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Green Synthesis and Characterization of Copper Nanoparticles Using *Azadirachta indica* (Neem) Leaf Extract and Evaluation of their Antimicrobial Activities

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Abstract
The present study demonstrates the green synthesis of copper oxide nanoparticles (CuONPs) utilizing aqueous extracts of Azadirachta indica *(neem) leaves. Fresh neem leaves were processed to obtain the extract, which served as a bioreducing and stabilizing agent for the synthesis of CuONPs via a simple aqueous-phase method using copper nitrate. The formation of CuONPs was indicated by a colour change from brown to sea green and confirmed through X-ray diffraction (XRD), scanning electron microscopy (SEM), and Fourier transform infrared spectroscopy (FTIR) analyses. XRD results revealed the monoclinic crystalline structure of CuO nanoparticles with an average crystallite size of approximately 3.3 nm. SEM analysis showed irregular, aggregated morphologies, while FTIR spectra identified functional groups responsible for the reduction and stabilization of nanoparticles. Antibacterial activity assessed by the well diffusion method indicated that the biosynthesized CuONPs exhibited substantial inhibitory effects against* Pseudomonas aeruginosa *and* Klebsiella oxytoca*, but no activity against* Staphylococcus aureus*. The findings highlight the potential application of neem-mediated CuONPs as eco-friendly antimicrobial agents, particularly targeting Gram-negative bacterial strains.*

Keywords: *Green synthesis, Eco-friendly, CuONPs, Bacterial strains, Multidrug-resistance*

INTRODUCTION
The global rise of multidrug-resistant (MDR) microbial infections poses a significant challenge to public health, necessitating the development of novel and effective antimicrobial agents. Metallic nanoparticles, particularly copper oxide nanoparticles (CuONPs), have emerged as promising candidates due to their potent antimicrobial, antifungal, and antiviral properties (Wahab *et al*., 2023). In addition to their bioactivity, CuONPs are known for their excellent catalytic efficiency, thermal conductivity, and optical properties, making them highly valuable in biomedical, environmental, and industrial applications (Pourmadadi *et al*., 2023;

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Akintelu *et al*., 2020). Compared to silver or gold nanoparticles, CuONPs are more cost-effective and abundant, enhancing their potential for scalable applications in diagnostics, drug delivery, wound healing, and antimicrobial coatings (Pourmadadi *et al*., 2023; Chaikali *et al*., 2025).

Traditional chemical and physical methods for CuONPs synthesis, however, involve hazardous reagents and high energy consumption, raising concerns about environmental toxicity and sustainability (Devaraji *et al*., 2024). In contrast, green synthesis using plant extracts offers a more eco-friendly approach, leveraging phytochemicals such as phenolics, flavonoids, and terpenoids to act as natural reducing and stabilizing agents (Singh *et al*., 2023). Among such plants, *Azadirachta indica* (neem) has been extensively studied for its broad-spectrum antimicrobial activity and rich phytochemical profile, yet its application in the green synthesis of CuONPs remains underexplored compared to other metals like silver and zinc (Din *et al*., 2023; Ujah *et al*., 2021).

Neem (*Azadirachta indica*) leaves are widely recognized for their rich phytochemical content, including flavonoids, terpenoids, alkaloids, and polyphenols, which make them suitable for the green synthesis of copper nanoparticles (CuNPs) (Ujah *et al*., 2021). These bioactive compounds serve dual functions as reducing agents, converting copper ions (Cu^{2+}) into copper nanoparticles, and as stabilizing agents, capping the nanoparticles to prevent agglomeration and enhance stability (Rajeshkumar and Bharath 2017; Singh et al., 2020). Moreover, neem itself possesses strong antimicrobial properties, which, when combined with the intrinsic bactericidal activity of copper, lead to synergistic antimicrobial efficacy (Ahmad et al., 2022). Copper nanoparticles synthesized using neem extract have been shown to effectively inhibit a broad range of microbial pathogens, including Gram-positive and Gram-negative bacteria, as well as fungi (Singh et al., 2020). This enhanced antimicrobial activity is attributed to the ability of copper to generate reactive oxygen species (ROS), disrupt microbial membranes, and interfere with DNA and protein functions, while neem phytochemicals further augment these effects (Ramesh et al., 2015). Additionally, the biocompatibility of neem-derived CuNPs makes them suitable for diverse applications such as medical device coatings, wound dressings, water purification, and antimicrobial packaging (Rajeshkumar & Bharath, 2017). Several studies have provided empirical evidence supporting the antimicrobial effectiveness of neem-synthesized CuNPs against common pathogens like *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*, reinforcing the potential of neem leaves as a sustainable and effective agent in nanomaterial synthesis (Ahmad et al., 2019; Singh et al., 2020).

It is worth nothing that despite the demonstrated bioactivity of neem-based nanoparticles, few studies have focused on the detailed physicochemical characterization and antibacterial efficacy of CuONPs synthesized from neem leaves. Moreover, the selective activity of these nanoparticles against Gram-positive and Gram-negative bacteria remains poorly understood. This research aims to fill this gap by (i) synthesizing CuONPs using *A. indica* leaf extract, (ii) characterizing them using XRD, SEM, and FTIR, and (iii) evaluating their antimicrobial activity against clinically important bacterial strains. This study not only promotes sustainable nanotechnology practices but also contributes to the development of plant-mediated alternatives to chemical antimicrobials in managing resistant infections.

MATERIALS AND METHODS

Study Area and Sample Collection

Fresh leaves of *Azadirachta indica* (neem) were collected from the vicinity of the Multi-Laboratory Complex at Kwara State University, Malete, situated in Moro Local Government Area of Kwara State, Nigeria. All experimental procedures and analyses were conducted at the Central Research Laboratory of the University of Ilorin, Ilorin, Kwara State, Nigeria.

Laboratory Analysis and Sample Preparation

Preparation of Plant Extract

The extract was prepared following a modified version of the method described by Saranyaadevi *et al*. (2014). The collected neem leaves were thoroughly washed with tap water and subsequently rinsed with deionized water to remove dust and other surface contaminants. A total of 30 g of sliced neem leaves was boiled in 100 cm^3 of deionized water for 10 minutes. The mixture was filtered through Whatman No. 1 filter paper, and the resulting filtrate was collected in a clean, dry conical flask and stored under sterile conditions for further use.

Synthesis of Copper Nanoparticles (CuONPs)

Copper oxide nanoparticles were synthesized by mixing the crude neem leaf extract with an aqueous solution of copper nitrate [$Cu(NO_3)_2 \cdot 3H_2O$] in a 1:4 volumetric ratio. Specifically, 25 cm^3 of the prepared plant extract was added to 100 cm^3 of 0.001 M $Cu(NO_3)_2 \cdot 3H_2O$ solution and incubated in the dark for 24 hours. A noticeable change in color from brown to sea green occurred within minutes, indicating the formation of copper nanoparticles. After 24 hours, the solution became darker, confirming nanoparticle formation, in line with the findings of Saranyaadevi *et al*. (2014).

Characterization of Copper oxide Nanoparticles

The synthesized nanoparticles were initially centrifuged at 400 rpm for 15 minutes. The supernatant was subsequently centrifuged at 14,000 rpm for 30 minutes. The resulting pellet was re-suspended in 0.5 cm^3 of deionized water and analyzed using X-ray Diffraction **(**XRD), Scanning Electron Microscopy (SEM) and Fourier Transform Infrared Spectroscopy (FTIR). X-ray Diffraction (XRD) was an analytical technique used to study the crystallographic structure, chemical composition, and physical properties of materials by measuring the way X-rays are diffracted (scattered) by the atoms in a material. SEM analysis was conducted using a JEOL JSM-6480-LV model to determine the morphological characteristics of the nanoparticles. FTIR analysis was performed to identify the functional groups involved in the reduction and stabilization of the copper ions. The model of the FTIR is FTIR spectrometer Nicolet IS Thermo Scientific model.

Antibacterial Activity Assay

The antibacterial efficacy of the synthesized copper nanoparticles was evaluated using the well diffusion method described by Krishnamoorthy and Jayalakshmi (2012). The test organisms included *Staphylococcus aureus, Klebsiella oxytoca*, and *Escherichia coli*. Mueller-Hinton Agar (MHA) was used as the growth medium. A volume of 20 cm^3 of sterilized MHA was poured into sterile Petri dishes and allowed to solidify. The bacterial cultures were uniformly spread on the surface of the solidified agar. Using a sterile cork borer, wells were created in the agar, and each well was filled with 0.1 cm^3 of the nanoparticle suspension. The plates were incubated at 37 °C for 24 hours in an upright position in an incubator. Following incubation, the diameters of the zones of inhibition were measured, and the average values were recorded for each nanoparticle treatment.

RESULTS

Structural and Morphological Characterization

The structural properties of the synthesized copper oxide nanoparticles were investigated using X-ray diffraction (XRD) analysis. The diffraction pattern exhibited a prominent peak at approximately 21.6 keV, corresponding to a 2θ value of ~35°, which is characteristic of monoclinic CuO. The full width at half maximum (FWHM) of the main diffraction peak was measured to be approximately 2.5°. Using the Scherrer equation with a shape factor (K) of 0.9 and Cu-Kα radiation (λ = 0.15406 nm), the average crystallite size was calculated to be approximately 3.3 nm, indicating the formation of nanosized copper oxide particles. Minor peaks observed between 2–4 keV may be attributed to other elements or phytochemicals from the neem extract acting as stabilizers or capping agents (Figure 1).

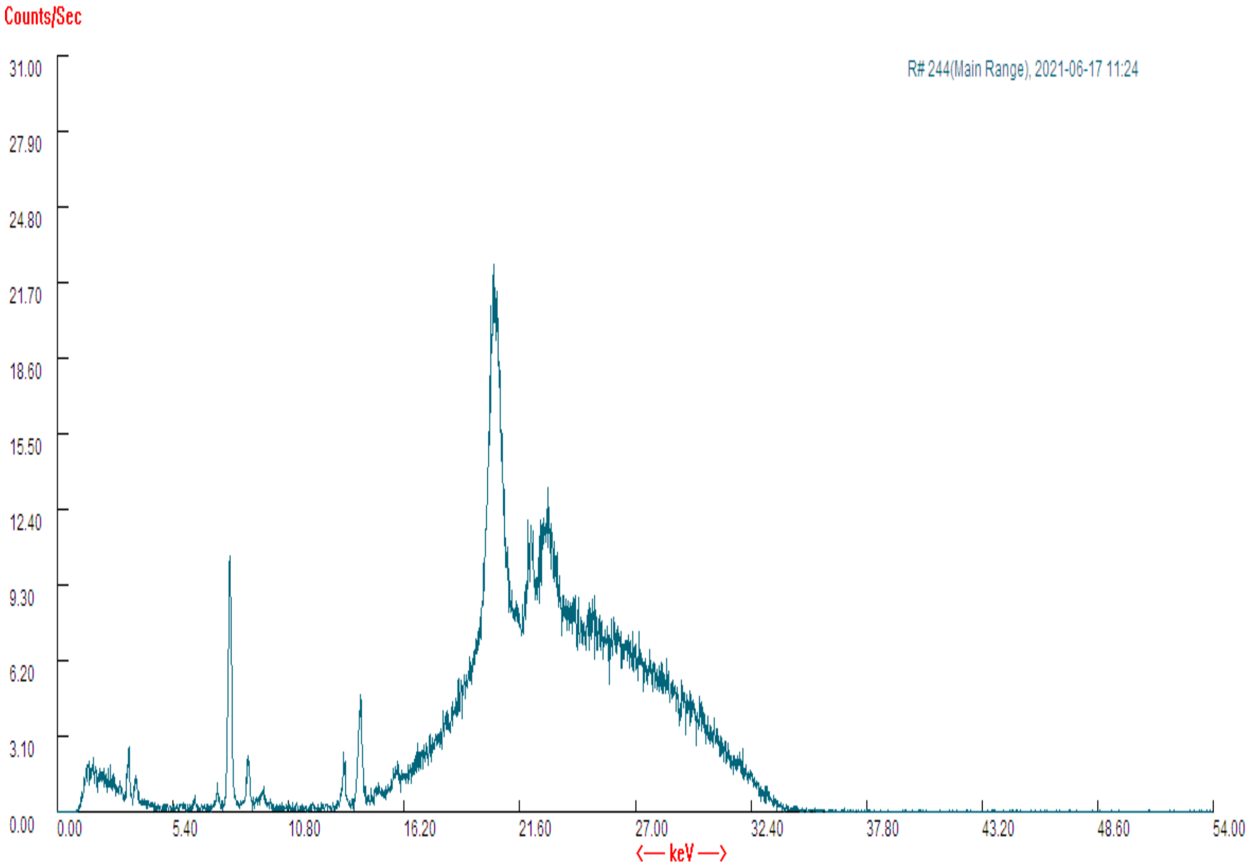

Figure1: XRD of the synthesized copper nanoparticles

Scanning electron microscopy (SEM) analysis of the synthesized CuONPs revealed the presence of irregularly shaped nanoparticle clusters, suggesting aggregation of smaller primary particles during synthesis. The absence of significant agglomeration and the uniform distribution observed in SEM images suggest the effectiveness of Azadirachta indica leaf extract in stabilizing nanoparticle formation (Figure 2).

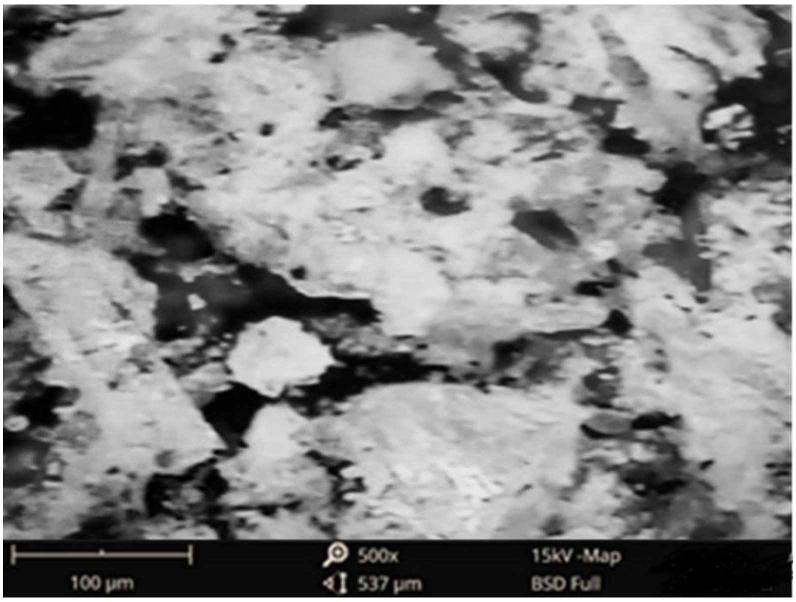

Figure2: Scanning electron microscopic images of the synthesized copper oxide nanoparticles

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectroscopy was employed to investigate the functional groups present in the synthesized copper oxide nanoparticles. Broad absorption bands were observed at 3954 cm⁻¹ and 3932 cm⁻¹, corresponding to O–H stretching vibrations. A bending vibration of water molecules was detected at 1655 cm⁻¹. Peaks at 1406 cm⁻¹ and 1076 cm⁻¹ were assigned to C–O stretching vibrations. A strong absorption band at 622 cm⁻¹ was attributed to Cu–O bond stretching, confirming the successful formation of copper oxide nanoparticles (Figure 3 and Table 1).

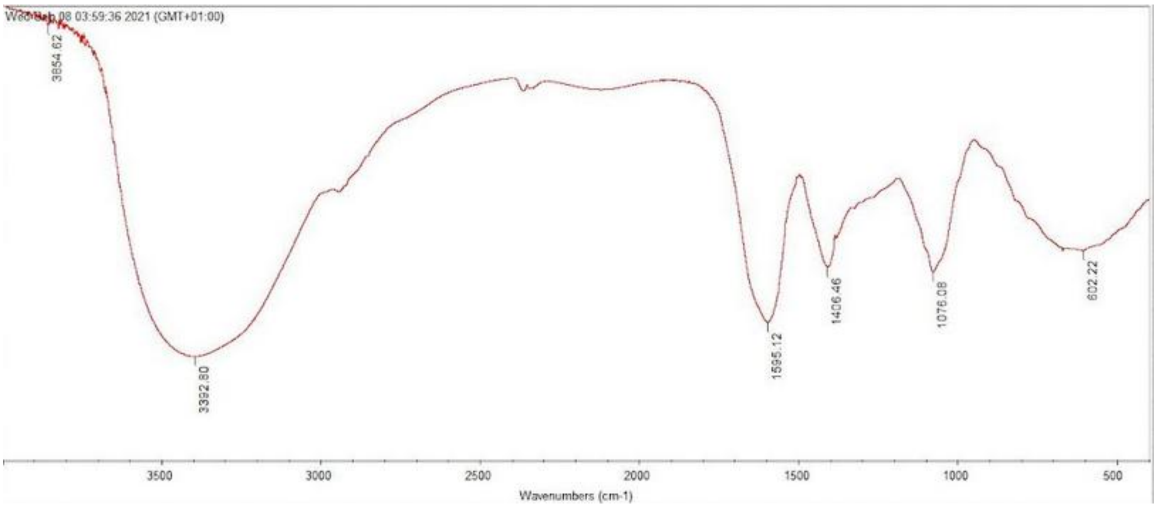

Figure 3: FTIR Analysis of the synthesized copper oxide nanoparticles

Table 1: Fourier Transform Infrared (FTIR) Spectroscopy characterization

Wavenumber (cm^{-1})	Functional group	Remark
3954, 3932	O–H stretching	Adsorbed moisture or surface hydroxyl groups
1655	H–O–H bending	Water molecules
1406	C–O stretching / Cu–O related	Minor carbonates or copper oxide vibrations
1076	C–O stretching	Possible carbonate species
622	Cu–O stretching	Characteristic of copper oxide nanoparticles

The combined XRD, SEM, and FTIR results confirm the successful synthesis of crystalline, nanoscale CuO particles.

Antibacterial efficacy of the synthesized copper oxide nanoparticles

The antibacterial efficacy of the synthesized copper nanoparticles (CuONPs) was assessed using the well diffusion method, and the results are summarized in Table 2. The nanoparticles exhibited no inhibitory effect on *Staphylococcus aureus* at either concentration (100 mg/mL or 200 mg/mL), while substantial zones of inhibition were recorded for *Pseudomonas aeruginosa* and *Klebsiella oxytoca*. The inhibition zones ranged from 13 mm to 15 mm, with the highest activity observed at the 200 mg/mL concentration.

Table 2: Zone of Inhibition (mm) of Copper Nanoparticles against Selected Bacterial Isolates

Isolate	200 mg/mL CuNPs	100 mg/mL CuNPs	Control (Sterile Water)
Staphylococcus aureus	0	–	0
Pseudomonas aeruginosa	13	14	0
Klebsiella oxytoca	15	14	0
Pseudomonas aeruginosa	13	14	0

DISCUSSION

The XRD results are in agreement with the findings posited by Mobarak *et al*. (2022), who worked on the synthesis and characterization of CuONPs from waste fish scale in Bangladesh, and also similar to the research of Naz *et al*. (2019). However, the nanoparticle size obtained in this study contrasts with the 30 nm size reported by Mali et al. (2019) for copper oxide nanoparticles synthesized using *Enicostemma axillare* (Lam.) leaf extract. The difference in results could be due to the difference in the plant used for the synthesis (Figure 1).

The SEM analysis aligns with the one observed by Mali *et al*. (2019). The rough surface morphology and aggregated structures are consistent with typical behavior observed in copper oxide nanoparticles, likely driven by high surface energy and van der Waals forces (Rabbani *et al*., 2014). Similar morphology and dispersity have been reported in studies utilizing other plant extracts (Nieto-Maldonado *et al*., 2022; Vincent *et al*., 2022), affirming the efficiency of green synthesis methods (Figure 2).

The FTIR findings are consistent with those reported by Alhalili (2022) and Andualem *et al*. (2020), confirming the presence of characteristic Cu–O stretching and adsorbed functional groups (Figure 3).

Similar antimicrobial effects were reported by Quiroz-Munoz *et al*. (2024) in targeted treatments against pathogenic bacteria of the mouth. These findings corroborate recent studies, such as Pourmadadi *et al*. (2024), which demonstrated that copper oxide nanoparticles synthesized using plant-based methods showed greater activity against Gram-negative bacteria than Gram-positive strains. The outer membrane of Gram-negative bacteria may facilitate the interaction of nanoparticles with the microbial cell wall, leading to increased permeability and oxidative stress (Iram *et al*., 2021). In contrast, the thick peptidoglycan layer in *S. aureus* may provide structural resistance to CuONP penetration, explaining its lack of susceptibility.

This selective activity suggests that biosynthesized CuONPs could serve as effective antimicrobial agents, particularly against Gram-negative pathogens, which are commonly involved in hospital-acquired infections and antibiotic resistance (Singh *et al.*, 2022). It is worth nothing too that there was no activity against Gram- positive pathogens. The lack of inhibition in the control group affirms the antimicrobial role of the synthesized CuONPs, excluding any inhibitory effects from the medium or solvents.

CONCLUSION

This study successfully achieved the green synthesis of copper oxide nanoparticles (CuONPs) using *Azadirachta indica* leaf extract, offering a cost-effective and environmentally sustainable approach to nanoparticle fabrication. Structural and morphological characterization confirmed the formation of nanosized, partially crystalline CuONPs with functional groups derived from plant metabolites aiding their stabilization. The synthesized CuONPs demonstrated pronounced antibacterial activity against Gram-negative bacteria (*Pseudomonas aeruginosa* and *Klebsiella oxytoca*), while showing no inhibitory effect on Gram-positive *Staphylococcus aureus*, likely due to differences in bacterial cell wall structure. These results suggest that plant-mediated CuONPs possess promising antibacterial properties and could serve as effective alternatives in the development of antimicrobial agents, particularly for combating infections caused by Gram-negative pathogens. Future studies should explore the cytotoxicity, stability, and broader biomedical applications of these biosynthesized nanoparticles.

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