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Antimicrobial and Antioxidant activity of Copper Nanoparticles Synthesised from *Centella asiatica*

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ABSTRACT

Copper nanoparticles can be synthesised using various methods, including chemical reduction, thermal decomposition, and green synthesis approaches. The present study focuses on the green synthesis of copper oxide nanoparticles from the leaf extract of *Centella asiatica*. The synthesised copper nanoparticles were characterised by a UV-visible spectrophotometer, which gave an optical absorbance spectrum at 204.8 nm, and by X-ray diffraction, Bragg's reflection 2ϴ was 30.334°. The synthesised copper nanoparticles exhibited good antimicrobial activity against pathogenic strains of *Bacillus velezensis, Klebsiella ozaenae, Pseudomonas aeruginosa, and Proteus vulgaris, with the* minimum inhibitory concentration of *Bacillus velezensis* and *Proteus vulgaris* being 0.6 µg/ml. The copper nanoparticles showed antioxidant activity with the DPPH method and the FRAP reducing assay.

Key words : Copper Nanoparticles, Antimicrobial, Antioxidant, X-Ray Diffraction, UV Visible Spectrophotometer

INTRODUCTION

Nanotechnology is a multidisciplinary field of research that integrates material science with bioscience and engineering [Tan *et al.*, 2020; Abu Rakheyet *al.*, 2022; Zhang *et al.*, 2019; Wei *et al.*, 2020]. Nanoparticles are materials, namely metals, metal oxides, or semiconductors (quantum dots), with sizes ranging from 1–100 nm. Metals such as gold, silver, platinum, zinc, or copper, due to their unreactive or less reactive nature, are commonly used for the synthesis of nanoparticles [Khatua *et al.*, 2019; Sharma *et al.*, 2018; Ganaieet *al.*, 2018; Rajini *et al.*, 2022; Hano & Abbasi, 2022]. Nanoparticles can be produced by physical, chemical, and biological methods, with the biological method using microorganisms or plant extracts being an eco-friendly approach. The use of plant extracts reduces the cost of microorganism isolation and culture media, enhancing the cost-competitive feasibility of nanoparticle synthesis by microorganisms. Green synthesis of metal nanoparticles finds applications in catalysis, electronics, optics, the environment, the medical and pharmaceutical industries, as well as biotechnology [Faisal *et al.*, 2020; Shafey, 2020].

Centella asiatica (family Apiaceae) is a psychoactive medicinal plant that has been used for centuries in the ayurvedic system of medicine as a *Medya Rasayana*. *Centella asiatica* has been used as a medicinal herb in India, China, Nepal, Sri Lanka, and Madagascar for many years. *It is* reported to possess various pharmacological activities, including wound healing, anti-microbial, anti-cancer, anti-inflammatory, hepatopoietic, insecticidal, and anti-oxidant activities [Torbati *et al.*, 2021]. *Centella asiatica* is rich in terpenoids, with asiaticoside being characterised for its pharmacological importance [Park, 2021].

Due to the unique properties of copper nanoparticles (CuNPs), such as high electrical conductivity, low electrochemical migration behaviour, a high melting point, and low cost, they can serve as alternatives to noble metals in applications like inkjet printing, organic transformations, gas phase catalysis, photocatalysis, and electrocatalysis (Tamilvanan, 2014). The solvents, stabilising agents, and reducing agent used for the biosynthesis of CuNPs should be non-toxic. This emphasizes that the synthesis of CuNPs is a bottom-up method. Copper nanoparticles find applications in industries such as medicine and electronics.

A nanocomposite of copper and manganese oxide, synthesised using *Centella asiatica* leaf extract, serves as a recyclable heterogenous catalyst capable of reducing dyes such as dinitrophenyl hydrazine, rhodamine B, methylene blue, and congo red in the presence of sodium borohydrate in water at room temperature. This demonstrates the efficacy of nano-particles in wastewater treatment, catalysis, and medicine (Nasrollahzadeh *et al.*, 2018). The primary objective of this study is to assess the synthesis, characterization, and investigate the antibacterial and antioxidant properties of copper nanoparticles derived from *Centella asiatica leaf extract.* Additionally, we aim to elucidate the potential applications of these nanoparticles in addressing bacterial infections and oxidative stress-related disorders, highlighting their potential as novel therapeutic agents with natural origins. This research contributes to the broader understanding of bio-based nanoparticles and their multifaceted properties for future biomedical and environmental applications.

MATERIALS AND METHODS

2.1 Preparation of *Centella asiatica* extract: Fresh leaves of *Centella asiatica* (Figure 1), collected from Manickal, Thiruvananthapuram, Kerala, were washed several times with tap water to remove the dust particles and dried in the shade for 7 days. The plant extract was prepared by mixing 20g of dried leaf powder with 100 ml of double-distilled water in a 250 ml glass beaker. The solution was then boiled at 100°C for 60 minutes until the colour of the solution changed. The extract was filtered with Whatman No. 1 filter paper, stored at 4°C, and used for further analysis.

Figure 1: Leaves of *Centella asiatica*

2.2 Synthesis of Copper Nanoparticles

An aqueous solution of 2 mM copper acetate was prepared and used for the synthesis of copper nano-particles. Five milliliters of the prepared leaf extract were mixed with 50 ml of 2mM aqueous copper acetate solution in a 100 ml conical flask and stirred for 10 hours using a magnetic stirrer at room temperature. The colour of the solution changed to a dark green. The solution was centrifuged for 30 minutes at 5000 rpm. The pellets obtained were washed, transferred to a petri plate, and dried in a hot air oven at 80°C for 2 hours.

2.3 Characterization of Copper Nanoparticles

2.3.1 UV-VIS Spectroscopic Analysis:

The copper nanoparticle was characterised using the Systronics M2202 double beam UV-visible spectrophotometer, operating between 200-800nm with a spectral bandwidth of 2nm.

2.3.2 X-ray Diffraction:

The X-ray diffraction measurements of copper nanoparticles were conducted using a Bruker D2 Phaser X-Ray Diffractometer. The diffraction pattern was recorded with a wavelength of 1.54 A° in the 2e range from 10.002 A° to 80.010 A°, with a step size of 0.020° and a time constant of 0.30 sec.

2.4 Antibacterial Activity of Copper Nanoparticles

To determine the antibacterial activity of copper nanoparticles, pathogenic strains of *Bacillus velezensis, Klebsiella ozaenae, Pseudomonas aeruginosa, and Proteus vulgaris* were used, following the agar-well diffusion method. The test organisms were prepared by inoculating a loop full of stock culture into a test tube containing approximately 5 ml of nutrient broth, and allowing it to incubate for 3 hours.

Muller-Hinton media were prepared, sterilised, and poured into the Petri plates. After solidification, the culture broth was inoculated onto agar plates using the swab method. Wellswere created using cock-borers. Copper acetate and gentamycin were employed as negative and positive controls, respectively. Copper nanoparticles, as well as positive and negative controls, were added to each well. The plates were then incubated for 24 hours at 37°C, and after incubation, the zone of inhibition was measured.

2.4.1 Minimum Inhibitory Concentration (MIC)

The MIC was determined by the agar-well diffusion method. The MIC was defined as the lowest concentration of an antimicrobial that would inhibit visible growth.

Mueller-Hinton medium was prepared, sterilised, poured into Petri dishes, and allowed to solidify. Test bacterial strains were inoculated onto the plates with the help of a sterile cotton swab. Wells were made aseptically using a corkborer. The different concentrations (0.2 µg/ml, 0.4 µg/ml, 0.6 µg/ml, 0.8 µg/ml, and 1 µg/ml) of 100 µl of nanoparticles were added to each well. The culture was incubated at 37°C for 24 hours. MIC was recorded as the lowest concentration of nanoparticle that showed a zone of inhibition.

2.4.2 Minimum Bactericidal Concentration (MBC)

Minimum bactericidal concentration is the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on an antibiotic-free medium. The determination of MBC is done by the dilution method. The test bacterial strains were inoculated onto Mueller-Hinton agar media plates, and different concentrations (0.2 µg/ml, 0.4 µg/ml, and 0.6 µg/ml) of nano-particles were spread onto them. The culture was incubated at 37°C overnight. The lowest concentration, which had no single colony of bacterial growth, was taken as MBC.

2.5 Antioxidant Activity of Copper Nanoparticles

Antioxidant activity of copper nanoparticles was determined using DPPH and FRAP assays.

2.5.1 DPPH Radical Scavenging Activity:

DPPH (2,2-diphenyl-1-picryl-hydrazyl hydrate) radical scavenging activity was measured according to the method of Brand-

Williams *et al*., (1995), with certain modifications. A methanolic solution of 0.1 mM DPPH (4 mg/100 ml) was prepared freshly. 0.3 ml of synthesised CuNPs were added to 2.5 ml of DPPH solution. Reaction mixtures were kept in the dark for 30 minutes, and a decrease in absorbance was measured at 515 nm. Ascorbic acid was taken as a standard.

The scavenging activity percentage (AA%) was determined according to the equation.

$$AA\% = \frac{\{A\,control - (A\,sample - A\,blank)\}100}{A\,control}$$

where A is the absorbance of the synthesised CuNPs.

A control is the absorbance of the control (methanol + DPPH).

A blank is the absorbance of blank (methanol).

2.5.2 Ferric Reducing Antioxidant Power (FRAP) Assay:

The FRAP assay was performed following the method outlined by Benzie and Strain (1996), with some modifications. A freshly prepared FRAP solution, consisting of 50 mL of 0.3M acetate buffer (pH 3.6), was combined with 5 ml of a 10 mM of tripyridyltriazine (TPTZ) solution in 40 mM HCl and 20mM $FeCl_3$ solution. Subsequently, 900 l of FRAP solution was mixed with 300 µl of sample and diluted to a final volume of 1500 µl using distilled water. The ferric chloride-reducing ability was assessed after a 15-minute incubation in darkness, and the absorbance was measured at595 nm.

$$\text{Reducing activity}(\%) = \frac{\{A\,control - (A\,sample - A\,blank)\}100}{A\,control}$$

A control is the absorbance of control.

A blank is the absorbance of blank.

RESULTS AND DISCUSSION

3.1 Characterization of Copper Nanoparticles:

The formation of copper nanoparticles in the leaf extract of *Centella asiatica* was visualised as a colour change from light green to dark due to the reduction of copper ions (Figure 2).

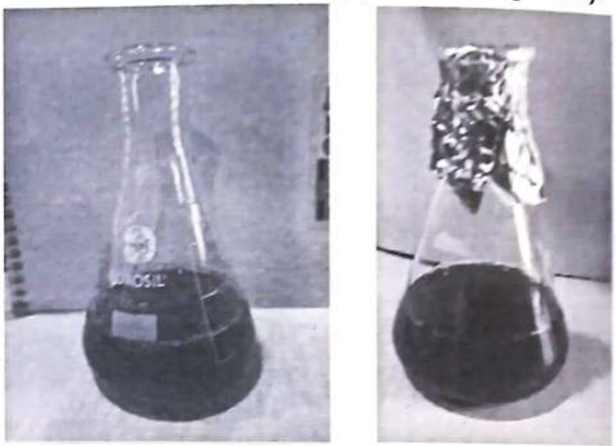

Figure 2: Leaf extract of *Centella asiatica* (left) and copper nanoparticles synthesised (right)

The copper nanoparticles in powder form displayed a dark green hue. To ascertain their configuration, which was attained through the reduction of aqueous metal ions when exposed to *Centella asiatica* leaf extract, we monitored them using UV-VIS spectroscopy. The UV Visible spectrum is depicted in Figure 3. The surface plasmon resonance absorption band of Copper nanoparticle was obtained at 204.8nm. This aligns with previous research findings, where the surface plasmon resonance absorption band of Cu nanoparticles typically falls within the range of 220-235nm, as demonstrated by Rehana *et al*., in their 2017 study. Furthermore, the UV-visible spectrum analysis of copper oxide nanoparticles conducted by Renuga *et al*. in 2020 also revealed a resonance peak at approximately 220nm. These consistent observations underscore the characteristic spectral behaviour exhibited by copper nanoparticles in this specific wavelength range.

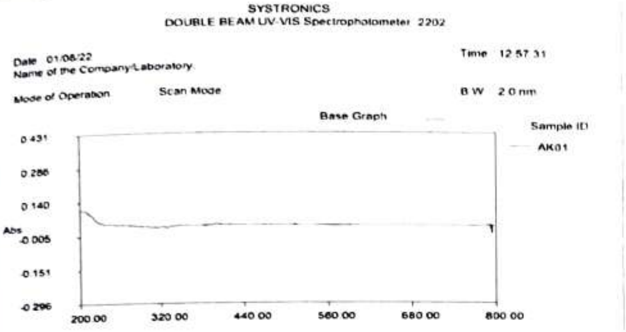

Figure 3. UV – VIS spectrum of CuNPs.

The verification of copper nanoparticles' formation, synthesised using the leaf extract, was further substantiated through X-ray diffraction (XRD) measurements. In this context, the XRD analysis revealed distinctive Bragg's reflections, with a 2angle of 30.334°, as illustrated in Figure 4, for the CuNPssynthesied from *Centella asiatica*. Notably, in a study conducted by Kavitha *et al.*, in 2022 involving copper nanoparticles derived from *Catharanthus roseus*, multiple Bragg's reflections were identified at specific 2ɵ angles, including 33.53°, 35.82°, 38.78°, 48.99°, 55.75°, 58.64°, 62.26°, 55.75°, 58.64°, 62.26°, and 67.34°. The X-ray diffraction pattern of copper nanoparticles derived from *Oscimum sanctum* leaves exhibited a broad halo in the 2ɵ range of 20 – 40^0, confirming their amorphous nature (Usha *et al.*, 2017). Copper nanoparticles synthesised from *Capparis zeylanica* exhibited distinct diffraction peaks at 39.1^0

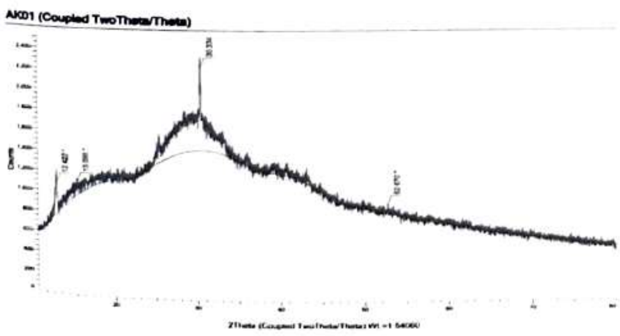

Figure 4: XRD spectrum of CuNPs.

and 68.3^0 in their XRD spectrum (Saranyaadevi*et al.*, 2014). In their XRD analysis, Fatma *et al.* (2017) observed specific peaks at 2ɵ values of 42.47^0 for CuNPs synthesised from *Passiflora foetida* leaf extract.

3.2 Antibacterial Activity of Copper Nanoparticles

The antibacterial activity of the synthesized copper nanoparticles from the leaf extract of *Centella asiatica* was assessed against two Gram-positive and Gram-negative pathogens; *Pseudomonas aeruginosa, Bacillus velezensis, Klebsiella ozaenae, and Proteus vulgaris. The results demonstrated* promising antibacterial activity against all of these pathogens (Figures 5, 6, 7, and 8). The zone of inhibition created by the copper nanoparticle indicated that they were more effective than the positive control used (Table 1).*Klebsiella ozaenae exhibited the lowest antibacterial activity.*

In a study by Netala *et al.*(2014), silver nanoparticles synthesised from the aqueous callus extract of *Centellaasiaticaexhibited* potent inhibitory activity against *Escherichia coli, Psuedomonas aeruginosa, Staphylococcus aureus,* and *Bacillus subtilis,* with corresponding inhibition zones measuring 16.2mm, 18.8mm, 21.3 mm, and 19.4 mm, alongside a distinctive surface plasmon resonance peak observed at 453nm.

Table 1. Antibacterial activity of CuNPs against different bacterial strains

Bacterial strain	Zone of inhibition		
	CuNPs (mm)	Positive control (mm)	Negative control (mm)
Bacillus velezensis	21 + 0.29	18 + 0.16	Nil
Proteus vulgaris	20 + 0.23	18 + 0.08	Nil
Pseudomonas aeruginosa	16 + 0.12	12 + 0.04	Nil
Klebsiella ozaenae	11 + 0.12	9 + 0.09	Nil

In contrast, zinc oxide nanoparticles, produced using an aqueous leaf extract of *Centellaasiatica*, demonstrated antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The respective zones of inhibition for these bacteria measured 10mm, 12 mm, and 8 mm. (Rajini *et al.*, 2022).

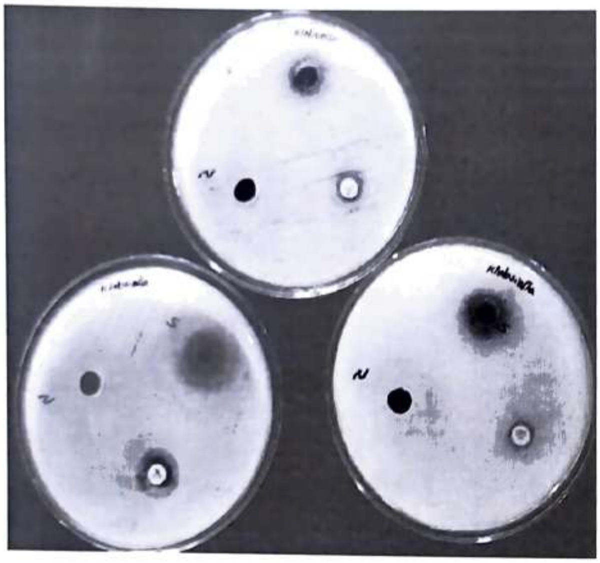

Fig.5 : Antimicrobial activity of synthesized synthesized CuNPs against *Klebsiella ozaenae*

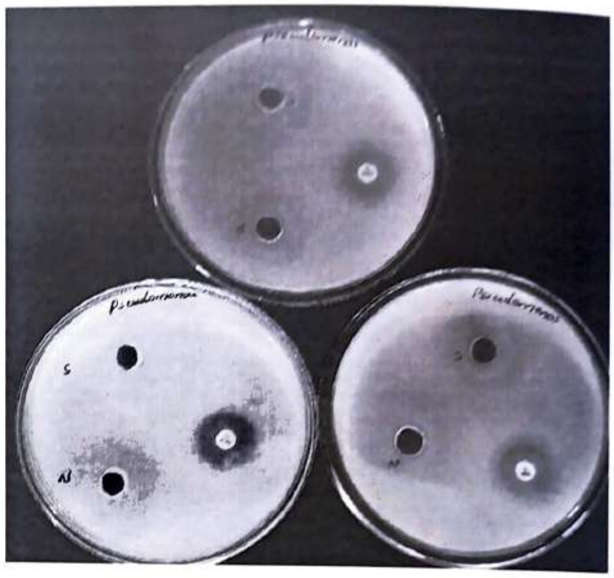

Fig.6: Antimicrobial activity of CuNPs against *Pseudomonas aeruginosa*

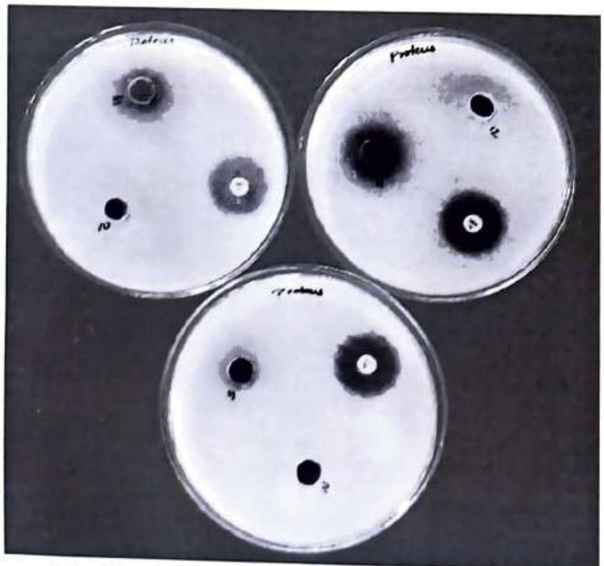

Fig.7 Antimicrobial activity of synthesized synthesizedCuNPs against *Proteus vulgaris*

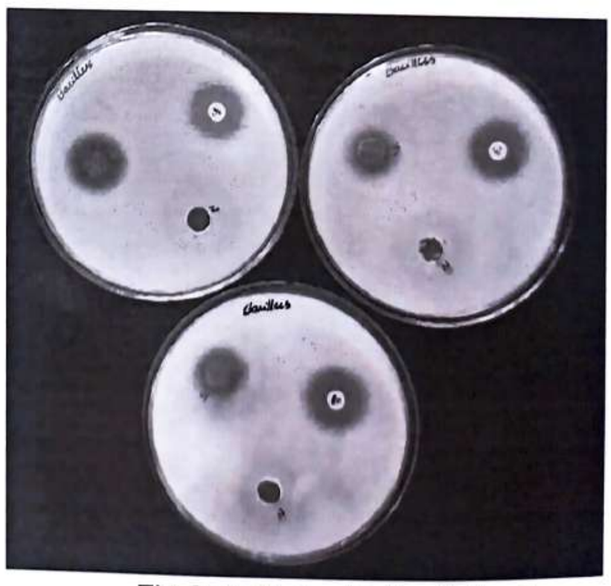

Fig.8: Antimicrobial activity of CuNPs against *Bacillus velezensis*

Fig.9: MIC of *Bacillus velezensis*

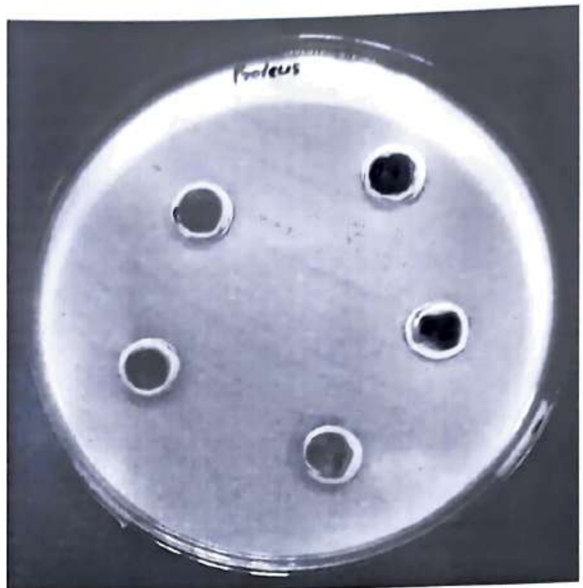

Fig. 10: MIC of *Proteus vulgaris*

Minimum inhibitory concentration of *Bacillus velezensis* and *Proteus vulgaris*: the lowest concentration of an antimicrobial that will inhibit the growth of a given strain of bacteria was 0.6 µg/ml (Figures 9 and 10; Table 2).

Minimum bactericidal concentration (MBC): The lowest concentration of antimicrobial that resulted in no colony growth was taken as the MBC. The MBC results revealed that the synthesised CuNPs significantly inhibited the growth of bacteria. The lowest MBC concentration was observed at 0.6 ug/ml for *Bacillus velezensis (Figure* 11).

Fig.11: MBC of *Bacillus velezensis*

Table 2. Zone of inhibition (minimum inhibitory concentration)

Concentration (µg/ml)	Zone of inhibition (mm)	
	Bacillus velezensis	*Proteus vulgaris*
0.2	Nil	Nil
0.4	3 + 0.08	2 + 0.06
0.6	6 + 0.06	4 + 0.04
0.8	4 + 0.05	3 + 0.02
1	2 + 0.12	3 + 0.06

3.3 **Antioxidant Activity of CuNPs:** The DPPH scavenging activity of ascorbic acid is 35.5%, while that of CuNPs is 12.67%. In the FRAP assay, the reducing activity of ferric chloride was 79%, whereas that of CuNPs was 41%. In both methods, the CuNPs derived from *Centella asiatica* exhibited significant antioxidant activity when compared to the standards. Green synthesis studies of nano-particle synthesis are in progress, and further research is needed in this aspect.

CONCLUSION

Copper nanoparticles were synthesised using leaf extracts of Centella asiatica. Structural analysis was conducted using UV-visible spectroscopy and XRD. The synthesised nanoparticles demonstrated antibacterial activity against Pseudomonas aeruginosa, Bacillus velezensis, Klebsiella ozaenae, and Proteus vulgaris with inhibition zones of 16±0.12, 21±0.29, 11±0.12, and 20±0.29 mm, respectively, surpassing the effectiveness of the positive control gentamycin. Copper nanoparticles also exhibited antioxidant activity, with a DPPH assay result of 12.67% and an FRAP-reducing activity of 41%. This study underscores the potential of medicinal plants for the synthesis of copper nanoparticles with robust antibacterial and antioxidant properties. Further investigations are necessary to identify the functional groups responsible for binding and stabilizing copper nanoparticles. Additionally, comparative studies with other metal nanoparticles in Centella asiatica leaf extracts could be conducted.

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