Original Article

Zinc Oxide Nanoflakes Mechanism of Action: A Future Prospective Nanomedicine Against CRE Infections

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Abstract

The emergence and spread of carbapenem-hydrolysing beta-lactamases among *Enterobacteriaceae* and Non-fermentative Gram-negative bacilli organisms like *Pseudomonas spp.* or *Acinetobacter baumannii* over the past ten years is a serious problem in hospital settings. This problem has been exacerbated by the fact that carbapenems are no longer as effective against these pathogens. Aside from that, numerous bacteria in the natural environment form biofilms. When opportunistic biofilm-forming pathogens cause infections, they can cause severe symptoms in many cases and death in people who don't have strong immune systems. No new class of antibiotic have been reported in the last several decades. In this context, Recent years have seen a rise in the importance of nanobiotechnology in the production of nanomaterials that function as antibacterial agents. It is possible for nanoparticles to be active molecules or compounds that react with bacterial cells to kill them. This has the potential to resolve issues associated with multidrug resistance and inhibit biofilm. In the present study, synthesized ZnO nanoflakes were utilized. These nanoflakes had an average crystallite size of 21.5 ± 4.8 nm, a thickness of 20-50 nm, and different characteristics. We have included 67 Carbapenem resistant Enterobacteriaceae (CRE) and Non-fermentative Gram-negative bacilli isolates, which were isolated from various clinical samples. ZnO-NPs showed strong antibacterial and antibiofilm action against clinical isolates, according to growth kinetics and antibiofilm assay. In addition, we looked into how ZnO-NPs interact with clinical bacterial strains to determine their mode of action. ZnO is thought to exert its effects via reactive oxygen species (ROS) production, which boosts membrane lipid peroxidation and ultimately results in the loss of reducing sugars through membrane leakage. These findings suggest that in future ZnO-NPs might be an effective alternate treatment of choice to treat infections caused by CR bacterial isolates.

Key Words: Biofilm, CRE, Nanoflakes, ROS, Superbugs

INTRODUCTION

The development of antimicrobial resistance (AMR) risks global health and development threats. It requires urgent multisectoral activities in support of the Sustainable Development Goals (SDGs). Antimicrobial resistance is a budding issue, as stated by the World Health Organization (WHO) is one of humanity's top ten global public health threat (1). The development and spread of drug-resistant organisms is a major public health concern, continue to threaten our ability to treat common infections. Multi- and pan-resistant bacteria, also called "superbugs," are spreading quickly worldwide. These bacteria cause infections that cannot be treated with existing antimicrobials like antibiotics (1). Due to the widespread irrational use of antibiotics, the number of deaths caused by multidrug-resistant (MDR) microorganisms is reported to be close to 700,000 annually round the globe (2,3).

In the hospital setting, the emergence and spread of carbapenem-hydrolysing beta-lactamases among *Enterobacteriaceae* and non-fermentative Gram-negative bacillary organisms like *Pseudomonas spp.* or *Acinetobacter baumannii* over the past ten years is a serious issue. It is important to note that carbapenemase-producing organisms, or CPOs, have primarily been identified in hospitals; nevertheless, at present, these organisms are also a more common cause of infections in the population (4). Existing pathways for carbapenem resistance in Gram-negative bacteria include modified porins, efflux pumps, enhanced natural cephalosporin activity, and the most prevalent carbapenemase-producing enzymes (5–8).

The carbapenemase hydrolyse almost all beta-lactams, are resistant to all therapeutically useful inhibitors, and are co-produced with other beta-lactamases. Moreover, carbapenemase genes are often a part of integrons, which carry diverse arrays of resistance gene cassettes (9). Important nosocomial pathogens like *Pseudomonas aeruginosa, Acinetobacter baumannii,* and members of the family *Enterobacteriaceae* can get transmissible enzymes in unpredictable ways. The

chromosomal enzymes can be found in less common pathogens like *Stenotrophomonas maltophilia*, *Aeromonas spp.*, *Chryseobacterium spp.*, and others (10).

Aside from that, numerous bacteria in the natural environment form biofilms. Biofilms are multicellular colonies of bacteria that are immobilized by an extracellular polymeric matrix created by the bacteria, which can be adhered to various biotic and abiotic surfaces (11,12). The extracellular matrix, which includes polysaccharides, proteins, enzymes, DNA, bacterial glycolipids, and water, accounts for 85 percent of this three-dimensional biofilm structure (13). Antibiotics and innate immune responses are shielded from biofilm microorganisms by the matrix (14). Drug resistance indicators and other virulence factors can spread faster in biofilms than in other environments (15). Pathogens that stay in a biofilm can cause infections that are hard to treat and last a long time, like upper respiratory infections, cystic fibrosis caused by *Pseudomonas aeruginosa* (16), urinary tract infections (17), periodontitis (18), and infections caused by catheters and other medical devices (19). When opportunistic biofilm-forming pathogens cause infections, they can cause severe symptoms and, in many cases, death in people who do not have strong immune systems (20).

No new classes of carbapenem antibiotics have been reported in the last few decades, so combination therapy can improve or expand the antimicrobial spectrum, prevent bacterial resistance during treatment, and achieve synergistic activity (21). In this context, Nano-biotechnology has recently gained significance in producing nanomaterial which acts as bacteria curing agent. Nanoparticles can be active molecules or compounds that react with bacterial cells to kill them. They might resolve issues associated with multidrug resistance and inhibit biofilm (22).

Metal oxides such as zinc oxide (ZnO) have recently received a great deal of attention due to their stability under various environmental conditions and manufacturing at low temperatures. ZnO particles have shown antimicrobial activity (23) against both Gram-positive and Gram-negative bacteria (24,25), as well as spores (24,25). ZnO NPs are thought to be non-toxic, safe, and compatible with living things (26). The carbapenem-resistant strains isolated from various clinical samples have not been tested to see the antimicrobial activity of ZnO NPs. ZnO NPs antibacterial mechanisms of action are unclear but several hypotheses have been offered, such as the formation of hydrogen peroxide could be the primary factor of antibacterial activity (27,28), or binding of ZnO particles on the bacterial surface due to electrostatic forces could be a mechanism. So, this study aims to use ZnO NPs and test how they will kill and inhibit the biofilm formation of clinical bacterial strains resistant to carbapenem (beta-lactam). The results of this study will help in finding a suitable replacement for carbapenem, which is used to treat the infections.

MATERIAL AND METHODS

Each and every experiment was carried out in accordance with the applicable laws/ Institutional guidelines and approved by the ethics committee of Index Medical College, Hospital & Research Centre affiliated with Malwanchal University, Indore (MP).

2.1 Chemicals

Zinc oxide nanoparticles that had been previously produced were utilized in this investigation. The culture media were purchased from Himedia Lab Pvt. Ltd., Mumbai, India. In this investigation, only high-quality analytical reagents were used.

2.2 Isolation of Carbapenem-Resistant (CR) clinical isolates

67 carbapenem-resistant *Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumanni*, and *Pseudomonas aeruginosa* clinical isolates were obtained from diverse clinical samples (Urine, Pus, Blood, Sputum, ET tips & body fluids). The automated system VITEK-2 was used for up to species level identification, and the disc diffusion method performed antibiotics sensitivity tests according to standard guidelines (29). The phenotypic (CDST, MHT, mCIM, and eCIM) and genotypic approaches were used to identify MBLs (CLSI, 2018). RT-PCR multiplex using ready-to-use Hi-PCR Carbapenemase Gene (multiplex) test PCR pack (MBPCR132) manufactured by Himedia lab Pvt. Ltd. India for identification of the targeted gene.

2.3 Preparation of Stock solution and dilutions

8 $mg.ml^{-1}$ stock solution of ZnO nanoflakes in sterile deionized water was prepared and sonicated for 20 min at 35°C using 24 Hz frequency and 400 rpm rotation through ultrasonication for removing agglomeration. The working solution was prepared to perform two-fold serial dilutions in sterile deionized water. Six MCT tubes was arranged and filled with 1000 µL sterile BHI broth except 1st tube and add 1000 µL of stock solution in second tube mix well and transfer the solution from second to third followed by last tube to get concentration of 4 $mg.ml^{-1}$, 2 $mg.ml^{-1}$, 1 $mg.ml^{-1}$, 0.5 $mg.ml^{-1}$, 0.25 $mg.ml^{-1}$, 0.125 $mg.ml^{1}$ solutions aseptically. After preparation of dilutions of ZnO NPs suspensions perform the experiment in tissue culture plate (**figure 1),** 100 µL ion adjusted Muller Hinton broth (MHB) was filled in each well, then add 50 µL of each ZnO suspension (diluted) in the first raw from top to below and added 50 µL bacterial suspension from left to right. Growth control use as a positive control without addition of any antimicrobial compounds and Broth control is used as a negative control for sterility check of BHI medium.

2.4 Antibacterial activity

2.4.1 Growth Kinetics & Antibacterial efficacy of ZnO nanoflakes

Micro broth dilution methods with few modifications were used to access the bacterial growth kinetics and antibacterial effect in the presence of ZnO NPs in various concentrations on CR isolates (29). An overnight culture of test strain in trypticase soya broth (TSB) was used. The optical density matched 0.5 McFarland turbidity standards resembling a $1.5x10^8$ CFU/mL bacterial count. It then diluted twenty folds bacterial suspension to reach $5x10^6$ CFU/ml and follow the procedure as per figure 1 and then tissue culture plate put on an orbital shaker with an incubator at 37 ℃. The absorbance was taken every three hours at 650 nm using a spectrophotometer (30).

2.5 Analysis of the mechanism of action of ZnO NPs (30)

2.5.1 Reactive Oxygen species (ROS) estimation

Reactive oxygen species (ROS) in the microbial cell were determined by following a published protocol (30). 500 ml of ZnO (final working concentration of 2 mM) were added to 100 ml of bacterial culture and incubated at 37º C with an orbital shaker. After 6 hours, the bacterium pellet was collected by centrifuging at 10,000g for 10 minutes at 4º C. The pellet was then dissolved in a two percent Nitro Blue Tetrazolium (NBT) solution, mixed, and incubated for an hour at room temperature in the dark. then centrifugation and discarded the supernatant. The pellet was rinsed with PBS, centrifuged for 2 minutes at 8000g, then rinsed with methanol, and centrifuged at 8000g for 2 minutes. The pellet was suspended in 2 M KOH for cell membrane rupture after centrifugation. To dissolve the formazan crystals, a 50 percent DMSO solution was added and incubated at room temperature for 10 minutes. It was then centrifuged for 2 minutes at 8,000g. After centrifugation, 100 µL of supernatant was added to 96 well plates, and absorbance at 620 nm was measured with an ELISA reader. Cultures with no treatment were used as controls, and luria bertani broth (LB) media was used as a blank (30).

2.5.2 Estimation of Membrane lipid peroxidation

In microbial cells, unstable lipid peroxides produce oxidative stress, decomposing to form reactive chemicals that cause cellular damage. For the detection of lipid peroxidation, thiobarbituric acid-reactive substances (TBARS) assay is performed (31,32). Malondialdehyde forms a compound with thiobarbituric acid in this assay, which may be measured spectrophotometrically. 100 mL bacterial culture was cultured in an orbital shaker at 37° C with 500 mL of ZnO NPs solutions (final concentration of 2 mM). After 6 hours the culture was centrifuged at 10,000g for 10 minutes at 4°C. The pellet was cleaned and redistributed in 10% -SDS (500 mL). This slurry was incubated for 10 minutes with 20% acetic acid. The solution received 250 mL of TBA buffer (0.8 percent TBA in 2 M NaOH). This reaction mixture was incubated at 95° C for 1 hour before being cooled to 25° C. The reaction mixture was centrifuged at 5000g for 15 minutes to remove the cell debris. Using an ELISA reader, absorbance was measured at 532 nm. Cultures with no treatment were used as controls, and LB media was used as a blank (30).

2.5.3 Quantification of Membrane Leakage of Reducing Sugars and Proteins

The effect of ZnO-NPs on membrane leakage was assessed by the estimation of reducing sugars and proteins released from inside cells after ZnO-NP treatment. 100 mL of LB broth culture was treated with 500 mL of ZnO NPs solutions (2 mM final concentration) and incubated at 37º C in an orbital shaker at 125 rpm. The culture was centrifuged for 30 minutes at 10,000g at 4º C after 24 hours of incubation. The resultant supernatant was kept at 20ºC. This sample is used to calculate reducing sugars and proteins. The dinitro salicylic acid (DNSA) assay was used to estimate the reducing sugar by colorimetric absorbance at 540 nm (33). Proteins were calculated using the Bradford technique, and absorbance at 595 nm was measured (Bradford, 1976) (30).

2.6 Antibiofilm assay

Similarly, the ZnO NP dilutions and bacterial suspensions were prepared as before in growth kinetics. Individual wells of sterile polystyrene 96-well flat-bottom tissue culture plates (TCP) were filled with 100 µl of Brain Heart Infusion (BHI) broth and 50 µl of overnight culture. 50 µl of ZnO NPs were immediately poured into the wells following vigorous vortex mixing to achieve final concentrations ranging from 31.25 µg/ml to 1000 µg/ml. Every well had a volume of 200 µl. The tissue culture plates were kept at 37ºC for 24 hours. The contents of each well were gently removed after incubation (34). Phosphate-buffered saline solution (pH = 7-7.2) was used to wash the wells four times to remove free-floating planktonic bacteria. Bacterial biofilms were preserved with 95% ethanol and stained with 0.1% (w/w) crystal violet. The extra stain was rinsed off by washing the plates several times with deionized water, and the plates were kept to dry. The optical densities (OD) of dyed adherent bacteria were measured with a microplate reader (model CS, Biotec) at 590 nm 15 minutes after the addition of 200 µl of glacial acetic acid (33%). Bacteria adhere to surfaces and build biofilms based on these OD values. The experiments were carried out three times to ensure accurate results, which were then averaged (34).

2.7 Statistical analysis

Statistical R-Software 4.1.2 was used to analyse the data. For quantitative data, descriptive statistics were used as Mean ± SD. Analytical variance (ANOVA) and Post hoc analysis were employed to examine the differences between and within the distinct groups. The Pearson correlation coefficient was used when looking for a link between two or more quantitative parametric variances. Using a two-tailed test, If the p-value was less than 0.05, all analyses were considered statistically significant.

RESULTS AND DISCUSSION

Beta-lactams, often known as carbapenems, are frequently prescribed by clinicians to treat various gram-negative infections. Carbapenems are considered to be a treatment option of very last resort for the infection that is being treated. The development of resistance to carbapenem drugs will lead to mortality and morbidity rates. So, now it's time to begin develop an alternate chemical molecule to combat CR infections. We used four different kinds of strains in this investigation. These strains were isolated from different clinical samples from patients hospitalized in tertiary care settings. These isolates exhibited extensive drug resistance (XDR), a lack of sensitive to at least one agent in all antimicrobial categories except for two or fewer. These strains have a minimum inhibitory concentration (MIC) of less than 32 mg/mL for carbapenem antibiotics such as Imipenem, Meropenem, Doripenem, and Ertapenem. For this investigation, synthesized ZnO nanoflakes were utilized. These nanoflakes had an average crystallite size of 21.5 ± 4.8 nm and a thickness of 20-50 nm without impurities.

3.1 Antibacterial activity and Growth kinetics

The growth of the CR strains was examined in the presence and absence of chemically produced ZnO. The treated bacterial culture's growth curves show a decline compared to the untreated one with time. This lends up credence to the idea that ZnO may affect the growth of bacteria **(Figure 2)**. According to the growth curves **(Figure 2)** ZnO NPs suppresses the growth of CR isolates at different concentrations. All four types of isolates exhibiting comparable growth suppression at 500 µg/ml ZnO NPs. ZnO nanoparticles at a concentration of 250 µg/ml had a moderate impact on bacterial growth suppression.

3.2 Effect of ZnO on ROS Production and Membrane Lipid Peroxidation

ZnO- NPs increase the formation of ROS, which damages bacterial cells. **Figure 3A,** shows increased ROS production treated with various concentrations of ZnO on CR pathogens compared to untreated. This increased ROS has numerous impacts on the bacteria, one of which is lipid peroxidation. The estimation revealed a twofold increase in lipid peroxidation following ZnO treatment (**Figure 3 B**). This lipid peroxidation has an impact on the bacterial membrane's integrity.

3.3 Effect of ZnO in Reducing Sugar Membrane Leakage

Figure 3 demonstrates the effect of different ZnO-NPs concentrations on membrane leakage of reducing sugars. The membrane leakage of reducing sugars was 1.5 times greater in ZnO-treated CR bacteria than in untreated bacteria **(Figure 4)**. As a control, cultures without any treatment were used.

3.4 Antibiofilm Activity of ZnO NPs

The results showed that **(Figure 5)** CR isolates could produce biofilm using the Tissue Culture Plate (TCP) test. ZnO NPs showed anti-biofilm activity on all bacteria, and the anti-biofilm activity increased with the rising concentration of nanoparticles. Treating these CR organisms with ZnO NPs at 1000- 31.25 µg/ml concentrations resulted OD reduction%, respectively **(Figure 5)**. According to the statistical data **(Table 1)** the effect of ZnO NPs on biofilm formation was significant at 1000 and 500 µg/ml concentrations ($p < 0.01$).

Treatment of bacterial infections is a major concern in recent years due to the growing problem of resistance to traditional antibiotics. MBL-producing Gram-negative microorganisms have now been identified in a variety of geographical areas (8) . The rise of GNB makes MBL a problem for microbiology labs because there are no standardized rules for how to find them. Plasmids easily spread MBLs, so they quickly spread through an institution and cause bad results when they infect someone (35–37).

The CR strain was employed in this investigation to test the antibacterial efficacy of produced ZnO NPs. Additionally, the mechanism of action of ZnO nanoparticles in CR isolates was evaluated using a variety of metrics, including ROS production, lipid peroxidation, and reduced sugar membrane leakage. Based on all of the findings, it is suggestive that ZnO works by producing ROS, which increases membrane lipid peroxidation and results in membrane leakage of reducing sugars, decreasing cell viability.

In the clinical setting, biofilms cause a wide variety of issues, including persistent and chronic infections that result from a decrease in the immune response and the effectiveness of antibacterial agents (38). The research that is conducted to develop novel substances that have an anti-biofilm action may provide strategies for the control of infections and issues that are associated with the production of biofilm.

Our findings show that CR isolates can form biofilm. All CR isolates' growth was found to be significantly inhibited, and ZnO NPs inhibited the development of biofilms. ZnO Nanoparticles showed the potential to suppress biofilm formation when increasing concentration from 0.25 mg/ml to 4 mg/ml. At 4 mg/ml concentration, biofilm formation was almost entirely inhibited. Some previous studies have shown that ZnO NPs have antimicrobial action. Sangani et al. (34) evaluated the effect of ZnO NPs with an average size of 20 nm on P. aeruginosa at different concentrations ranging from 50 to 350 µg/ml. At a concentration of 350 µg/ml, biofilm formation was almost entirely inhibited. Saadat et al.(39) studied the effect of ZnO NPs with sizes ranging from 30-90 nm on P. aeruginosa. They found a MIC of 300 µg/ml. Another studies observed the antibacterial activity of ZnO NPs with an average size of 25 nm against various bacteria. The MIC for P. aeruginosa was 156.25 µg/ml (40). Similar findings by Lee et al. show that ZnO NPs inhibits biofilm formation and

the generation of virulence factors by the P. aeruginosa (41). ZnO nanoparticle-coated surfaces have been demonstrated by Applerot et al. to reduce bacterial biofilm formation and boost antibiotic sensitivity. They demonstrated that the coated surface's hydroxyl radicals played a significant part in the anti-biofilm action (42). Khameneh et al. (43) reported the high efficacy of nanosilver particles (SNPs) in eliminating biofilms using a pour plate assay. According to the findings, enhancing the anti-biofilm efficacy of SNPs required more significant SNP concentrations and longer incubation durations. According to antibacterial mechanistic investigations by Pati et al., ZnO NPs can damage bacterial cell membrane integrity, decrease cell surface hydrophobicity, and suppress the transcription of oxidative stress-resistance genes in bacteria (44).

CONCLUSION

As a result, the current investigation allows us to give the following conclusion, ZnO-NPs have the potential as an alternative for carbapenem (beta-lactam), which inhibits carbapenem-resistant growth CR bacteria by producing ROS and causing membrane damage. In light of this, using ZnO nanoparticles to treat CR strains may emerge as a more promising future prospect.

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AUTHORS CONTRIBUTIONS

All authors equally contributed to the present study.

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Dilution of ZnO NPs

Well	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
8 mg/ml Stock sol.	4 mg/ml	2 mg/ml	1 mg/ml	0.5 mg/ml	0.25 mg/ml	0.125 mg/ml	Growth Control (Positive Control)	Broth Control (Negative control)
ZnO Sol. added	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	-------	-------
Bacterial Suspension	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	-------
BHI Broth	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	150 µl	200 µl
Total Volume	200 µl	200 µl	200 µl	200 µl	200 µl	200 µl	200 µl	200 µl
Final Concentration of ZnO NPs	1000 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml	62.50 µg/ml	31.25 µg/ml	---------	--------

Figure 1 Demonstrate dilution processes and getting final concentration of ZnO for evaluation of antimicrobial action.

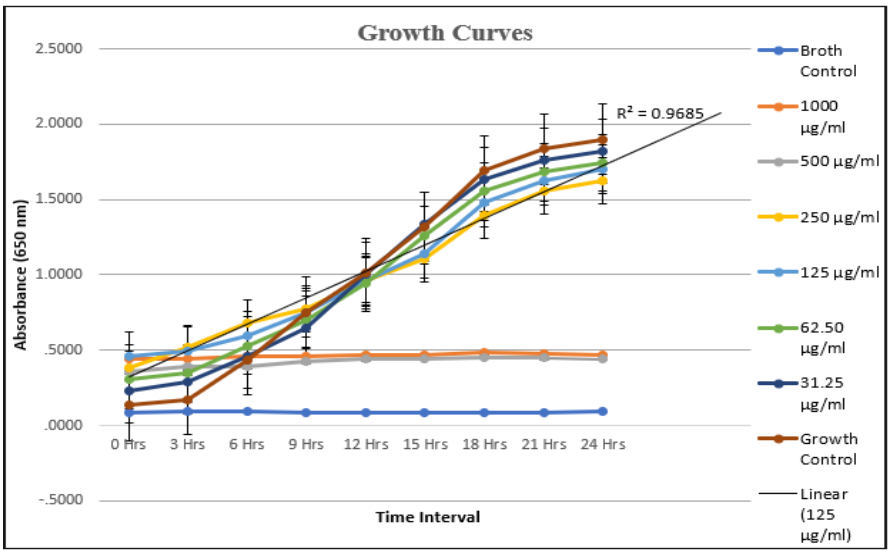
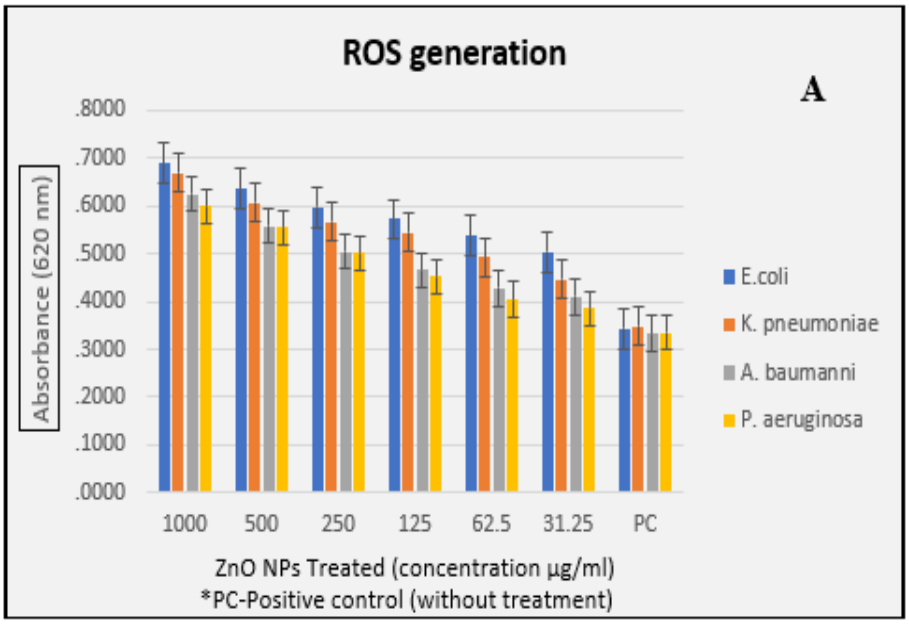


Figure 2 CRE isolate growth curves in the presence and absence of various ZnO NP concentrations. The data are presented as Mean ± SD.



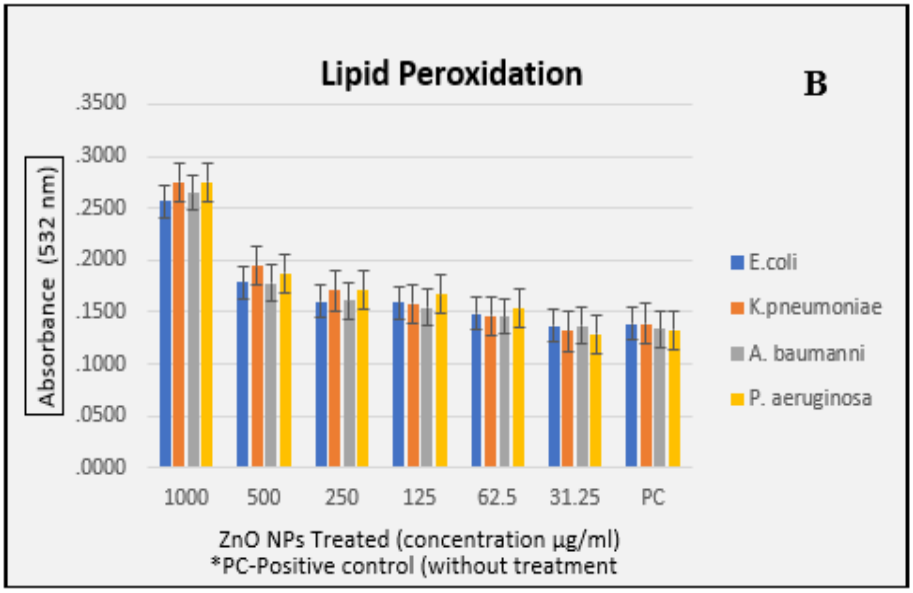


Figure 3. Effect of ZnO-NPs on CRE isolates' production of ROS **(A)** and lipid peroxidation **(B).** The data are shown as Mean± SD.

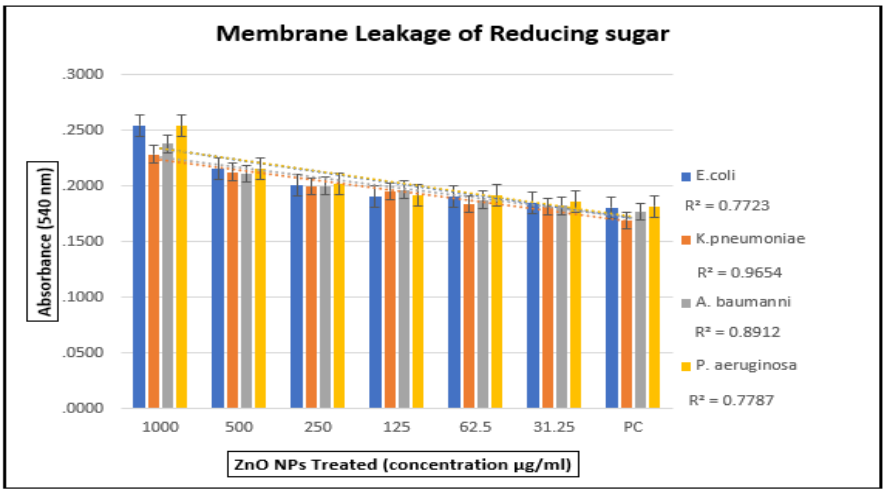


Figure 4 Membrane leakage in the CRE strain after treatment with ZnO-NPs. A quantitative comparison of sugar leakage. The control is untreated samples. The data is provided as Mean ±SD.

Conc. of ZnO NPs (µg/ml)	Total Isolates	Minimum	Maximum	Mean ± SD	F	Sig.
1000	67	0.43	0.55	0.47 ± 0.04	7.87	0.01
500	67	0.39	0.49	0.44 ± 0.03	0.65	0.01
250	67	1.45	1.81	1.63 ± 0.12	35.14	0.02
125	67	1.55	1.86	1.70 ± 0.12	13.20	0.06
62.5	67	1.59	1.89	1.74 ± 0.10	21.20	0.08
31.25	67	1.68	1.96	1.82 ± 0.10	8.60	0.09
GC	67	1.84	1.98	1.90 ± 0.05	23.40	0.19

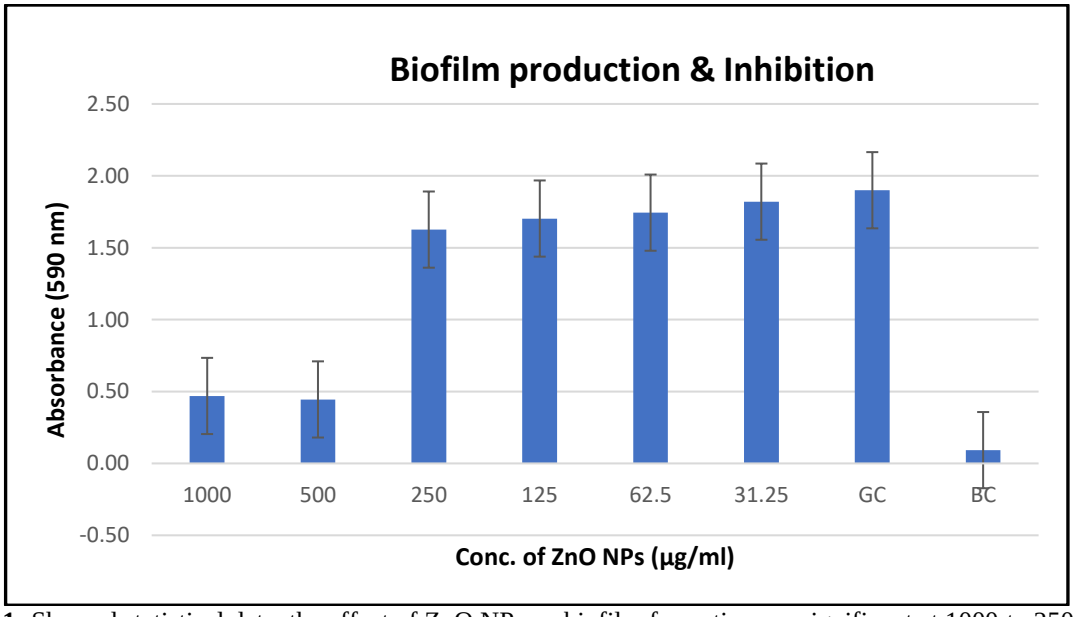


Table 1- Showed statistical data, the effect of ZnO NPs on biofilm formation are significant at 1000 to 250 µg/ml concentrations ($p < 0.05$). Growth control (GC) as Positive control (bacterial growth without any treatment)