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ORIGINAL RESEARCH ARTICLE

Silver nanoparticles functionalized in situ with D-limonene: Effect on antibacterial activity

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ABSTRACT

This study focused on the formulation and characterization of silver nanoparticles (AgNP) functionalized with d-limonene. The nanoparticles were functionalized by phase inversion and the synthesis of the nanoparticles was performed in situ; particle size was determined by laser diffraction, zeta potential and optical colloidal stability using Multiscan 20 for a period of 24 hours at 37 °C; the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the formulated material on *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Klebsiella oxytoca* ATCC 700324, *Enterococcus casseliflavus* ATCC 700327, *Escherichia coli* BLEE, carbapenem-resistant *Pseudomona aeruginosa* were determined. The nanoparticles showed colloidal stability at a d-limonene concentration of 3.93%, silver ions at 1.61×10^{-3}%, non-ionic adjuvant at 24% and ascorbic acid at 5.88%; citric acid/citrate (1:1) 0.48M for a pH of 4.5 was used as a buffer system. The formulation was classified as a polydisperse system (PD = 0.0851), with a zeta potential of −11.6 mV and average particle size of 81.5 ± 0.9 nm. A particle migration velocity of −0.199 ± 0.006 mm·h^{-1}, a constant transmission profile and backscattering profile with variations of 10% were evidenced, which represents a stable formulation. The nanoparticles presented an MIC and an MBC of 28 µg·mL^{-1} (5.6×10^{-2}% d-limonene and 4.7×10^{-5}% AgNP) against all tested bacteria.

Keywords: Silver Nanoparticles; Phase Inversion; Bacterial Resistance; Minimum Inhibitory Concentration

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1. Introduction

Due to the indiscriminate use of artificial chemical compounds such as antibiotics and disinfectants for the treatment of infectious diseases in humans and other species, for livestock production, cleaning and disinfection of environments, food production and preservation, many exposed microorganisms quickly develop resistance to these compounds, becoming a worldwide public health problem by generating infections that cannot be treated; additionally, artificial compounds can directly cause diseases in humans and other living beings and contaminate the environment.

In 2017, the World Health Organization pointed out that the battery of antimicrobials available to combat multidrug-resistant bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Mycobacterium tuberculosis* and *Staphylococcus aureus*, is insufficient to mitigate their proliferation and cause infection in humans; the design of innovative products is urgently required to control disease-causing microorganisms that endanger human life and increase treatment costs[1–6]. There is a growing trend towards the research and use of natural extracts or essen-

tial oils (EO) that have been demonstrated in vitro the ability to inhibit the growth of clinically important bacteria, such as antibiotic or disinfectant agents[4,5,7]. Additionally, the development of new formulations using inorganic compounds has gained importance, being metal ions, which are known to be very toxic to bacterial cells[3,8]. Metal nanoparticles present a better performance to eliminate bacteria by increasing the surface area to react[3,8,9]. The scientific community should contribute to the solution of the problem by researching and carefully selecting active agents and formulations that target multiple bacterial sites and present modes of action less likely to confer cross-resistance.

Antimicrobial resistance is defined as the acquisition of resistance by a microorganism to an antimicrobial drug to which it was previously sensitive. The acquisition of resistance by bacteria poses a threat to public health; the greatest concern is the increasing spread of multidrug-resistant pathogenic bacteria worldwide, due to the misuse and abuse of antibiotics[10,11]. Among the mechanisms expressed by bacteria to resist various groups of antibiotics is the production of enzymes such as extended-spectrum beta-lactamases (ESBL) that confer resistance to oxymino-cephalosporins and monobactams (aztreonam), antibiotics that act by inhibiting the synthesis of the bacterial cell wall. When a bacterium is identified as a ESBL producer, a group of antibiotics called carbapenems are used as a therapeutic alternative; these act on the cell wall and are highly resistant to hydrolysis against ESBL[12]. But bacteria have also created multiple mechanisms to avoid the action of carbapenems, becoming a threat to world health, since for many years they have been the most stable and active antibiotics against bacteria with multiple resistance[13].

Citrus plants have high concentrations of EO in the peel of their fruits; its main component is the terpene d-limonene, a molecule with recognized inhibitory power against bacterial growth in vitro. In Colombia, citrus fruits are considered the second most important fruit species after bananas, and there is sufficient raw material in Colombia to obtain EOs with high limonene content[4,14–17]. The antimicrobial activity of d-limonene on the in vitro growth of *Streptococcus uberis*, *Sthapylococcus aureus*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Pseudomonas fragi*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhimurium*, *Salmonella enteritidis* and *Listeria monocytogenes* has been reported[14,16,18–21]. These results demonstrate the potential advantages of using d-limonene as a naturally occurring antimicrobial. Among metal compounds, silver "$Ag_{(s)}$" is a common element in nature and has been used by humans as a disinfecting agent[3,22–24]. Silver nanoparticles (AgNP) have a strong bactericidal potential due to their higher surface-to-volume ratio, presenting on average a size of 10–100 nm, and being highly reactive molecules can be incorporated as an active ingredient of drugs and disinfectants, offering distinct advantages such as reduced toxicity, overcoming resistance and reduced cost compared to conventional antibiotics[2–4,8,25]. AgNPs can act as an antimicrobial agents against nearly 650 species, including antibiotic-resistant bacteria[5,22], exhibit good in vitro performance against Gram-positive bacteria such as *Staphylococcus aureus*, *Streptococcus pyogenes* and *Bacillus subtilis* and Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhi*[2,3,8,22–26]. The mechanisms of action of AgNP on bacteria begin with binding to the cell membrane, membrane proteins and negatively charged nucleic acids, blocking the respiratory chains, generating reactive oxygen species, which lead to functional changes in the cell until it is destroyed[4,3,22,24,26]. On the other hand, some authors report bacterial strains that present mechanisms of adaptation and/or resistance to AgNP[22,24].

In recent studies, it has been demonstrated that nanoparticles functionalized with essential oils increase their antibacterial effect and biocompatibility[4,5,8,27]; the antibacterial effects of AgNP in combination with the EO of the Zataria multiflora plant have been evaluated, observing that AgNP with EO present synergistic effect against the growth of Staphylococcus epidermidis and Staphylococcus aureus. However, no reports were found indicating the inhibitory potential of bacterial growth of for-

mulations combining d-limonene and AgNP; in this study it is proposed to produce a nanoemulsion containing AgNP functionalized with d-limonene and evaluate its effect on antibacterial activity, hoping to obtain a formulation that offers an alternative for the control and eradication of bacterial agents of clinical importance, especially microorganisms that manifest mechanisms to avoid the action of antibiotics and disinfectants currently used.

2. Method

The process of formulation, characterization and microbiological evaluation of AgNp functionalized with d-linomenon is described below.

2.1 Formula

Silver nitrate (>99%), ascorbic acid (>99%), citric acid (>99.5%), sodium citrate (>99%), and Tween 20® (for synthesis) were imported by Sigma-Aldrich Co (St. Louis, MO). The d-limonene was donated by the Fundación de Apoyo a la Investigación en el Grupo Interdisciplinario de Estudios Moleculares- FUNDAGIEM of the Universidad de Antioquia. The formulations were prepared in a 50 mL Falcon tube, adding non-ionic coadjuvant (Tween 20®; Glycerin and ethyl alcohol) and d-limonene with continuous agitation; then, silver nitrate solution and the solid mixture composed of citric acid/sodium citrate were slowly added. Subsequently, ascorbic acid was slowly added with continuous stirring in Vortex (Thermo Scientific) for the reduction of the silver ion; the volume was made up to 50 mL with deionized water.

Formula F1 represents AgNP functionalized with d-limonene, formula F2 represents AgNP without limonene addition and formula F3 represents nanoemulsion with d-limonene and absence of AgNP.

2.2 Particle size and z-potential analysis

The formulations were diluted 1:10 with sterile water for injection. They were then analyzed on the Malvern NanoSight 300, which uses a technique ideal for polydisperse systems and yields a particle tracking analysis, allowing characterization of nanoparticles from 10 nm to 2,000 nm.

2.3 Colloidal stability analysis

The colloidal stability of the nanosuspensions was evaluated by the DataPhysics Multiscan 20. Each formulation was stored in closed 40 mm clear glass bottles, where the products were subjected to periodic analysis at 37 °C for 24 hours. The objective was to accelerate the destabilization processes and to detect potentially unstable products at the earliest possible stage to consequently reduce the time for new product development.

2.4 Microbiological analysis

For antibacterial activity tests, the following bacteria were used: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Klebsiella oxytoca* ATCC 700324, *Enterococcus casseliflavus* ATCC 700327, BLEE-producing *Escherichia coli*, *Pseudomona aeruginosa*, which showed impermeability to carbapenemics, the latter two isolated from urine samples. All strains were provided by the Clinical Hematology Laboratory. Bacteria cryopreserved on BHI Brain Heart Infusion agar, glycerol and fetal bovine serum, were thawed and Gram-negative bacilli were seeded by depletion on MacConkey agar (Biomériux) and Gram-positive cocci on Columbia CNA Biomériux agar. They were incubated for 24 hours at 37 °C; the genus and species of each growth were identified by means of the Vitek 2 Compac Biomériux kit. Once the genus and species were confirmed, the MIC was determined.

2.5 Agar dilution method

This method made it possible to quantify the in vitro activity of an antimicrobial by determining the growth of a microorganism in a series of dilutions of the antibiotic mixed with culture medium. The agar dilution method made it possible to determine the minimum bactericidal concentration CMB, defined as the lowest concentration of the antimicrobial agent necessary to eliminate 99% of the initial inoculum, and the minimum inhibitory concentration CMI, defined as the lowest concentration of substance that can inhibit the visible growth of a microorganism. To make the dilutions of the anti-

bacterial product under study, the amount of the antibacterial agent to be analyzed was dispersed in an Erlenmeyer flask, then a known amount of sterile agar still molten (50 °C) was added; Müeller-Hinton agar is usually used, which allows the development of Gram-negative bacilli and Gram-positive cocci. The mixture is homogenized and poured into an empty sterile Petri dish, thus obtaining a Müeller-Hinton agar plate with the antibiotic diluted to a certain concentration ready to be inoculated[12,28–31]. The MIC was that dilution at which no growth of the tested bacteria was observed. The CMB is established by taking a sample with a sterile swab from the surface of the solid agar containing the dilution that allowed the MIC to be established and the sample is cultured on Müeller-Hinton agar. It is incubated for 48 hours at 37 °C, waiting for no bacterial growth to be observed[28]. Müeller-Hinton Agar is a solid, non-selective medium that allows antimicrobial susceptibility testing of aerobic, anaerobic and microaerophilic bacteria[30]. It has a low inhibitory power and high reproducibility; it should be prepared at a pH between 7.2 and 7.4 which can be adjusted with Ca^{2+} (20–25 mg·L^{-1}) and Mg^{2+} (10–12.5 mg·L^{-1})[31].

2.6 Determination of Minimum Inhibitory Concentration (MIC)

The MIC of formulations F1, F2, F3 was determined by the agar dilution method described in CLSI guideline M-7 2018[32], with some modifications; after 24 hours of incubation at 37 °C, three to four colonies of each bacterium are taken with a sterile wooden stick and suspended in 0.85 % saline, until a turbidity standard of 0.5 McFarland (1.5 × 10^8 CFU m·L^{-1}) is reached. Each suspension was tested with Densichek equipment (Biomériux). Mueller- Hinton agar (Becton Dickinson, USA) was used to perform the dilutions, incorporating the determined amount of the nanoemulsion in the agar when it is still in liquid phase (50 °C), always keeping a final volume of 10 mL; the first dilution contains 1 mL of formulated + 9 mL of agar, the final dilution contains 100 µl of formulated + 900 µl of sterile distilled water + 9 mL of agar. The mixture is deposited in sterile plastic Petris dishes and allowed to cool to gel the culture medium. Once the mixture is in its solid phase, surface inoculation is performed, 10 µL of each 0.5 McFarland suspension is taken and deposited as a dot on the medium; incubate for 24 hours at 37 °C. A growth control is performed by inoculating 10 µL of each bacterial suspension on a Petri dish with 10 mL of Mueller-Hinton agar (Becton Dickinson, USA); a sterility control is performed by leaving in incubation for 24 hours at 37 °C a Petri dish with 10 mL of the previously prepared Mueller-Hinton agar without seeding. As method control the commercially used antibiotic Amoxicillin (SIGMA) is used starting from the concentration 512 µg·mL^{-1} up to the concentration of 2 µg·mL^{-1} according to the “Preparation of dilutions of antimicrobial agents for use in agar dilution susceptibility testing”, available in the CLSI guide M100 E28[33]. Each analysis is performed in triplicate.

The MIC of two commercial disinfectant detergents produced by Spartan Chemical Campany, Inc, Clean By Peroxy based on hydrogen peroxide and Super HDQ Neutral based on quaternary ammonium were determined for comparison with the MIC of F1. First, the commercial disinfectants were prepared at the dilution recommended on the label, 1:32 for Clean By Peroxy and 1:250 for Super HDQ Neutral; distilled water was used as diluting agent. Dilution in agar was performed to determine the MIC of each product on the same group of bacteria previously tested. To evaluate the effect of temperature on the formulations, taking into account that they are added to the liquid phase agar which is at 50 °C, the direct effect of the nanoemulsion at a temperature of 30 °C on the selected bacteria was determined.

2.7 Determination of minimum bactericidal concentration (MBC)

From the Petri dish containing the agar with the dilution of formulated that allowed the MIC to be established, a sample is taken from the agar surface with a sterile swab and seeded on a new sterile Mueller-Hinton agar; incubate for 48 hours at 37 °C and read.

2.8 Statistical analysis

For the systematization of the information and analysis of the results, the statistical software SPSS version 24, licensed by the University of Antioquia, was used. The descriptive tables of the information were constructed in this software. For each of the formulations and commercial disinfectants, the Chi-square test was applied to observe the relationship between product concentration and bacterial growth, finding in all experiments the concentration value that allowed no bacterial growth in the three replicates, with no growth in the tested concentrations higher than the MIC, which shows the stability of the formulations. Subsequently, the Student's t-test was applied, taking as reference the average concentration at which the F1 formulation (with AgNP and d-limonene) acted, and it was established whether there were statistically significant differences with the means of the other products evaluated. The "p" values less than or equal to 0.05 were taken as statistically significant in both tests.

3. Results

3.1 Particle stability

Table 1 presents the colloidal characterization results of the formulations. F1 (5.6×10^{-2}% d-limonene, 4.7×10^{-5}% AgNP) presented smaller average particle size, lower polydispersity and higher electrostatic stability.

Figures 1, **2** and **3** show the particle size distributions of the formulations and the effect of in situ d-limonene functionalization on the AgNP synthesis process.

Table 1. Colloidal characterization of formulations evaluated

	F1	F2	F3
Average diameter (nm)	81.5 ± 0.9	116.4 ± 9.2	133.7 ± 1.5
Fashion (nm)	69.5 ± 1.7	81.8 ± 4.8	9.4 ± 2.7
D10 (nm)	60.5 ± 0.5	72.1 ± 5.6	86.5 ± 0.8
D50 (nm)	75.1 ± 0.8	99.7 ± 8.6	121 ± 3
D90 (nm)	107.8 ± 2.4	186.3 ± 18.2	198.4 ± 4.8
Polydispersity	0.0851	0.495	0.0928
Zeta potential (mV)	−11.6 ± 0.3	−8.3 ± 0.4	−5.1 ± 0.5

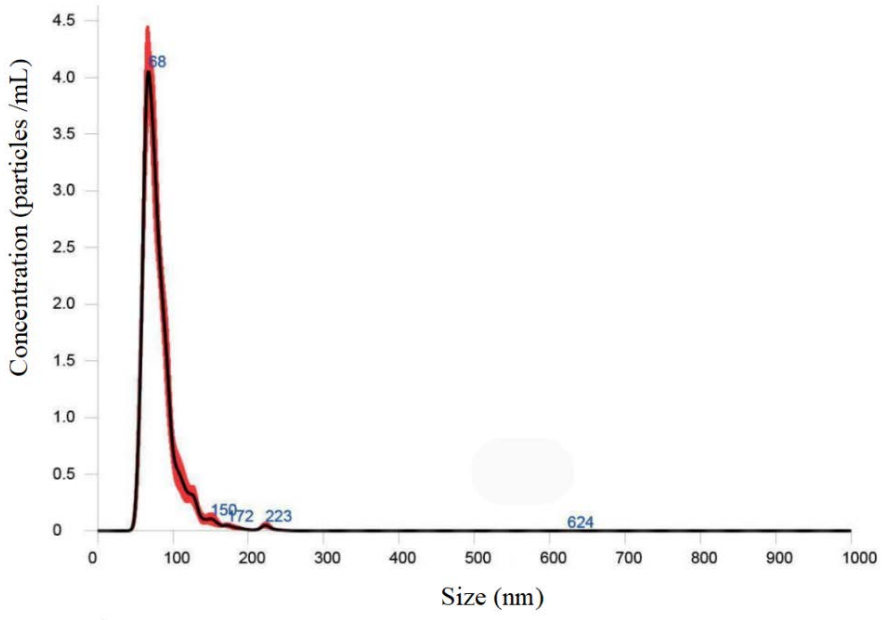

Figure 1. Particle size distribution of formulation F1.

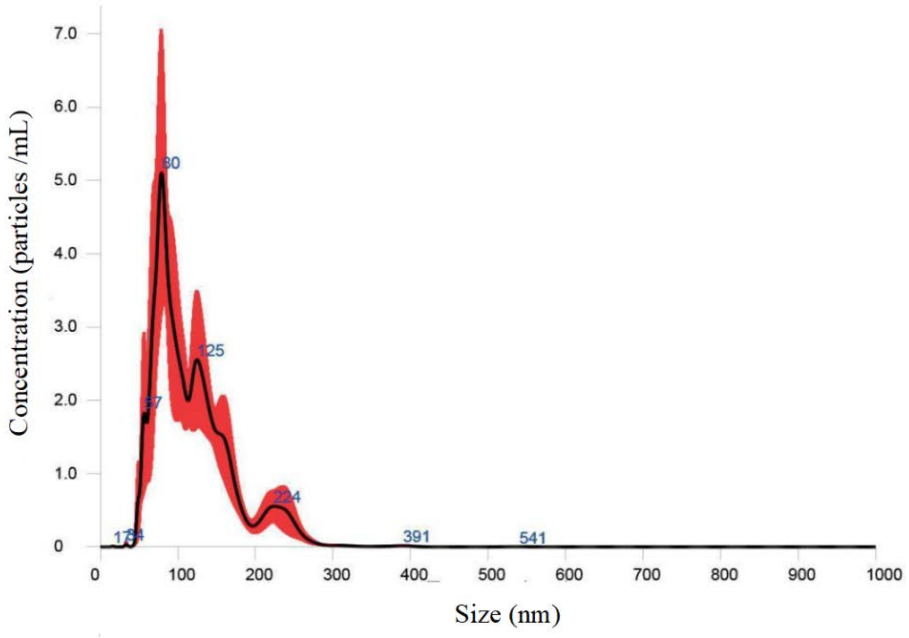

Figure 2. Particle size distribution of the formulation F2.

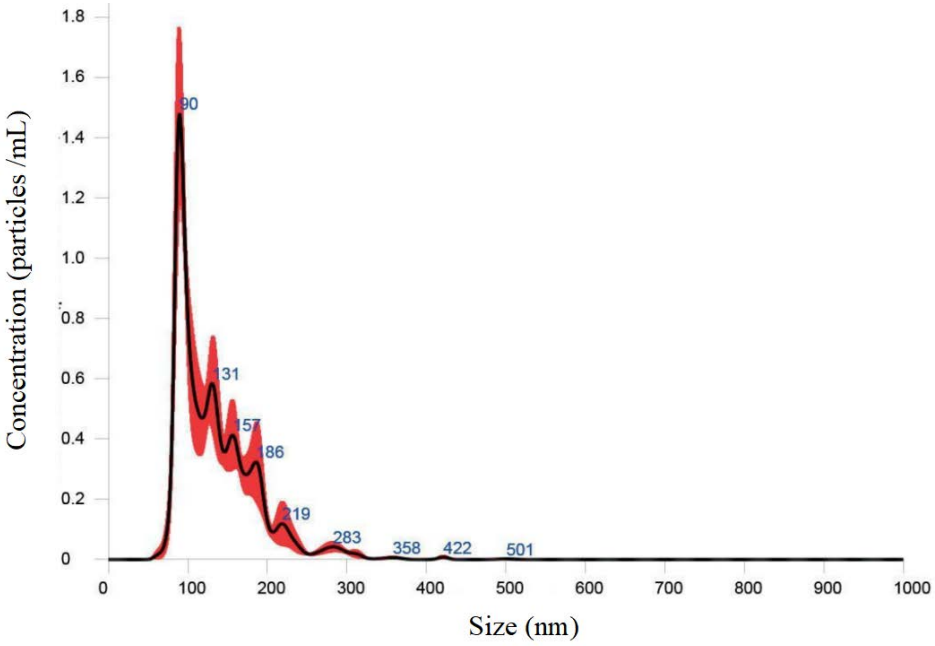

Figure 3. Particle size distribution of the formulation F3.

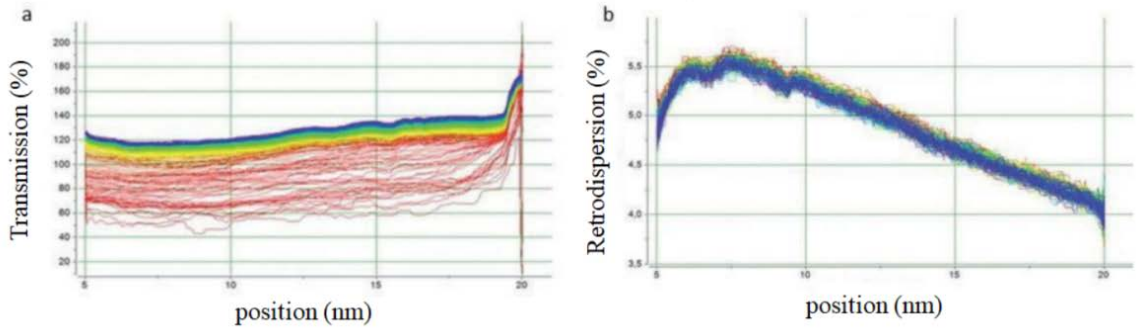

Figure 4. Colloidal stability of formulation F1: **a**) Transmission profile; **b**) Backscattering profile.

For the colloidal stability of formulation 1, a transmission that remains constant along the height of the vial is observed (**Figure 4a**) which means that no particle migration (creaming or sedimentation) is evidenced during the 24 hours of sample analysis. On the other hand, **Figure 4b** shows the backscattering profile in absolute form; although no isosbestic point is observed, there is no variation in particle size since the backscattering profile is within the ±2% range.

3.2 Microbiological

Bacterial Identification**.** Results of Vitek 2 Compac indicate that all bacterial strain identifications achieve an average of 96% probability.

Results of MIC and BMC of the formulations. Formulation F1 containing AgNP functionalized with d-limonene presented a MIC and WBC of 28 $\mu g \cdot mL^{-1}$ with a concentration of d-limonene and AgNP of 5.6×10^{-2}% and 4.7×10^{-5}%, respectively, against all tested bacteria; the MIC and MIC for *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Klebsiella oxytoca* ATCC 700324, *Escherichia coli* producing extended-spectrum beta-lactamase BLEE was 28 $\mu g \cdot mL^{-1}$, against *Pseudomona aeruginosa* was 22 $\mu g \cdot mL^{-1}$ and against *Enterococcus casseliflavus* was 24 $\mu g \cdot mL^{-1}$. **Table 2** shows the results of each formulation on the bacteria tested. The growth control yielded a positive result in all three replicates and the method control reproduced in the expected MIC range of Amoxicillin on each bacterium. The commercially available disinfectants based on Clean By Peroxy hydrogen peroxide and Super HDQ Neutral quaternary ammonium ammonium had MIC of 68 $\mu g \cdot mL^{-1}$ and 36 $\mu g \cdot mL^{-1}$, respectively.

The percentage of AgNP and d-limonene in the CMB of the formulations, taking into account that the minimum amount of the product tested to eliminate the total bacteria tested was 28 $\mu g \cdot mL^{-1}$ for F1, 34 $\mu g \cdot mL^{-1}$ for F2 and 50 $\mu g \cdot mL^{-1}$ for F3, is reported in **Table 3**.

Table 2. Minimum inhibitory/bactericidal concentration of the formulations on the tested bacteria

Formulated disinfectant	**Bacteria - CMI CMB $\mu g \cdot mL^{-1}$***					
	E.coli ATCC 25922	**E.coli BLEE**	**P. aeruginosa R Carb**	**K. oxytoka ATCC 700324**	**S. aureus ATCC 29213**	**E. casseliflavus ATCC 700327**
F1 AgNP+ d-limonene	28	28	22	28	28	24
F2 AgNP only	30	30	22	34	34	26
F3 d-limonene only	40	40	32	46	50	34
Hydrogen peroxide (A)	68	68	46	46	46	44
Quaternary ammonium (B)	20	20	18	36	20	18

A: Hydrogen peroxide-based disinfectant detergent, Clean by Peroxy, Spartan.
B: Quaternary ammonium-based disinfectant detergent for medical devices, Super HDQ Neutral, Spartan.
* Average of the results of three different experiments.

Table 3. Percentage of d-limonene and AgNP in the CMB

	F1	F2	F3
d-limonene (%)	5.6×10^{-5}	n/c*	0.1
AgNP (%)	4.7×10^{-5}	5.7×10^{-5}	n/c

*n/c: does not contain

Table 4 shows the descriptive statistics for the behavior of each disinfectant. The one with the widest range was the hydrogen peroxide-based disinfectant, with a value of 2.4 $\mu g \cdot mL^{-1}$. The maximum concentration used in this was 68 $\mu g \cdot mL^{-1}$. The one with the smallest range of action was the combination of AgNP and d-limonene, presenting a minimum value of 22 $\mu g \cdot mL^{-1}$ and a maximum of 28 $\mu g \cdot mL^{-1}$, which shows greater stability among the agents evaluated. When observing the behavior of the mean, it is found that the disinfectants that required on average less volume to inhibit the evaluated agents are the quaternary ammonium-based disinfectants, with a mean of 22 $\mu g \cdot mL^{-1}$, followed by F1 (AgNP and d-limonene) 26.3 $\mu g \cdot mL^{-1}$. The two disinfectants with the highest MIC were F3 (d-limonene) and hydrogen peroxide-based disinfectant with means of 40.3 $\mu g \cdot mL^{-1}$ and 53 $\mu g \cdot mL^{-1}$ respectively.

Table 4. Descriptive statistics for the formulated volume of disinfectant

Formulation	N (Bacteria evaluated)	Range (μg·mL^{-1})	Minimum (μg·mL^{-1})	Maximum (μg·mL^{-1})	Mean (μg·mL^{-1})	Deviation (μg·mL^{-1})
F1 AgNP + d-limonene	6	0.6	22	28	26.3	2.6
F2 AgNP only	6	1.2	22	34	29.3	4.7
F3 d-limonene only	6	1.8	32	50	40.3	6.9
A. Disinfectant based on hydrogen peroxide	6	2.4	44	68	53	12
B. Disinfectant based on quaternary ammoniums	6	1.8	18	36	22	7

Table 5. Student's t-test of means

Formulation	Test value = 26.3			
	t-test	gl	Sig. (bilateral)	Difference in means
AgNP + limonene	0.000	5	1.000	0.0
AgNP only	1.571	5	0.177	3.0
Limonene only	4.999	5	0.004	14.0
Disinfectant based on hydrogen peroxide	5.609	5	0.002	26.7
Quaternary ammonium-based disinfectant	−1.532	5	0.186	−4.3

Having the behavior of the average concentrations necessary to inhibit the growth of the different bacteria, Student's t-test was performed taking as reference the mean given for formulation F1 (AgNP and d-limonene) 26.3 μg·mL^{-1}.

When performing the Student's t-test with this parameter (see **Table 5**), statistically significant differences were found when comparing F1 with F3, the d-limonene-only formulation ($p = 0.004$) and the hydrogen peroxide-based disinfectant ($p = 0.002$). In contrast, the F2 formulations of AgNP alone and the quaternary ammonium-based disinfectants did not show statistically significant differences compared to the AgNP+d-limonene formulation.

4. Discussion

The bacteria analyzed in this study are considered human pathogens; reference strains provided by the American Type Culture Collection ATCC and bacteria that have shown *in vivo* and *in vitro* antibiotic resistance mechanisms, such as BLEE-producing *Escherichia coli*, are included, enzymes produced by bacteria with the ability to inactivate third-generation cephalosporins (ceftriaxone, cefotaxime, ceftazidime) and aztreonam[12], *Pseudomona aeruginosa* with resistance to carbapenemics, a group of antibiotics used for the treatment of infections caused by BLEE-producing bacteria[13]. The mixtures used to prepare the nanoemulsions allowed achieving a final formulation called F1, whose physical and chemical properties demonstrate that it is a stable mixture, containing particles with an average size of 81.5 ± 0.9 nm, characteristics that enhance the effect of AgNP and d-limonene in microbiological tests; F1 presented the same effect on *E. coli* and multidrug-resistant *E. coli* and an MIC against carbapenem-resistant *P. aeruginosa*, lower than that obtained against the other 5 bacteria tested; this suggests that AgNP functionalized with d-limonene perform with the same power on bacteria without resistance and bacteria multidrug-resistant to antibiotics. The combination of AgNP with essential oils has already demonstrated a synergistic effect against multidrug-resistant bacteria[8].

The MIC is considered the amount of antimicrobial that allowed the complete inhibition of growth of all the bacteria tested in all the assays. The formulation named F1 showed an outstanding broad-spectrum antimicrobial activity since it acted similarly on Gram-positive cocci and Gram-negative bacilli, eliminating the in vitro growth of all the bacteria tested with a MIC and

BMC of 28 μg·mL^{-1} with an average of 26.3 μg·mL^{-1}; the formulation named F2 presented an MIC and a WBC of 34 μg·mL^{-1} and formulation F3 showed an MIC and a WBC of 50 μg·mL^{-1}; these data indicate that AgNPs functionalized in situ with d-limonene in a nanoemulsion type formulation present an antimicrobial additive effect, being necessary less quantity of formulation F1 to eliminate 99.9% of the tested bacteria, compared to F2 and F3. Elements such as Silver (Ag), Gold (Au), Zinc (Zn), Platinum (Pt), Iron (Fe) and Copper (Cu) have been used in combination with EC to evaluate their antimicrobial activity, showing a synergistic effect[35]. According to the statistical analysis, F1 presents significantly different MIC values compared to F3, a formulation containing only d-limonene, which indicates that F1 acts better at lower concentrations compared to F3; with F2, which only contains AgNP, there are no statistically significant differences. Despite this, as shown in **Table 4**, a lower amount of the formulation is necessary when combining AgNP with d-limonene to inhibit the growth of 5 of the 6 bacteria tested; the percentage of AgNP necessary to inhibit 100% of the bacteria tested in F1 was 4.7 × 10^{-5}% and that of F2 was 5.7 × 10^{-5}%, achieving a decrease of 1 × 10^{-5}% when combining AgNP with d-limonene. This low decrease of one unit may signify a reduction in the toxicity of the product to eukaryotic cells, reflecting that limonene acts as a stabilizer of the silver nanoparticles and, when combining the two antibacterial agents, their growth inhibitory power is not affected. The effect of F1 (AgNP with d-limonene) compared to the F2 formulation (AgNP alone) is more noticeable on *K. oxytoka* and *S. aureus*, bacteria known for their high pathogenicity and ability to generate and transmit resistance to antibacterials. This finding is important because it proves the addictive effect generated by limonene on AgNPs.

In the comparative tests of the antibacterial effect of the disinfectants versus the F1 formulation, the quaternary ammonium-based disinfectant presented the best inhibitory effect against the bacteria *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 2921, *Enterococcus casseliflavus* ATCC 700327, *Escherichia coli* BLEE+, carbapenem-resistant *Pseudomona aeruginosa*, compared to formulation F1 and Clean By Peroxy disinfectant. But the MIC (36 g·L^{-1}) against *Klebsiella oxytoca* ATCC 700324 was higher than the MIC (28 g·L^{-1}) presented by the F1 formulation on the same bacteria. According to this data, the F1 formulation is considered more stable in its effect on the total bacterial group tested, presenting a significant difference with both disinfectants, since a minimum of 28 μg·mL^{-1} of F1, 36 μg·mL^{-1} of quaternary ammonium and 68 μg·mL^{-1} of hydrogen peroxide are needed to eliminate the 6 bacterial genera. The hydrogen peroxide-based disinfectant presented lower inhibitory effect than the F1 formulation on all tested bacteria. These findings suggest that F1 has a broad-spectrum action as a disinfectant agent by acting evenly and at low concentrations on Gram-positive and Gram-negative bacteria. When analyzing the minimum and maximum values, it is noted that the formulation that had a smaller range of action was the combination of AgNP and d-limonene, presenting a minimum value of 22 μg·mL^{-1} and a maximum of 28 μg·mL^{-1}, which shows a greater stability among the agents evaluated. F1 behaved very similar to the commercial quaternary ammonium-based disinfectant; this product is used in places such as laboratories, intensive care units, food industries among others to control dangerous pathogens, therefore, it is concluded that F1 presents a potential as a disinfectant agent that is at the level of the latest generation disinfectants with the advantage that its effect is more even on a heterogeneous group of bacteria compared to quaternary ammonium.

The combination of AgNP with EO to achieve greater antimicrobial effect against bacteria has been tested in several studies. The additive effect between AgNP and the terpene thymol to disinfect vegetative tissue of Bermudagrass plant has been reported[36]. AgNPs combined with the essential oil of *Oreganum spp* exhibited an addictive effect against multidrug-resistant bacteria[34]. The mixture of AgNPs with the EO of *Oreganum spp*. showed antimicrobial stability against Gram-positive bacteria[37]; AgNPs functionalized with essential oils of the plants *Cymbopogon citra-*

tus, *C. martini*, *Eucalyptus globules*, *Azadirachta indica*, *Ocimum sanctum* showed effect against *S. aureus* bacteria[38]. This is the first report of the in vitro antibacterial effect of a mixture of AgNPs and d-limonene; the results suggest that the additive effect of AgNPs functionalized with d-limonene can be used to prevent the growth of bacteria including multi-resistant bacteria that are considered pathogenic for humans and other species; further research on combinations of AgNPs with products of natural origin is necessary to provide more alternatives against the phenomenon of bacterial multi-resistance.

The mechanisms of action of EOs on bacterial cells vary depending on their composition and the bacterial strain exposed; EOs are characterized by their hydrophobicity and lipophilic nature, which allows them to interact easily with the fatty acids of the microbial cell membrane; they act on cell membrane integrity by changing permeability, leading to electrolyte leakage and loss of vital intracellular contents such as proteins, reducing sugars, while inhibiting energy generation, leading to cell destruction[39–42]. D-limonene derived from citrus essential oil acts on the cytoplasmic membranes of microorganisms, causing a loss of membrane integrity, inhibition of respiratory enzymes and dissipation of proton motive force[27].

The mechanism of action of AgNPs on bacteria begins with binding to the cell membrane, increasing permeability, producing the release of lipopolysaccharides, membrane proteins and subsequent binding to nucleic acids, blocking the respiratory chains, generating reactive oxygen species, which lead to functional changes in the cell leading to cell death[3,5,22–24,26,35,44]. The effect depends on the surface area that is increased by the presentation in nanometric size, being able to interact in greater proportion with molecules such as enzymes and nucleic acids, causing greater structural changes and deformation in bacterial walls and membranes[2,21,43]. On the other hand, some authors report bacterial strains that present mechanisms of adaptation and or resistance to AgNP[22,24], so it is important to continue evaluating whether the mixture of AgNP with essential oils with antibacterial power with d-limonene counteracts the resistance effect that has been presented on AgNP. However, the results show that the addition of d-limonene to the formulation requires a lower amount of AgNP, which decreases the toxicity due to the presence of silver in eukaryotic cells.

According to the mechanisms of action of EOs and AgNPs, it can be suggested that the F1 nanoemulsion presents a combined and synergistic mechanism of action; d-limonene by its lipophilic nature interacts easily with the fatty acids of the microbial cell membrane, damaging the integrity of the membrane, AgNPs also affect the membrane, allowing easy entry of AgNPs and limonene into the cell cytoplasm, there they disrupt the electron transport process inhibiting the secretion of toxins into the environment, causing dysfunction of ribosomes, interact with the genetic material until degrading it and finally achieving cell lysis[35].

5. Conclusions

The problem of bacterial resistance is growing at an alarming rate, considerably increasing morbidity and mortality rates worldwide. The level of evolution of bacteria to survive and multiply in environments with high concentrations of commercially available antibiotics and disinfectants, in the last decade, is occurring at a much higher rate than the evolution in the development of antimicrobials by scientists. There is a need for the development of new products that demonstrate a microbicidal effect on all types of pathogenic bacteria, especially those with higher resistance mechanisms. AgNPs are considered as a real alternative for the development of antimicrobials against multidrug-resistant bacteria because of their high toxicity to bacterial cells. However, bacteria have been reported to present resistance mechanisms to AgNPs and it is therefore important to test mixtures of AgNPs with other agents that present microbicidal potential in search of synergy, in order to achieve a better antibacterial effect and counteract resistance. Some EO derivatives have shown broad-spectrum antibacterial effect; d-limonene as the main component of citrus EO showed a good bactericidal effect at low concentra-

tions, becoming an alternative to combat germs of clinical importance.

The formulation containing AgNPs functionalized with d-limonene produced an additive effect to eliminate the growth of pathogenic bacteria, compared to the nanoemulsion containing only AgNP or the nanoemulsion containing only limonene. F1 presented an effect similar to that obtained with commercially available disinfectants; this suggests that AgNP can be enhanced when mixed with essential oils and thus provide better effects against bacterial cell integrity.

Conflict of interest

The authors declared no conflict of interest.

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