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Antibacterial and Post-biofilm Effects of Biosynthesized Copper Oxide Nanoparticles Using *Pseudomonas aeruginosa* Pyocyanin

Amna Emad Hamed*, Hassan Majeed Rasheed
Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

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Abstract

The increasing resistance of bacteria to conventional antibiotics has driven the search for alternative antimicrobial agents, such as metal nanoparticles. Copper oxide nanoparticles exhibit strong antibacterial and antibiofilm properties due to their high surface-to-volume ratio and reactive oxygen species generation. The biosynthesis of nanoparticles (NPs) using microorganisms offers an eco-friendly and cost-effective approach compared to chemical methods. *Pseudomonas aeruginosa*, a pathogenic bacterium, produces pyocyanin, a redox-active pigment with metal-reducing properties, which can facilitate the synthesis of CuO NPs. Pyocyanin acts as a stabilizing and reducing agent, enabling the formation of NPs with enhanced biocompatibility and antimicrobial efficacy. The research involves the isolation of *P. aeruginosa*, extraction of pyocyanin, and subsequent biosynthesis of copper oxide nanoparticles. The findings are expected to provide insights into novel therapeutic strategies for combating resistant bacterial strains, addressing a critical public health need. Samples were collected from Baghdad-Al-Yarmouk Teaching Hospital, Ghazi Al-Hariry Hospital, and Al-Imamein Al-Khadumein Teaching. The copper oxide nanoparticles were synthesized from *P. aeruginosa* using the biological method and characterized using Atomic Force Microscopy (AFM), Field Emission Scanning Electron Microscopy (FE-SEM), and Energy-dispersive X-ray spectroscopy (EDX). These confirmed that it is a nanoparticle. The antimicrobial efficiency of CuO NPs was determined for Six multidrug-resistant bacterial isolates using a well diffusion assay (WDA). The maximum inhibition zone of *P. aeruginosa* was 34 mm at a concentration of 500mg/ml of CuO NPs. CuO NPs showed consistent inhibition on all strains. This study aims to assess the effectiveness of copper nanoparticles (CuO NPs) biosynthesized using pyocyanin, as an antibacterial agent and antibiofilm after formation against *P. aeruginosa*, there is a proportional relationship between the concentration of nanoparticles and their effect on non-planktonic cells within the biofilm texture.

Keywords: *Pseudomonas aeruginosa*, Copper Oxide nanoparticles, Antibiofilm, Pyocyanin, Antibacterial

التأثيرات المضادة للبكتيريا وما بعد تشكل الأغشية الحيوية للجسيمات النانوية لأكسيد النحاس المُنتجة حيوياً باستخدام صبغة السيانين الزائفة

امنة عماد حامد*, حسن مجيد رشيد

قسم علوم الحياة, كلية العلوم, جامعة بغداد, بغداد, العراق

*Email: aamna.emad2302@sc.uobaghdad.edu.iq

الخلاصة

أدى التزايد المستمر في مقاومة البكتيريا للمضادات الحيوية التقليدية إلى تعزيز البحث عن عوامل مضادة للميكروبات بديلة، مثل الجسيمات النانوية المعدنية .تتميز جسيمات أكسيد النحاس النانوية بخصائص مضادة للبكتيريا والغشاء الحيوي الحيوي قوية، وذلك بسبب نسبة مساحة السطح إلى الحجم المرتفعة وقدرتها على توليد أنواع الأكسجين التفاعلية.تقدم الطرق الحيوية لتصنيع الجسيمات النانوية باستخدام الكائنات الدقيقة نهجاً صديقاً للبيئة واقتصادياً مقارنة بالطرق الكيميائية التقليدية. تنتج البكتيريا الممرضة الزائفة الزنجارية صبغة البيوسيانين ، وهي صبغة نشطة أكسدة–اختزال تمتلك خصائص اختزال للمعادن، مما يمكنها من تسهيل تخليق جسيمات أكسيد النحاس النانوية. يعمل البيوسيانين كعامل مختزل ومثبت في نفس الوقت، مما يمكنه من تكوين جسيمات نانوية تتمتع بدرجة عالية من التوافق الحيوي وفعالية مضادة للميكروبات.البحث يتضمن زراعة عزلات من بكتيريا الزائفة الزنجارية، واستخلاص البيوسيانين من البكتريا المعزولة، ومن ثم التخليق الحيوي لجسيمات أكسيد النحاس النانوية.من المتوقع أن توفر النتائج رؤى حول استراتيجيات علاجية جديدة لمكافحة السلالات البكتيرية المقاومة، مما يعالج حاجة ملحة في الصحة العامة. تم جمع العينات من مستشفى بغداد اليرموك التعليمي، ومستشفى غازي الحريري، ومستشفى الإماميين الكاظميين التعليمي، تم تخليق جسيمات أكسيد النحاس النانوية باستخدام بكتيريا الزائفة الزنجارية باستخدام الطريقة الحيوية وتم تعريفها باستخدام مجهر القوة الذرية ، مجهر إلكتروني ماسح ذو انبعاث ميداني وطيف الأشعة السينية المشتتة للطاقة .تم تحديد كفاءة مضاد الميكروبات لجسيمات أكسيد النحاس النانوية ضد ست عزلات بكتيرية مقاومة للأدوية المتعددة باستخدام اختبار الانتشار في الحفر تم تسجيل أعلى منطقة تثبيط لبكتيريا الزائفة الزنجارية بقطر 34 ملم عند تركيز 500 ملغ/مل من جسيمات أكسيد النحاس النانوية. أظهرت جسيمات أكسيد النحاس النانوية تثبيطًا ثابتًا على جميع السلالات. تهدف هذه الدراسة الى تقييم فعالية الجسيمات النانوية النحاسية التي تم تخليقها بيولوجيا بأستخدام المستخلص كعامل مضاد للبكتيريا ومضاد لتكوين الاغشية الحيوية بعد التكوين ضد بكتريا الزائفة الزنجارية .توجد علاقة تناسبية بين تركيز الجسيمات النانوية وتأثيرها على الخلايا غير العوالقية داخل نسيج الاغشية الحيوية .

1. Introduction

Bacterial infections have a long history, and records of infectious disorders date back to 3000 B.C. Over the period of several centuries, several notable pandemics brought on by bacteria have been reported. Despite recent advancements in medical research and therapies, infectious diseases continue to rank among the world's leading causes of death in the twenty-first century [1]. Antimicrobial resistance claims the lives of 700.000 people worldwide and is expected to claim 10.000.000 lives by 2050 [2].

Pseudomonas aeruginosa is a Gram-negative bacterium that is known to cause severe infections in humans, particularly in individuals with weakened immune systems or compromised barriers [3,4]. This opportunistic pathogen is responsible for a wide range of infections, including pneumonia, urinary tract infections, and sepsis. *P. aeruginosa* 's most distinctive feature is the production of soluble pyocyanin pigment, a water-soluble blue-green phenazine chemical with numerous applications [5]. In addition, the pyocyanin pigment is a significant virulence component. It is produced during biofilm development and plays an important role in both acute and chronic bronchitis. It is also an antibiotic chemical that acts as an oxidizing agent, allowing bacteria to grow without oxygen [6].

Nanotechnology is one of the most active areas of research in materials science [7]. Nanotechnology is a growing multi-disciplinary approach centered on producing molecules in the nanoscale size range. One innovative approach that shows promise for addressing the challenges with chemical processes is green synthesis, or the utilization of extracts from natural products [8]. Due to their affordability, environmental friendliness, and lack of

requirement for particular conditions, biological methods are preferred [9]. Copper nanoparticles (NPs) have been seen as a competitive substitute for gold and silver NPs due to lower cost and higher natural abundance [10,11]. One of the strongest effects of metal nanoparticles against bacteria is the ability to attach to the cell membrane and cell wall by electrostatic interaction and disrupt them. In addition, ions and ion channels can be disrupted by their association with them. Through electrostatic interactions, these NPs can break DNA double strands, obstruct ribosome assembly, and inhibit enzyme activity. Reactive oxygen species (ROS), which can harm proteins, lipids, and DNA, are also known to increase in concentration when metal NPs are present [12].

This study holds particular significance for Iraq, where the escalating prevalence of antibiotic-resistant bacterial infections, particularly those caused by *P. aeruginosa*, poses a serious challenge in clinical and hospital settings. The biosynthesis of copper oxide nanoparticles (CuO NPs) using pyocyanin derived from *P. aeruginosa* presents a sustainable and innovative solution, offering a cost-effective, locally producible alternative for combating drug-resistant infections and recalcitrant biofilms, which are major contributors to patient complications. Given Iraq's pressing need for advanced yet economically viable medical technologies, this eco-friendly synthesis method aligns with national healthcare priorities by reducing dependence on expensive imported pharmaceuticals. Furthermore, this research could catalyze the development of a domestic nanotechnology-based pharmaceutical industry, leveraging locally available microbial resources. Such advancements would strengthen Iraq's healthcare infrastructure while addressing critical issues related to antimicrobial resistance (AMR). By integrating green nanotechnology with infection control strategies, this study contributes to global AMR mitigation efforts while specifically addressing Iraq's unique healthcare challenges, potentially improving treatment outcomes and reducing the economic burden associated with resistant infections [13].

2. Material and Method

The study followed an in vitro antimicrobial susceptibility testing protocol based on the CLSI guidelines. The steps of the work-study are illustrated in Figure 1.

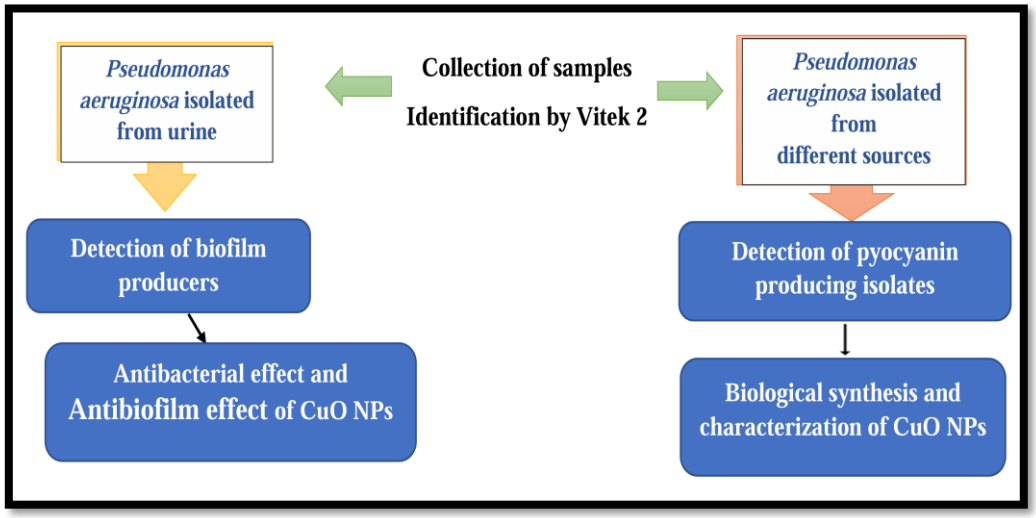


Figure 1: Study design chart

2.1 Collection, isolation, and identification of bacteria

During the period from November 2023 to April 2024, 228 burn wound swabs and urine specimens were collected from three Baghdad hospitals, including Al-Yarmouk Teaching Hospital, Ghazi Al-Hariry Hospital, and Al-Imamein Al-Khadumein Teaching Hospital. All

specimens were cultured on MacConkey agar as a selective medium of Gram-negative bacteria, and their ability to lactose fermenter, which is recognized as a differential character of the Enterobacteriaceae members, then cultured on nutrient agar to show their distinguishing pigments. After that, the isolates were screened with cetrimide agar, which is used as a selective media for *P. aeruginosa*. The isolates were incubated for 24-48 hr. at 37˚C. Isolates were confirmed by the VITEK-2 compact system.

2.2 Detection of pyocyanin-producing isolate

Pyocyanin-producing isolates were screened by culturing on nutrient agar in standard Petri dishes (90 mm diameter). After 48–72 hours of incubation at 37°C, distinct blue-green pigmentation (indicative of pyocyanin production) was observed. Pyocyanin, a phenazine-derived secondary metabolite synthesized by *P. aeruginosa* during the late stationary phase, serves as a key phenotypic marker due to its characteristic chromogenesis [14]. Isolates exhibiting the most intense pigmentation were selected for downstream analysis. Preliminary screening identified 14 pyocyanin-producing isolates (out of the total tested cohort).

2.3 pyocyanin production

This method was performed according to Elbargisy [15]. Briefly, *Pseudomonas aeruginosa* isolates were cultured on nutrient agar and incubated at 37°C for 72 hours. Colonies producing pyocyanin (evident by blue-green pigmentation) were selected and subcultured in nutrient broth, followed by incubation at 37°C for 3–7 days. Pyocyanin production was confirmed by the appearance of the blue-green color either directly or after flask agitation.

2.3 Extraction of pyocyanin

Extraction of pyocyanin was done by centrifuging the nutrient broth that contained blue-green color (pyocyanin) at 5000 rpm for 15 minutes. Then, the supernatant was taken and poured into a separating funnel, and chloroform absolute was added at a 2:1 ratio (200 ml chloroform for every 100 ml supernatant). The mixture was shaken and kept undisturbed for 5 minutes. Directly collect the blue-colored chloroform layer without drying. Subsequently, the pyocyanin solution was analyzed using a UV-visible spectrophotometer (Shimadzu, Japan). Ultraviolet (UV) spectroscopy was employed to determine the optical absorbance spectra of the pyocyanin solution, as shown in Figure 2.

(a) **(b)** **(c)**

Figure 2: a) Nutrient broth before incubation, b) Nutrient broth with *Pseudomonas aeruginosa* after 5 days, c) The chloroform containing pyocyanin.

2.4 Synthesis of copper oxide nanoparticles (CuO NPs) from pyocyanin pigment

The nanoparticle preparation involves dissolving 15g copper chloride and pyocyanin in 150 ml chloroform, followed by 24-hour shaking to facilitate nanoparticle formation through reduction and stabilization processes. Centrifugation at 5000rpm for 15 minutes serves the critical function of separating the formed nanoparticles (pellet) from reaction byproducts and excess reagents (supernatant), with the supernatant being discarded to eliminate impurities. Subsequent washing with 98% ethanol removes residual contaminants adsorbed on nanoparticle surfaces, and a second centrifugation (5000rpm, 10 minutes) ensures complete purification. The final nanoparticle pellet is dried at 37°C to obtain a stable powder for characterization. This centrifugation-based purification is essential for obtaining monodisperse nanoparticles free from unreacted precursors, with the pellet constituting the desired product and all supernatants being discarded as waste throughout the process.

2.5 Characterization of copper oxide nanoparticles (CuO NPs)

Various techniques were employed to characterize CuO nanoparticles, including colour change, Atomic Force Microscopy (AFM), Energy Dispersive X-ray microanalysis (EDX), UV-Vis. spectrophotometer, and Field Emission Scanning Electron Microscopes (FE-SEM) [15].

2.6 Detection of biofilm using Congo red agar

The detection medium was composed of Brain-Heart Infusion (BHI) broth 37g, Sucrose 50g, and Agar-agar 15g. They were melted in 900 ml of distilled water. Sterilized by autoclave at 121°C /15 pounds/inche² for 15 minutes, cooling to 55°C, and then the volume was completed to 1000 ml by Congo red solution, then poured into sterile Petri dishes. The plates of Congo red agar were streaked with overnight culture isolates and incubated at 37ºC for 24 hours. The formation of a black-pigmented colony indicated a positive result [16].

2.7 Preparation of copper oxide nanoparticles concentrations

The antimicrobial evaluation employed a two-phase dilution approach: initial screening using two-fold serial dilutions by using deionized distilled water (500, 250, 125, 62.5, 31.25, and 15.63 mg/mL) to establish the bioactive concentration range, followed by precision four-fold dilutions (62.5, 46.88, 35.16, 26.37 and 19.78 mg/mL) within the active spectrum for accurate minimum inhibitory concentration (MIC) determination. This systematic method covered a 256-fold concentration gradient while maintaining analytical precision through triplicate testing and appropriate controls, enabling a comprehensive assessment of the nanoparticles' dose-dependent antibacterial effects [17].

This optimized protocol combines the advantages of broad-range screening with high-resolution MIC determination while maintaining compliance with standardized antimicrobial testing guidelines for nanoparticle suspensions. The methodology proved particularly effective for assessing the dose-dependent antibacterial effects of CuO NPs against *P. aeruginosa* biofilms.

2.8 Antibacterial test of copper oxide nanoparticles

The agar well diffusion technique was used to determine the antibacterial activity of biologically generated CuO NPs against the selected isolate [18]. In this case, a sterile medium, prepared from Müller Hinton agar, was poured into pre-cleaned Petri dishes and allowed to set overnight in the laboratory. The agar medium containing the cultivated test species was then spread using the sterile cotton swab method. The NPs dilutions were introduced into the pre-drilled wells, and the plates were further incubated for 24 hours at 37

ºC. The plates were examined for inhibition zone after incubation. The inhibition zones were then measured using callipers and recorded. The test was repeated three times to ensure reliability [19].

2.9 Detection of post-biofilm effect

The bacterial isolates were sub-cultured in BHI broth and incubated at 37°C for 18 hours. Following incubation, the turbidity of the bacterial suspension was adjusted to match the 0.5 McFarland standard. A series of concentrations of copper oxide nanoparticles (CuO NPs) were prepared from a stock solution. To determine the minimum inhibitory concentration (MIC), a 96-well polystyrene microtiter plate was utilized, with two technical controls included: the first consisting of BHI broth inoculated with bacterial isolates and the second containing only sterile BHI broth. A 100 µL aliquot of the standardized inoculum of *P. aeruginosa* isolates was added to each well, and the plate was incubated at 37°C for 48 hours. After the incubation period, 50 µL of sterile BHI broth was added to each well. Subsequently, 50 µL of the serially diluted CuO NPs was added to each well, bringing the final volume in each well to 200 µL. The MIC values were determined after an additional 24 hours of incubation at 37°C and were defined as the lowest concentration of CuO NPs that completely inhibited bacterial growth. Finally, the absorbance of each well was measured at 630 nm using an ELISA reader.

2.10 Statistical analysis

The Statistical Packages of Social Sciences-SPSS (2019) [20] program was used to detect the effect of different groups in study parameters. The least significant difference and T-test were used to compare significantly between means, whereas Statistical Test Factorial; ANOVA-LSD. The chi-square test was used to significantly compare between percentages (0.05 and 0.01 probability) in this study.

3. Results and discussion

3.1 Isolation and identification of Pseudomonas aeruginosa

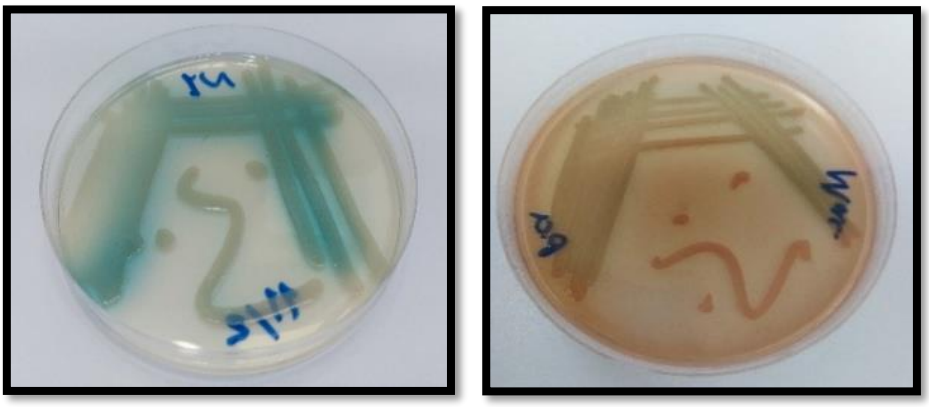
A total of 228 samples were subjected to various examinations. Initially, *P. aeruginosa* cells exhibited a negative Gram reaction and appeared as single bacterial cells or in small pairs, forming rod shapes. The cultural characteristics of *P. aeruginosa* were assessed on MacConkey agar and cetrimide agar. On MacConkey agar, the colonies appeared pale due to *P. aeruginosa* being a non-lactose fermenting bacterium. In contrast, on cetrimide agar, the colonies displayed a greenish-yellow color, indicating the bacterium's ability to thrive in the presence of the toxic cetrimide compound [21], as shown in Figure 3. In total, only 61 isolates were identified as *P. aeruginosa*. Some strains that are isolated from urinary tract infections appear as mucoid due to alginate production [22]. This result has been matched with Kim *et al.,* [21].

Bacterial strain characterization was performed using the VITEK® 2 Compact System (bioMérieux, France) with identification card GN-ID (Ref. 21341) and AST card GN-AST-N202 (Ref. 413221). The analysis yielded identification code 1515179, confirming a multidrug-resistant (MDR) phenotype with resistance to:

- β-lactams (penicillin MIC ≥32 µg/mL, ceftazidime MIC ≥16 µg/mL)
- Fluoroquinolones (ciprofloxacin MIC ≥4 µg/mL)
- Aminoglycosides (gentamicin MIC ≥8 µg/mL)

The selection of multidrug-resistant (MDR) isolates for nanoparticle testing was based on their clinical relevance and utility in evaluating antimicrobial efficacy. These strains, verified by VITEK® 2 with standardized AST profiles, represent worst-case infection scenarios, ensuring stringent assessment of nanoparticle performance. By targeting bacteria resistant to

≥3 antibiotic classes, this approach rigorously tests whether nanoparticles can overcome complex resistance mechanisms. The choice of phenotypically confirmed MDR pathogens enhances translational potential, as successful inhibition would suggest broad applicability against treatment-resistant infections. This methodology balances scientific rigor with clinical significance, providing a robust foundation for developing novel antimicrobial strategies while maintaining reproducibility through automated, standardized testing protocols. Future studies may expand to molecular characterization to further elucidate resistance-nanoparticle interactions.



(a) **(b)**

Figure 3: *Pseudomonas aeruginosa* on a) Nutrient agar, b) MacConkey agar

3.2 Characterization of pyocyanin

3.2.1 UV-Visible analysis

The pyocyanin extracted from *P. aeruginosa* was characterized by scanning a UV-visible spectrophotometer to detect the maximum absorption (Figure 4). Absorbance is measured at 330 nm, a similar result by Ohfuji *et al.,* [24] that indicates the UV-visible of pyocyanin is 318.5 nm.

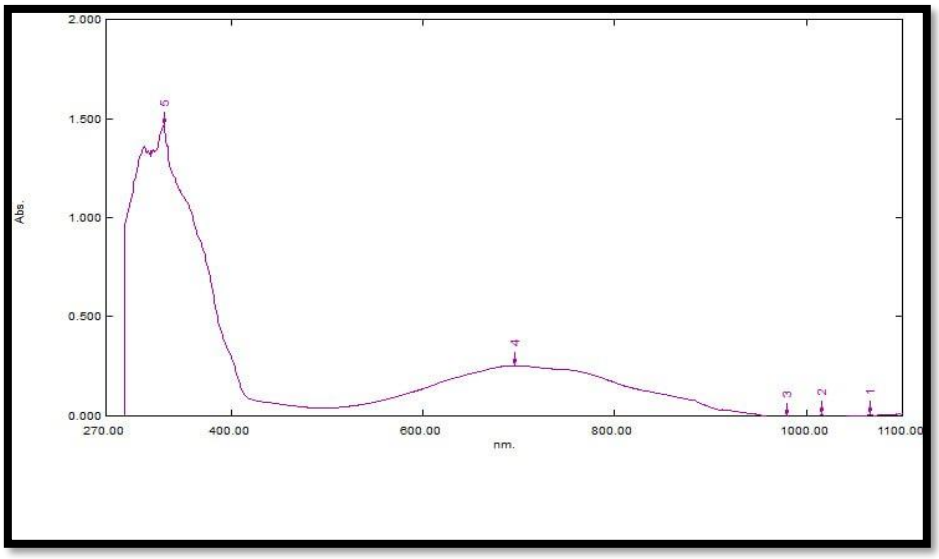


Figure 4: UV-visible of *Pseudomonas aeruginosa* pyocyanin

3.3 Synthesis of CuO NPs using pyocyanin pigment

When salt powder of copper chloride $CuCl_2$ was added to the solution of chloroform containing pyocyanin, the aqueous solution was placed on the ultrasonic, and then it was

transferred to the shaker. It was noticed that the color of the aqueous solution had changed, which indicates that it had turned into copper oxide nanoparticles. It was identified by a color change [25]. It is observed that the color of the solution turned from blue to colorless with a green precipitate. The reaction indicated the formation of copper oxide nanoparticles synthesized from the chloroform containing pyocyanin pigment. Finally, a light green powder of copper nanoparticles was obtained, as shown in Figure 5.

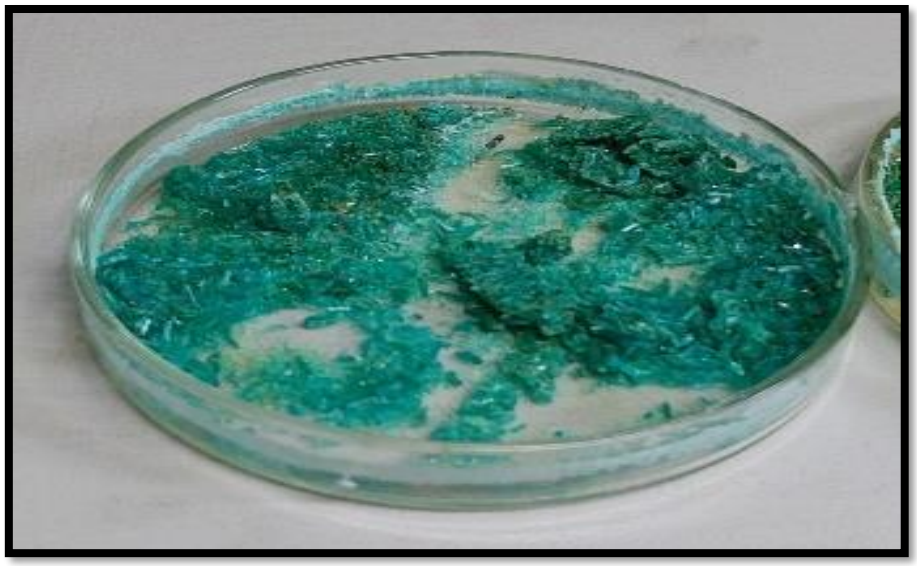


Figure 5: Copper Oxide nanoparticles

3.4 Characterization of CuO NPs

3.4.1 EDX analysis

Energy-dispersive X-ray spectroscopy (EDX) is a technique for analysing solid material composition, providing quantitative and qualitative data on near-surface composition. However, high-quality standards are required for accurate interpretation, and technical constraints may affect resolution [26]. The chemical composition of CuO NPs was determined to have a copper content of 37.10 wt.% and an oxygen content of 12.38 wt.%, as shown in Figure 6.

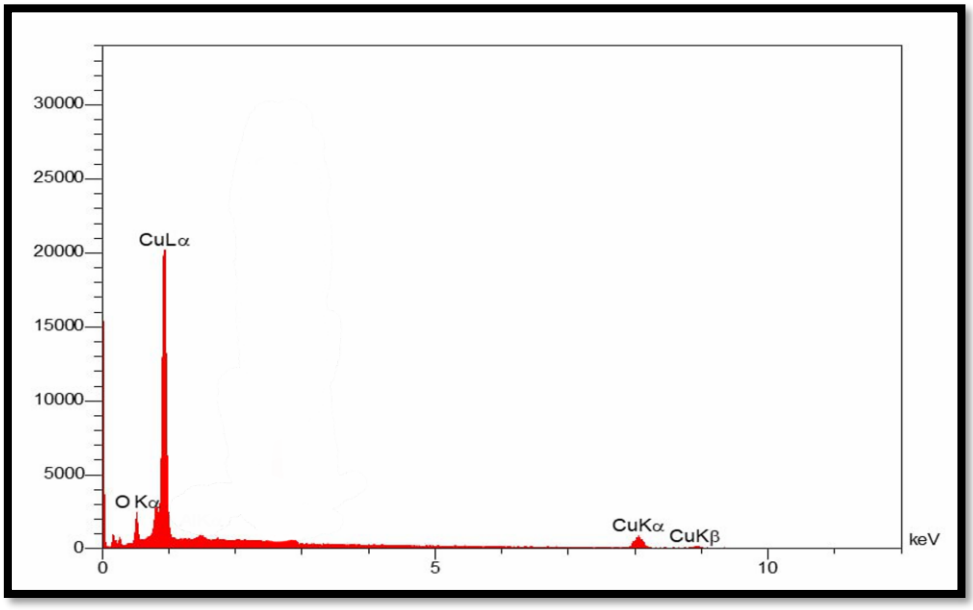


Figure 6: EDX peaks components of Copper Oxide Nanoparticles

3.4.2 AFM analysis

AFM identified the morphology and topography of the surface of nanoparticles, providing a two- and three-dimensional image of the surface of nanoparticles at an atomic level [27]. In nanoscale size, the average particle diameter was calculated, as shown in Figure 7. The particle size distribution histogram of CuO NPs showed that the average diameter was 81.85 nm.

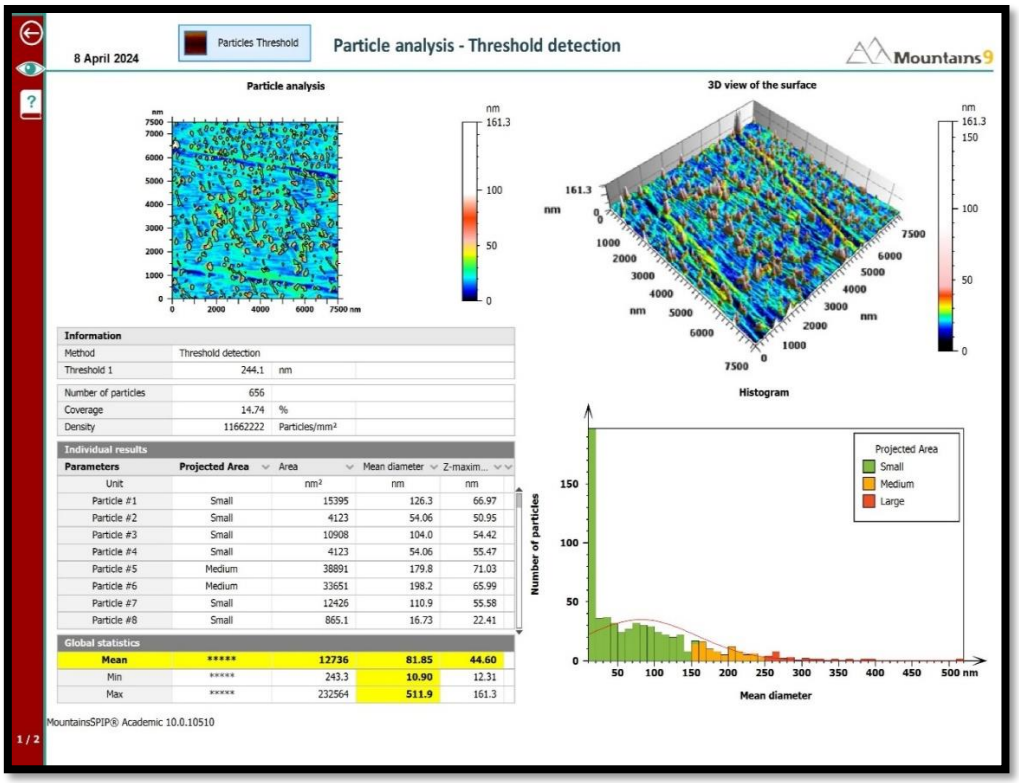


Figure 7 : AFM of CuO NPs

3.4.3 FE-SEM analysis

The SEM images demonstrated that although the produced NPs are primarily spherical, they have a variety of shapes. The effective function of the bioactive components of pyocyanin as capping and stabilizing agents is confirmed by smaller NPs as tiny as 10 nm. Otherwise, NPs would have aggregated to produce larger elongated form NPs [28]. The results recorded near-spherical particles sized 80 nm ,as shown in Figure 8.

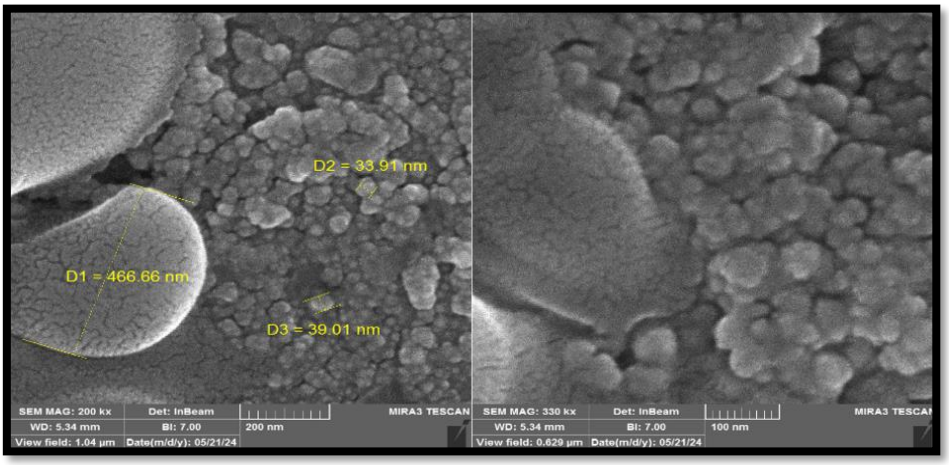


Figure 8: FE-SEM images of copper oxide (CuO) nanoparticles

3.5 Antibacterial activity of biosynthesized CuO NPs

Generally, biosynthesized CuO NPs had a remarkably antibacterial effect against *P. aeruginosa* used in the current study, which was multidrug-resistant, and significant differences between concentrations of CuO NPs were observed.

This laboratory-based study employed a targeted approach using six clinically significant isolates pre-screened with the VITEK® system, all confirming multidrug-resistant (MDR) phenotypes through standardized antimicrobial susceptibility testing (AST). These strains were specifically selected based on dual critical criteria: (I) resistance to ≥3 antimicrobial classes via automated AST, and (II) demonstrated robust biofilm-forming capacity (OD630 > 2.0 in crystal violet assays). While resistance classifications (e.g., MRSA/ESBL) weren't molecularly characterized, the VITEK®-confirmed MDR status ensured clinically relevant, high-risk pathogens were investigated. The limited sample size (n=6) reflected a purposeful sampling strategy to enable in-depth characterization of biofilm dynamics in phenotypically verified MDR strains, balancing the technical feasibility of resource-intensive assays (e.g., qPCR, confocal microscopy) with scientific rigor. Although this design provides strong internal validity for biofilm-MDR correlations, future work would benefit from expanded cohorts and molecular characterization of resistance mechanisms (e.g., β-lactamase gene profiling) to enhance clinical translatability.

The results revealed that the inhibition zone of *P. aeruginosa* increased with the increasing CuO NPs concentration, as shown in Figure 9. The maximum inhibition zone of *P. aeruginosa* was 34 mm, determined at a concentration of 500mg/ml of CuO NPs, while the minimum one was 10 mm and determined at a concentration of 31.25 mg/ml of CuO NPs, as illustrated in Table 1.

Heavy metal ions exert diverse inhibitory effects on bacterial cellular functions. In the case of copper (Cu^{2+}), the primary mechanism of toxicity involves the induction of oxidative stress through Fenton-like reactions, leading to the generation of ROS that damage cellular components [29]. Similarly, copper oxide nanoparticles (CuO NPs) exhibit potent antibacterial activity via multiple pathways. Khatoon *et al.,* demonstrated that exposure to CuO NPs causes severe cytotoxic effects in *Escherichia coli*, including (I) DNA strand breaks, (II) protein carbonylation, and (III) membrane lipid peroxidation all hallmarks of ROS-mediated damage [30].

Notably, CuO NPs have also shown significant antibacterial and antibiofilm activity against *P. aeruginosa*, a clinically relevant pathogen known for its drug resistance. Studies confirm that the antimicrobial efficacy of CuO NPs follows a concentration-dependent trend, with higher nanoparticle concentrations (typically in the range of 50–200 μg/mL) achieving more pronounced bacterial growth inhibition and biofilm disruption. This dose-response relationship underscores their potential for therapeutic or surface-coating applications where controlled release is feasible.

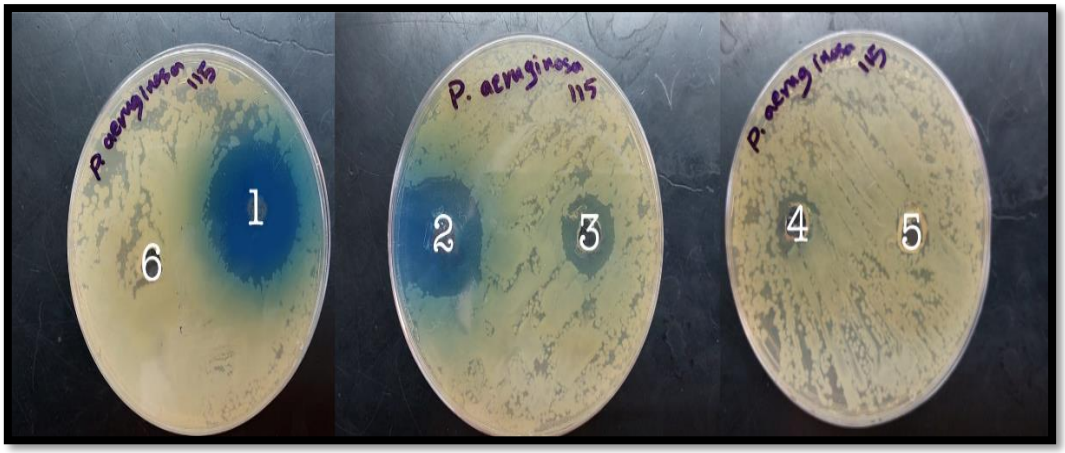


Figure 9 : Effect of different concentrations from copper oxide nanoparticles on *Pseudomonas aeruginosa:* numbers 1 to 6 correspond to CuO NPs concentration, 1) 500 mg/ml, 2) 250 mg/ml, 3) 125 mg/ml,4) 62.5 mg/ml, 5) 31.25 mg/ml, 6) 15.625 mg/ml.

Table 1 : Inhibition zone of *Pseudomonas aeruginosa* by using Copper oxide nanoparticles

Inhibition zone(mm)							Overall means
NPs 15.625 (mg/ml)	NPs 31.25 (mg/ml)	NPs 62.5 (mg/ml)	NPs 125 (mg/ml)	NPs 250 (mg/ml)	NPs 500 (mg/ml)	L.S.D. value	
0	9	10	17	27	34	7.02 *	19.4
* (P≤0.05)							

The data presented in Table 1 demonstrate statistically significant differences ($p < 0.05$) in inhibition zone diameters corresponding to varying concentrations of CuO NPs. A clear dose-dependent response was observed, with higher nanoparticle concentrations producing progressively larger zones of bacterial growth inhibition.

3.6 Post-biofilm activity of biosynthesized CuO NPs

This investigation utilized the same six clinically isolated, VITEK®-confirmed MDR bacterial strains (previously characterized for antibiotic susceptibility and robust biofilm formation, OD630>2.0) to evaluate nanoparticle efficacy against both planktonic and biofilm-embedded states, ensuring direct comparability between conventional antibiotic resistance profiles and nanomaterial performance while maintaining experimental consistency across all antimicrobial assessments.

The results in Table 2 revealed that the addition of nanoparticles had an impact on the bacterial concentration in comparison to the positive control, which was made up only of bacteria and BHI broth. This indicates that the bacteria's growth was inhibited after biofilm formation.

The series of diluted concentrations had a gradual effect, reaching a point where the final concentration did not inhibit any of the bacteria, and they continued to grow to high concentrations compared to the positive control. This indicates that the bacteria were not affected by the biosynthesized copper oxide nanoparticles.

This study aims to assess the effectiveness of copper nanoparticles (CuO NPs) biosynthesized using pyocyanin, as an antibacterial and post-biofilm effective agent against *P. aeruginosa*, there is a proportional relationship between the concentration of nanoparticles and their effect on non-planktonic cells within the biofilm texture.

Moreover, the purpose of this modification is to examine the growth of bacteria in the presence of copper oxide nanoparticles after biofilm formation. Considering that this revised study is the first of its kind. This modification of the assay indicates that the conventional method has been altered to an alternative approach targeting non-planktonic cells after biofilm formation, in contrast to standard testing methods where evaluation occurs prior to biofilm development.

Table 2- Effect of copper oxide nanoparticles after formation biofilm showed by the optical density of each related *Pseudomonas aeruginosa* isolates.

Isolates	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6	L.S.D. value	Average
Negative control (Only BHI broth)	0.052	0.058	0.048	0.044	0.054	0.056	0.031 NS	0.052
Positive control (BHI broth + bacterial isolate)	1.045	1.193	1.148	1.132	1.069	1.198	0.297 NS	1.131
NP Conc.1 (62.5 mg/ml)	1.022	1.149	1.135	1.098	1.104	0.965	0.344 NS	1.079
NP Conc.2 (46.88 mg/ml)	1.199	1.011	0.988	1.018	1.091	1.199	0.361 NS	1.084
NP Conc.3 (35.16 mg/ml)	1.156	1.096	1.065	1.001	1.121	1.089	0.278 NS	1.088
NP Conc.4 (26.37 mg/ml)	1.318	1.025	1.298	1.392	1.092	1.293	0.327 NS	1.236
NP Conc.5 (19.78 mg/ml)	1.271	1.087	1.202	0.992	1.252	1.355	0.366 NS	1.193
L.S.D. value	0.438 *	0.422 *	0.507 *	0.451 *	0.498 *	0.527 *	---	0.472 *

*** (P≤0.05), NS: Non-Significant.**

Conclusion

This study demonstrates that biosynthesized CuO NPs exhibit dose-dependent antibacterial and antibiofilm effects against multidrug-resistant *P. aeruginosa*, with inhibition zones increasing from 10 to 34 mm at concentrations of 31.25–500 mg/mL. The antimicrobial mechanism involves ROS-mediated cellular damage. Notably, the research provides new insights into biofilm inhibition, identifying a critical concentration threshold for efficacy. While the eco-friendly synthesis using pyocyanin highlights translational potential, further work is needed to elucidate molecular mechanisms, assess cytotoxicity, and evaluate antibiotic synergies. These findings support CuO NPs as a promising alternative for treating resistant biofilm-associated infections.

Ethical clearance

This research was ethically approved according to the approval with reference number CSEC/0424/0035 issued by the Ethical Committee of the University of Baghdad, College of Science.

Conflict of interest

The authors declare that they have no conflicts of interest.

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