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Synergistic Effect of ZnO Nanoparticles with β-lactam Antibiotic against β-lactamase Producing Pathogenic *Pseudomonas aeruginosa*

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ABSTRACT

Nanomedicine among several approaches is considered of potential therapeutic value. So, zinc oxide nanoparticles (ZnO-NPs) conjugated with clinically approved drugs (β-lactam Antibiotic) was synthesized with the aim to investigate the effect of ZnO NPs and ZnO NPs-antibiotics combinations against clinically isolated *Pseudomonas aeruginosa* that showed significant resistance to β-lactam antibiotic carbenicillin and detecting the level of *ampC* gene expression before and after ZnO NPs exposure. A total of, 10 isolates of *P. aeruginosa* were identified. The minimum inhibitory and bactericidal concentrations (MIC and MBC, respectively) of ZnO NPs and carbenicillin were determined using the colony counting method. Quantitative real-time PCR (RT-qPCR) was performed to estimate the expression of *ampC*. ZnO NPs with nanoscale size were effectively synthesized using the sono-chemical co-precipitation method as a simple and cost valuable method. ZnO NPs were highly effective against clinically isolated *P. aeruginosa* in a dose-dependent pattern. Moreover, the combination of ZnO NPs with carbenicillin increased the antibacterial activity of carbenicillin. Finally, the expression of *ampC* was down regulated due to ZnO NPs treatment and the down regulation was inversely proportional. Combination of ZnO NPs and carbenicillin highly induced down regulation of *ampC* gene without significant differences among different concentrations used.

Key words: ZnO NPs, *ampC*, *Pseudomonas aeruginosa*, β-lactam antibiotic

INTRODUCTION

The primary cause of hospital and community-associated infections in people is the bacterium known as *Pseudomonas aeruginosa*, which is considered to be a serious medical issue that requires treatment with antibiotics. In order to minimize the clinical outcome, the use of antibiotics against resistant strains of this bacterium was conducted.

The main strategies of bacterial resistance against antibiotic attack were categorized into three main categories: intrinsic, acquired and adaptive resistance (Arzanlou *et al.*, 2017). In the case of *P. aeruginosa*, the intrinsic resistance involves the reduction of its outer membrane permeability and the production of deactivating enzymes (Pang *et al.,* 2019). On the other hand, the acquired resistance is caused by the development of a resistance gene that can be transferred to the bacteria through a combination of genetic and phenotypic changes (Pachori *et al.*, 2019). In the case of adaptive resistance, the formation of a thick layer of a protective film in the lungs of infected patients helps limit the interaction of antibiotics with the bacterial cells (Kamali *et al.*, 2020; Langendonk *et al.*, 2021).

The increased level of intrinsic resistance in *P. aeruginosa* to antibiotics is high due to the development of efflux systems that allow the bacteria to release antibiotics from their outer membrane (Nishino *et al.*, 2021). On the other hand, the development of an inducible *ampC* gene that allows the bacteria to produce deactivating enzymes has additionally been known to contribute to the development of this resistance (Berrazeg *et al.*, 2015). Due to the presence of multidrug-resistant strains, they can form a type of biofilm that can lead to prolonged and frequent infections (Silva *et al.*, 2021).

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Nanotechnology provides an innovative strategy for developing new combinations based on metallic nanoparticles like silver, titanium, zinc and others that exhibit antibacterial properties with combination of antibiotics (Sánchez-López *et al.*, 2020). Interactions of metallic nanoparticles with antibiotics are commonly used to test the combined action of nanoparticles with antibiotics against pathogenic bacteria, in addition, many studies reported that the efficacy of antimicrobial agents could be boosted by compounding antibiotics with metallic nanoparticles against different pathogens, including *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Slavin *et al.*, 2017; Gudkov *et al.*, 2021). Zinc supplementation has numerous therapeutic roles in different diseases. Moreover, zinc oxide has been found to display potential antimicrobial activities. Previous studies have revealed the advanced action of ZnO nanoparticle (ZnO NPs) when used in conjugation with β-lactam antibiotics and others against different pathogenic microorganisms (Mendes *et al.,* 2022).

According to increasing antibiotic resistance of *P. aeruginosa* and frequent infections, this study aimed at investigating the effect of ZnO NPs and ZnO NPs-antibiotic combinations against clinically isolated *P. aeruginosa* that showed significant resistance to β-lactam antibiotic carbenicillin and detecting the level of *ampC* gene expression before and after ZnO NPs exposure.

MATERIALS AND METHODS

A total of 10 isolates of *P. aeruginosa* were kindly provided from Central Public Health Laboratories. All isolates were isolated from different clinical samples including sputum, urine, blood, abdominal drainage and body fluids. Isolates were morphologically and biochemically identified as *P. aeruginosa*. The isolates were cultivated in nutrient broth (Himedia Laboratories Ltd., India) at 37°C for 24 h in shaker at 200 rpm. For solid medium, 1.5 g of agar (Himedia Laboratories Ltd., India) was added to 100 ml nutrient broth.

ZnO NPs were synthesized depending on sonochemical-coprecipitation method (Molnár *et al.*, 2020). In brief, $ZnCl_2$ solution (0.2 M) was gently dripped into 25% ammonium hydroxide solution. After complete dissolution, the mixture was heated to 60°C under vigorous stirring (120 rpm) to ensure complete precipitation. The precipitate was subjected to ultrasonic bath for 30 min to obtain ZnO NPs. The precipitate was filtered (0.22 µm), washed with deionized water and dried overnight at 100°C.

The synthesized ZnO NPs were characterized using various analytical techniques including, UV-Vis spectrophotometry, Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction and Field emission scanning electron microscope (SEM). The UV-Vis absorbance spectrum was recorded in Shimadzu NIR spectrophotometer using water as a reference solvent. The FT-IR spectrum was recorded in transmission mode with a Bruker Optik QCL system in the range of 400-4000/cm. XRD analysis of ZnO NPs was detected using Shimadzu XRD6000 equipped with power diffractometer with source of Cu Kα radiation and wavelength of 1.5405. Samples were scanned with range from 10° to 80° and mean crystalline size of ZnO NPs was calculated using Scherrer's formula of full width at half maximum (FWHM) equation of the intense X-ray diffraction peaks (Vorokh, 2018). Surface morphology of ZnO NPs was determined using Zeiss SIGMA VP-500.

The antimicrobial activity of ZnO NPs and carbenicillin (individual and combined) was investigated using colony counting method (Arora *et al.*, 2015). Fresh colonies from each *P. aeruginosa* strain were cultivated in 3 ml nutrient broth overnight in shaker incubator at 37°C. After incubation, bacterial cells were centrifuged at 3000 rpm for 10 min at 4°C and washed twice using phosphate buffer saline PBS (pH: 7.2). Bacterial cells were resuspended in PBS and bacterial turbidity was adjusted to 0.5 McFarland standard (Absorbance 0.1 at 600 nm) which corresponded cell density of 10^8 Colony Forming Unit per milliliter (CFU/ml). Aliquot of 100 µl of bacterial suspension (10^{-3} dilution) was added to 1 ml of nutrient broth containing 10, 20 and 30 µg/ml, nutrient broth containing 30 µg/ml carbenicillin, nutrient broth containing combined ZnO NPs and carbenicillin. Media containing different treatments without bacterial inoculation were used as control. All cultures were incubated at 37°C overnight. After incubation, 50 µl of

bacterial suspension was spread over nutrient agar plates and the colonies formed were counted. The rate of inhibition (%) was calculated as:

Inhibitory rate (%) = 100 x [(CFU Control – CFU Sample)]/CFU control

The expression of *ampC* in *P. aeruginosa* strains was determined using RT-qPCR. Total RNA was extracted from bacterial culture using AccuPrep® Bacterial RNA Extraction Kit (Bioner) according to the manufacturer's instructions. QuantiTect Reverse Transcription Kit (QIAGEN) was used to reverse transcription to cDNA. Primers used in reverse transcription were: (F) 5′-CGCCGTACAACCGG TGAT and (R) 5′-GAAGTAATGCGGTTCTC CTTTCA (Gupta *et al.*, 2014). The resulting cDNA was used to detect the expression of *ampC* in ABI PRISM 7500 (Applied Biosystem) and the expression was normalized with housekeeping gene *ropD* (F: 5′-CAGCAATCTCGTCTGA AAGAGTTG; R: 5′-TTGATCCCCATGTCG TTGATC). Means of threshold cycle (Ct) values were calculated and the differences in fold of expression were determined using $2^{-\Delta\Delta Ct}$ method.

Data were expressed as mean±standard deviation. One-way ANOVA was used to determine differences between different groups. $P < 0.05$ was considered significant. GraphPad Prism (GraphPad Software, California, USA) version 9.0 was used to analyze the results.

RESULTS AND DISCUSSION

UV-Vis analysis was conducted to confirm the formation of ZnO with nanoscale range. In Fig. 1 absorption spectra of ZnO NPs at room temperature showed that a characteristic absorption peak of ZnO at wavelength 345 nm could be considered as the intrinsic absorption peak of ZnO due to electron transitions from the equivalence band to the conduction band ($O_{2p} \rightarrow Zn_{3d}$). In addition, the appearance of sharp peak with ultraviolet range confirmed the formation of ZnO NPs in the mixture (Muhammad *et al.*, 2019).

The FT-IR spectrum of ZnO NPs was detected within the range of 500 to 4500/cm. Results in Fig. 2 show a significant vibration area ranging from 500 to 1270/cm with the presence of significant peak at 575/cm that represented Zn-O binding. The vibration

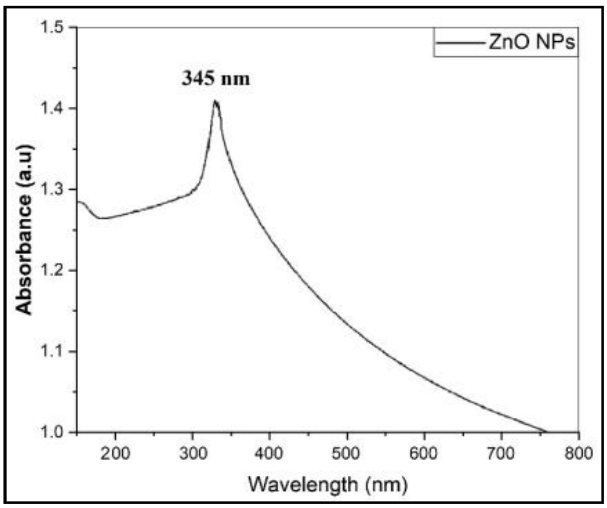

Fig. 1. Uv-Vis spectra of ZnO NPs. The measurement was obtained 24 h after preparation.

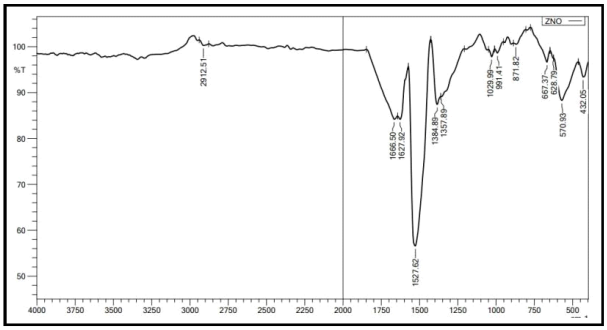

Fig. 2. FT-IR spectra of ZnO NPs. The experiment was performed at room temperature.

resulted beyond 2000/cm represented the stretching of hydroxyl group which confirmed the existing of water molecules in which ZnO NPs dissolved (Arumai Salvan *et al.*, 2021).

The crystalline structure and size of ZnO NPs detected X-ray diffraction (Fig. 3). Based on XRD analysis, the resulted diffraction peaks were detected at 2 Theta: 32.1°, 34.7°, 36.8°, 47.3°, 56.7°, 63.6°, 65.1°, 68.2° and 69.4° which

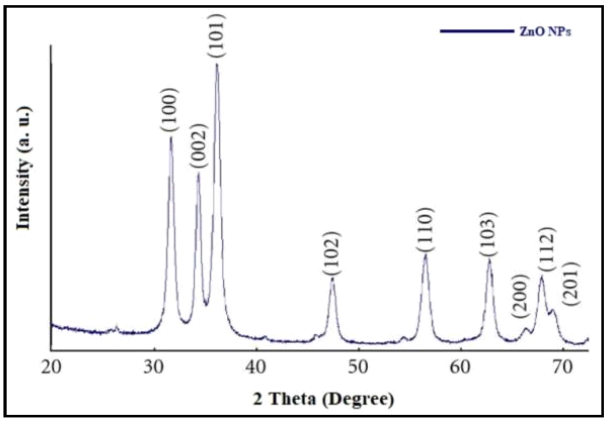

Fig. 3. XRD pattern of ZnO NPs.

corresponded to 100, 002, 101, 102, 110, 103, 200, 112 and 201 as hexagonal crystals (JCPSD No. 01-007-2551). The average size of ZnO NPs calculated from XRD based on Scherrer equation was 30.21 nm.

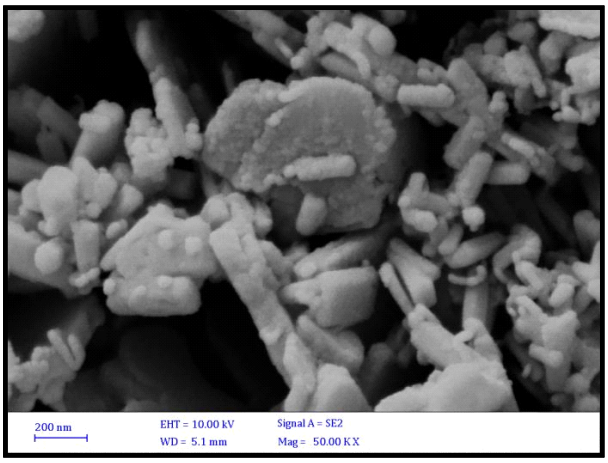

Fig. 4. ZnO NPs morphologies using scanning electron microscope (Scale 200 nm).

Finally, SEM was used to identify the morphology of ZnO NPs. Fig. 4 shows individual aggregations of ZnO NPs, however, ZnO NPs appeared as spherical structures with cumulations of nano-crystallites.

The bactericidal activity of ZnO NPs and carbenicillin was estimated against clinical isolates of *P. aeruginosa* using colony count method. Results (Fig. 5) showed that ZnO NPs treatment caused significant ($P<0.05$) reduction in CFU in dose-dependent pattern,

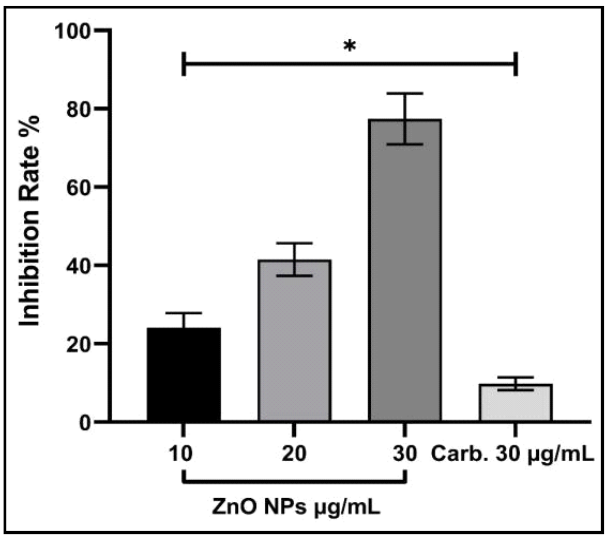

Fig. 5. Mean (±SD) inhibition of *P. aeruginosa* colony formation after treatment with ZnO NPs (10, 20 and 30 µg/ml) compared with carbenicillin treatment (30 µg/ml).

in which ZnO NPs at concentration 10 µg/ml caused reduction in viability by 24.1±3.71%, 41.49±4.14% at 20 µg/ml and highest sensitivity to ZnO NPs was recorded at 30 µg/ml with maximum inhibition of 77.35±6.48%. On the other hand, carbenicillin exhibited the least inhibitory effect against *P. aeruginosa* not exceeding 9.79±1.63%. It was indicated that ZnO NPs synthesized with the size of less than 100 nm can be effective against β-lactam resistant *P. aeruginosa* used in this study. The relationship between the bactericidal effect of ZnO NPs and concentration was proportionally increased by increasing the dose, in which at concentration 30 µg/ml of ZnO NPs all isolates were killed by approximately 80%. These results are in agreement with other studies using pathogenic multidrug-resistant *P. aeruginosa* (Arora *et al.,* 2015; Ali *et al.,* 2020). It was reported that pathogenic bacteria like methicillin resistance *Staphylococcus aureus* (MRSA) and *Escherichia coli* were markedly affected by ZnO NPs prepared within 30 nm in diameter (Kadiyala *et al.,* 2018).

The combination of different concentrations of ZnO NPs and carbenicillin were tested on all clinically isolated *P. aeruginosa*. The combination between ZnO NPs and antibiotic significantly ($P<0.05$) enhanced the inhibition effect in comparison to individual carbenicillin on tested *P. aeruginosa*. The combination treatments increased the inhibition rate of ZnO NPs by 33.34, 39.94 and 18.03% at 10, 20 and 30 µg/ml ZnO NPs with carbenicillin, respectively.

Carbenicillin β-lactam was first introduced as antipseudomonal antibiotic. However, carbenicillin is less stable to β-lactamase hydrolysis (Bush and Bradford, 2016). According to structure, carbenicillin consisted of hydroxy and amide moieties, the ionic interaction between the reduced ZnO NPs and active moieties of carbenicillin stabilized the antibiotic and enhanced its antimicrobial activity (Kadiyala *et al.*, 2018). In addition, Zn working as chelating metal that interfered with bacterial membrane allowing the increase access of carbenicillin to bacterial cells (Fadwa *et al.*, 2021).

The expression of *ampC* gene was detected in *P. aeruginosa* isolates treated with different concentrations of ZnO NPs, carbenicillin and ZnO NPs combined with carbenicillin compared with untreated cells as control. The average

of fold change was calculated depending on $^{\Delta\Delta}$Ct method. Results in Table 1 show significant differences (P< 0.05) in *ampC* gene expression between different groups. Over expression was detected in *P. aeruginosa* isolates treated with carbenicillin by 4.21 fold compared with untreated. A decrease in *ampC* gene expression was observed in ZnO NPs treated isolates and the effectiveness was dose-dependent by 2.73, 1.78 and 1.26 fold for treatments 10, 20 and 30 µg/ml, respectively.

Table 1. The effect of ZnO NPs at different concentrations with average of folds of gene (*ampC*) expression

Treatments	Average of fold of expression
Untreated *P. aeruginosa*	1.0[a]
Carbenicillin	4.21[b]
ZnO NPs 10 µg/ml	2.73[c]
ZnO NPs 20 µg/ml	1.78[d]
ZnO NPs 30 µg/ml	1.26[e]
ZnO NPs 10 µg/ml + Carbenicillin	0.51[ae]
ZnO NPs 20 µg/ml + Carbenicillin	0.45[e]
ZnO NPs 30 µg/ml + Carbenicillin	0.43[e]

Different superscripts (a, b, c, d, e) considered as significant differences at P<0.05.

The combination of ZnO NPs and carbenicillin significantly down regulated the expression of *ampC* gene, however, no significant differences were observed among combination treatments.

The effects of ZnO NPs markedly affected the expression of *ampC* gene by reducing the susceptibility of carbenicillin to β-lactamase digestion. The gene *ampC* not only caused hydrolysis of carbenicillin but also affected wide spectrum of β-lactam antibiotics. It was reported that *ampC* gene highly increased the minimum inhibitory concentration of β-lactam antibiotics (Mirsalehian *et al.*, 2014). Although the mechanism of ZnO NPs not directly affected the genes responsible to degrading β-lactam antibiotics, ZnO NPs were well known for its ability to induce the over expression of different stress genes and thus caused accumulation of intracellular free radicals which at the end caused cell death (Jiang *et al.*, 2018). Moreover, over expression of stress genes significantly caused down regulation of many virulence genes and genes involved in antibiotic susceptibility (Musarrat *et al.*, 2015). In summary, ZnO NPs with nanoscale size were effectively synthesized using sonochemical-coprecipitation method as a simple and cost valuable method. ZnO NPs were highly effective against clinically isolated *P. aeruginosa* in dose-dependent pattern. Moreover, combination of ZnO NPs with carbenicillin increased anti-bacterial activity of carbenicillin. Finally, the expression of *ampC* was down regulated due to ZnO NPs treatment and the down regulation was inversely proportional. Combination of ZnO NPs and carbenicillin highly induced down regulation in *ampC* gene without significant differences among different concentrations used.

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