Research Article

Antibacterial Activity of Green Synthesized Copper Oxide Nanoparticles Using *Muntingia calabura* Leaf Extracts against *Staphylococcus aureus* and *Escherichia coli*

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ABSTRACT

Background: The surge in bacteremia and healthcare-associated infections, driven by aging populations and antimicrobial resistance, prompted research into nanotechnology. Copper Oxide Nanoparticles (CuONPs) have emerged as a promising candidate due to their potent antibacterial properties. *Muntingia calabura*, renowned for its medicinal uses, presents a novel avenue for CuONP synthesis. This study aimed to evaluate the antibacterial efficacy of CuONPs against *Staphylococcus aureus* and *Escherichia coli*. **Materials and Methods:** *M. calabura* leaves extracts were utilized to synthesize CuONPs from Copper (II) Sulfate Pentahydrate. CuONPs morphology was characterized by Scanning Electron Microscopy. Subsequently, the antibacterial properties of the CuONPs with various concentrations of 25%, 50%, 75% and 100% were analyzed against *S. aureus* and *E. coli* using agar well diffusion and MIC determination *vis-à-vis* gentamicin as the control. **Results:** The results showed antibacterial potential with 75% leaf extract concentration showing the highest Zone of Inhibition (ZOI) and 50% as Minimum Inhibitory Concentration (MIC) for *S. aureus*. While, 50% of the concentration showed the highest ZOI and MIC at 25% for *E. coli*. ANOVA and Tukey's *post-hoc* determined a significant difference between the different concentrations of the CuONPs (*p*-values=0.000 and 0.035). However, only the antibacterial activity of the CuONPs against *E. coli* showed no significant difference with the positive control (*p*-value=0.125). **Conclusion:** Although the synthesized CuONPs are deficient compared to positive control gentamicin, it manifests significant antibacterial potential against *S. aureus* and *E. coli* and is recognizable as a potential alternative in combating antibacterial resistance.

Keywords: Bacteriemia, Antibacterial resistance, Copper oxide nanoparticles, *Muntingia calabura*, *Staphylococcus aureus*, *Escherichia coli*.

INTRODUCTION

The rise in bacteremia and Healthcare-Associated Infections (HAIs) in developed nations, linked to aging populations and increased healthcare demands, emphasizes *S. aureus* and *E. coli* as major causative agents.[1] *S. aureus,* a gram-positive bacterium, is a major contributor to infections acquired in the community and healthcare settings, posing a threat to health when entering the bloodstream or tissues. It commonly resides in the environment and human flora, particularly on the skin and mucous membranes like the nasopharynx and spreads through direct or indirect contact. Moreover, its

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antibiotic resistance has been escalating, contributing to its growing infection prevalence.[2]

Conversely, *E. coli*, a gram-negative bacterium, naturally resides in the intestinal flora and encompasses numerous strains linked to a broad spectrum of diseases. Its manifestations range from various diarrheal illnesses such as dysentery to conditions like traveler's diarrhea. *E. coli* ranks among the primary pathogens causing cystitis, pneumonia, bacteremia and abdominal infections like spontaneous peritonitis. Typically, infection stems from bacterial translocation from the intestine and treatment strategies vary based on the strain's characteristics and pathogenicity.[3] According to a World Health Organization report,[4] HAIs and antimicrobial resistance result in significant mortality rates, with over 24% of patients with sepsis and 52.3% in intensive care units succumbing annually. This underscores the urgency for innovative nanotechnological approaches to combat antibiotic-resistant bacterial infections.

Nanotechnology, with its widespread adoption across industries, has notably seen significant growth in nano-scale markets in recent years.[5] The synthesis of Copper Oxide Nanoparticles (CuONPs) is particularly interesting, renowned for their exceptional antibacterial properties. The application of copper-based materials in healthcare exhibits toxicity against various microorganisms, including bacteria, fungi and viruses. Plant-mediated, microbial and enzymatic synthesis emerge as eco-friendly methods for nanoparticle production, offering sustainability and cost-effectiveness.[6,7]

M. calabura, known as Aratiles locally, is abundant in the Philippines despite its origin in tropical America. Apart from being enjoyed for its sweet fruits, it holds multiple health benefits, including antioxidant and anticancer properties attributed to its leaves.[8] Traditionally, *M. calabura* have been used to address health issues like loose bowel movement and bacterial infections, often in the form of tea prepared from its leaves.[9] Additionally, decoctions from *M. calabura* flowers are believed to alleviate abdominal cramps due to their antiseptic and anti-inflammatory properties.[10] Despite its medicinal use, *M. calabura* have not been explored as a natural reagent for green synthesis of nanoparticles, particularly CuONPs. This study aims to evaluate the antibacterial efficacy of CuONPs synthesized from *M. calabura* against *S. aureus* and *E. coli*.

This study was generally conducted to determine if the biosynthesis of Copper Oxide Nanoparticles (CuONPs) from *M. calabura* is possible using known and modified protocols and to identify its antibacterial activity against *S. aureus* and *E. coli*. Different research has shown positive results in the biosynthesis of CuONPs using various organic sources, although *M. calabura* has been used to synthesize nanoparticles to test bactericidal effects, there are currently no studies that synthesized CuONPs specifically. This study is mainly focused in investigating the effectiveness of the green synthesized CuONPs, using *M. calabura* leaves as a biosource, in inhibiting *S. aureus* and *E. coli*.

MATERIALS AND METHODS

Preparation of *M. calabura* Leaf Extract

Fresh *M. calabura* leaves were thoroughly washed in sequence with tap and deionized water to remove any dust particles. The leaves were dried in an oven at 100ºC for 30-45 min, then ground using a mortar and pestle until they turned into a fine powder. The 12.5 g of *M. calabura* powder was boiled with 250 mL of deionized water for 30 min maintained at 60ºC. After boiling, it was allowed to cool at room temperature (around 27ºC), then the extract was initially filtered using cheesecloth to remove excess leaves. It was then filtered again using Whatman No. 1 filter paper.[11]

Biosynthesis of CuONPs

Four concentrations were prepared: 25%, 50%, 75% and 100%. These concentrations were achieved using varying ratios of Leaf Extract (LE) and Deionized Water (DIW). As a source of CuONPs, 3.25 g of analytical grade Copper (II) Sulfate Pentahydrate ($CuSO_4.5H_2O$) was dissolved in 125 mL of distilled water to form a solution.[12] For the concentrations, 25% concentration was a 1:4 ratio of LE to DIW; 50% concentration utilized a 2:4 ratio; 75% concentration consisted of a 3:4 ratio; and the 100% concentration was a 4:4 ratio. All of the concentrations were combined with 25 mL of $CuSO_4.5H_2O$.[11]

Characterization of the green synthesized CuONPs

The size, shape, average size, size distribution and image of the freshly obtained green synthesized CuONPs from *M. calabura* leaves extract was determined and described using a Scanning Electron Microscope from the Department of Science and Technology - Advanced Technology and Materials Testing Laboratory.[13,14]

Preparation of Bacterial Inoculum

Bacterial inoculum was prepared using fresh isolates of *S. aureus* and *E. coli,* through sterile inoculating loops into two sterile test tubes that contained 10 mL of Normal Saline Solution (NSS). The concentration of the bacterial inoculum was meticulously assessed utilizing the McFarland standard with a Wickerham card to ensure both consistency and accuracy.[15]

Agar Well Diffusion Method

The prepared bacterial inoculum of *S. aureus* was swabbed across the entire surface of the 90 mm Mueller Hinton Agar plate using a sterile cotton swab. This process was repeated for *E. coli*. A triplicate was performed, with a total of three plates for each bacteria. Following inoculation, allow the bacterial lawn to air dry. Then, pipette tips with a diameter of 6mm were used for the carving of wells. Four wells in each agar were created for the different concentrations of the CuONPs. Using an automated pipette with sterile pipette tips, 10µL of MHA was transferred into each well to seal the bottom of the well. Subsequently, 20µL of the prepared concentrations (100%, 75%, 50% and 25%) were dispensed to each well. A gentamicin disc (10 µg/mL) was placed in a separate MHA plate as a control. Lastly, the plates were then placed into the incubator for 24 hr at 37ºC.[11]

Following the incubation, the inoculated plates were examined using a calibrated caliper for accurate measurement of the zones of inhibition. The measurements of the zones of inhibition for each concentration of CuONPs and the control disk were recorded in millimeters as shown in Figure 1. This data included three measurements for each disk to ensure precision and reliability.[16]

Determining the Minimum Inhibitory Concentration (MIC) using Dilution

A modified protocol was adapted to determine the Minimum Inhibitory Concentration (MIC) of *S. aureus* and *E. coli* by utilizing the method of dilution.[11,17] The MIC of the bacteria was determined starting from the lowest concentration at which the bacterial activity is totally suppressed. Different concentrations of the green synthesized CuONPs were prepared through various dilutions of the mother concentration of CuONPs to the sterile Mueller-Hinton Broth (MHB). The 25% concentration tube was a 1:4 ratio of CuONPs to MHB. The 50% concentration tube utilized a 2:2 ratios. The 75% concentration tube followed a 3:4 ratios. And lastly, the 100% concentration tube consists of 5 mL of the CuONPs. The assay for the positive control, gentamicin and the negative control was also performed without the presence of the CuONPs. These were done for both bacteria. To ensure accuracy, a triplicate was made. The tubes were incubated for 24 hr at 37ºC and then observed for turbidity to determine the MIC.

Statistical Treatment

One-way Analysis of Variance (ANOVA) was used in the study to assess the significant difference among the zones of inhibition of green synthesized CuONPs with different concentrations (25%. 50%, 75% and 100%) and a positive control using gentamicin (10 µg/mL) against *S. aureus* and *E. coli*. Upon determining that there is a significant difference between the zones of inhibition, Tukey's *post hoc* analysis was also used for the specific assessment of differences among the concentrations.

RESULTS

Biosynthesis of Copper Oxide Nanoparticles

The present study deals with the green synthesis of copper oxide nanoparticles using *M. calabura* leaf

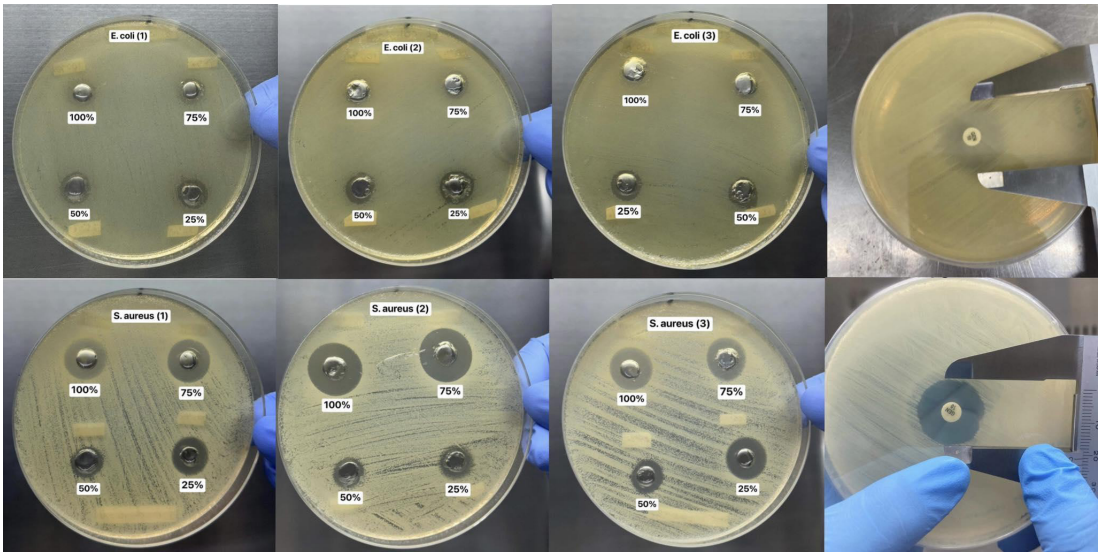

Figure 1: The results of the Agar Well Diffusion Test that shows the Zone of Inhibition of *S. aureus* and *E. coli* against the Different Concentrations of the green synthesized CuONPs and control.

extract. During the synthesis process, there was a visible change of color, varying from green to brownish-green, depending on the concentration. The color became darker as the concentration increased from 25% to 100% as shown in Figure 2.[18]

Characterization of Green Synthesized Copper Oxide Nanoparticles

Scanning Electron Microscope analysis was utilized to analyze the morphological characteristics of the copper oxide nanoparticles synthesized from *M. calabura* leaf extract. The micrograph in Figure 3 shows the nanoclusters of CuONPs. Still, the high magnification power (50,000x) reveals the spherical shape of the CuONPs and a significant variation in particle size, with an average diameter of approximately 65.38 nm.[19]

Antibacterial Activity

Zone of Inhibition

Through measuring the zones of inhibition on both *S. aureus* and *E. coli* against different concentrations of the CuONPs, the antibacterial activity was determined. Respectively on Figure 1, presents the agar well diffusion test and four concentrations of green synthesized CuONPs (25%, 50%, 75% and 100%) for evaluating the ability and effectiveness of the said concentrations

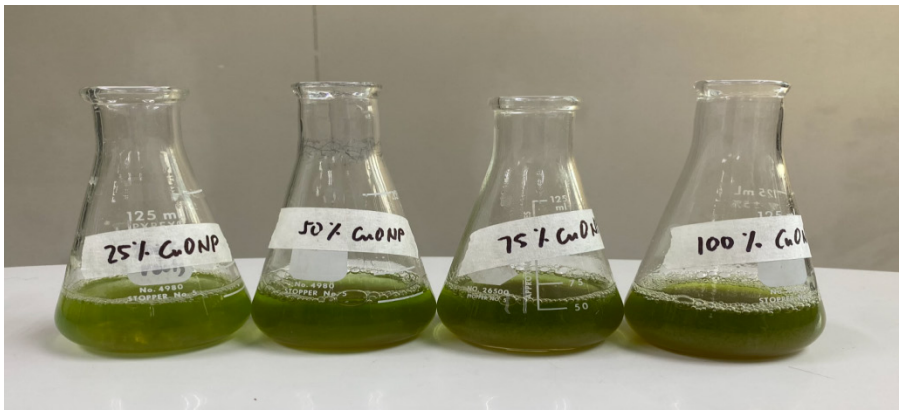

Figure 2: Green synthesis of copper oxide nanoparticles using *M. calabura* leaf extract at various concentrations.

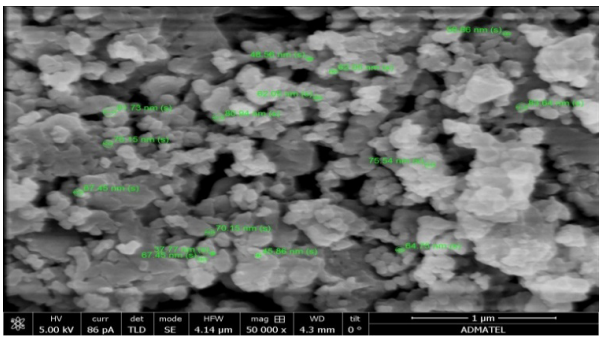

Figure 3: Photomicrograph of SEM analysis of CuONPs analysis synthesized CuONPs at 50,000x magnification scale in the size range of 1000 nm.

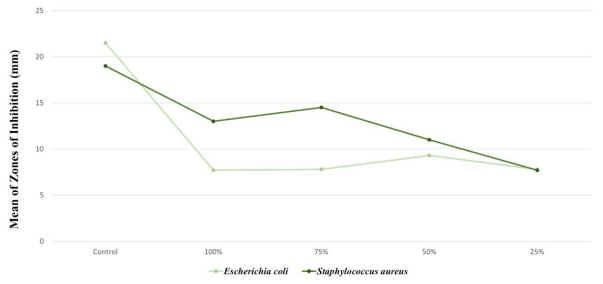

Figure 4: Antibacterial activity of the different concentrations of CuONPs against selected bacterial strains.

through its inhibition of growth presented against the pathogenic bacteria. Moreover, Figure 4 shows the average antibacterial activity of each concentration against both bacterial strains. The triplicate results show that the zone of inhibition was more prominent against *S. aureus* at 75% concentration 14.5 mm±1.32. Meanwhile, CuONPs at 50% concentration showed the largest zone of inhibition against *E. coli* at an average of 9.33 mm±1.04 for 50%.[16,20,21]

Minimum Inhibitory Concentration

The minimum inhibitory concentrations of the green synthesized CuONPs against *S. aureus* and *E. coli* were assessed using the broth dilution method.[11] The results revealed that 50% concentration of leaf extract in CuONPs is the minimum inhibitory concentration for *S. aureus*, while 25% is for *E. coli*. Figure 5 shows the graphical representation of the antibacterial activity of different concentrations against both bacterial strains.

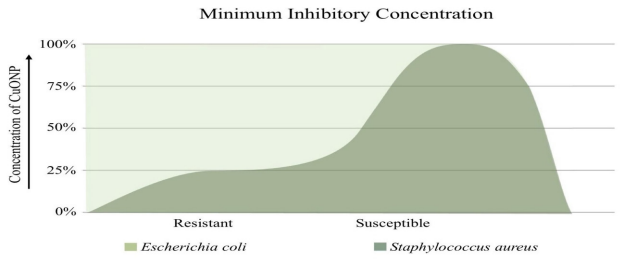

Figure 5: Minimum Inhibitory Concentration of CuONPss against selected bacterial strains.

Difference between different concentrations

This study also assessed the impact of the varying concentrations of *M. calabura* leaf extract on the antibacterial activity of the CuONPs. As shown in Figure 6, the statistical analysis revealed a significant difference in antibacterial activity against *S. aureus* between most extract concentrations, especially when comparing 25% to higher concentrations with *p*-value<0.000. While there is a significant difference between 50% and 100%, the *p*-value (0.035) suggests a weaker effect than the other comparisons. Similarly, the antibacterial activity of the CuONPs against *E. coli* all exhibited significant differences (*p*-value=0.035) between the different concentrations.

Difference between concentrations and control

The antibacterial capability of CuONPs with varying concentrations of *M. calabura* leaf extract against *S. aureus* and *E. coli* was also compared to a control, gentamicin (10 ug). Based on the results of the statistical analysis shown in Figure 7, the different concentrations of CuONPs collectively showed higher significance than the conventional antibiotic against *S. aureus*, with *p*-value of 0.004. However, when used against *E. coli*, there is no significant difference (*p*-value=0.125) between the antibacterial activity of the varied concentrations and the control.

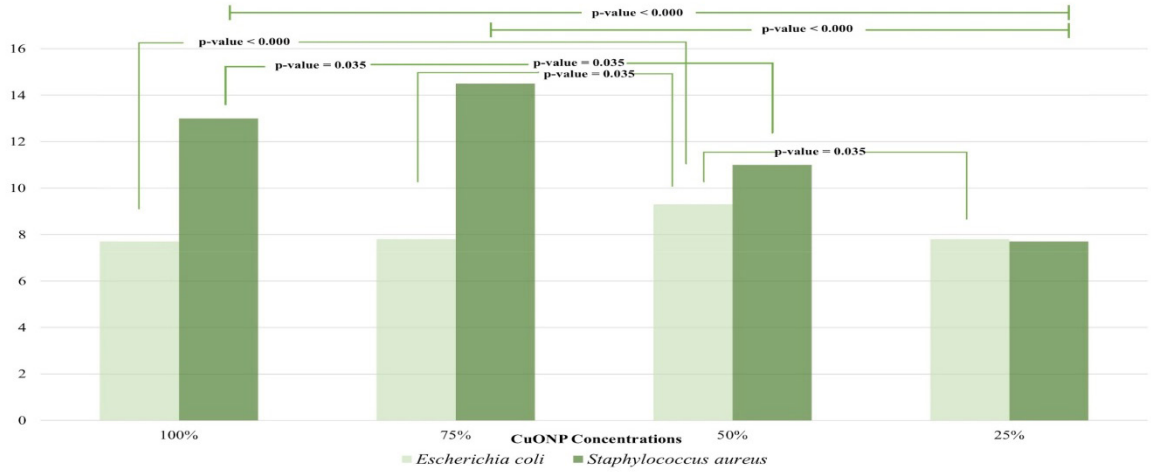

Figure 6: Statistical analysis of the difference between different concentrations.

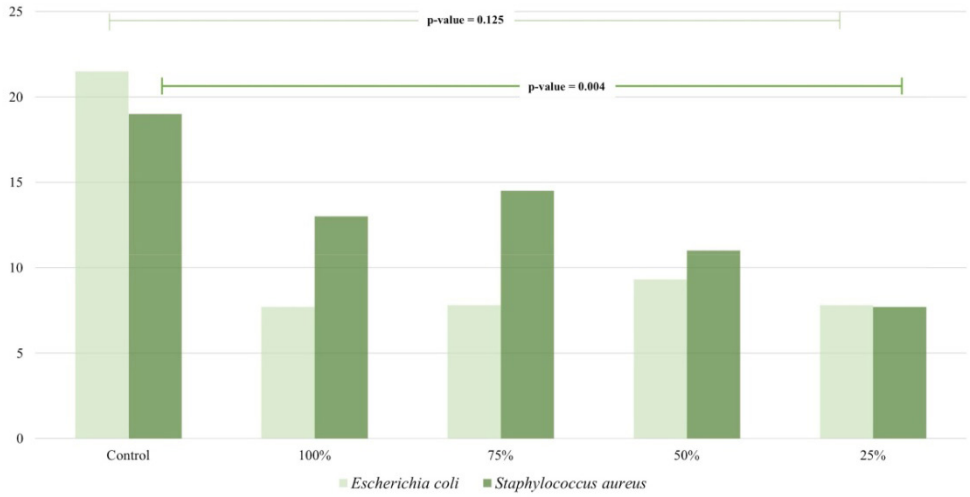

Figure 7: Statistical analysis of the difference between the concentrations and control.

DISCUSSION

This study explored an alternative approach to address the rising threat of antibiotic resistance. Specifically, the investigation focused on the potential of green-synthesized copper oxide nanoparticles derived from *M. calabura* leaves extract to combat *S. aureus* and *E. coli*, two common bacteria associated with healthcare-acquired infections, through various antimicrobial susceptibility tests. Based on the results, the CuONPS displayed significant antibacterial activity against both strains, with specific concentrations proving most effective, even when compared to the potency of the conventional antibiotic gentamicin.

This study will mainly explore the possibility of synthesizing CuONP using *M. calabura* leaves. This will include the characterization using a Scanning Electron Microscope (SEM). The evaluation of the antibacterial activity of the green synthesized CuONP, specifically targeting *S. aureus*, a gram-positive bacterium and *E. coli,* a gram-negative bacillus, by utilizing agar well diffusion test. Concurrently, MIC will employ a dilution method. In the antibacterial activity testing against *S. aureus* and *E. coli*, only gentamicin with a concentration of 10 µg/mL will be employed as the positive control while evaluating the efficacy of the green-synthesized nanoparticle. This study will not utilize other parts of the Aratiles plant to synthesize CuONP. It will be limited to the bacteria mentioned above for antibacterial testing and the results will only be compared to the output of the standard antibiotic.

These findings highlight the promising potential of plant-derived CuONPs as a weapon in the battle against antibiotic-resistant bacteria. Further research to optimize their potency and ensure safety could pave the way for a novel and much-needed therapeutic approach in the fight against the increasingly concerning pathogens.

The plant *M. calabura* has different applications in the field of medicine, especially the leaves. Its phytochemical analysis exhibits the presence of bioactive compounds that are significant in the treatment of various diseases.[8,22] Thus, in the green synthesis of copper oxide nanoparticles, they were subjected to color change, which is green to brownish green, depending on different concentrations. This demonstrates the formation of copper oxide nanoparticles and the complete reduction of copper ions, indicating the attainment of biosynthesis similarly discussed in the study of Sivaraj *et al.*[11]

The SEM analysis revealed the average size of the green synthesized CuONPs, 65.38 nm from a total of 30 nanoparticles measured for greater accuracy. This size range suggests that the CuONPs have good antibacterial potential which is similar to the study of Amin *et al.* and Balela and Amores.[20,23] A similar study by Priya *et al.* that also synthesized CuONPs confirms that smaller nanoparticles typically have a larger surface area and potentially better penetration into bacteria[12], contributing to more substantial antibacterial effects. Moreover, Rajeshkumar *et al.*[14] pointed out that the spherical shape of CuONPs can indirectly influence other factors relevant to the antibacterial activity as supported by the study of Ramzan *et al.*[24] that showed spherical and symmetric nanoparticles. Its spherical shape reduces aggregation which could benefit antibacterial activity by allowing individual nanoparticles to interact more effectively with bacteria.

The evaluation of the antibacterial effect of CuONP through agar well diffusion revealed notable findings regarding its efficacy across varying concentrations. Through agar well diffusion, the antibacterial effect of CuONP was evaluated with different concentrations.[12,16,20,21] Despite the discrepancies that were presented, all concentrations were able to inhibit the bacteria. For *E. coli,* the lowest Zone of Inhibition (ZOI) was 100% and for *S. aureus*, the biggest ZOI was 75% and 25%, respectively, for the smallest. It was emphasized that there was a dose-dependent relationship between CuONP concentration and the zone of inhibition against various pathogens, hence the result of the well diffusion method, which was also highlighted in the study of Ali *et al.* and Vinnothkana *et al.*[16,25] However, the greater efficacy of the green synthesized CuONPs in inhibiting the gram-positive *S. aureus* in comparison to the gram-negative *E. coli* is due to the fact Copper ions can easily penetrate the thick layer of peptidoglycan leading to the denaturation of protein, or its cell wall, leading to cell death as explained by Ijaz *et al.*[26]

The Minimum Inhibitory Concentration (MIC) via broth dilution correspondingly demonstrated the potent antibacterial property of the green-synthesized CuONP, with varying efficacy against different bacterial strains. The broth dilution presented 50% leaf extract as the MIC for *S. aureus* and 25% for *E. coli*. The findings highlight the antibacterial capability of the green synthesized CuONPs even at significantly low concentrations. However, the effectiveness of the green synthesized CuONPs varies on bacterial strains since the inhibition of a gram-negative *E. coli* only required a 25% concentration while 50% was needed to inhibit the gram-positive *S. aureus*.[17] Nonetheless, it further supports the mechanism of CuONPs' ability as a potential antimicrobial agent that can be further studied as an effective antibiotic, particularly among gram-negative bacteria.[11]

Examining the pivotal influence of different concentrations, the varying concentrations of *M. calabura* leaf extract significantly influence the antibacterial activity of CuONPs.[27] CuONPs exhibited potent antibacterial activity against *E. coli*, with a 75 g/ml concentration resulting in a notable inhibitory zone of 22.20±0.16 mm.[14] This efficacy was attributed to the electrostatic interactions between the CuONPs' surface and the bacterial cell wall, particularly effective against gram-negative bacteria like *E. coli* due to the peptidoglycan layer's appearance. Similarly, the antibacterial activity of Sesbania grandiflora leaf extract-synthesized CuONPs was investigated against *E. coli*.[28] It was found that CuONPs at concentrations of 100 and 125 µg/mL showed substantial zones of inhibition, indicating dose-dependent inhibition of bacterial growth.

As a final consideration, the effectiveness of varying concentrations of *M. calabura* leaf extract on the antibacterial activity of CuONPs was evaluated compared to a control, gentamicin.[29] The disc diffusion method assessed their antimicrobial activity against various human pathogenic bacteria. The results indicated that there is no significant difference between the control and the varying concentrations of the green synthesized CuONPs against *E. coli*. It is suggested that the green synthesized CuONPs were able to dissipate the cell wall of the bacteria, which led to its filamentation.[30]

CONCLUSION

The green synthesized copper oxide nanoparticles show significant antibacterial potential against *S. aureus* and *E. coli*. Thus, the study recognizes the capacity of biosynthesized nanoparticles in combating the emerging problem of antibacterial resistance. This extended the interest in nanotechnology research and supported its diagnostic progression. Consequently, it is imperative to conduct *in vivo* and cytotoxicity studies using animal models to maximize its antibacterial potential.

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AUTHORS' CONTRIBUTIONS

SZSA, CAGB and PRB were involved in planning and conceptualizing the research, PRB supervised the overall research process. IGOB, AMGB and KALB performed the data acquisition/collection; AMGB organized the protocols and kept track of the research expenses. SZSA, CAGB and KALB communicated with supply acquisition sources and other external affairs, SZSA, CAGB and AMGB designed the figures and RDTA performed parameter calculations, while IGOB and AMGB aided. All performed the experimental procedures, analyzed data, drafted and took part in critically revising the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AgNPs: Silver Nanoparticles; **ANOVA:** Analysis of Variance; **CuONPs:** Copper Oxide Nanoparticles; **DIW:** Deionized water; **HAIs:** Healthcare-associated infections; **LE:** Leaf Extract; **MIC:** Minimum Inhibitory Concentration; **MHA:** Mueller Hinton Agar; **MHB:** Mueller Hinton Broth; **NSS:** Normal Saline Solution; **SEM:** Scanning Electron Microscope; **ZOI:** Zone of Inhibition.

SUMMARY

This study explored an alternative approach to address the rising threat of antibiotic resistance. Specifically, the investigation focused on the potential of green-synthesized copper oxide nanoparticles derived from M. calabura leaves extract to combat S. aureus and E. coli, two common bacteria associated with healthcare-acquired infections, through various antimicrobial susceptibility tests. Based on the results, the CuONPS displayed significant antibacterial activity against both strains, with specific concentrations proving most effective, even when compared to the potency of the conventional antibiotic gentamicin.

These findings highlight the promising potential of plant-derived CuONPs as a weapon in the battle against antibiotic-resistant bacteria. Further research to optimize their potency and ensure safety could pave the way for a novel and much-needed therapeutic approach in the fight against the increasingly concerning pathogens.

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