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Antibacterial Activity against *Staphylococcus aureus* and *In Vitro* Cytotoxicity of a *Pimpinella anisum* Essential Oil Nanoemulsion

Actividad antibacteriana frente a *Staphylococcus aureus* y citotoxicidad *in vitro* de una nanoemulsión de *Pimpinella anisum*

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Highlights:

1. *Pimpinella anisum* nanoemulsion showed colloidal stability and antibacterial activity under the evaluated conditions.
2. Nanoemulsion cytotoxicity was concentration-dependent.
3. The findings support further exploration of this system as a complementary approach for microbiological control.

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ABSTRACT

Introduction. Microbial resistance to disinfectants is an increasing challenge because it can promote pathogen persistence on surfaces and devices. *Staphylococcus aureus* is characterized by its ability to form biofilms and cause healthcare-associated infections and contamination in food-related environments. In this context, essential oils such as that of *Pimpinella anisum* L. and their nanoemulsions have attracted interest because of their potential antimicrobial properties. **Objective.** To evaluate the antimicrobial activity and cytotoxicity of a *Pimpinella anisum* essential oil nanoemulsion against *Staphylococcus aureus* and Vero cells. **Materials and Methods.** A *P. anisum* nanoemulsion (NEPa) was prepared and colloidally characterized. Cytotoxicity was assessed in Vero cells using the MTT colorimetric assay. Antimicrobial activity against *S. aureus* was evaluated by a direct-contact method using concentrations of 300 and 600 mg/L and exposure times of 1, 3, 5, and 10 min. **Results.** A monodisperse NEPa with a nanoscale hydrodynamic diameter was obtained. The nanoemulsion showed a CC50 of 185.3 mg/L and a CC90 above 740.74 mg/L. At 600 mg/L, NEPa reduced *S. aureus* growth by 82.3% after 1 min and by 86.4% after 10 min of exposure. **Conclusions.** *P. anisum* NEPa showed antimicrobial activity against *S. aureus* and concentration-dependent cytotoxicity. These findings support its potential as an antimicrobial system; however, further studies are required to optimize concentration and exposure conditions and to establish an appropriate balance between antimicrobial efficacy and cytotoxicity.

Keywords: essential oil; *Pimpinella anisum*; nanoemulsion; cytotoxicity; antimicrobial activity; *Staphylococcus aureus*.

RESUMEN

Introducción. La resistencia microbiana frente a los desinfectantes constituye un desafío creciente, ya que puede favorecer la persistencia de patógenos en superficies y dispositivos. *Staphylococcus aureus* se caracteriza por su capacidad para formar biopelículas y causar infecciones asociadas a la atención en salud y contaminación en entornos relacionados con alimentos. En este contexto, los aceites esenciales, como el de *Pimpinella anisum* L., y sus nanoemulsiones han despertado interés por sus potenciales propiedades antimicrobianas. **Objetivo.** Evaluar la actividad antimicrobiana y la citotoxicidad de una nanoemulsión de aceite esencial de *Pimpinella anisum* frente a *Staphylococcus aureus* y en células Vero. **Materiales y métodos.** Se preparó y caracterizó coloidalmente una nanoemulsión de *P. anisum* (NEPa). La citotoxicidad se evaluó en células Vero mediante el ensayo colorimétrico MTT. La actividad antimicrobiana frente a *S. aureus* se evaluó mediante un método de contacto directo, empleando concentraciones de 300 y 600 mg/L y tiempos de exposición de 1, 3, 5 y 10 min. **Resultados.** Se obtuvo una NEPa monodispersa con diámetro hidrodinámico en escala nanométrica. La nanoemulsión presentó una CC50 de 185,3 mg/L y una CC90 superior a 740,74 mg/L. A 600 mg/L, la NEPa redujo el crecimiento de *S. aureus* en 82,3% al minuto 1 y en 86,4% a los 10 min. **Conclusiones.** La NEPa de *P. anisum* mostró actividad antimicrobiana frente a *S. aureus* y citotoxicidad dependiente de la concentración. Estos hallazgos respaldan su potencial como sistema antimicrobiano; sin embargo, se requieren estudios adicionales para optimizar la concentración y las condiciones de exposición y establecer un balance adecuado entre eficacia antimicrobiana y citotoxicidad.

Palabras clave: aceite esencial; *Pimpinella anisum*; nanoemulsión; citotoxicidad; actividad antimicrobiana; *Staphylococcus aureus*.

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INTRODUCTION

Surface disinfection is a fundamental practice for preventing and controlling the spread of microbial infections in different environments. However, in recent years, a significant increase in the resistance of pathogenic microorganisms to disinfectant agents has been reported [1], representing a serious global public health threat. The rise in microbial resistance compromises the effectiveness of disinfectant products and hinders pathogen elimination, which may facilitate the spread of infections or disease, particularly in hospital settings. In these environments, the presence of multidrug-resistant bacteria can interfere with disinfection processes and increase the risk of healthcare-associated infections [2]. In particular, the ESKAPE group includes pathogens of major clinical relevance because they are responsible for infections that are difficult to treat [3]. Among these pathogens, *Staphylococcus aureus* is recognized as one of the leading causative agents of healthcare-associated infections. This microorganism is of considerable microbiological importance because of its ability to colonize the skin, mucous membranes, and inert surfaces, as well as its broad range of virulence factors that enable adaptation to different ecological niches. In addition, the emergence and dissemination of multidrug-resistant strains, such as methicillin-resistant *S. aureus* (MRSA), have made this microorganism a major global public health concern. Its persistence in hospital, industrial, and food-related environments promotes cross-contamination and increases the risk of transmission and infection [4]. Like other ESKAPE pathogens, *S. aureus* is able to form biofilms, which represent an important strategy for microbial persistence on surfaces. These structured communities are embedded in an extracellular matrix that limits the penetration of antimicrobial agents and disinfectants, thereby favoring microbial persistence even after routine cleaning procedures. In *S. aureus*, biofilm formation contributes substantially to persistence on medical devices, food-processing equipment, and other frequently contacted surfaces, increasing tolerance to different microbiological control strategies [5]. In this context, there is growing interest in exploring alternatives with antimicrobial potential. Anise (*Pimpinella anisum* L.) is an aromatic plant cultivated for its seeds, which are rich in essential oils, and it has been used since ancient times in food and traditional medicine. It is recognized for its antioxidant, antimicrobial, anti-inflammatory, and antidiabetic properties and also provides B-complex vitamins and essential minerals that contribute to its nutritional and therapeutic value [6]. In particular, previous studies have shown that anise essential oil and its major chemical constituent, trans-anethole, together with other phenolic and terpenic compounds, exhibit activity against Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli,* and *Pseudomonas aeruginosa*, supporting its broad-spectrum antimicrobial activity and potential application in microbiological control

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strategies [7,8]. However, the direct application of essential oils is limited by their volatility, low water solubility, susceptibility to oxidation, and variability in bioavailability. In this context, nanoemulsions have emerged as encapsulation systems capable of improving the physicochemical stability of bioactive compounds, enhancing their dispersion in aqueous media, and increasing their interaction with microbial cells. These characteristics may enhance the antimicrobial activity of essential oils and broaden their potential application in disinfection processes [9]. Accordingly, the aim of this study was to evaluate the antimicrobial and cytotoxic effects of a *Pimpinella anisum* nanoemulsion against *Staphylococcus aureus* and in mammalian cells.

MATERIALS AND METHODS

Mammalian Cells and Microorganisms

Vero cells (ATCC CCL-81), donated by the Chemotherapy Laboratory of the Center for Research in Tropical Diseases at Universidad Industrial de Santander, were cultured in DMEM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco) at 37 °C, 5% CO_2, and 95% relative humidity in a controlled-atmosphere incubator. The *Staphylococcus aureus* ATCC 29213 strain, donated by the strain collection of the Bacteriology and Clinical Laboratory Program at Universidad de Santander, was cultured on blood agar and Baird-Parker agar at 37 °C for 24 h.

Preparation of the Nanoemulsion (NEPa)

An oil-in-water nanoemulsion was prepared from *P. anisum* essential oil (AEPa), obtained by microwave-assisted hydrodistillation and characterized by gas chromatography coupled to mass spectrometry (GC-MS). The NEPa was prepared using a high-energy method. Initially, the oil phase (EO) was stirred at 500 rpm for 10 min. Subsequently, the surfactant polysorbate 80 (Tween 80, Sigma-Aldrich) was added at a 1:3 ratio, while maintaining constant stirring at 700 rpm for an additional 20 min. Distilled water was then added under continuous stirring at 1200 rpm for 20 min. The resulting mixture was emulsified using an ultrahomogenizer (Nvymen) with 5-s pulses followed by 3-s rest intervals for 20 min at a power of 60 W. Finally, the NEPa was filtered through a 0.25 µm membrane and stored at 8 °C.

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Colloidal Characterization of NEPa

The NEPa was characterized by determining the hydrodynamic diameter (HD) and polydispersity index (PDI) using dynamic light scattering. In addition, zeta potential (ZP) was determined by electrophoretic mobility. These parameters were measured using a Zetasizer Nano ZS90 instrument (Malvern). Formulation stability was evaluated by measuring its colloidal properties during storage at 8 °C at two time points over a 6-month period.

In Vitro Cell Viability Assessment

To determine the effects of the essential oil and NEPa on cell viability, Vero cells (5×10^4 cells/mL) were cultured as monolayers in 96-well plates for 24 h at 37 °C, 5% CO_2, and 95% relative humidity. The cell monolayer was exposed to NEPa concentrations ranging from 0.3 to 740 mg/L, EO concentrations ranging from 22.2 to 600 µg/mL, and sodium hypochlorite concentrations ranging from 61.72 to 5000 mg/L for 72 h under the same incubation conditions. Cell viability was evaluated using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT, Sigma-Aldrich) at a concentration of 5 mg/mL. The resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO, Carlo Erba), and optical density was measured at 595 nm using a microplate reader (Varioskan™ LUX, Thermo Scientific). Sodium hypochlorite was also evaluated as a reference compound.

Evaluation of the Disinfectant Activity of NEPa

A direct-contact suspension test with colony-forming unit (CFU) counting was performed. After determining the cytotoxicity profile of NEPa, two concentrations (300 and 600 mg/L) were evaluated. Sodium hypochlorite at concentrations of 1000, 2500, and 5000 mg/L was used as the reference compound. The procedure was performed using a 9:1 direct-contact ratio, with a final tube volume of 10 mL, consisting of disinfectant solution (9 mL) and *Staphylococcus aureus* suspension (1 mL). The inoculum was standardized by turbidimetry (McFarland 2.0 ≈ 6×10^8 CFU/mL) without confirmation by viable count. The suspensions were directly exposed to each concentration for 1, 3, 5, and 10 min. An untreated control was included at each exposure time (1, 3, 5, and 10 min) to calculate relative growth reduction. For sodium hypochlorite, once the exposure period was completed, neutralization was performed using 5% sodium thiosulfate. After each exposure time, 1 mL was collected and subjected to serial tube dilutions from 1:10 to 10^6 in sterile saline solution. Subsequently, 100 µL was surface-plated onto Baird-Parker agar and incubated at 37 °C for 24 h. Finally, *S. aureus* colony-forming units (CFU) were counted.

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Statistical Analysis

Biological activity assays were performed at least in triplicate in independent experiments. The 50% and 90% cytotoxic concentrations (CC_{50} and CC_{90}) were calculated by sigmoidal regression using XLFit statistical software. Statistical analyses were performed in RStudio (version 2024.04.2+764) [10]. Both CFU counts and their logarithmic transformations showed a non-normal distribution, as determined by the Shapiro-Wilk test. Therefore, the nonparametric Kruskal-Wallis test was used, followed by Dunn's post hoc test with Bonferroni correction to determine statistically significant differences, with a significance level of $p < 0.05$. The relative activity of the nanoemulsion was calculated as the percentage reduction based on the medians of the log-transformed values using the following equation:

$$\%\ de\ reduction = \frac{(median\ log10\ CFU/ml_{control} - median\ log10\ CFU/ml_{treatment})}{median\ \ log10\ CFU/ml_{control}} x\ 100$$

RESULTS

Preparation and Characterization of the Nanoemulsion

The chemical composition of the AEPa used in this study was expressed as relative abundance (%). Tentative identification of its components was based on mass spectra (EI, 70 eV), fragmentation patterns, and comparison with the Adams, Wiley, and NIST databases. A total of 17 compounds were identified, as shown in the reconstructed total ion chromatogram (**Figure 1**). Among them, trans-anethole (83.1%) was the major component, followed by γ-himachalene (3.7%), ϱ-anisaldehyde (3.2%), 4-methoxy-2-(prop-1-en-1-yl)phenyl 2-methylbutanoate (2.7%), and estragole (2.2%), among others.

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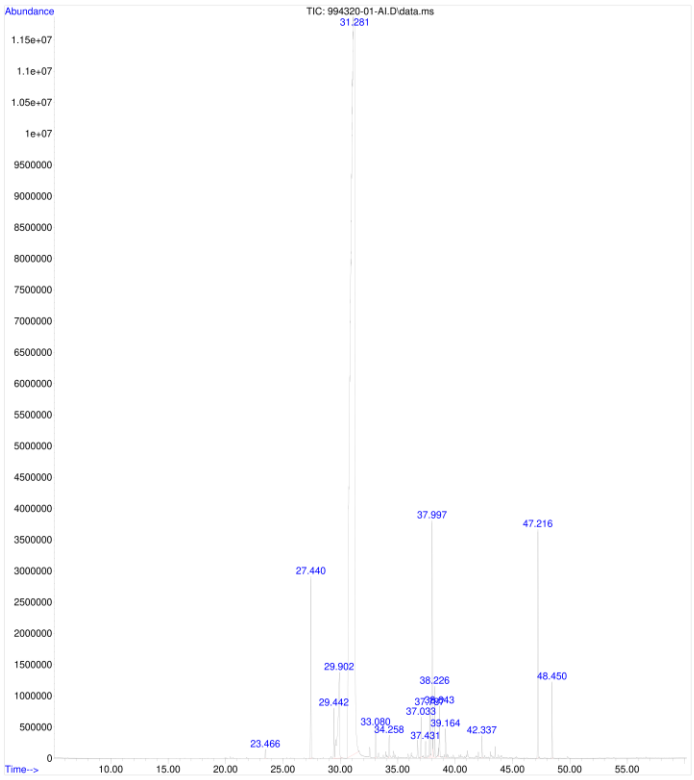


Figure 1. Chromatographic profile (reconstructed total ion current) of anise essential oil obtained by GC-MS. Nonpolar DB-5MS column (60 m); EI, 70 eV; full-scan mode.

The numbers assigned to each peak correspond to the compounds identified in (**Table 1**). The oil-in-water NE was obtained using the high-energy method and exhibited a hydrodynamic diameter (HD) of 12.9 ± 0.04 nm, a polydispersity index (PDI) of 0.10 ± 0.008, and a zeta potential of −6.02 ± 2.11 mV. In addition, NEPa maintained its colloidal characteristics during six months of storage, with no appreciable changes in the evaluated parameters.

Cytotoxic Activity of the Nanoemulsion

Evaluation of NEPa cytotoxicity in Vero cells using the MTT colorimetric assay showed a concentration-dependent response (**Figure 2**). Within the range of 0–250 mg/L, NEPa showed a moderate cytotoxic effect, with inhibition percentages below 40%. However, at higher concentrations, cytotoxicity increased markedly, reaching a maximum inhibition of 92% at the highest concentration evaluated (740 mg/L).

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Table 1. Chemical composition of anise essential oil (*Pimpinella anisum*) obtained by MW-HD and analyzed by GC-MS.

Peak number	Retention time	Compound	Relative area (%)
1	23.5	Linalool	0.1
2	27.4	Estragole	2.2
3	29.4	cis-Anethole	0.6
4	29.9	ρ-Anisaldehyde	3.2
5	31.3	trans-Anethole	83.1
6	33.1	Eugenol	0.3
7	34.3	1-(ρ-Methoxyphenyl)propan-2-one	0.3
8	37.0	α-Himachalene	0.4
9	37.4	8,9-Dehydro-neoisolongifolene	0.2
10	37.8	ar-Curcumene	0.6
11	38.0	γ-Himachalene	3.7
12	38.2	α-Zingiberene	0.7
13	38.6	β-Bisabolene	0.7
14	39.2	Sesquiterpene, M+ 202	0.3
15	42.3	Isospathulenol	0.2
16	47.2	4-Methoxy-2-(prop-1-en-1-yl)phenyl 2-methylbutanoate	2.7
17	48.5	4-Methoxy-2-(3-methyl-2-oxiranyl)phenyl 2-methylbutanoate	0.7

Note: Compounds highlighted in gray correspond to the major components of AEPa, whereas the remaining compounds were detected at lower relative proportions according to the GC-MS relative area percentages.

Based on dose-response curve fitting using a sigmoidal regression model, the 50% cytotoxic concentration (CC50) of NEPa was determined to be 185.3 mg/L. This result indicates a considerable increase in cytotoxic potential compared with the free, non-emulsified essential oil, which showed a substantially higher CC_{50} (>600 mg/L). Likewise, the 90% cytotoxic concentration (CC_{90}) was estimated to be greater than 740.74 mg/L. In contrast, sodium hypochlorite, used as the reference compound, showed CC_{50} and CC_{90} values of 163 mg/L and 814.3 mg/L, respectively.

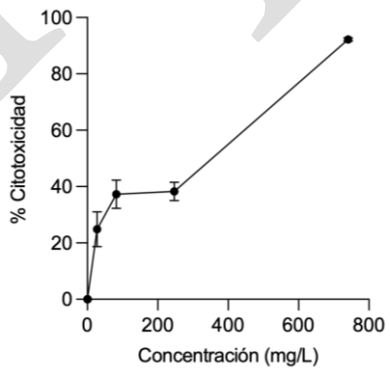


Figure 2. Effect of anise essential oil nanoemulsion (*Pimpinella anisum* L.) on Vero cells. A concentration-dependent pattern in the percentage of cytotoxicity was observed. Error bars represent the standard deviation of the experiments.

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Determination of Antimicrobial Potential

The antimicrobial effect of NEPa was evaluated at two concentrations (300 and 600 mg/L) and different exposure times (1, 3, 5, and 10 min), showing that antimicrobial efficacy depended on both variables. At 600 mg/L, high relative inhibitory activity was observed over time (**Figure 3**). During the first minute of exposure, NEPa reduced *S. aureus* growth by 82.3%, indicating an immediate antimicrobial effect. However, at 3 and 5 min, a slight decrease in activity was observed (69% and 62.4%, respectively). Antimicrobial activity subsequently increased again, reaching 86.4% inhibition at 10 min.

Figure 3. Antimicrobial activity of the anise nanoemulsion (NEPa) and sodium hypochlorite (NaOCl) against *Staphylococcus aureus*. In contrast, the 300 mg/L concentration showed an increasing trend over time (**Figure 3**). At 1 min, moderate inhibition (50.6%) was observed, followed by a slight increase at 3 min and a more pronounced increase at 5 and 10 min. These results indicate that, at a lower concentration, a longer exposure time was required to achieve higher inhibition percentages.

Table 2. Effect of NEPa on microbial growth at different concentrations and exposure times.

Treatment	Concentration (mg/L)	Time (min)	CFU/mL (median)	CFU/mL (IQR)	Log10 (median)
Control	0	1	4.20×10^8	2.7×10^8–5.9×10^8	8.62
		3	4.40×10^8	1.9×10^8–6.9×10^8	8.64
		5	5.20×10^8	4.1×10^8–6.6×10^8	8.72
		10	1.45×10^9	5.0×10^8–2.25×10^9	9.16
NEPa	300	1	1.91×10^8	7.8×10^7–3.8×10^8	8.28
		3	2.51×10^8	1.26×10^8–5.7×10^8	8.40
		5	4.17×10^8	7.6×10^7–8.5×10^8	8.62
		10	2.18×10^8	8.4×10^7–3.8×10^8	8.34*
NEPa	600	1	6.40×10^7	4.0×10^7–1.3×10^8	7.81*
		3	1.09×10^8	5.6×10^7–2.7×10^8	8.04
		5	1.95×10^8	7.2×10^7–3.2×10^8	8.29
		10	1.91×10^8	8.6×10^7–3.2×10^8	8.28*

Note: Values marked with an asterisk (*) differed significantly from the control at the same exposure time ($p < 0.05$).

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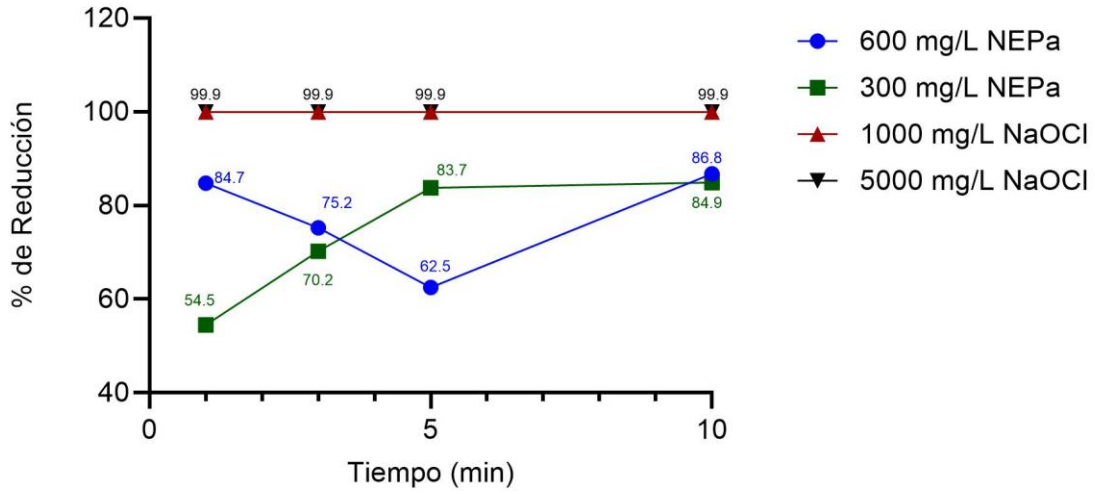


Figure 3. Antimicrobial activity of the anise nanoemulsion (NEPa) and sodium hypochlorite (NaOCl) against *Staphylococcus aureus*.

Sodium hypochlorite, used as the reference compound, also reduced microbial viability. A pronounced effect was observed at concentrations of 5000 mg/L and 1000 mg/L, with a reduction of 99.9%, demonstrating high antimicrobial efficacy under these experimental conditions (**Figure 3**). CFU counts were expressed as medians because the data were not normally distributed. Statistically significant differences were observed between the evaluated NEPa concentrations and the control after 1 min ($p = 0.029$) and 10 min ($p = 0.024$) of exposure (**Table 2**). In contrast, no significant differences were detected at 3 or 5 min ($p > 0.05$). Concentration-specific analysis showed that, at 1 min, NEPa at 600 mg/L significantly reduced the number of colony-forming units (CFU) compared with the control, demonstrating rapid antimicrobial activity against *S. aureus*. At 10 min, both 300 and 600 mg/L NEPa showed significant differences compared with the control (**Table 2**), indicating a substantial reduction in bacterial load. Overall, NEPa antimicrobial activity was more evident at the beginning and end of the evaluation period, whereas no significant effects were observed at the intermediate time points

DISCUSSION

The increasing prevalence of resistant microorganisms has created a need to explore alternatives to conventional chemical disinfectants. In this context, the use of nanobiotechnology to encapsulate active compounds represents a

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promising strategy because it may provide advantages related to stability, efficacy, and biocompatibility, among others. Nanoemulsions are lipid-based systems composed of two immiscible liquids and characterized by kinetic stability. They can enhance bioavailability and permeability while protecting essential oils (EOs) against oxidation, volatilization, and degradation [11, 12]. In addition, their antimicrobial efficacy has been reported [13,14,15,16], positioning them as an environmentally friendly alternative for microbiological control in healthcare, industrial, and food-related settings.

In this study, NEPa was prepared and characterized to determine physicochemical parameters associated with its stability and biological behavior. Properties such as hydrodynamic diameter, size distribution, and zeta potential may influence the availability of encapsulated bioactive compounds, their interaction with microbial and eukaryotic cells, and the reproducibility of antimicrobial and cytotoxicity assays. Several studies have reported that smaller nanostructured systems provide a larger surface area and more efficient interaction with biological systems [17, 18]. In this regard, NEPa exhibited colloidal properties characterized by a small droplet size and homogeneous distribution. Although the negative zeta potential close to zero does not support electrostatic repulsion as the primary mechanism responsible for the observed stability, Tween 80 may have contributed to steric stabilization by forming an interfacial layer around the droplets. Taken together, these characteristics may have contributed to the physicochemical stability observed during storage [19]. Therefore, the reported stability should be interpreted exclusively in physicochemical terms, since neither antimicrobial activity nor the cytotoxicity profile was evaluated throughout the storage period.

Similarly, Hashem et al. [20] reported the synthesis of an oil-in-water nanoemulsion containing *P. anisum* essential oil; however, their nanocarrier exhibited different colloidal characteristics, including a particle size 16-fold larger, moderate dispersity, and a negative potential indicative of electrostatic stabilization. The differences between that formulation and the system obtained in the present study may be mainly attributed to variations in formulation parameters and emulsification conditions. It should be noted that preparation methods, as well as the selection and proportions of surfactants and cosurfactants, are key factors influencing droplet size, colloidal distribution, and interfacial stability in nanoemulsions [21,22]. In contrast, the nanoscale size obtained for our NEPa could, according to the literature, favor biological activity; however, this relationship was not directly evaluated. These differences may explain the discrepancies observed relative to the system developed in the present study. Nevertheless, the properties of the system reported by Hashem conferred notable insecticidal efficacy [23].

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Regarding antimicrobial activity, NEPa significantly inhibited *Staphylococcus aureus*, particularly at 600 mg/L, at which a reduction of 82.3% was achieved during the first minute and 86.4% after 10 min of exposure. These findings are consistent with those reported by Ali et al. [24], who demonstrated, using the agar diffusion method, that nanoemulsions prepared from anise essential oil exhibit marked antimicrobial activity against *Staphylococcus aureus*, reaching a 99% reduction in colony-forming units (CFU) after 2 h of exposure. This supports the concept that the antimicrobial efficacy of nanoemulsions depends on both exposure time and concentration, indicating that the intensity and duration of contact with the compound are important determinants of antibacterial activity.

The pattern observed in this study, characterized by marked initial inhibition of *Staphylococcus aureus*, followed by an intermediate decrease and subsequent recovery, suggests that antimicrobial activity may depend not only on the total concentration of essential oil incorporated into the NE, but also on dynamic release, partitioning, and physicochemical stability phenomena inherent to nanoemulsified systems [25]. The pronounced initial inhibition could be related to the early availability of bioactive compounds, particularly anethole, which may diffuse rapidly from nanoscale droplets into the aqueous phase. This release may be favored by the high specific surface area of the nanoemulsion, thereby increasing the freely available fraction of the active compound and facilitating interaction with the *S. aureus* cell membrane, in agreement with reports describing the early bactericidal activity of essential oil nanoemulsions [26]. The intermediate decrease in activity could be associated with repartitioning of hydrophobic compounds into the droplets, oxidative degradation of anethole, or interaction with proteins and polysaccharides present in the medium, temporarily reducing the availability of the active compound [27]. In addition, a transient adaptive response by *S. aureus* cannot be ruled out, as this bacterium is capable of modulating membrane lipid composition, activating efflux pumps, and employing other tolerance mechanisms against antimicrobial compounds [28].

The recovery of the inhibitory effect toward the end of the exposure period could be explained by delayed release occurring after the nanoemulsion reaches a new physicochemical equilibrium, thereby allowing renewed diffusion of the essential oil into the medium

Nanoemulsions have been reported to exhibit delayed or prolonged release profiles related to interfacial reorganization and surfactant stabilization, which can modulate the availability of bioactive compounds in the surrounding medium [29]. Nevertheless, the potential occurrence of secondary release and its impact on bacterial

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inhibition dynamics require specific evaluation in future studies. Likewise, renewed antimicrobial pressure could exceed the adaptive capacity of the bacterium and lead to the re-establishment of growth inhibition.

In addition, the cytotoxic activity of NEPa showed a concentration-dependent response, with a low percentage of cytotoxicity at concentrations <30 mg/L and a considerably greater effect at higher concentrations. This concentration-related cytotoxic behavior may be explained by the increased availability of active molecules capable of interacting with cells. At higher concentrations, increased amounts of the active compound may enhance contact with the cell membrane, resulting in more evident cumulative damage, whereas at lower concentrations the effect is less pronounced. This pattern has been described for other nanoemulsions, in which low doses showed little or no toxicity, whereas increasing concentrations resulted in progressive cellular damage, suggesting that greater availability of the encapsulated compound enhances interactions with cell membranes and promotes adverse effects [30].

These findings indicate that the cytotoxicity observed with nanoemulsions is closely associated with the administered concentration and the level of cellular exposure to the active compound. Unencapsulated *P. anisum* essential oil has been shown to exert a cytotoxic effect on the AGS gastric adenocarcinoma cell line, with the highest activity observed at concentrations close to 15 μg/mL after approximately 48 h of exposure [31]. In addition, NEPa has shown differential cytotoxic activity against hepatocellular carcinoma (HepG2) and breast cancer (MCF-7) cell lines, with particularly low IC_{50} values in formulations containing lower oil loads, indicating high cytotoxic potency under those conditions [24]. Taken together, these findings suggest that nanoemulsion composition and essential oil content directly influence its ability to compromise cell viability [32], indicating that nanoemulsion composition and structure may modify the interaction of the essential oil with eukaryotic cells. In the present study, NEPa showed a lower CC_{50} than the free essential oil, indicating that nanoformulation did not reduce cytotoxicity under the evaluated conditions. This behavior may be related to changes in the dispersion and availability of bioactive compounds; however, the underlying mechanisms were not directly investigated.

Although a relatively high CC_{50} was determined in this study, the concentration associated with this value was lower than that at which the greatest antimicrobial activity was observed. This indicates that achieving adequate antimicrobial efficacy may require a dose above that associated with effects on mammalian cells, posing a challenge in terms of safety and selectivity. Nevertheless, the CC_{90} was considerably higher than the concentration showing the greatest

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antimicrobial activity, suggesting that, although there is a moderate risk once the CC_{50} is exceeded, a concentration range remains before extreme cytotoxicity is reached, potentially allowing use under controlled conditions or after further formulation optimization.

When sodium hypochlorite and NEPa were compared, notable differences were observed in both antimicrobial intensity and cytotoxic effects. The reference compound produced nearly complete microbial inhibition together with marked cytotoxicity toward the cell line, consistent with its strong oxidative capacity and its damaging effects on membranes, proteins, and DNA, as described by Aguilera et al. [33]. In contrast, NEPa showed antimicrobial activity under the evaluated conditions; however, its cytotoxicity profile highlights the need to optimize concentration and exposure conditions before practical applications can be considered. Although sodium hypochlorite remains useful for intensive disinfection, formulations such as NEPa may represent a complementary approach by providing a different mechanism of action and potentially reducing reliance on conventional disinfectants. From this perspective, their incorporation into alternative disinfection strategies warrants further investigation, particularly in contexts where reducing the selective pressure associated with antimicrobial resistance is desirable.

It is important to highlight that the mechanism underlying both the disinfectant potential and the cytotoxic effect of NEPa may be related to the chemical composition of its essential oil. *trans*-Anethole is the major chemical component of anise essential oil and may play a key role in its antimicrobial activity [34]. In addition, it has been shown to activate programmed cell death processes through the generation of reactive oxygen species (ROS), DNA damage, activation of apoptotic pathways, and induction of autophagy and oxidative stress mechanisms [35,36]. Complementarily, other phenolic compounds, such as propenyl-anisum, limonene, and anisole, have also been reported to contribute to the antimicrobial activity of the oil [8].

In this context, the chemical composition of anise essential oil provides the nanoemulsion with potential as a natural disinfectant agent that could complement conventional disinfectants in settings requiring effective microbiological control. Finally, the results of this study should be interpreted as a relative reduction compared with the untreated control at each time point, indicating that contact time significantly influenced the antibacterial activity of the NE. However, future studies should validate the efficacy of the biodisinfectant according to international standards.

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CONCLUSIONS

In this study, anise essential oil showed a profile rich in bioactive compounds, with *trans*-anethole as the predominant constituent. The developed NEPa exhibited appropriate colloidal properties and remained stable throughout the evaluated period, supporting its performance as an encapsulation system. In addition, NEPa cytotoxicity was concentration-dependent, with lower effects at low concentrations, and antimicrobial activity against *S. aureus* was demonstrated. However, although antimicrobial activity was confirmed, the most active concentration exceeded the CC50 determined in Vero cells, indicating the need to optimize the formulation and conditions of use to improve its biological selectivity. Therefore, although the results demonstrate the potential of the nanoemulsion as a naturally derived antimicrobial system, its possible application as a disinfectant requires further studies to more precisely establish the balance between efficacy and safety.

ETHICAL CONSIDERATIONS

This study was an experimental *in vitro* investigation approved under ethical opinion No. 02192024-3614022024 by Universidad Santo Tomás. It did not involve the direct participation of humans or animals. The Vero cell line and the bacterial strain *Staphylococcus aureus* were obtained from authorized sources and handled in accordance with current biosafety standards and good laboratory practices. All procedures were conducted in accordance with the ethical principles applicable to the handling of biological materials, ensuring the safety of the personnel involved and the appropriate disposal of generated waste.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTIONS

Arenas DA: Methodology, investigation, formal analysis, and writing—original draft. **Herrera LV:** conceptualization, investigation, supervision, validation, data curation, formal analysis, manuscript editing, and final manuscript review. **Cervantez Díaz M:** Conceptualization, project administration, funding acquisition, and final manuscript review. **Bastidas SE:** Methodology and investigation. **Leal Pinto SM:** Conceptualization, investigation, supervision, formal analysis, manuscript review and editing, and final manuscript review.

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