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Antimicrobial Effect of Zinc Oxide Nanoparticles against Multidrug-Resistant *Acinetobacter baumannii*

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ABSTRACT

Introduction: Increased multidrug-resistant (MDR) *Acinetobacter baumannii* (*A. baumannii*) infections pose a significant challenge in hospital settings. Enhanced resistance to antibiotics like fluoroquinolones and β-lactams necessitates adopting alternative treatment strategies such as metal oxide nanoparticles. This study investigated the synergistic effect of zinc oxide nanoparticles (ZnO-NPs) on ciprofloxacin and ceftazidime activity against MDR *A. baumannii*. **Methods:** We examined 30 MDR *A. baumannii* isolates from intensive care unit (ICU) patients in Iran. ZnO-NPs were synthesized via the solvothermal method and characterized using X-ray diffraction (XRD) and field emission scanning electron microscopy (FESEM) to ascertain their crystalline structure and morphology. Antibacterial activity was evaluated by determining minimum inhibitory concentrations (MICs) and inhibition zones through broth microdilution and disk diffusion methods, using concentrations of ciprofloxacin and ceftazidime in combination with ZnO-NPs. **Results:** ZnO-NPs combined with ciprofloxacin 8 µg/mL and ceftazidime 32 µg/mL exhibited inhibition growth percentage (GI%) increases of 44.9% and 31.65%. **Conclusion:** The enhanced *in vitro* antibacterial effects of combined ZnO-NPs and antibiotics against MDR *A. baumannii* indicate a synergy. Considering the limited number of isolates, comprehensive research incorporating *in vivo* models and clinical trials is warranted to evaluate the practicality of this approach in overcoming antibiotic resistance.

INTRODUCTION

Nosocomial infections caused by multidrug-resistant *Acinetobacter baumannii* have increased in recent decades, posing a significant threat to hospitalized patients worldwide [1]. *A. baumannii* is a Gram-negative, non-fermentative opportunistic and aerobic bacterium. Infection with this agent poses a significant risk for critically ill patients in ICUs, especially those undergoing treatment with broad-spectrum antibiotics [2]. This pathogen is capable of causing infections in multiple organs, including the bloodstream, lungs, urinary tract, and skin [3].

Fluoroquinolones, such as ciprofloxacin, and β-lactams, including ceftazidime, are antibiotic choices to combat *A. baumannii* infections> still, the resistance to these drugs is steadily increasing [4]. Resistance to β-lactams is attributed to enzymatic degradation by β-lactamases, alongside non-enzymatic mechanisms, such as alterations in the structure and reduced outer membrane proteins that limit antibiotic uptake, overexpression of efflux pumps that actively expel antibiotics from the cell, and changes in penicillin-binding proteins, such as reduced binding affinity for β-lactams and altered levels of expression [5]. In *A. baumannii*, two mechanisms primarily contribute to resistance to quinolones: (i) mutations in the quinolone resistance-determining regions (QRDR) of the *gyrA* and *parC* genes, leading to structural changes in DNA gyrase — an enzyme critical for DNA replication; and (ii) the action of multidrug efflux pumps, such as AdeABC and AdeM, which actively transport quinolones out of the cell and reduce the intracellular accumulation of the drug, thereby diminishing its efficacy [6, 7].

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Studies have shown the antimicrobial properties of nanoparticulate forms of metals, metal oxides, metal halides, and bimetal [8-10]. Zinc oxide nanoparticles (ZnO-NPs) are extensively utilized in nanobiotechnology and are recognized for their effective antibacterial properties. These nanoparticles are biologically safe for specific medical applications and exhibit significant antibacterial activity due to their inherent nanoscale size [11]. The antibacterial mechanisms of ZnO-NPs involve the production of reactive oxygen species, lipid peroxidation, and releasing cellular contents such as sugars, proteins, and DNA from the bacterial membrane [14]. ZnO-NPs have demonstrated toxicity to pathogenic bacteria while being low toxic to human cells, supporting their continued assessment for use as antibacterial agents in the pharmaceutical industry [11]. Combining metal nanoparticles with conventional antibiotics produces a synergistic effect. This approach is promising as it allows lower antibacterial dosage, prevents bacterial resistance, and reduces adverse side effects, as evidenced by preliminary studies [8].

This study investigated the ZnO-NPs effect combined with ciprofloxacin and ceftazidime against MDR *A. baumannii*.

In recent decades, the incidence of nosocomial infections specifically caused by multidrug-resistant *Acinetobacter baumannii* has increased, posing a significant threat to hospitalized patients worldwide. The increasing incidence of nosocomial infections caused by *A. baumannii* is a significant cause for concern. It has led to heightened research focus within the medical community [1]. *A. baumannii* is a Gram-negative, non-fermentative bacterium, indicating its metabolic inflexibility to ferment sugars, and is opportunistic and aerobic. It represents a particular risk for severe nosocomial infections in critically ill patients in ICUs, especially those undergoing treatment with broad-spectrum antibiotics [2]. This pathogen is capable of causing infections in multiple organs, including the bloodstream, lungs, urinary tract, and skin [3].

Fluoroquinolones, such as ciprofloxacin, and β-lactams, including ceftazidime, have been the antibiotics of choice to combat *A. baumannii* infections. Still, the prevalence of resistance to these drugs is steadily increasing [4]. Resistance to β-lactams is attributed to enzymatic degradation by β-lactamases, along with non-enzymatic mechanisms, including alterations in the structure and reduced presence of outer membrane proteins that limit antibiotic uptake, overexpression of efflux pumps that actively expel antibiotics from the cell, and changes in penicillin-binding proteins, such as reduced binding affinity for β-lactams and altered levels of expression [5]. In *A. baumannii*, two mechanisms primarily contribute to resistance to quinolones: (i) mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA* and *parC* genes, leading to structural changes in DNA gyrase — an enzyme critical for DNA replication; and (ii) the action of multidrug efflux pumps, such as AdeABC and AdeM, which actively transport quinolones out of the cell and reduce the intracellular accumulation of the drug, thereby diminishing its efficacy [6, 7].

Several studies have shown that the nanoparticulate form of metals, metal oxides, metal halides, and bimetals exhibit significant antimicrobial properties, as bacteria currently exhibit low resistance levels to such particulate materials [8-10]. Zinc oxide nanoparticles (ZnO-NPs) are extensively utilized in nanobiotechnology and are recognized for their effective antibacterial properties. These nanoparticles are biologically safe for specific medical applications and exhibit significant antibacterial activity due to their inherent nanoscale size [11]. The antibacterial mechanisms of ZnO-NPs involve the production of reactive oxygen species, lipid peroxidation, and releasing cellular contents such as sugars, proteins, and DNA from the bacterial membrane [14]. ZnO-NPs have demonstrated toxicity to pathogenic bacteria, while evidence suggests they show low toxicity to human cells, supporting their continued assessment for use as antibacterial agents in the pharmaceutical industry [11]. Combining metal nanoparticles with conventional antibiotics has been shown to produce a synergistic effect attributable to the enhanced antimicrobial activity from the combined action of antibiotics and the targeted release of metal ions from the nanoparticles. This approach is promising because it may allow for more effective use of lower antibacterial agent doses than when each is administered alone, with the potential to overcome bacterial resistance problems and reduce adverse side effects, as evidenced by preliminary studies [8].

This study was undertaken due to the emerging role of nanoparticles in potentially overcoming or mitigating antibiotic resistance in certain pathogenic microbes. We tested ZnO-NPs for their antibacterial efficacy against strains of *A. baumannii* isolated from ICU patients.

MATERIAL AND METHODS

Collection of isolates. Over six months, we selected 30 *A. baumannii* isolates during diagnostic culture tests on various clinical samples such as blood, respiratory, wound, and urine at Taleghani University Hospital, Tehran, Iran. The isolates exhibited multidrug resistance to at least three antibiotic classes by disk diffusion testing. All standard chemicals and media used in our experiments were purchased from Merck (Darmstadt, Germany).

This study was approved by the Student Research Committee of Islamic Azad University, Lahijan Branch (approval No.IR.IAU.Lahijan.REC.1398.008).

Producing ZnO Nanofluids. With modifications, we prepared ZnO nanofluids using the solvothermal process outlined by Ashtaputre *et al*. (2005) [13]. Zinc nitrate and hexamethylenetetramine were dissolved in ethylene glycol with vigorous stirring while heating to 60°C. This

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solution was transferred to a Teflon-lined autoclave and heated at 140°C for 12 h. The resulting ZnO nanoparticles were washed thoroughly with acetone and dispersed in glycerol by sonication for 30 min. Appropriate amounts of ammonium citrate were added to the ZnO nanofluids as a stabilizing agent. Glycerol was the base fluid, and ammonium citrate was added to stabilize the ZnO nanoparticles within the fluid, preventing agglomeration, which is crucial for consistent antimicrobial evaluations. We combined ZnO-NPs and ammonium citrate in equimolar amounts and then dispersed the mixture into the glycerol solution to achieve a final concentration of 0.5% w/v, stirring continuously with a magnetic stirrer for 24 h at 20–25 °C [14].

ZnO-NPs Characterization. The synthesized ZnO-NPs were characterized using X-ray diffraction (XRD). XRD with Cu Kα radiation was employed to determine the crystal structure of the nanoparticles over a 2θ range of 20–80 degrees. The XRD analysis was conducted at a controlled temperature of 25°C using a PANalytical X'Pert Pro diffractometer with Cu Kα radiation (λ = 1.54056 Å, voltage: 40 kV, current: 40 mA), scanning from 10° to 90° (2Θ) at a rate of 0.02°/s, a range commonly used to encompass the primary diffraction peaks of ZnO. Morphology of the ZnO-NPs was visualized using a Zeiss Sigma VP Field Emission Scanning Electron Microscope (FESEM) on samples sputter-coated with gold to enhance conductivity [15].

Species identification. The isolates were cultured on brain heart infusion agar and incubated at 35 °C for 24-48 h, and identified as *A. baumannii* using standard biochemical methods including catalase, oxidase, glucose fermentation, citrate utilization, motility, indole production, urease, and growth on MacConkey agar, along with macroscopic and microscopic examinations [12]. *A. baumannii* ATCC 19606 was included in all assays as a positive control. Species identification was further confirmed by sequencing the hypervariable V3-V4 region of the *16S rRNA* gene as described by others [16].

Antibiotic susceptibility testing. Antibiotic susceptibility was assessed using the disk diffusion method following the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) M02-A12, 2022 edition [17]. The antibiotic disks were purchased from a commercial company (Mast Diagnostics, UK) included ampicillin/sulbactam (10/10 µg), amikacin (30 µg), tobramycin (10 µg), ciprofloxacin (5 µg), ceftazidime (30 µg), cefepime (30 µg), gentamicin (10 µg), and imipenem (10 µg). We seeded Mueller Hilton agar plates with a standardized bacterial inoculum adjusted to the 0.5 McFarland standard using a densitometer (1.5×10^8 CFU/mL). We measured the inhibition zone diameters using calipers, with *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 as controls.

ZnO-NPs susceptibility testing. Based on the antibiotic susceptibility results, the antibacterial activity of ZnO-NPs alone and combined with ceftazidime and ciprofloxacin antibiotics was analyzed using the two-fold broth microdilution method and the disk diffusion method. Bacteria at a concentration of 10^6 CFU/mL were incubated with various concentrations of ZnO-NPs (0.0625 to 2 mg/mL), ceftazidime (2 to 64 mg/mL) and ciprofloxacin (1 to 16 mg/mL) for 24 h. Bacterial growth was monitored by measuring the optical density at 630 nm (OD630) for 24 h at 2-h intervals using an ELx800 Absorbance Microplate Reader (BioTek Instruments Inc., Winooski, VT). The minimum inhibitory concentration (MIC) was calculated from the OD630 measurements as the lowest concentration that prevented visible growth [18].

Synergistic effects of ZnO-NPs. The bacteria were exposed to antibiotics with concentrations ranging from 16 to 64 µg/mL for ceftazidime and 4 to 16 µg/mL for ciprofloxacin combined with the subinhibitory concentration of ZnO-NPs (0.25 mg/mL, representing 1/2 × MIC for the test strain), over 24 h. Bacterial growth was monitored using the optical density method as previously described. An inoculated nutrient broth without ZnO-NPs and antibiotics was used as a positive control. Experiments were performed in triplicate, with the antibacterial activity assessed by measuring the MIC and calculating the mean growth inhibition percentage (GI%) relative to the untreated positive control. The GI% for each concentration was determined by the equation below, as described by others [3].

$$\mathrm{GI\%} = (100 - \frac{\mathrm{OD630\ in\ the\ presence\ of\ antibacterial\ agents\ (s)}}{\mathrm{OD630\ of\ positive\ control}} \times 100$$

DNA fragmentation analysis. To assess cellular apoptosis and damage, log-phase *A. baumannii* cultures grown in Mueller Hinton broth with a 10^9 CFU/mL concentration were exposed to ceftazidime (32 µg/mL) or ciprofloxacin (8 µg/mL) alone, and in combination with a subinhibitory concentration of ZnO nanoparticles (0.25 µg/mL) for one h at 37 °C. Genomic DNA was then isolated using a Genomic DNA Isolation Kit (Favorgene, catalog no. XYZ123, Taiwan), and DNA fragmentation was evaluated by agarose gel electrophoresis (using a specific gel percentage) [19].

Statistical analyses. Descriptive statistical analysis, such as calculating percentages, means, medians, and standard deviations to characterize the data, was performed using SPSS version 22 (IBM Corp, Armonk, NY, USA).

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RESULTS

Characterization of ZnO-NPs. The XRD pattern revealed numerous Bragg reflections indexed to the (100), (002), (101), (102), (110), (103), (200), (112), and (201) planes, consistent with the wurtzite hexagonal phase of ZnO-NPs. The diffraction peaks were consistent with those listed in the International Centre for Diffraction Data (ICDD) reference (Card No. 89-1397) (Fig. 1). The FESEM images of the ZnO-NPs indicated that the nanoparticles predominantly exhibit spherical shapes with smooth surfaces, with an average size of 100 ± 68.68 nm (Fig. 2).

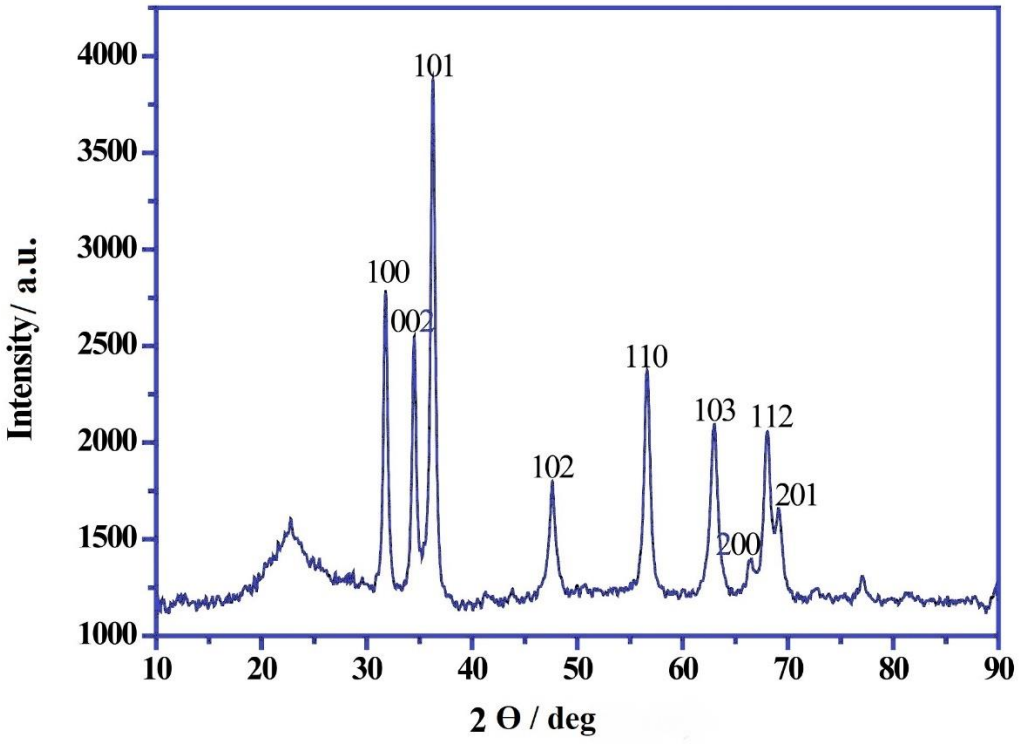

Fig. 1. X-ray diffraction (XRD) patterns of ZnONPs.

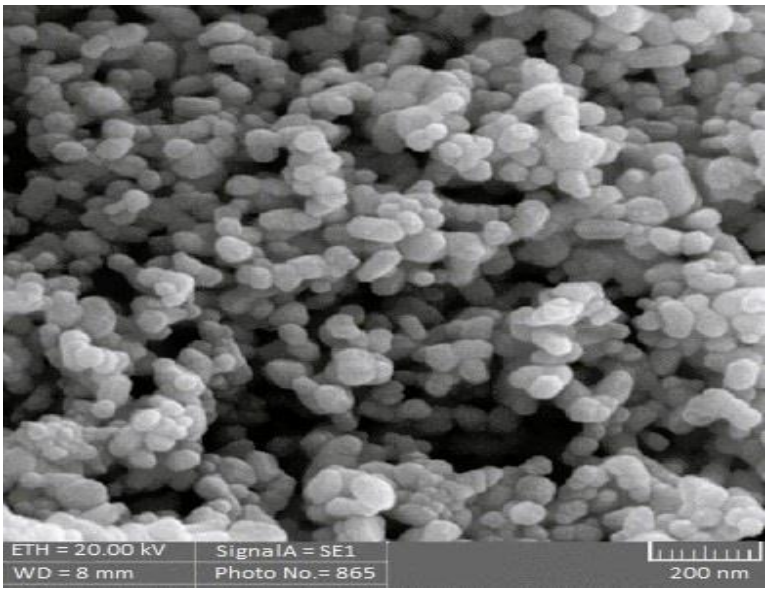

Fig. 2. Field Emission Scanning Electron Microscopy (FESEM) image of ZnO-NPs.

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Antibacterial susceptibility testing. The *A. baumannii* isolates showed various resistance rates to tested antibiotics, with the highest (100%) to ciprofloxacin and ceftazidime (Table 1).

Table 1. Antibacterial susceptibility testing for *A. baumannii* isolates.

Antibiotics (µg)	Number of *A. baumannii* isolates (%)
Ampicillin/sulbactam (10/10)	28 (93)
Amikacin (30)	24 (80)
Tobramycin (10)	26 (87)
Ciprofloxacin (5)	30 (100)
Ceftazidime (30)	30 (100)
Cefepime (30)	21 (71)
Gentamicin (10)	26 (87)
Imipenem (10)	27 (90)

Ceftazidime and ciprofloxacin at tested concentrations did not inhibit bacterial growth, while ZnO-NPs significantly inhibited bacteria growth compared to the control condition (Table 2).

Table 2. Growth inhibition of *A. baumannii* treated with ZnO-NPs, ceftazidime, and ciprofloxacin individually.

Treatment	Concentration	Mean GI%
ZnO-NPs	0.0625 mg/mL	9.75 ± 0.4
ZnO-NPs	0.125 mg/mL	14.1 ± 0.81
ZnO-NPs	0.25 mg/mL	21.8 ± 1.81
Ceftazidime	16 µg/mL	0
Ceftazidime	32 µg/mL	0
Ceftazidime	64 µg/mL	0
Ciprofloxacin	4 µg/mL	0
Ciprofloxacin	8 µg/mL	0
Ciprofloxacin	16 µg/mL	0

GI: Growth inhibition

The MIC for ZnO-NPs was 0.5 mg/mL, indicating that the inhibitory effect on bacterial growth increased with higher nanoparticle concentrations. The mean values of GI% for *A. baumannii* at ZnO-NPs concentrations of 0.0625, 0.125, and 0.25 mg/mL were 9.75 ± 0.4, 14.1 ± 0.81, and 21.8 ± 1.81, respectively. Control tests showed that neither the base fluid nor the dispersant used for ZnO-NPs affected *A. baumannii* growth at subinhibitory concentrations. Consequently, to isolate the effect of ZnO-NPs on bacterial growth, the impact of these substances was not included in the final analysis of antibacterial activity. The microdilution and disk diffusion methods were utilized to evaluate the effects of ZnO-NPs against *A. baumannii* when combined with ceftazidime and ciprofloxacin. The GI% of antibiotics alone and combined with a subinhibitory concentration of ZnO-NPs are reflected in Table 3.

Table 3. Growth Inhibition Percentages (GI%) for *A. baumannii* exposed to ceftazidime, ciprofloxacin, and combined with ZnO-NPs over 24 h.

ZnO concentration (mg/mL)	Ceftazidime concentration (µg/mL)				Ciprofloxacin concentration (µg/mL)			
	0	16	32	64	0	4	8	16
0	0	1.25±0.01	2.28±0.3	1.76±0.38	0	1.21±0.020	2.51±0.075	1.86±0.027
0.25	18.70±0.09	4.75±0.08	31.65±0.52	8.2±0.42	19.9±0.075	11.42±0.04	44.90±0.021	21.9±0.71

The antibacterial activity of ciprofloxacin and ceftazidime was enhanced when combined with 0.25 mg/mL of ZnO-NPs. Notably, this enhancement was most pronounced at antibiotic concentrations of 8 mg/mL for ciprofloxacin and 32 mg/mL for ceftazidime. Table 4 shows the diameters of the inhibition zones for ciprofloxacin and ceftazidime disks, respectively. Despite *A. baumannii*'s resistance to both antibiotics, their combination with ZnO-NPs resulted in larger inhibition zones than the antibiotics used alone.

Table 4. Inhibition zones for ceftazidime and ciprofloxacin alone and combined with ZnO-NPs against *A. baumannii*.

Antibiotic	Zone (mm)	
	Without ZnO-NPs	With ZnO-NPs
Ceftazidime	0	34
Ciprofloxacin	12	38

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DNA ladder assay. DNA concentrations in both untreated and ZnO nanofluid and/or antibiotic-treated *A. baumannii* cells were quantified via agarose gel electrophoresis. ImageJ software (US National Institutes of Health, Bethesda, MD) was employed to quantify the genomic DNA bands. Treatment with either ZnO nanofluids, antibiotics, or their combination did not lead to quantifiable changes in DNA concentration. Moreover, analysis of the DNA electropherograms did not reveal detectable DNA fragmentation or degradation within the tested samples.

DISCUSSION

This study evaluated the efficacy of ZnO-NPs in enhancing the antibacterial action of antibiotics against MDR *A. baumannii* isolates. The results show that ZnO-NPs, whether alone or in conjunction with ceftazidime or ciprofloxacin, effectively controlled *A. baumannii* infection. The augmented antibacterial activity observed with ceftazidime and ciprofloxacin in the presence of ZnO-NPs suggests a disruption of bacterial membranes and subsequent increased accumulation of antimicrobial agents within the bacterial cells.

Our results indicated a high prevalence of MDR *A. baumannii* among the isolates examined. In particular, 100% of the isolates were resistant to all major antibiotic classes commonly used to treat their infections. These high resistance rates were similar to findings from previous studies by Noori *et al*. (2019), Ghasemi *et al*. (2016), Maraki *et al*. (2016), and Vakili *et al*. (2014), all indicating that over 90% of *A. baumannii* isolates were MDR [8, 20-22]. The study revealed that all isolates were resistant to ciprofloxacin, ceftazidime, and ampicillin. Specifically, Shokrollahi *et al*. (2021), Ghasemi *et al*. (2016), Al-Naqshbandi *et al*. (2019), Maraki *et* al. (2016), and Lv *et al*. (2019) observed 100% resistance to these antibiotics across all tested isolates [8, 21, 23-25].

Our results indicated that ZnO-NPs can mitigate resistance to ciprofloxacin and ceftazidime. These observations corroborate the findings of Thati et al. (2010), which reported the amplified antibacterial effects of cephalosporins and aminoglycosides when used in conjunction with ZnO-NPs (80 nm) against Staphylococcus aureus, suggesting potential broader applicability of ZnO-NPs in combating antibiotic resistance [26]. In studies on different bacteria, including *S. aureus* and *E. coli*, ZnO-NPs (20–45 nm) combined with various antibiotics demonstrated variable impacts on bacterial resistance [27, 28]. Other studies in Iran have also revealed the ability of ZnO-NP to enhance the antimicrobial actions of fluoroquinolones (ciprofloxacin) and cephalosporins (ceftazidime) against MDR *A. baumannii* [23, 29]. Moreover, the sub-inhibitory concentration of ZnO-NPs did not affect the activity of ciprofloxacin against *A. baumannii* but enhanced ceftazidime activity [30].

The precise mechanisms by which ZnO-NPs enhance the antibacterial effects of antibiotics have yet to be fully elucidated [31]. Studies suggest that the synergistic effect of nanoparticles with conventional antibiotics may stem from their ability to inhibit the efflux of antibacterial agents from or to facilitate their entry into bacterial cells by compromising membrane integrity [32]. Previous studies suggested that ZnO-NPs may cause genotoxic effects either indirectly, through the induction of oxidative stress, or directly, by damaging four nitrogenous bases of DNA or inducing cross-links between thymine and tyrosine [33]. Still, our results showed no fragmentation or degradation of *A. baumannii* cells when exposed to ZnO-NPs.

In contrast to using NPs and antibiotics alone, combining ZnO-NPs with ciprofloxacin and ceftazidime significantly enhanced antibacterial activity against MDR *A. baumannii*. Consequently, ZnO-NPs may serve as an adjunct to traditional antibiotics to restore antimicrobial effectiveness against resistant strains of bacteria. One potential limitation of our study was the small sample size. Furthermore, our *in vitro* results might not directly apply to clinical settings. *In vitro* studies are instrumental in uncovering mechanisms of action and gauging intervention efficacy, yet they do not fully simulate human body complexity. Future *in vivo* studies are required to confirm the synergistic effect of ZnO-NPs on antimicrobial agents.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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