RESEARCH PAPER

" Enhanced Antimicrobial Activity of Eco-Friendly Synthesized Copper Oxide Nanoparticles and Streptomyces azureus Against Multidrug-Resistant Bacterial Pathogens"

Hiba A. Jasim[1*], Bayan Shakir Mahmood Al-Saeedi[2], Talib S. A-Rubaye[3]

[1*]Lecturer, Department of Biotechnology, College of science, University of Baghdad, Baghdad, Iraq. Email: hiba.jasim@sc.uobaghdad.edu.iq; Orcid Id: https://orcid.org/0009-0002-9429-9535

[2]Assistant Lecturer, Department of Biotechnology, College of science, University of Baghdad, Baghdad, Iraq

[3]Lecturer, Department of Biotechnology, College of science, University of Baghdad, Baghdad, Iraq

***Corresponding Author:** Hiba A. Jasim; hiba.jasim@sc.uobaghdad.edu.iq

Abstract

This study evaluated the antimicrobial activity of green-synthesized copper oxide nanoparticles (CuO-NPs) and metabolites produced by Streptomyces azureus against multidrug-resistant (MDR) bacteria. CuO-NPs were synthesized using an aqueous extract of Camellia sinensis leaves as reducing and capping agents. UV–Vis, FTIR, SEM, and AFM analyses confirmed the formation of stable, spherical nanoparticles with an average size of 70 nm. A potent isolate obtained from Iraqi soil sediments was identified as Streptomyces azureus (TA12) by 16S rRNA sequencing. GC–MS analysis revealed several antimicrobial compounds, including Tetradecane, 1-iodo-, and Azetidine derivatives. The minimum inhibitory concentration (MIC) of CuO-NPs against Staphylococcus aureus and Escherichia coli was 54.6 µg/m. CuO-NPs exhibited strong activity against S. aureus (20 mm inhibition zone) but weak activity against E. coli (10 mm). In contrast, the S. azureus extract showed broad-spectrum antibacterial activity, producing inhibition zones of 17 mm against both pathogens. The combination of CuO-NPs and Streptomyces azureus extract exhibited enhanced antimicrobial activity against both S. aureus and E. coli, producing inhibition zones of 24 and 20 mm, respectively, suggesting a synergistic interaction and highlighting its potential for combating MDR bacterial pathogens.

Keywords: nanomaterials, Streptomyces, Nanobiotechnology, MDR, Bioactive metabolites

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Introduction

The increasing prevalence of multidrug-resistant (MDR) bacteria represents a major global health challenge in the twenty-first century. Pathogens such as methicillin-resistant Staphylococcus aureus (MRSA) and extended-spectrum β-lactamase (ESBL)-producing Escherichia coli have reduced the effectiveness of conventional antibiotics, leading to higher mortality rates and healthcare costs[1]. Consequently, considerable attention has been directed toward discovering novel antimicrobial agents and developing alternative therapeutic strategies[2]. The genus Streptomyces, a group of filamentous Gram-positive bacteria within the phylum Actinobacteria is recognized as one of the most important sources of bioactive secondary metabolites. More than two-thirds of clinically used antibiotics, including streptomycin, tetracycline, and chloramphenicol, are derived from Streptomyces species[3]. Owing to their metabolic diversity, soil-dwelling Streptomyces produce a wide range of compounds with potent antimicrobial properties, making them attractive targets for bioprospecting[4]. In parallel, nanotechnology has emerged as an effective approach for combating antimicrobial resistance. Copper nanoparticles (CuNPs) have attracted significant interest because of their unique physicochemical properties, cost-effectiveness, and broad-spectrum antimicrobial activity[5,6]. Green synthesis using plant extracts, such as Camellia sinensis, provides an environmentally friendly and biocompatible alternative to conventional methods, as plant phytochemicals act as reducing and stabilizing agents during nanoparticle formation[7]. Although both Streptomyces azureus metabolites and CuNPs exhibit promising antimicrobial effects individually, their combined application may produce synergistic interactions capable of enhancing antibacterial activity and overcoming the limitations of single-agent therapies[8]. Therefore, this study aimed to isolate and identify a potent Streptomyces strain from Iraqi soil, synthesize and characterize CuNPs using Camellia sinensis leaf extract, and evaluate the individual and combined antimicrobial activities of these agents against MDR Staphylococcus aureus and Escherichia coli[9].

Materials and Methods

Isolation and Characterization of Soil-Derived Streptomyces

Fifty-seven soil samples were collected from different locations across Baghdad, Iraq. Streptomyces isolates were recovered using the serial dilution method on Starch Casein Agar (SCA) supplemented with nystatin (50 µg/mL) to suppress fungal contamination. The inoculated plates were incubated at 30°C for 4–7 days. Colonies exhibiting the typical morphological characteristics of Streptomyces, including a chalky and powdery appearance with well-developed aerial mycelia, were selected and repeatedly sub-cultured on International Streptomyces Project medium 2 (ISP-2) to obtain pure isolates10,11.

Conventional and Molecular Identification of the Isolates

The selected isolate were characterized according to their macroscopic and microscopic characteristics, including colony morphology, pigment production, and the structural features of aerial and substrate mycelia12,13. Molecular identification was performed by extracting genomic DNA using a commercial extraction kit (Geneaid, Taiwan). The bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) employing the universal primers 27F and 1492R. Subsequently, the amplified products were sequenced, and the resulting nucleotide sequences were analysed

using the Basic Local Alignment Search Tool (BLASTn) against the National Center for Biotechnology Information (NCBI) GenBank database to determine the closest phylogenetic affiliations and identify the isolates at the species level14.

Detection of Antimicrobial Activity and Metabolic Profiling

Primary screening of the isolated Streptomyces strains for antimicrobial activity was initially conducted using the cross-streak assay against Staphylococcus aureus and Escherichia coli15. Isolates exhibiting significant inhibitory activity were subsequently subjected to secondary screening employing the agar well diffusion method using both cell-free culture supernatants and crude ethyl acetate extracts. The most active isolate was selected for metabolite production and cultivated in Starch Casein Broth (SCB) under optimized conditions (30°C and pH 7.0)16. The crude extract was then analysed by Gas Chromatography–Mass Spectrometry (GC–MS) using a PerkinElmer Clarus 500 system to identify the major bioactive secondary metabolites responsible for the observed antimicrobial activity17.

Biogenic Synthesis of CuO Nanoparticles

A total of 200 g of air-dried Camellia sinensis leaves was extracted with 500 mL of 80% aqueous methanol (methanol:water, v/v) using a Soxhalet apparatus for 8 h. The resulting extract was filtered and concentrated under reduced pressure at 45°C using a rotary evaporator18. The crude extract was weighed to determine the extraction yield and stored at 4°C until use. Green synthesis of copper oxide nanoparticles (CuO-NPs) was carried out using the plant extract as a reducing and stabilizing agent. Briefly, 10 mL of Camellia sinensis extract was mixed with 90 mL of 2 mM copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) solution under continuous stirring19. A control sample was prepared by replacing the plant extract with deionized water. The reaction mixtures were incubated in a rotary shaker in the dark at room temperature for 3 h to ensure homogeneous mixing and facilitate nanoparticle formation. The synthesis of CuO-NPs was initially confirmed by the observed color change in the reaction mixture. The nanoparticles were subsequently collected by centrifugation at 10,000 rpm for 15 min, washed repeatedly with deionized water to remove residual impurities, dried at 60°C, and stored at room temperature for further characterization and biological applications20,21

Physicochemical Characterization of Synthesized CuO Nanoparticles22

The synthesized copper oxide nanoparticles (CuO NPs) were characterized using different analytical techniques to determine their physicochemical properties, including particle size, morphology, and surface functional groups. Ultraviolet–visible (UV–Vis) spectroscopy was employed to confirm the formation of CuO nanoparticles through their characteristic absorption peak. Fourier-transform infrared spectroscopy (FTIR) analysis was performed to identify the functional groups responsible for the reduction and stabilization of the nanoparticles. Furthermore, scanning electron microscopy (SEM) was used to investigate the surface morphology and particle size, while atomic force microscopy (AFM) analysis provided detailed information regarding the surface topography and size distribution of the synthesized CuO nanoparticles.

In Vitro Evaluation of Antimicrobial Activity

The antimicrobial activity of copper oxide nanoparticles (CuO NPs), the crude extract of Streptomyces azureus, and their combination was evaluated against multidrug-resistant (MDR) clinical isolates of Staphylococcus aureus and Escherichia coli23. The antibacterial activity was initially assessed using the agar well diffusion method. Briefly, bacterial suspensions were adjusted to 0.5 McFarland standard and uniformly spread onto Mueller–Hinton agar plates. Wells of 6 mm diameter were aseptically punched into the agar and loaded with 50 µL of CuO NPs (54.6 µg/mL), S. azureus crude extract, or a 1:1 mixture of both agents. Following incubation at 37°C for 24 h, the diameters of the inhibition zones were measured in millimeters24,25. In addition, the minimum inhibitory concentration (MIC) of CuO NPs was determined by the broth microdilution method in sterile 96-well microplates according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, using resazurine as a colorimetric indicator of bacterial growth. Furthermore, the interaction between CuO NPs and the S. azureus extract was evaluated by comparing the antibacterial activity of the combined treatment with that of each component alone in order to determine whether the interaction exhibited synergistic, additive, or antagonistic effects26,27,28.

Results and Discussion

Isolation and Identification of a Potent Streptomyces Strain

A total of 75 sediment soil samples collected, 70 samples (93%) were suspected to be Streptomyces species. From these samples, 60 isolates (86%) exhibiting diverse morphological characteristics were successfully recovered. Presumptive actinomycete colonies were carefully subcultured on Starch Casein Agar (SCA) and International Streptomyces Project medium 2 (ISP2) to obtain pure cultures. The purified isolates were characterized by variations in the colour of both aerial and substrate mycelia and displayed distinct colony morphologies, including dry surfaces, rough or smooth textures, regular or irregular margins, and predominantly convex elevations 10,11 (Fig. 1).

Figure 1. Isolation of Streptomyces spp. from soil sample dilutions on starch casein agar following incubation at 30°C for 4–7 days.

Sequencing analysis was performed to identify the isolated Streptomyces spp. strains. The amplified PCR products were sent to Bioneer Company (Korea) for DNA sequencing. The obtained nucleotide sequences were subsequently compared with those deposited in the GenBank database using the Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov). The sequences showing the highest similarity scores were retrieved and used for further phylogenetic analysis (Fig. 2).

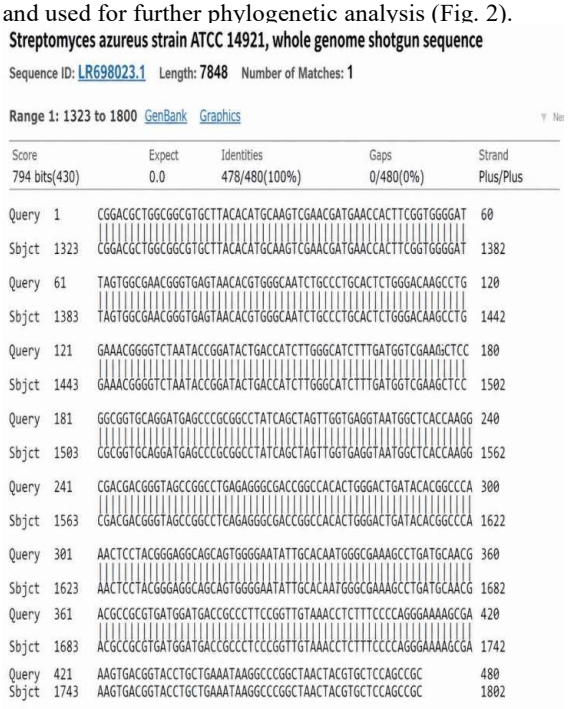

Figure 2: Sequence Analysis and Taxonomic Identification of Streptomyces azureus Through NCBI BLAST

Metabolomic Analysis of Streptomyces azureus Using GC–MS

GC–MS analysis of the ethyl acetate extract of Streptomyces azureus (TA12) revealed the presence of several bioactive secondary metabolites, including Tetradecane, 1-iodo- ($C_{14}H_{29}I$), Dodecane ($C_{12}H_{26}$), azetidine derivatives, fatty acids, phenolic compounds, and esters (Table 1). These compounds have been previously reported to exhibit antimicrobial activity against a wide range of pathogenic microorganisms. In particular, fatty acids and phenolic compounds can disrupt bacterial cell membranes, whereas azetidine derivatives possess antibacterial and antioxidant properties. The antimicrobial activity observed in the crude extract is likely due to the synergistic action of these metabolites. Similar findings were reported by[29], confirming the potential of Streptomyces species as a promising source of novel antimicrobial agents.

Table 1: Major bioactive compounds identified by GC–MS analysis of the ethyl acetate extract of Streptomyces azureus (TA12) and their reported biological activities.

1	Tetradecane, 1-iodo-	$C_{14}H_{29}I$	Antimicrobial and antifungal activities
2	Dodecane	$C_{12}H_{26}$	Antibacterial activity
3	Azetidine derivatives	Variable	Antimicrobial and antioxidant activities
4	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	Antibacterial and anti-inflammatory activities
5	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	Antimicrobial activity
6	9,12-Octadecadienoic acid (Z,Z)	$C_{18}H_{32}O_2$	Antibacterial and antioxidant activities
7	Octadecanoic acid	$C_{18}H_{36}O_2$	Antimicrobial activity
8	Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{14}H_{22}O$	Antibacterial and antioxidant activities
9	Cyclo-(L-leucyl-L-phenylalanyl)	$C_{17}H_{24}N_2O_2$	Antibacterial and antifungal activities
10	Eugenol	$C_{10}H_{12}O_2$	Broad-spectrum antimicrobial activity

Spectroscopic and Morphological Characterization of CuNPs UV–Vis Spectroscopy

The UV–Vis spectrum of the biosynthesized CuO nanoparticles displayed a distinct absorption peak at 295 nm, confirming that CuO-NPs had formed successfully. Its sharpness points to relatively stable, well-dispersed nanoparticles with minimal aggregation. A characteristic UV–Vis absorption band for green-synthesized copper nanoparticles was also reported by 30, and the agreement with that finding supports the

successful reduction of copper ions by biological extracts. It also indicates that the biosynthetic method used here produced CuO nanoparticles with optical properties comparable to those described previously. Small differences in the precise absorption wavelength between studies may result from variation in the biological reducing agents, precursor concentration, pH, temperature, and other reaction conditions, which affect nanoparticle size and optical properties. (Fig 3).

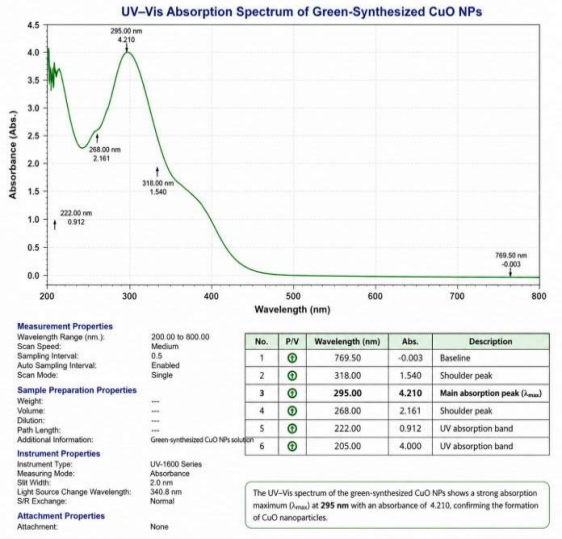

Figure 3. UV–Vis spectrum of green-synthesized CuO-NPs showing the absorption peak at 295 nm.

FTIR Analysis

The FTIR spectrum confirmed the successful biosynthesis of CuO nanoparticles by identifying the functional groups involved in both nanoparticle formation and stabilization. The broad O–H stretching band at 3412.78 cm^{-1} indicates the presence of hydroxyl groups from biomolecules and adsorbed water, while the C–H, C=O, C=C, C–N, and C–O absorption bands suggest that proteins, phenolic compounds, and other metabolites acted as reducing and capping agents during nanoparticle synthesis. These biomolecules likely enhanced nanoparticle stability by preventing aggregation. Most importantly, the characteristic absorption bands at 582.30 and 428.65 cm^{-1}, assigned to Cu–O stretching vibrations, provide direct evidence for the formation of crystalline CuO nanoparticles. These findings are consistent with those reported by 31, who identified similar FTIR bands corresponding to Cu–O bonds and biomolecule-associated functional groups in green-synthesized CuO nanoparticles. The agreement between the present results and previous studies confirms the effectiveness of the biosynthetic method and demonstrates the essential role of biological metabolites in the reduction, stabilization, and successful formation of CuO nanoparticles(Fig.4).

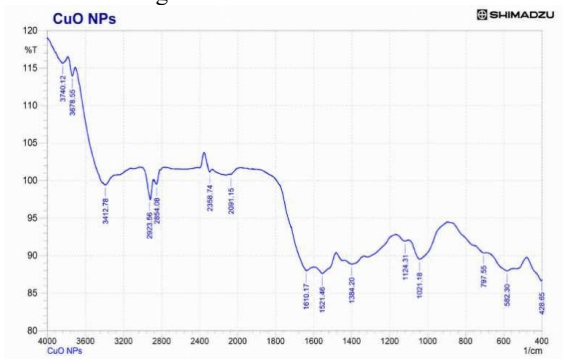

Figure 4. FTIR spectrum of biosynthesized CuO nanoparticles (CuO NPs).

SEM and AFM

The SEM and AFM analyses confirmed the successful formation of CuO nanoparticles with nanoscale dimensions and a heterogeneous surface morphology. SEM images revealed predominantly spherical to irregular particles with an average size of(73.8 nm), while AFM analysis demonstrated an uneven surface topology and the presence of aggregated nanoparticles. The observed aggregation is a common characteristic of CuO nanoparticles and is mainly attributed to their high surface energy and strong interparticle interactions. The measured particle size falls within the typical nanoscale range reported for biologically synthesized CuO nanoparticles. These findings are consistent with the study of 32 who reported that green-synthesized CuO nanoparticles exhibit aggregated structures with irregular morphologies and complex surface architectures at the nanoscale. Therefore, the agreement between the present study and the published report confirms the effectiveness of the biosynthetic approach in producing CuO nanoparticles with morphological characteristics comparable to those previously described (Fig 5).

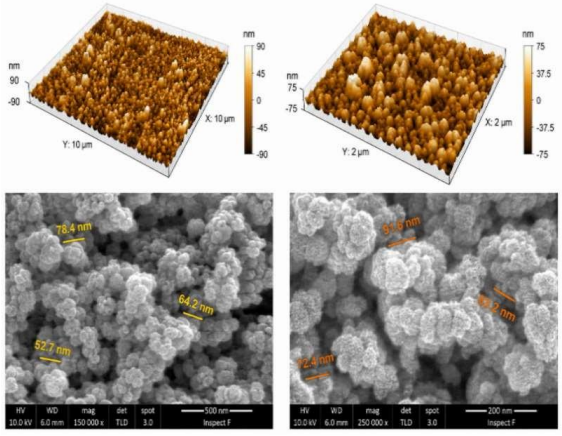

Figure 5. SEM and AFM images of the biosynthesized CuO nanoparticles (CuO NPs).

Assessment of Antibacterial Activity and Combined Effects

The antimicrobial results demonstrated that the antibacterial activity of CuO nanoparticles varied according to the bacterial species. CuO-NPs exhibited greater efficacy against the Gram-positive bacterium Staphylococcus aureus than against the Gram-negative Escherichia coli, which is likely due to the outer

lipopolysaccharide membrane of Gram-negative bacteria that limits nanoparticle penetration and reduces susceptibility. The crude extract of Streptomyces azureus showed broad-spectrum antibacterial activity against both pathogens, reflecting the presence of bioactive secondary metabolites. The combined treatment produced a larger inhibition zone against S. aureus, indicating a synergistic interaction, possibly resulting from enhanced membrane disruption by CuO-NPs that facilitates the uptake of antimicrobial metabolites. In contrast, the reduced activity observed against E. coli suggests an antagonistic interaction, which may be attributed to physicochemical interactions between nanoparticles and bioactive compounds that decrease their antimicrobial effectiveness (Table 2). These findings are partially consistent with the study of33, who reported that metal nanoparticles can enhance antibiotic activity through synergistic mechanisms, particularly against Gram-positive bacteria, although antagonistic interactions may also occur depending on the nanoparticle type, antimicrobial agent, and target microorganism. Therefore, the present results highlight the importance of evaluating nanoparticle–metabolite combinations individually for different bacterial species before their therapeutic application (Fig 6).

Table 2. Antimicrobial and combinatorial effects of CuONPs and S. azureus

CuO nanoparticles (CuONPs)	54.6	20	10
Streptomyces azureus crude extract	—	17	17
CuONPs + S. azureus crude extract	—	24	20

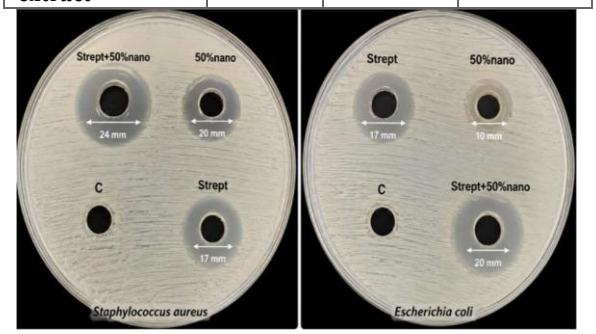

Figure 6. Synergistic antibacterial effects of CuO-NPs and Streptomyces azureus metabolites against Staphylococcus aureus and Escherichia coli.

Conclusion

This study demonstrated the successful green synthesis of CuO nanoparticles using Camellia sinensis leaf extract, yielding stable nanoparticles with confirmed physicochemical characteristics. Streptomyces azureus (TA12), isolated from Iraqi soil sediments, produced bioactive secondary metabolites with broad-spectrum antibacterial activity. Both CuO-NPs and the S. azureus extract exhibited inhibitory effects against multidrug-resistant Staphylococcus aureus and Escherichia coli, although CuO-NPs were more effective against the Gram-positive strain. The combined treatment enhanced antibacterial activity, particularly against S. aureus, indicating a synergistic interaction between CuO-NPs and Streptomyces metabolites. These findings suggest that integrating biologically synthesized CuO nanoparticles with microbial secondary metabolites represents a promising strategy for improving antimicrobial efficacy against MDR pathogens. Further studies are recommended to investigate the underlying mechanisms of synergy, evaluate cytotoxicity, and assess the therapeutic potential of this combined approach in in vivo models.

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