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Anti-Virulence and Cytotoxic Behavior of Copper Nanoparticles against antibiotics-resistant *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is one of the most common and distributed pathogens. *S. aureus* was resistant to 11 antibiotics including four categories based on their functions. Synthesis of copper nanoparticles (CuNPs) from $CuSO_4.5H_2O$ using ascorbic acid was performed. Properties of CuNPs using UV and TEM were measured. Results of characterization observed absorption peak at 592 nm and CuNPs cubic in shape with average size 3.5 nm. The activity of CuNPs as bactericidal agent against *S. aureus* was determined using agar well diffusion method. CuNPs showed greater antibacterial activity and MIC was 40 µg/ml. The anti-biofilm activity of CuNPs was assayed in 96-well polystyrene plates and 3 ml polyethylene tube. Obviously, remarkable progressive inhibition of biofilm formation was noticed with increasing of CuNPs concentrations. Determination of CuNPs cytotoxicity on WI-38 cells using MTT protocol was performed. The toxicity observed at concentration 62.5 was 23% with IC50 value 134.65 µg ml^{-1}.

Key words: CuNPs, *Staphylococcus aureus*, bactericidal, Biofilm, cytotoxicity.

1. Introduction

Staphylococcus aureus is one of the most virulent Staphylococci species encountered. Myocarditis, osteomyelitis, bacteremia, pneumonia, pericarditis, meningitis, encephalitis, mastitis, and scalded skin syndrome are all caused by it [1]. Multidrug-resistant *S. aureus*, first identified in the 1960s, emerged as a source of hospital infections in the latter period, implicated in rapidly progressing latent deadly diseases such as hostile life pneumonia, fasciitis, osteomyelitis, severe sepsis, endocarditis, necrotizing and toxinoses. A safe and effective alternative is critical given the constant emergence of life-threatening resistant pathogens [2].

Biofilm infections are notoriously difficult to eliminate, even more so when multidrug resistant organisms are involved [3]. Recent years have shown a rise in the number of infections associated with emerging antibiotic resistance bacteria [4]. Surprisingly, interactions between bacteria and hosts have been shown to increase the probability of infections in which pathogens swiftly kill the host. On medical equipment such as catheters, artificial heart valves, and prosthetic joints, both Gram-positive and Gram-negative bacteria can develop a biofilm [5]. Biofilm-associated diseases are often chronic infections with a slow growth rate, and they are capable of resisting both the host's immune system and a transitory response to antimicrobial therapy [6].

Due to the visual, catalytic, mechanical, and electrical capabilities of copper nanoparticles, they have garnered substantial attention. Additionally, its low cost, excellent yields, and short reaction periods under standard reaction conditions are benefits in the creation of green nanoparticles [7]. Copper nanoparticles offer a wide range of uses as superstrong materials, sensors, and antibacterial materials due to their high reactivity and ability to interact well with other materials. Numerous studies have established copper nanoparticles' superior antibacterial efficacy against a variety of bacterial and fungal species. [8].

Copper-based active powders or pigments in fabrics, paints, or coatings [9], aqueous copper solutions [10], complex copper species [11], or copper-containing polymers have all been used as antifungal agents due to their antibacterial activity. The antibacterial mechanism of nanomaterials can be understood by examining their particular binding to

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microorganisms' surfaces and subsequent metabolism within microorganisms. There is currently a dearth of information regarding the antibacterial capabilities of copper-based nanoparticles and nanocomposites [12].

The toxicological effects are a major concern as they are highly reactive, cause oxidative stress, and generate free radicals [13]. Metal oxides NPs have been receiving considerable attention for a large variety of applications such as optoelectronics, nanodevices, nanoelectronics, nanosensors, information storage, and catalysis [14]. However, these nanomaterials are potential toxicants and few trials have been conducted to evaluate their ecotoxicity in biological systems [15]. NPs of some metal oxides can pass through physiological barriers resulting in an increase in inflammatory responses, and can cause severe damage in DNA and protein structures, therefore causing mutations [16].

At this research, authors aimed to synthesis the CuNPs using chemical method. tested the CuNPs antibacterial efficient, anti-virulence as antibiofilm against *S. aureus*. Our study involves the cytotoxicity of CuNPs on human cell lines.

2. Experimental

2.1. *Staphylococcus aureus* and antibiotic susceptibility test

The clinical isolate *Staphylococcus aureus* identified using biochemical and molecular methods in pervious study, and then sub cultured on mannitol salt agar and blood agar medium. The antibiotic resistance test of the *S. aureus* strain was carried according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) was examined using a disc diffusion method [17]. The following 24 antibiotics covered the main four categories based on their targets cell wall, protein synthesis, DNA synthesis and nucleic acid targeting were tested: kanamycin, vancomycin, novobiocin, cefadroxil, oxacillin, azithromycin, linezolid, ertapenem, tobramycin, cefuroxime, linomycin, rifamycin, cefotaxime, cephradine ceftazidime, streptomycin, ceftriaxone, amoxicillin, cefadroxil, erythromycin, cephalothin and cephalexin. Tested plates were incubated at 37°C for 24 h, and the results were read and reported based on the inhibition zone diameter as sensitive or resistant [18].

2.2. Preparation and Characterization of CuNPs

Chemical reduction was used to manufacture the CuNPs, with $CuSO_4.5H_2O$ as the precursor salt and starch as the capping agent. The preparation procedure begins by vigorously swirling 0.1 M $CuSO_4.5H_2O$ solution into 120 mL of starch (1.2 percent) solution for 30 minutes. The second step involves adding 50 mL of 0.2 M ascorbic acid solution to the synthesis solution while rapidly swirling continuously. Following that, 30 mL of a 1 M sodium hydroxide solution was gradually added to the produced solution while stirring constantly and heated to 80^o C for 2 hours. The solution's colour changed from yellow to ocher. After the reaction was complete, the solution was removed from the heat and allowed to settle overnight before discarding the supernatant solution gingerly. The precipitates were filtered from the solution and washed three times with deionized water and ethanol to remove excess starch attached to the nanoparticles. Nanoparticles were dried and then stored in a glass vial for subsequent investigation.

UV–Vis spectrophotometry was used to determine the absorbance of CuNPs over a range of 300–800 nm until no further variations in absorbance were recorded. Transmission electron microscopy (TEM) images provide detailed information about the morphological aspects of objects, such as their size and shape.

2.3. Antibacterial activity and MIC of CuNPs against *Staphylococcus aureus*

The antibacterial activity of CuNPs against *Staphylococcus aureus* was determined using agar well diffusion method [19]. A pure microbial colony of *S. aureus* was allowed to grow in nutrient broth medium. *S. aureus* suspensions were spread on the nutrient agar plates using a sterile cotton swab, and then wells were made using a sterilized cork borer, and CuNPs concentrations (0, 10, 20, 30, 40 and 50 µg/ml) were added. Plates were incubated for 24h at 37 ^{o}C and finally the inhibition zones were measured.

2.4. Anti-biofilm Assay of CuNPs using two methods

As previously described [20], an antibiofilm experiment of CuNPs was done using a 3 ml polyethylene tube. CuNPs were injected into *S. aureus* cells at concentrations of 0, 10, 20, 30, 40, and 50 g/ml and incubated for 24 hours static. Crystal violet was used to stain polyethylene tubes, and the findings provided are the averages of at least three repeats.

As previously described [21], static biofilm development was measured on 96-well polystyrene plates. To summarise, cells with a turbidity of 0.05 at 600 nm in Sabouraud broth were inoculated and cultivated for 24 hours at 37^{o}C with or without CuNPs. To assess total biofilm production, biofilms in 96-well plates were dyed for 20 minutes with 0.1 percent crystal violet, dissolved in 95 percent ethanol, and their absorbance at 570 nm was measured. Additionally, cell proliferation was determined at 620 nm in 96-well plates.

2.5. MTT assay of CuNPs

Determination of CuNPs cytotoxicity on Normal Homo sapiens, lung tissue fibroblast (WI-38) cells using MTT protocol was performed. The method was done as follow: - To grow a full monolayer sheet, the tissue culture plate of 96 well was inoculated with 1 X 10^5 cells/ml (100 µl / well) and incubated at 37^{o}C for 24 hours. After forming a confluent sheet of cells,

growth medium was poured from 96 well microtiter plates, and with wash media the cell monolayer was washed twice. The d dilutions of the checked sample were made in RPMI medium. Three wells were left as controls, receiving only maintenance medium, and 0.1 ml of each dilution was measured in separate wells. The plate was incubated and tested at 37°C. Any physical symptoms of toxicity, such as partial or complete loss of the cell granulation, rounding, shrinkage, or monolayer were examined in the cells. The solution (5 mg/ml in PBS) was prepared. Then a solution in the amount of 20 ul was provided to each well. Then, place on a shaking table at 150 rpm for 5 minutes. Then, to allow the MTT to be metabolized incubate at (37^oC, 5% CO_2) for 1-5 hours. The media dump off (to remove residue if necessary). In 200 ul DMSO, resuspend formazan (MTT metabolic product). Then, mix the formazan into the solvent, shaking for 5 minutes at 150 rpm. At 560 nm, read the optical density at 620 nm [22].

3. Results and Discussion

3.1. *Staphylococcus aureus* antibiotics sensitivity

Staphylococcus aureus was resistant to 11 antibiotics including four categories based on their functions. Moderate resist observed to 8 antibiotics, and finally studied strain revealed sensitive to only 4 antibiotics Azithromycin, Fusaric acid, Piperacillin tazobactam and Linezolid with inhibition diameter 3.0, 2.66 , 2.63 and 3.2 cm respectively.

Table 1: Antibiotics sensitivity of *Staphylococcus aureus*

Antibiotic	Disc potency(µg)	symbol	I Z diameter (cm)
Azithromycin	**15**	**AZM**	**3.06 (S)**
Erythromycin	**15**	**E**	**2.4 (M)**
Streptomycin	**10**	**S**	**1.76 (M)**
Vancomycin	**30**	**VA**	**2.1 (M)**
Fusaric acid	**10**	**FA**	**2.66 (S)**
Novobiocin	**30**	**NV**	**2.2 (M)**
Piperacillin tazobactam	**110**	**TZP**	**2.63 (S)**
Kanamycin	**30**	**K**	**2.53 (M)**
Linezolid	**30**	**LZD**	**3.2 (S)**
Rifamycin	**30**	**RF**	**1.7 (M)**
Tobramycin	**10**	**TOB**	**2.06 (M)**
Cephalexin	**30**	**CL**	**0.93 (M)**
Oxacillin	**1**	**OX**	**– R**
Amoxicillin	**25**	**AX**	**– R**
Cephalothin	**30**	**KF**	**– R**
Cefuroxime	**30**	**CXM**	**– R**
Ceftriaxone	**30**	**CRO**	**– R**
Ertapenem	**10**	**ETP**	**– R**
Lincomycin	**2**	**L**	**– R**
Cefotaxime	**30**	**CTX**	**– R**
Ceftazidime	**30**	**CAZ**	**– R**
Cefpirome	**30**	**CE**	**– R**
Cefadroxil	**30**	**CFR**	**– R**

3.2. Preparation and Characterization of CuNPs

This spectra is obtained shortly following particle production. At 592 nm, an absorption peak was seen, indicating the production of CuNPs in solution [23]. The shape of produced nanoparticles was investigated using TEM examination. Fig. 1 illustrates the morphology of the Cu nanoparticles. The TEM images revealed that the produced CuNPs are cubic in shape and ranged in size from 2 to 5 nm with an average of 3.5 nm. Stabilized nanoparticles may also cluster and gravitate toward one another. Individual nanoparticles, on the other hand, are contained in the stabilising agent and can be re-dispersed. Stabilizers are critical in controlling the particle size distribution and limiting clustering and flocculation. The current study reveals that starch is an excellent capping agent for the synthesis of nanoparticles of various sizes. The structure of nanoparticles is determined by the competition between several processes such as growth, nucleation, aggregation, and impurity adsorption. [24].

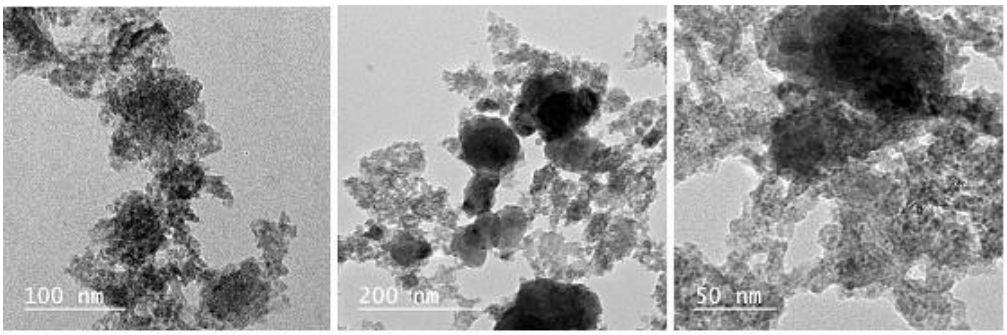
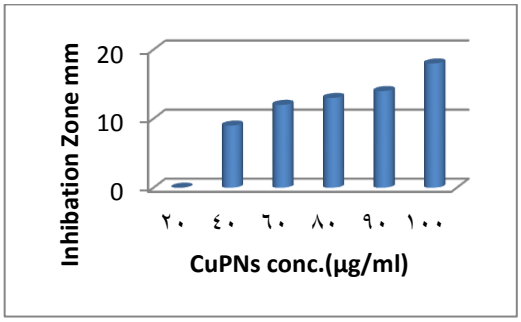
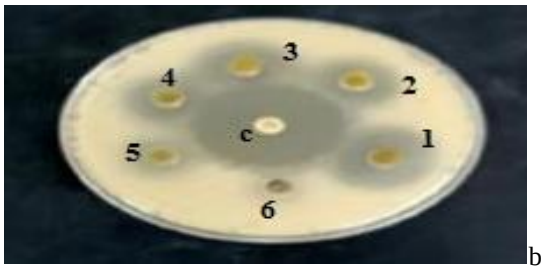


Figure 1: TEM images of CuNPs.



a



b

Figure 2: a. The MIC activity of CuNPs against *Staphylococcus aureus*,b. photo of MIC plate with antibiotic control disc Azithromycin CuNPs concentrations (100, 90, 80, 60, 40, and 20 µg/ml) coded from 1 to 6 respectively.

3.3. Antibacterial and MIC activity of CuNPs

In general, CuNPs synthesized from $CuSO_4.5H_2O$ showed greater antibacterial activity with inhibition zone diameter 18 mm at 100 µg/ml compared to those made with the antibiotic control Azithromycin 30 mm. The results, of MIC, showed that the inhibition zone decreased with lower CuNPs concentrations (100, 90, 80, 60, 40, and 20 µg/ml) coded from 1 to 6 respectively, until 40 µg/ml with 9 mm zone of inhibition that consider MIC for CuNPs studied.

3.4. Inhibition of *Staphylococcus aureus* biofilm formation

The capacity of CuNPs to suppress the formation of Staphylococcus aureus biofilms was determined using a microtiter plate. CuNPs were used in this investigation to inhibit biofilm formation by biofilm-producing S. aureus. Clearly, a notable gradual suppression of biofilm formation was observed as CuNPs concentrations increased (figure 3). In comparison to (C) without CuNPs, the test tube approach demonstrated that concentrations of 20 to 35 g/ml can suppress biofilm growth. Similar antibiofilm activity was seen in free-living bacteria, where Gram-positive biofilms were more susceptible to CuNPs at various doses than Gram-negative biofilms. The antibiofilm activity of biofabricated CuNPs can be exerted in a series of sequential stages beginning with the inhibition of planktonic forms (the initial stage of biofilm formation), followed by the inhibition of exopolysaccharide formation that adheres to and aggregates sessile cells (the second stage), and finally by the inhibition of quorum sensing activity (the third stage) [25].

3.5. Cytotoxicity of CuNPs on human cell lines

The synthesized CuNPs cytotoxicity was tested *in vitro* on lung tissue fibroblast WI-38 using MTT to explore their selective toxicity towards bacterial cells over mammalian cells with various concentrations like 31.25, 62.5, 125, 250, 500 and 1000 μgml^{-1} respectively. The toxicity observed at concentration 62.5 was 23% with IC50 value 134.65 μg ml^{-1} table 2. Results showed that toxicity increases when CuNPs concentration increases. These results suggest CuNPs cause cytotoxicity above IC50 62.5 μg ml−1.

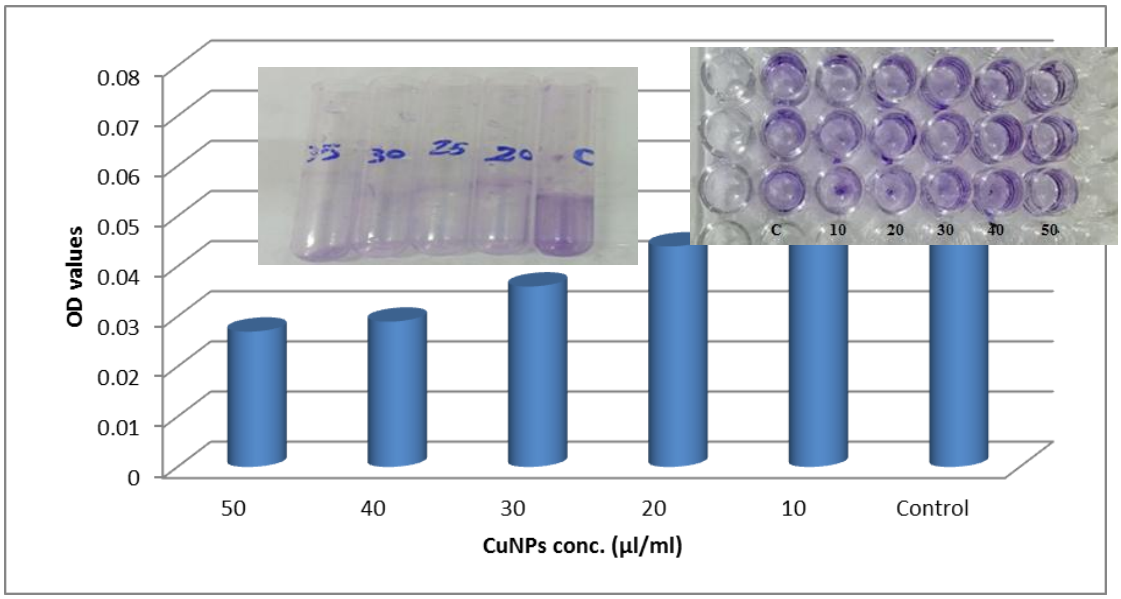


Figure 3: Biofilm inhibition effect of Cu NPs

Examination was performed using microscopy to noticeable change in morphology of cells as appeared in figures 4, cell shape in the control remained normal; the cells adhered well, with most attaching. Most cells were polygonal, with transparent cytoplasm, better scattering and a few newly cells during the process of adhering. Although cells adhered, they could not spread, and some lost the polygonal and became rounded shape. Viability assays are essential for evaluating the cellular response to toxicants. Cytotoxicity of CuNPs against WI-38 Cell Line was carried out to determine the side effect on normal cells. The ratio of IC50 and antibacterial concentration, suggest that CuNPs have a higher selectivity value, means the nanoparticles were toxic for pathogenic bacteria and save for human cells.

Conclusion

The cubic CuNPs in average 3.5 nm are more effective at minimum inhibitory concentration against *S. aureus*, which resist too many antibiotics. The CuNPs have impact as anti-virulence and inhibit *S. aureus* biofilm formation. The CuNPs concentrations used as bactericidal and anti-biofilm were observed lees than toxic concentration at IC50 value 134.65 μg ml^{-1}.

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Consent for Publication

Not applicable.

Conflict of Interests

The authors declare no conflict of interests, financial or otherwise

CuNPs Conc. μg /ml	Toxicity %	IC50 μg /ml
1000	94.20731707	134.65
500	93.29268293	
250	89.93902439	
125	58.84146341	
62.5	23.57723577	
31.25	0	

Table 2: Effect of CuNPs on the Viability of Wi38 cell line

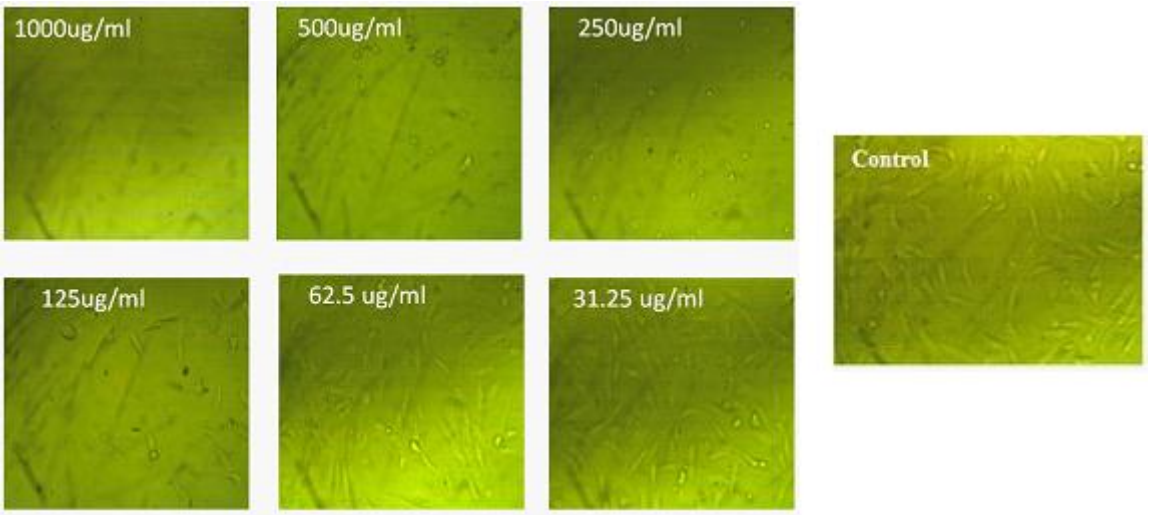

Figure 4: Morphology changes in Wi38 cells after exposure to CuNPs

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