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# Clove essential oil extraction and the evaluation of the bactericidal properties and *in vitro* toxicity of its water emulsions

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ABSTRACT

It is well known that clove essential oil exhibits antimicrobial properties due to its high content of eugenol, a phenylpropanoid. However, clove essential oil can also present cytotoxic effects on cell membranes of human tissues. In this manner, clove essential oil was obtained from hydrothermal distillation of clove to avoid any traces of solvent that could interfere with biological characterization. The clove essential oil was characterized by Gas Chromatography-Mass Spectrometry (CG-MS) and Fourier-Transformed Infrared Spectroscopy (FTIR) to determine its specific composition. Then, clove essential oil-water emulsions were prepared by ultrasound at concentrations of 2.5, 5, 7.5, 10, and 12.5% w/w (mass of oil/mass of water). Then, the bactericidal properties of these emulsions were evaluated using the disk diffusion and broth microdilution methods against *E. coli* and *S. aureus*. In parallel, cell viability in human monocytes and porcine dermal fibroblasts was assessed by the MTT assay. The chemical composition of clove essential oil indicates that the main components are eugenol, trans-isoeugenol, and caryophyllene, which together account for 98.39%. The disk diffusion and broth microdilution methods indicate that the bactericidal concentration (>90%) is 2.5%. However, the cell viability test exhibits that concentrations even below a 2.5% have cytotoxic effects on monocytes and fibroblasts, leading to a cell viability of 20 to 40%, indicating that its use can cause tissue damage. Despite the outstanding bactericidal properties of clove essential oil, its high toxicity limits its therapeutic application.

**Keywords:** Clove Essential Oil; Water Distillation; Ultrasound; Oil-Water Emulsions; *E. coli*; *S. aureus;* Antibacterial Properties; Porcine Dermis Fibroblasts; Human Monocytes; Cell Viability.

## 1.0. Introduction

Antimicrobial resistance is the ability of bacteria, viruses, fungi, and parasites to resist antibiotics, leading to chronic infections and increased risk of death. According to the World Health Organization (WHO) antimicrobial resistance is among the top three major public health threats. Even though antimicrobial resistance is a natural process due to the genetic evolution of microbes, this has increased over the past three decades due to the use and misuse of antibiotics in pharmacological treatment for human beings, food for livestock farming to induce growth, and prophylactic therapeutics systems in agriculture to prevent pests [1], [2].

The slow development of new antimicrobials to treat infections caused by resistant pathogens has raised the percentage of healthcare-associated infections [3]. Then, the scientific community has focused on green antimicrobial agents such as phytochemicals (plant-derived), green-synthesized nanoparticles (plant, fungal, or bacterial-derived), antimicrobial peptides and bacteriophages, and essential oils [4], [5], [6].

Since ancient times, essential oils have been used by humanity as ingredients of perfumes, to aromatize food, and in folk medicine for their antimicrobial properties [6], [7], [8]. Essential oils have an inherent chemical composition comprising a mix of terpenes, terpenoids, phenols, and phenylpropanoids, which confer bactericidal properties [6], [7], [8]. The antibacterial effect of essential oils can be to inhibit bacterial growth (bacteriostatic) or to destroy bacterial cells (bactericidal) [7].

Among essential oils, clove oil is reported to have a chemical composition with a major content of eugenol, β-caryophyllene, and eugenol acetate [9]. Also, clove essential oil has been reported to possess anti-inflammatory, analgesic, antiparasitic, antioxidant, anti-carcinogenic, and antimicrobial properties [10], [11].

However, to the best of our knowledge, there has been no report about the evaluation of anti-bacterial properties using only water as a solvent. Commonly, this kind of experiment uses clove essential oil emulsions, with the addition of a surfactant or a solvent such as dimethyl sulfoxide (DMSO) [12], [13]. Thus, clove essential oil was extracted by hydrodistillation to avoid any sources or traces of organic solvents that could interfere with the antibacterial study. Then, water-clove essential oil emulsions were obtained using ultrasound, the diffusion disk method, and the spectrophotometric growth inhibition assay (Figure 1) and were used to assess the antibacterial properties of the oil-water emulsions. Additionally, the in vitro cell viability of human monocytes and porcine dermal fibroblasts was evaluated to assess the cytotoxicity of clove essential oil emulsions.

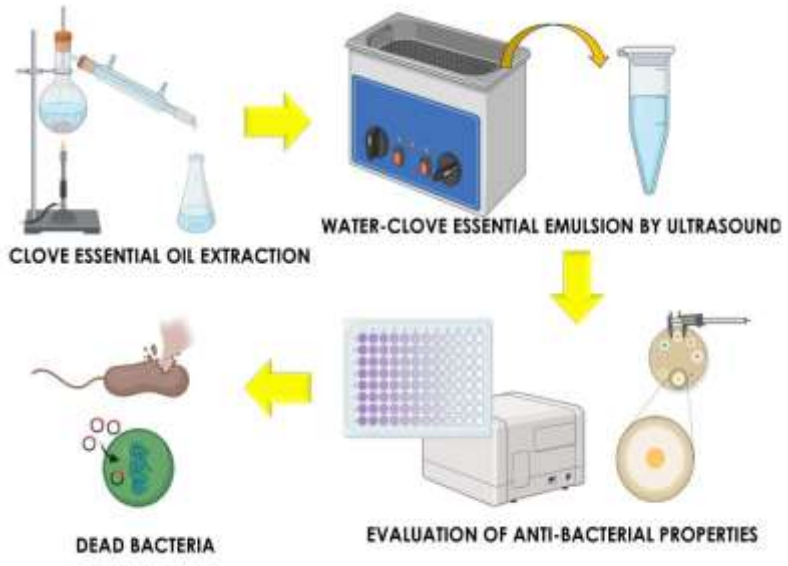


**Figure 1.** Overview of clove essential oil extraction, ultrasonic oil-water emulsification, and antibacterial evaluation via disk diffusion and spectrophotometric growth inhibition assays. *Created in BioRender. Cabrera, D. (2026) https://BioRender.com/a54dmw7*

### 1.1. Study objectives

1) Obtaining essential oil by hydrodistillation, to avoid the use of organic solvents.
2) Characterization of the chemical composition of clove essential oil by FTIR and CG-MS.
3) Evaluate the antibacterial properties of a clove essential oil microemulsion in water, in both solid and liquid media.
4) Assess the in vitro cytotoxicity of these emulsions in human monocytes and porcine dermal fibroblasts, analyzing cell viability by MTT assay.
5) The objective was to find a suitable concentration necessary to obtain bactericidal properties, but without damaging beneficial cells such as monocytes and fibroblasts.

## 2.0. Literature

The most recent research works related to the evaluation of clove essential oil-water emulsification are summarized and presented in Table 1. In practice, clove essential oil-water emulsifications are typically formed using ultrasound energy and surfactant and co-surfactant agents, with Tween 80 typically used as the surfactant. The suitable amount of surfactant can vary from 5-45 % of the total mass of the emulsion, the rest being water. A high concentration of surfactants leads to a smaller droplet size, which ranges from 6 nm to 300 nm. This is important to note, as a lower droplet size can more easily penetrate the bacterial cell membrane, which has a minimum diameter of 7 nm [14], and thus a Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) are needed.

It is worth noting that, in practice, the biological characterization of clove essential oil-water emulsions commonly focuses on demonstrating antibacterial properties by disk diffusion assay and/or determining MIC and MBC by broth microdilution, but only a few studies evaluate in vitro cell viability upon exposure to clove essential oil-water emulsions.

Thus, the aim of the present research work is to evaluate the antibacterial properties of clove essential oil-water emulsifications formed by ultrasound energy without any addition of surfactant or solvent, as well as the parallel evaluation of their biocompatibility by hemolysis and in vitro cell viability, the latter being our contribution to the body of knowledge in frontier science.

**Table 1.** Most recent clove essential oil-water emulsification research works evaluating their antibacterial and biocompatibility properties.

| **Oil percentage** | **Surfactant** | **Droplet size** | **Biological characterization** | **Reference** |
|---|---|---|---|---|
| 5-40 wt. % | Tween 20,<br>Tween 80,<br>(5-45%) | 6-27 nm | Disk diffusion assay<br>*S. aureus.* -There is no reported value | [12] |
| 2.5-15 wt. % | Tween 80,<br>Span 80 (2.5-7.5 %) | 50 nm | Broth microdilution assay<br>*E. coli*.-<br>MIC: 16 µg/mL<br>MBC: 32 µg/mL<br>*B. cereus*.-<br>MIC: 16 µg/mL<br>MBC: 64 µg/Ml | [15] |
| 0-25 wt. % | Montanov 202 (4%) | 303.67 ± 4.03 nm | A percentage beyond 15%, the cell viability of Mouse skin fibroblast decreased below a 80%<br>(Cell viable concentration of 10 %) | [16] |

| | | | | |
|---|---|---|---|---|
| 0.25-2.33 mg of oil per mL of emulsion | None | Not mentioned | Broth microdilution assay<br>*E. coli* and *B. subtilis*.-<br>MIC: 2.33 mg/mL | [17] |
| 0.25-2.25 wt. % | Tween 80,<br>Span 80 (<10%) | 120-210 nm | Broth microdilution assay<br>*E. coli*. -<br>MIC: 0.5 mg/mL<br>MBC: 1 mg/mL<br>*S. aureus*. -<br>MIC: 0.25 mg/mL<br>MBC: 2 mg/mL | [18] |
| 1-10 wt. % | Whey protein concentrate (0.1-1%) | 279.0 ± 8.43 nm | Broth microdilution assay<br>*E. coli* and<br>*B. subtilis*. -<br>MIC: 50 µL<br>MBC:90 µL | [19] |
| 1 % v/v | Tween 80 (3 % v/v) | 30.76 nm | *E. coli*. -<br>MIC.-7.75 mg /mL<br>*S. aureus*. -<br>MIC.-3.87 mg/mL<br>*P. aeruginosa*. -<br>MIC.-3.87 mg/mL | [20] |
| 10-25 wt. % | Tween 80,<br>Transcutol P (1:1 w/w)<br>40-55% of the emulsion | <20 nm | *E. coli*. -<br>MIC.-10-42 µg /mL<br>*S. aureus*. -<br>MIC.-5-42 µg/mL | [21] |

## 3.0. Methodology

### 3.1. Clove oil extraction

The clove essential oil was obtained by hydrodistillation, a green method that avoids the use of organic solvents [22]. In a typical procedure, 100 grams of dried clove buds were transferred into a 2-liter round-bottom flask, then 500 mL of deionized water was added to the flask, which was connected to a water-cooled condenser. The heating mantle was turned on, and the mixture was then left to gently boil for 4 hours. The steam carried the clove essential oil into the condenser, and the water-and-oil suspension was collected in a beaker. The suspension was transferred to a separating funnel; the clove essential oil was the heavy phase. Finally, sodium sulfate was added to the clove essential oil in a 25 wt. % of the mass of essential oil to avoid any moisture, then left overnight. The purified clove essential oil was stored in a refrigerator in a glass vial covered with aluminum foil.

### 3.2. Characterization of clove oil

Qualitative and quantitative analyses of the chemical components of clove essential oils were performed using GC-MS. A sample of 10 µL of clove essential oil was taken and dissolved in 1 mL of grade High-Performance Liquid Cromatography (HPLC) ethanol for further analysis using a CLARUS 580/SQ8S Perkin Elmer CG-MS. The injector temperature was 250 °C, and the gas carrier (He) flow rate was 0.8 mL/min, using an Elite-5 MS column (30 m x 0.32 mm x 0.25 µm).

To analyze the functional groups in clove essential oil, a sample was studied by infrared spectroscopy using a PerkinElmer Frontier spectrophotometer coupled to an attenuated total reflectance (ATR) accessory. The spectrum was collected from 4000 to 600 $cm^{-1}$ with a resolution of 4 $cm^{-1}$.

### 3.3. Water microemulsions

Every emulsion of water-clove essential oil was prepared using Eppendorf tubes of 2 mL, to every formulation, a volume of 1.5 mL of deionized water was fixed, and then volumes of 36, 71, 107, 141, and 180 µL of clove essential oil were added to formulate 2.5, 5, 7.5, 10, and 12.5% w/w (weight of oil/weight of water). Then, the suspension was placed in an ultrasound bath for 30 minutes, leading to whitish emulsions. The ultrasound energy reduces the drop size of clove essential oil, thereby favoring the formation of homogeneous emulsions. The Eppendorf tubes were protected of sunlight using aluminum foil and were kept under refrigeration conditions until the biological experiments were performed.

### 3.4. Disk diffusion test

Eosin methylene blue agar and tryptone glucose extract agar were used as bacterial culture media for *E. coli* and *S. aureus*, respectively. Each sterile culture medium was poured into Petri dishes, and once solidified, inoculated with the corresponding bacteria using a sterile loop. To evaluate the antimicrobial activity, the disk diffusion method was used with sterile filter paper disks. The disks were impregnated with the water-clove essential oil emulsions prepared at different concentrations and collocated in triplicate in each petri dish. Afterward, the petri dishes were incubated at 37 °C for 24 hours, and then the inhibition halo was measured. As a control, filter paper disks were impregnated with Gentamicin solution prepared at 20 ppm. The growth inhibition capacity of *E. coli* and *S. aureus* was calculated by comparing the diameter of the halo formed by the disk with the diameter generated by the positive control (Equation 1) [23]:

$$\text{Growth inhibition, \%} = \frac{\text{Emulsion diameter}}{\text{Control diameter}} \times 100 \qquad (1)$$

### 3.5. Spectrophotometric growth inhibition test

*E. coli* and *S. aureus* were suspended in eosin methylene blue agar and tryptone glucose extract agar culture medium, respectively. Then, 100 µL of each suspension was added to the wells of a 96-well microplate, and another 100 µL of the emulsions was added; this was done in triplicate. As a control, 100 µL of gentamicin at 20 ppm was used. The 96-well microplate was incubated at 37ºC for 24 h. The absorbance of the solutions was measured at 600

nm using a MultiSkan Sky spectrophotometer (Thermo Scientific). The inhibition percentage was calculated using Equation 2 [24]:

$$\text{Inhibition}, \% = \frac{\text{Absorbance}_{\text{Bacteria}} - \text{Absorbance}_{\text{Sample}}}{\text{Absorbance}_{\text{Bacteria}} - \text{Absorbance}_{\text{Antibiotic}}} \quad (2)$$

### 3.6. Evaluation of *in vitro* toxicity

The cell viability of human monocytes and dermal porcine fibroblasts was evaluated under contact with clove essential oil emulsions using the MTT assay. In a typical procedure, 100 µL of water-clove essential oil emulsion was placed in a well of a 96-well plate together with 100 µL of the appropriate cell suspension culture. Later, the 96-well plates were incubated at 24 and 48 h. Once the incubation period had passed, 10 µL of MTT solution was added to each well, and the 96-well plate was incubated for 3 additional hours to allow metabolically active cells to reduce MTT and form insoluble purple formazan crystals. Thereafter, the culture medium was carefully removed, and 100 µL of isopropanol was added to each well to solubilize the formazan crystals. Afterward, 30 µL of each sample was transferred to a new 96-well plate, followed by the addition of 170 µL of isopropanol. The absorbance of the samples was measured using a MultiSkan Sky spectrophotometer at 570 nm. The cell viability was calculated using Equation 3 [25]. The control was 100 µL solution of Phosphate Buffer Solution 1X (PBS 1X sterilized) instead of an emulsion sample:

$$\text{Cell viability}, \% = \frac{\text{Absorbance of emulsion}}{\text{Control absorbance}} \text{x} 100 \quad (3)$$

## 4.0. Results and Discussions

### 4.1. Clove essential oil characterization

Table 2 indicates the chemical composition of clove essential oil found by CG-MS. It is found that eugenol, trans-isoeugenol, and caryophyllene are the three major components, accounting for 98.39% in total. In contrast to other reports, eugenol acetate was not detected in this sample [26], [27]; instead, methyl salicylate was quantified, as in other studies [11]. However, this clove essential oil contains a high level of eugenol and its isomer, totalling 91.32%.

**Table 2.** Qualitative and quantitative chemical composition of clove essential oil analyzed by CG-MS.

| No. | Chemical compound | Percentage, % |
|---|---|---|
| 1 | Eugenol | 84.07 |
| 2 | Trans-isoeugenol | 7.25 |
| 3 | Caryophyllene | 7.07 |
| 4 | Humulene | 0.90 |
| 5 | Methyl salicylate | 0.28 |
| 6 | 14-hydroxycaryophyllene | 0.25 |
| 7 | Phenol-4-(2-propenyl) | 0.09 |
| 8 | Eucalyptol | 0.06 |
| 9 | 3-allyl-6-methoxyphenol | 0.02 |
| 10 | Phenol-2-methoxy-4-(1-propenyl)-z | 0.02 |

The clove essential oil spectrum (Figure 2) exhibits a broad band at 3518 $cm^{-1}$, which can be attributed to the stretching vibration of hydroxyl groups associated with phenolic compounds such as eugenol. The bands at around 2900 and 2940 $cm^{-1}$ are related to the asymmetric and symmetric stretching of C-H bonds in $-CH_2$ and $-CH_3$ groups. The vibrational band at 1765 cm-1 is attributed to methyl salicylate, a benzoate ester identified in CG-MS analysis [26], [27].

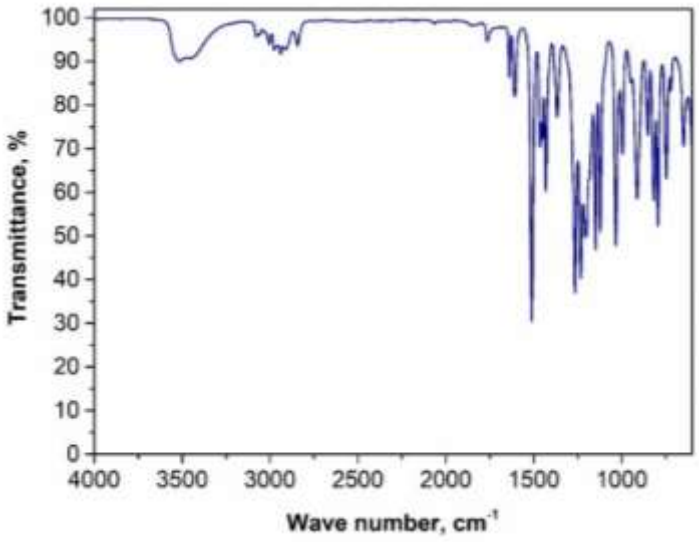


**Figure 2.** ATR-FTIR spectrum of clove essential oil

### 4.2. Bactericidal properties of water-clove essential oil microemulsions

The disk diffusion assay was performed to determine if *E. coli* is susceptible or resistant to clove essential oil emulsions. Figure 3 shows the percentages of halo inhibition, indicating that 2.5% w/w inhibits the growth of E. coli by 90.7% compared with antibiotics. When the percentage of clove essential oil is increased to 5%, the growth inhibition is superior to that of the antibiotic applied, gentamicin, and there is no statistically significant difference between 7.5% and 10% w/w ($p>0.05$).

Similarly, Figure 4 shows the growth inhibition percentages for S. aureus, indicating the strain's resistance or susceptibility to clove essential oil emulsions compared with gentamicin. The results indicate that there is no statistically significant difference between clove essential oil emulsions of 2.5, 5, 7.5, and 10% w/w with $p>0.05$. Thus, 2.5% w/w is sufficient to inhibit the growth of S. aureus, with 97.9% inhibition compared to gentamicin.

Hence, *E. coli* requires a higher concentration than *S. aureus* to inhibit bacterial growth; this result is due to differences in the membranes of the two bacteria. *E. coli* is a Gram-negative bacterium; this implies a thin peptidoglycan layer protected with an outer membrane chemically composed of lipopolysaccharides. While S. aureus is a Gram-positive bacterium with a thick layer of peptidoglycan crosslinked with teichoic acids. As mentioned above, 91.32% of this clove essential oil consists of eugenol and its isomer, indicating a high degree of hydrophobicity. The protective outer membrane of *E. coli*, made of lipopolysaccharides (amphipathic molecules), makes it more resistant to the penetration of hydrophobic molecules such as eugenol. In the case of *S. aureus*, the lack of an outer membrane, along with the behavior of the thick peptidoglycan layer, which acts as a porous barrier, makes the cytoplasmic membrane more susceptible to hydrophobic compounds like eugenol [28], [29].

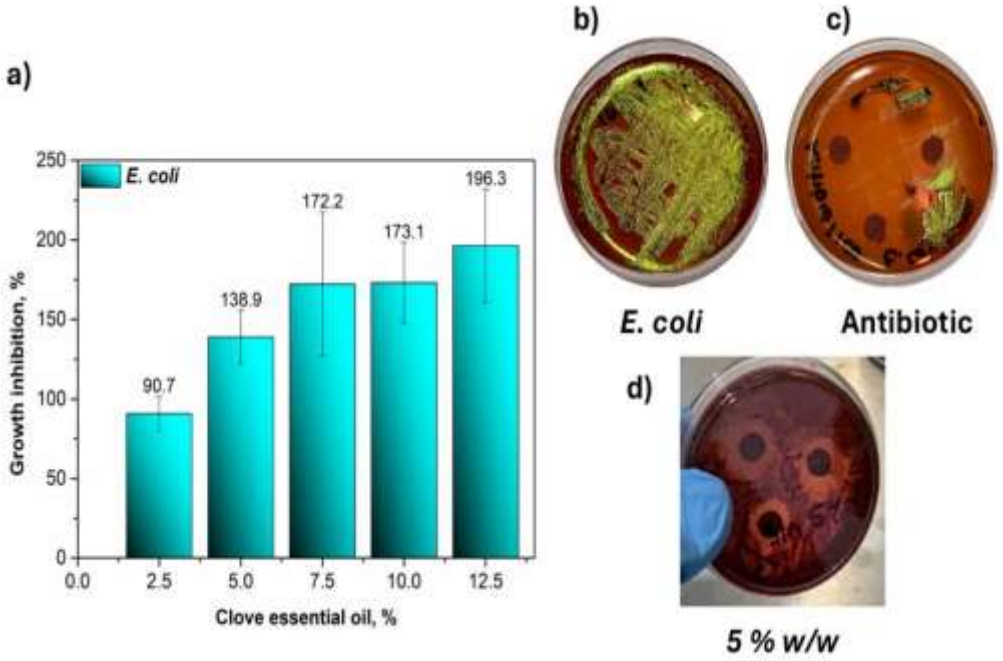


**Figure 3.** a) Growth inhibition of *E. coli* calculated by the diffusion disk method, b) Petri dish with bacteria, c) Petri dish with gentamicin, and d) petri dish with emulsion of 5 w/w %.

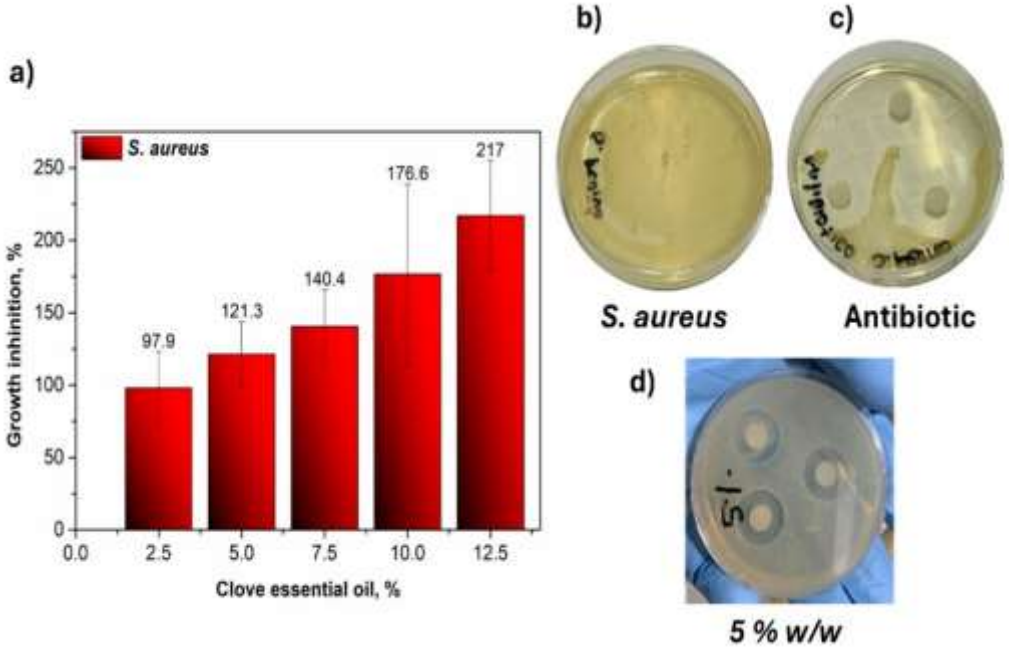


**Figure 4.** a) Growth inhibition of *S. aureus* calculated by the diffusion disk method, b) Petri dish with bacteria, c) Petri dish with gentamicin, and d) Petri dish with emulsion of 5 w/w %.

The disk diffusion method is a qualitative assay that measures only bacterial inhibition zones. Then, bacterial inhibition was followed by spectrophotometry, which provides a quantitative assay in liquid broth to track total bacterial biomass by measuring optical density, a more accurate method [24].

Figure 5 depicts the inhibition percentage *of E. coli* and *S. aureus* after 24 h of incubation. The results indicate that the emulsion of clove essential oil with a 2.5% w/w is enough to inhibit the bacteria's growth in a similar way to the

antibiotic (gentamicin) for *E. coli* and *S. aureus.* This is probably because, at lower clove essential oil concentrations, the droplets generated by ultrasound are smaller than at higher concentrations, which facilitates eugenol permeability in both bacteria [30]. According to the inhibition percentages, S. aureus is more susceptible to eugenol than E. coli; this is related to the low particle size at low clove essential oil concentration (2.5 %w/w) and to the outer membrane of *E. coli.* This result is in line with the disk diffusion assay.

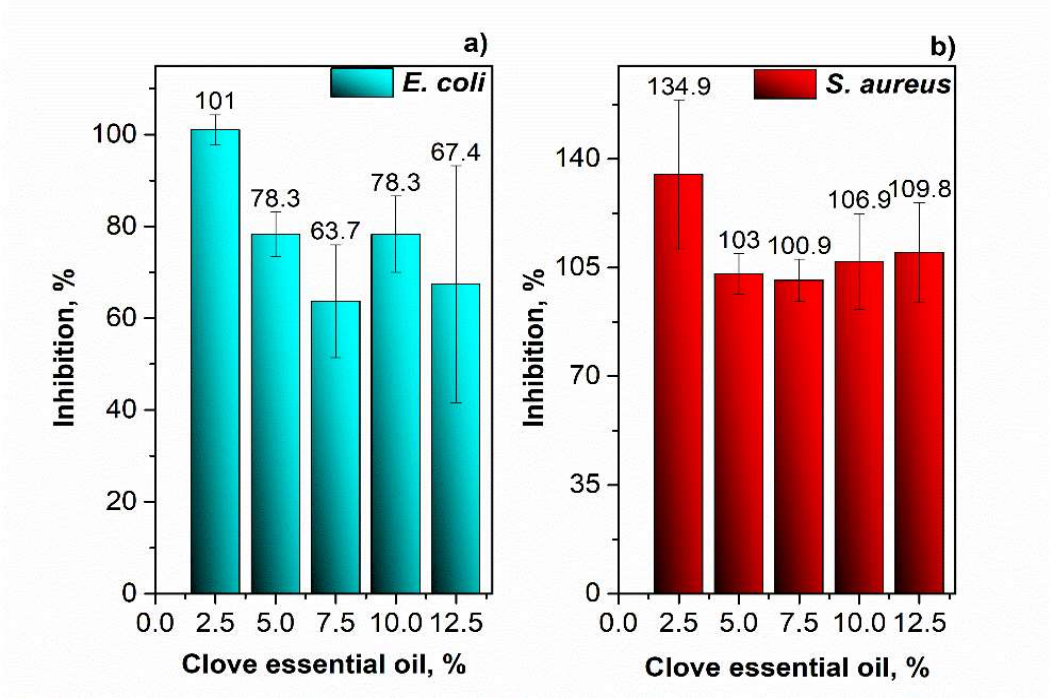


**Figure 5.** a) Inhibition of *E. coli*, and b) *S. aureus* quantified by UV-Vis spectrophotometry

### 4.3. Evaluation of in vitro toxicity of water-clove essential oil microemulsions

To analyze the effect of cytotoxicity of water-clove essential oil microemulsions. Human monocytes and porcine dermal fibroblasts were exposed to clove essential oil emulsion for 24 and 48 h. The MTT assay measures cellular metabolic activity and indicates cell viability, proliferation, and cytotoxicity. The chemical reaction occurs only in living cells, and an increase over time indicates proliferation (cell division), while a decrease indicates cell death or a cytotoxic effect [31]. ISO 10993-5 specifies that a percentage below 70% indicates a cytotoxic effect of a chemical substance [32].

Figure 6 plots the results of cell viability, adding the result of an emulsion with clove essential oil at a 0.5 % w/w. The results indicate that the lowest concentrations of clove essential oil (0.5, 2.5, and 5% w/w) exhibit cytotoxic effects at 24 and 48 h in monocytes and fibroblasts, likely due to the small droplet size of clove essential oil generated during ultrasound, as mentioned above [30]. When the clove essential oil concentration increases to 7.5%, the toxic effect remains marked in human monocytes. However, when the concentration rises to 10 and 12.5%, these emulsions are categorized according to ISO 10993-5 as non-cytotoxic at 24 h, but over time, at 48 h, the cell viability decreases for monocytes and fibroblasts, which is indicative of the probable toxicity of water-clove essential oil emulsion to healthy tissues. Thus, clove essential oil should be encapsulated to achieve a controlled diffusion rate, thereby avoiding cell damage and killing targeted bacteria.

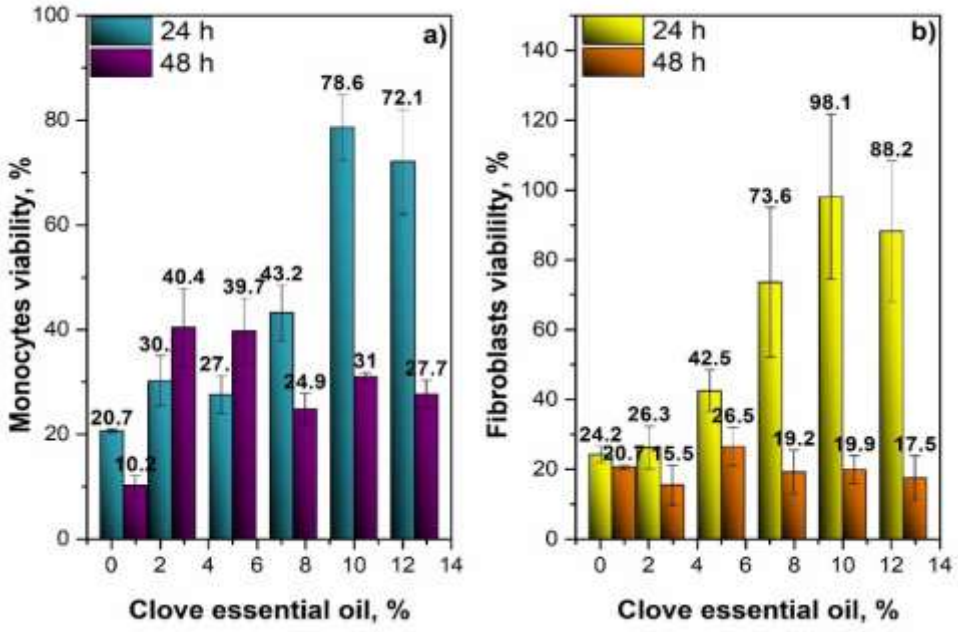


**Figure 6.** Cell viability of a) human monocytes, and b) porcine dermal fibroblasts

## 5.0. Conclusion and Future Recommendations

The quantitative and qualitative characterization of clove essential oil indicates high purity obtained by hydrodistillation, with eugenol and its isomer as the major components, accounting for 91.32%. The evaluation of antibacterial properties in solid and liquid media indicates that a clove essential oil emulsion at 2.5% w/w is as effective as gentamicin in inhibiting the growth of E. coli and S. aureus.

However, the cytotoxic effect measured by the MTT assay suggests that the lowest concentrations are cytotoxic due to the smallest clove essential oil droplet size when the emulsion was generated under ultrasound. In addition, after 48 h of cell contact, cellular metabolic activity begins to decrease, indicating cytotoxic effects even at higher concentrations.

Future work recommendations are:

1. The encapsulation of clove essential oil in a hydrogel for sustained delivery, to avoid cytotoxic effects, and to synthesize wound dressings.
2. Create a microemulsion of clove essential oil with tween 80, a surfactant.
3. Evaluate the effect of the concentration of microemulsion in hydrogel: physical, chemical, and biological properties.
4. Use biopolymers for hydrogel synthesis, such as hyaluronic acid, gelatin, chitosan, or collagen.
5. The evaluation of the antibacterial properties of these hydrogels via disk diffusion, spectrophotometric growth inhibition test, determination of Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC).

**Declarations**

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**Conflict of Interest**

The authors declare no conflicts of interest.

**Consent for Publication**

The authors declare that they consented to the publication of this study.

**Authors' contributions**

DACM and JACR conceived and designed the study. DEHG and LKUD performed the experiments and collected the data. LFCS and TEFG analyzed the data. DACM wrote the first draft of the manuscript. All authors read, revised, and approved the final manuscript.

**Informed Consent**

Not applicable.

**Availability of data and material**

All data is included within the article.

**Institutional Review Board Statement**

Not applicable.

**Ethical Approval**

Not applicable.

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**Declaration of Artificial Intelligence**

Not applicable.

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