

Comparative Assessment of Anti-biofilm Potency and Gene Suppression by Green-Synthesized Silver and Copper Oxide Nanoparticles on Urinary *Pseudomonas aeruginosa* Isolates in Zahedan (2025)

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ABSTRACT

Background: Biofilm-forming *Pseudomonas aeruginosa* strains, particularly those forming robust biofilms, severely complicate urinary tract infections (UTIs). Green nanotechnology offers a sustainable solution to this crisis.

Methods: In this cross-sectional study (2025), 103 *P. aeruginosa* isolates were collected from UTI patients in Zahedan, Iran. Following phenotypic and molecular (16S rRNA) identification, biofilm formation was assessed via microtiter plate assay. Silver nanoparticles (AgNPs) and copper oxide nanoparticles (CuONPs) were biosynthesized using *Mesembryanthemum nodiflorum* extract and characterized by UV-Vis, TEM, and XRD. Antimicrobial and antibiofilm activities were evaluated against strong biofilm producers. Expression levels of *lasI* and *pelA* genes were quantified by Real-time PCR, and cytotoxicity was tested on HEK293 cells.

Results: Characterization confirmed stable, spherical AgNPs (~45 nm) and CuONPs (~15 nm). Despite the larger size of AgNPs, they exhibited superior efficacy with lower MIC values (6.25–12.5 µg/mL) and higher biofilm inhibition (72.8–75.4%) compared to smaller CuONPs (MIC 25–50 µg/mL; 55.3–60.1% inhibition). AgNPs significantly downregulated *lasI* (up to 4.7-fold) and *pelA* (up to 4.0-fold) genes, outperforming CuONPs. Both agents maintained >85% cell viability at therapeutic doses.

Conclusion: Although smaller nanoparticles are typically expected to be more reactive, this study revealed that AgNPs possessed markedly stronger antibiofilm and gene-suppressive activities than the smaller CuONPs. This suggests that intrinsic material properties override particle size in determining efficacy. These green-synthesized nanoparticles offer a promising, biocompatible strategy for coating urinary catheters to combat strong biofilm-producing infections.

KEYWORDS: *Pseudomonas aeruginosa*; Biofilm Inhibition; *Mesembryanthemum nodiflorum*; Gene Expression; Nanotherapeutics.

1. Introduction

Urinary tract infections (UTIs) continue to pose a significant challenge to healthcare systems worldwide, often complicated by opportunistic pathogens that exploit vulnerable populations. Among these, *Pseudomonas aeruginosa* emerges as a particularly tough adversary, especially in catheter-associated

cases where treatment failures are common. This bacterium's tenacity stems mainly from its capacity to build biofilms; intricate, multicellular structures shielded by a self-produced extracellular polymeric substance (EPS) [1]. Bacteria embedded within these biofilms can endure antibiotic doses hundreds of times higher than those needed to eliminate free-

floating cells, fueling chronic and recurring infections that strain clinical resources [2].

This shift from a wandering, planktonic lifestyle to an anchored, biofilm-based existence is not random; rather, it is finely tuned by quorum sensing (QS) systems, which allow bacteria to communicate and coordinate behaviors based on population density. The *las* system, spearheaded by the *lasI* gene, serves as a key regulator, orchestrating the expression of virulence factors that promote biofilm development [3]. Concurrently, the structural backbone of the biofilm relies on exopolysaccharides, with the *pelA* gene being essential for crafting the glucose-laden matrix that binds the community together [4]. Consequently, breaking down these biofilms demands more than mere bactericidal action; it requires interventions capable of infiltrating the EPS and sabotaging these precise genetic controls.

In an era where traditional antibiotics are losing ground, colloidal science and nanotechnology have stepped up as innovative allies in the fight against microbial resistance. Metallic nanoparticles, such as Silver nanoparticles (AgNPs) and copper oxide nanoparticles (CuONPs), stand out for their multifaceted antimicrobial prowess, driven by their nanoscale size and high surface reactivity [5]. These particles disrupt bacterial cells through several overlapping mechanisms: they puncture cell membranes, unleash reactive oxygen species (ROS) that trigger oxidative damage, and release metal ions (Ag^+ or Cu^{2+}) that bind to critical enzymes and DNA, halting essential processes like respiration and replication [6-8]. From a colloidal perspective, their stability in suspension; often enhanced by surface coatings; enables better dispersion and interaction with biological targets, making them particularly adept at navigating the dense, protective layers of biofilms [7].

Beyond their core antimicrobial effects, AgNPs and CuONPs have found diverse biomedical applications. Silver nanoparticles, prized for their potent broad-spectrum activity, are commonly integrated into wound dressings, catheter coatings, and drug delivery systems to prevent infections and promote healing [8]. Copper oxide nanoparticles, meanwhile, offer cost-effective alternatives with strong anti-biofilm properties, and they are increasingly utilized in antimicrobial surfaces for hospitals, water purification, and agricultural settings to combat plant pathogens [9]. Both types of nanoparticles show promise in targeting resistant strains like *P.*

aeruginosa, where their ability to interfere with QS and matrix formation could revolutionize treatments for chronic infections [10, 11].

Recent studies, both in Iran and internationally, have shed light on the anti-microbial, anti-biofilm, and gene-suppressive capabilities of these nanoparticles, often highlighting their edge over conventional therapies. For instance, research in Iran has demonstrated that green-synthesized AgNPs effectively inhibit biofilm formation in clinical *P. aeruginosa* isolates by downregulating QS genes like *lasI*, leading to notable reductions in virulence factors [10]. Globally, investigations have shown CuONPs disrupting EPS production and suppressing genes such as *pelA*, leading to weakened biofilm structures in biofilm-forming strains [9, 12]. Comparative analyses further reveal that AgNPs often outperform CuONPs in gene expression inhibition, though both exhibit low toxicity at therapeutic doses, paving the way for safer, targeted interventions [10-12].

While the antibacterial traits of AgNPs and CuONPs are well documented, traditional chemical synthesis methods frequently rely on hazardous solvents, raising safety and environmental concerns for clinical use. Green synthesis addresses this by harnessing plant extracts as natural reducing and stabilizing agents, yielding eco-friendly, biocompatible particles [13, 14]. *Mesembryanthemum nodiflorum* L., a resilient, fleshy-leaved succulent halophyte characterized by its cylindrical, papillose leaves and small white flowers, is plentiful in arid regions like Iran and boasts a wealth of antioxidant phenolics and flavonoids. These phytochemicals excel in this role, potentially boosting nanoparticle stability and mitigating mammalian cell toxicity [15].

Despite these advancements, gaps remain in the current literature. Most studies evaluate nanoparticles individually without a direct comparative framework on their molecular mechanisms, particularly concerning QS gene suppression [16]. Furthermore, there is a lack of head-to-head comparative studies evaluating two distinct green-synthesized monometallic nanoparticle systems against MDR strains from specific locales such as Zahedan, where local antibiotic practices and arid environmental conditions may foster unique resistance profiles. Therefore, establishing whether nanoparticle physical size or intrinsic material chemistry dictates the anti-biofilm efficacy remains a critical research gap.

This study seeks to fill these voids by biosynthesizing stable AgNPs and CuONPs utilizing *M. nodiflorum*,

characterizing their physicochemical properties, and evaluating their comparative impact on biofilm inhibition and *lasI/pelA* downregulation in clinical *P. aeruginosa* urinary isolates collected in 2025. The specific aim of the current study is to provide a detailed comparative assessment of these two distinct nanoparticles to determine if intrinsic biochemical properties override physical particle size in disrupting the genetic and structural foundations of robust biofilms.

2. Experimental

2.1. Isolation and Identification of Bacterial Strains

From January to December 2025, a total of 103 clinical isolates of *Pseudomonas aeruginosa* were gathered from patients diagnosed with UTIs at Imam Ali, Social Security, and Khatam hospitals in Zahedan, Iran. Initial identification relied on standard biochemical tests, including Gram staining, oxidase activity, and pigment production on selective media [17]. For definitive confirmation, the 16S rRNA gene was amplified using universal primers, followed by sequencing and analysis via the BLAST tool, which verified 89 isolates as *P. aeruginosa* [18].

2.2. Preparation of Plant Extract

Fresh aerial parts of *Mesembryanthemum nodiflorum*, collected from local arid regions in Zahedan, were thoroughly washed with deionized water and shade-dried at room temperature (25–30 °C) to preserve bioactive compounds. The plant samples were taxonomically identified and authenticated by a botanist using standard regional flora references (Flora Iranica), and a voucher specimen was deposited in the institutional herbarium of the University of Sistan and Baluchestan for future reference. The dried material was pulverized using an electric grinder. Subsequently, 10 g of the powder was boiled in 100 mL of deionized water for 30 minutes to yield the aqueous extract [19]. The mixture was filtered through Whatman No. 1 filter paper and centrifuged at 10,000 rpm for 10 minutes to remove structural debris. The clear supernatant was stored at 4 °C for subsequent use.

2.3. Biosynthesis of Nanoparticles

Silver and copper oxide nanoparticles were biosynthesized using a one-pot green method,

with the *M. nodiflorum* extract serving as both the reducing and capping agent [20]. For AgNPs, 10 mL of the filtered extract was mixed with 90 mL of 1 mM silver nitrate (AgNO_3) solution and incubated in the dark at 25 °C until a dark brown color appeared, signaling the reduction of Ag^+ ions. For CuONPs, 10 mL of the extract was added dropwise to 90 mL of 1 mM aqueous copper (II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) solution under continuous stirring at 60 °C for 2 h. The gradual appearance of a dark greenish-brown color indicated the formation of a copper–phytochemical complex and its subsequent conversion to CuO nanoparticles [21]. Both colloidal suspensions were centrifuged at 12,000 rpm for 20 minutes, and the resulting pellets were washed thrice with deionized water before oven-drying at 50 °C to obtain the final nanoparticle powders.

2.4. Nanoparticle Characterization

The synthesized nanoparticles were characterized using standard techniques to confirm their formation, size and morphology. UV-Vis spectrophotometry (Jenway 6715 spectrometer, UK), was employed to detect surface plasmon resonance (SPR) peaks, scanning from 300 to 800 nm using a 1 cm quartz cuvette [22]. Transmission electron microscopy (TEM; Philips CM120, 100 kV) operating at an accelerating voltage of 100 kV, provided high-resolution images of particle shape and size distribution. Samples were prepared by drop-casting ultrasonicated and diluted suspensions onto carbon-coated copper grids and allowing them to air-dry at room temperature prior to imaging. X-ray diffraction (XRD; D8-Advance, Bruker, Germany) was utilized to analyze crystallinity, recording patterns from $2\theta = 10^\circ$ to 80° using Cu-K α radiation to identify lattice planes [23].

2.5. Biofilm Profiling

All 89 confirmed isolates were screened for biofilm formation capabilities using the standard crystal violet microtiter plate assay [24]. Overnight bacterial cultures in tryptic soy broth (TSB) were diluted to a 0.5 McFarland standard, inoculated into 96-well flat-bottom plates, and incubated at 37 °C for 24 hours. The wells were then washed with phosphate-buffered saline (PBS), stained with 0.1% crystal violet, and solubilized in 30% acetic acid for optical density (OD) measurement at 570 nm. Strains were classified based on calculated cutoff values (OD_c) (mean OD of negative control + 3× standard deviation), and two

strong biofilm producers (PA-Q6 and PA-Q41) were selected for downstream molecular and anti-biofilm studies.

2.6. Antimicrobial and Anti-biofilm Assays

Minimum inhibitory concentrations (MICs) were determined via the broth microdilution method in Mueller-Hinton broth, strictly adhering to CLSI guidelines [25]. For anti-biofilm evaluation, the selected strong-producing strains were treated with sub-inhibitory doses ($0.5 \times \text{MIC}$) of the synthesized nanoparticles in 96-well plates. Biofilm reduction was quantified as the percentage decrease in OD₅₇₀ relative to the untreated control groups after a 24-hour incubation period. The minimum bactericidal concentration (MBC) was determined by sub-culturing 10 μL from each well showing no visible growth onto Mueller–Hinton agar plates; the lowest concentration yielding $\geq 99.9\%$ killing (no colony growth after 24 h at 37°C) was recorded as the MBC [24].

2.7. Biocompatibility Assay

In vitro cytotoxicity was assessed on the HEK293 human embryonic kidney cell line using the MTT assay [26]. Cells were seeded at a density of 1×10^4 cells/well and exposed to varying concentrations of the nanoparticles (up to $200 \mu\text{g/mL}$) for 24 hours. Cell viability was measured colorimetrically at 570 nm following MTT reagent incubation.

2.8. Gene Expression Quantification

Total RNA was extracted from both treated ($0.5 \times \text{MIC}$) and control bacterial cultures utilizing a commercial RNA extraction kit (RNeasy Mini Kit) [27]. Following reverse transcription to synthesize cDNA (Thermo Fisher RevertAid First Strand cDNA Synthesis Kit), real-time PCR (utilizing SYBR Green chemistry) (Applied Biosystems) was performed to quantify the relative expression levels of the *lasI* and *pelA* virulence genes. The *rpoD* housekeeping gene

served as the internal control, and relative fold changes were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method [28].

2.9. Statistical Analysis

All quantitative data were analyzed using GraphPad Prism software (version 9.0). Results are presented as the mean $\pm$ standard deviation (SD) derived from three independent, biological replicates. Differences between experimental groups were evaluated using a one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. Statistical significance was defined as $P < 0.05$ [29].

3. Results and Discussion

3.1. Isolate Characterization and Prevalence

Our screening of 89 confirmed *P. aeruginosa* isolates revealed a substantial prevalence of biofilm formation (Table 1). Overall, 64.0% of isolates were biofilm producers, including 16.8% classified as strong biofilm producers. This finding is consistent with the recognized clinical importance of biofilm-associated persistence in *P. aeruginosa* urinary isolates [1,17]. The proportion of strong biofilm-forming isolates in the present cohort underscores the need for locally relevant anti-biofilm strategies. However, direct comparisons with studies from other regions should be interpreted cautiously because biofilm assays, cutoff definitions, patient populations, and isolate sources differ across studies [30].

3.2. Nanoparticle Synthesis and Characterization

The use of *M. nodiflorum* extract successfully mediated the green synthesis of AgNPs and CuONPs. UV–Vis spectroscopy showed distinct absorption bands at approximately 420 nm for AgNPs and 580 nm for CuONPs (Fig. 1a). TEM images demonstrated predominantly spherical AgNPs with an average diameter of approximately 45 nm, whereas CuONPs were smaller, with an

Table 1. Distribution of biofilm formation capabilities among *P. aeruginosa* isolates (n=89).

Biofilm Category	Optical Density (OD 570nm)	Number of Isolates	Percentage (%)
Strong Producer	$\text{OD} > 4\text{ODc}$	15	16.8
Moderate Producer	$2\text{ODc} < \text{OD} \leq 4\text{ODc}$	22	24.7
Weak Producer	$\text{ODc} < \text{OD} \leq 2\text{ODc}$	20	22.5
Non-Producer	$\text{OD} \leq \text{ODc}$	32	36.0

ODc = cutoff optical density (mean OD of negative control + $3 \times$ standard deviation).

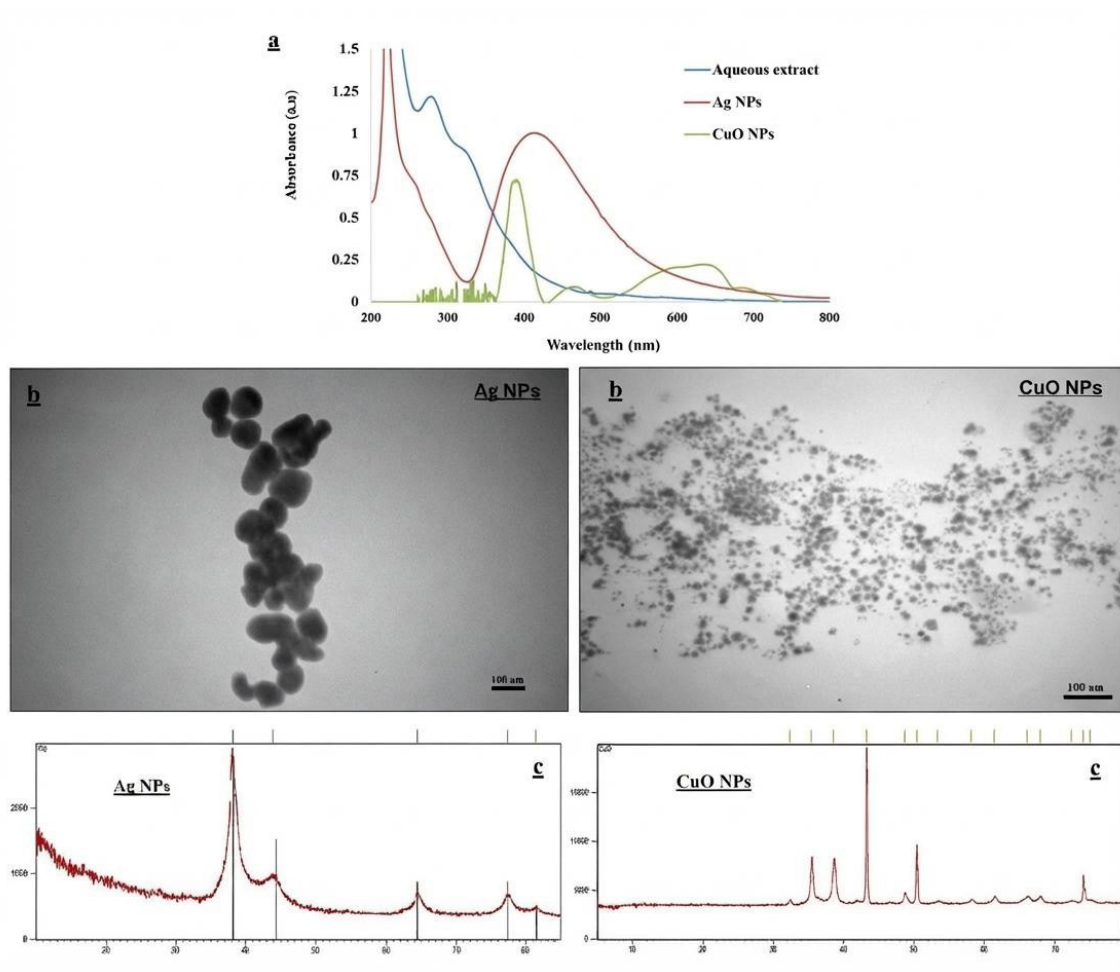


Fig. 1. Characterization of green-synthesized silver and copper oxide nanoparticles: (a) UV–Vis absorption spectra showing surface plasmon resonance bands; (b) transmission electron micrographs illustrating particle morphology and size distribution; and (c) X-ray diffraction patterns.

average diameter of approximately 15 nm (Fig. 1b). XRD patterns confirmed the crystalline nature of both nanomaterials (Fig. 1c). AgNPs exhibited characteristic reflections at $2\theta \approx 38.1^\circ$, 44.3° , 64.5° , and 77.4° , corresponding to the (111), (200), (220), and (311) planes of face-centred cubic metallic silver (JCPDS No. 04-0783). CuONPs displayed peaks at $2\theta \approx 32.5^\circ$, 35.5° , 38.7° , 48.7° , 53.4° , and 58.3° , corresponding to the (110), (-111), (111), (-202), (020), and (202) planes of monoclinic CuO (tenorite; JCPDS No. 48-1548). The average crystallite sizes estimated using the Debye–Scherrer equation were approximately 41 nm for AgNPs and 13 nm for CuONPs, in agreement with the TEM results. The absence of marked agglomeration in TEM images may reflect surface passivation by phytochemicals present in the plant extract, including phenolic and flavonoid compounds. However, this interpretation

remains tentative because FTIR spectroscopy and zeta-potential measurements were not performed [15, 31, 32].

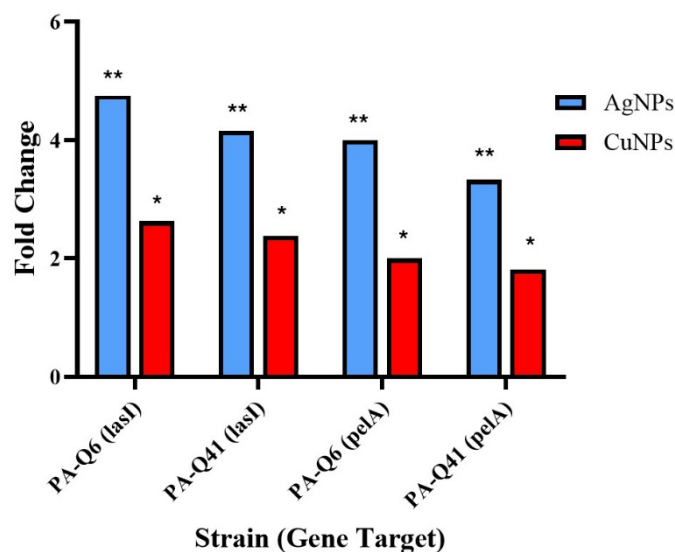
3.3. Antimicrobial and Antibiofilm Efficacy

AgNPs demonstrated superior antibacterial and antibiofilm activity, with MIC values of 6.25–12.5 $\mu\text{g/mL}$ and biofilm reduction rates of 73–75%, compared with CuONPs, which showed MIC values of 25–50 $\mu\text{g/mL}$ and biofilm reduction rates of 55–60% (Table 2). This result is noteworthy because CuONPs were smaller than AgNPs; however, the larger AgNPs showed greater biological activity. Thus, under the tested conditions, antibacterial efficacy appeared to depend more strongly on the intrinsic chemistry of the nanomaterial than on particle size alone. Silver nanoparticles can damage bacterial membranes, generate oxidative stress, release Ag^+

Table 2. Comparative antimicrobial (MIC, MBC) and antibiofilm activity of green-synthesized AgNPs and CuONPs against strong biofilm-producing *P. aeruginosa* strains.

Strain	Treatment	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)	Biofilm Inhibition at $0.5\times\text{MIC}$ (%)
PA- Q6	AgNPs	12.5	25.0	75.4 ± 3.2
	CuONPs	50.0	100.0	60.1 ± 2.8
PA- Q41	AgNPs	6.25	12.5	72.8 ± 4.1
	CuONPs	25.0	50.0	55.3 ± 3.5

*Values represent mean $\pm$ SD from triplicate experiments.



* $P < 0.05$ and ** $P < 0.001$ indicate significance compared to the untreated control group.

Fig. 2. Comparative fold changes of *lasI* and *pelA* gene expression in *P. aeruginosa* strains (PA-Q6 and PA-Q41) treated with sub-MIC doses of AgNPs and CuONPs.

ions, and interact with proteins, thiol groups, and nucleic acids, collectively disrupting essential cellular processes [6–8]. The antibiofilm activity of AgNPs observed in this study is consistent with reports describing the inhibitory effects of phytosynthesized AgNPs against biofilm-forming *P. aeruginosa* [10, 11, 33]. The activity of CuONPs may likewise be related to Cu^{2+} release, membrane disruption, oxidative stress, and interactions with the biofilm matrix [9, 12]. Nevertheless, direct comparisons among studies should be made cautiously because nanoparticle composition, phytochemical capping, particle size, exposure time, bacterial strain, and biofilm model differ substantially.

3.4. Differential Gene Expression Analysis

Real-time PCR analysis confirmed that AgNPs produced substantial downregulation of *lasI* (4.1- to 4.7-fold) and *pelA* (3.3- to 4.0-fold), whereas CuONPs produced a more modest reduction of

approximately 2.5-fold (Fig. 2). These findings suggest that AgNPs were more effective than CuONPs at disrupting QS-associated regulation and EPS-related biofilm architecture under the tested conditions. The *las* system plays a central role in the hierarchical QS network of *P. aeruginosa*, whereas *Pel* polysaccharide contributes to biofilm structure and persistence [3, 4, 36]. However, because only two strong biofilm-producing isolates were evaluated, the generalizability of these molecular findings should be confirmed using a larger and genetically diverse isolate collection.

3.5. Cytotoxicity and Biocompatibility

The MTT assay on HEK293 cells (Fig. 3) revealed a favourable safety profile, with cell viability remaining above 85% at therapeutically relevant concentrations ($\leq 50 \mu\text{g/mL}$) for both nanoparticles. A concentration-dependent decline in viability became apparent only at the highest tested concentration (200 $\mu\text{g/mL}$), which is 4- to 32-fold greater than

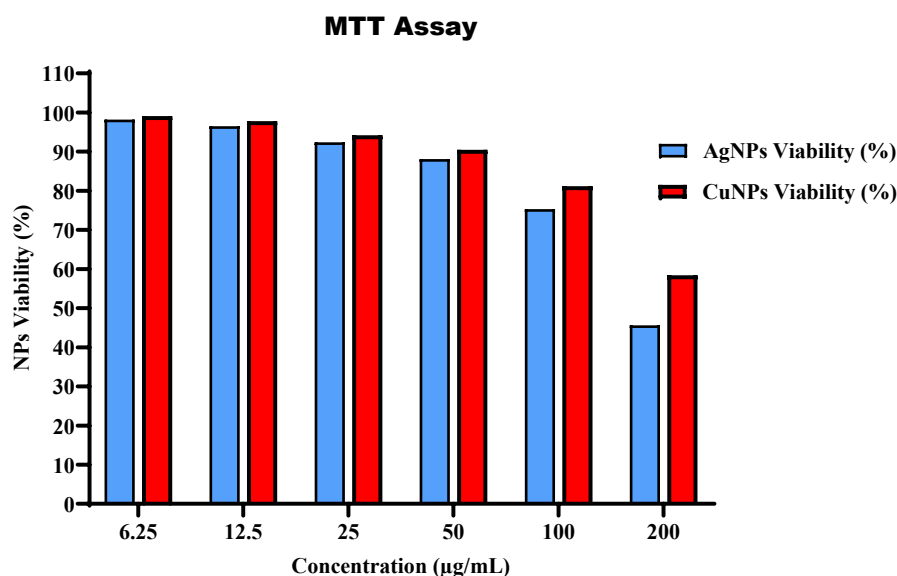


Fig. 3. In vitro cytotoxicity evaluation of AgNPs and CuONPs on HEK293 cells via the MTT Assay.

the determined MIC values. The observed viability of HEK293 cells at concentrations relevant to the antibacterial activity provides preliminary evidence of in vitro cytocompatibility. However, additional testing using multiple relevant cell types, prolonged exposure periods, hemocompatibility assays, and in vivo models is required before clinical translation can be considered [7].

3.6. Study Limitations

Several limitations should be acknowledged. First, colloidal characterization was restricted to UV-Vis, XRD and electron microscopy; dynamic light scattering, zeta-potential and FTIR analyses were not performed, and therefore the hydrodynamic size distribution and the precise nature of the phytochemical capping layer could not be established. Second, gene expression and anti-biofilm assays were limited to two representative strong biofilm-producing isolates, which may not capture the full genotypic diversity of the local *P. aeruginosa* population. Third, biofilm inhibition was evaluated during biofilm formation in a static microtiter-plate model; mature preformed biofilms and dynamic flow-cell systems that better mimic catheter-associated conditions were not evaluated. Fourth, cytotoxicity was assessed on a single cell line over a 24-h exposure, so long-term and in vivo biocompatibility remain to be determined.

4. Conclusion

In conclusion, this study successfully demonstrated the green synthesis of stable silver and copper oxide nanoparticles using *Mesembryanthemum nodiflorum* extract. A direct comparison revealed that despite their larger physical size, AgNPs exhibited remarkably superior antimicrobial, antibiofilm, and gene-suppressive capabilities against regional strong biofilm-producing *P. aeruginosa* isolates compared to CuONPs. These findings strongly indicate that the intrinsic material properties and biochemical reactivity of the metallic core override particle size in determining therapeutic efficacy. Given their excellent biocompatibility on mammalian cells, these green-synthesized AgNPs, represent promising candidates for further development as antimicrobial surface-coating materials, particularly for functionalizing urinary catheters to combat resilient biofilm-associated infections [8, 35].

Abbreviations:

MDR: Multidrug-resistant; UTIs: Urinary Tract Infections; AgNPs: Silver Nanoparticles; CuONPs: Copper Oxide Nanoparticles; SPR: Surface Plasmon Resonance; TEM: Transmission Electron Microscopy; XRD: X-ray Diffraction; MIC: Minimum Inhibitory Concentration; QS: Quorum Sensing; EPS: Extracellular Polymeric Substance; OD: Optical Density.

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CONFLICT OF INTEREST

The author declare has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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