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Antibacterial effect of biosynthesized Copper Oxide nanoparticles against *Staphylococcus aureus*

Amna Emad Hamed[1]*, Hassan Majeed Rasheed[1]

[1] Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

Abstract

Background. Multidrug-resistant (MDR) infections, particularly those caused by *Staphylococcus aureus*, pose a critical global health threat, necessitating innovative antimicrobial strategies such as the biosynthesis of copper oxide nanoparticles (CuO NPs) via pyocyanin from *Pseudomonas aeruginosa*. **Aim.** This study aims to evaluate the efficacy of copper oxide nanoparticles (CuO NPs), biosynthesized using pyocyanin, as an antibacterial agent against *Staphylococcus aureus*. **Methods.** The research involves the cultivation of *Pseudomonas aeruginosa* isolates, extraction of pyocyanin, and subsequent biosynthesis of CuO NPs. The findings are expected to provide insights into novel therapeutic strategies for combating resistant bacterial strains, addressing a critical public health need. Sample collection from Baghdad-Al-Yarmouk Teaching Hospital, Ghazi Al-Hariry Hospital, and Al-Imamein Al-Khadumein Teaching Hospital involved 228 clinical samples from burn wounds, otitis media, and urinary tract infections. The Copper Oxide Nanoparticles were synthesized by *P. aeruginosa* pyocyanin using the biological method and characterized using Atomic Force Microscopy (AFM), Ultraviolet-Visible Spectroscopy (UV-VIS), Field Emission Scanning Electron Microscopy (FE-SEM) and Fourier Transform Infrared Spectroscopy (FTIR) These confirmed that it is nanoparticle. **Results.** The antimicrobial efficiency of Copper Oxide nanoparticle was determined for six multidrug-resistant bacterial isolates using a well diffusion assay (WDA). The maximum inhibition zone of *Staphylococcus aureus* was 40 mm at concentration 500mg/ml of CuO NPs. **Conclusion.** The study concludes that biosynthesized copper oxide nanoparticles (CuO NPs) using pyocyanin exhibit potent antibacterial activity against multidrug-resistant *Staphylococcus aureus*, highlighting their promise as a novel therapeutic strategy for resistant infections.

Keywords: *Pseudomonas aeruginosa*, Copper Oxide nanoparticles, *Staphylococcus aureus*, Pyocyanin, Antibacterial

Corresponding author: (E-mail: Aamna.emad2302@sc.uobaghdad.edu.iq)

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].

Nanotechnology is one of the most active areas of research in materials science [3]. Nanotechnology is a growing multidisciplinary approach centered on the production of molecules in the nanoscale size range [4]. One innovative approach that shows promise for addressing the challenges with chemical processes is green synthesis, or the utilization of extracts from natural products [5]. Due to their affordability, environmental friendliness, and lack of requirement for particular conditions, biological methods are preferred [6]. Copper NPs have been seen as a competitive substitute for gold and silver NPs due to lower cost and higher natural abundance [7], [8]. Copper NPs' antibacterial qualities have been the subject of several reports (Lee *et al*., [9]; Ahamed *et al*., [10]; Awwad *et al*., [11]; Sutradhar *et al.,* [12]). One of the most potent effects of metal nanoparticles against bacteria is their ability to attach to the cell membrane and cell wall through electrostatic interactions, thereby disrupting 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) are highly reactive molecules capable of damaging proteins, lipids, and DNA. Their concentration is known to increase in the presence of metal nanoparticles (NPs), potentially exacerbating their harmful effects [13]. Urinary tract infections (UTIs) are considered a serious health issue, affecting millions of people annually [14]. These infections are characterized by the presence of microorganisms, then multiplication and invasion in the uroepithelial cells that may occur at different sites of the urinary tract. Bacterial infections can affect the lower urinary tract, including the bladder, leading to cystitis, or may extend to the kidneys and renal pelvis, resulting in pyelonephritis [15].

Materials and Methods

Collection, isolation and identification of bacteria

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, a selective medium for Gram-negative bacteria. Their inability to ferment lactose was recognized as a differential characteristic from the Enterobacteriaceae family. Subsequently, they were cultured on Nutrient agar to display their distinguishing pigments. Finally, the isolates were screened using Cetrimide agar, a selective medium for *P. aeruginosa* [16] .Mannitol salt agar was a selective medium for the cultivation and isolation of Gram-positive bacteria. *Staphylococcus aureus* Lactose-fermenting colonies were identified morphologically, When *Staphylococcus aureus* is cultured on blood agar, it typically forms golden-yellow colonies surrounded by a clear zone of beta-hemolysis (complete hemolysis), aiding in its differentiation from other bacteria. The incubation for isolates was at 37˚C for 24-48 hr. Isolates were confirmed by the VITEK-2 compact system.

Detection of pyocyanin-producing isolate of Pseudomonas aeruginosa

This method was done according to Elbargisy [17] with some modifications. For the determination of pyocyanin-producing isolates, firstly, the nutrient agar was cultured with Pseudomonas aeruginosa and incubated for 72 hr. at 37 ºC; if the bacteria produced pyocyanin (blue-green color), then a single colony selected from nutrient agar and inoculated into the nutrient broth and incubated at 37 ºC for 3-7 days until the blue-green color appears directly or after shaking the flask then the pyocyanin is extracted by using chloroform.

Extraction of pyocyanin from producing isolates

The pyocyanin extraction method involved taking Nutrient broth containing blue-green color (pyocyanin) and centrifuged at 5000 rpm for 15 minutes. Then, the supernatant was taken and poured into a separating funnel. Then, chloroform was added to it at a 2:1 ratio (about 200 ml chloroform for every 100 ml of supernatant). The mixture was shaken and then left undisturbed for 5 minutes. Directly collect the blue-colored chloroform layer without drying, this method was modified by researcher, as shown in Figure 3.

Synthesis of Copper Oxide Nanoparticles (CuO NPs) from pyocyanin pigment

The process of nanoparticle preparation involves preparing a flask with 150 ml of chloroform containing pyocyanin and 15g of copper chloride. The flask is shaken for 24 hours and then poured into tubes. The tubes are centrifuged at 5000 rpm for 15 minutes, washed with ethanol 98%, and centrifuged again. The solution is then dried in an incubator before being tested for its characteristics.

Characterization of Copper Oxide Nanoparticles (CuO NPs)

Various techniques were employed to characterize CuO nanoparticles, including ultra-violate visible light (UV-Vis), Field Emission Scanning Electron Microscopes (FE-SEM), Atomic Force Microscopy (AFM), Fourier transforms infrared (FTIR) and Energy Dispersive X-ray microanalysis (EDX) [18].

Antibacterial test of Copper Oxide Nanoparticles

The agar well diffusion technique was employed to determine the minimum inhibitory concentration (MIC) of biologically generated CuO nanoparticles for their antibacterial properties against the selected isolate [19]. 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. Subsequently, solutions of Copper Oxide nanoparticles at varying concentrations (100%, 50%, 25%, 12.5%, and 6.25%) were introduced into the pre-drilled wells. At 37 degrees Celsius, the obtained plates were incubated for 24 hours. Following incubation, the plates were inspected for the presence of inhibition zones. The diameters of these zones were measured using calipers and documented. To ensure reliability, the experiment was repeated three times [20].

Statistical analysis

The Statistical Packages of Social Sciences (SPSS) (2019) [21]. A statistical program was used to analyze the effects of different groups on the study parameters. The least significant difference (LSD) and T-test were applied to compare means, while the chisquare test was utilized to compare percentages. Significance was assessed at probability levels of 0.05 and 0.01.

Results

Isolation and identification of bacterial samples

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 [22]. 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 [23], as shown in Figure 1. 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 [24]. This result has been matched with Kim [25].

Staphylococcus aureus

Staphylococcus aureus bacteria were tested using gram-stain and mannitol salt agar, with nine isolates tested. The results confirmed the standard results for S. aureus. The bacteria were found to have small circular, smooth, and mucoid colonies on Nutrient agar, β hemolytic colonies on Blood agar, and golden yellow colonies on Mannitol salt agar due to their ability to ferment mannitol sugar, as shown in Figure 2. Microscopic examination revealed purple gram-positive cocci, occurring in pairs or short chains. These findings support the standard results for S. aureus that agreed with El Nagdy et al., [26].

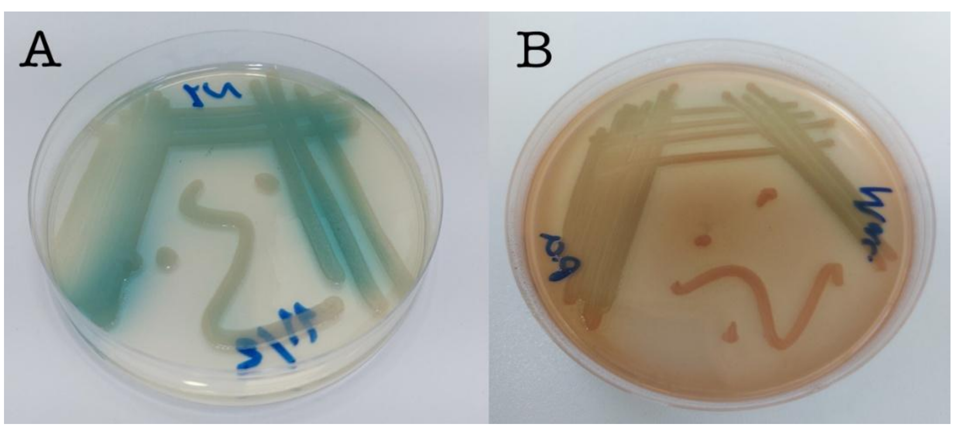


Figure 1. ***Pseudomonas aeruginosa*** **on A) Nutrient agar, B) MacConkey agar**

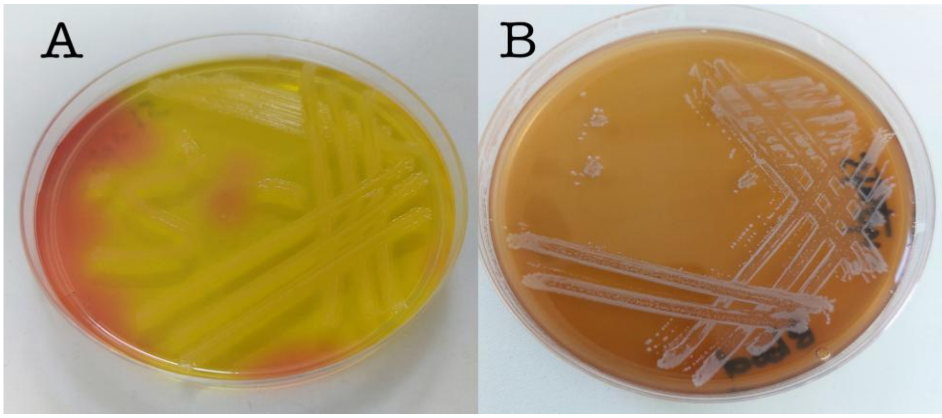


Figure 2. ***Staphylococcus aureus*** **isolate on (A) Mannitol Salt Agar, (B) Blood agar.**

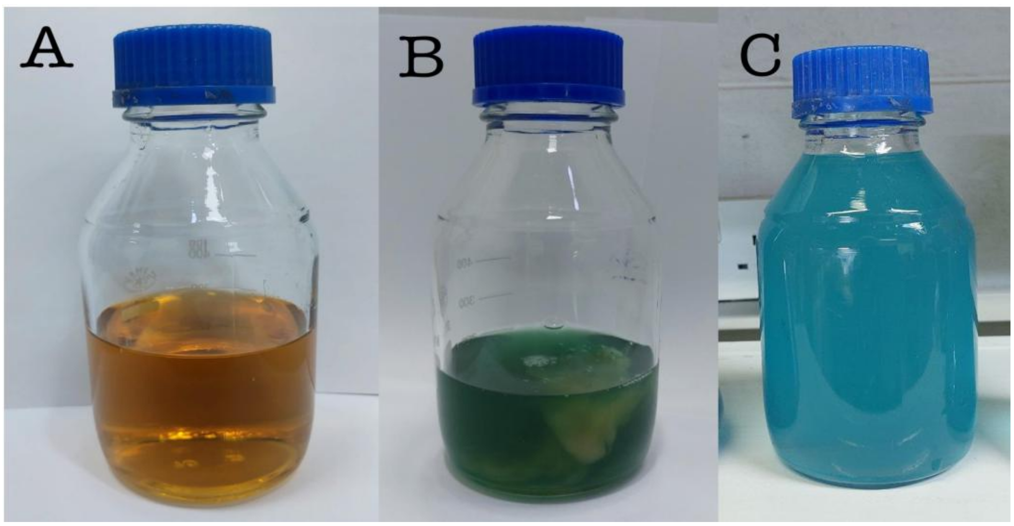


Figure 3. A) nutrient broth before incubation, B) nutrient broth with *Pseudomonas*, C) Chloroform containing pyocyani

Characterization of pyocyanin

UV-Visible analysis

The pyocyanin extracted from *Pseudomonas aeruginosa* was characterized using a UV-visible spectrophotometer (Shimadzu, Japan) as shown in Figure 4 to detect the maximum absorption. Absorbance was measured at 330 nm, this finding is consistent with the results reported by Ohfuji *et al.* [27], indicating that the UV-visible absorption of pyocyanin is 318.5 nm.

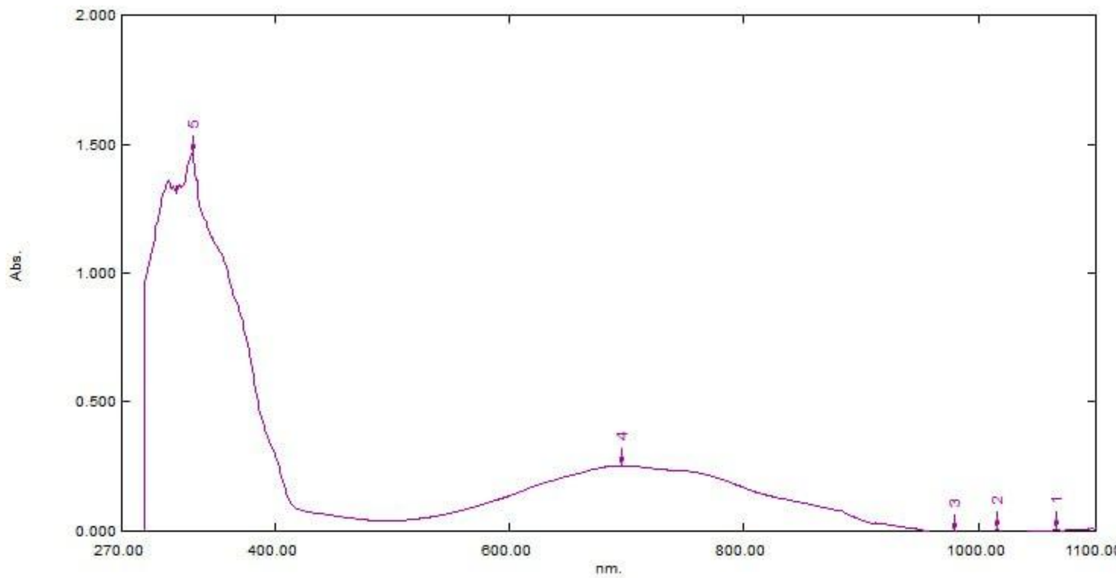


Figure 4. UV-visible of pyocyanin

FT-IR analysis

The chloroform containing pyocyanin pigment exhibits intense peaks at 3410.27 cm-1, attributed to O-H stretching in the alcohol compound class. Additional peaks were observed at 2956.86 - 2925.08 cm-1 for O-H stretching, 1627.36 cm-1 for C-C stretching, and 1462.48-1379.95 cm-1 for C=H bending, as shown in Figure 5. The carboxylate groups present in the chloroform containing pyocyanin pigment are responsible for binding proteins to the Cu surface, thereby stabilizing the biosynthesized CuO nanoparticles [28].

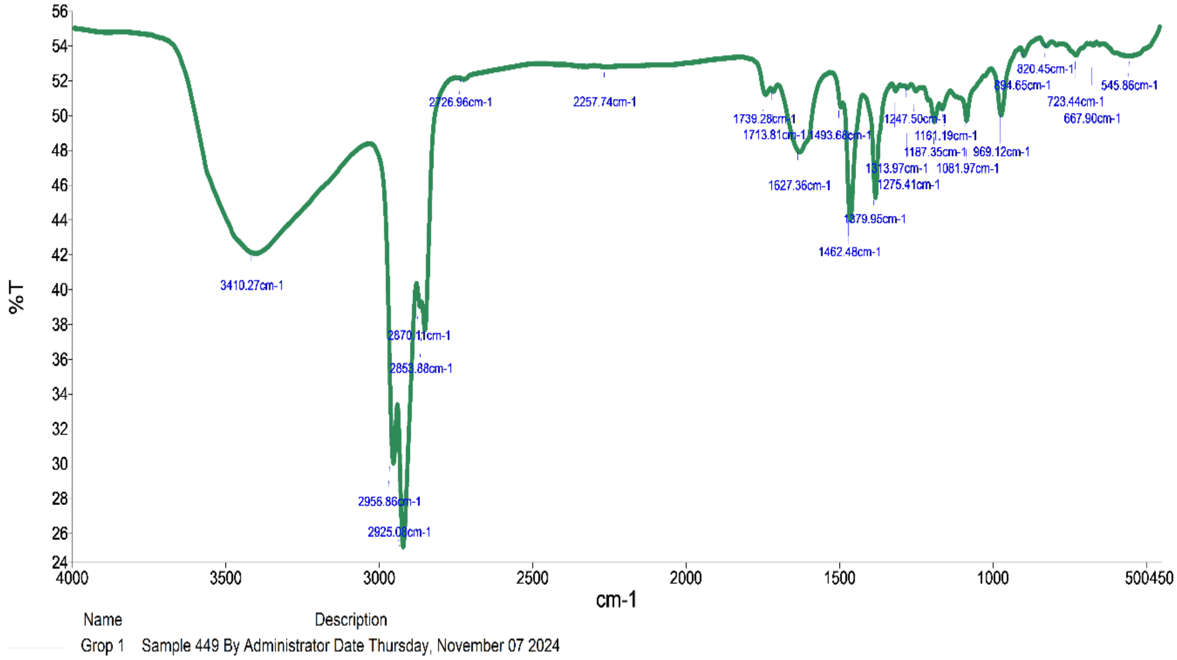


Figure 5. Fourier Transform Infrared of pyocyanin pigment

Synthesis of CuO NPs by using pyocyanin pigment

When salt powder of Copper chloride $CuCl_2$ was added to the solution of chloroform containing pyocyanin, the aqueous 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 [29]. 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, Figure 6.

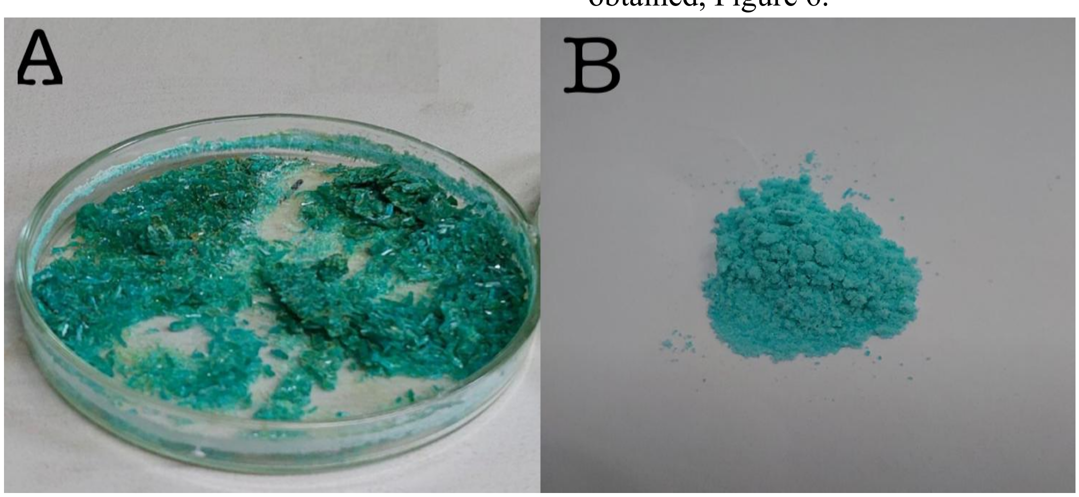


Figure 6. Show Copper Oxide nanoparticles: A) after drying, B) after gridding.

Characterization of CuO NPs

UV-Visible analysis

The biosynthesis of copper oxide nanoparticles (CuO NPs) was characterized using a UV-visible spectrophotometer (Shimadzu, Japan) to determine the maximum absorption. The biosynthesized CuO NPs exhibited a distinct absorption peak at 288 nm (Figure 7), confirming the formation of cupric oxide nanoparticles. This result aligns with the findings of Al-Mohaimeed *et al.* [30], who reported a similar absorbance peak at 280 nm for CuO NPs.

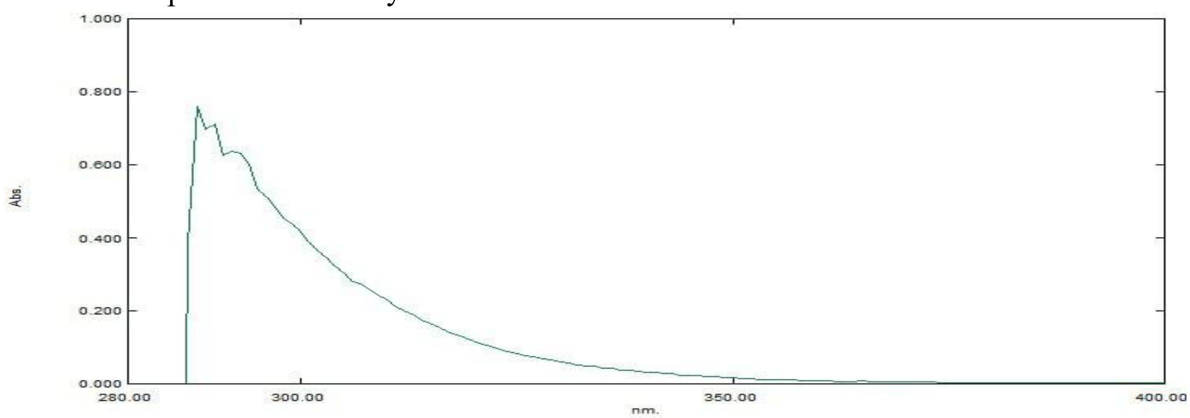


Figure 7. UV-vis spectra of copper Oxide nanoparticles

FTIR analysis

Fourier Transform Infrared (FTIR) spectroscopy is a critical technique for identifying functional groups involved in stabilizing synthesized nanoparticles. In this study, FTIR was used to analyze the interaction of functional groups in pyocyanin during the reduction of $CuCl_2$ to form copper oxide nanoparticles (CuO NPs) and their subsequent capping by chloroform containing the pyocyanin pigment. The FTIR spectra of both chloroform with pyocyanin and CuO NPs were recorded within the infrared radiation (IR) range of 4000/cm to 500/cm.

The FTIR spectra of CuO NPs revealed a peak at 667.15 cm^{-1}, confirming the presence of metal ions, consistent with findings reported by Ali *et al.* [31]. Additional peaks were observed at 3436.71 cm^{-1} and 1623.27 cm^{-1}, indicative of interactions between Cu and pyocyanin biomolecules. These peaks, along with those corresponding to $CuCl_2$, chloroform with pyocyanin, and CuO NPs, suggest the presence of higher alcohol concentrations, C-N stretches of amines, phenols, alkenes, and aldehydes. Furthermore, an absorption peak at 771.22 cm^{-1} was attributed to C=C bending vibrations, providing further evidence of the chemical interactions involved in nanoparticle stabilization.

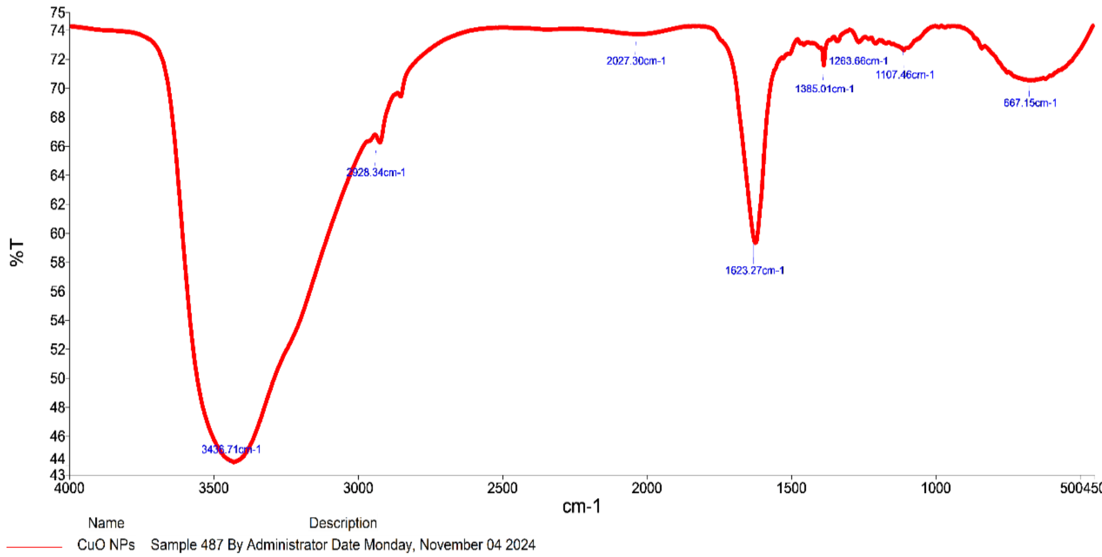
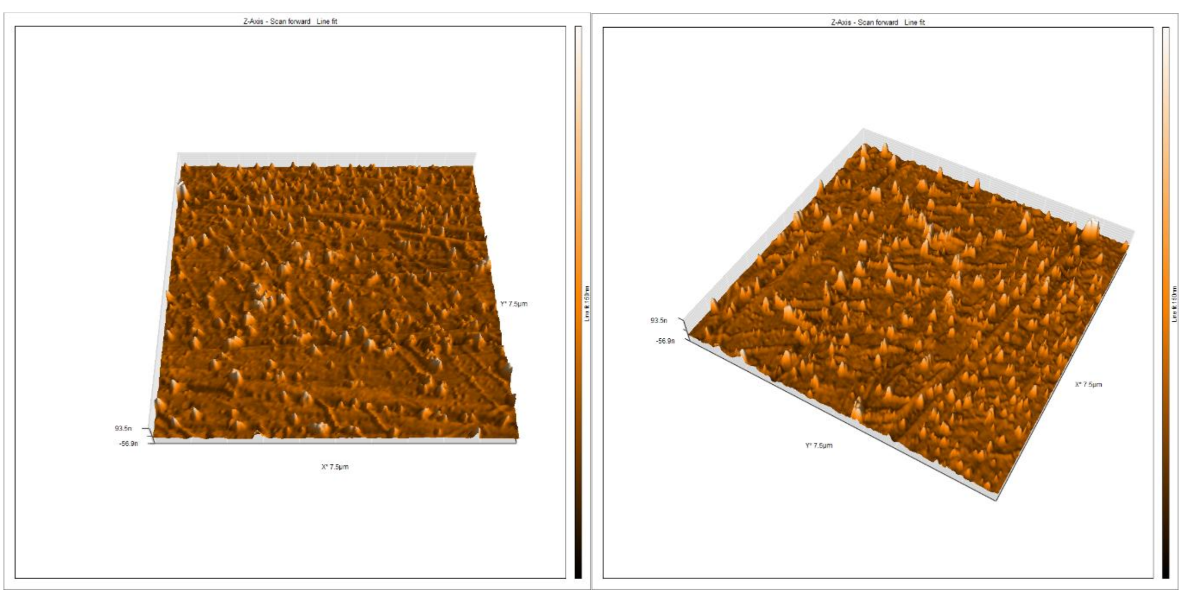


Figure 8. Fourier Transform Infrared spectra of copper Oxide nanoparticle

AFM analysis

Atomic Force Microscopy (AFM) was employed to analyze the morphology and topography of the nanoparticle surfaces, generating both two-dimensional and three-dimensional images at an atomic resolution. The average particle diameter was determined to be in the nanoscale range (Figure 9). The particle size distribution histogram revealed that the CuO nanoparticles (NPs) had an average diameter of 81.85 nm



(A) **(B)**

Figure 9. Atomic Force Microscopy of copper oxide nanoparticles: A) 2Dimention, B) 3Dimention

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 [32]. The results recorded near-spherical particles sized 80 nm.

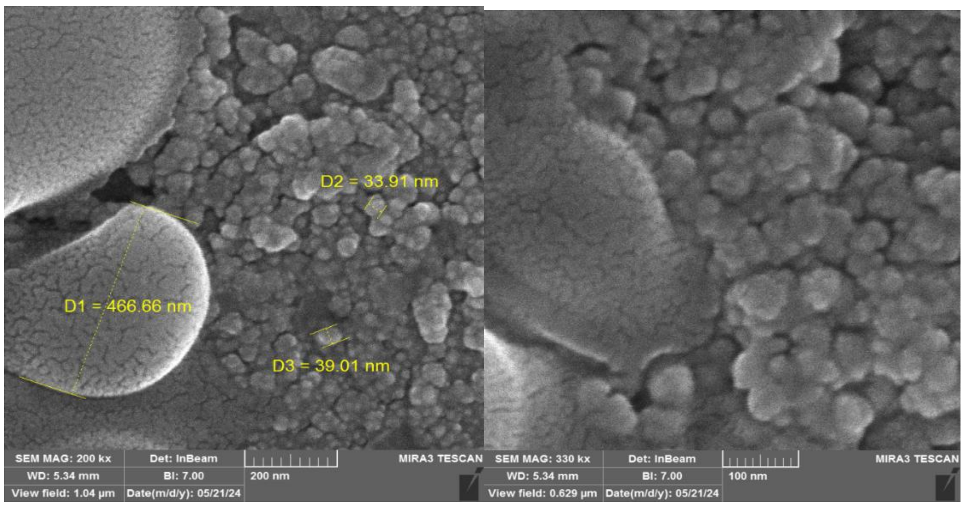


Figure 10. Images of the copper oxide nanoparticles by Field Emission Scanning Electron Microscopy

Antibacterial activity of biosynthesized CuO NPs

In this study, biosynthesized copper oxide nanoparticles (CuO NPs) demonstrated significant antibacterial activity against multidrug-resistant *Staphylococcus aureus*. The results indicated a concentration-dependent effect, with the inhibition zone increasing as the concentration of CuO NPs increased (Figure 11). The maximum inhibition zone observed was 40 mm at a concentration of 500 mg/mL, while the minimum inhibition zone was 17 mm at a concentration of 31.25 mg/mL.

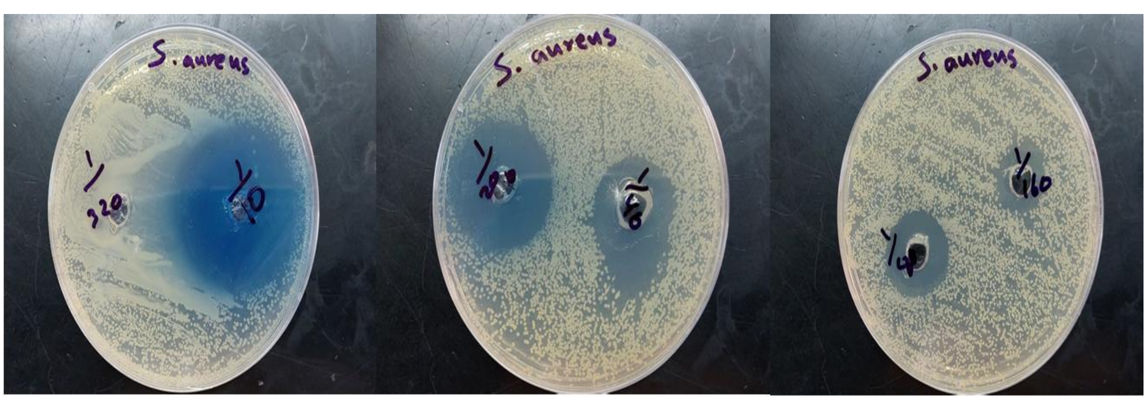


Figure 11. Effect of copper oxide nanoparticles on *Staphylococcus aureus*

Table 1. The inhibition zone of *Staphylococcus aureus* using copper oxide nanoparticles.

Bacteria	The Inhibition Zone (mm)							Average
	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	
Staphylococcus aureus	**0**	**17**	**20**	**27**	**30**	**40**	**8.51 ***	**26.8**
*** (P≤0.05), NS: Non-Significant.**								

Discussion

The identification of *Pseudomonas aeruginosa* and *Staphylococcus aureus* was consistent with standard microbiological characteristics and aligned with previous sections, validating the accuracy of the isolation process. Pyocyanin pigment was successfully extracted and characterized, confirming its dual role as a reducing and stabilizing agent in the biosynthesis of copper oxide nanoparticles (CuO NPs). Spectroscopic analyses, including UV-Vis and FTIR, further substantiated the formation of CuO NPs, with functional groups derived from pyocyanin contributing to their stabilization. Complementary SEM analysis revealed nanoscale particles, predominantly spherical, with bioactive pyocyanin effectively preventing aggregation, thereby ensuring structural integrity and enhanced bioactivity of the synthesized nanoparticles. Current study agreement with Al-Jubouri *et al*. [33] were synthesis Copper Nanoparticles from *Myrtus communis* leaves extract and recorded a significant result against *S. aureus*. Also, the antibacterial activity of CuO NPs using Mint extract was checked against *Escherichia coli* and *Bacillus subtilis* by Aziz *et al*. [34]. Jarallah *et al*. [35] were indicated antibacterial effect against *S. aureus*, and Zebari *et al*. [36] against *S. aureus*, *Clostridium*, *P. aeruginosa*, *Escherichia coli*, and *Klebsiella*, prepared CuO NPs from *Malva sylvesteris* extract.

Abbas *et al*. [37] prepared CuO NPs using extract of *Cordia myxa* and recorded a high diameter of inhibition zone for both *P. aeruginosa* and *klebsiella.*

The significant differences in inhibition diameter zones were due to significant different interactions between CuO NPs and bacteria. In the current study, CuO NPs synthesized from the Pyocyanin indicated bactericidal effects when applied to treat *S. aureus* bacteria, moreover Gram-positive bacteria lack an outer layer of membranes due to the structure of their bacterial cell wall. This means that materials entering the cell can pass through the cell more easily than materials entering Gram-negative bacteria Al-Mossawei *et al*. [38]. The thickest cell wall may cease the permeation of nanoparticles within cells [39]. The antimicrobial activity of copper nanoparticles is widely reported; it is associate with ions that are deliver from these nanoparticles. Their compact size and high surface area to volume ratio allow them to contact closely with the microbial membranes, further enhancing the activity.

The current study agreed with Majumder [40], which found that the CuO NPs formed using the fungi *Fusarium oxysporum* were very stable and effectively inhibited the growth of both Gram-class bacteria. As well, Yoon *et al* describe the antibacterial properties of copper and silver nanoparticles using a single, distinctive strain of *E. coli*, where the copper nanoparticles outer formed the silver nanoparticles in terms of antibacterial activity [41]. Additionally, heavy metal ions have diverse effects on bacterial cell function. For copper ions, the mechanism may involve oxidative stress. The mechanism of antibacterial activity of CuO NPs studied by Chatterjee *et al*. [42] who found that CuO NPs treatment resulted in several toxic consequences in *E. Coli* cells, including DNA destruction, protein oxidation, and lipid peroxidation.

Antimicrobial capability of nanoparticles is due to its tendency to alternate between their cuprous - Cu [I] and cupric - Cu [II] oxidation states that lead to the generation of hydroxyl radicals, which attach to DNA molecules and cause cross-linking between and within the nucleic acid strands to disrupt the helical shape of the molecules. Additionally, by attaching to the carboxyl and sulfhydryl amino groups of amino acids, damage vital proteins. Similarly, surface proteins that are required for material transport across cell membranes are eliminated, compromising the integrity and lipids of the membrane [43]. Biochemical activities are also disrupted by copper ions present in bacterial cells.

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.

Conclusion

Copper oxide nanoparticles biosynthesized by pyocyanin demonstrated effective antibacterial activity against *Staphylococcus aureus*. The antibacterial activity of CuO nanoparticles is clearly concentration-dependent.

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