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ORIGINAL ARTICLE

Modulation of antibiotic resistance in *Pseudomonas aeruginosa* by ZnO nanoparticles

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ABSTRACT

Background and Objectives: Bacterial resistance to conventional antibiotics has become a widespread public health problem. The aim of this study was to investigate the influence of zinc oxide nanoparticles (ZnO NPs) on the antibacterial activity of several conventional antibiotics against *Pseudomonas aeruginosa.*

Materials and Methods: ZnO NPs were prepared by solvothermal method and dispersed in glycerol with the help of ammonium citrate as a dispersant. The antibacterial effects of the resulting ZnO nanofluid, ceftazidime, tobramycin, and ciprofloxacin were investigated against two *P. aeruginosa* strains, including one clinical isolate and *P. aeruginosa* ATCC 9027 using microdilution method. For the evaluation of the combined effect of ZnO nanofluid and antibiotics, the fractional inhibitory concentration indices were calculated and isobolograms were plotted.

Results: Clinical strain in comparison to standard strain of *P. aeruginosa* showed more resistance to ZnO nanofluid and the antibiotics. ZnO nanofluid acted synergistically with ceftazidime and tobramycin against both strains. Combination of ZnO nanofluid and ciprofloxacin displayed synergistic and partial synergistic activity against clinical and standard strains of *P. aeruginosa,* respectively.

Conclusion: The results suggest that bacterial resistance to antimicrobials could be reduced by the synergistic action of ZnO NPs.

Keywords: Ceftazidime, Ciprofloxacin, *Pseudomonas aeruginosa,* Tobramycin, ZnO nanoparticles

INTRODUCTION

Pseudomonas aeruginosa is a frequent cause of nosocomial pneumonia, hospital-acquired urinary tract infections, wound infections, and blood stream infections (1, 2). *P. aeruginosa* is also responsible for opportunistic infections in immunocompromised patients (1). Three major classes of antibiotics are commonly used against this pathogen, including aminoglycosides (tobramycin), β-lactams (ceftazidime), and quinolones (ciprofloxacin) (3). Quinolones and β-lactams inhibit DNA gyrase and cell wall peptidoglycan-assembling transpeptidases, respectively, while aminoglycosides inhibit protein synthesis by binding to the 16S rRNA within the 30S ribosomal subunit (4-6). *P. aeruginosa* exhibits intrinsic resistance to many antibacterial agents, and moreover tends to acquire additional resistance during therapy (7). There are several mechanisms for antibiotic resistance, which include low intrinsic cell wall perme-

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ability, efflux systems, inactivation and modification of antibiotics, and changes in targets of antibiotics (4). A common approach to overcome antibiotic resistance is to use other compounds in combination with antibiotics (7).

Over the past decade, some of the nanometer-sized metal oxides were found to be cytotoxic against bacteria (12). Antibacterial effects of zinc oxide nanoparticles (ZnO NPs) on a large number of microorganisms have been reported to be size-dependent (13-16). ZnO NPs are biosafe up to a certain amount, but may be hazardous at higher concentrations. Reddy et al. found no reduction in cell viability of human T cells following exposure to ZnO NPs at concentrations below 5 mM (17). Yang et al. also reported no hemolysis of red blood cells exposed to ZnO quantum dots in concentration above 1600 µg/ml (18). It is at present difficult to determine the threshold limits for various forms of ZnO due to a lack of sufficient data. The antibacterial mechanisms of ZnO NPs include binding to and damaging the bacterial membrane, penetrating into the bacterial, and generating reactive oxygen species (ROS) (19, 20).

In the present study, we investigated the influence of ZnO NPs on the antibacterial activity of tobramycin, ceftazidime, and ciprofloxacin against *P. aeruginosa*. The combination effects between ZnO nanofluid and the antibiotics under investigation were also evaluated.

MATERIALS AND METHODS

The studies were performed on a standard strain of *P. aeruginosa* (ATCC 9027) and a clinical isolate of this bacterium (kindly supplied by Faculty of Veterinary Medicine, Ferdowsi University of Mashhad). Ceftazidime, tobramycin, and ciprofloxacin were purchased from Sigma.

Preparation and characterization of ZnO NPs. ZnO NPs were synthesized by dissolving 0.001 mol of zinc acetate dehydrate in 920 ml of deionized water. Then, 80 ml of 0.02 M sodium hydroxide was added to the solution drop-wise to zinc acetate dehydrate solution under magnetic stirring at 0 °C. The formed transparent $Zn(OH)_4^{2-}$ solution was incubated into a water bath at 65 °C for 2 h and at room temperature for 3 days. Afterwards, ZnO NPs were separated from the suspension by centrifugation, washed several times by deionized water and ethanol and finally dried in a vacuum oven at 40 °C for 10 h (21). The X-ray diffraction (XRD) pattern of ZnO NPs was recorded using D8 Advance diffractometer (Bruker) with Cu Kα radiation (λ= 0.15406 nm). The average crystallite size can be calculated using Debye Scherrer equation:

$$D_{hkl} = \frac{k \times \lambda}{\beta_{hkl} \times \cos\theta_{hkl}}$$

where D_{hkl} is the crystallite size perpendicular to the normal line of (hkl) plane, k is a constant (0.9), λ is the wavelength of X-ray, β_{hkl} is the full width at half maximum of the (hkl) diffraction peak and θ_{hkl} is the Bragg angle of (hkl) peak.

Preparation of stable ZnO nanofluid. ZnO nanofluid was prepared by dispersing ZnO NPs in glycerol as the base fluid with ammonium citrate as dispersant. The weight ratio of nanoparticles to ammonium citrate was kept 1:1 (21). The ZnO suspension was continuously stirred for a few hours to give a stable suspension with uniform dispersion of ZnO NPs. The particle size distribution (PDS) of the prepared ZnO NPs was examined by dynamic light scattering (DLS) from Malvern Zetasizer Nano-ZS instrument.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination. MIC was expressed as the lowest concentration of antibacterial agent that caused complete inhibition of growth during 24 hours (22). The MIC of ZnO nanofluid, ceftazidime, tobramycin, and ciprofloxacin were obtained by the broth microdilution method in a microplate. Serial two-fold dilutions of ZnO nanofluid and all the antibiotics with the following concentrations: ZnO nanofluid (5.8-375 µg/ml), ceftazidime (0.25-32 µg/ml), tobramycin (0.25-16 µg/ml), and ciprofloxacin (0.25-16 µg/ml) were prepared with nutrient broth medium (23, 24). A suspension of exponentially growing bacteria was added to each medium, to attain a final bacterial concentration of 10^5 CFU/ml. Samples were placed in 96-well microtiter plates and incubated for 24 hours at 37 ºC with shaking about 120 rpm. Bacterial growth was monitored every 2 h by measuring the absorbance at 630 nm (OD_{630}) until 24 h. The medium without antibacterial agents as positive control and medium without bacteria as negative control were included

for each assay. For evaluation of antibacterial activity of ZnO NPs, antibacterial effect of glycerol and ammonium citrate was also assayed and considered as control. The antibacterial activity of the base fluid and dispersant was excluded from the antibacterial activity of ZnO nanofluid.

The MBC of ZnO nanofluid and the antibiotics were also determined. Briefly, 10 µl aliquots of each above sample, demonstrating no visible growth, were plated on culture agar plates. After 24 hours incubation at 37 ºC, colony formation was investigated. MBC was defined as the lowest concentration of antibacterial agent that kills more than 99% of the bacteria (22).

After determining the MIC of ZnO NPs and antibiotics alone, we examined combinations of ZnO NPs with ceftazidime, tobramycin, and ciprofloxacin at subinhibitory concentrations. The following concentrations were tested: 1/16 × MIC – 1/2 × MIC for ZnO NPs and 1/8 × MIC – 1/2 × MIC for antibiotics (25, 26). The percentage of growth inhibition for both microorganisms in comparison with positive controls was determined using Eq. 1:

$$GI\% = (100 - \frac{OD_{630} \text{ at the presence of antibacterial agent (s)}}{OD_{630} \text{ of positive control}} \times 100)$$

To avoid potential optical interference of the growing cultures caused by the light-scattering properties of the NPs, the same liquid medium without bacteria, but containing the same concentration of NPs was incubated under the same conditions and used as the blank control.

Fractional inhibitory concentration index (FICI) determination and isobologram analysis. The combined action of ZnO nanofluid and each of the antibiotics was studied by checkerboard broth microdilution method (11). The MIC of antibiotics and ZnO nanofluid in combination was determined and the fractional inhibitory concentration index (FICI) was calculated using the following formula:

$$FIC_A = \frac{MIC_A \, combination}{MIC_A alone}$$

$$FIC_B = \frac{MIC_B combination}{MIC_B \, alone}$$

$$FICI = FIC_A + FIC_B$$

where MIC_A combination is the MIC of drug A in the presence of drug B and vice versa for MIC_B combination; MIC_A and MIC_B are MIC of A and B drugs alone, respectively. FICI was interpreted as follow: FICI ≤ 0.5 synergy, FICI > 0.5 and FICI < 1 partial synergy, FICI = 1 additive, FICI ≥ 2 and FICI < 4 indifferent, and FICI > 4 antagonism (3, 10). Moreover, the combination effect of ZnO nanofluid with the antibiotics was evaluated by isobologram analysis. Briefly, in a two-coordinate plot with one coordinate representing concentration of ZnO nanofluid and other representing concentration of the antibiotic (tobramycin, ceftazidime, and ciprofloxacin), MIC of ZnO nanofluid and the antibiotic are located on the x and y-axes, respectively. These two points are connected by the line of additively. The combination concentration of ZnO nanofluid and antibiotic that caused complete inhibition of bacterial growth, are pointed in the plot. As this point is located below, on, or above the additively line indicates synergy, additively or antagonism, respectively (27).

Data Analysis. The results of percentage of growth inhibition of the tested antibiotics alone and in combination with ZnO NPs are expressed as mean ± standard error mean (SEM). Every experiment was repeated at least three independent times. All data were analyzed and compared utilizing one-way ANOVA Tukey test (SPSS software) and differences with $p < 0.05$ were considered significant. The obtained FICI values for each combination were compared through Student's t test (28).

RESULTS AND DISCUSSION

Characterization of the ZnO NPs. The XRD pattern of ZnO NPs is shown in Fig. 1 and confirmed that ZnO NPs are crystalline. A number of strong Bragg reflections can be seen which correspond to the (100), (002), (101), (102), (110), (103), (200), (112), and (201) reflections of wurtzite hexagonal phase of ZnO (29). The diffraction peaks match well with those in the JCPDS card (Joint Committee on Powder Diffraction Standards, Card No. 89-1397). The crystallite size of the ZnO NPs estimated using Scherrer formula (Eq. 5) was 20.2 nm.

The average particle size determined by DLS was 181.9 nm (Fig. 2). Clearly, the mean particle size obtained by DLS is greater than of the actual particle size since the hydrodynamic radius is probed with DLS.

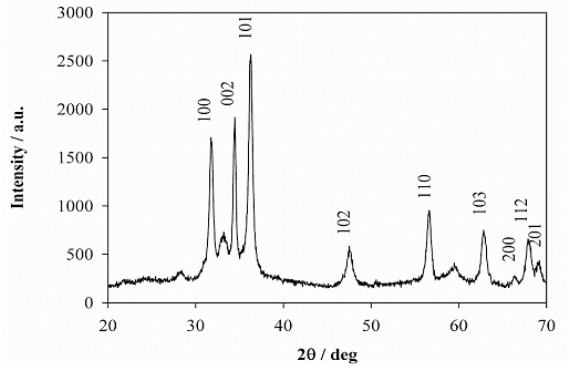

Fig. 1. Powder X-ray diffraction of the ZnO nanostructures

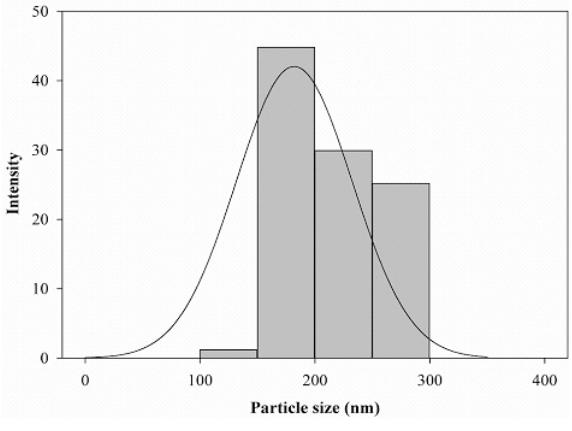

Fig. 2. Particle size distribution of ZnO NPs

MIC and MBC of ZnO NPs, tobramycin, ceftazidime, and ciprofloxacin. The antimicrobial activity of ZnO NPs and antibiotics alone and in combination were tested at different concentrations against standard and clinical *P. aeruginosa* strains in nutrient broth medium. The results showed that *P. aeruginosa* ATCC9027 was more susceptible to ZnO NPs than the clinical isolate of this bacterium with MIC values of 93.7 and 375 µg/ml for standard and clinical strains, respectively. The MBC/MIC values of ZnO NPs against the standard and clinical strains were, respectively, 1 and 2. The MIC values for ceftazidime against standard and clinical isolates of *P. aeruginosa* were 0.5 to 8 µg/ml, for tobramycin 0.5 to 2 µg/ml, and for ciprofloxacin 0.0625 to 0.5 µg/ml. The MBC values of these three antibiotics were equal with the MIC values of them. These findings show that mechanism of antibacterial activity of ZnO NPs, tobramycin, ceftazidime, and ciprofloxacin against both strains is bactericidal (22). As shown in Tables 1 and 2, growth of standard and clinical strains of *P. aeruginosa* decreased as the concentration of ZnO NPs and the tested antibiotics increased.

FICI and isobologram analysis. The synergistic effect between ZnO nanofluid and the antibiotics were evaluated using the checkerboard assay and

Table 1. The percentage of *P. aeruginosa* ATCC 9027 growth inhibition in the presence of the tested antibiotics alone and in combination with ZnO NPs at subinhibitory concentrations (1/2 and 1/4 × MIC)

Antibiotic Concentration (µg/ml)	ZnO NPs Concentration (µg/ml)		
	0	23.4	46.8
Ceftazidime			
0	0	23.29 ± 0.02	47.41 ± 0.28
0.0625	47.44 ± 1.74	50.94 ± 0.54	100
0.125	57.63 ± 2.53	59.27 ± 1.86	100
0.25	70.81 ± 1.94	100	100
Tobramycin			
0	0	23.29 ± 0.04	47.41 ± 0.48
0.0625	50.82 ± 0.59	57.84 ± 2.83	100
0.125	59.83 ± 0.77	68.72 ± 1.51	100
0.25	68.63 ± 2.36	100	100
Ciprofloxacin			
0	0	23.75 ± 4.64	47.14 ± 3.82
0.0078	32.91 ± 1.93	36.74 ± 6.52	100
0.0156	54.62 ± 0.54	55.11 ± 4.49	100
0.0312	66.80 ± 0.94	64.87 ± 2.91	100

ZnO NPs: ZnO nanoparticles

Table 2. The percentage of clinical isolate of *P.aeruginosa* growth inhibition in the presence of the tested antibiotics alone and in combination with ZnO NPs at subinhibitory concentrations (1/2 - 1/16 × MIC)

Antibiotic Concentration (µg/ml)	ZnO NPs Concentration (µg/ml)				
	0	23.4	46.8	93.7	187.5
Ceftazidime					
0	0	32.48 ± 2.08	50.57 ± 3.38	76.78 ± 3.03	93.29 ± 0.82
1	12.29 ± 2.83	46.52 ± 0.47	95.49 ± 1.48	98.32 ± 0.32	99.8 ± 0.75
2	22.96 ± 1.12	93.57 ± 1.60	98.12 ± 0.12	98.88 ± 0.11	100
4	47.52 ± 7.69	99.20 ± 0.46	100	100	100
Tobramycin					
0	0	42.44 ± 1.59	57.54 ± 0.67	79.24 ± 1.61	93.15 ± 0.60
0.25	7.68 ± 2.76	81.03 ± 3.07	92.24 ± 1.95	97.12 ± 0.24	97.12 ± 0.24
0.5	25.10 ± 3.24	85.45 ± 1.46	96.51 ± 0.80	100	100
1	62.31 ± 3.03	92.03 ± 0.82	100	100	100
Ciprofloxacin					
0	0	45.01 ± 2.64	54.05 ± 1.45	75.87 ± 2.37	94.81 ± 1.65
0.0625	42.51 ± 3.48	74.66 ± 1.16	94.15 ± 0.82	98.59 ± 0.09	98.57 ± 0.25
0.125	78.40 ± 6.10	94.66 ± 0.50	100	100	100
0.25	96.96 ± 0.87	100	100	100	100

ZnO NPs: ZnO nanoparticles

FIC index was calculated. FICI is an indicator of degree of interaction between ZnO nanofluid along with tobramycin, ceftazidime, and ciprofloxacin for standard and clinical *P. aeruginosa* strains (Table 3). The FICI of the combination of ZnO nanofluid plus ceftazidime or tobramycin was calculated as 0.375, showing synergistic interaction of ZnO nanofluid with these antibiotics against the two strains under investigation. The combination of ZnO nanofluid with ciprofloxacin against the standard and clinical strains is estimated as partial synergism and synergism, respectively.

Combination effect between ZnO nanofluid and the antibiotics under investigation against both *P. aeruginosa* strains was also evaluated by isobologram analysis (Fig. 3 and 4). The points of combination concentrations of the two antibacterial agents in each assay are located on or below the additively line,

Table 3. Fractional inhibitory concentration index (FICI) of the tested antibiotics alone and in combination with ZnO NPs against *P. aeruginosa* ATCC 9027 and clinical isolate of *P.aeruginosa* as determined by checkerboard method

Strain	MIC in combination								
	ZnO NPs + CAZ			ZnO NPs + CIP			ZnO NPs + TOB		
	ZnO NPs (µg/ml)	CAZ (µg/ml)	FICI	ZnO NPs (µg/ml)	CIP (µg/ml)	FICI	ZnO NPs (µg/ml)	TOB (µg/ml)	FICI
Clinical isolate of *P.aeruginosa*	93.7	2	0.5	23.4	0.125	0.3125	46.8	0.5	0.375
P.aeruginosa ATCC 9027	23.4	0.0625	0.375	46.8	0.0078	0.625	23.4	0.0625	0.375

ZnO NPs: ZnO nanoparticles, CAZ: Ceftazidime, CIP: Ciprofloxacin, TOB: Tobramycin, FICI: Fractional inhibitory concentration index

showing additively or synergy effect, respectively.

Our cytotoxic analysis showed that combined use of ZnO nanofluid with the antibiotics potentiated their antibacterial activity in both test strains. Permiabilizing agents are reported to have the capability of increasing the susceptibility of *P. aeruginosa* to some antibiotics (30). A mechanism of antibacterial activity of ZnO NPs was reported to damage to the cell membrane and leak out contents of bacterial cell (31, 32). Therefore, it is probable that ZnO NPs by increasing the permeability of cell membrane may cause the enhancement of antibiotic efficacy. Anoth-

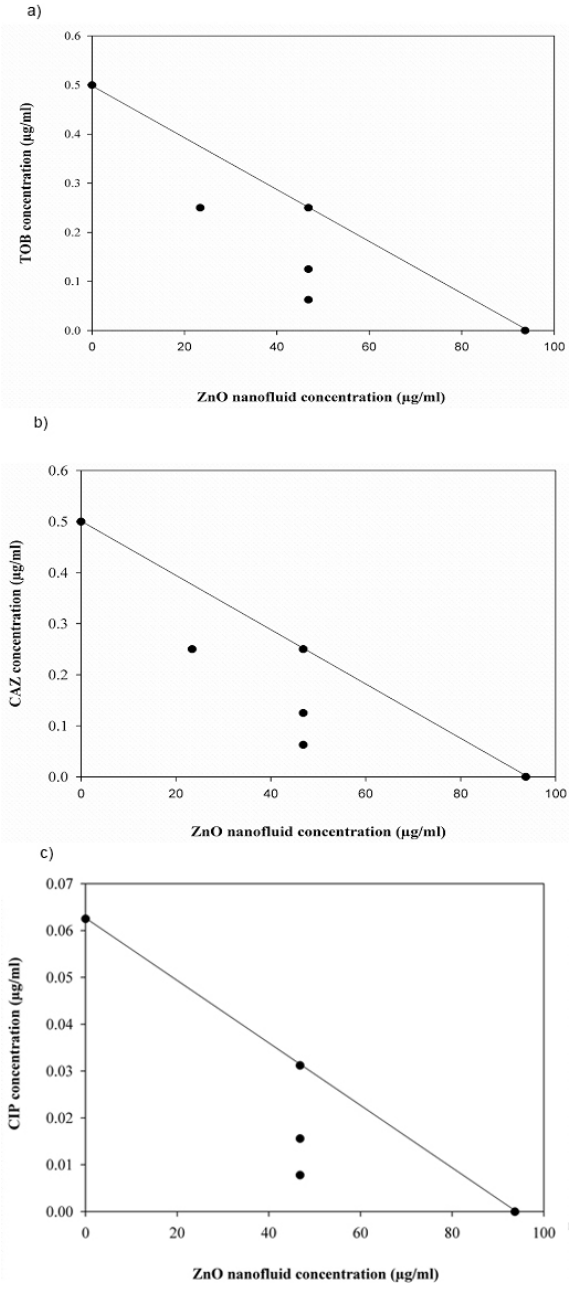

Fig. 3. Isobologram analysis for the combinations of ZnO nanofluid and antibiotics against *P. aeruginosa* ATCC 9027. (a) tobramycin (TOB), (b) ceftazidime (CAZ) and (c) ciprofloxacin (CIP)

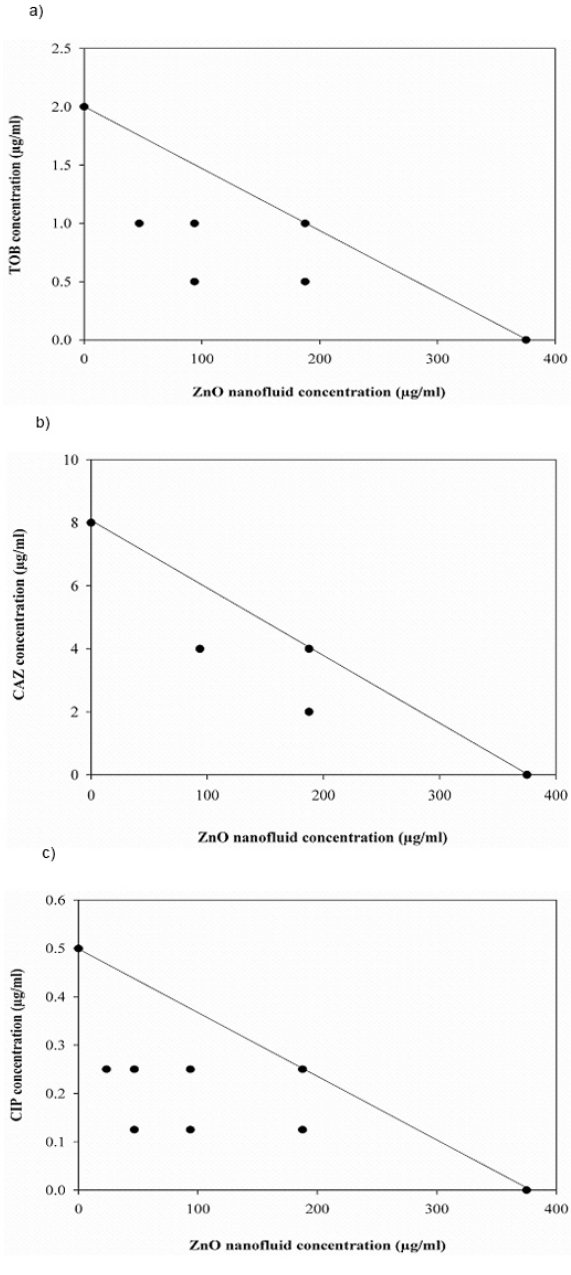

Fig. 4. Isobologram analysis for the combinations of ZnO nanofluid and antibiotics against clinical isolate of *P. aeruginosa.* (a) tobramycin (TOB), (b) ceftazidime (CAZ) and (c) ciprofloxacin (CIP)

er explanation for these findings would be that ZnO NPs may interfere with pumping activity of efflux systems (33).

Metal ions may play a very important role in the mechanism of action of these antibiotics (34-36). The interaction of several metal ions with quinolones is reported (37, 38). For example, carbonyl group, carboxylic oxygens, and fluore atom in ciprofloxacin may interact with Zn atom in ZnO NPs. These interactions may stabilize ciprofloxacin-ZnO NPs system. Ionic interaction between protonated nitrogen atoms in ciprofloxacin and hydroxylated surface of ZnO NPs may also stabilize ciprofloxacin-ZnO NPs system (33). Anacona et al. showed that the copper (II) and zinc (II) complexes of ciprofloxacin had higher antibacterial activity than ciprofloxacin against *P. aeruginosa* (39).

In this study, it is probable that ciprofloxacin may form complex with Zn^{2+} ions released from the surface of ZnO NPs and increase its antibacterial activity. ZnO NPs may also inhibit the efflux transporters and thereby increase the efficacy of antibiotics against *P. aeruginosa*.

CONCLUSION

In the present paper, we report for the first time the combination effect of ZnO nanofluid with antibiotics tobramycin, ceftazidime, or ciprofloxacin against *P. aeruginosa.* Our findings suggest that application of ZnO nanofluid combined with these three antibiotics can enhance their antibacterial effects.

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