

Copper Nanoparticles Enhance Bactericidal Activity of 70% Ethanol Against Multidrug-Resistant *Serratia marcescens*.

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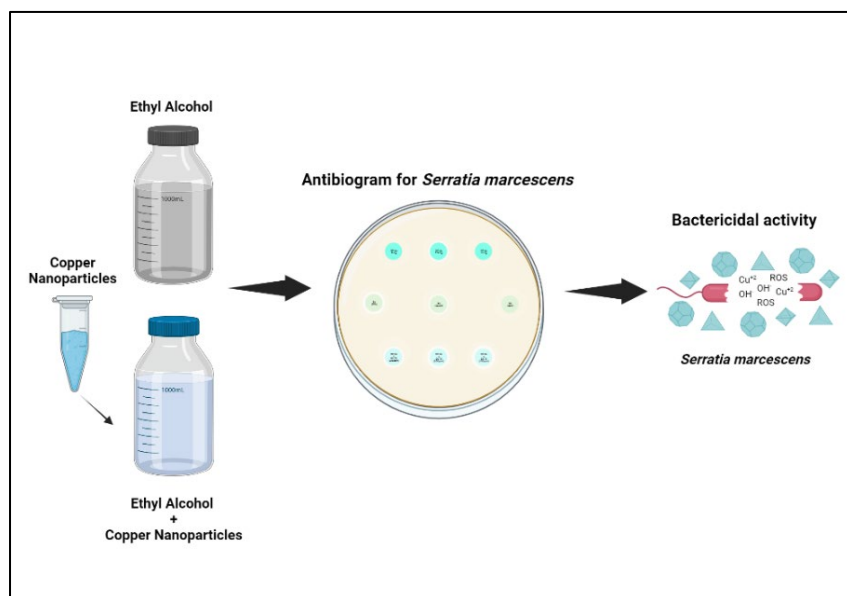
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ABSTRACT

Copper nanoparticles (CuNPs) were synthesized by an adaptation of the chemical reduction method, using cupric sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) and sodium borohydride (NaBH_4) as a reducing agent, to combat bacterial resistance that leads to the proliferation of hospital infections caused by the bacterium *Serratia marcescens* through the potentiation of 70% ethyl alcohol (EA70) as a bactericidal agent through the addition of CuNPs. Characterization was performed with Fourier transform infrared spectroscopy (FTIR), UV-visible spectrophotometry, scanning electron microscopy (SEM), and energy dispersive spectroscopy (EDS), such that the CuNPs reached a diameter of 20-50 nm. The bactericidal activity was performed by inoculating the bacterium in Petri dishes with TM MEDIA Mueller Hinton agar, and its effectiveness was verified with susceptibility testing discs. The analysis was conducted at concentrations of 100, 300 and 500 mg/L; the current results have demonstrated that the lowest concentration shows a better inhibition halo, with a maximum of 13 mm, thus observing the synergism between both substances.

Keywords: Nanoparticles, antibiogram, inhibition, antimicrobial, nosocomial infection, synergism.



INTRODUCTION

Antiseptics are topical substances that eliminate microorganisms on the skin, mucous membranes, and wounds to prevent infectious diseases¹. Bacterial resistance to these chemical agents is a problem that is scarcely considered worldwide, reducing the effectiveness of epidemic control and giving rise to microorganisms resistant to antibiotics and biocides in general^{2,3}. This is generated by inadequate use or use of low concentrations of the same due to their ability to transfer antimicrobial resistance genes⁴. One of the most widely used antiseptics in the clinical and medical area is EA70, as it allows skin disinfection prior to blood draws and is used to perform prophylaxis of central venous catheters, highlighting that it kills up to 90% of bacteria when it remains on the skin for at least 2 minutes without drying after its application^{5,6,7}. EA70 fulfills a bactericidal function against certain vegetative microorganisms, whose mechanism of action is based on high-impact damage to the phospholipid layer of the cell membrane and to intracellular proteins, causing their denaturation^{8,9}. Having portrayed this, it is essential to mention *Serratia marcescens*. This gram-negative bacillus belongs to the *Enterobacteriaceae* family as a bacteria of interest in the hospital environment since it has been shown that one of its reservoirs is the antiseptics used in this area, against which it has acquired resistance^{10,11,12}. This results in a significant health problem since the reduction in the bactericidal activity of these substances against this etiological agent allows it to be the cause of nosocomial infections, which are those contracted during a health care process in a health facility and whose main characteristic is that it was not present or in the incubation period before the patient's admission^{13,14}. Within these cases, this opportunistic microorganism has a prevalence of 1-2% and can cause urinary tract, central nervous system, eye and bloodstream infections, focusing its principal place of transmissibility in ICUs (Intensive Care Units) and newborn ICUs^{15,16,17}. In the hospital context, a generally effective bactericidal agent is copper (Cu), which is used in the disinfection of high-contact surfaces; it is worth mentioning in this section that the United States Environmental Protection Agency has stated that Cu was the first element to act as an antimicrobial, also verifying its efficacy in the sterilization of wounds^{18,19}. Derivatives of this metal are not far behind, and among these are copper nanoparticles (CuNPs), which are tiny particles with effective bactericidal activity thanks to their surface-to-volume ratio, measuring 1-100 nanometers and covered by an interfacial layer composed of organic and inorganic molecules and ions^{20,21}. The mechanism of action of CuNPs in bacterial cells is based on cell damage through the generation of reactive oxygen species (ROS), the release of Cu(II) ions, and the replacement or binding of native cofactors in metalloproteins. Also, Cu participates in innate immunity and can give way to the synthesis of ROS in the explosion reaction that occurs internally in phagocytes, increasing bactericidal activity in the process of bacterial phagocytosis^{22,23}. Despite this, there are strains of *S. marcescens* that are resistant to Cu (II), as these often originate from environments rich in heavy metals such as soil and water from mining deposits, for which they must adapt through different tolerance and elimination strategies such as intra and extracellular ion sequestration, the transformation of toxic metal species into non-toxic forms such as sulfides or metal oxides, development of permeability barriers, enzymatic detoxification, biosorption, precipitation, etc^{24,25}. Given the acquired resistance of this commensal to the chemical agents described above, a solution that was considered viable to combat it is the addition of CuNPs to a specific antiseptic also used in hand disinfection, which in this case is EA70, taking into account that this last aspect reduces the rate of nosocomial infections by 40%, all in order to observe the synergistic behavior of both substances and thus increase their bactericidal power against this *Enterobacteriaceae*, this being the main focus of the present study, thus laying significant foundations in the fight against bacterial resistance to bactericides²⁶.

MATERIALS AND METHODS

Reagents

CuSO₄·5H₂O 98% from the brand Sigma Aldrich, NaBH₄ 95% manufactured by Thermo Fisher Scientific, distilled water, gaseous nitrogen, EA70, sample of *S. marcescens* provided by the Laboratory of Biochemical and Microbiological Analysis of the Polytechnic School of Chimborazo, sterile saline solution 0.9%, BD Difco Nutrient Broth and TM MEDIA Mueller Hinton agar.

Synthesis of Copper Nanoparticles

A modification of the chemical reduction method²⁷ synthesized the CuNPs. 0.5000 g of CuSO₄·5H₂O were weighed and filled to the mark in a 100 mL Florence flask with water treated with gaseous nitrogen. The solution was then sonicated with the Florence flask covered in the ultrasound equipment for 5 minutes. Subsequently, the contents were placed in a 250 mL beaker and agitated in the shaker for 30 min, uniformly adding 10 ml of a 0.8 M NaBH₄ reducing mixture. The mixture sat for 48 hours, covered with aluminum foil. After this time, the samples were centrifuged for 5 min at 3000 rpm in a Digtor 21 C Ortoalresa centrifuge and washed with distilled water, 70% antiseptic alcohol and distilled water two more times using the same process. Lastly, the CuNPs were left to dry in an air circulation oven for 24 hours at 33 °C.

UV-Visible Spectrophotometry

The characterization of optical properties was carried out through scanning in a Thermo Fisher Scientific Evolution 220 spectrophotometer with an analysis range of 500 to 190 nm for the CuNPs and from 300 to 190 nm for the EA70 with CuNPs, where everything was operated with a resolution of 1 nm. The purpose of this equipment is to determine the wavelength at which the substances analyzed in this study absorb light significant way²⁸.

Fourier Transform Infrared Spectroscopy (FTIR)

The structural characterization of the copper nanoparticles was carried out by infrared spectroscopy with Fourier transform (FTIR), for which a JASCO FT/IR-4100 infrared spectrometer was used. This instrumental technique makes it possible to detect the rotation and vibration of molecules that respond to infrared radiation under a defined wavelength using characteristic peaks of the bonds of each compound in an analysis range of 4000 to 520cm⁻¹²⁹.

Scanning Electron Microscopy (SEM) with Energy Dispersive Spectroscopy (EDS)

The nanostructural characterization was carried out using a scanning electron microscope SEM, JSM-IT100LA, manufactured by JEOL, operated at 20.0 kV. This tool directs a beam of low-energy electrons toward the analyzed materials and observes its surface³⁰. Coupled with this, the elemental composition analysis of the concentration and weight of CuNPs was carried out employing EDS³¹.

Evaluation of bactericidal activity

To evaluate bactericidal activity, a strain of *S. marcescens* was inoculated in BD Difco Nutrient Broth for enhancement and incubated at 37°C for 24 hours. Once this time had passed, an inoculum was prepared with sterile saline solution with 0.9% turbidity similar to scale 0.5 McFarland 10⁸ CFU/ml and a massive inoculation was carried out with a sterile swab in quadruplicate in petri dishes with TM MEDIA Mueller Hinton agar³². Thermo Fisher susceptibility test discs with a diameter of 6 mm with 5, 7.5 and 10 mg CuNPs were placed in the culture medium, impregnated with a jaw press. Next, with the help of a micropipette, using the disc diffusion technique, 20 uL of 70%, 52.5% and 35% ethyl alcohol solutions were placed, respectively, and 20 uL of a prepared mixture of 100, 300 and 500 mg/L of CuNPs with EA70³³. For the interpretation of results, the inhibition halo of each of the disks was measured with a conventional ruler.

RESULTS AND DISCUSSION

UV-Visible Spectrophotometry

The UV-visible spectrophotometry of the CuNPs gave a spectrum shown in Figure 1a, which peaked at 265 nm due to the absorption phenomenon of the surface plasmon, which originates from the joint oscillation of

free electrons in the conduction band and which was stimulated by the incoming UV radiation; it is also important to note that the wavelength obtained is within a characteristic range, which is 200-300 nm, thus determining the presence of CuNPs³⁴. Moreover, in the characterization of CuNPs with EA70, an absorption spectrum with an average peak of ≈ 193 nm was obtained for the three samples analyzed, which is observed in Figure 1b, and worth mentioning that the fact of adding alcohol as a solvent caused the absorption wavelength to decrease by approximately ≈ 72 nm, which is also known as hypochromic change, which occurs due to the π - π^* stacking interaction when both substances react with each other³⁵.

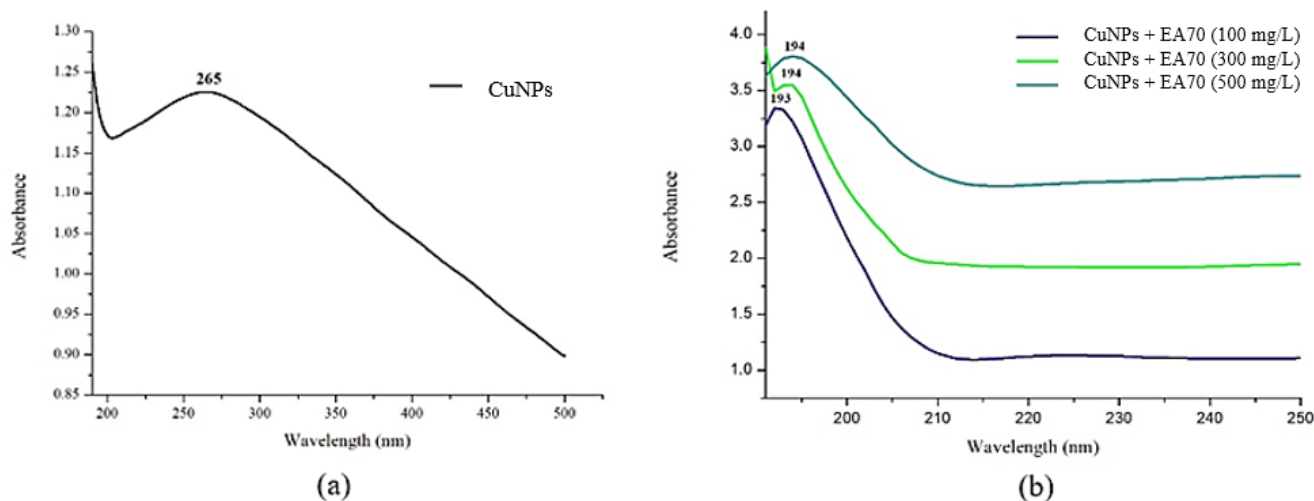


Figure 1. a) UV-visible spectrum of CuNPs; b) UV-visible spectrum of CuNPs + EA70 at different concentrations.

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectrum shows a band characteristic of the stretching vibrations of the Cu-O bond, where the wavenumber associated with this typical peak is 598 cm^{-1} ³⁶. Two peaks that are found at approximately 791 and 876 cm^{-1} indicate metal-oxygen (M-O) stretch vibrations; on the other hand, the bending and stretching vibrations that occur due to the moisture content on the surface of the CuNPs were determined by the peaks located approximately at 1655 and 3402 cm^{-1} ³⁷. Bands between 800 and 1250 cm^{-1} could be part of the vibration modes of the remaining $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ that did not react in the process³⁸. Figure 2 shows the spectrum with the characteristics described in this section.

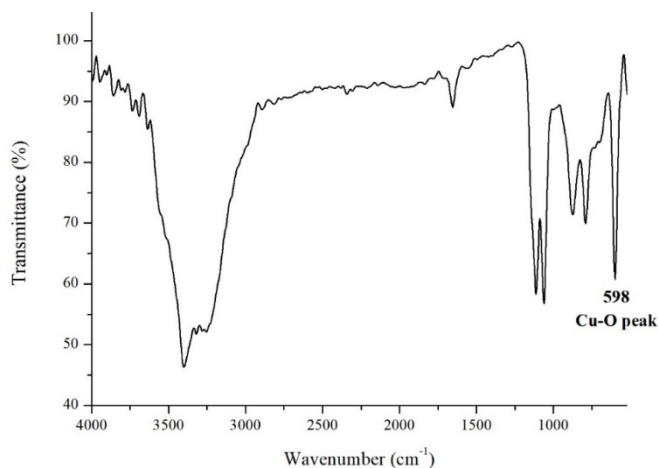


Figure 2. Fourier Transform Infrared Analysis Spectrum of CuNPs.

Scanning Electron Microscopy (SEM)

Figure 3 shows the nanoparticles microscopically at a scale of 1 μm , with a diameter range of 20-50 nm and different polyhedral shapes, as well as considerable agglomeration ²².

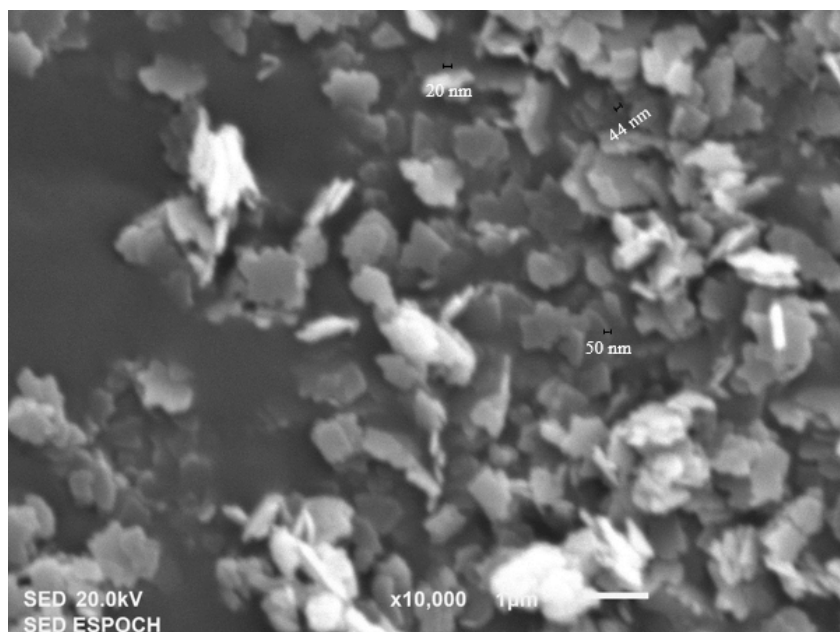


Figure 3. Analysis of CuNPs morphology using scanning electron microscopy.

Energy Dispersive Spectroscopy (EDS)

Samples were previously coated with gold through spray coating prior to observations to avoid the loading effect. The energy-dispersive spectroscopy presented a spectrum shown in Figure 4, where peaks of Cu are shown, confirming the presence of this metal. The mass ratio between Cu and O was 5.65% and 1.08%, respectively, while their atomic composition was 50.23% and 38%. Moreover, the presence of sulfur in 7.74% is a byproduct since the synthesis was made with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. The presence of other metals, such as aluminum, are traces that arose during synthesis and do not intervene in the process. ³⁵

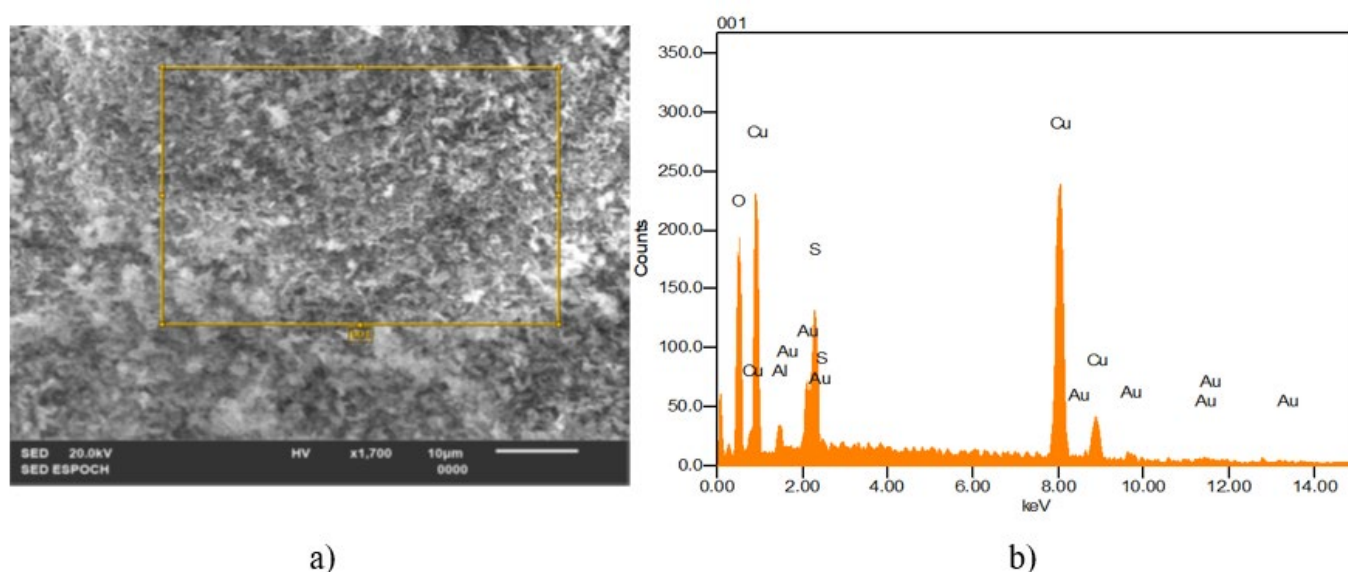


Figure 4. (a) Sample analysis area for (EDS); b) Spectrum of peaks of elements that make up the CuNPs sample

Bactericidal activity

When analyzing the results of the bactericidal activity of the different substances against *S. marcescens*, it was possible to observe that, in the case of CuNPs, no inhibition halos were obtained for 5 and 10 mg; however, for 7.5 mg, a confirmatory antimicrobial susceptibility test was performed again because the presence of a slight halo could be evidenced, where in this repetition of the procedure an inhibition zone of 8 mm was obtained. Additionally, in evaluating the bactericidal activity of ethyl alcohol (EA), inhibition halos of 6, 9 and 10 mm were obtained for 35%, 52.5% and 70%, respectively. Lastly, the bactericidal activity of EA70 in conjunction with CuNPs resulted in inhibition zones of 13, 10 and 9 mm for 100, 300 and 500 mg/L. Figure 5 and Figure 6 show the results photographically and statistically, respectively.

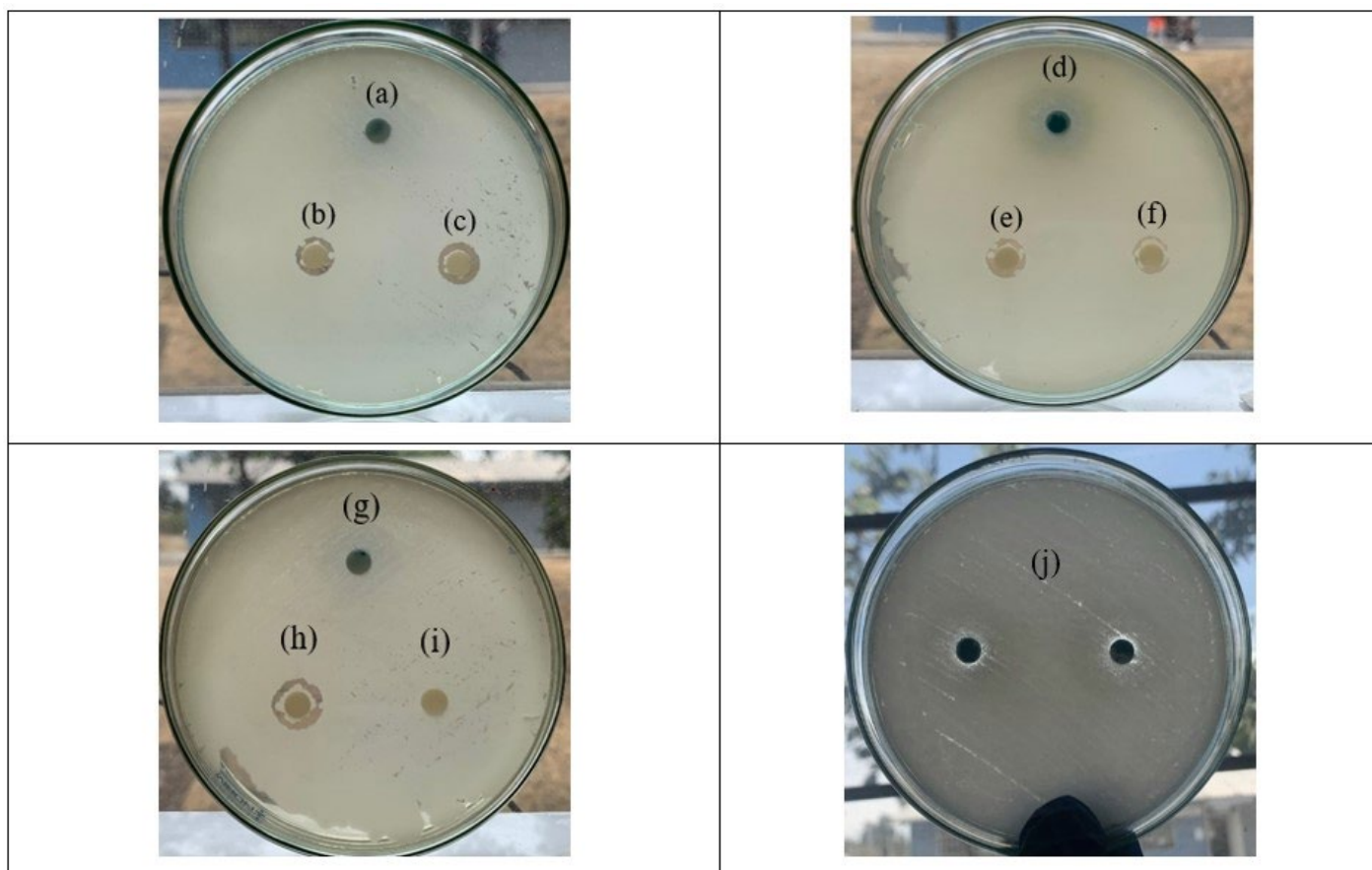


Figure 5. Comparison of the bactericidal activity of CuNPs, EA70 + CuNPs and EA at different concentrations against *S. marcescens* (a) 10mg of CuNPs, (b) 500 mg/L of EA70 + CuNPs c) EA70, (d) 7.5mg of CuNPs, (e) 300 mg/L of EA70 + CuNPs, (f) EA at 52.5% (g) 5mg of CuNP, (h), 100 mg/L of EA70 + CuNPs (i) EA at 35%, (j) 7.5 mg of CuNPs.

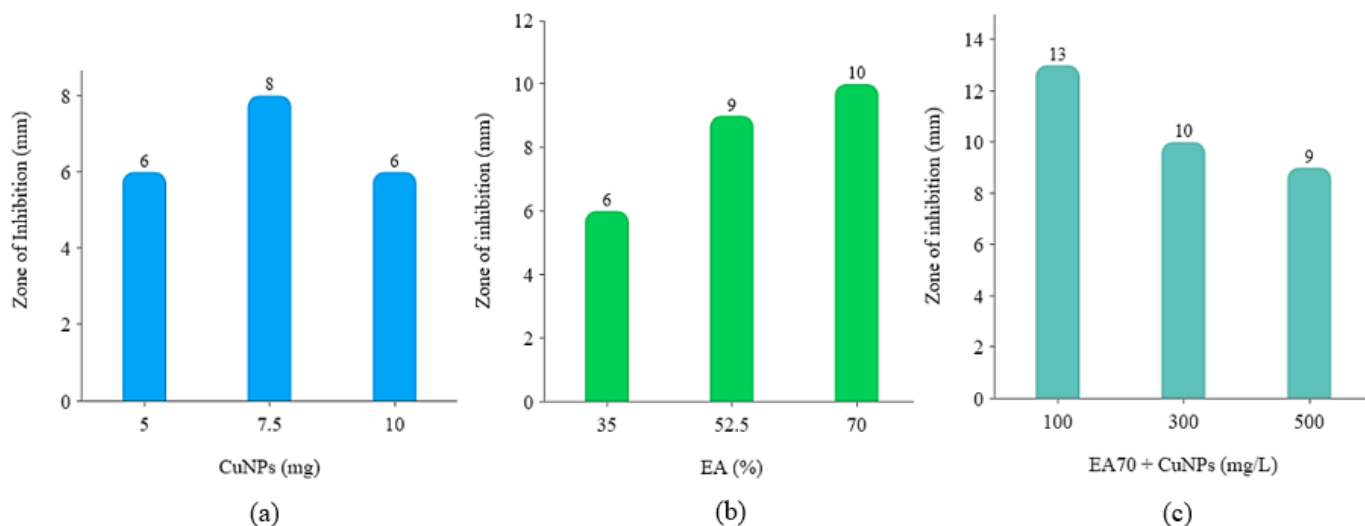


Figure 6. Zone of inhibition concerning the concentration of the bactericidal agents used. (a) CuNPs. (b) EA. (c) EA70 + CuNPs

S. marcescens was chosen as the study subject due to its well-known resistance to several biocides¹². The present research demonstrated that CuNPs alone exhibit significantly reduced bactericidal activity, possibly due to their stability being affected by the culture medium used for performing the antibiogram. It is essential to mention that stabilizing agents such as surface coating are necessary to avoid this. On the other hand, it was observed that the ideal concentration to potentiate EA70 by adding CuNPs was 100 mg/L or less; however, further studies are needed to confirm this. Therefore, it is possible to determine that increasing the concentration does not necessarily enhance the bactericidal effect. This occurs because high amounts of nanoparticles in the solvent tend to agglomerate with each other. It was considered that fewer agglomerations result in a larger available surface area, which induces more excellent ROS production and, thus an increase in their interaction with the bacterial membrane and the release of Cu (II) ions in the surrounding matrix. This phenomenon does not occur to a great extent in this case³⁹.

The uniqueness of this study lies in its focus on the effectiveness of CuNPs combined with EA70 against *S. marcescens*, a bacterium that can be found in hospital antiseptics. The research results highlight the significant resistance of this bacterium to the mentioned substances, thus challenging conventional asepsis strategies. However, it is emphasized that adding CuNPs to EA70 shows promising potential to overcome this resistance and enhance the antiseptic's efficacy. These findings pave the way for new avenues of research in developing innovative solutions in the fight against resistant bacteria in clinical settings.

CONCLUSIONS

The characterization of the CuNPs by UV-visible spectrophotometry showed a characteristic peak at 265 nm due to the absorption phenomenon of the surface plasmon. Moreover, in the characterization of CuNPs with EA70, an absorption spectrum with an average peak of ≈ 193 nm was obtained for the three samples analyzed. Fourier transform infrared spectroscopy provided a characteristic peak at 598 cm^{-1} , indicating a stretching vibration due to the presence of the Cu-O bond.

Scanning electron microscopy revealed the size of the CuNPs, whose length was approximately 20-50 nm, thus evidencing their nanometric size. The EDS was also performed, where peaks of Cu and O were observed.

CuNPs alone cannot significantly inhibit *S. marcescens*, as evidenced by the antimicrobial susceptibility test, given the formation of a small-diameter halo.

The EA showed no bactericidal power against *S. marcescens* at a concentration of 35% and slight effectiveness at a concentration of 52.5%.

An increased concentration of CuNPs in EA70 does not necessarily imply an improvement in bactericidal activity, as the agglomeration effect causes this. Using stabilizing agents in CuNPs is essential to ensure complete uniform dispersion and enhanced stability in the solution, which helps maintain their bactericidal activity and prevents particle aggregation.

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