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The Influence of Biologically Synthesized Copper Nanoparticles on the Biofilm Produced by *Staphylococcus haemolyticus* 1solated from Seminal Fluid

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Abstract:

Staphylococcus haemolyticus is one of the most frequently isolated coagulase-negative staphylococci. The ability to form biofilm is considered as one of the most important virulence factors of coagulase negative staphylococci. There is only limited knowledge of the nature of *S. haemolyticus* biofilms**.** This study was aimed at evaluating the ability of *S. haemolyticus* strains to produce biofilm in the presence of copper oxide nanoparticles (CuONPs). The biological synthesis of nanoparticles is an environmentally friendly approach for large-scale production of nanoparticles. Copper oxide nanoparticles were produced in the current study from the *S. haemolyticus* viable cell filtrate. UV-visible (UV-Vis) spectroscopy, X-ray diffraction (XRD), atomic force microscope (AFM), field emission scanning electron microscope (FE-SEM), energy-dispersive X-ray spectroscopy (EDX) and zeta potential (ZP) analysis were used to analyze the newly synthesized CuONPs. Our findings revealed that at minimum inhibitory concentration (MIC), CuONPs showed remarkable inhibition on the biofilms produced by multidrug resistant (MDR) *S. haemolyticus* isolates.

Keywords: Green synthesis, Anti-biofilm, CuONPs

تأثير جزيئات اوكسيد النحاس النانوية المصنعة حيويا على الغشاء الحيوي للمكورات العنقودية الحالة للدم المعزولة من السائل المنوي

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الخلاصة

تعتبر المكورات العنقودية الحالة اكثر انواع المكورات العنقودية ا السالبة لأنزيم المخثر للبلازما شيوعا. تعتبر القدرة على تكوين الغشاء الحيوي من اهم عوامل ضرواة المكورات العنقودية السالبة لإنزيم المخثر للبلازما. توجد معلومات محدودة على الطبيعة التكوينية للأغشية الحيوية للمكورات العنقودية الحالة. هذه الدراسة هدفت الى التحري على قدرة عزلات المكورات العنقودية الحالة على تكوين الغشاء الحيوي مع وجود جزيئات النحاس النانوية. التصنيع البايولوجي للجزيئات النانوية هي طريقة صديقة للبيئة للأنتاج الواسع المدى. جزيئات اوكسيد النحاس النانوية صنعت في هذه الدراسة من راشح الخلايا الحية لبكتريا المكورات العنقودية

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الحالة. تم استخدام الاختبارات التالية: مطياف الاشعة المرئية وفوق البنفسجية، حيود الاشعة السينية، مجهر القوة الذري، مجهر الماسح الالكتروني، مطياف تشتت الطاقة بالأشعة السينية وجهد الزيتا لتحليل جزيئات اوكسيد النحاس المصنعة حديثا. اظهرت النتائج ان اقل تركيز تثبيطي لجزيئات اوكسيد النحاس النانوية ذو فعالية تثبيطية على الاغشية الحيوية المنتجة بواسطة المكورات العنقودية الحالة المقاومة المتعددة للمضادات الحيوية.

Introduction:

Nanomaterials (NMs) exhibit unique physical and chemical properties and, hence, they have received much attention from scientists and researchers in different areas of sciences, specifically as antimicrobial and anti-biofilm agents. It has been shown that various NMs, such as polymeric or hybrid NMs, gold and magnetic nanoparticles (NPs) respond to stimuli from the external resulting in temporally regulated macromolecule release. For these reasons, synthetic NMs have been thoroughly studied and applied in pharmacology and medicine over the past 20 years, especially for diagnostic and therapeutic purposes [1]. Copper (Cu) nanoparticles are the ideal among all metal nanoparticles because of their distinctive chemical and physical characteristics, including excellent thermal conductivity, electrical conductivity and biological activity [2]. CuNPs exhibit potent antimicrobial activity against harmful pathogens and infectious diseases such as cholera [3]. Due to its excellent features including no harmful chemicals, being cost-effective and environmentally friendly , CuNPs green synthesis approaches are ideal for use in biomedical applications [4].

S. haemolyticus is the second-most common species of coagulase-negative Staphylococci (CoNS) that has been linked to infections of the respiratory tract, otitis, peritonitis and septicemia [5]. *S. haemolyticus* is known for its multidrug resistance and for earlier development of methicillin and glycopeptide antibiotic resistance [6]. A community of structured bacterial cells known as a biofilm is encased in an extracellular matrix (ECM) [7]. They form a material called polysaccharide intercellular adhesion(PIA), an extracellular matrix. which has been found to be an essential component of *Staphylococcus* spp. biofilms [8]. These biofilms provide mechanical stability and extracellular environment protection. They can be constructed from a matrix that contains different polymeric elements such proteins, extracellular DNA and polysaccharides. Bacterial biofilms that develop on foreign implanted devices or chronic wounds can cause persistent infections that are more challenging to treat with antibiotics [9]. Metal oxide nanoparticles are effective in fighting against multidrug resistance biofilm producing pathogenic bacteria, where CuONPs are found to be more effective than iron oxide and nickel nanoparticles [10]. Therefore, the goal of the current study was to biosynthesize CuONPs utilizing the culture supernatant broth of *S. harmolyticus.* In addition, the characterization of biosynthesized CuONPs was also assessed using UV-Vis, atomic force microscope (AFM), field emission scanning electron microscope (FE-SEM), energy-dispersive X-ray spectroscopy (EDX), X-ray diffraction (XRD) and ZP analysis. And finally, evaluation of their antibacterial, anti-biofilm and cytotoxicity activities.

Materials and Methods

S. haemolyticus Isolation and Identification:

In this study, one hundred and fifty Specimens of urine and seminal fluids were collected during September 2022 to December 2022 from patients in Baghdad medical city hospital in Baghdad, Iraq. The specimens were cultured on mannitol salt agar and blood agar then incubated for 24-48 hrs at 37°C under aerobic conditions, suspected colonies were identified morphologically and biochemically [11]. Isolates were subsequently confirmed as *S.*

haemolyticus by using the Vitek 2 compact system (BioMe`rieux/France), following the instructions provided by the manufacturer.

Antibiotic Sensitivity Test:

The antibiotic sensitivity test was done for all 60 isolates by Vitek 2-compact system using card containing 16 different antibiotics benzylpencillin, oxacillin, gentamicin, levofloxacin, moxifloxacin, erythromycin, clindamycin, linezolid, teicoplanin, tetracycline, vancomycin, tigecycline, nitrofurantoin, fusidic acid, rifampicin and trimethoprim/sulfamethoxazole

Suspension of *S. haemolyticus*:

Bacteria were cultured in Mueller Hinton Broth to prepare a suspension that was incubated for 18 h at 37°C. At 4° C, the bacteria were centrifuged at 10,000 g for 10 min. The supernatant was collected after the precipitate was removed to synthesize copper nanoparticles [12].

Biosynthesis of Copper oxide Nanoparticles:

For the green synthesis of CuO Nps a volume of 15 ml of 1mM copper acetate was added to 15 ml of *S. haemolyticus* cells free supernatant after centrifugation and removal of the bacterial cells precipitate and the suspension was used to synthesize CuoNps in 100 ml Erlenmeyer flasks. The flasks were incubated at 30°C–35°C for three days and any color change was recorded. After incubation, the reaction mixture was centrifuged at 6000 rpm for 25 min. at 4°C to remove the supernatant. The supernatant was then replaced with deionized water and the centrifugation process was repeated three more times under the same conditions to remove any remaining supernatant. The pellet-shaped collection of nanoparticles were the transferred into a hot air oven set at 120°C to evaporate off all the liquid content.The dried powder was carefully gathered and kept in storage for additional analysis.

Only the copper acetate was employed as a negative control and no color change was observed over time [13] (Figure 1).

(a) (b) (c) (e) (f) (g)

Figure 1: Synthesis of CuONPs by biological methods: (a) Copper acetate (negative control), (b) Cell free supernatant of *S. haemolyticus*, (c) Mixture of copper acetate and cell supernatant after incubation for three days, (e) Centrifugation of reaction mixture, (f) Supernatant replaced with deionized water for further centrifugation and (g) The pellet-shaped CuONPs.

Characterization of biosynthesized CuO NPS:

Using the following techniques, the morphology and size of the Cu/CuO NPs were investigated, such as UV-VIS spectroscopy, AFM, EDX, FESEM, XRD and zeta potential analysis ZP.

•UV-VIS Spectroscopy

The CuONps was confirmed by measuring the wavelength of the reaction mixture in the UV-VIS spectrum of the spectrophotometer (Shimadzu/Japan). The scanning range for the samples was 300-900 nm at a scan speed 500nm/min, by using a blank reference for correction of the spectrophotometer [14].

•Atomic Force Microscopy

AFM (UNICCO/USA) was used to determine the size and surface morphology of CuONPs nanoparticles. A thin film of the prepared CuONPs was deposited on a silica glass plate by dropping few drops of the CuONPs on it which was then allowed to dry at room temperature in the dark. The deposited film glass plate was then scanned with the AFM [15].

•Field Emission Scanning Electron Microscope

Scanning electron microscope technique (Hitachi Ltd. /Japan) was used to characterize the mean particle morphology and diameter of nanoparticles. The dried sample of CuONPs solution was sonicated with distilled water. A small drop of this sample was placed on glass slide and was then allowed to dry. After that, a thin layer of platinum was coated to make the samples conductive [16].

•Energy-dispersive X-ray Analysis

EDX analysis (Bruker/Germany) is utilized to determine the compositions of various elements in a given sample. It depends on a sample and an X-ray excitation source interacting [17].

•X-ray Diffraction

The crystal structure of CuONPs was measured by XRD (Shimadzu/Japan). Powdered sample of CuONPs was prepared for XRD by grinding, which can be accomplished by several different methods. The best used method is Sample Slurry/Smear Slide Mount for Small Sample Amounts [18].

•Zeta Potential Analysis

The zeta potential of copper oxide nanoparticles was estimated using electrophoretic light scattering technology (Brookhaven/USA). A concentration of 1 mg/ml of nanoparticle suspension was made in Millipore water in a 900 µL Zetasizer disposable cell. The suspension was analyzed 60 times per scan for three scans [19

Biofilm Activity of *S. haemolyticus*:

Briefly, a sterile brain heart infusion (BHI) broth was inoculated with 18-hour-old cultures of the selected strains at a final cell concentration of 10^8 CFU/ml. One-eighty µl of 1% glucose-containing brain heart infusion broth was added to sterile 96-well polystyrene

microtiter plates. Three sterile 96-well polystyrene microtiter plates were filled with a volume of 20 µl of bacterial suspension. As a negative control, six wells containing brain heart infusion broth free of bacteria were taken into account. The prepared plates were incubated at 37°C for 24 hours to allow bacteria to form biofilms. A crystal violet assay was performed to measure the intensity of the biofilms after they had developed. The cultures were carefully discarded before being thoroughly washed with 1X Phosphate buffer saline (PBS). The adherent biofilms were fixed with 150 µl of methanol for 15 minutes and the excess was discarded. After 10 minutes of drying at room temperature, the plates were dyed for 15 minutes with 250 µl of 0.2% (w/v) crystal violet solution. After washing off any excess crystal violet with distilled water, the plates were allowed to dry for 30 minutes. A concentration of 95% ethanol (v/v) was used to dissolve the adhered crystal violet from the biofilms which then was incubated for 15 minutes to read optical density(OD) at 630 nm [20].

Testing the Antibacterial Activity of CuONPs:

Mueller Hinton agar was used for the agar diffusion assay. Sterilized cotton swabs were used to swab the test isolates of *S. haemolyticus* onto Muller hinton agar plates. A sterile cork borer was used to create wells which were then filled with 100 µl of varied concentrations (64-8) µg/ml). The plates were subsequently incubated for an additional 24 hours at 37°C, after which the zones of inhibition were determined the following day [21].

Determination of Minimum Inhibition Concentration (MIC) of Vancomycin and CuO NPs

Vancomycin and CuoNPs antibacterial activity were evaluated for *S. haemolyticus* using the broth microdilution method [22]. *S. haemolyticus* isolates were inoculated into nutrient broth and cultured at 37°C for 24 hours. In Mueller-Hinton Broth (MHB) medium, the stock solution of vancomycin and CuoNPS (512 g/ml) was diluted to produce two-fold serial dilutions ranging from 2 µg/ml to 64 µg/ml. Vancomycin and CuO NPS dilutions were transferred to microplate wells and inoculated with a bacterial sample with a turbidity of 0.5 McFarland standard. MHB medium was used as a negative control, while bacterial suspension with any addition was utilized as a positive control. For 24 hours, the microplate was incubated at 37°C. Then, in each well, bacterial growth was examined and the minimum concentration that prevented noticeable growth, was considered as MIC. In accordance with The Clinical & Laboratory Standards Institute guidelines (CLSI), 2022, the MIC of vancomycin for each isolate was calculated.

Screening for Antibiofilm Activity of CuONPs and Vancomycin

Vancomycin and CuONPs were tested individually for their antibiofilm action using a modified method for the microtitre plate assay [23]. Ten milli liters of brain heart infusion with bacteria were incubated for 24 hours at 37°C. After which, using Densicheck (BioMe`rieux/France), the cultures were diluted to 1.5×10^8 CFU/ml,and 10 µl of the diluted culture was added to 96-well microplates containing 180 µl of BHI with 1% glucose. To each well of the microplate, 200 µl of media, 10 µl of an incubated bacterial culture, 10 µl of MIC-concentrated CuONPs, and 10 µl of vancomycin solution were added. As a control, only the sterile liquid medium was utilized. Following incubation, 0.2 ml of phosphate-buffered saline (PBS, pH 7.2) was used to wash the wells four times. The biofilms in the wells were fixed with 95% (v/v) methanol and dyed with 0.2% crystal violet. Following the removal of any excess dye with distilled water, 200 µl of 95% (v/v) ethanol was added once the wells were dried. The following formula was used to determine the biofilm inhibition activity after which the optical density at 630 nm was measured in a multi-plate reader [24].

% biofilm inhibition = (OD of untreated isolate – OD of untreated isolate/ OD of treated isolate) ×100

Cytotoxicity Assay of CuONPs

To evaluate the cell viability of various CuONPS concentrations (400, 200, 100, 50, and 25 µg/ml), as previously reported, the MTT test was utilized against normal fibroblast cells (HdFn cells) and malignant cells (PC3 cells). The percent viability of cells exposed to treatments was calculated using the following equation and the concentration that inhibits 50% of cell growth was used as a parameter for cytotoxicity [25]:

Cell Viability (%) = (Mean OD of Treated Cells/Mean OD of Control Cells) × 100

Results and Discussion

Isolation of *S. haemolyticus*

In this study, one hundred and fifty urine and semen samples were examined and only sixty isolates were identified as *S. haemolyticus*. Smaller than the characteristic yellow pinhead colonies of S. aureus, the colonies on mannitol salt agar exhibited varied reactions. Some strains displayed yellow fermenting colonies while others displayed whitish pink non-fermenting ones [26.27]. Whilst the isolates on blood agar displayed beta hemolysis yellow-gray colonies that are (4–3) mm in diameter (Figure 2)

(a) (b) (c)

Figure 2: *S. haemolyticus on* (a) MSA (Mannitol fermenters isolate), (b) MSA (Mannitol non-fermenter isolate) and (c) Blood agar at 37°C for 24 hrs.

Antibiotic Susceptibility Test

According to the findings, all 60 (100%) isolates displayed a high level of resistance towards oxacillin and benzyl-penicillin, respectively followed by Erythromycin (90%), fusidic acid (86.6%) and tetracycline (85%). Resistance to gentamicin and clindamycin was present in 71.6% isolates. A moderate degree of resistance was observed among isolates to moxifloxacin (58.3%) and trimethoprim/sulfamethoxazole (43.3%). A lower degree of resistance was seen to teicoplanin (28.3%), levolfloxacin and rifampicin (26.6%) followed by vancomycin (20%) and nitrofurantoin (3%). All (100%) isolates showed high level of sensitivity toward tigecycline and linzolid (Figure 3).

The findings approximately matched a study by Naqid *et al*. [28] which found that 100% and 95% of isolates were resistant to oxacillin and benzylpenicillin. Another investigation by Al-Naqshbandi *et al*. [29] showed that isolates were resistant to oxacillin (82.05%) and benzylpenicillin (94.87%) correspondingly.

The findings of Al-Salmani *et al*. [30], who revealed that the majority of isolates were erythromycin resistant (100%), were compatible with the resistance of isolates to erythomycin

(90%) of isolates. Almost all isolates were resistant to trimethoprim/sulfamethoxazole (43.3%) and fusidic acid (86.6%). Similar results were found by Luty and his colleagues [31 who also found resistance to trimethoprim/sulfamethoxazole (62%), and fusidic acid (95%). Furthermore, resistance to tetracycline was demonstrated in the Khudheir study with a resistance rate of 52.6% [32]. The percentage of isolates that were resistant to gentamicin was somewhat consistent with the research by Al-Tulaibawi [33] which showed that (66%) of isolates were resistant.

Teicoplanin, levolfloxacin, rifampicin, vancomycin and nitrofurantoin had the lowest rates of resistance discovered in this study. A study by Al-Sultany & Al-Charrakh [34] showed that 26.7% and 6.7% were resistant to levofloxacin and rifampicin respectively. While all isolates were sensitive to teicoplanin, vancomycin and nitrofurantoin which disagrees with this current study. Tigecycline and linzolid were in the first place as the most effective and sensitive drugs against *S. haemolyticus* isolates in the current study. This result agreed with Al-Khafaji & Al-Khataua [35] who found that the sensitivity to tigecycline (100%). The results of the current also resemble the results of the by Alla-werdi & Mahdi [36] who documented that all of *S. haemolyticus* isolates (100%) were sensitive to linezolid.

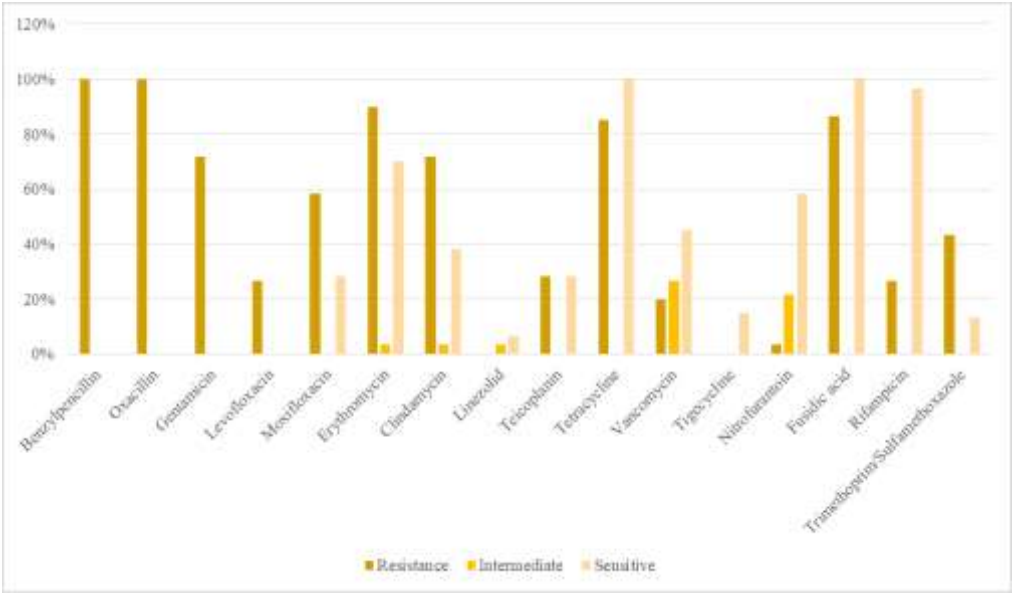


Figure 3: Percentage of antibiotic profile for *S. haemolyticus* isolates.

The findings revealed that 95% of the isolates were multi-drug resistant. *S. haemolyticus* can develop multi-resistance to a variety of antimicrobial agents. A key characteristic of *S. haemolyticus* and other coagulase negative staphylococci, including *S. saprophyticus, S. hominis*, and *S. cohnii*, is biofilm formation. This phenotype is associated with high levels of antibiotic resistance [37].

Determination of Biofilm Formation before CuONPs Treatment

Biofilm formation of the MDR *S. haemolyticus* isolates (n = 55) was assessed for its ability to produce biofilms using quantitative method by microtiter plates (MTP). The results demonstrated that isolates of *S. haemolyticus* biofilm production were 1(1.7%) non producer, 1 7(30.3%) isolates produced weak biofilm, 29(51.7%) isolate as moderate biofilm and 9 (16.07%) isolates were strong biofilm producers (Figure 4), 5) and Table 1).

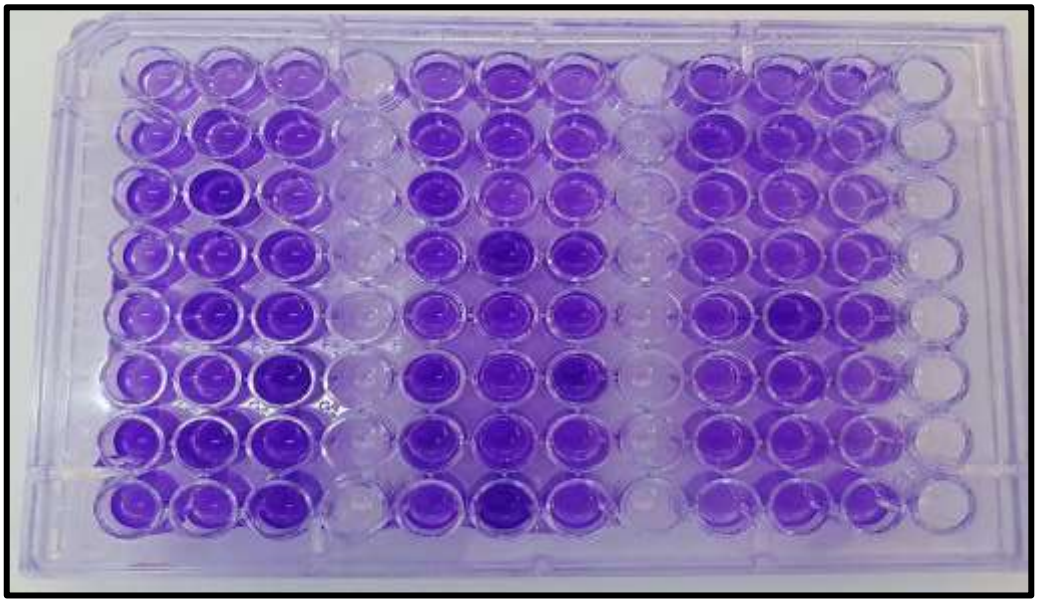


Figure 4: Biofilm forming examination results of *S. haemolyticus* isolates by micro-titerplate method.

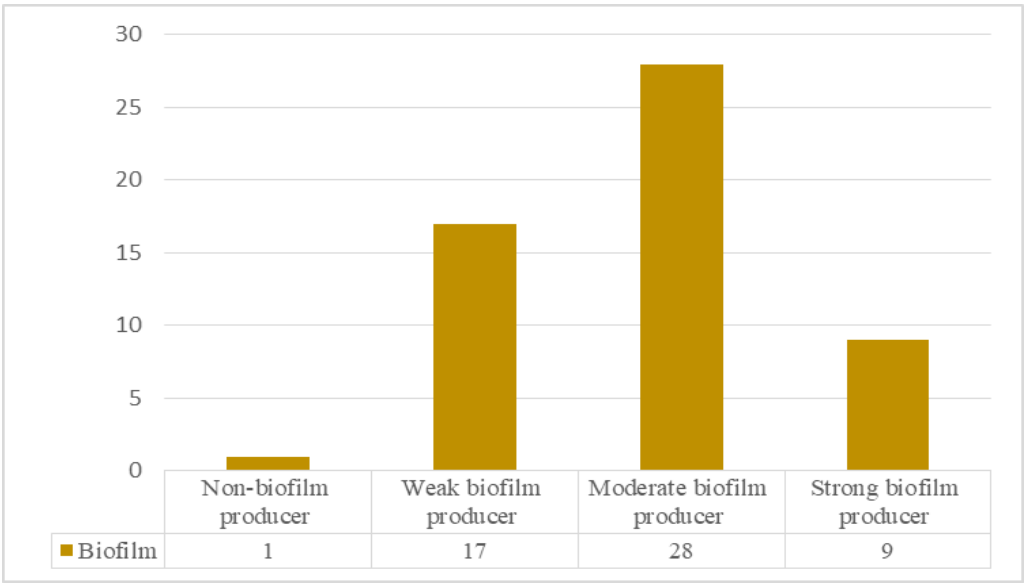


Figure 5: Percentage of biofilm values of *S. haemolyticus* isolates by microtiter plate method.

Table 1: Biofilm formation of isolates of *S. haemolyticus.* *(+/- SD + : high ; - : low)

Isolates Code	O.D	± SD*	Biofilm Response	Isolates Code	O.D	± SD*	Biofilm Response
A1	0.27	±0.04	Weak	A30	1.153	±0.11	Strong
A2	0.462	±0.15	Moderate	A31	0.211	±0.01	Weak
A3	0.293	±0.07	Weak	A32	0.392	±0.006	Moderate
A4	0.358	±0.17	Moderate	A33	0.257	±0.08	Weak
A5	0.302	±0.04	Weak	A34	0.253	±0.09	Weak
A6	0.149	±0.01	Non-producer	A35	0.695	±0.26	Strong
A7	0.482	±0.31	Moderate	A36	0.281	±0.04	Weak
A8	0.375	±0.23	Moderate	A37	0.854	±0.03	Strong
A9	0.221	±0.05	Weak	A38	0.445	±0.15	Moderate
A10	0.496	±0.2	Moderate	A39	1.315	±0.17	Strong
A11	0.402	±0.05	Moderate	A40	0.559	±0.27	Strong
A12	0.251	±0.06	Weak	A41	0.393	±0.17	Moderate
A13	0.199	±0.01	Weak	A42	0.359	±0.03	Moderate
A14	0.479	±0.09	Moderate	A43	0.427	±0.12	Moderate
A15	0.378	±0.12	Moderate	A44	0.517	±0.18	Moderate
A16	0.215	±0.03	Moderate	A45	1.263	±0.63	Strong
A17	0.483	±0.43	Moderate	A46	0.324	±0.02	Moderate
A18	0.275	±0.18	Weak	A47	0.646	±0.05	Moderate
A19	0.396	±0.25	Moderate	A48	0.579	±0.12	Moderate
A20	0.194	±0.01	Weak	A49	1.531	±0.48	Strong
A21	0.548	±0.14	Moderate	A50	0.248	±0.03	Weak
A23	0.323	±0.07	Weak	A51	0.252	±0.07	Weak
A24	0.229	±0.07	Weak	A52	0.499	±0.08	Moderate
A25	0.7	±0.14	Strong	A53	0.453	±0.09	Moderate
A26	0.28	±0.07	Weak	A54	0.467	±0.08	Moderate
A27	0.649	±0.13	Moderate	A55	0.38	±0.11	Moderate
A28	0.462	±0.02	Moderate	A56	1.268	±0.11	Strong
A29	0.597	±0.23	Moderate	Control	0.150	±0.005	

Almost all *S. haemolyticus* isolates were found to be biofilm producers and these results were compatible with the local study by Al-mousawi *et al*. [38] who concluded that the bacteria can produce strong biofilm. Also, in line with Alhusain *et al.s* study which revealed that all the isolates of *S. haemolyticus* were capable of yielding biofilms, one of which displayed a high accumulation of biofilm (O.D 0.7) [39].

The current study is also quite similar to a study by Fredheim *et. al.* [40] who found that 74% of the *S. haemolyticus* tested isolates could produce biofilms. In contrast another local study by Khudheir [32] showed that strong biofilm producers were 10 (52.6%), weak biofilm were 2 10.6% and 7 (36.8%) were non-biofilm producers. These results disagreed with this current study.

Variations in biofilm development is caused by the presence of associated genes, as well as different phenotypic methods, could be attributed to the influence of different culture media, pH, temperature and osmotic pressure. Stress and host immune system chemicals may also play a role in discrepancies between biofilm production *in vivo* and *in vitro* [41].

Biosynthesis of Copper Oxide Nanoparticles

Copper oxide nanoparticles were biosynthesized from *S. haemolyticus*. A yellow to blue color change and the production of a light blue precipitate were indicators of the formation of nanoparticles. After centrifugation, the precipitate was greenish blue in color, and after microwave drying, a shiny blue powder was obtained (Figure 7). In recent years, an obvious coordination to use bacteria to synthesizes nanomaterials (mainly silver, zinc, gold and copper) with remarkable properties has been observed in order to develop antimicrobials with *in vitro* activities against pathogenic bacteria other than antibiotics [42].

Bacteria is an easy culturing microorganism with short generation time, a characteristic that makes bacteria an ideal source for nanoparticle synthesis as they have extra cellular reduction enzymes [43].

Several bacterial types such as *Escherichia coli*, *Pseudomonas fluoresces, Serratia sp*., and *Pseudomonas stutzeri* proved their ability to reduce certain metal ions and produce nanoparticles [44].

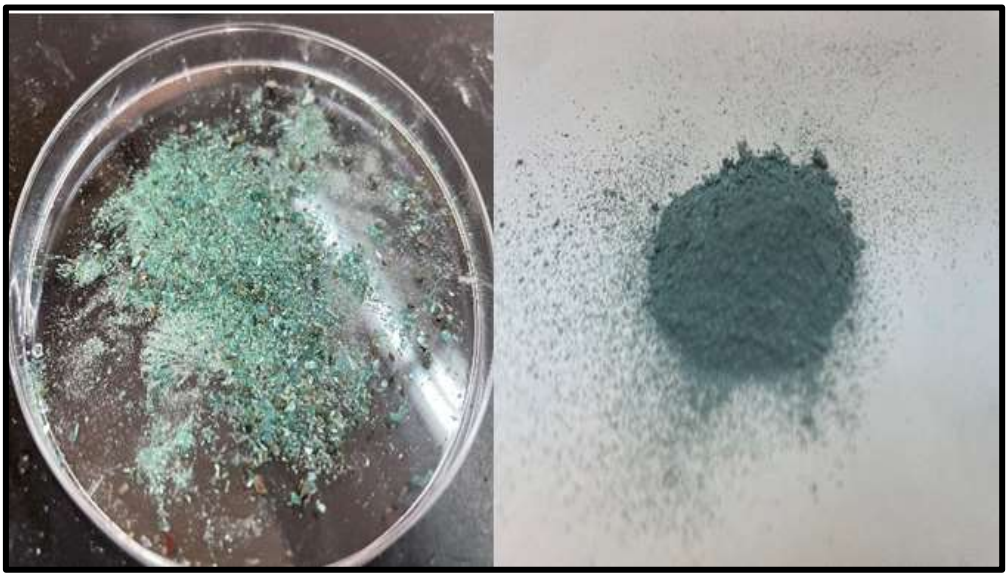


Figure 7: Biosynthesis of CuO nanoparticles by *S. haemolyticus*

Characterization of CuO NPs:

1- UV–Vis Spectral Analysis

Based on the findings, biosynthesized CuoNps displayed a highest peak (absorption peak) at 275 nm. (Figure 8). However, smaller particle size was indicated previously at sharp peak observation at 270 nm as reported by Turakhia *et. al.* [45]. While absorption spectroscopy UV examination of zinc oxide showed a high absorption band at approximately 355 nm [46].

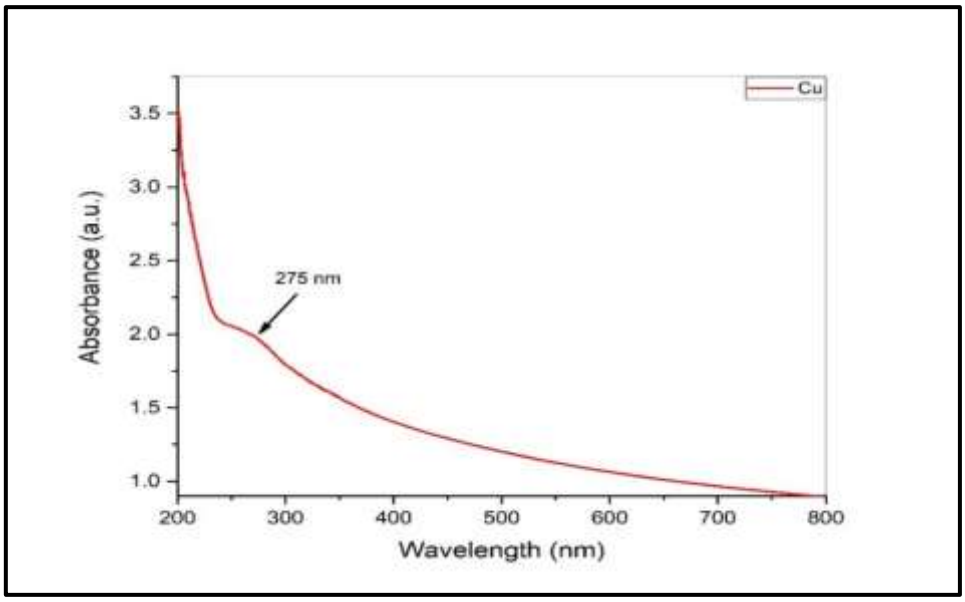


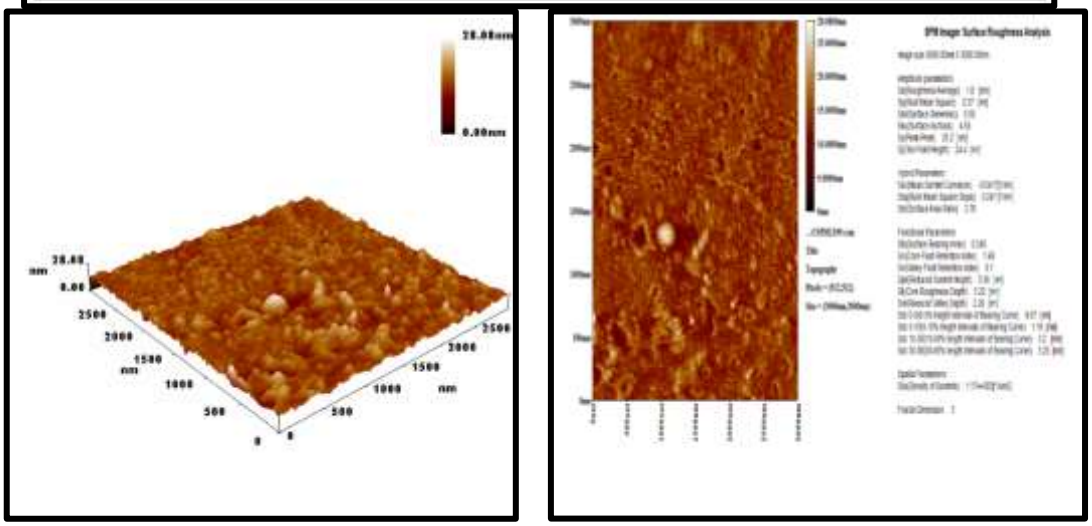
Figure 8: UV–Vis spectrophotometry of synergistic CuO Nps

2- Atomic Force Microscopy (AFM) Analysis

AFM was used to identify and characterize nanoparticle distributions in CuONPs. The copper nanoparticles in Figure 9a and Table 2) have an average diameter of 35 nm. According to the two-dimensional and three-dimensional images of all CuONPs, all the nanoparticles were found to be of same size and shape (Figure 9b) [47].

Table 2: The cumulation size of copper oxide nanoparticles biosynthesis by *S. hamolyticus* measured by AFM technique.

Sample: Sample Name	**Code:**Sample Code
Line No.:lineno	**Grain No.:**6909
Instrument:SPM	**Date:**2022-11-22
Avg. Diameter:35.48 nm	**<=10% Diameter:18.00 nm**
<=50% Diameter:32.00 nm	**<=90% Diameter:56.00 nm**

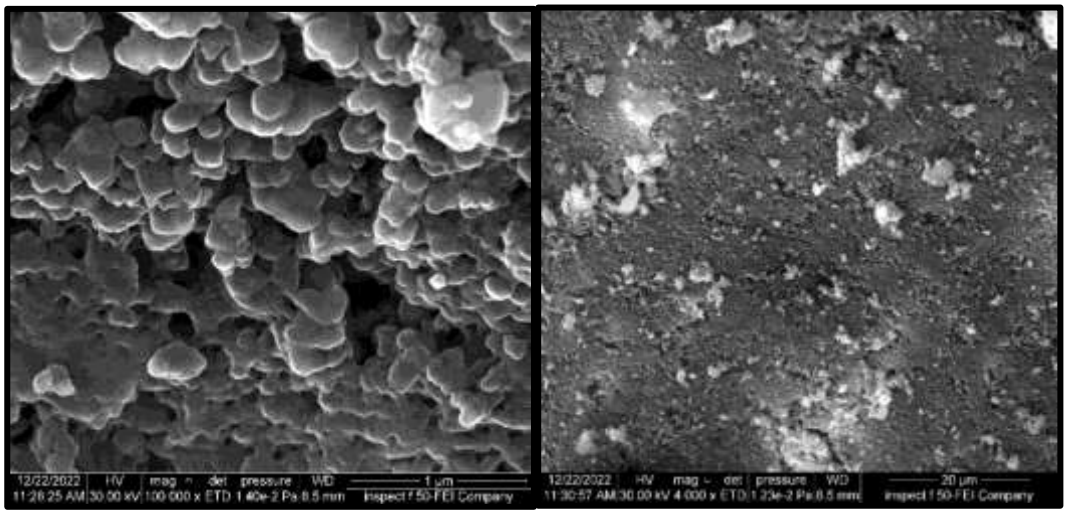


(a) **(b)**

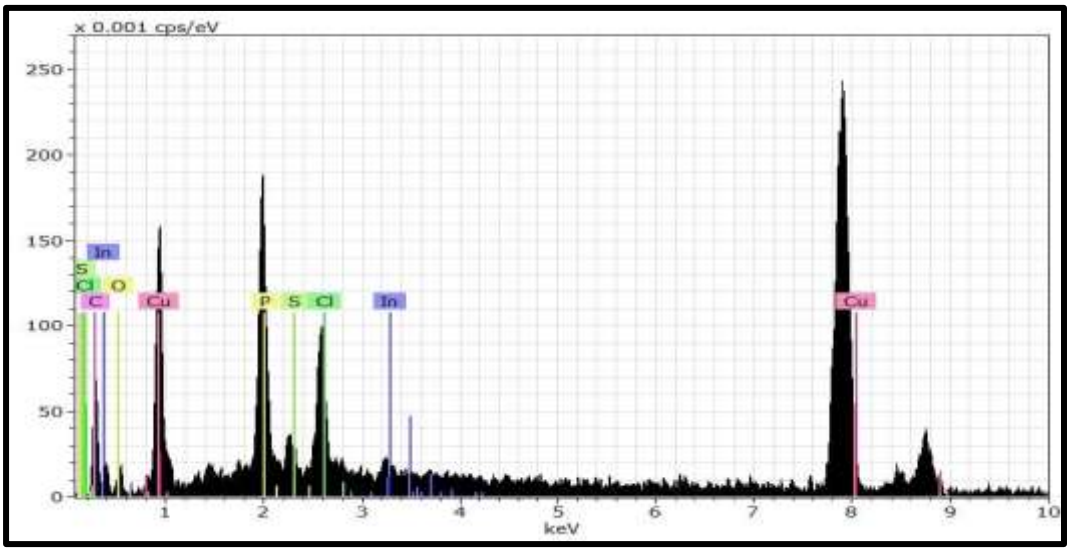
Figure 9: AFM images of CuONPs. (a) Average diameter of CuONPs(b) Two-dimensional image (2D) and three-dimensional image (3D).

3- Field Emission Scanning Electron Microscope (FESEM) and Energy dispersive X-ray (EDX)

The elemental and structural composition of NPs samples, as well as their morphology and size, were all examined using SEM analysis. Figure 10a shows the spherical shape of CuO NPs at 3000kv and 40000 magnification power. SEM images revealed that it was essentially spherical and uniform in appearance with the diameter ranging from 15 to 19nm. In comparison with EDX-ray, FESEM allowed to determine the presence of different components in the examined model [48]. The CuONPs components were examined using EDX. The results showed the EDX spectra together with the major elemental peak at 8 keV that is unique to the Cu metal and the composition percentage. Other additional minor peaks for *S. haemolyticus* biomolecules were detected [49] (Figure 10b).



(a)



(b)

Figure 10: a) SEM Images of CuO NPs b) EDX of CuO NPs

4- X-ray Diffraction (XRD)

The XRD spectra of CuONps powder is shown in Figure 11 which confirmed formation of hexagonal structure of the CuO NPs by revealing six prominent peaks that corresponded to the diffraction peaks (16.414, 18.414, 22.278, 23.983, 26.113, and 43.537).

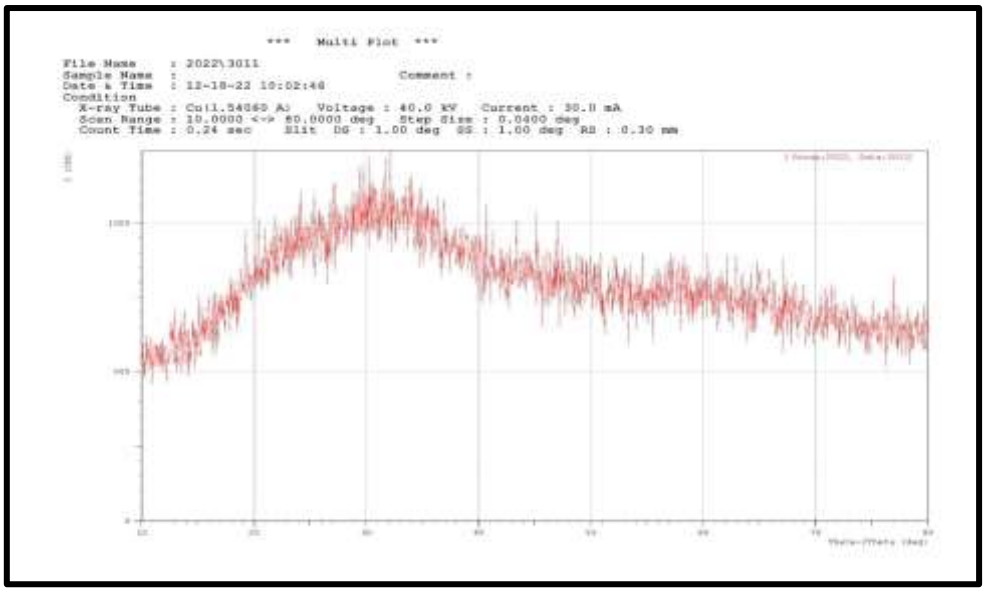


Figure 11: XRD analysis of green synthesized CuO NPs

According to a recent study, manufactured copper nanoparticles exhibited the same peaks on their XRD patterns as copper nanoparticle samples that were exposed to air for 24 hours [50]. The crystalline point characterization was carried out by comparison with crystallographic standard data, according to the International Center of Diffraction Data.

5- Zeta Potential (ZP) :

Zeta potential is considered to be an important indication of a colloid internal stability. It should be mentioned that CuNPs particles were considered stable if they were more positive than +30 mV or more negative than 30 mV. It may be concluded that the biologically produced CuONPs were remarkably stable based on Figure 12 which displays the zeta potential of the CuONPs in this work to be -22.47 mV [51].

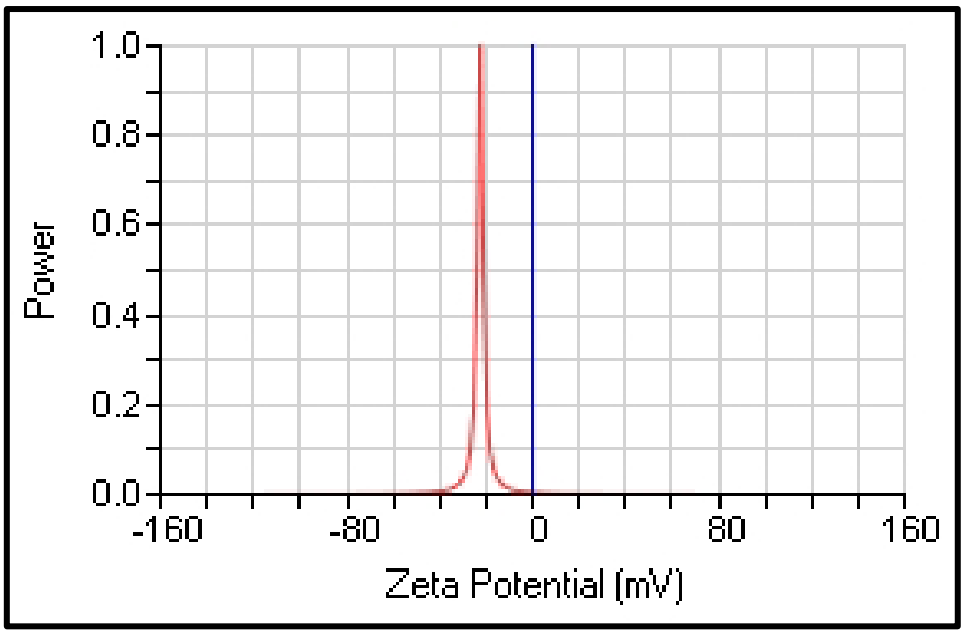


Figure 12: Zeta potential of the green synthesized CuO NPs.

Antibacterial Activity of CuONPs

From the diameters of zones of inhibition observed in Figure 13, it can be stated that the CuO nanoparticles had significant antibacterial activity against *S. haemolyticus*.

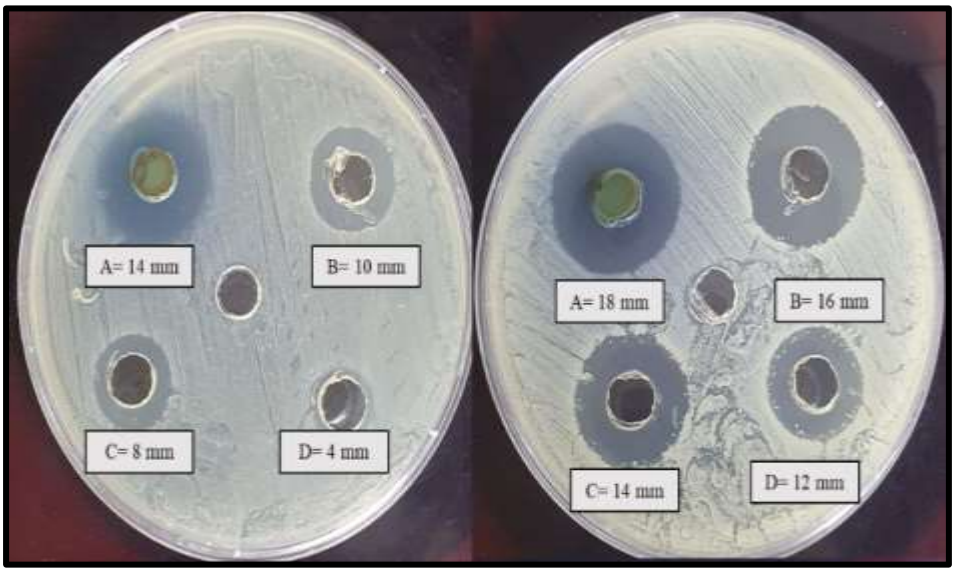


Figure 13: Antibacterial activity of CuONPs against *S. haemolyticus* isolates (A, 64 µg/ml; B, 32 µg/ml; C, 16 µg/ml; D, 4 µg/ml)

According to the findings, CuONPs exhibited an increase in inhibition zone diameter proportional to concentration. The maximum inhibitory zone was seen at a concentration of 64 g/ml, with a diameter equal to 14 and 18 mm respectively. Vancomycin, used as a positive control, displayed an inhibition zone of 10 mm and 14 mm respectively, at a concentration of 64 g/ml. A previous research points to various pathways for metallic NPs antibacterial activity. CuONPs cause a dramatic decline in the integrity of bacterial cell membrane which leads to the leakage of several cytoplasmic macromolecules such as protein, amino acids and carbohydrates, which is the major cause of bacterial cell death [52]. According to another study, exposure to CuONPs and the subsequent release of reactive oxygen species (ROS) would result in the generation of superoxide species, which then facilitates the subsequent destruction of biomolecules (lipid peroxidation and protein oxidation) [53].

Minimum Inhibitory Concentration of Vancomycin and CuONPs

The lowest MIC of vancomycin and CuONPs for two *S. haemolyticus* isolates from semen (A30) and urine (A42) was determined for each isolate (Table 3).

Table 3: The minimal inhibitory concentration of vancomycin and CuONPs.

Bacterial isolate NO.	MIC of Vancomycin (µg/ml)	MIC of CuONPs (µg/ml)
1(A30)	32	32
2(A42)	2	4

The CuONP concentrations, regarded as MIC, are the ones where no detectable growth is observed. In Muller Hinton broth, higher CuONP concentrations (64 g/ml) had a consequence effect on bacterial cell viability and resulted in a 100% reduction in optical density. According to Amiri *et al*. [54], CuO NPs MIC50 value was found to be between 1 and 10 g/ml. This finding is in line with previous studies on the minimum inhibitory concentration of CuONPs.

Determination of Biofilm Formation after CuONPs Treatment

Biofilm production significantly reduced after 24 hours of incubation in 37 °C with MIC of vancomycin and CuONPs. OD measurement of the same strains of *S. haemolyticus* decreased from strong to weak production. The % inhibition of biofilm formation induced by vancomycin and CuONPs was calculated as follows: Percentage inhibition = 100 - [OD after treatment / OD before treatment x 100] (Table 3 a & b and Figure 15).

Table 4: Antibiofilm activity of a) Vancomycin and b) CuONPs.

a)

Bacterial Isolate NO.	Biofilm Capacity before Vancomycin Treatment	Biofilm Capacity after Vancomycin Treatment	O.D before Vancomycin Treatment ± SD	O.D after Vancomycin Treatment ± SD	Percentage of Inhibition
1(A30)	Strong	Weak	0.773 ± 0.19	0.197 ± 0.08	74.6%
2(A42)	Moderate	Weak	0.367 ± 0.004	0.121 ± 0.01	67.1%

b)

Bacterial isolate NO.	Biofilm capacity before CuONPs treatment	Biofilm capacity after CuONPs treatment	O.D before CuONPs treatment ± SD	O.D after CuONPs treatment ± SD	Percentage of inhibiton
1(A30)	Strong	Weak	0.773 ± 0.19	0.141 ± 0.02	81.8%
2(A42)	Moderate	Weak	0.367 ± 0.004	0.131 ± 0.006	64.4%

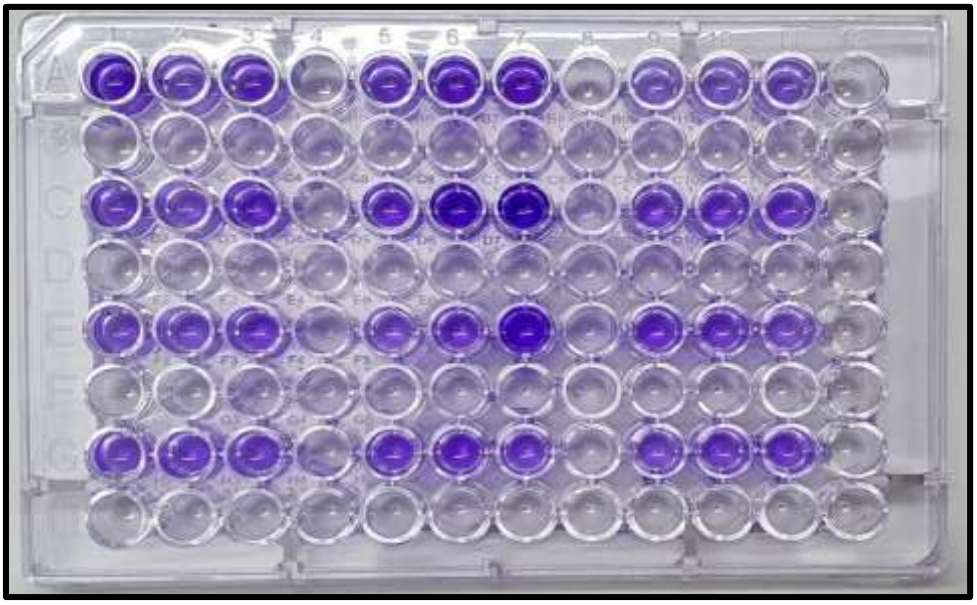


Figure 14: Biofilm production after treatment with CuONPs MIC.

Vancomycin reduced biofilm mass of *S. haemolyticus* isolates by 74.6% and 67.1% respectively. We observed that vancomycin lowered the biofilm formation of vancomycin resistant *S. haemolyticus* (VRSH) with efficiency lesser than CuO NPs biofilm inhibition capacity. Vancomycin does not completely penetrate staphylococcal biofilms, according to previous studies. Since the components of biofilms are negatively charged. It is thought that the electrostatic interaction between antimicrobial agents and biofilms plays a key role in determining the permeability of biofilms. Under physiological circumstances, vancomycin has a positive charge [55].

Antimicrobial action can be explained due to the small particle size of CuONPs because smaller materials can enter bacterial cell membranes and cause cell damage. One research detailed that NPs seem to be more effective when they are smaller than 39 nm of diameter because they have a greater surface of interaction and in turn can reach the intra cellular space of bacteria straightforwardly [56]. According to Dehkordi *et al*. [57] findings the inhibition of bacterial adhesion by CuONP led to significant reduction in biofilm formation on surfaces coated with NPs which could be attributed to their inhibitory effect.

Cytotoxic Effect of CuONPs

The cytotoxic effect of copper oxide nanoparticles on the PC3 cell line was also investigated (Figure 15). PC3 cells are a prostate-cancer-derived cell line. The IC50 value was around 61.25 g/mL. These NPs had toxic effects on the BC3 cell line, and at a concentration of more than 400 g/mL, the cytotoxic effects were about 66.4%.

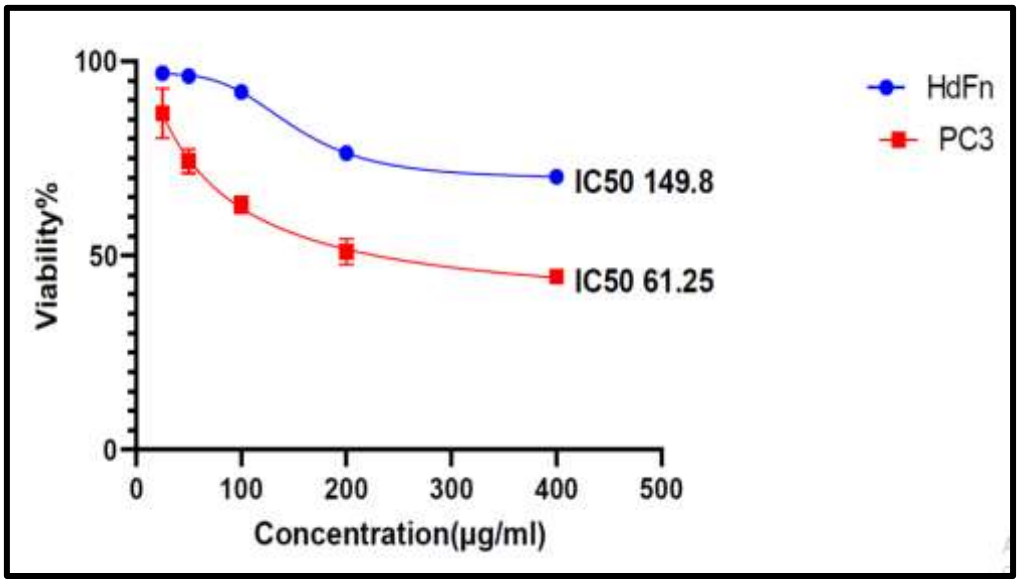


Figure 15: Cytotoxic effects of CuO NPs on HdFn cells and PC3 cells.

CuONPs were tested for *in vitro* cytotoxicity against PC3 cell lines at 25, 50, 100, 200, and 400 g/ml concentrations which resulted in cellular toxicity values of 86.65, 74.3, 63.08, 50.9, and 44.5% respectively. The findings demonstrated that CuONPs have strong cytotoxic effect on cancerous cells (PC3 cell line) [58].

Conclusions

Multi-resistant bacterial strains have created an alarming situation that has encouraged the development of new therapies for infections and diseases brought on by bacterial biofilms. CuONPs were effectively created using a simple, direct, low-cost, high yield and environmentally friendly approach. When tested against several *S. haemolyticus* isolates, CuONPs showed an outstanding antimicrobial activity. In order to stop biofilm-producing microbes from attaching to and colonizing indwelling medical devices, surfaces on a variety of substrates can be coated with CuONPs. The nature of nanoparticle-biofilm interactions can be modulated by making copper oxide nanoparticles extremely selective towards one component of the biofilm via bioconjugation technologies.

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