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Antibacterial Efficacy of Zinc oxide nanoparticles against *Serratia marcescens* (ATCC 43862) and *Enterococcus faecalis (*ATCC 29121)

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KEYWORDS

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Zinc oxide nanoparticles

Serratia marcescens

Enterococcus faecalis

Minimum inhibitory concentration

ABSTRACT

Zinc oxide nanoparticles (ZnO NPs) are a novel and alternative biomaterial for active biomedical applications among all metal and metallic oxide nanoparticles due to less toxicity and biocompatibility with human cells. In this study, we studied the growth curve of *Serratia marcescens* and *Enterococcus faecalis* to identify the mid-log phase of the bacterial growth to perform the exposure with ZnO NPs for investigating the antibacterial efficacy. The INT assay was used to determine the anti-bactericidal efficiency of ZnO NPs against *S. marcescens* and *E. faecalis.* The results showed that both the test bacteria attained the mid-log phase at the 5th hour. The determination of minimum inhibitory concentration (MIC) demonstrated a higher efficacy of ZnO NPs on the Gram-positive bacterium *E. faecalis* compared to the Gram-negative bacterium *S. marcescens*. The present study reports a higher susceptibility of Gram-positive bacterium over Gram-negative bacterium to the treatment of ZnO NPs.

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1 Introduction

Antibiotics have been used for over 70 years to combat bacterial infections around the world. High numbers of pathogenic bacteria are becoming resistant to antibiotics; it is well known that more than 50 years ago the bacteria were resistant to antibiotics. According to Stapleton and Taylor (2002), most of the *Staphylococcus aureus* isolates were resistant to penicillin. The development of new antibiotics, such as vancomycin and methicillin, leads to the emergence of antibiotic-resistant bacteria (Aslam et al. 2018). However, bacteria developed resistance mechanisms against many antibiotics and in 2019, World Health Organization considered antimicrobial resistance as one of the top ten human global health threats (Mancuso et al. 2021). This is a serious concern, which can result in up to a 25% mortality rate among patients with severe infections (Oerther and Oerther 2020; Boucher 2020). In general, nanotechnology is a tool that uses science at the nanoscale level to manipulate materials. The combination of biology and nanotechnology revolutionizes biomedical research using new phenomena and properties (physical, chemical, and biological) of materials present at the nanoscale and by the direct application of nanomaterials with biological targets (Menaa 2011). As it grows more sophisticatedly, it has found numerous applications in medical technology such as diagnostics, drug delivery, patient monitoring, and treatment (Zhang et al.2008).

Metal oxide nanoparticles (MONPs) and carbon-based 2D nanostructures are considered to be potential antiviral/antibacterial agents (Ray and Bandyopadhyay 2021). Thus, with the emergence of drug resistance in the treatment of microbial diseases, the development of new antibacterial materials is essential. Currently, metal oxide NPs (Ag NPs, ZnO NPs, CuO NPs, FeO NPs, etc.,) can be used in various biomedical applications (Jaison et al. 2022). ZnO NPs are extensively applied as an effective anti-bacterial agent against pathogenic bacteria**.** Thus, nanoparticles might serve as a promising candidate against multidrug-resistant bacterial infections and also as a novel antimicrobial agent of their unique target-specific binding property. This can be achieved by developing nanomaterials as implants and wound dressing complexes in clinical practices (Siemer et al. 2019; Gao and Zhang 2021). The emerging area is autonomous nano-objects that enhance the effect of antibiotics or impart bactericidal activity without the use of antibiotics. Another new area is nanostructured surfaces that prevent bacterial adhesion or kill bacteria through physical and mechanical interactions with bacteria (Shaikh et al. 2020).

In particular, a large number of studies have shown that ZnO NPs have high anti-bacterial potential against pathogenic bacteria due to the biological activity of zinc ions and the anti-inflammatory effects of ZnO NPs. Due to excellent broad-spectrum antibacterial properties, long-term effects, and excellent biocompatibility, ZnO nanomaterials can become a suitable effective alternative to traditional antibiotics (Silva et al. 2019). However, the antibacterial effects of these nanoparticles are depending on the cell wall assembly of the target organism. Based on this characteristic feature, ZnO NPs exhibit an extensive range of antibacterial activities against a variety of microorganisms, including *Escherichia coli*, *Bacillus subtilis*, etc. (Alam 2021). Zinc ions are responsible for the antibacterial activity caused by ZnO and also particles of these metal oxide pass through the cell membrane and cause oxidation of the cells by the excess production of ROS, which results in cell death (Djearamane et al. 2020). Further, ZnO NPs also have a wide range of photo-catalytic as well as high light absorption capacity in the UVA (315-400 nm) and UVB (280-315 nm) regions, which help in inducing bacterial toxicity (Lakshmipriya and Gopinath 2021). ZnO NPs are superior to compare with other MONPs due to their biocompatibility and excellent antibacterial potential. Hence, the present study focused on finding the antibacterial activity of ZnO NPs against *Serratia marcescens* and *Enterococcus faecalis*.

2 Materials and Methods

2.1 Chemicals and Nanoparticles

Zinc Oxide Nanoparticles (ZnO NPs) powder was obtained from Sigma- Aldrich, USA. The antibiotics Chloramphenicol and Ampicillin used as positive control were purchased from Bio Basic Inc, Canada. Luria Bertani (LB) medium was purchased from Laboratories CONDA, Spain.

2.2 Characterization of ZnO NPs

The previous publication of the same author (Djearamane et al. 2022) studied the surface topology of ZnO NPs using scanning electron microscopy and reported it as the mixed rod and spherical-shaped particles with an average size of 59.1 nm.

2.3 Bacteria Culture

The pure culture of Gram-negative bacterium *S. marcescens* (ATCC 43862) and Gram-positive bacterium *E. faecalis (*ATCC 29121) were obtained from the Faculty of Science, Universiti Tunku Abdul Rahman and subcultured to mid-log phase in LB broth for further utilization.

2.4 Preparation of ZnO NPs Suspension

A stock solution of ZnO NPs (320 µg/mL) was prepared by suspending ZnO NPs powder in Luria Bertani broth, mixed homogenously by vortex, and prepared the required concentrations of 5, 10, 20, 40, 80, and 160 µg/ mL of ZnO NPs by adding extra amount of LB Medium.

2.5 Growth Curve of Bacteria

The growth curves were plotted to identify the mid-log phase for both the test bacteria *S. marcescens* and *E. faecalis.* The bacteria were grown in Luria Bertani broth in 15 mL falcon tubes and incubated at 37 ^{O}C for *S. marcescens* and 35°C for *E. Faecalis* without shaking. The turbidity of bacteria was determined by spectrophotometer at 600 nm for every 1 hr interval from 0 to 8 hr, and then from 24 to 72 hr.

2.6 Growth Inhibition Test

2.6.1 INT Assay

The INT dye was used to determine the bacteriostatic concentration of ZnO NPs against *S. Marcescens* and *E. faecalis*. Upon reaching the mid-log phase at the 5th hour, the bacteria were incubated with different concentrations of mixed-shaped ZnO NPs (5, 10, 20, 40, 80, and 160 µg/ mL) for 24 hours, 1 mL of test bacteria suspension from each concentration, positive control (*S. marcescens* and *E. faecalis* after treating with 0.08 mg/ mL of chloramphenicol and 1.0 µg / mL of ampicillin respectively) and negative control (*S. marcescens* and *E. faecalis* without any treatment) were added separately to the microcentrifuge tubes and washed twice with 1 mL of 1x PBS at 6000g for 10 min and resuspended with 1 mL of 1x PBS. After that, 100 µL of the washed bacterial suspension was added with 20 µL of INT dye in the 96 wells plate and incubated for 20 min to observe colour change. The INT assay was done in a dark condition since INT dye is light-sensitive.

3 Results and Discussion

3.1 Growth Curve of Bacteria

The bacteria growth curve of *S. marcescens* was plotted to identify the growth pattern of the bacterial cells over time. According to figure 1, the lag phase was observed at the first two hours, while the log phase was found between 3rd to 7th hours, hence, the mid-log phase of *S. marcescens* was identified to be at 5th hour. The turbidity continued to increase after 8 hours of incubation until it came to the death phase at 48 hours of incubation.

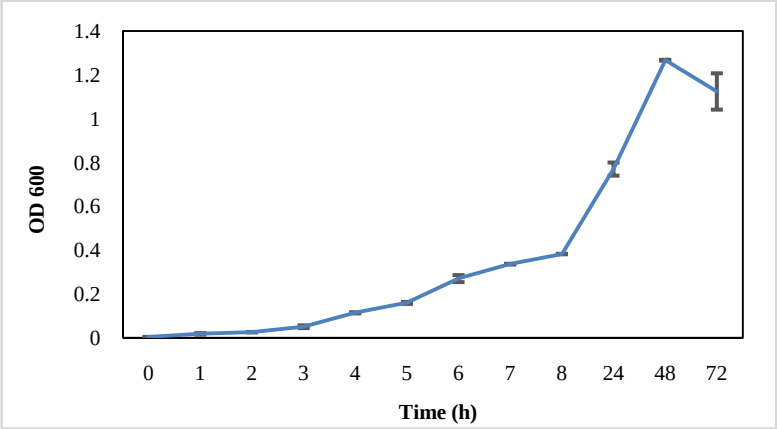

Figure 1 Growth curve of *S. marcescens* in Luria Bertani broth incubated at 37^{O} C

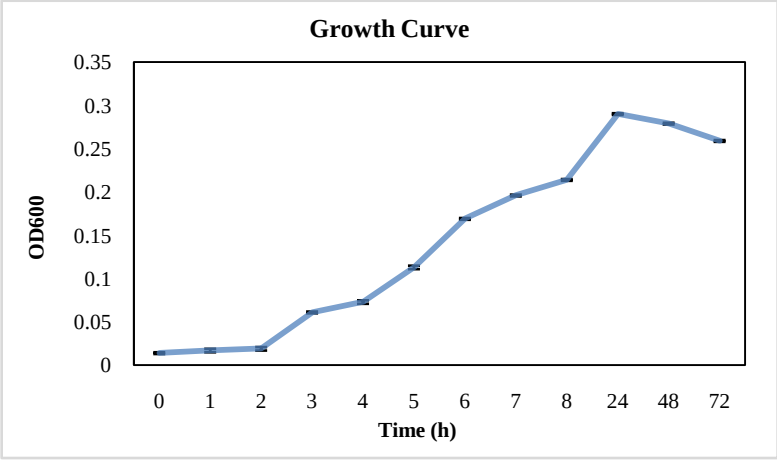

Figure 2 Growth curve of *E. faecalis* in Luria Bertani broth (LB) at 35°C

Similarly, the growth curve of *E. faecalis* indicated that the log phase of *E. faecalis* began at 2 hours and lasted until 7 hours (Figure 2). The mid-log phase of *E. faecalis* was then identified at 5 h. The growth of *E. faecalis* started to decline from 24 to 72 hrs indicating the decline phase of the growth curve. During the lag phase, the bacterial cells do not increase in number and also on the size as they take this duration to adapt to the new environment. Whereas the bacterial cells multiply actively in the log phase resulting in many folds increase in the cell population, this phase continues until the availability of favorable conditions and adequate nutrients. At the death phase, the cells lose their capacity for cell division and progress to death. The antibacterial studies are conducted during the mid-log phase of bacterial growth since they are most active in metabolism and cell division during this stage (Brazas and Hancock 2005; Kathleen and Arthur 2012; Dhanasegaran et al. 2021).

3.2 INT Assay

INT dye was used to identify the minimum inhibitory concentration (MIC) which is defined as the lowest concentration of an antibacterial substance that can inhibit bacteria growth (Dzotam et al. 2018). Iodonitrotetrazolium (INT) is an indicator dye that turns pink/purple to indicate the reduction of the dye by viable bacteria by the activity of dehydrogenases. The wells which show the purple or pink colour indicate the viable bacterial cells. The lowest concentration of ZnO NP displaying no visible purple or pink colour was recorded as the MIC value. According to figure 3, pink colour was observed until 160 µg/mL of ZnO NPs for *S. marcescens*. On the other hand, as shown in Figure 4, no pink colour formation was observed at 160 µg/mL of ZnO NPs after incubating with INT dye for 20 minutes indicating that 160 µg/mL as the MIC that can inhibit the growth of *E. faecalis*. Further, it is also visually observed that the intensity of the pink colour is decreased as the concentration of ZnO NPs increased for both the tested bacteria (Figure 3 & 4) indicating the dose-dependent bacteriostatic effect of ZnO NPs against *S. marcescens* and *E. faecalis.* In line with these findings, our earlier publication reported a dose-dependent growth inhibition of ZnO NPs on *S. marcescens* and *E. faecalis* with more percentage of growth inhibition on *E.* faecalis with the reported values of 5.42, 16.69, 23.74, 32.07, 54.73, and 63.50 % compared with 1.34, 5.65, 16.03, 21.73, 32.1 and 51.27 % of growth inhibition on *S. marcescens* for 5, 10, 20, 40, 80, and 160 µg/ml of ZnO NPs respectively at 24 hours (Djearamane et al. 2022).

Antibacterial agents are more effective when their MIC value is lower. In the present study, ZnO NPs showed a MIC value of >160 µg/ mL against the Gram-negative bacterium *S. marcescens* and MIC of 160 µg/ mL against Gram-positive bacterium *E. faecalis*, evidencing the higher sensitivity of Gram-positive bacterium towards ZnO NPs treatment compared with Gram-negative bacterium. Similar results on the higher sensitivity of Gram-positive bacterium to ZnO NPs were reported by the earlier studies, and this might be due to the presence of the liposaccharide in the outer membrane (Sukri et al. 2019; Demissie et al. 2020).

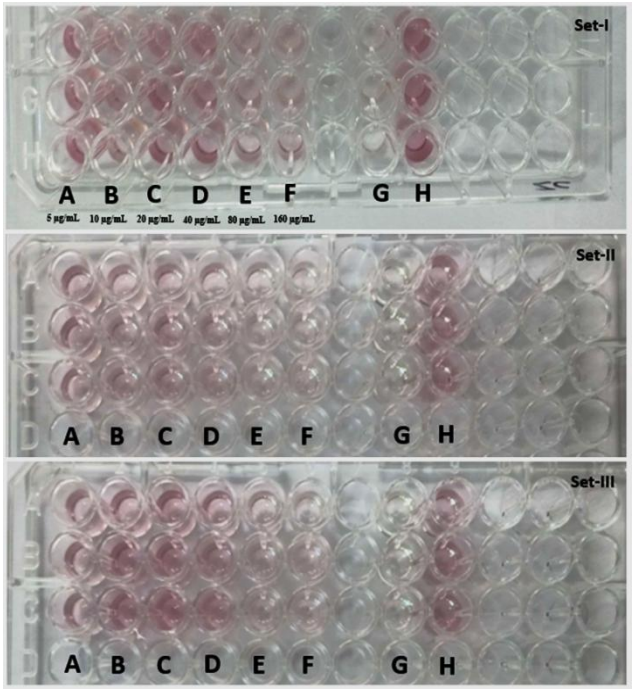

Figure 3 The colour changes of *S. marcescens* upon treating with ZnO NPs with (A) 5 µg/ mL; (B) 10 µg/ mL; (C) 20 µg/ mL; (D) 40 µg/ mL; (E) 80 µg/ mL; (F) 160 µg/ mL as compared to (G) positive control and (H) negative control

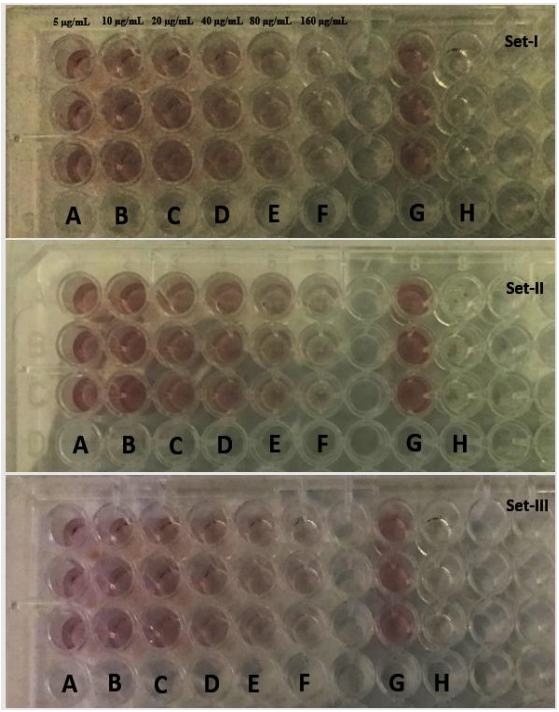

Figure 4 The colour changes of *E. faecalis* after being treated with (A) 5 µg/mL; (B) 10 µg/mL; (C) 20 µg/mL (D) 40 µg/mL; (E) 80 µg/mL; (F) 160 µg/mL of ZnO NPs as compared to the negative control (G) and positive control (H)

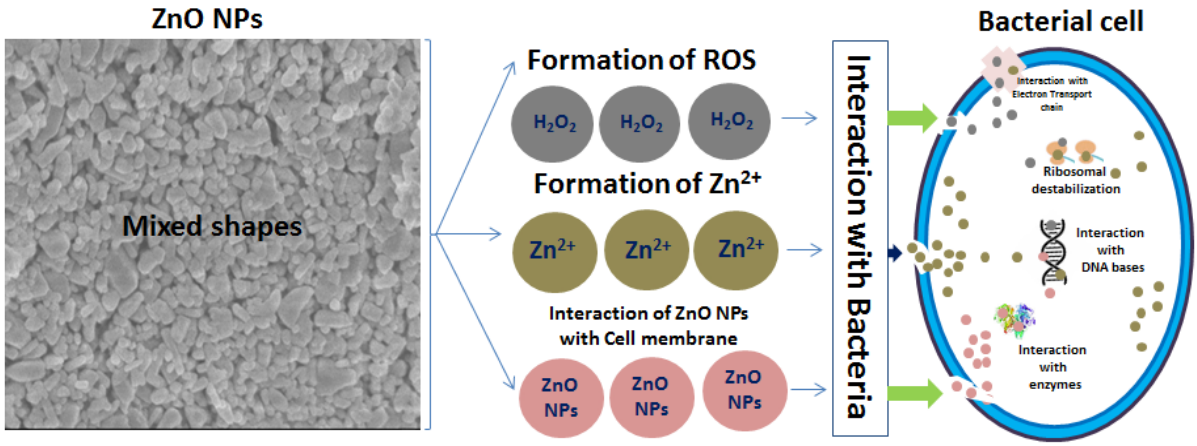

Figure 5 Mechanisms of antibacterial action of ZnO NPs

Earlier studies on the antibacterial properties of ZnO NPs reported good antibacterial efficacy against antibiotic-resistant Gram-negative bacillus of *Acinetobacter baumannii* in growth kinetics and disc diffusion assay (Tiwari et al. 2018). About 20nm in size and spherical shape ZnO NPs revealed significant antibacterial properties against *Staphylococcus aureus* and *Salmonella typhimurium*, and this might be due to the surface topological modifications by ZnO NPs which made bacterial cells pitted and distorted (Akbar et al. 2019). Similarly, Gupta et al. (2018) demonstrated the hexagonal wurtzite shapes of ZnO NPs synthesized from leaf extract of *Catharanthus roseus* caused a significant antibacterial effect against *Escherichia coli* MTCC (40), *S. pyogenes* (MTCC 1926), *Proteus mirabilis* (MTCC 3310), *P. aeruginosa* (MTCC 424), *S. aureus* (MTCC 9760) and *Bacillus cereus* (MTCC 430). The size, shape, and concentration of ZnO NPs have a big impact on their antibacterial activity. Metal ions created by NPs bind to active enzymes in the respiratory chain, causing ROS to develop and damage the cell wall membrane, and DNA, and disturb protein synthesis which eventually result in bacterial cell death (Dimapilis et al. 2017; Roberta et al. 2019; Liang et al. 2020; Gudkov et al. 2021). Figure 5 depicts the probable antibacterial mechanisms of ZnO NPs.

Conclusion

The present study identified the existence of a mid-log phase for *S.marcescens* (ATCC 43862) and *E. faecalis* (ATCC 29121) at the 5th hour. Based on the MIC values reported from the INT assay, it is observed that the Gram-positive bacterium *E. faecalis* showed higher sensitivity to the treatment of ZnO NPs compared to the Gram-negative bacterium *S. marcescens*. Further, the visual examination of the INT assay evidenced a dose dependent decrease in the viable cells of both the tested bacteria as the concentration of ZnO NPs increased. Hence, the findings of this study indicate the potential of ZnO NPs as an antibacterial agent.

Conflicts of interest

The authors affirm that they do not have any conflict of interest.

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