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Anti-capsular activity of CuO nanoparticles against *Acinetobacter baumannii* produce efflux pump

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ABSTRACT

Copper oxide nanoparticles are modern kinds of antimicrobials, which may get a lot of interest in the clinical application. This study aimed to detect the anti-capsular activity of CuO nanoparticles against *Acinetobacter baumannii* produce efflux pump. Thirty-four different clinical *A. baumannii* isolates were collected and identified by the phenotypic and genetic methods by the *recA* gene as housekeeping. Antibiotic sensitivity and biofilm-forming ability, capsular formation were carried out. The effect of CuO nanoparticles on capsular isolates was detected, the synergistic effects of a combination CuO nanoparticles and gentamicin against *A. baumannii* were determined by micro broth checkerboard method, and the effect of CuO nanoparticles on the expression of *ptk*, *espA* and *mexX* genes was analyzed. Results demonstrated that CuO nanoparticles with gentamicin revealed a synergistic effect. Gene expression results show reducing the expression of these capsular genes by CuO nanoparticles is major conduct over reducing *A. baumannii* capsular action. Furthermore, results proved that there was a relationship between the capsule-forming ability and the absence of biofilm-forming ability. As bacterial isolates which were negative biofilm formation were positive in capsule formation and vice versa. In conclusion, CuO nanoparticles have the potential to be used as an anti-capsular agent against *A. baumannii*, and their combination with gentamicin can enhance their antimicrobial effect. The study also suggests that the absence of biofilm formation may be associated with the presence of capsule formation in *A. baumannii*. These findings provide a basis for further research on the use of CuO nanoparticles as a novel antimicrobial agent against *A. baumannii* and other bacterial pathogens, also to investigate the potential of CuO nanoparticles to inhibit the production of efflux pumps in *A. baumannii*, which are a major mechanism of antibiotic resistance.

1. Introduction

Among common species found in the environment such as water, soil, animals, foods and sewage. *Acinetobacter*, where some of its species are found which are associated with various habitats moist tissues are infected by *A. baumannii* throughout wound or injury. For that, there is an urgent need to enhance antimicrobial effects against pathogenic bacteria [1]. Nanoparticle applications as therapeutic agents are enhanced recently [2]. The major reason for hospital-acquired infections is *A. baumannii* [3]. Efflux pumps are proteins located on the cell membrane of bacteria that can pump out antibiotics and other toxic compounds, reducing their effectiveness [3].

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Virulence factors of *A. baumannii* include systems of siderophore-mediated iron acquisition, motility, biofilm formation and the capacity of acquiring and rearrangement of genetic determinants. These factors are involved in the infection process and pathobiology, like binding to host cells damage of cells, serum resistance and invasion. The major reason of *A. baumannii* virulence is surface carbohydrates which include capsular polysaccharides, lipo-polysaccharides and poly-β-(1–6)-*N*-acetylglucosamine (exopolysaccharide (PNAG)). Bacterial cells are enveloped by a layer composed of high molecular weight hydrophilic polymers. In the case of *A. baumannii* capsules, the structure is composed of tightly packed repeating oligosaccharide subunits (K units), typically consisting of 4–6 sugars [4,5]. The capsules represent a protective shield against immune components as they limit the interactions between pathogenic immunogenic surface structures and defenses of the host, that's leading to invasion of the immune system and serum resistance [6]. Furthermore, the production of the capsule participates in anti-bacteriolytic and anti-phagocytic activity, the capsule prevents phagocytes from adhering because of the negatively charged capsule, so, the capsule is an important virulence factor for *Acinetobacter* and understanding its role in infection can inform strategies for prevention and treatment [7,8]. The capsule of *Acinetobacter* is a protective layer that surrounds the bacterial cell. It helps the bacteria to evade the host's immune system and resist the effects of antibiotics. This capsule has been linked to the persistence of *Acinetobacter* infections, which are difficult to treat and can persist for long periods of time despite antibiotic treatment. The capsule provides a physical barrier that prevents antibiotics from penetrating the bacterial cell, allowing the bacteria to survive and continue to cause infection. Additionally, the capsule can also prevent the host's immune system from recognizing and attacking the bacteria, further contributing to persistence [6]. Many genes play an essential role in adhesion and capsule formation including *ptk* gene, The *Ptk* gene encodes for a protein tyrosine kinase found in the cytoplasm, while the *EpsA* gene encodes for a polysaccharide export membrane protein in the outer layer of the bacteria's structure. Both of these genes play a crucial role in the polymerization of the capsule, a protective layer formed around the bacteria (Maqusood et al., 2014). While *mexX* gene encodes for a membrane-associated protein that is a component of a multidrug efflux system. This system can transport various chemical compounds outside the bacterial cell, including antibiotics, which contributes to antibiotic resistance in the bacteria [8].

Nanoparticles of metal oxides have attention due to their applications in nanoelectronics, nanodevices, optoelectronics, information storage, catalysis and Nano-sensors. CuO nanoparticles among these various metal oxides which is the simplest member of the copper compounds family. CuO nanoparticles have a range of physical properties like electron correlation effects, spin dynamics and high-temperature superconductivity. Furthermore, they are used in batteries, gas sensors, solar energy, heat transfer fluids and catalysis (Maqusood et al., 2014). In addition to their antibacterial properties, copper oxide (CuO) nanoparticles have also been shown to have anti-biofilm and anti-virulence effects against *A baumannii*, a multidrug-resistant bacteria that causes hospital-acquired infections. Copper oxide nanoparticles also have biomedical applications because of their toxic effect on mammalian cells as well as on vertebrates and invertebrates. Their toxicity belongs to reactive oxygen species (ROS) production. For that, they induce oxidative stress in pulmonary epithelial cells in humans and promote toxicity and DNA and mitochondria damage [9]. However, like with AgNPs, the potential toxicity of CuO nanoparticles is a concern. Studies have shown that they can induce oxidative stress, DNA damage, and inflammation in human cells. Therefore, further research is needed to determine the optimal dose and exposure time of CuO nanoparticles to minimize their toxicity while maximizing their antibacterial effects [9].

Copper oxide nanoparticles are strong candidates as a therapeutic agent due to their antimicrobial activity [10]. Recently, several studies included the antibacterial activity of CuO nanoparticles against Gram-negative bacteria like *P. aeruginosa* and *E. coli* and Gram-positive bacteria such as *S. aureus* and *B. subtilis*. These nanoparticles also have multi-toxicity against a broad spectrum of bacterial species which include several multi-drug resistant bacteria (Fisseha et al., 2020).

However, there was no study with details about the anti-capsular potential of CuO nanoparticles against clinical *A. baumannii-produce* efflux pump has been investigated in Iraq. **The goal of this study** is to assess the impact of CuO nanoparticles against capsule formation, biofilm formation, efflux pumps, and study the synergistic effects of a combination of CuO nanoparticles and gentamicin against this pathogenic bacteria. So, CuO nanoparticles have the potential as a novel approach to combat antibiotic-resistant bacteria like *A. baumannii* by inhibiting efflux pumps. However, more research is needed to fully understand their safety and effectiveness before they can be used clinically. Our study discus the decrease in capsule production from bacteria that produce efflux pumps with capsules together by using nanoparticles as antimicrobial agents.

Mechanisms of Anti-capsular of CuO NPs: CuO nanoparticles have been shown to have anti-capsular potential against *Acinetobacter baumannii*. This is thought to occur through a mechanism involving the production of efflux pumps. Efflux pumps are transporters that are able to pump out toxic compounds from the bacterial cell [8], including antibiotics and other antimicrobial agents. CuO nanoparticles have been shown to induce the production of these pumps, which can help to remove the protective capsule and increase the susceptibility of the bacteria to antibiotics. In addition, CuO nanoparticles can also directly interact with the capsule and disrupt its structure, further weakening the bacterial defense mechanism. These mechanisms may ultimately lead to improved treatment outcomes for *Acinetobacter* infections (Maqusood et al., 2014); [6].

2. Materials and methods

2.1. Bacterial isolates

A number of 34 isolates of *A. baumannii* were obtained from different hospitals in Baghdad from October to December 2021, from different sources of UTI, wound, injury and blood. The bacterial isolates were identified by using biochemical tests, culture on CHROMagar Acinetobacter medium and confirmed by genetic method by *recA* gene as a housekeeping gene. The sequence of the primers is listed in Table 1.

2.2. Synthesis of copper oxide nanoparticles (CuO NPs)

All substances were acquired from British Drug Houses (BDH) without purification. The photoirradiation method (photolysis) was employed to prepare CuO nanoparticles. As shown in Fig. 1, a photocell was utilized to irradiate copper acetate as a source of copper oxide nanoparticles. A 125 W mercury medium pressure lamp is employed as an immersed UV source, with a maximum light intensity of 365 nm. A quartz tube functions as a jacket for the immersion UV source in the copper acetate solution of the cell. A Pyrex tube serves as the reactor. An ice bath keeps the reactor cool [13,14]. Accordingly, 50 ml of 2 mmol urea is slowly (drop per second) injected into 50 ml of 1 m mol Cu $(CH_3COO)_2$ in a magnetic stirrer at 20 °C for 20 min. Then, the copper \ urea solution (1:1) was irradiated with cooling by a photocell for ½ h. The blue Precipitate was formed, separated, and rinsed numerous times with deionized water (D.W). The precipitate was dried at 90 °C overnight, then calcined for 3 h at 450 °C. Copper oxide nanoparticles as a black powder were obtained.

2.3. Antibiotic susceptibility tests

The disk diffusion method was performed in triplicate, according to the 2017 [15] (CLSI) guidelines. A concentration of 5×10^8 CFU/ml of bacterial suspension was prepared and a bacterial lawn was added to the

Table 1
PCR oligonucleotide primers used in this study.

Primer	Target gene	Sequences (5′_3′)	Size product bp	Annealing temp (°C)	Reference
mexX-	efflux pump	F- TGA AGG CGG CCC TGG ACA TCA GC R- GAT CTG CTC GAC GCG GGT CAG CG	326	62	[11]
ptk	Capsule	F-ATTTCAGGGCTT ATTGGTC R-TCATAAGCAGCAACGGCAG	474	55	[12]
EpsA	Capsule	F-ACAAACTTCTTCTGTAGCACC R-AAAAATACTCTGCCATAGGG	348	52	[12]
recA	Housekeeping gene	F- CCTGAATCTTCYGGTAAAAC R-TCTGGGCTGCCAAACATTA	425bp	54	[11]

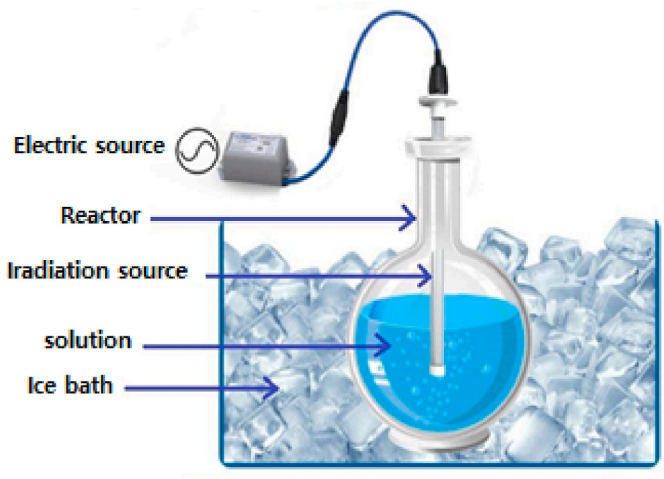

Fig. 1. Photo-cell of prepared CuO nanoparticles.

surface of Mueller-Hinton agar (MHA; Merck, Darmstadt, Germany). Next, antibiotic discs were placed on the inoculated MHA surface, and all the plates were incubated for 18–24 h at 37 °C. Next, the growth inhibition zones around the antibiotic disks were measured. Resistant or susceptible bacteria were determined based on each disk zone diameter and interpreted based on the 2020 CLSI. The antibiotic disks included gentamicin (30 μg), amikacin (30 μg), tobramycin 10 μg (10 μg) and kanamycin (30 μg).

2.4. Biofilm formation assay

The capacities of biofilm formation were measured by microtiter plate method as characterized in details through many previous studies [2,16].

2.5. Hypermucoviscosity assay

Hypermucoviscosity was carried out using the string test. Briefly, isolates were grown overnight at 37 °C on LB agar. A single colony was lifted with a loop to evaluate the formation of a viscous string between the loop and the colony. A positive string test was defined as a string length ≥5 mm. A centrifugation assay was also used to assess hmv. Centrifugation of overnight bacterial cultures (5 ml LB) was performed at 3220×*g* for 10 min. hmv-positive isolates were identified qualitatively by the persistence of turbidity [17].

2.6. Antimicrobial agent stock preparation

A stock solution of the gentamicin antibiotic was prepared at a final concentration of 10 mg/ml. Stock solutions of gentamicin were prepared by dissolving 0.1 g from an antibiotic in 10 ml of distilled water, followed by sterile filtration using a 0.22 μm membrane filter. Furthermore, the stock solution of CuO NPs was prepared in the same way by dissolving 0.1 g of CuO NPs in 10 ml distilled water to make a stock solution of a concentration of 10.000 μg/ml [18].

2.7. Antibacterial activity of CuO nanoparticles and gentamicin

2.7.1. Well-cut diffusion method

A few colonies of *A. baumannii* isolates from overnight growth were suspended in 5 ml normal saline to make an inoculum of 1.5×10^8 CFU/ml and then picked and spread on Mueller-Hinton agar plates. Wells of 3 mm were punched out on the media and 100 μL of CuO nanoparticles, gentamicin and CuO nanoparticles with gentamicin together were pipetted into the wells. Then incubated at 37 °C for 18 h to measure the inhibition zone in mm [19,20]. All the tests were repeated in triplicate.

2.7.2. Microtiter checkerboard method

The synergistic effects of a combination CuO nanoparticles and gentamicin against *A. baumannii* isolates were determined by the micro broth checkerboard method [21,22].

The micro-dilution method using a 96-well polystyrene microtiter plate was carried out to determine the MICs of CuO nanoparticles and CuO nanoparticles -gentamicin combination on *A. baumannii.* Briefly, 5 μL of bacterial suspension was added to 95 μl of Mueller- Hinton broth and placed in a microtiter plate. The stock solution of gentamicin was prepared, then serially diluted giving 50 μl of gentamicin solution added with concentrations of 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 μg/ml, respectively. The gentamicin solution of the combination was serially diluted along the first column, while the CuO nanoparticles solution was serially diluted along the first row. Dilutions were started from the last well with a higher concentration towards the first well for the two drugs. These concentrations were then diluted along the ordinate (vertical axis) and abscissa (horizontal axis) to obtain varying concentrations of the combined. The resulting checkerboard contained the combination of each agents, with wells that contain the highest concentration at opposite corners. Microtiter plates were covered with a lid and incubated at 37 °C for 24 h. The MIC was determined at a concentration which no visible growth could be observed after adding 10 μl of resazurin dye and observing the change in color. Fig. 2 presents a schematic view of the checkerboard assay procedure. Calculation of the fractional inhibitory concentration FIC Index, interaction between the gentamicin and CuO nanoparticles was assessed algebraically by determining the FIC index which was calculated as follows:

The FIC Index and interaction between the gentamicin and CuO nanoparticles was calculated as follows:

$$FICI = \frac{MIC\ of\ drug\ A\ in\ combination}{MIC\ of\ drug\ A\ alone} + \frac{MIC\ of\ drug\ B\ in\ combination}{MIC\ of\ drug\ B\ alone}$$

where: A being gentamicin, B being CuO nanoparticles.

This index interpreted the type of relationship between gentamicin and CuO nanoparticles as follows: FIC values 0.5 were considered synergistic, those from 0.5 to 4 were considered indifferent, and those ≥4, were antagonistic [23].

Furthermore, from the sub-MIC concentration of the CuO nanoparticles, a loopful of bacterial suspension was examined by using a field

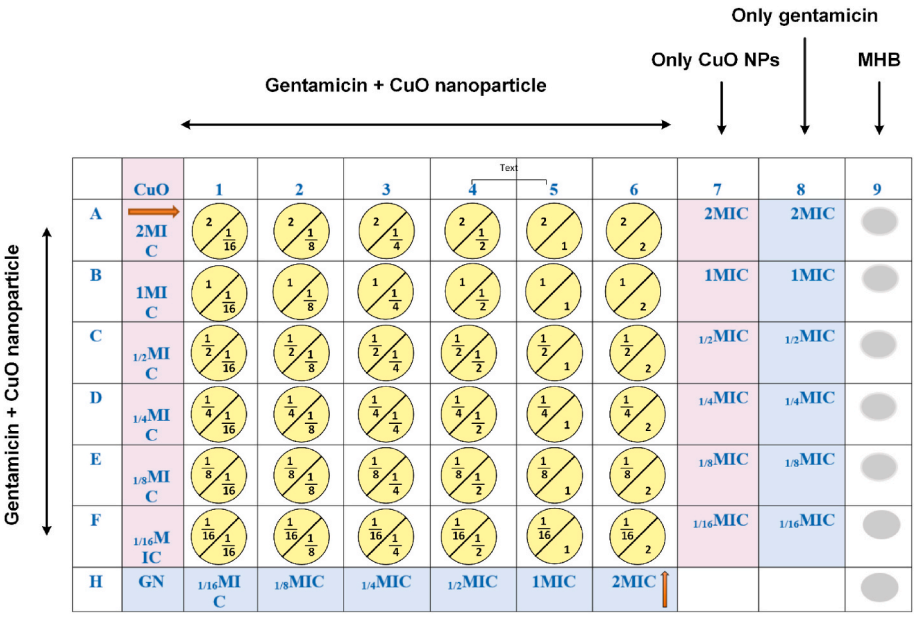

Fig. 2. The schematic view of microtiter plate in checkerboard microdilution assay, all concentrations of gentamicin and CuO NPs are presented in details.

emission scanning electron microscope (FE-SEM) to investigate the morphological changes of bacteria caused by CuO nanoparticles. All the tests were repeated in triplicate.

2.8. Evaluating the expression of capsule gene in isolate producing efflux pump

RT-PCR analysis was completed for the identification of gene expression of capsule formation before and after adding CuO nanoparticles, which had sever capsule inhibition formation for *A. baumannii* isolates which resist to gentamicin. RT-PCR was applied to evaluate the gene expression of *ptk*, *espA* and *mexX*, which are capsule related to these genes. The oligonucleotide sequences are listed in Table 1. The experimental of expression for targets genes was determined in triplicate by using SYBR green PCR master mix in QPCR instrument (Applied Biosystems, Foster City, USA) and normalized using endogenous control by *recA* gene as housekeeping gene. A default reaction cycle was used as provided in QPCR instrument. A melting curve was completed to ensure the purity of target gene amplification through specific melting temperatures using the dissociation Curve Software. Ct value differences (delta Ct) were referred as absolute gene expression fold changes relative to endogenous control, which was calculated as follows: relative copy number (Rc) = 2ΔCt, where Δ Ct = Ct Target–Ct Endo, All the tests were repeated in triplicate.

2.9. Statistical analysis

All the obtained results were statistically analyzed with the GraphPad Prism (version 6.0, GraphPad, San Diego, CA, USA) and the statistical software package for the Social Sciences software (SPSS, version 16). Frequencies and percentages are used to display categorical data. The Chi-square test is performed to determine the relationship between provisional diagnosis and specific data, Significant was defined as a *P*-value equal or less than 0.05.

3. Results

3.1. Identification of Acinetobacter baumannii and antibiotics resistance

Among 34 isolates of *A. baumannii* were obtained from different hospitals in Baghdad from October to December 2021, from different sources: 15 isolates from UTI, 3 from the wound, 2 from injury and 14 from blood. Ten (29%) isolates out of 34 were resistant to amikacin, while 24 (70%) were resistant to gentamicin, twenty-nine (85%) isolates resist tobramycin and all isolates (100%) gave resistance to kanamycin.

3.2. Capsular formation

Results showed that among fifteen gentamicin-resistant isolates, two *A. baumannii* isolates were able to form capsules as the turbidity presence in the isolates which were positive to string test after their centrifugation (Table 2). Furthermore, results proved that there was a relationship between the capsule-forming ability and the absence of biofilm-forming ability. As bacterial isolates which were negative biofilm formation were positive in capsule formation and vice versa. Although there was a wide range of the minimum inhibitory concentration value for different bacterial isolates with high significant differences p-value equal to 0.01.

Table 2
Screening of resistant isolates whose capsular forming.

Isolate No.	GN disk sensitivity	MIC value to GN	Biofilm formation	Capsule formation
ATCC	**S**	1μg/ml	Negative	Negative
4	R	32μg/ml	Negative	Positive
5	R	128μg/ml	Negative	Positive
6	R	32μg/ml	Negative	Positive
8	R	64μg/ml	Negative	Positive
9	R	32μg/ml	Negative	Positive
10	R	512μg/ml	Negative	Positive
11	R	128μg/ml	Negative	Positive
12	R	128μg/ml	Negative	Positive
14	R	64μg/ml	Negative	Positive
15	R	128μg/ml	Negative	Positive
17	R	64μg/ml	Negative	Positive
18	R	128μg/ml	Negative	Positive
19	R	64μg/ml	Negative	Positive
20	R	64μg/ml	Negative	Positive
25	R	512μg/ml	Negative	Positive
Total 15	15(%100) R	32 μg/ml	128μg/ml	P value
		3(%20) isolates	5(%33.3) isolates	0.01
	0(%100) S	64μg/ml	512μg/ml	
		5(%33.3) isolates	2(13.4) isolates	

3.3. Characterization of synthesis of copper oxide nanoparticles (CuO NPs)

X-ray diffraction (XRD) with λ = 0.154 nm of Cu–K radiation source evaluated the specimens' composition of synthesized nano-powder by photolysis method. Fig. 3 show the XRD patterns of the Nano-sample following calcination at 450 °C. The XRD pattern demonstrates that all refraction peaks coincide with the conventional refraction details (JCPDS NO.48-1548). The pattern supports a crystalline monoclinic structure. CuO nanoparticles have a miller (110), (002), (111), (20−2), (020), (202), (11−3), (31−1), (113), (311), and (004) phases with diffraction angles of 32.45°, 35.46°, 38.68°, 48.70°, 53.50°, 58.3°, 61.49°, 66.14°, 67.86°, 72.38°, and 74.89°. There are no peaks for any contaminant in nano-synthesized, indicating that the sample is highly clean. The Scherrer equation [24–27] was used to estimate the crystallite size of the sample. The average crystallite size of the CuO NPs determined for the highest peaks at 35.46°, and 38.68° was around 24 nm. The formation of nanocrystalline CuO is suggested by the resolution of sharp peaks with obtained crystallite sizes.

The nano-powder of CuO chemical composition was determined using the EDX technique. Fig. 4 depicts the resulting spectrum, demonstrating that only Cu and O were present. The percentage amount of Cu and O was shown in the inner table. The molar ratio of the two elements was determined to be 1:1, which is compatible with the theoretical CuO ratio. The discovery proved the presence of the CuO crystal phase in the sample produced.

Fig. 5 show the SEM and TEM pictures of as-prepared CuO nanoparticles. Fig. 5 (a) demonstrates that the CuO nanoparticles are rectangular. Some aggregations can be observed in the images due to preparing the sample and its measurement. The TEM image of as-prepared nanoparticles is shown in (Fig. 5 b). The particle size detected in the TEM image is in the 33–42 nm range, which agrees well with the Scherrer formula determined using XRD.

Fig. 6 depicts the photoluminescence spectra of as-prepared CuO nanoparticles at ambient temperature. CuO emits at two wavelengths: 282 nm (violet) and 570 nm (green). The first is associated with band-edge emission. The second is caused by recombining a photo generated hole with a singly ionized electron in the valence band, resulting in CuO materials' green emission. The band gap obtained from equation 1240/(nm) is 2.7 eV [28,29].

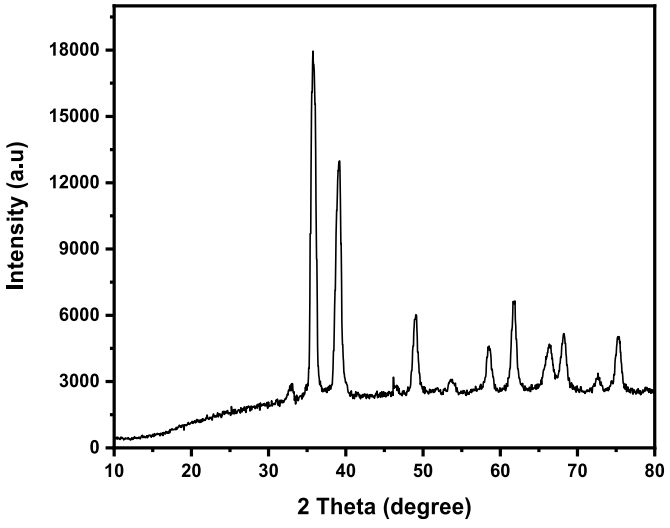

Fig. 3. XRD pattern of nano-powder CuO calcinated at 450 °C.

Fig. 4. EDX spectrum of CuO nanoparticles.

3.4. Antibacterial activity of CuO nanoparticles and gentamicin

3.4.1. Well-cut diffusion method

The agar-well diffusion method was used to screen the antibacterial efficacies of CuO nanoparticles and gentamicin against *A. baumannii* bacterial growth. Exposure to 100 μg/ml AgNPs produced marked inhibition zones in all tested bacterial strains (mean = 16 mm and range = 6–27 mm). The inhibition zones of CuO nanoparticles, gentamicin, and the CuO nanoparticles with gentamicin were (19 mm, 14 mm, 23 mm), respectively.

3.4.2. Combined effect of CuO nanoparticles and gentamicin (microdilution checkerboard method)

The antibacterial activities of CuO nanoparticles and gentamicin nanoparticles was studied against *A. baumannii* isolates number: 24 and 85 using the standard microdilution method, and interaction between the nanoparticle and the antibiotic was estimated by calculating the fractional inhibitory concentration (FIC index) of the combination through checkerboard assay. Results demonstrated that CuO nanoparticles revealed the maximum antibacterial effect, the improvement in antibacterial effect was seen when combined with gentamicin. Synergistic effect was found (Table 3).

3.5. Investigation the morphological changes caused by CuO nanoparticles

The field emission scanning electron microscopy (FE-SEM) images for the bacterial shape during the sub-MIC concentrations of the CuO nanoparticles was detected, and the results (Fig. 7) disclosed that the integrity of *A. baumannii* cell membranes was deteriorated and destroyed. The difference was noticed in control bacterial isolates that had not been treated with CuO nanoparticles, implying that the cell membranes were unaffected. Furthermore, the morphological changes in the whole bacterial shape were easily observed as there was an overlapping and merging with each other among the colonies. These feedbacks indicate that CuO nanoparticles can impair the outer membrane, causing disfigured, impotent, secretion of cellular components as well as altering the regular shape *A. baumannii*.

3.6. Modulation of capsule-related genes expression

Before and after the addition of CuO nanoparticles, RT-PCR analyses were performed to assess the expression of the *ptk*, *espA* and *mexX* genes. The differences in gene folding were (3.506423, 6.773962, 3.41054) for *ptk*, *espA* and *mexX* in treated samples, while were (1.580083, 0.582367, 1.140764) in untreated samples, respectively. The presence of CuO nanoparticles resulted in a significant low folding as shown in Fig. 8 and Table 4.

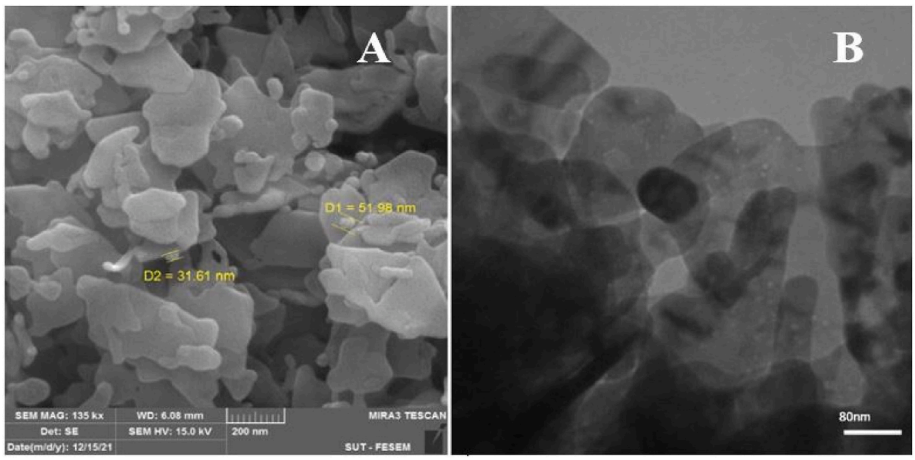

Fig. 5. The electron microscope Images of CuO nanoparticles (a) SEM and (b) TEM.

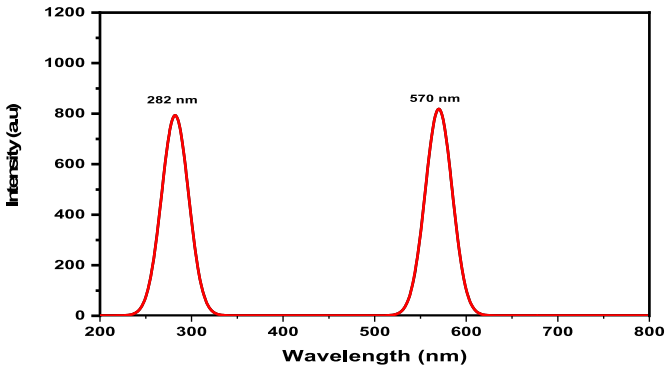

Fig. 6. PL spectrum of CuO nanoparticles.

Table 3
Combined activity of CuO nanoparticles with gentamicin against *A. baumannii* isolates compare with *A. baumannii* ATCC 17978.

	Bacterial isolates		
	ATCC	*A. baumannii* (25)	*A. baumannii* (10)
MIC of CuO nanoparticles (μg/ml)	–	12,500	12,500
Con. of CuO nanoparticles in combination (μg/ml)	–	781.25	781.25
MIC of gentamicin (μg/ml)	0.5	32	16
MIC of gentamicin in combination (μg/ml)	0.5	8	4
FIC	–	0.3125	0.3125
Kind of interaction	–	Synergistic	Synergistic

4. Discussion

In the last few years, there has been a significant increase in serious infections caused by antibiotic-resistant bacteria. Multidrug-resistant bacteria can develop phenotypic resistance, implementing themselves further fatal than another pathogenic bacteria [30]. Diseases triggered from *A. baumannii* are common in intensive care units, in which exceptionally subjected individuals are handled. Many research have found that *A. baumannii* are unsusceptible towards numerous types of antibiotics because of improper usage of drugs, in addition to the inhibiting ways, like their formation [31]. *A. baumannii* possesses less small porins and efflux pumps than another Gram-negative bacteria, making it fewer susceptible to antibiotics while also removing antibacterial agents from bacterial cell membranes. Regardless of *A. baumannii's* unique features, some antibacterial agents are still productive [32].

According to the present study, 34 isolates of *A. baumannii* were obtained from different hospitals in Baghdad. Overall, 29% of these bacteria were resistant to amikacin, 70% were resistant to gentamicin, 85% isolates resistant to tobramycin, and 100% were resistant to kanamycin.

Compared to other metal oxide nanoparticles, CuO nanoparticles are more stable, electron correlation effects, spin dynamics and high temperature superconductivity (Maqusood et al., 2014). Nanoparticles are able to produce radial oxygen molecules, which can destroy cell walls and ultimately kill microbes. Multiple investigations had indicated the antimicrobial actions toward Gram-negative and Gram-positive bacteria such as *S. aureus*, *E. coli*, *P. aeruginosa*, and multi-drug resistant *A. baumannii* [33–35].

In the present study, the inhibition zones of most isolates were almost the same. As the largest inhibition zone for CuO nanoparticles was (27 mm), and the MIC of CuO nanoparticles were 12,500 μg/ml.

Generally, the checkerboard assay is a common and simple method for studying interactions between two antimicrobials [36]. In this context, researchers noticed a synergistic impact of some plants extracts and vancomycin against *S. aureus*, to be suggested through the FIC indexes [37]. A different approach research investigated at the synergistic activities of metal nanoparticles and multiple antibiotics, which includes amikacin, ampicillin, amoxicillin, oxacillin, clavulanic acid, methicillin, gentamicin, and cloxacillin. Metal nanoparticles paired with these antibiotics were found to improve the diameter of the inhibition zone toward *S. aureus* [8,38].

In the current study, the interactions of gentamicin in combination with CuO nanoparticles were investigated against two gentamicin resistant isolates compare with *A. baumannii* ATCC 17978; the FIC values of these isolates were 0.3125 and 0.3125, respectively. According to the FIC index, synergistic interactions were found, demonstrated that CuO nanoparticles revealed the maximum antibacterial effect, the improvement in antibacterial effect was seen when combined with gentamicin. In addition, the FE-SEM images for the bacterial shape throughout the sub-MIC levels of the CuO nanoparticles revealed disturbances, overlapping, and amalgamating among the colonies, suggesting that CuO nanoparticles can disrupt the outer membrane, leading to damaged, ineffective, production of cellular parts, and changing the typical shape. *A. baumannii*. So, these feedbacks are new and give a novelty characteristics to research.

Recent studies have shown that copper oxide (CuO) nanoparticles have potential as an antimicrobial agent against A. baumannii. CuO nanoparticles have been shown to disrupt the bacterial cell membrane and inhibit bacterial growth. Additionally, CuO nanoparticles have been

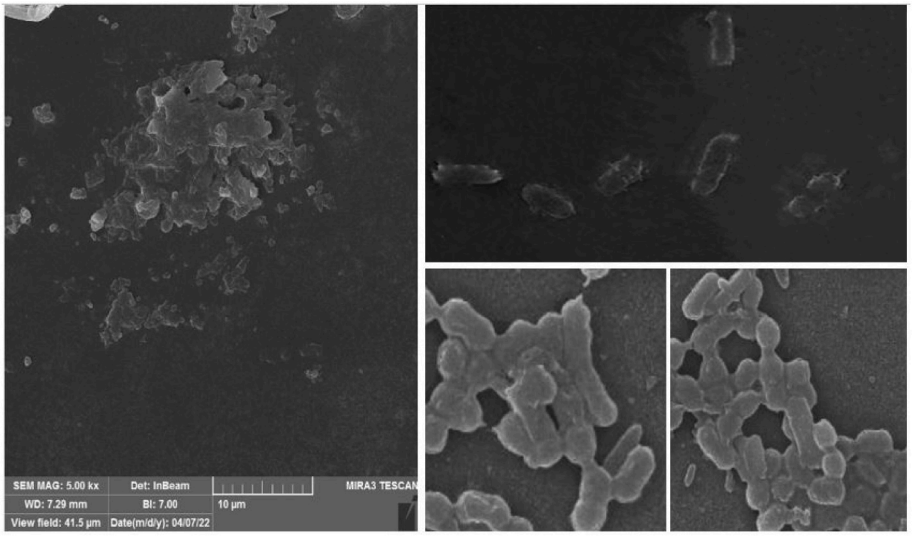

Fig. 7. FE-SEM images of capsular *A. baumannii* isolates after treated with CuO nanoparticles.

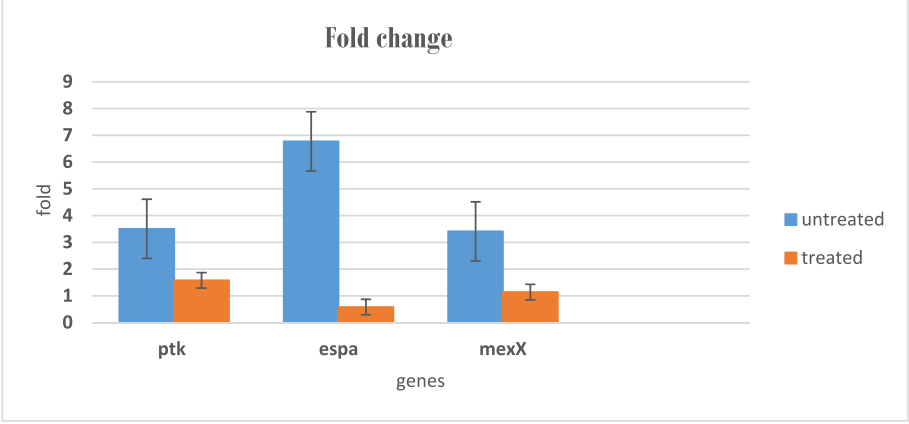

Fig. 8. The differences in gene folding between treated and untreated samples.

Table 4
The results of q PCR according to Ct and folding value for three genes and reference gene.

	Ct of target	Ct of 16s	delta ct	2ˆ-delta ct	fold	ct *ptk*	ct 16 treated	delta ct	2ˆ-delta ct	fold
ptk	16.78	18.49	−1.71	3.271608	3.506423	27.43	25.89	1.54	0.343885	1.580083
espa	15.68	18.34	−2.66	6.32033	6.773962	28.22	25.24	2.98	0.126745	0.582367
mex	16.83	18.5	−1.67	3.182146	3.41054	27.78	25.77	2.01	0.248273	1.140764
control	16.5	16.4	0.1	0.933033	1	27.4	25.2	2.2	0.217638	1

shown to inhibit the production of efflux pumps in A. baumannii, which may contribute to their antimicrobial activity. Efflux pumps are a major mechanism of antibiotic resistance in A. baumannii, and targeting these pumps may be an effective strategy for combating antibiotic resistance. CuO nanoparticles may have the potential to inhibit the production of efflux pumps in A. baumannii, which could enhance the effectiveness of antibiotics against this bacterium [39,40].

The current study's real-time PCR evaluations reveal variations in gene folding between CuO nanoparticle-treated and untreated samples. This is the first report in Iraq demonstrating the capacity of CuO nanoparticles to inhibit the expression of the *ptk*, *espA*, and *mexX* genes, adding to the identified mechanisms through which CuO interferes with microbial growth. The distinctions in gene folding were (3.506423, 6.773962, 3.41054) in treated samples and (1.580083, 0.582367, 1.140764) in untreated samples for *ptk*, *espA*, and *mexX*, accordingly At last, we concluded that reducing the expression of these genes by CuO nanoparticles is a major conduct over reducing *A. baumannii* capsular action, which may aid in the reduction of antibiotic resistance processes.

CuO nanoparticles can have an effect on the capsule of bacteria because they have a high surface area to volume ratio, which allows them to interact with the bacterial surface and disrupt the capsule. However, the configuration of bacteria refers to their overall shape and structure, which is not affected by CuO nanoparticles.

If MIC declines with the use of synergistic CuO and gentamicin, it suggests that the combination has a stronger bactericidal effect than either compound alone. Bacteriostatic compounds inhibit bacterial growth, while bactericidal compounds kill bacteria. The exact effect of the CuO-gentamicin combination would depend on the concentration of each compound and the specific bacterial strain being targeted.

5. Conclusion

In conclusion, our study confirms the impacts of gentamicin and CuO nanoparticles to show synergistic anti-capsular potential activity. Beside the capacity of CuO nanoparticles to cause disturbances, overlapping, and amalgamating among *A. baumannii* colonies, suggesting that CuO nanoparticles can disrupt the outer membrane, leading to damaged, ineffective, production of cellular parts, and changing the typical shape, additionally, CuO nanoparticles have been shown to inhibit the production of efflux pumps in A. baumannii, which may contribute to their antimicrobial activity. *A. baumannii*. So, these feedbacks are novel and interesting. Furthermore, results proved that there was a relationship between the capsule forming ability and the absence of biofilm forming ability. As bacterial isolates which were negative biofilm formation were positive in capsule formation and vice versa.

This study highlights the potential of CuO nanoparticles as a promising antimicrobial agent against *A. baumannii*, a notorious multidrug-resistant bacterium. The synergistic effect of CuO nanoparticles and gentamicin opens up new avenues for the development of combination therapies to combat antibiotic resistance. This study found that treatment with CuO nanoparticles significantly reduced the expression of efflux pump genes in A. baumannii, suggesting that they could be used as an adjunct therapy to enhance the effectiveness of antibiotics. Briefly, our study discus the decrease in capsule production from bacteria that produce efflux pumps with capsules together by using nanoparticles as antimicrobial agents.

However, further research is needed to fully understand the underlying mechanisms and evaluate the safety and efficacy of CuO nanoparticles as an antimicrobial agent.

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Ethical approval

Mustansiriyah University oversaw and approved this study by their the Ethics Committee.

CRediT authorship contribution statement

Israa M.S. Al-Kadmy: Writing – original draft, Supervision, Project administration. **Sarah Naji Aziz:** Writing – original draft, Software, Data curation. **Ahmed Mahdi Rheima:** Formal analysis, Data curation. **Suhad Abbas Abid:** Investigation. **Ahmed Suhail:** Writing – review & editing, Visualization. **Israa Hussein Hamzah:** Methodology. **Eman N. Naji:** Resources, Methodology. **Alexandros Besinis:** Writing – review & editing, Formal analysis. **Helal F. Hetta:** Resources, Data curation.

Declaration of competing interest

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

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