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

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Abstract: The activity against microbes of Streptomyces azureus metabolites and green-synthesized copper oxide nanoparticles (CuO-NPs) against multidrug-resistant (MDR) bacteria was evaluated in the present study. In the synthesis of CuO-NPs, an aqueous extract of Camellia sinensis leaves, was used as a reducing and capping agent. UV–Vis, FTIR, SEM, and AFM examination substantiated the creation of stable, spherical nanoparticles with an average size of 70 nm. Streptomyces azureus (TA12), using 16S rRNA sequencing, was determined in a strong isolate from sediments of Iraqi soil. Various antibacterial compounds, such as 1-iodo-, Tetradecane, and Azetidine derivatives, were identified using GC-MS analysis. The minimum inhibitory concentration (MIC) of CuO-NPs against Escherichia coli and Staphylococcus aureus was 54.6 µg/m. CuO-NPs showed a potent activity against S. aureus (20 mm inhibition zone) but fragile activity against E. coli (10 mm). Equally, the S. azureus extract showed broad-spectrum antibacterial activity, producing inhibition zones of 17 mm against both pathogens. Correspondingly, the combination of CuO-NPs and Streptomyces azureus extract revealed an improved antimicrobial activity against both S. aureus and E. coli, producing inhibition zones of 24 and 20 mm, suggesting a synergetic interaction and demonstrating its potential for combating MDR bacterial pathogens.

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

Introduction

In the current century, the recurrent surge of multidrug-resistant (MDR) microbes has developed into a major health issue globally. Pathogens, such as methicillin-resistant Staphylococcus aureus (MRSA) and extended-spectrum β-lactamase (ESBL)-producing Escherichia coli, have moderated the efficiency of regular antibiotics, increasing the healthcare expenses and higher mortality rates[1]. As a result, finding original antimicrobial agents and creating alternative therapeutic approaches has received considerable attention [2]. More recently, one of the most important recognised sources of bioactive secondary metabolites is the genus Streptomyces, which belongs to a phylum of filamentous Gram-positive bacteria within the group. A significant percentage

of antibiotics used in clinics, such as tetracycline, chloramphenicol, and streptomycin, are sourced from Streptomyces species [3]. Due to their metabolic variability, soil-dwelling Streptomyces produce varieties of compounds with effective antimicrobial attributes, establishing them as one of the most attractive phenomena for biodiscovery [4].

Correspondingly, nanotechnology has evolved into an effective technique for combating the antimicrobials resistance. Owing to the distinctive physicochemical properties, broad-spectrum antimicrobial activity, and cost efficiency of copper nanoparticles (CuNPs), they have received considerable attention[5][6] In the nanoparticle formation period, plant phytochemicals function as reducing and stabilising agents', addressing the limitations of traditional methods and making green synthesis through plant extracts, particularly Camellia sinensis, an eco-friendly and biocompatible alternative to conservative approaches[7]. Despite Streptomyces azureus metabolites and CuNPs showing signs of antimicrobial properties independently, applying them simultaneously may bring synergistic relationships capable of augmenting antibacterial activity and subduing the therapies[8]. As a result, the present research intended to separate and categorise an effective Streptomyces strain from Iraqi soil, synthesize and describe CuNPs using Camellia sinensis leaf extract, and assess the individual and collective 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 isolates[10][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 mycelia[12][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 level[14]

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 coli*[15]. 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 activity[17]

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 evaporator[18] 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 stirring[19]. 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 applications[20][21]

Physicochemical Characterization of Synthesized CuO Nanoparticles[22]

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 coli*[23]. 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 millimetres[24][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 effects[26][27][28].

Results and Discussion

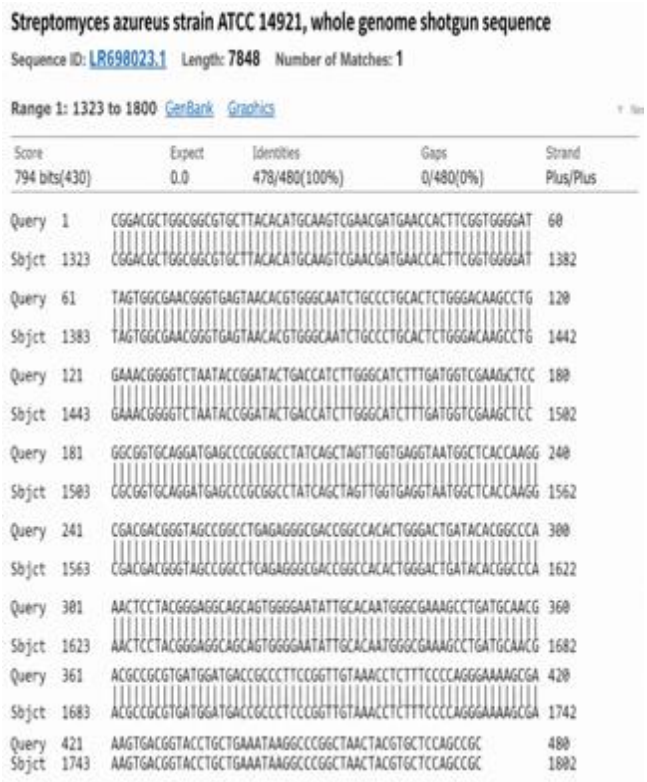
Isolation and Identification of a Potent *Streptomyces* Strain

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 (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. For DNA sequencing, the improved PCR products were submitted to Bioneer Company (Korea). Basic Local Search Tool (BLAST), accessed through: https://www.ncbi.nlm.nih.gov, the website of the National Center for Biotechnology Information (NCBI) was used to compare the collected nucleotide sequences with those kept in the GenBank database. The sequences with highest similarity score were retrieved and utilised for more phylogenetic analysis (Fig. 2).



Streptomyces azureus strain ATCC 14921, whole genome shotgun sequence

Sequence ID: LR698023.1 Length: 7848 Number of Matches: 1

Range 1: 1323 to 1800 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
794 bits(430)	0.0	478/480(100%)	0/480(0%)	Plus/Plus

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Query  1     CGGACGCTGGCGGCGTGCTTACACATGCAAGTCGAACGATGAACCACTTCGGTGGGGAT  60
Sbjct  1323  CGGACGCTGGCGGCGTGCTTACACATGCAAGTCGAACGATGAACCACTTCGGTGGGGAT  1382

Query  61    TAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCTG  120
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Sbjct  1743  AAGTGACGGTACCTGCTGAAATAAGGCCCGGCTAACTACGTGCTCCAGCCGC  1802
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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). In the previous report, these compounds have been confirmed to exhibit antimicrobial activity against various pathogenic microorganisms. More particularly, while phenolic compounds and fatty acids can interrupt bacterial cell membranes, azetidine derivatives have antioxidant and antibacterial patterns. However, the antimicrobial activity detected in the crude extract is expected to be the results of the synergistic action of such metabolites. Correlated findings were reported by[29], proving the likelihood of Streptomyces species as a latent source of new 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.

No.	Compound	Molecular Formula	Reported biological activity
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)

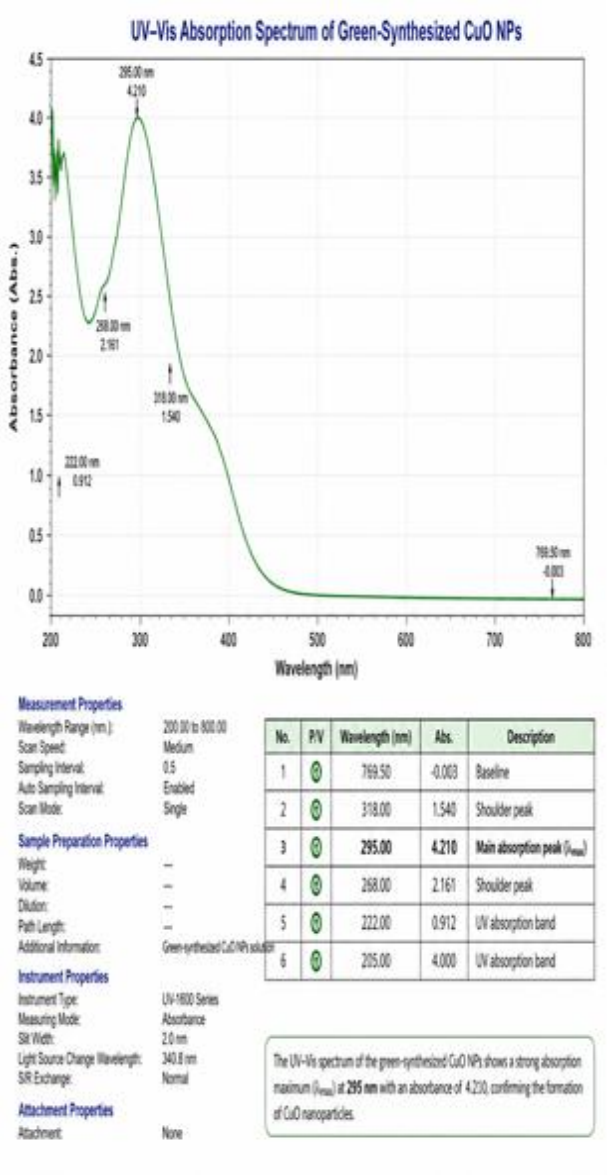


Figure 3. UV–Vis spectrum of green-synthesized CuO-NPs showing the absorption peak at 295 nm.

FTIR Analysis

By finding the functional groups contributing to the formation and stabilization of nanoparticle, the FTIR spectrum established the effective biosynthesis of CuO nanoparticles. While the broad O–H stretching band at 3412.78 cm ^{1} shows the existence of hydroxyl groups $^{-}$ from biomolecules and adsorbed water, the C–H, C=O, C=C, C–N, and C–O absorption bands signify those proteins, phenolic compounds, and other metabolites acted as reducing and capping agents in the period of nanoparticle synthesis. Such biomolecules show potential in enhancing the nanoparticle stability by thwarting aggregation. Notably, the characteristic absorption bands at 582.30 and 428.65 cm, attributed to Cu–O stretching $^{-}$ vibrations, offer direct suggestions for the formation of crystalline CuO nanoparticles. These findings are consistent with those reported by[30] 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).

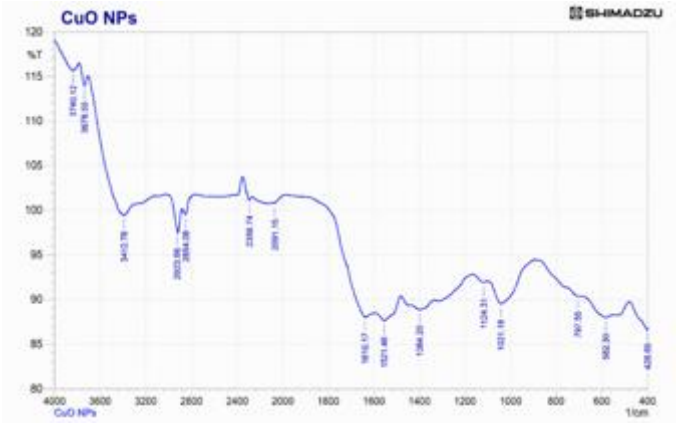


Figure 4. FTIR spectrum of biosynthesized CuO nanoparticles (CuO NPs).

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[31] 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).

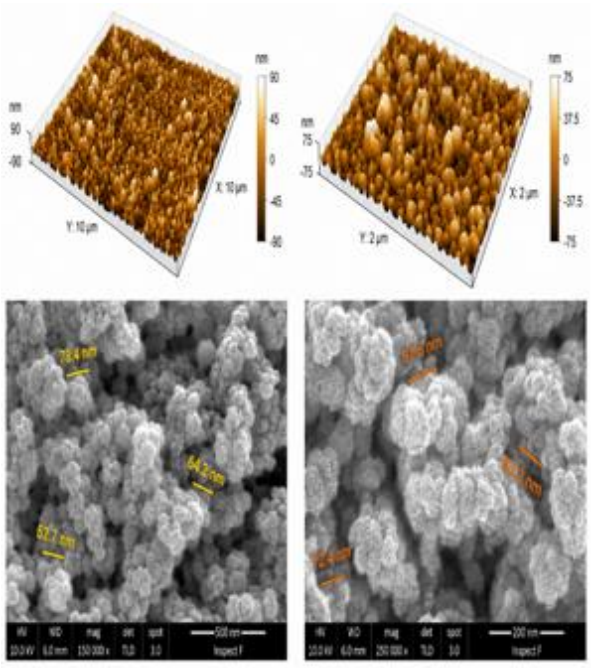


Figure 5. SEM and AFM images of the biosynthesized CuO nanoparticles (CuO NPs).

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 reduced bioactive 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[32]. Conversely, the reduced activity detected against E. coli denotes an antagonistic interaction, which may conceivably be due to the physicochemical interactions of nanoparticles and bioactive compounds that reduce their antimicrobial efficacy (Table 2). These findings are to some extent in line with the study of 33 that posited that through synergistic mechanisms, especially against Gram-positive bacteria, metal nanoparticles can increase antibiotic activity, even though antagonistic interactions may also happen according to the nanoparticle type, antimicrobial agent, and target microorganism[33]. 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*

Treatment	MIC (μg/mL)	Inhibition Zone against *S. aureus* (mm)	Inhibition Zone against *E. coli* (mm)
CuO nanoparticles (CuONPs)	54.6	20	10
Streptomyces azureus crude extract	—	17	17
CuONPs + *S. azureus* crude extract	—	24	20

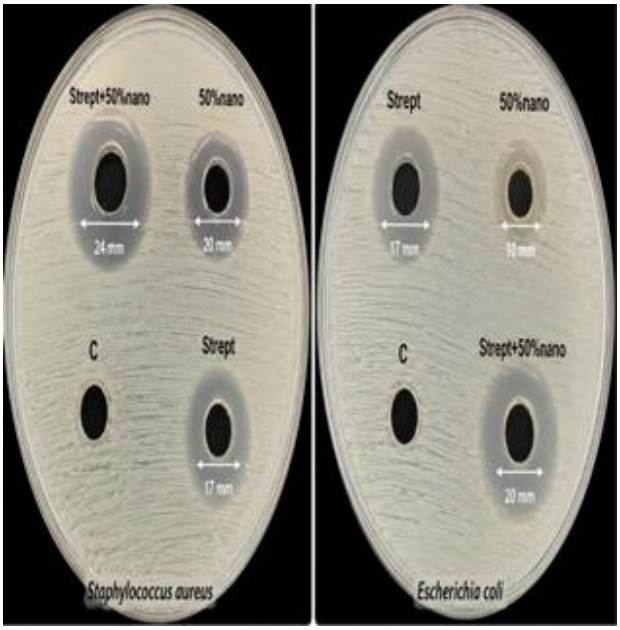


Figure 6. Synergistic antibacterial effects of CuO-NPs and *Streptomyces azureus* metabolites against *Staphylococcus aureus* and *Escherichia coli*.

Conclusion

This research established the positive green synthesis of CuO nanoparticles by extracting the leaf of the Camellia sinensis plant, producing stable nanoparticles with physicochemical characteristics. Isolated from sediments of Iraqi soil, Streptomyces azureus (TA12) yielded bioactive secondary metabolites with broad-spectrum antibacterial activity. Though CuO NPs were more active

against the Gram Submission ID trn:oid:::1:3685729082 positive strain, both CuO-NPs and the S. azureus extract demonstrated inhibitory effects against multidrug-resistant Staphylococcus aureus and Escherichia coli. The collective treatment boosted antibacterial activity, especially against S. aureus, signifying a coactive collaboration between confirmed CuO-NPs and Streptomyces metabolites. Further, the findings show that synthesized biological CuO nanoparticles integrated with microbial secondary metabolites demonstrates a potential antimicrobial efficacy strategy for improving treatment against MDR pathogens. Future studies are also recommended to investigate the primary mechanisms of synergy, assess cytotoxicity, and evaluate the therapeutic potential of this combined approach in vivo models.

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