INSIGHTS-JOURNAL OF
HEALTH AND REHABILITATION

INSIGHTS-JHR
INSIGHTS-JOURNAL OF HEALTH AND REHABILITATION

A COMPREHENSIVE STUDY ON THE SYNTHESIS, CHARACTERIZATION, AND BIOACTIVITY OF PHYTOGENIC COPPER OXIDE NANOPARTICLES: DALBERGIA SISSOO LEAF EXTRACT AS A POTENT CAPPING AGENT FOR ANTIBACTERIAL, ANTIBIOFILM, AND ANTIOXIDANT APPLICATIONS

Original Research

Hafsa Munir[1], Muhammad Naeem[2], Usman Naseer[3], Meryem Mehmood[4], Tanveer Aslam[5], Areej Safdar[6], Muhammad Hassam Saleem[2], Shazia Aslam[7]*

[1]Department of Microbiology, Cholistan University of Veterinary and Animal Sciences, Bahawalpur, Pakistan.
[2]Department of Allied Health Sciences, Islamic International Institute of Sciences, Multan, Pakistan.
[3]Department of Chemistry, University of Agriculture Faisalabad, 38040, Pakistan.
[4]Faculty of Rehabilitation and Allied Health Sciences (FRAHS), Riphah International University, Al-Mizan Campus, Rawalpindi, Pakistan.
[5]Department of Physics, Division of Science and Technology, University of Education, Lahore, Multan Campus, Pakistan.
[6]Department of Microbiology and Molecular Genetics, The Women University, Multan, Pakistan.
[7]Department of Chemistry Education, Universitas Negeri Yogyakarta, Indonesia.

Corresponding Author: Shazia Aslam, Department of Chemistry Education, Universitas Negeri Yogyakarta, Indonesia, shazia0041fmipa.2023@student.uny.ac.id

Acknowledgement: The authors acknowledge the support and laboratory assistance provided by the Department of Microbiology, CUVAS, Pakistan.

Conflict of Interest: None

Grant Support & Financial Support: None

ABSTRACT

Background: The global rise of multidrug-resistant pathogens poses a critical challenge to public health, necessitating the development of novel, sustainable antimicrobial strategies. Nanotechnology-based interventions, particularly metal oxide nanoparticles synthesized through environmentally friendly processes, offer a promising alternative to conventional antibiotics. Plant-mediated "green synthesis" methods provide biocompatible and cost-effective pathways to produce nanoparticles with enhanced antimicrobial and antioxidant potential.

Objective: This study aimed to synthesize copper oxide nanoparticles (CuO NPs) using Dalbergia sissoo leaf extract and to evaluate their antibacterial, antibiofilm and antioxidant activities against multidrug-resistant bacterial strains.

Methods: CuO NPs were biofabricated using aqueous D. sissoo extract and characterized through UV–Visible spectroscopy, XRD, FTIR and SEM analyses. Antibacterial activity was assessed using broth microdilution and agar well diffusion methods. Cell membrane disruption was quantified by measuring leakage of DNA, proteins and reducing sugars after exposure to increasing NP concentrations. Antibiofilm activity was evaluated using a 96-well crystal violet assay, while antioxidant potential was investigated using DPPH, ABTS and H_2O_2 radical scavenging assays across concentrations of 62.5–1000 µg/ml.

Results: Synthesized CuO NPs showed a UV absorbance peak at 290 nm, crystalline structure on XRD, distinct functional groups on FTIR and partial agglomeration in SEM imaging. MIC values ranged from 62.5–125 µg/ml, with maximum inhibition zones of 24 mm. Membrane disruption increased markedly at higher concentrations, with measurable leakage of DNA (0.3–0.72 µg/ml), proteins (5.6–12.2 µg/ml) and reducing sugars (11.4–69.1 µg/ml). Biofilm inhibition reached 68.4–75.8% at 1× MIC. Antioxidant activity demonstrated strong radical-scavenging efficiency: DPPH (73.6%), ABTS (68%) and H_2O_2 (63%) at 1000 µg/ml.

Conclusion: Biofabricated CuO NPs derived from D. sissoo exhibited potent antibacterial, antibiofilm and antioxidant properties, supporting their potential as eco-friendly nanomaterials for combating multidrug-resistant infections and oxidative stress-related conditions.

Keywords: Antibacterial activity Antioxidants, Biofilms, Copper oxide nanoparticles, Dalbergia sissoo, Green synthesis, Nanoparticles.

INTRODUCTION

The global rise of multidrug-resistant (MDR) microorganisms has emerged as one of the most pressing public health concerns of the modern era, accounting for more than 16% of hospital-acquired infections and driven by factors such as infectious disease exposure, self-medication, inappropriate antimicrobial use, and prophylactic antibiotic administration in livestock (1). The World Health Organization has identified MDR organisms as one of the top three critical threats to human health, signaling an urgent need for alternative therapeutic strategies (2). Compounding this crisis is the ability of many pathogenic bacteria to form biofilms—complex, surface-attached microbial communities that dramatically increase resistance to conventional antibiotics and host immune responses (3). As biofilm-forming MDR pathogens continue to proliferate, the limitations of existing antimicrobial therapies become increasingly evident, underscoring the necessity for innovative, sustainable antimicrobial agents. Nanotechnology has emerged as a promising frontier in this context, offering unique physicochemical properties that can be harnessed for potent antimicrobial activity (4,5). Metallic and metal-oxide nanoparticles (NPs), in particular, demonstrate broad-spectrum bactericidal effects due to their high surface-area-to-volume ratio and distinctive magnetic, optical, chemical, and structural characteristics that differ markedly from their bulk counterparts (6). These nanoscale dimensions facilitate intimate interaction with bacterial membranes and promote localized ion release, ultimately disrupting critical cellular functions (7). Among the diverse metal-based nanomaterials explored—such as iron, silver, titanium, zinc, gold, and palladium—copper oxide nanoparticles (CuO NPs) have gained considerable attention for their unique structural and biological properties, lower toxicity, biocompatibility, cost-effectiveness, and wide applicability across biomedical domains (8). Copper itself is an essential trace element required for metabolic and enzymatic processes within the human body (9), and the nanoscale form exhibits high reactivity, enabling strong conjugation with biomolecules and enhancing antimicrobial, antioxidant, and polymer-forming potential (10).

Traditional physical and chemical synthesis methods for metallic nanoparticles, however, require high temperatures, toxic reagents, extended reaction times, and generate hazardous by-products that limit biomedical applicability (11,12). Green synthesis approaches have therefore gained momentum as environmentally friendly, cost-effective, and safer alternatives (13). In particular, plant-mediated nanoparticle fabrication is advantageous because plant extracts are widely available, rich in metabolites, and contain phytochemicals—including flavonoids, terpenoids, amino acids, saponins, and carbohydrates—that act as natural reducing and capping agents (14). These biomolecules not only facilitate the conversion of metal ions into nanoparticles but also impart functional groups that enhance nanoparticle stability, biocompatibility, and potential for conjugation with antibiotics or polymers. Such conjugation has been shown to reduce antibiotic toxicity, lower required doses, and produce synergistic antibacterial effects (15). Several plant species have been explored for the green synthesis of CuO NPs, including Euphorbia escula (12), Cymbopogon citratus (16), and Nerium oleander (17). Dalbergia sissoo, commonly known as Shisham or Indian Rosewood, is a medicinally important member of the Fabaceae family with documented antimicrobial, antioxidant, anticancer, analgesic, and antipyretic properties attributable to its diverse phytochemical profile (15-17). Importantly, Dalbergia sissoo extracts have been shown to be non-toxic and non-mutagenic (11), making them a suitable candidate for nanoparticle fabrication. Despite its therapeutic potential, the use of Dalbergia sissoo leaf extract for the synthesis of CuO NPs has not been previously reported, representing a notable research gap.

Given the escalating clinical burden of MDR and biofilm-forming bacteria, the search for safe, cost-effective, and potent antimicrobial agents is critical. Utilizing Dalbergia sissoo as a green reductant and stabilizer offers a sustainable method of producing CuO NPs with potential therapeutic relevance. In the present study, Dalbergia sissoo leaf extract was employed to biosynthesize CuO NPs using aqueous extraction to avoid harmful organic solvents. The synthesized nanoparticles were evaluated for antibacterial and antibiofilm activity against Gram-positive and Gram-negative antibiotic-resistant clinical isolates, as biofilm formation contributes significantly to therapeutic failure. Additionally, antioxidant activity was assessed using DPPH, H_2O_2, and ABTS assays to explore broader biomedical applicability. Finally, interactions between CuO NPs and commonly used antibiotics were examined to investigate possible synergistic effects that may enhance antimicrobial efficacy. Building upon existing gaps in the literature and the growing need for eco-friendly antimicrobial nanomaterials, this study aimed to synthesize copper oxide nanoparticles using Dalbergia sissoo leaf extract and to evaluate their antibacterial, antibiofilm, antioxidant, and synergistic antibiotic-enhancing potential.

INSIGHTS-JHR
INSIGHTS-JOURNAL OF HEALTH AND REHABILITATION

METHODS

Bacterial Strains and Growth Conditions

The study employed multidrug-resistant clinical bacterial isolates including *Acinetobacter baumannii* (Accession No. KY228372), *Staphylococcus aureus* (Accession No. KX685332), *Escherichia coli* (Accession No. KY305421), and *Klebsiella pneumoniae* (Accession No. MF953599), which were obtained from the Department of Microbiology, Cholistan University of Veterinary and Animal Sciences (CUVAS), Bahawalpur, Pakistan. All bacterial strains were revived and maintained in Luria–Bertani (LB) broth and LB agar under standardized microbiological conditions. Only strains confirmed as MDR through prior antibiograms were included, while non-resistant strains or those contaminated during revival were excluded. Cultures were incubated at 37°C for 18–24 hours before experimental use. Ethical approval for the use of microbial isolates was obtained from the Institutional Bioethics Committee of CUVAS, and all laboratory procedures adhered to institutional biosafety guidelines.

Plant Extract Preparation

Fresh leaves of *Dalbergia sissoo* (Shisham) were collected from local surroundings and taxonomically verified by the Department of Botany, CUVAS. Leaves free from physical damage or fungal contamination were selected. Ten grams of washed leaves were boiled in 100 ml of deionized water for 30 minutes at 60–80°C using a controlled water bath. The extract was filtered, and its volume was adjusted to 250 ml with distilled water. Only aqueous extraction was used to ensure safety and avoid organic solvent residues. The extract was cooled, stored at 4°C, and used within 24 hours of preparation.

Copper Oxide Nanoparticles (CuO NPs) Bio fabrication and Characterization

Bio-fabrication of CuO NPs followed the procedure with minor modifications (12). Analytical-grade copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, 5 mM) was gradually dissolved in the freshly prepared leaf extract using a magnetic stirrer. The reaction mixture was heated at 130°C for 7 hours under continuous stirring until a visible color change and black precipitate formation indicated nanoparticle synthesis. The mixture was cooled, and the supernatant was decanted. The precipitate was repeatedly washed, centrifuged at 3500 rpm for 20 minutes, and dried at 120°C for 6 hours. The dried nanoparticles were finely ground and stored in airtight containers. Characterization was performed using UV–Visible spectroscopy (200–800 nm), scanning electron microscopy (SEM) for morphology, energy-dispersive X-ray (EDX) spectroscopy for elemental profiling, Fourier transform infrared spectroscopy (FTIR) for functional-group identification (4000–600 cm^{-1}), and X-ray diffraction (XRD) to confirm crystalline structure.

Antibacterial Susceptibility Testing of Bio fabricated Copper Oxide Nanoparticles

The antibacterial activity of CuO NPs was assessed through broth microdilution (13). Serial two-fold dilutions (0–1000 µg/ml) of NPs were prepared in Mueller–Hinton (MH) broth using sterile microtiter plates. A standardized 24-hour bacterial suspension (0.5 McFarland) was added to each well and incubated at 37°C for 24 hours. Nitro-blue tetrazolium (NBT; 5 mg/ml) was added to determine cell viability; the MIC was defined as the lowest concentration preventing the NBT color change from yellow to blue. To determine the minimum bactericidal concentration (MBC), aliquots from MIC wells and higher concentrations were plated onto MH agar and incubated for 24–48 hours. The lowest NP concentration showing no growth was recorded as the MBC. Agar well diffusion was performed as a second antibacterial assay (14). Bacterial suspensions were lawn-cultured on MH agar, wells were created, and 100 µl of CuO NPs (125–1000 µg/ml prepared in 0.1% DMSO) were added. Plates were incubated at 37°C overnight and inhibition zones were measured in millimeters.

Growth Kinetic Assay

Growth kinetics were evaluated by monitoring optical density at 600 nm over 24 hours. Bacterial cultures adjusted to 0.5 McFarland were incubated in LB broth containing CuO NPs at 0.25× MIC, 0.5× MIC, and 1× MIC. Samples were collected every four hours, and OD values were compared with untreated controls.

Cell Membrane Disruption Assay

To assess membrane integrity, leakage of cellular components including DNA, proteins, and reducing sugars was quantified. Bacterial cultures (0.5 McFarland) were exposed to CuO NPs at 0.5×, 1×, and 2× MIC and incubated at 37°C for 24 hours in a shaking incubator. After centrifugation (10,000 g, 4°C, 30 minutes), supernatants were collected for biochemical analysis. Protein leakage was quantified

INSIGHTS-JHR
INSIGHTS-JOURNAL OF HEALTH AND REHABILITATION

using the Bradford assay (15), DNA leakage was assessed using absorbance at 260 nm, and reducing sugars were measured via the phenol-sulfuric acid method (16). Untreated cultures served as controls.

Antibiofilm Effect of CuO NPs

The ability of CuO NPs to inhibit biofilm formation was evaluated using a 96-well microtiter assay. Standardized bacterial inocula (0.5 McFarland) were added to LB broth containing NPs at 0.25×, 0.5×, and 1× MIC. Plates were incubated for 24 hours at 37°C, non-adherent cells were discarded, and attached biofilms were fixed with methanol. Wells were stained with 0.1% crystal violet, washed, and solubilized with 33% glacial acetic acid. Absorbance was recorded at 575 nm. Biofilm inhibition (%) relative to untreated controls was calculated (17).

Synergistic Studies

Synergy between CuO NPs and antibiotics (erythromycin and cefixime) was assessed using a checkerboard microdilution assay (18). Two-fold serial dilutions of antibiotics and CuO NPs (starting from 1000 µg/ml) were arranged along orthogonal axes of the microtiter plate. Standardized bacterial inoculum (20 µl, 0.5 McFarland) was added to each well, and plates were incubated at 37°C for 24 hours. Fractional inhibitory concentration index (FICI) values were calculated using the accepted formulas. Synergy was defined as FICI ≤ 0.5, additive interaction as > 0.5–1.0, indifference as > 1–4, and antagonism as > 4.

Antioxidant Activities of CuO NPs

CuO NPs were evaluated for antioxidant activity using DPPH, ABTS, and hydrogen peroxide radical scavenging assays across concentrations ranging from 62.5–1000 µg/ml. Ascorbic acid was used as the reference standard for all assays.

DPPH Radical Scavenging Assay

The DPPH free-radical scavenging activity followed the procedure of Sowndhararajan and Kang with minor modifications (19). CuO NPs (10 µl) were added to 0.1 mM DPPH solution and incubated at 37°C for 30 minutes in the dark. Absorbance was measured at 517 nm and scavenging (%) was calculated relative to control.

ABTS Radical Scavenging Activity

ABTS activity was measured. $ABTS^{+}$ solution was prepared using potassium persulfate and incubated for 12 hours. After dilution to an absorbance of 0.7 at 743 nm, it was mixed with nanoparticle suspensions and incubated for 6 minutes before reading at 734 nm.

Hydrogen Peroxide Radical Scavenging Assay

The hydrogen peroxide scavenging assay followed the method of Keshari (20). Nanoparticles were mixed with 2 mM H_2O_2 in phosphate buffer (pH 7.4), incubated for 10 minutes at room temperature, and absorbance was measured at 230 nm.

RESULTS

Characterization of Bio fabricated CuO NPs

Bio fabricated CuO NPs synthesized using *Dalbergia sissoo* leaf extract showed a distinct absorption peak at 290 nm in the UV–Visible spectrum, consistent with the formation of copper oxide nanoparticles. X-ray diffraction analysis demonstrated characteristic diffraction peaks at 2θ values of 31.36°, 43.51°, 45.31°, 51.47° and 60.35°, indicating a crystalline CuO phase. The FTIR spectrum exhibited prominent bands at 3296, 2920, 1728, 1642, 1392, 1128, 828 and 610 cm^{-1}; the peak at 3296 cm^{-1} corresponded to O–H stretching of phenolic groups, while bands at 1642 cm^{-1} and 1392 cm^{-1} were associated with alkene and C–N stretching of amines, respectively. The absorption band at 610 cm^{-1} was attributed to Cu–O stretching vibrations, confirming the presence of copper oxide bonds. Energy-dispersive X-ray spectroscopy revealed strong elemental copper signals with absorption peaks around 0.3, 1 and 8 keV, consistent with CuO composition. Scanning electron microscopy showed predominantly agglomerated nanoparticles, with only a fraction of discrete, approximately spherical particles having an average diameter of less than 100 nm.

Antibacterial Susceptibility Testing of Bio fabricated CuO NPs

The broth microdilution assay demonstrated that CuO NPs inhibited all tested MDR bacteria at relatively low concentrations. The minimum inhibitory concentration (MIC) was 125 µg/ml for *Staphylococcus aureus*, *Acinetobacter baumannii* and *Escherichia coli*,

INSIGHTS-JHR
INSIGHTS-JOURNAL OF HEALTH AND REHABILITATION

and 62.5 µg/ml for *Klebsiella pneumoniae*. The minimum bactericidal concentration (MBC) was 250 µg/ml for *S. aureus* and *K. pneumoniae*, and 500 µg/ml for *A. baumannii* and *E. coli*. In the agar well diffusion assay, CuO NPs produced clear dose-dependent inhibition zones ranging from 8 to 24 mm across the tested concentrations (125–1000 µg/ml). At 125 µg/ml, inhibition zones were 8 ± 0.59 mm for *S. aureus*, 8 ± 0.84 mm for *A. baumannii*, 9 ± 1.19 mm for *E. coli* and 10 ± 1.02 mm for *K. pneumoniae*. At 250 µg/ml, inhibition zones increased to 12 ± 1.19 mm, 11 ± 1.02 mm, 14 ± 0.59 mm and 16 ± 1.32 mm, respectively. At 500 µg/ml, zones further increased to 16 ± 0.84 mm for *S. aureus*, 15 ± 1.19 mm for *A. baumannii*, 17 ± 1.03 mm for *E. coli* and 20 ± 1.19 mm for *K. pneumoniae*. At the highest concentration of 1000 µg/ml, inhibition zones reached 20 ± 0.59 mm, 19 ± 1.02 mm, 22 ± 1.45 mm and 24 ± 1.32 mm for *S. aureus*, *A. baumannii*, *E. coli* and *K. pneumoniae*, respectively, indicating the strongest growth suppression at the maximum tested dose.

Growth Kinetic Assay

Exposure of MDR bacteria to CuO NPs altered their growth kinetics in a concentration-dependent manner. Across all strains, cultures treated with 1× MIC of CuO NPs showed the greatest reduction in optical density at 600 nm over time, indicating marked inhibition of bacterial proliferation compared with untreated controls. Cultures treated with 0.5× MIC showed intermediate growth suppression, whereas those exposed to 0.25× MIC exhibited only modest reductions in growth, with absorbance values closer to control levels. Among control (untreated) cultures, *A. baumannii* showed the highest cell density over the monitoring period, followed by *E. coli*, *S. aureus* and *K. pneumoniae*.

Cell Membrane Disruption Assay

Quantification of cellular content leakage showed that CuO NPs disrupted bacterial membrane integrity in a concentration-dependent manner. DNA released into the supernatant after treatment ranged from 0.3 to 0.72 µg/ml, with significantly higher values at 2× MIC compared with both the control and 0.5× MIC-treated cells ($p < 0.05$). Protein leakage ranged from 5.6 to 12.2 µg/ml, with a significant increase at 2× MIC relative to the control and 0.5× MIC, whereas protein levels in 0.5× MIC-treated cultures were comparable to untreated controls ($p < 0.05$ for 2× MIC versus both other groups). Reducing sugars released into the supernatant were in the range of 11.4–69.1 µg/ml, and increased with higher nanoparticle concentrations, further supporting concentration-dependent membrane damage.

Antibiofilm Effect of CuO NPs

CuO NPs reduced biofilm formation by all tested MDR strains in a dose-dependent pattern. When biofilms were grown in the presence of 0.5× MIC of CuO NPs, biofilm biomass decreased by approximately 38.9–49.3% compared with the untreated control. At 1× MIC, biofilm inhibition increased to 68.4–75.8%. The order of biofilm inhibition, from greatest to least, was *K. pneumoniae*, *A. baumannii*, *S. aureus* and *E. coli*. Even subinhibitory concentrations therefore substantially lowered biofilm-forming capacity relative to untreated conditions.

Synergistic Studies

In checkerboard assays, combinations of CuO NPs with cefixime or erythromycin modified MIC values for both components. For the combination of CuO NPs with cefixime, MICs for *S. aureus* were reduced from 125 µg/ml (NPs alone) and 250 µg/ml (cefixime alone) to 15.625 µg/ml and 62.5 µg/ml, respectively, yielding a fractional inhibitory concentration index (FICI) of 0.375. For *A. baumannii*, MICs decreased from 125 µg/ml (NPs) and 500 µg/ml (cefixime) to 31.25 µg/ml and 125 µg/ml, with a FICI of 0.5. For *E. coli*, MICs were reduced from 125 µg/ml (NPs) and 500 µg/ml (cefixime) to 31.25 µg/ml and 125 µg/ml, also producing a FICI of 0.5. For *K. pneumoniae*, MICs changed from 62.5 µg/ml (NPs) and 250 µg/ml (cefixime) to 31.2 µg/ml and 125 µg/ml, with a FICI of 0.9. For combinations of CuO NPs with erythromycin, *S. aureus* MICs changed from 125 µg/ml (NPs) and 125 µg/ml (erythromycin) to 31.25 µg/ml for both components, with a FICI of 0.49. For *A. baumannii*, MICs changed from 125 µg/ml (NPs) and 125 µg/ml (erythromycin) to 31.25 µg/ml and 62.5 µg/ml, respectively, with a FICI of 0.75. For *E. coli*, MICs decreased from 125 µg/ml (NPs) and 250 µg/ml (erythromycin) to 15.62 µg/ml and 62.5 µg/ml, producing a FICI of 0.375. For *K. pneumoniae*, MICs for NPs and erythromycin remained 62.5 µg/ml and 125 µg/ml in combination, resulting in a FICI of 2.0. Overall, FICI values for the different strain–antibiotic–NP combinations ranged from 0.375 to 2.0.

Antioxidant Activities of CuO NPs

CuO NPs exhibited measurable antioxidant activity in all three radical scavenging assays, with a clear dose-dependent pattern. In the DPPH assay, scavenging activity increased from 23 ± 1.26% at 62.5 µg/ml to 38 ± 1.34% at 125 µg/ml, 46.5 ± 1.02% at 250 µg/ml, 66

INSIGHTS-JHR
INSIGHTS-JOURNAL OF HEALTH AND REHABILITATION

± 1.15% at 500 µg/ml and 73.6 ± 1.12% at 1000 µg/ml. In the ABTS assay, scavenging activity was 18 ± 1.22% at 62.5 µg/ml, 28 ± 1.20% at 125 µg/ml, 47 ± 1.23% at 250 µg/ml, 55 ± 0.07% at 500 µg/ml and 68 ± 1.23% at 1000 µg/ml. In the hydrogen peroxide assay, scavenging activity ranged from 20.3 ± 1.50% at 62.5 µg/ml to 23 ± 1.50% at 125 µg/ml, 38.3 ± 1.31% at 250 µg/ml, 47 ± 1.62% at 500 µg/ml and 63 ± 1.73% at 1000 µg/ml. These findings demonstrated a consistent increase in radical scavenging capacity with rising nanoparticle concentration across all three assay systems.

Table 1: Minimum Inhibitory Concentration and Minimum Bactericidal Concentrations (µg/ml) of bio fabricated CuO NPs against MDR bacteria.

Bacterial Strains	MIC (µg/ml)	MBC (µg/ml)
S. aureus	125	250
A. baumannii	125	500
E. coli	125	500
K. pneumonia	62.5	250

Table 2: Diameter of inhibitory zones (mm) for bio fabricated CuO NPs against MDR bacteria.

Concentration (µg/ml)	S. aureus	A. baumannii	E. coli	K. pneumonia
125	8±0.59	8±0.84	9±1.189	10±1.02
250	12±1.189	11±1.02	14±0.59	16±1.32
500	16±0.84	15±1.189	17±1.029	20±1.189
1000	20±0.59	19±1.02	22±1.45	24±1.32

Table 3: Combined Effect of CuO NPs and antibiotics against MDR bacteria.

Bacterial strains	Single component		Combination		FICI	Single component		Combination		FICI
	MIC(µg/ml)					MIC (µg/ml)				
	NPs	Cefixime	NPs	Cefixime		NPs	Erythromycin	NPs	Erythromycin	
S. aureus	125	250	15.625	62.5	0.375	125	125	31.25	31.25	0.49
A. baumannii	125	500	31.25	125	0.5	125	125	31.25	62.5	0.75
E. coli	125	500	31.25	125	0.5	125	250	15.62	62.5	0.375
K. pneumoniae	62.5	250	31.2	125	0.9	62.5	125	62.5	125	2

INSIGHTS-JHR
INSIGHTS-JOURNAL OF HEALTH AND REHABILITATION

Table 4: Free radical scavenging activities of CuO NPs.

Concentration (µg/ml)	Scavenging activity (%)		
	H2O2	DPPH	ABTS
1000	63±1.73	73.6±1.12	68±1.23
500	47±1.62	66±1.15	55±0.07
250	38.3±1.31	46.5±1.02	47±1.23
125	23±1.5	38±1.34	28±1.2
62.5	20.3±1.5	23±1.26	18±1.22

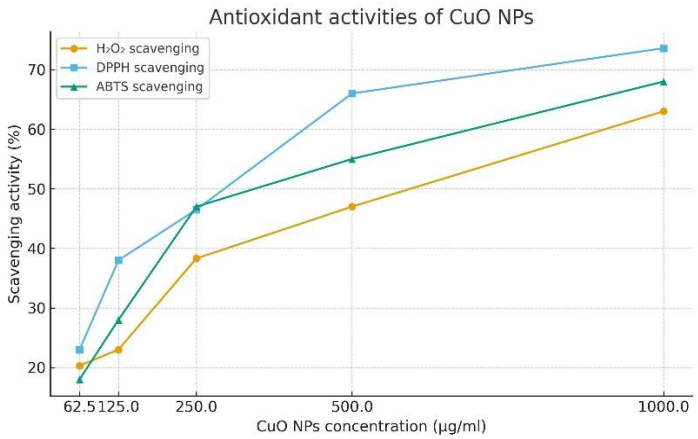


Figure 2 Antioxidant Activities of CuO NPs

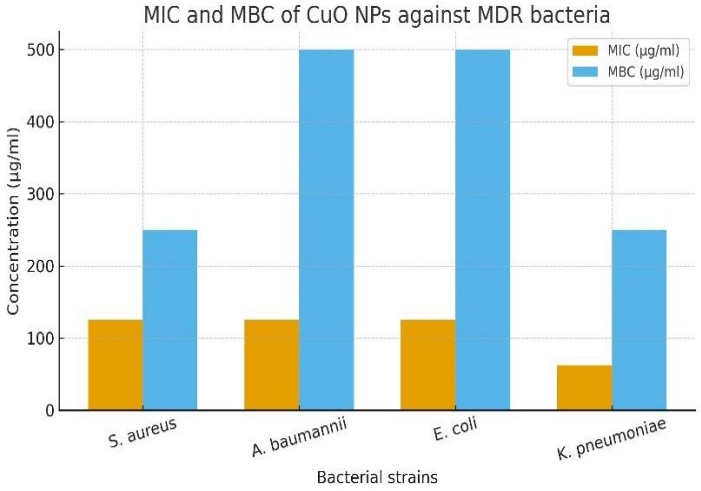
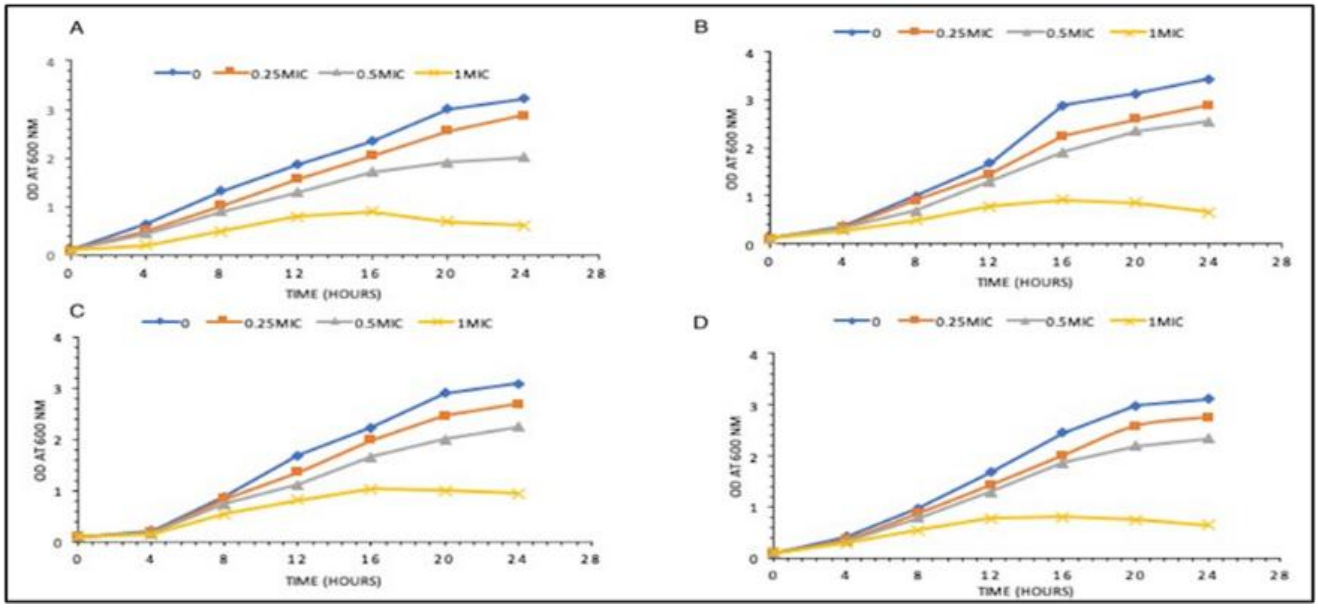
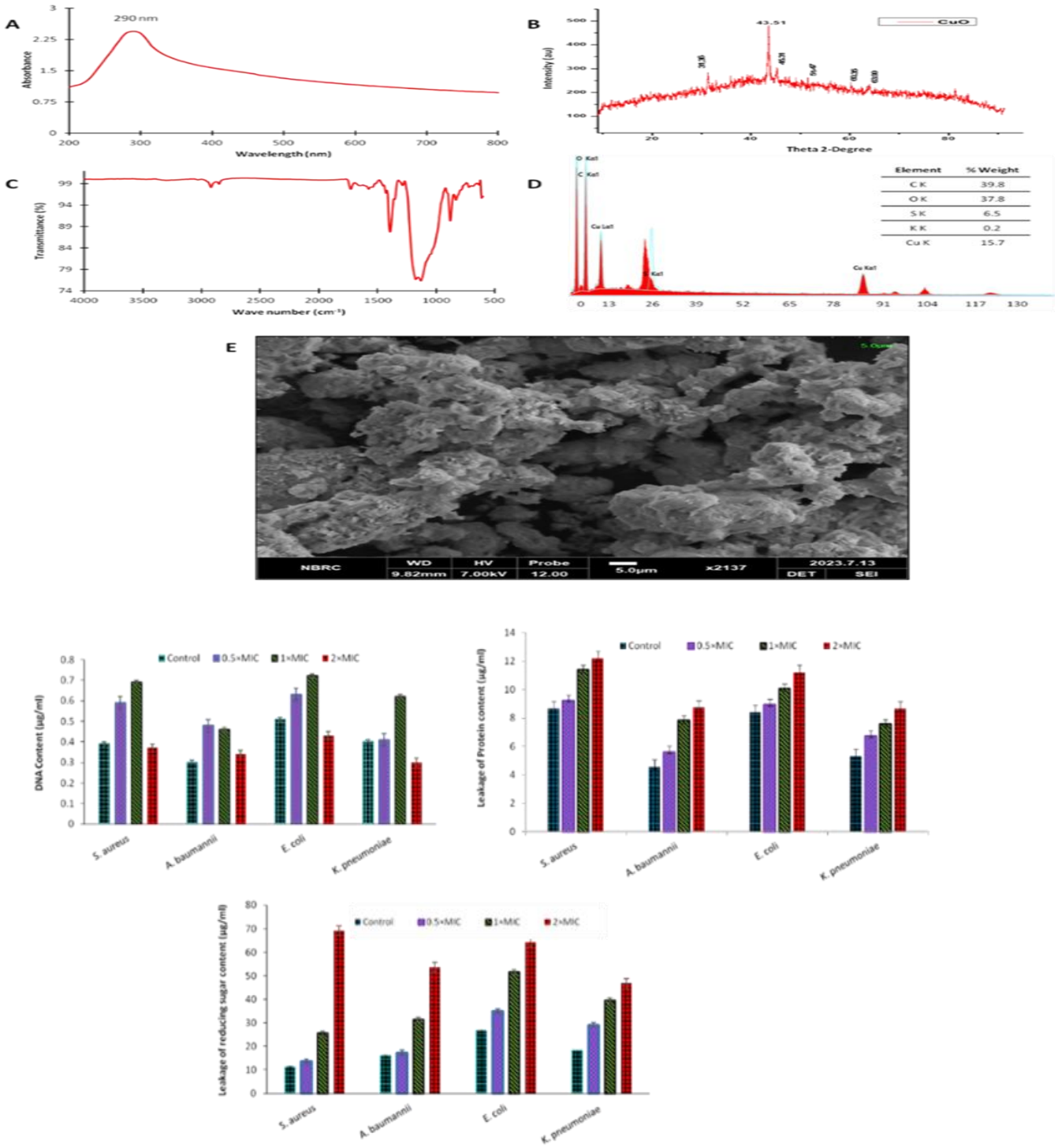


Figure 2 MIC and MBC of CuO NPs Against MDR Bacteria



INSIGHTS-JHR
INSIGHTS-JOURNAL OF HEALTH AND REHABILITATION



DISCUSSION

The present investigation demonstrated that bio fabricated CuO NPs synthesized using *Dalbergia sissoo* leaf extract possessed substantial antibacterial, antibiofilm and antioxidant activities against clinically relevant MDR bacteria, with additional synergistic interactions when combined with selected antibiotics. These findings aligned with the broader global effort to identify alternative strategies against the rapidly growing burden of infections caused by multidrug-resistant pathogens, which continued to threaten global health despite advances in antimicrobial development (1–3). Within this context, nanotechnology provided a rational and adaptable

INSIGHTS-JHR
INSIGHTS-JOURNAL OF HEALTH AND REHABILITATION

platform in which metallic oxide nanoparticles offered promising options to counteract resistance mechanisms in both Gram-positive and Gram-negative bacteria (4,5). The synthesis approach adopted in this study relied on an eco-friendly, organic solvent-free "green" route, using aqueous *D. sissoo* leaf extract as both reducing and stabilizing medium. This strategy was supported by previous work where plant-mediated CuO NPs demonstrated superior biomedical properties compared with those synthesized by conventional chemical methods, due to the involvement of benign biological molecules and improved biocompatibility (6,7). The FTIR spectrum in the current work indicated the involvement of hydroxyl and carboxylate groups of polyphenolic compounds from *D. sissoo* in the reduction and stabilization of copper ions, consistent with reports in which plant extracts rich in sugars, terpenoids, tannins, phenols and flavonoids participated in nanoparticle formation (6-8). The formation of a solid black product and the presence of characteristic Cu–O stretching at 610 cm^{-1}, in addition to other phytochemical-related bands, further supported the role of plant-derived metabolites in the fabrication process.

The optical and structural characterization corroborated the successful synthesis of CuO NPs. The UV–Visible absorption peak at 290 nm fell within the reported range of 250–300 nm for CuO NPs, in agreement with previous work on plant-mediated copper oxide nanomaterials (9). Minor variability in absorption maxima across studies could be attributed to differences in precursor salts, plant matrices, reaction conditions and nanoparticle size distribution. The EDX profile confirmed copper as the principal metallic element, while SEM images primarily showed agglomerated structures with some discrete spherical nanoparticles below 100 nm. Agglomeration has been commonly reported for metal oxide nanoparticles synthesized by green methods and might be related to partial oxidation, residual biomolecules or drying conditions (10). The FTIR and XRD patterns indicated that the nanoparticles were crystalline and stabilized by phytochemicals, consistent with earlier findings on biogenic CuO nanostructures (11,12). Collectively, these physicochemical features suggested that *D. sissoo*-mediated CuO NPs were structurally comparable to other biogenic copper oxide formulations, while leveraging a locally available, pharmacologically relevant plant source. In terms of antibacterial performance, the bio fabricated CuO NPs demonstrated MIC values between 62.5 and 125 µg/ml and MBC values between 250 and 500 µg/ml against MDR *S. aureus*, *A. baumannii*, *E. coli* and *K. pneumoniae*. These values were broadly in line with previously reported MIC ranges for green-synthesized CuO NPs, although some studies documented slightly higher or lower MICs depending on nanoparticle size, surface charge and the nature of phytochemical capping (13-15). The inhibitory zone diameters of 9–24 mm observed in the agar well diffusion assay confirmed a dose-dependent and broad-spectrum antibacterial effect against both Gram-positive and Gram-negative bacteria, supporting the concept that CuO NPs could interfere with multiple microbial targets and bypass traditional resistance mechanisms. Comparative reports where biogenic CuO NPs produced inhibition zones of approximately 20–25 mm against clinical isolates further reinforced these observations (16). The membrane disruption and leakage assays provided mechanistic insight into the antibacterial action of the synthesized nanoparticles. The release of nucleic acids, proteins and reducing sugars into the extracellular environment increased with nanoparticle concentration, with particularly high leakage recorded for *S. aureus* at 250 µg/ml, where reducing sugars and proteins in the supernatant reached 69.1 µg/ml and 12.2 µg/ml, respectively. These findings were consistent with earlier quantitative assessments in which metal-based nanoparticles, including CdO and Ag NPs, induced marked leakage of cellular macromolecules from both Gram-positive and Gram-negative bacteria following membrane perturbation (17,18). The current data supported the concept that CuO NPs compromised cell envelope integrity, promoted permeability changes and facilitated efflux of intracellular components, likely contributing to bactericidal outcomes in conjunction with oxidative stress and direct interactions with DNA and proteins (19).

Biofilm inhibition represented another key outcome with clinical relevance. Since biofilm formation is a major factor underpinning chronic infection, antibiotic tolerance and device-associated complications, any strategy that undermines biofilm development carries therapeutic significance (20). In the present work, CuO NPs reduced biofilm biomass by approximately 38.9–49.3% at 0.5× MIC and 68.4–75.8% at 1× MIC, with the greatest inhibition observed against *K. pneumoniae* followed by *A. baumannii*, *S. aureus* and *E. coli*. These levels of inhibition aligned with previous studies where biogenic CuO NPs produced substantial reductions in biofilm formation in different clinical and environmental strains (21,22). The observation that even subinhibitory concentrations attenuated biofilm formation suggested that the nanoparticles interfered with early adhesion, quorum-sensing or matrix synthesis rather than acting solely as bactericidal agents. This feature was particularly relevant for preventing biofilm establishment on indwelling medical devices and surfaces. An important strength of the study lay in the exploration of synergistic interactions between CuO NPs and conventional antibiotics. The checkerboard assays revealed that combinations of CuO NPs with cefixime or erythromycin frequently reduced MIC values for both components, with FICI values as low as 0.375. These data indicated that in several strain–drug combinations, CuO NPs and antibiotics acted in a synergistic or additive manner, enhancing overall antibacterial efficacy. Earlier investigations had reported that CuO NPs combined with different antibiotics improved bacterial killing, downregulated efflux pumps or altered resistance-associated gene expression, consistent with the general concept that nanoparticle–antibiotic combinations could re-sensitize MDR pathogens and

INSIGHTS-JHR
INSIGHTS-JOURNAL OF HEALTH AND REHABILITATION

reduce required antibiotic doses (23,24). The present findings therefore supported nanoparticle–antibiotic combination therapy as a plausible strategy to improve current treatment regimens, preserve antibiotic effectiveness and potentially mitigate the selection pressure driving resistance (25).

The antioxidant activity of the bio fabricated CuO NPs further complemented their antimicrobial profile. The dose-dependent scavenging of DPPH, ABTS and H_2O_2 radicals indicated that the nanoparticles possessed significant redox-modulating capacity, likely conferred by phytochemical capping from *D. sissoo* extract. Several studies had highlighted that plant-derived CuO NPs exhibited enhanced antioxidant activity compared with uncapped or chemically synthesized counterparts, primarily due to the presence of phenolics, flavonoids and other secondary metabolites on their surface (26). The scavenging percentages reported in this work were broadly comparable to previously documented ranges, where DPPH and other radical assays showed activity spanning from approximately 19% to over 90% across increasing nanoparticle concentrations (27). From a biomedical perspective, such antioxidant properties might mitigate oxidative damage in certain applications; however, the same redox activity could also contribute to antibacterial effects via ROS generation, emphasizing the dual and context-dependent nature of nanoparticle redox behavior. Despite these encouraging findings, several limitations of the present study were apparent. The work was confined to in vitro assays and focused on a limited panel of MDR clinical isolates, which restricted generalizability across species, strain diversity and infection sites. No in vivo evaluation or cytotoxicity assessment on mammalian cells was included, which constrained conclusions regarding safety, biodistribution and therapeutic window. The synthesis protocol involved relatively high temperatures and prolonged reaction times that might have contributed to nanoparticle agglomeration; more refined control of reaction parameters, purification steps and drying conditions could potentially yield narrower size distributions and improved dispersibility. The mechanistic analyses were limited to leakage and indirect inference; more detailed molecular studies, including ROS quantification, gene expression profiling and ultrastructural imaging, would further clarify the antibacterial and antibiofilm mechanisms. In addition, although synergy with two antibiotics was assessed via FICI, dynamic time-kill curves, resistance development studies and evaluation against a broader panel of antibiotics and clinical isolates would strengthen the evidence for combination therapy.

The study, however, had several notable strengths. It employed a fully green synthesis method using a medicinally important and locally available plant, thereby aligning with sustainable and low-cost nanotechnology approaches. It combined comprehensive physicochemical characterization with multiple biological endpoints, including antibacterial, antibiofilm, membrane disruption, synergy and antioxidant assays, providing a multidimensional perspective on the functional potential of *D. sissoo*-mediated CuO NPs. The use of clinically relevant MDR strains enhanced the translational relevance of the findings, and the demonstration of activity at relatively low MICs, together with substantial biofilm inhibition at subinhibitory doses, underscored the promise of this nanomaterial as an adjunct to existing therapies. Future research needed to move beyond in vitro testing and should include detailed cytotoxicity profiling using mammalian cell lines, followed by in vivo infection models to assess efficacy, safety and pharmacokinetics. Optimization of synthesis conditions to reduce agglomeration, narrow size distribution and tune surface chemistry would be important for improving reproducibility and clinical suitability. Expanded synergy studies with a wider range of antibiotics, including last-line agents, and investigation of resistance development under prolonged exposure to nanoparticle–antibiotic combinations would further inform clinical applicability. Finally, surface functionalization strategies that harness specific phytochemicals from *D. sissoo* or other medicinal plants might enable targeted delivery, enhanced selectivity and improved therapeutic indices. Overall, the present study supported the concept that bio fabricated CuO NPs derived from *Dalbergia sissoo* leaf extract represented a promising multifunctional platform with antibacterial, antibiofilm, antioxidant and synergistic properties against MDR pathogens, while also highlighting the need for rigorous mechanistic, toxicological and in vivo validation before translation into clinical practice.

CONCLUSION

The current study demonstrated that copper oxide nanoparticles synthesized through a cost-effective, eco-friendly approach using *Dalbergia sissoo* leaf extract possess substantial antibacterial, antibiofilm and antioxidant properties, underscoring their potential as a versatile nanomaterial to address the growing challenge of antimicrobial resistance and hospital-acquired infections. By establishing the biological efficacy of these biogenic nanoparticles, the study provides a strong foundation for exploring their broader therapeutic relevance. Further research, particularly in vivo evaluation and testing against a wider range of clinically important pathogens, will be essential to validate their safety, optimize their performance and advance their application toward practical biomedical use.

INSIGHTS-JHR
INSIGHTS-JOURNAL OF HEALTH AND REHABILITATION

AUTHOR CONTRIBUTION

Author	Contribution
Hafsa Munir	Substantial Contribution to study design, analysis, acquisition of Data Manuscript Writing Has given Final Approval of the version to be published
Muhammad Naeem	Substantial Contribution to study design, acquisition and interpretation of Data Critical Review and Manuscript Writing Has given Final Approval of the version to be published
Usman Naseer	Substantial Contribution to acquisition and interpretation of Data Has given Final Approval of the version to be published
Meryem Mehmood	Contributed to Data Collection and Analysis Has given Final Approval of the version to be published
Tanveer Aslam	Contributed to Data Collection and Analysis Has given Final Approval of the version to be published
Areej Safdar	Substantial Contribution to study design and Data Analysis Has given Final Approval of the version to be published
Muhammad Hassam Saleem	Contributed to study concept and Data collection Has given Final Approval of the version to be published
Shazia Aslam*	Writing - Review & Editing, Assistance with Data Curation

REFERENCES

1. Bai, X. et al. The Basic Properties of Gold Nanoparticles and their Applications in Tumor Diagnosis and Treatment. Int. J. Mol. Sci. 21, 2480 (2020).

2. Nasrollahzadeh, M., Sajjadi, M., Iravani, S. & Varma, R. S. Green-synthesized nanocatalysts and nanomaterials for water treatment: Current challenges and future perspectives. J. Hazard. Mater. 401, 123401 (2021).

3. Huq, M. A. & Akter, S. Biosynthesis, Characterization and Antibacterial Application of Novel Silver Nanoparticles against Drug Resistant Pathogenic Klebsiella pneumoniae and Salmonella Enteritidis. Mol. Basel Switz. 26, 5996 (2021).

4. Ostovar, N., Mohammadi, N. & Khodadadeh, F. Photocatalytic, antioxidant and antibacterial potential of bio-synthesized ZnO nanoparticles derived from espresso spent coffee grounds: optimization by central composite design. Inorg. Nano-Met. Chem. (2023)

5. Montiel Schneider, M. G. et al. Biomedical Applications of Iron Oxide Nanoparticles: Current Insights Progress and Perspectives. Pharmaceutics 14, 204 (2022).

6. Crisan, M. C., Teodora, M. & Lucian, M. Copper Nanoparticles: Synthesis and Characterization, Physiology, Toxicity and Antimicrobial Applications. Appl. Sci. 12, 141 (2022).

7. Amin, F. et al. Green Synthesis of Copper Oxide Nanoparticles Using Aerva javanica Leaf Extract and Their Characterization and Investigation of In Vitro Antimicrobial Potential and Cytotoxic Activities. Evid.-Based Complement. Altern. Med. ECAM 2021, 5589703 (2021).

8. Sukumar, S., Rudrasenan, A. & Padmanabhan Nambiar, D. Green-Synthesized Rice-Shaped Copper Oxide Nanoparticles Using Caesalpinia bonducella Seed Extract and Their Applications. ACS Omega 5, 1040–1051 (2020).

9. Mahboub, H. H. et al. Protective effects of Allium hirtifolium extract against foodborne toxicity of Zinc oxide nanoparticles in Common carp (Cyprinus carpio). Comp. Biochem. Physiol. Toxicol. Pharmacol. CBP 257, 109345 (2022).

10. Ssekatawa, K. et al. Phyto-Mediated Copper Oxide Nanoparticles for Antibacterial, Antioxidant and Photocatalytic Performances. Front. Bioeng. Biotechnol. 10, (2022).

11. Hayat, S. et al. Biofabrication of ZnO nanoparticles using Acacia arabica leaf extract and their antibiofilm and antioxidant potential against foodborne pathogens. PLOS ONE 17, e0259190 (2022).

12. Verduzco-Chavira, K. et al. Antibacterial and Antibiofilm Activity of Chemically and Biologically Synthesized Silver Nanoparticles. Antibiotics 12, 1084 (2023).

13. Morsy, M. A. et al. Screening and Molecular Docking of Novel Benzothiazole Derivatives as Potential Antimicrobial Agents. Antibiotics 9, 221 (2020).

14. Muzammil, S. et al. Aluminium oxide nanoparticles inhibit EPS production, adhesion and biofilm formation by multidrug resistant Acinetobacter baumannii. Biofouling 36, 492–504 (2020).

15. Vila, J., Moreno-Morales, J. & Ballesté-Delpierre, C. Current landscape in the discovery of novel antibacterial agents. Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis. 26, 596–603 (2020).

16. G, S. et al. Green and chemical synthesis of CuO nanoparticles: A comparative study for several in vitro bioactivities and in vivo toxicity in zebrafish embryos. J. King Saud Univ. - Sci. 34, 102092 (2022).

17. Restrepo, C. V. & Villa, C. C. Synthesis of silver nanoparticles, influence of capping agents, and dependence on size and shape: A review. Environ. Nanotechnol. Monit. Manag. 15, 100428 (2021).

18. Alhalili, Z. Green synthesis of copper oxide nanoparticles CuO NPs from Eucalyptus Globoulus leaf extract: Adsorption and design of experiments. Arab. J. Chem. 15, 103739 (2022).

19. Upadhyay, S. K. et al. Efficient removal of total arsenic (As3+/5+) from contaminated water by novel strategies mediated iron and plant extract activated waste flowers of marigold. Chemosphere 313, 137551 (2023).

20. Tshireletso, P., Ateba, C. N. & Fayemi, O. E. Spectroscopic and Antibacterial Properties of CuONPs from Orange, Lemon and Tangerine Peel Extracts: Potential for Combating Bacterial Resistance. Molecules 26, 586 (2021).

21. Ramzan, M. et al. Green synthesis of copper oxide nanoparticles using Cedrus deodara aqueous extract for antibacterial activity. Mater. Today Proc. 36, (2020).

22. Atri, A. et al. Green synthesis of copper oxide nanoparticles using Ephedra Alata plant extract and a study of their antifungal, antibacterial activity and photocatalytic performance under sunlight. Heliyon 9, e13484 (2023).

23. Alhalili, Z. Metal Oxides Nanoparticles: General Structural Description, Chemical, Physical, and Biological Synthesis Methods, Role in Pesticides and Heavy Metal Removal through Wastewater Treatment. Molecules 28, 3086 (2023).

24. Ali, M. et al. Biogenic Synthesis, Characterization and Antibacterial Potential Evaluation of Copper Oxide Nanoparticles Against Escherichia coli. Nanoscale Res. Lett. 16, 148 (2021).

25. Bhavyasree, P. G. & Xavier, T. S. Green synthesised copper and copper oxide based nanomaterials using plant extracts and their application in antimicrobial activity: Review. Curr. Res. Green Sustain. Chem. 5, 100249 (2022).

26. Azam, Z. et al. Microbial synthesized cadmium oxide nanoparticles induce oxidative stress and protein leakage in bacterial cells. Microb. Pathog. 144, 104188 (2020).

27. Shehabeldine, A. M. et al. Potential Antimicrobial and Antibiofilm Properties of Copper Oxide Nanoparticles: Time-Kill Kinetic Essay and Ultrastructure of Pathogenic Bacterial Cells. Appl. Biochem. Biotechnol. 195, 467–485 (2023).