoDock Vina and AutoDock4 AMDock v.1.5.2 to analyze the interactions between the IL-1 β , IL-6, IL-8, IL-10 and TNF- α and the eugenol. To evaluate the data, one-way analysis of variance (ANOVA) was used, followed by Tukey's test, using GraphPad Prism 8.0.1 software (GraphPad Software Inc, San Diego, CA, USA). Nanoemulsion had a mean droplet size of 117 nm, a low polydispersity index, and a zeta potential of -7.9 mV. Antimicrobial activity tests demonstrated 0.78 mg/ml MIC and 1.56 mg/mL MBC against *Streptococcus. mutans*. The nanoemulsion also effectively eradicated and inhibited biofilm in vitro . Genotoxicity and cytotoxicity tests confirmed the safety of the nanoemulsion for dental use. In molecular docking, eugenol demonstrated higher binding affinity to the TNF- α and lower affinity to the IL-10. This study highlights the potential of this nanoemulsion as antimicrobials in dentistry, emphasizing its natural origin and confirmed safety by the tests.

Keywords Biofilms · Nanostructures · Oils volatile · Molecular docking simulation · Eugenol · Toxicity

Introduction

Dental caries is a chronic and multifactorial disease that affects a large part of the global population, often culminating in toothache and chewing and speaking difficulties, affecting people's quality of life [1]. Its etiology consists of the accumulation of pathogenic biofilm on the dental surface, conceptually described as a complex microbiological community, in which bacteria adhere to a substrate and enter a matrix of self-produced extracellular polysaccharides

[2–4]. Among the most prevalent bacteria in this biofilm is *Streptococcus mutans*, a gram positive, facultative anaerobic, acidogenic and aciduric microorganism [5].

In Dentistry, chlorhexidine 0.12% remains one of the most used antimicrobials, being considered the gold standard in the control of dental biofilm and gingival inflammation [6]. Although it is widely used, one of its limitations lies in dental staining, which is unfavorable due to the aesthetic impacts on the patient's oral health as well as changes in taste [7]. Furthermore, this substance has an unpleasant

taste, can cause mucosal peeling and, in some cases, trigger allergic reactions.

Then, biofilm formation disruption using natural compounds, exploring phytotherapy, has been searched to develop products with effectivity and substantivity as efficient as chlorhexidine but with lower adverse effects [8]. Therefore, *Eugenia caryophyllus* essential oil, popularly known as clove leaf essential oil [9], emerges as an alternative to work in this microbial community [10]. The selection of *E. caryophyllus* for this study was based on its well-documented antimicrobial properties, particularly its efficacy against oral pathogens [11, 12], highlighting its potential as a natural alternative for combating dental caries and associated infections [13, 14]. The main constituent of essential oil is eugenol, topically used as an analgesic, germicide, antiseptic and local anesthetic in dentistry [15]. However, the physicochemical characteristics of oils promote handling difficulties and potential damage that alters their activities when preparing formulations [16].

One alternative is using nanoemulsions (NE), dispersions with droplet size on the nanometric scale ranging between 10 and 100 nanometers [17, 18]. That might reduce the concentration to obtain an effect, such as antimicrobial action, equivalent to or higher than conventional emulsions [19]. Therefore, this study developed a *E. caryophyllus* nanoemulsion and analyzed its performance against *S. mutans*.

Materials and methods

Essential oil *E. caryophyllus*

Eugenia caryophyllus essential oil (Batch 0239) was obtained commercially from VIA AROMA (Porto Alegre, Rio Grande do Sul, Brazil) and extracted from the plant by drag steam. The industry realized preview characterization, but additional analytical characterization was performed using a gas chromatograph supplied with a flame ionization detector (GC-FID; Varian Star 3400), an autosampler (Varian 8100) and gas chromatograph coupled to a mass spectrometer (Shimadzu GCMS QP-2010 Ultra). Initially, quantitative analysis of the major constituents was conducted using gas chromatography with flame ionization detection (GC-FID; Varian Star 3400, autosampler Varian 8100). The essential oil was diluted 1:100 in hexane, and 1 μ L was injected in split mode (1:20) at an injector temperature of 250 °C. Hydrogen was used as carrier gas (15 psi), and separation was achieved using a BPX5 capillary column (25 m $\times$ 0.22 mm; 0.25 μ m film thickness). The temperature program was set from 40 °C (1 min), increasing at 2 °C $\cdot$ min⁻¹ to 120 °C, then rising at 10 °C $\cdot$ min⁻¹ to 250 °C (held for 2 min). The FID detector operated at 250 °C.

Quantification was expressed as relative percentage based on normalization of chromatographic peak areas.

Subsequently, qualitative confirmation of compound identity was performed using gas chromatography coupled to mass spectrometry (GC-MS; Shimadzu GCMS QP-2010 Ultra) under the same chromatographic conditions. The mass spectrometer operated in electron ionization (EI) mode with a scan range of 35–400 m/z. The GC/MS interface and ion source temperatures were maintained at 250 °C and 200 °C, respectively. Compound identification was achieved by comparison of experimental mass spectra with the NIST library database and by calculation of retention indices using a homologous series of alkanes, with comparison to literature data [20].

Nanoemulsion development and characterization

Nanoemulsions containing 5% clove leaf essential oil were developed in triplicate using the high agitation method, following the methodology by da Silva Gundel et al. [16]. The oily phase comprised clove leaf essential oil (5%) and the sorbitan monooleate surfactant (Span 80[®]) (2%), and the aqueous phase included the polysorbate 80 surfactant (Tween 80[®]) (2%) and ultrapure water. Both phases were weighed separately and homogenized by magnetic stirring (without heating) for 15 min. Next, the aqueous phase agitated at 10,000 rpm in Ultra Turrax[®] equipment for 15 min. Then, the oily phase was injected into the aqueous phase. The formulation continued stirring at 17,000 rpm for 30 min. The nanoemulsions process had a controlled temperature (ice bath) to prevent the volatilization and/or degradation of the constituent oil. The physicochemical characterization of nanoemulsions occurred by determining the mean droplet diameter and polydispersity index using dynamic light scattering in Zetasizer equipment (Zetasizer[®] nano-ZS model ZEN 3600, Malvern), diluting the formulations 500 times in ultrapure water previously filtered with a syringe and membrane with a 0.45 μ m-diameter porosity. The same equipment evaluated the zeta potential by applying the laser Doppler Microelectrophoresis and diluting the formulation in sodium chloride (10 mM NaCl) previously filtered through a 0.45 μ m membrane. All analyzes occurred triplicate. The pH measurements were performed directly on the formulations, without previous dilution, in a previously calibrated potentiometer (DM-22[®], Digimed - Brazil). All analyses were performed in triplicate and results expressed as mean and standard deviation.

For evaluation of nanoemulsion stability analysis, three batches of nanoemulsion were prepared and stored at ambient temperature (25 $\pm$ 2 °C), climatic chamber (40 $\pm$ 2 °C and 65 $\pm$ 2% humidity), and refrigeration (5 $\pm$ 2 °C) for 15 days. The formulations were characterized soon after preparation

and throughout the study (0, 7, 15 days after preparation). Thus, the parameters of mean droplet diameter, polydispersity index, zeta potential, pH, and essential oil composition were evaluated. At the end of the study the values of each parameter and period were compared with those obtained immediately after preparation to verify possible changes related to storage time and temperature [19].

Antimicrobial activity tests

The antimicrobial activity assay were performed using *S. mutans* (ATCC 25175) strain acquired from the American Type Culture Collection. The microorganism remained on a culture medium with glycerol and cooled to -80°C . The strains were thawed, inoculated on brain heart infusion (BHI) broth, and incubated at 37°C for 24 h prior to testing. The microorganism can form biofilms, already recognized in the literature [21]. The minimum inhibitory concentration (MIC) was determined by the broth microdilution method using standardized 96-well plates according to the M07-M09 protocol approved by the Clinical and Laboratory Standards Institute [22]. All wells received Brain Heart Infusion (BHI) broth and nanoemulsion at a concentration of 50 mg/mL, and serial dilution was carried out to 0.04883 mg/mL. After the dilution, each well received 10 μL of the bacterial inoculum, establishing the positive control (inoculum and BHI broth) and negative control (only BHI broth). The inoculum containing *S. mutans* prepared at 0.5 on the McFarland scale (equivalent to 1.5×10^8 CFU/ml). The plate was incubated for 24 h at 37° under microaerophilic conditions. The assay occurred in triplicate and used resazurin (0.15 mg/mL). MIC was the lowest concentration without color changes. To determine the minimum bactericidal concentration (MBC), aliquots from wells showing no visible growth in the MIC assay were plated onto BHI agar and incubated at 37°C for 24 h under microaerophilic conditions. The MBC was defined as the lowest concentration at which no visible colony growth was observed [23].

The antibiofilm activity was evaluated under previously optimized conditions described by Sandberg et al., with modifications [24]. The crystal violet (CV) assay and colony-forming unit (CFU) were performed. Flat-bottom 96-well plates (NuncloTMSurface D, Nunc, Roskilde, Denmark) containing 100 μL of BHI broth supplemented with 2% sucrose received 15 μL of the fresh, exponentially grown *S. mutans* culture and were incubated at 37°C for 24 h under microaerophilic conditions to allow biofilm formation. After this period, 100 μL of nanoemulsion solution containing *E. caryophyllus* essential oil at concentrations to MIC, 2xMIC and 4xMIC (0.78 mg/ml, 1.56 mg/ml and 3.12 mg/ml) was added, followed by an additional 24 h incubation under the same conditions. Positive and negative

controls consisted of BHI with inoculum and BHI alone, respectively.

Biofilm biomass was quantified with the crystal violet staining method as described by Kouidhi et al. [25]. After treatment, supernatants were removed, and wells were washed three times with sterile saline. Plates were dried at 60°C for 60 min to fix the biofilm and stained with 200 μL of crystal violet (1%) for 15 min.

Excess stain was removed by washing with distilled water, and the retained dye was solubilized with 95% ethanol for 10 min (200 μL). An aliquot of 100 μL was transferred to a new plate, and absorbance was measured at 570 nm using a microplate reader (TP-Reader; ThermoPlate, Goiás, Brazil). Antibiofilm activity was calculated by comparing the optical density of treated groups with the positive control.

To evaluate inhibition of biofilm formation, subinhibitory concentrations of the nanoemulsion (0.39, 0.195, and 0.0975 mg/mL) were added to BHI supplemented with 2% sucrose prior to inoculation with *S. mutans*. After incubation for 24 h at 37°C under microaerophilic conditions, biofilms were fixed with 95% methanol and stained with 0.1% crystal violet for 15 min. Following washing, ethanol was added to dissolve the dye, and absorbance was measured at 540 nm. The inhibition rate was calculated as $\text{OD Test/OD Control} \times 100$ [26].

An in vitro biofilm model using bovine enamel was also employed [27]. Specimens measuring 5 mm $\times$ 5 mm $\times$ 1 mm were prepared from bovine incisors, measured with a digital caliper, and sterilized by autoclaving. To allow initial pellicle formation, specimens were immersed in sterile artificial saliva (Kin Hidrat[®]) for 30 min. They were then transferred to microtubes containing BHI supplemented with 2% sucrose and inoculated with *S. mutans*, forming the positive control (culture medium, inoculum, and specimen). After incubation at 37°C for 24 h under microaerophilic conditions, pre-formed biofilms were treated with nanoemulsion at MIC, 2xMIC, and 4xMIC concentrations for 6 h.

Following treatment, bacterial viability was assessed by transferring specimens to microtubes containing sterile saline and subjecting them to sonication for 15 min to detach adherent bacteria. Samples were vortexed (300 rpm) for 30 s, serially diluted (1:100), and aliquots were plated on BHI agar using the spread plate method. Plates were incubated at 37°C for 48 h under microaerophilic conditions, and colony-forming units were counted using a digital colony counter by an experienced researcher [28].

Finally, scanning electron microscopy (SEM) was performed to qualitatively evaluate the structural effects of the nanoemulsion on biofilm architecture. Biofilms were formed on bovine enamel specimens under the same conditions described above and treated with MIC, 2xMIC, and 4xMIC concentrations. Specimens were rinsed with distilled water,

dried at 60 °C for one hour, and sputter-coated with gold (108 Å; Cressington Scientific Instruments Inc., Watford, UK). Images were obtained using a JSM-6360 scanning electron microscope (JEOL, Tokyo, Japan).

Compound safety profile

The genomodifying/genotoxic potential of the tested compounds was evaluated using the GEMO assay. This method employs calf thymus double-stranded DNA (dsDNA), the dsDNA-specific fluorescent dye PicoGreen[®], and the test compound. The assay is based on the equation: Fluorescence = dsDNA + test compound + PicoGreen[®]. Fluorescence intensity is directly proportional to the amount of intact dsDNA present in the sample. In cases of dsDNA degradation, fluorescence decreases, since the PicoGreen[®] fluorophore selectively binds to double-stranded DNA. Fluorescence readings were performed at an excitation wavelength of 480 nm and an emission wavelength of 520 nm, as previously described [29]. All experiments were conducted in independent triplicates on three different days to ensure reproducibility.

To complement the safety evaluation, cytotoxicity was assessed using the human keratinocyte HaCaT cell line, commercially obtained from the Rio de Janeiro Cell Bank. Cells were cultured in DMEM supplemented with 20% fetal bovine serum, 1% penicillin/streptomycin, and 1% amphotericin B, and maintained under sterile conditions at 37 °C in a humidified atmosphere containing 5% CO₂. Subculturing was performed when cells reached approximately 80% confluence, until the required density for the experiments was achieved. For the assays, a cell concentration of 1×10^4 cells/mL was used. After 24 h of plating, cells were exposed to the respective treatments.

Cell viability was determined using the neutral red uptake assay. Following treatment, cells were incubated for three hours with a 50 µg/mL neutral red solution. The dye was then removed, a desorption solution was added to solubilize the incorporated dye, and absorbance was measured at 540 nm using a spectrophotometer. This method evaluates cytotoxicity based on lysosomal integrity and the ability of viable cells to incorporate neutral red; therefore, higher dye incorporation indicates lower toxicity of the tested compound [30].

In addition, *in silico* analyses were performed according to the methodology described by Malta et al. [31] to evaluate the safety profile and molecular interactions of eugenol. All chemical and structural information regarding the compound was retrieved from the PubChem[®] molecular database, maintained by the National Center for Biotechnology Information (NCBI) of the United States National Library of Medicine (NLM). The physicochemical and structural

Table 1 IUPAC nomenclature and SMILES of tested compounds

Compounds		
Eugenol	IUPAC	2-methoxy-4-prop-2-enylphenol
	SMILES	COC1=C(C=CC(=C1)CC=C)O
Data extracted from PubChem [®] (https://pubchem.ncbi.nlm.nih.gov/). IUPAC=International Union of Pure and Applied Chemistry. SMILES=Simplified molecular input line entry specification		

Table 2 Results of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the *Eugenia caryophyllus* nanoemulsion

Microorganism	MIC (mg/ml)	MBC (mg/ml)
<i>S. mutans</i> ATCC 25175	0.78	1.56

Table 3 Predictions of the tested compounds through computational simulation

Properties	Compound Eugenol
Number of violations ¹	0
Maximum tolerated dose (log mg/kg/dia) ²	1.024
Hepatotoxicity ^{2,3}	No ² , ND ³
Cytotoxicity ³	No ³
Carcinogenicity ^{3,4}	No ³ , Yes ⁴
Mutagenicity ^{3,4}	No ³ , Yes ⁴
Irritant ⁴	Yes ⁴

¹Molinspiration Cheminformatics; ²pkCSM; ³ProTox-II; ⁴OSIRIS; ND=not determined

characteristics of the compound analyzed are summarized in Tables 1, 2, 3, 4, 5).

In silico evaluation of bioavailability

Bioavailability prediction was performed using the Molinspiration Cheminformatics software (<https://www.molinspiration.com/cgi/properties>). The bioavailability analysis was based on Lipinski's "Rule of Five," which establishes key criteria to predict the bioavailability of bioactive compounds. According to this rule, a compound is expected to present favorable absorption when it meets at least four of the following criteria: $\text{miLogP} \leq 5.00$, molecular weight $\leq 500 \text{ g} \cdot \text{mol}^{-1}$, polar surface area $\leq 140 \text{ Å}^2$, total number of hydrogen bond donors and acceptors < 12 , hydrogen bond acceptors < 10 , and hydrogen bond donors < 5 . Compounds that violate more than one of these parameters may exhibit potential limitations in bioavailability [32].

In silico toxicological testing

To further characterize the safety profile, *in silico* toxicological predictions were carried out using the pkCSM (<http://biosig.unimelb.edu.au/pkcsml/>), ProTox-II (http://tox.charite.de/protox_II/), and OSIRIS Property Explorer (<http://www.organic-chemistry.org/prog/peo>) platforms. These

Table 4 Binding affinity, estimated Ki and ligand efficiency values and ligand box for the best docking configurations of each complex with Autodock Vina (Via AMDock v.1.5.2)

Complex Ligand	Protein	Affinity (kcal/mol)	Esti- mated Ki (mM)	Ligand efficiency (kcal mol)	Box (x, y,z)
Eugenol	IL-1	−5.0	0.22	−0.42	47.95 −5.96 17.94
					−15.10 5.12 −3.99
					28.25 13.42 13.11
	IL-6	−5.5	0.093	−0.46	8.61 23.75 19.65
					17.05 53.37 37.77
	IL-8	−5.5	0.093	−0.46	
	IL-10	−4.8	0.30	−0.40	
	TNF-α	−6.1	0.033	−0.51	

μM: 10^{−6} m; mM: 10^{−3} m

Autodock4 AMDock v.1.5.2 - Using a RMSD-tolerance of 2.0Å

All executions protonated and with box definition (T_BOX_MODE) in automatic

computational tools enable the prediction of biological and toxicological properties of organic molecules in the human body [33].

The pkCSM platform was used to estimate the maximum tolerated dose in humans and hepatotoxicity. ProTox-II provided predictions regarding hepatotoxicity, cytotoxicity, carcinogenicity, and mutagenicity, while the OSIRIS server was used to evaluate carcinogenic, mutagenic, and irritant risks. In ProTox-II, predictions with reliability lower than 70% are automatically excluded and classified as “not determined” (ND), ensuring greater robustness of the reported data.

Molecular docking analyses were conducted to investigate the potential interactions between eugenol and selected inflammatory mediators, following the methodology described by Malta et al. [31]. The simulations were performed using AutoDock Vina and AutoDock4 through the AMDock v.1.5.2 interface. AutoDock is widely used for computational modeling of biomolecular interactions,

enabling the prediction of three-dimensional conformations and binding affinities between a ligand and a macromolecular target [34]. AutoDock Vina applies an empirical scoring function to estimate protein–ligand binding energies and identify the most stable binding poses, whereas AutoDock4 employs a genetic algorithm to explore ligand conformational space in greater detail. Ten independent docking runs were performed to refine and validate the predicted binding conformations.

The docking study focused on evaluating the interactions between eugenol and the cytokines IL-1β (PDB ID: 1I1B), IL-6 (PDB ID: 1IL6), IL-8 (PDB ID: 1QE6), IL-10 (PDB ID: 2H24), and TNF-α (PDB ID: 1TNF), whose crystallographic structures were obtained from the Protein Data Bank (PDB) [35]. These well-characterized structures allow reliable simulation of molecular interactions and identification of potential binding regions. For each protein, a docking box was defined to delimit the region of interest, with central coordinates corresponding to the active or functionally relevant sites and dimensions adjusted according to ligand size.

Prior to docking, protein and ligand structures were preprocessed using PyRx software (The Scripps Research Institute, La Jolla, San Diego, USA). This preparation included the addition of hydrogen atoms, assignment of partial charges, and optimization of molecular geometries to ensure proper configuration for interaction analysis. These steps are essential to simulate realistic molecular interactions consistent with the biological characteristics of the target proteins.

Binding affinity was estimated from the free energy of binding (FEB), expressed as Gibbs free energy (ΔG), which represents the sum of van der Waals interactions (ΔG_{vdw}), hydrogen bonding (ΔG_H), electrostatic interactions (ΔG_{electrost}), and intramolecular ligand interactions (ΔG_{int}), as shown in Eq. 1:

$$FEB \approx \Delta G(kcal/mol) = \Delta G_{vdw} + \Delta G_H + \Delta G_{electrost} + \Delta G_{int} \quad (1)$$

Affinity values ≥ 0 kcal/mol were considered energetically unfavorable, indicating negligible or absent interaction.

Table 5 Binding affinity, estimated Ki and ligand efficiency values and ligand box for the best docking configurations of each complex with Autodock4 (Via AMDock v.1.5.2)

Complex Ligand	Protein	Affinity (kcal/mol)	Estimated Ki (mM)	Ligand efficiency (kcal mol)	Box (x, y,z)
Eugenol	IL-1	−5.83	0,05328	−0.49	49.52 −17.77 29.89
	IL-6	−5.09	0.19	−0.42	−21.63 −0.37 10.08
	IL-8	−5.12	0.18	−0.43	11.91 42.57 32.13
	IL-10	−4.95	0.24	−0.41	7.09 34.55 13.09
	TNF-α	−6.18	0,02951	−0.52	17.05 53.37 37.77

μM: 10^{−6} m; mM: 10^{−3} m. All executions protonated and with box definition (T_BOX_MODE) in automatic

Values lower than -0.5 kcal/mol were considered more favorable, suggesting medium to high docking affinity [34, 36].

The inhibition constant (K_i) was estimated from the binding energy using the thermodynamic relationship between ΔG and K_i , where R is the gas constant (1.987 cal/mol·K) and T is the absolute temperature (298 K):

$$K_i = \exp\left(\frac{\Delta G}{RT}\right)$$

In addition, ligand efficiency (LE) was calculated to normalize binding energy according to ligand size, providing a size-adjusted metric of interaction strength. LE was determined using the formula:

$$LE = \frac{\Delta G}{N_{\text{heavy atoms}}}$$

where ΔG is the binding energy (kcal/mol) and $N_{\text{heavy atoms}}$ represents the number of non-hydrogen atoms in the ligand. Higher LE values indicate more efficient binding relative to molecular size, representing a more promising interaction profile.

Statistical analysis

For the antimicrobial activity assays, statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test, using GraphPad Prism 8.0.1 software (GraphPad Software Inc., San Diego, CA, USA). This approach allowed comparison among the different tested concentrations and the respective control groups. Statistical significance was established at $p < 0.001$.

For the evaluation of the compound safety profile, including cytotoxicity and genotoxicity assays, data were analyzed using one-way ANOVA followed by Dunnett's post hoc test, which compares each experimental group directly with the control group. In this case, statistical significance was considered at $p < 0.05$. All experiments were performed in triplicate, and results were expressed as mean $\pm$ standard deviation.

Results

Characterization of the nanoemulsion

After preparation, the physicochemical characterization of the nanoemulsion containing *E. caryophyllus* essential oil was performed showing a mean diameter of 117 ± 2.6 nm,

polydispersity index less than 0.26, zeta potential -7.9 ± 0.4 and pH 5.56. The nanoemulsion maintains the same compounds characteristics of the *E. caryophyllus* essential oil. The formulations were monitored for average droplet size, polydispersity index, zeta potential, pH, and concentration of majority constituents after 15 days with controlled storage conditions (ambient temperature $(25 \pm 2^\circ\text{C})$, climatic chamber $(40 \pm 2^\circ\text{C}$ and $65 \pm 2\%$ humidity), and refrigeration $(5 \pm 2^\circ\text{C})$ for 15 days, all parameters remained stable.

Antimicrobial activity

The broth microdilution assay demonstrated that the *E. caryophyllus* nanoemulsion exhibited antimicrobial activity against *S. mutans* ATCC 25,175, with a minimum inhibitory concentration MIC = 0.78 mg/ml (MIC) of 0.78 mg/mL and a minimum bactericidal concentration (MBC) of 1.56 mg/mL (Table 2).

The ability of the nanoemulsion to eradicate preformed biofilm was evaluated by quantifying biomass through crystal violet staining. Treatment with 3.12 mg/mL and 1.56 mg/mL reduced preformed biofilm biomass by approximately 68% ($p < 0.0001$) and 25% ($p < 0.0062$), respectively. The mean absorbance values were 2.09 ± 0.10 for 1.56 mg/mL and 0.86 ± 0.16 for 3.12 mg/mL (Fig. 1A), demonstrating a concentration-dependent reduction in biofilm biomass.

In addition to biofilm eradication, the nanoemulsion significantly inhibited biofilm formation at sub-inhibitory concentrations. A reduction of approximately 70.7% ($p < 0.0001$) was observed at 0.39 mg/mL. The mean absorbance values were 2.10 ± 0.08 for 0.0975 mg/mL, 1.83 ± 0.28 for 0.195 mg/mL, and 0.81 ± 0.36 for 0.39 mg/mL (Fig. 1B), indicating substantial interference with biofilm development even below the MIC.

To further assess antimicrobial effects, colony-forming units (CFU) were quantified. The nanoemulsion reduced the number of viable biofilm-associated cells by approximately 20% ($p < 0.0001$) compared with the positive control. Mean CFU values were 7.10 ± 0.13 at 0.78 mg/mL, 6.79 ± 0.09 at 1.56 mg/mL, and 6.16 ± 0.10 at 3.12 mg/mL (Fig. 2), indicating a moderate but statistically significant reduction in viable bacterial cells. Morphological analysis by scanning electron microscopy (SEM) supported these findings. The images (Fig. 3) showed marked disruption and reduction of the biofilm structure following treatment with the nanoemulsion, corroborating the quantitative results obtained in the biofilm eradication and inhibition assays.

Compound safety profile

Regarding the compound safety profile, genotoxicity and cytotoxicity assays demonstrated that the *E. caryophyllus*

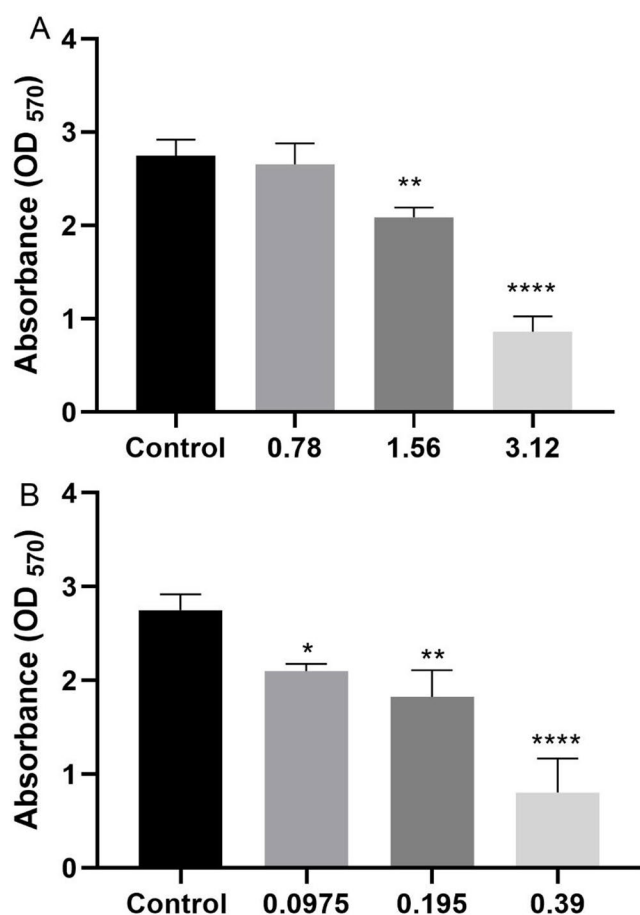


Fig. 1 Biofilm quantification of preformed biofilm by crystal violet staining after treatment with *E. caryophyllus* nanoemulsion (0.78, 1.56, 3.12 mg/mL) (A). The efficiency of biofilm inhibition of *E. caryophyllus* nanoemulsion in sub-inhibitory concentrations (0.39, 0.195 and 0.0975 mg/mL) (B). An analysis of variance (ANOVA) followed by Dunnett test was used considering values $p < 0.001$ (***) statistically significant compared with the Control. Data was expressed as the Mean $\pm$ Standard deviation

nanoemulsion did not differ statistically from the negative control at MIC, 2×MIC, and 4×MIC concentrations (0.78, 1.56, and 3.12 mg/mL, respectively), indicating absence of detectable DNA damage or cytotoxic effects under the tested conditions. Mean genotoxicity values were 1129.75 ± 14.13 at 0.78 mg/mL, 1536.27 ± 16.88 at 1.56 mg/mL, and 1528.27 ± 1.71 at 3.12 mg/mL (Fig. 4).

In silico analyses further complemented the experimental findings. Bioavailability and toxicity predictions indicated that the tested compound did not violate Lipinski's rule-of-five parameters according to Molinspiration Cheminformatics. The maximum tolerated dose predicted by pkCSM for eugenol was 1.024 log (mg/kg/day). No significant hepatotoxicity or cytotoxicity was predicted. However, carcinogenicity and mutagenicity predictions varied among platforms: eugenol was classified as non-carcinogenic and non-mutagenic by pkCSM and ProTox-II, whereas OSIRIS

indicated possible carcinogenic and mutagenic potential, and suggested irritant properties (Table 3).

Molecular docking simulations performed using AutoDock Vina and AutoDock4 evaluated potential interactions between eugenol and inflammatory mediators (IL-1 β , IL-6, IL-8, IL-10, and TNF- α) (Table 4 and 5). All systems demonstrated energetically favorable docking. Among the targets, TNF- α (PDB: 1TNF) exhibited the highest binding affinity with eugenol (−6.1 kcal/mol and −6.2 kcal/mol), the lowest predicted K_i values (0.03378 mM and 0.02951 mM), and the best ligand efficiency (−0.51 kcal/mol), indicating stronger interaction in both software platforms (Fig. 5). Conversely, IL-10 (PDB: 2H24) showed the lowest affinity (−4.8 kcal/mol and −4.95 kcal/mol), higher K_i values (0.30 mM and 0.24 mM), and lower binding efficiency (−0.40).

Detailed interaction analysis revealed that eugenol binding to TNF- α is stabilized primarily by hydrophobic and π -stacking interactions. Hydrogen bonding with PRO C:117 contributes to specificity and stability of the complex. Additional π -alkyl and π -sigma interactions with proline and isoleucine residues, along with π - π T-shaped interactions with tyrosine residues, suggest favorable accommodation of the aromatic ring and aliphatic chain of eugenol within hydrophobic pockets of the binding site. The combination of these interactions, including van der Waals forces, indicates efficient binding of eugenol to the target protein.

Discussion

To the best of our knowledge, this is the first study to evaluate an *E. caryophyllus* nanoemulsion against *S. mutans* in both planktonic form and organized as a biofilm, combining in vitro, ex vivo, and in silico approaches. The physicochemical characterization confirmed that the developed system meets the expected parameters for nanoemulsions. According to Charri et al. [37], droplet sizes typically range from 50 to 500 nm, remaining within the nanoemulsion classification even at the upper limit [38]. In the present study, the mean droplet size (117 ± 2.6 nm) indicates a well-defined nanometric system, likely influenced by the use of Tween 80 as a surfactant. Similar findings have been reported by da Silva Gündel et al. [16], who observed droplet sizes below 200 nm using the same surfactant. In addition, Tween 80 has been associated with negative zeta potential values, which is consistent with the results obtained in this study [19, 39].

The polydispersity index (PDI) further supports the homogeneity and stability of the formulation [38]. As described by Bhattacharjee [40], PDI values between 0.1 and 0.4 indicate moderate polydispersity, which is generally acceptable for pharmaceutical applications. The observed PDI (0.26) reflects a uniform size distribution, minimizing

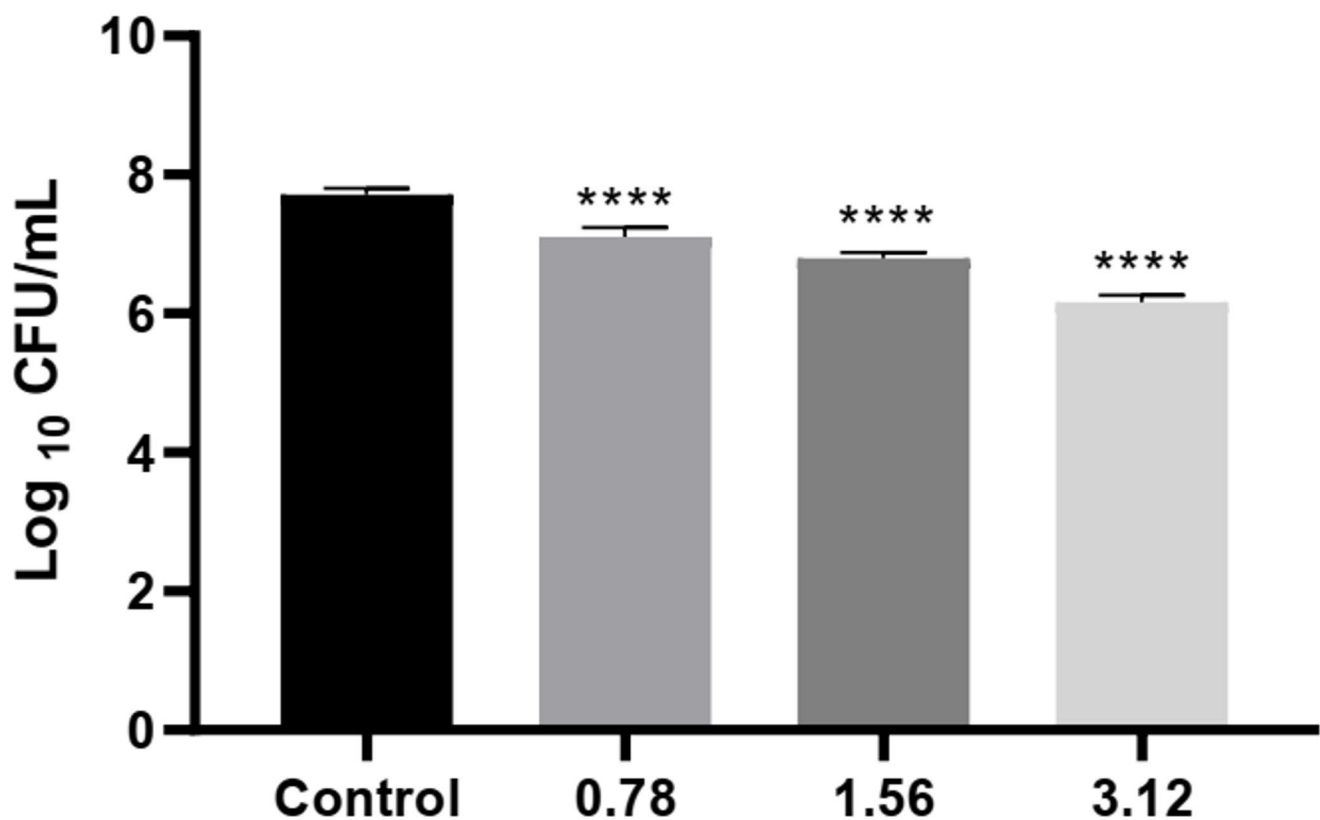


Fig. 2 Effect of *E. caryophyllus* nanoemulsion (0.78, 1.56 and 3.12 mg/mL) on viable cells of *S. mutans* in the biofilm. An analysis of variance (ANOVA) followed by Dunnet test was used considering values

$p < 0.01$ (**) and $p < 0.001$ (***) statistically significant comparing with Control. Data was expressed as the Mean $\pm$ Standard deviation

risks of aggregation and ensuring consistent drug release [41–43]. The zeta potential (-7.9 ± 0.4 mV), although relatively low in magnitude, is consistent with nanoemulsions stabilized by nonionic surfactants. In such systems, steric stabilization predominates over electrostatic repulsion, as surfactant molecules form a protective interfacial layer that prevents droplet coalescence [43–46]. The slightly acidic pH (~ 4) may be associated with oxidative degradation of essential oil components or partial hydrolysis of surfactants during processing [46].

Stability analysis over 15 days demonstrated that the nanoemulsion maintained its physicochemical integrity, including droplet size, PDI, zeta potential, pH, and concentration of major constituents. This stability can be attributed to the reduced droplet size, which enhances Brownian motion and counteracts gravitational separation, as well as to the steric stabilization conferred by the surfactant [47, 48]. Importantly, the preservation of the chemical composition suggests that the high-agitation homogenization method was effective in protecting volatile and thermosensitive compounds. These findings reinforce nanoemulsification as a valuable strategy for improving the stability and functionality of essential oils in pharmaceutical applications [19, 39].

The antimicrobial evaluation using *S. mutans* is highly relevant, given its well-established role as a key cariogenic pathogen. Although not a primary colonizer, *S. mutans* contributes significantly to biofilm virulence due to its acidogenic and aciduric properties, which drive enamel demineralization and caries progression [49–51]. In this context, nanostructured systems containing essential oils have emerged as promising alternatives for controlling oral biofilms [52, 53]. The nanoemulsion developed in this study demonstrated significant antimicrobial activity, including inhibition of bacterial growth, bactericidal effects, inhibition of biofilm formation, and disruption of pre-formed biofilms.

The observed MIC (0.78 mg/mL) and MBC (1.56 mg/mL) values are consistent with previous reports on clove oil nanoemulsions [54]. Although these values are higher than those typically reported for chlorhexidine [55], they fall within the expected range for essential oil-based systems [56, 57], supporting their therapeutic relevance as alternative antimicrobial agents. The antimicrobial activity can be attributed to the chemical composition of the oil, particularly eugenol, eugenol acetate, and E-caryophyllene [58]. These compounds exhibit intrinsic antimicrobial properties, which may be enhanced by synergistic interactions within the complex phytochemical matrix [59, 60]. Furthermore,

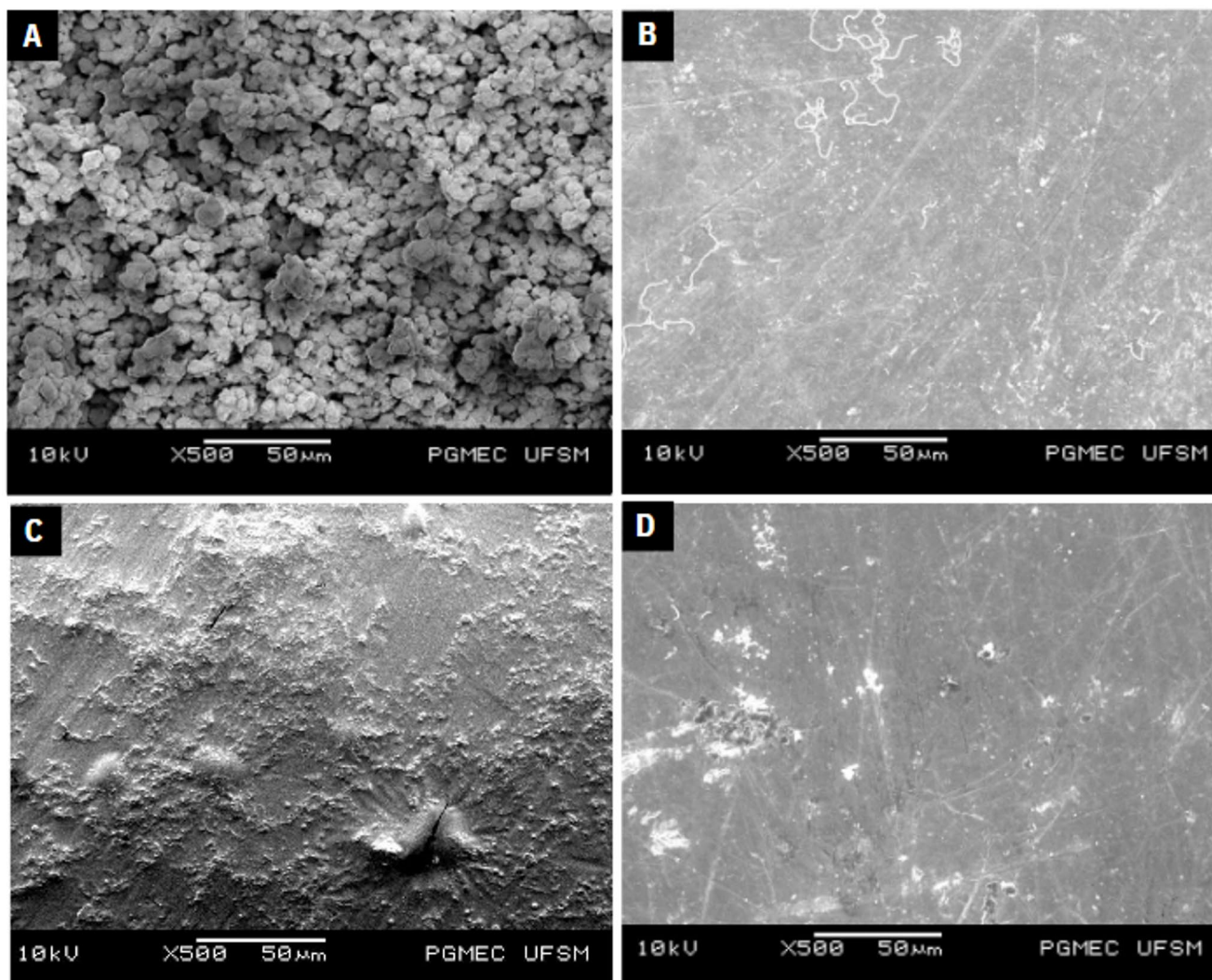


Fig. 3 Scanning electron microscopy of specimens treated with different concentrations of *E. caryophyllus* nanoemulsion. **A** Positive control; **B** treatment with 0.78 mg/ml (MIC) concentration; **C** treat-

ment with a concentration of 1.56 mg/ml (2x MIC); **D** Treatment with 3.12 mg/ml (4x MIC) concentration

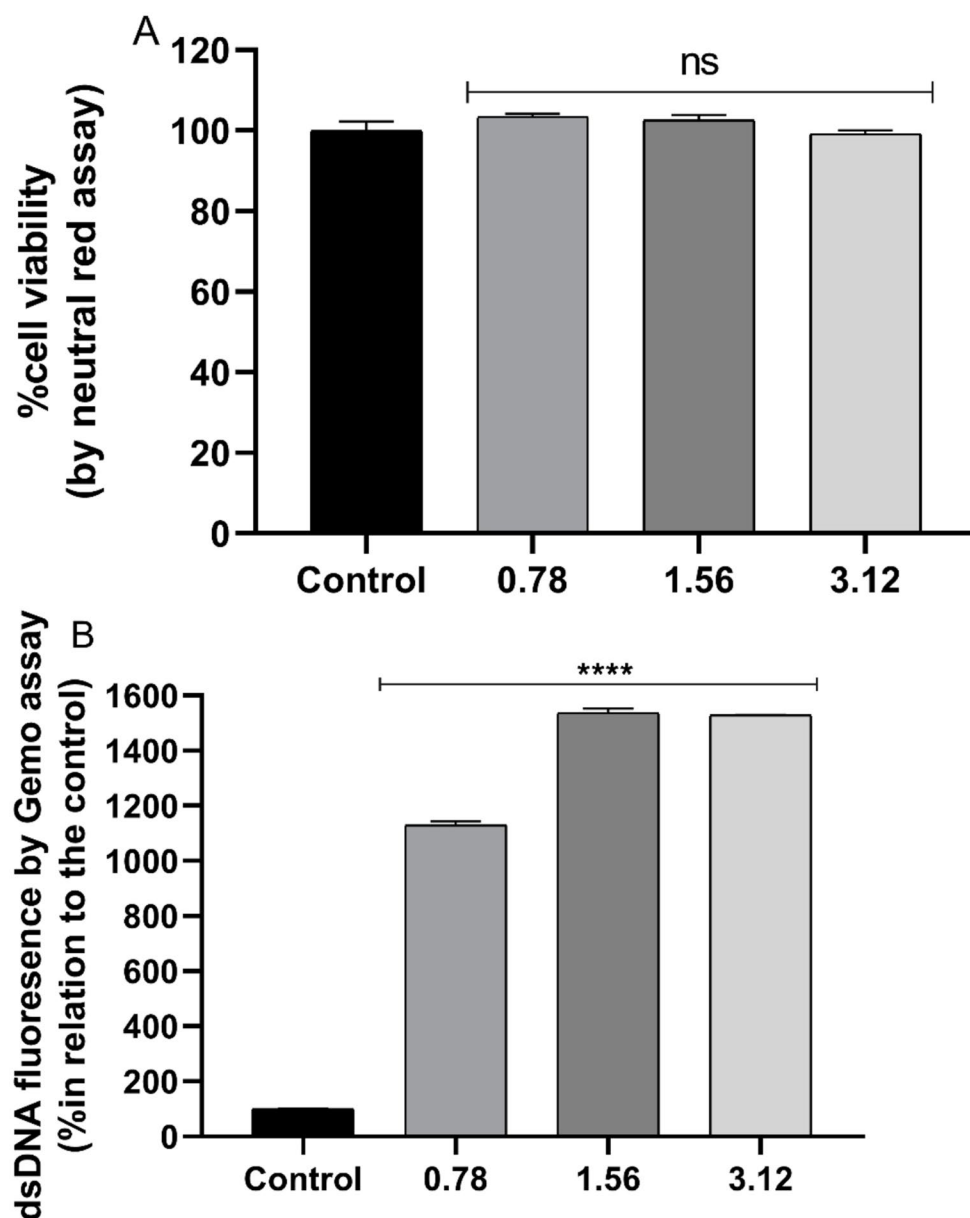
nanoencapsulation likely potentiates these effects by increasing surface area and facilitating interaction with microbial membranes, thereby enhancing permeability and cellular disruption [61].

A comprehensive assessment of antibiofilm activity was achieved through the combined use of crystal violet (CV) staining and colony-forming unit (CFU) quantification. These methods provide complementary information: while the CV assay measures total biofilm biomass, including extracellular polymeric substances (EPS) and both viable and non-viable cells, CFU counts reflect only viable cultivable bacteria [62]. In the present study, a marked reduction in biofilm biomass was observed, accompanied by a comparatively smaller decrease in CFU counts. This pattern suggests that the nanoemulsion primarily disrupts biofilm structure and matrix integrity rather than exerting an

exclusively bactericidal effect. Such findings are consistent with mechanisms involving EPS destabilization, reduced adhesion, and biofilm detachment, ultimately leading to structural disorganization [63].

These results were further supported by scanning electron microscopy (SEM), which revealed pronounced morphological alterations, reduced biofilm density, and, in some cases, the apparent absence of bacterial cells following treatment. The observed structural disruption is consistent with the proposed mechanism of action of eugenol, which involves membrane permeabilization and leakage of intracellular components such as ATP and potassium ions. Thus, SEM analysis provided qualitative confirmation of the antibiofilm effects detected by quantitative assays, reinforcing the robustness of the findings [64, 65]

Fig. 4 Cytotoxicity assay after 24 h of incubation. Results are expressed as a percentage of the negative control (100%). Data are expressed as mean $\pm$ standard deviation (SD) (A). Genotoxicity by GEMO assay in dsDNA after exposure to nanoemulsion. Bars represent the means (B). Analyses were performed by one-way ANOVA followed by Tukey Dunnett's post hoc test. Values with $p < 0.0001$ were considered statistically significant (****). The concentrations correspond to those found in the dilution of the *Eugenia caryophyllus* nanoemulsion (0.78, 1.56 and 3.12 mg/mL) in cito (A) and genotoxicity (B)



Regarding safety, the absence of cytotoxicity and genotoxicity observed in this study is consistent with the established safety profile of eugenol, which is recognized by regulatory agencies such as the FDA as a generally safe compound [16]. Similar results have been reported in studies evaluating eugenol-based nanoemulsions in human cells, demonstrating low toxicity and good biocompatibility [16]. Complementing these findings, the in silico analysis suggested a favorable safety margin, with a predicted maximum tolerated dose indicative of low systemic toxicity. Previous studies also support the safety of eugenol at therapeutic doses, although adverse effects may occur at higher concentrations [65, 66].

It is important to note that the literature presents conflicting evidence regarding the hepatotoxicity of eugenol. While

some studies report liver damage at high doses [67–69], others demonstrate hepatoprotective effects depending on dosage and experimental conditions [70]. These findings highlight the dose-dependent nature of eugenol's biological effects and underscore the importance of controlled application. Similarly, computational predictions of carcinogenicity and mutagenicity showed variability among different models, reflecting known limitations of in silico approaches and the complexity of eugenol's pharmacological profile [71].

The molecular docking analysis provided additional insights into the potential biological activity of eugenol. The interactions observed, including hydrogen bonding and hydrophobic interactions such as π -alkyl and π - π stacking, suggest a stable binding to the target protein, supporting

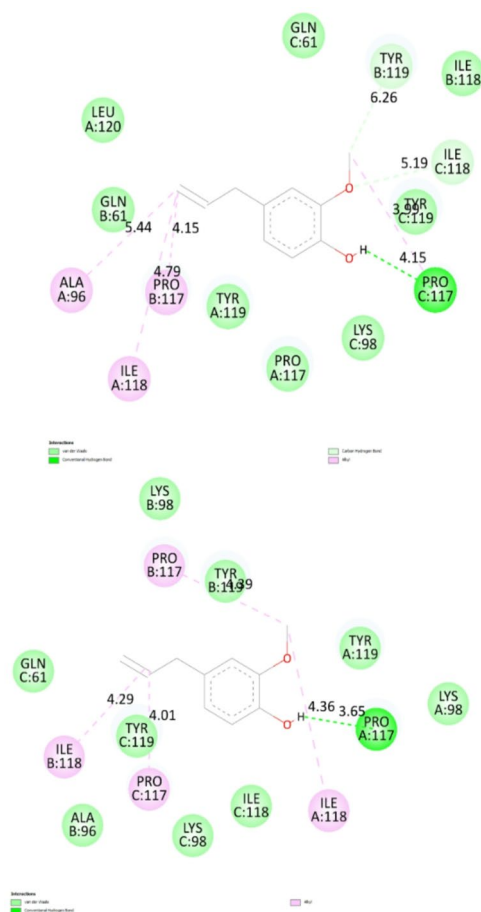


Fig. 5 Graphical representation of the molecular docking interactions of eugenol with the 1TNF protein. (A) Results obtained with AutoDock Vina software showed hydrophobic interactions occur with residues of lysine (LYS B:98, LYS A:98, LYS C:98), proline (PRO B:117, PRO C:117, PRO A:117), tyrosine (TYR B:119, TYR A:119, TYR C:119), isoleucine (ILE B:118, ILE C:118, ILE A:118), alanine (ALA B:96) and glutamine (GLN C:61), with distances ranging from 4.01 to 4.36 Å. A hydrogen bond is formed between one of the hydroxyl groups of eugenol and the proline residue (PRO A:117), with a distance of 3.65 Å. (B) Results obtained with AutoDock4 software, present a conventional hydrogen bond is observed with the residue PRO C:117 at a distance of 4.15 Å. Pi-Alkyl hydrophobic interactions were detected with GLN B:61 (5.44 Å), PRO B:117 (4.79 Å), ILE C:118 (5.19 Å) and TYR C:119 (3.98 Å and 4.15 Å). There are also Pi-Sigma interactions with PRO B:117 (4.15 Å) and Pi-Pi T-shaped interactions with TYR B:119 (6.26 Å) and TYR A:119 (5.77 Å). Other residues, such as ALA A:96, LEU A:120, ILE A:118, PRO A:117, LYS C:98, and GLN C:61, participate in van der Waals interactions with the ligand, indicated by the light green dotted lines

a possible modulatory effect on inflammatory pathways. This is particularly relevant in the context of oral diseases, where inflammation plays a central role alongside microbial colonization. Therefore, the dual functionality of eugenol, combining antimicrobial and potential anti-inflammatory effects, may represent an important therapeutic advantage.

Despite these promising findings, several limitations must be acknowledged. The study was conducted under

controlled laboratory conditions, which do not fully replicate the complexity of the oral environment. The use of bovine enamel, although standardized and comparable to human enamel, limits direct clinical extrapolation. Additionally, the monospecies biofilm model does not reflect the multispecies nature of oral biofilms. Regarding safety, although genotoxicity and cytotoxicity assays were performed, additional tests such as MTT could further strengthen the evaluation. Furthermore, the *in silico* findings should be interpreted as complementary theoretical evidence rather than definitive conclusions. Future studies should include *in vivo* and clinical investigations, as well as multispecies biofilm models, to enhance the translational relevance of these results.

Overall, the findings of this study demonstrate that the *E. caryophyllus* nanoemulsion exhibits promising antimicrobial and antibiofilm activity, combined with a favorable safety profile. These results support its potential as an alternative therapeutic strategy for the control of cariogenic biofilms and encourage further investigation toward clinical application.

Conclusion

Therefore, the *E. caryophyllus* nanoemulsion presented a satisfactory performance against *S. mutans*. However, *in situ* and *in vivo* tests are required to allow the clinical applicability of the compound.

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Data availability The data underlying this article are not available due to ethical restrictions.

Declarations

Competing interests The authors report there are no competing interests to declare. All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Ethical approval Not applicable.

Informed consent Not applicable.

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