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Research Article

Studying the remedial action of zinc oxide nanoparticles on *Salmonella typhimurium*

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Abstract

In recent years, zinc oxide nanoparticles (ZnO NPs) have become the main research attention due to their wide range of applications, including incorporation into cosmetics products and wound dressings. The present study aimed to determine the antibacterial properties of ZnO NPs on the Gram-negative, foodborne pathogen, *Salmonella typhimurium* by investigating the growth inhibition assay, surface interaction on bacterial cell wall and morphological analysis of bacteria. The surface morphology and elemental composition of the ZnO NPs were characterized using a scanning electron microscope (SEM) with energy dispersive X-ray (EDX) spectroscopy. The binding of ZnO NPs to the bacterial cell wall was evaluated by Fourier transform infrared (FTIR) spectroscopy. The results of the present study demonstrate that ZnO NPs exhibit a dose-dependent growth inhibitory effect on *S. typhimurium*. FTIR analysis revealed the involvement of functional groups such as alcohols, amide I, carboxylic acids, and phosphates in the interaction between ZnO NPs and the bacterial cell surface. SEM-EDX analysis confirmed membrane rupture and the accumulation of ZnO NPs on the bacterial surface. These findings suggest that ZnO NPs inhibit bacterial growth by inducing membrane deformities, ultimately leading to cell death. Based on these results, ZnO NPs hold promise for future applications in antimicrobial coatings for medical devices and other healthcare-related products to control bacterial infections.

Keywords: zinc oxide nanoparticle, *Salmonella typhimurium*, growth inhibition, anti-bacterial

Introduction

Nanotechnology has been receiving much attention in various fields, including cosmetics, electronics, engineering, food industries, and medical fields [1-5]. Currently, scientists are conducting vast research on the usage of nanoparticles that have a size of not more than 100nm. With such nano-sized particles, better penetration of the nanoparticles (NPs) through the bacterial cell wall can be achieved and thus inducing higher toxicity towards the bacteria as compared to micro-scaled NPs due to the higher surface-to-volume ratio [6-7]. Among all the metal oxide nanoparticles, zinc oxide nanoparticles (ZnO NPs) have attracted the attention of researchers as compared to other metal nanoparticles such as copper and titanium oxide nanoparticles owing to their enhanced antibacterial properties and higher biocompatibility and hence induce less toxicity to human cells [8-9].

ZnO NPs also possess UV-blocking properties due to their high optical absorption in the UVA and UVB region, which allows them to be incorporated into cosmetics products such as sunscreens [10-12]. Several studies also showed that ZnO NPs help in stimulating skin regeneration, and re-epithelisation, enhancing wound healing while imposing low toxicity [13-15]. Hence, a lot of research has been done to investigate the biomedical properties of ZnO NPs and showed significant results [16-18]. Multi-drug resistance (MDR) has been existing many years and has become the stumbling block for the treatment of infection by *Salmonella typhimurium* using antibiotics. *S. typhimurium* is resistant to several antibiotics used as a first-line treatment before the emergence of MDR for examples are ampicillin, chloramphenicol, streptomycin, and tetracycline. According to CDC (2020), *S. typhimurium,* isolated from clinical settings, exhibited high resistance towards tetracycline and streptomycin with 46% and 45.6%, respectively [19].

Multiple drug resistance is acquired when the bacterial cells acquire spontaneous mutation of the existing genes and acquire another type of resistance genes and express the resistance genes towards the treatment of antimicrobial drugs. This situation especially occurs in any setting that causes selective pressure in favor of drug resistance. For instance, in clinical settings, poor patient compliance with the antimicrobial treatment against bacterial infection could allow the microbes to survive the treatment and eventually develop resistance to the drug. In the poultry sector, where antimicrobial drugs are widely used to prevent contamination by foodborne pathogens, the microbes though being inhibited; however, they were not eradicated. This could be due to the bacteriostatic effect of the drug rather than bactericidal [20-25].

Gram-negative bacteria generally exhibit higher resistance to antimicrobial agents compared to Gram-positive bacteria, primarily due to differences in their cell wall architecture. Gram-positive bacteria have a thick peptidoglycan layer enclosed by a single cytoplasmic membrane. In contrast, Gram-negative bacteria possess a much thinner peptidoglycan layer that is located between two membranes-an inner cytoplasmic membrane and an outer membrane. The presence of this additional outer membrane acts as a selective barrier, limiting the penetration of many antimicrobial agents. Consequently, Gram-negative bacteria are often more resistant to antimicrobial treatments than their Gram-positive counterparts. [20,23,26].

Salmonella typhimurium, a self-limiting Gram-negative bacterium, is a common causative agent of gastroenteritis and, in severe cases, can lead to extraintestinal infections. Given the limited studies on its susceptibility to ZnO NPs, the present study aimed to evaluate the antibacterial activity of ZnO NPs against *S. typhimurium*. The findings of this study may contribute to the potential application of ZnO NPs as an effective antibacterial agent for the treatment of salmonellosis caused by *S. typhimurium*. In addition, ZnO NPs exhibit strong antimicrobial properties, making them promising candidates for use as coating agents in the healthcare and food industries. Their incorporation into surface coatings and packaging materials can effectively reduce microbial contamination and inhibit the spread of pathogenic microorganisms, thereby enhancing hygiene and safety standards in these sectors.

Materials and Methods

Chemicals

Zinc oxide nanoparticles (ZnO NPs) powder and glutaraldehyde were purchased from Sigma-Aldrich, Iodonitrotetrazolium chloride (INT) powder was purchased from Himedia. Absolute ethanol was purchased from Chemsol, kanamycin sulfate was purchased from Bio Basic, phosphate buffer saline (PBS) was purchased from Oxoid, potassium bromide was purchased from Fisher Scientific, and tryptic soy agar (TSA), and tryptic soy broth (TSB) were purchased from Merck KGaA.

Preparation and physicochemical characterizations of ZnO NPs

A stock solution of ZnO NPs was prepared by suspending ZnO NPs powder in tryptic soy broth (TSB), mixed homogenously by vortexing, and diluted using TSB to prepare the working concentrations of ZnO NPs. Next, a scanning electron microscope with energy dispersive X-ray (SEM-EDX) (JEOL, JSM-6710F) was used to characterize the morphology and size of ZnO NPs through SEM operated at an acceleration voltage of 4 kV with a working distance of 4.7 nm, while the elemental composition of ZnO NPs was confirmed by EDX analysis.

Bacterial culture

Salmonella typhimurium (ATCC 14028) was obtained from the Faculty of Science, Universiti Tunku Abdul Rahman, and sub-cultured to the mid-log phase in TSB for further study.

Bacterial exposure to ZnO NPs

A 50 µL of mid-log phase suspension of *S. typhimurium* with an optical density of 0.5 at 600 nm (OD_{600}) was exposed to 50 µL of ZnO NPs to obtain the final concentrations of 5, 10, 20, 40, 80, 160, 320, 640, 1280 µg/mL of ZnO NPs in 96-well plate and incubated for 24 hours at 37°C. Bacterial suspension without ZnO NPs served as a negative control, while bacterial suspension treated with 50 µg/mL of kanamycin sulfate was used as the positive control [27].

Growth inhibition test

A microdilution test was conducted to investigate the antibacterial effect of ZnO NPs against *S. typhimurium* by determining the optical density of bacterial suspension treated with different concentrations of ZnO NPs by a microplate reader (FLUOstar Omega) at 600 nm along with positive and negative controls. TSB was used as the blank. The absorbance of the respective concentration of ZnO NPs was subtracted from the test readings to avoid interference by ZnO NPs. Then, the absorbance values were used to calculate the percentage of bacterial growth inhibition using equation 1 (Eq. 1).

Percentage of growth inhibition (%)

$$= \frac{OD_{negative\ control} - OD_{test}}{OD_{negative\ control}}\ x\ 100 \qquad (1)$$

Investigation of surface interaction of ZnO NPs on bacterial cell wall

FTIR spectroscopy was used to investigate the involvement of functional groups in the binding of ZnO NPs to the bacterial cell wall. A 30 mL of bacterial suspension treated with 1280 µg/mL of ZnO NPs along with negative control was centrifuged for 10 mins at 6000 g to obtain the pellet. The pellet was then washed thrice with 1X PBS to remove the unbound NPs, freeze-dried, and analyzed using FTIR within the range of 4000 cm^{-1} to 400 cm^{-1}.

Morphological analysis of bacteria treated

The SEM images were used to observe the morphological changes in bacteria after treatment with ZnO NPs, while EDX was used to confirm the accumulation of ZnO NPs on bacterial cells. A 5 mL of bacterial suspension treated with 1280 µg/mL of ZnO NPs along with negative control was centrifuged for 10 mins at 6000 g to obtain the pellet. Then the pellet was washed thrice with 1X PBS and treated overnight with 2.5% of glutaraldehyde for fixation purposes. After fixation, the samples were centrifuged and washed thrice with PBS for 10 mins at 6000 g. Then, the washing process continued using distilled water. The samples were then dehydrated using a series of ethanol (50%, 75%, and absolute ethanol). Washing with absolute ethanol was repeated three times, followed by freeze-drying and sputter coating. The samples were then analyzed under SEM-EDX (JEOL, JSM-6710F).

Statistical analysis

Statistical analysis was done to identify the variances caused by ZnO NPs upon bacterial cells. Each test was done in triplicates (n=3), and the data are presented as mean and standard deviation. One-way analysis of variance (ANOVA) test was then used to analyze the result with a significance value of $p<0.05$ using SPSS (ver.24).

Results and Discussion

Physicochemical characterizations of ZnO NPs

Zinc oxide nanoparticles were observed with a mixture of rod, hexagonal and spherical shapes with an average size of 66.5 nm (**Figure 1A**). The EDX spectrum confirmed the presence of zinc and oxygen, while the presence of carbon could be due to carbon tape used for sample preparation (**Figure 1B**).

Bacterial growth inhibition

The absorbance of the treated bacterial suspension with different concentrations of ZnO NPs was measured at 600 nm along with the positive and negative controls. The treatment of ZnO NPs showed a concentration-dependent bacterial growth inhibition whereby a higher concentration of ZnO NPs induced a higher percentage of growth inhibition of *S. typhimurium* (**Figure 2** & **Table 1**).

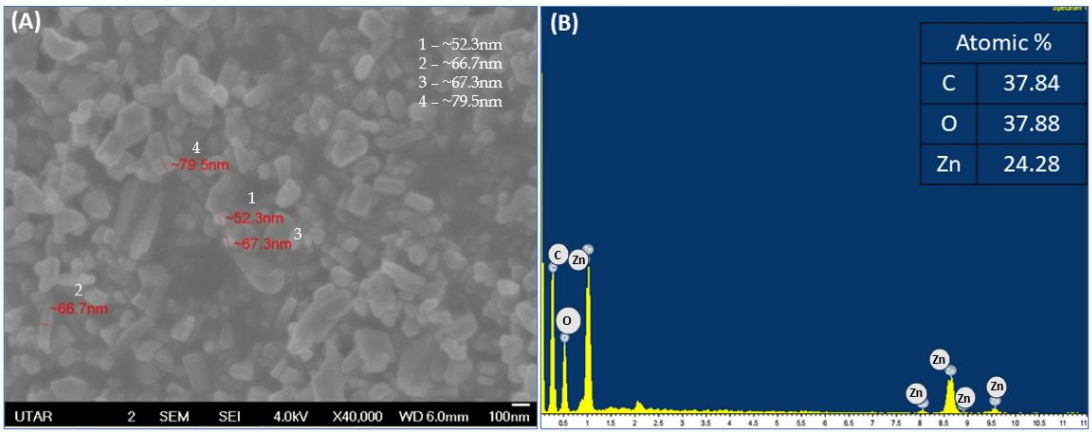

Figure 1. (A) SEM image of ZnO NPs and (B) EDX spectrum of ZnO NPs

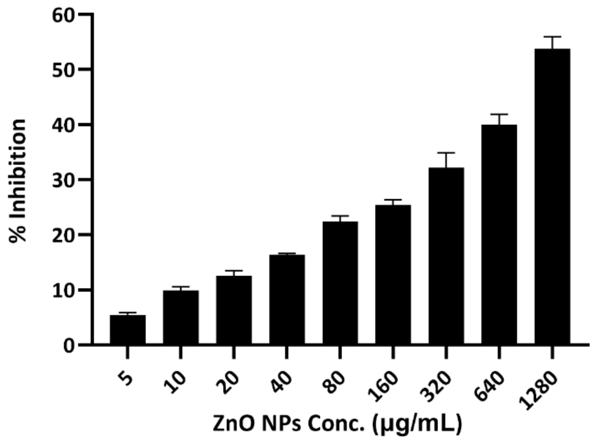

Figure 2. Growth inhibition test on *Salmonella typhimurium* by microdilution method using different concentrations of ZnO NPs. All concentrations showed significant growth inhibition

Table 1. Growth inhibition of ZnO NPs on *Salmonella typhimurium*

Concentration (µg/mL)	**Percentage of Growth Inhibition**
5	5.46 ± 0.42
10	9.94 ± 0.64
20	12.59 ± 0.95
40	16.35 ± 0.27
80	22.43 ± 0.99
160	25.41 ± 0.93
320	32.16 ± 2.71
640	40.00 ± 1.85
1280	53.79 ± 2.19

The percentage of growth inhibition of *S. typhimurium* at different concentrations of ZnO NPs is presented in **Table 1**. The results of the present study indicate that ZnO NPs exhibit dose-dependent antibacterial activity against *S. typhimurium*, as an increase in ZnO NP concentration corresponded with a higher percentage of bacterial growth inhibition. Previous studies have also reported concentration-dependent growth inhibition of *S. typhimurium* using various methods, including the agar well diffusion assay and turbidity measurements, supporting the findings of the present study [28-30].

Surface interaction study of ZnO NPs on bacterial cell wall

The FTIR spectrum displayed in **Figure 3** showed the functional groups that were involved in the interaction of ZnO NPs with the cell wall of *S. typhimurium* (**Table 2**).

The treated *S. typhimurium* showed peak shifts from 3429 to 3444 cm^{-1}, 2925 to 2928 cm^{-1}, 1644 to 1647 cm^{-1}, 1398 to 1400 cm^{-1}, and 1082 to 1084 cm$^{-1,}$ which corresponded to the stretching of O – H, C – H, C = C, COO^{-}, and P = O bonds, respectively. The region between 3425 and 3444 cm^{-1} was due to the O-H and N-H group stretching of proteins and polysaccharides as well as the formation of intermolecular hydrogen bonds [31, 32]. The peaks 2925 and 2928 cm^{-1} were dominated by the C-H stretching of the fatty acids. In comparison, the region between 1647 and 1644 cm^{-1} was ascribed to the stretching of C=C and C=O of proteins and peptides. Weak peaks observed at 1398 and 1400 cm^{-1} could be contributed to COO^{-} of proteins and carbohydrates, while peaks at 1082 and 1084 cm^{-1} could be attributed to the P=O stretching of nucleic acids [31, 33-36]. The peaks that shifted from 623 to 558 cm^{-1} were attributed to Zn-O stretching. A study conducted by Akbar et al. demonstrated that peaks between 400-700 cm^{-1} could be attributed to Zn-O stretching [30].

This agreed with a previous study in which the carboxyl, amide, phosphate, hydroxyl groups, and carbohydrate-related moieties in the bacterial cell wall might involve in binding with metal oxide NPs [37]. ZnO NP have the ability to interact with bacterial cell walls, leading to the disruption of cell wall-associated proteins and polysaccharides [38]. The binding of ZnO NPs is assisted by the electrostatic force between the Zn^{2+} ions released from the ZnO NPs and the negatively charged bacterial surface [39]. The accumulation of ZnO NPs on the bacterial surface causes a change in membrane potential and depolarization of the bacterial membrane, resulting in loss of integrity and increased membrane permeability. As a result of the loss of membrane integrity, the bacterial transport system becomes imbalanced, cellular respiration be impeded, and energy transduction within the bacterial cells can be interrupted [40, 41]. Consequently, cell lysis occurs, eventually leading to cell death [39, 40, 41]. Next, the surface accumulation of ZnO NPs on the cell wall of *S. typhimurium* was analyzed using EDX by comparing the elemental composition present on the surface of the treated and untreated bacterial suspension. **Figure 4A** showed the EDX spectrum of negative control, which depicted the existence of carbon, oxygen, sodium, phosphorus, and sulfur. **Figure 4B** showed the existence of carbon, oxygen, phosphorus, sulfur, and zinc in the bacterial suspension after treatment with 1280 µg/mL of ZnO NPs. The EDX spectrum obtained proved that the ZnO NPs accumulated on the surface of bacterial cells.

Table 2. The functional groups and the corresponding biomolecules involved in the binding of ZnO NPs to the bacterial cell wall by FTIR analysis

Peak Shift (cm^{-1})	Molecular Motion	Functional Groups	Biomolecules
3429 - 3444	O – H and N – H stretching	Alcohol, amide	Proteins and polysaccharides
2925 - 2928	C – H stretching	Alkene	Fatty acids
1644 - 1647	C = C and C = O stretching	Alkene	Proteins and peptides
1398 - 1400	COO- stretching	Carboxylic group	Proteins, carbohydrates, fatty acids
1082 - 1084	C – O and P = O stretching	Phosphate group	Nucleic acids, polysaccharides
623 - 558	Zn – O stretching	Zinc oxide	Fingerprint region

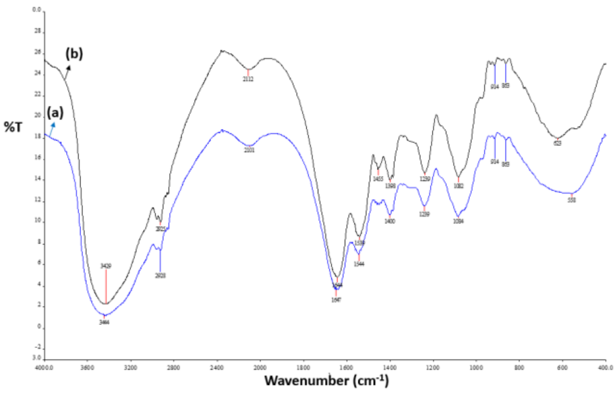

Figure 3. FTIR spectrum of bacterial cells (a) treated with 1280 µg/mL of ZnO NPs (black line) and (b) negative control (blue line)

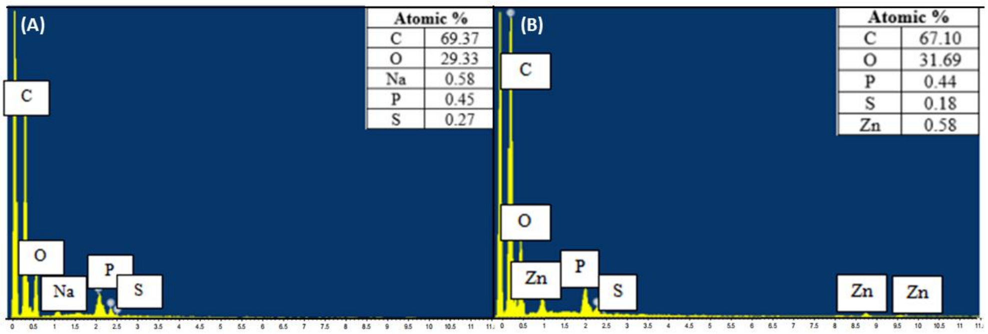

Figure 4. EDX analysis of *Salmonella typhimurium* (A) negative control and (B) bacterial cells treated with 1280 µg/mL of ZnO NPs

The SEM image of negative control of *S. typhimurium* showed an intact bacterial cell wall (**Figure 5**). However, *S. typhimurium* treated with 1280 µg/mL of ZnO NPs for 24 h, depicted the attachment of NPs on the surface of bacterial cells, membrane deformity, and pore formation (**Figure 6**).

Similar findings were reported by Tayel et al., in which after the exposure of ZnO NPs to *S. typhimurium* and *S. aureus*, both strains demonstrated membrane distortion and leakage of cellular components when observed under SEM [27]. Other experiments performed using *E. coli*, *S. aureus*, *P. aeruginosa*, and *Proteus mirabilis* also showed membrane distortion such as cell shrinkage, cell wall crumpling, and rupture and pitted cell membrane upon treatment of ZnO NPs [38, 42, 43].

The major cause of cell wall disruption was hypothesized to be the generation of ROS, for instance, superoxide anion (O_2^-), hydroxyl ion (OH^-), and hydrogen peroxide (H_2O_2), from the surface of ZnO NPs. These ROS can exert mechanical and oxidative stress on the cell membrane, thus compromising bacterial membrane integrity. Hydrogen peroxide but not O_2^- and OH^- can penetrate the cell membrane and cause lipid peroxidation and damage to cellular components due to the negatively charged membrane. Eventually, these events result in cell rupture and cell death [43-45]. **Figure 7** represents the possible mechanisms of ZnO NPs on pathogenic bacteria, the interaction of nanoparticles with the bacterial surface membrane triggers structural alterations that ultimately compromise the integrity of the bacterial cell wall. The penetration of ZnO NPs into the bacteria cell triggers the production of reactive oxygen species (ROS), which in turn results in the inhibition of protein synthesis, damage to ribosomes, disruption of mRNA and DNA activities, mitochondrial impairment, damage of proton effluent pump, alter membrane permeability and among various other effects and consequently, it causes the demise of microbial cells [46,47].

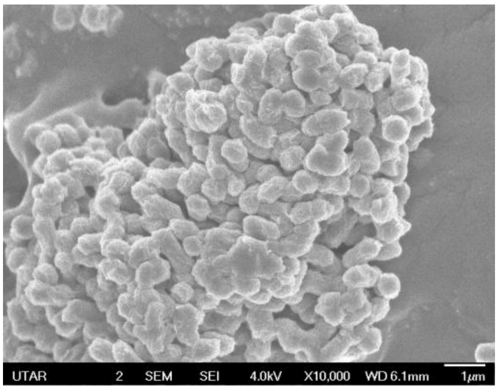

Figure 5. SEM image of *Salmonella typhimurium* (negative control)

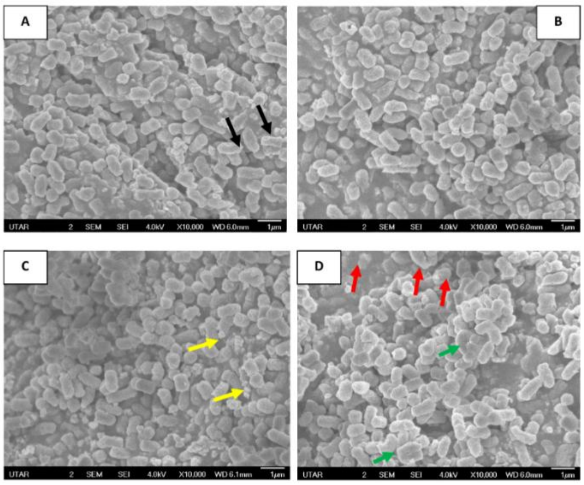

Figure 6. SEM images of *Salmonella typhimurium* treated with 1280 µg/mL of ZnO NPs. Bacterial cells showed membrane deformity (black arrow), pore formation (red arrow), accumulation of ZnO NPs (yellow arrow), and cell bending (green arrow)

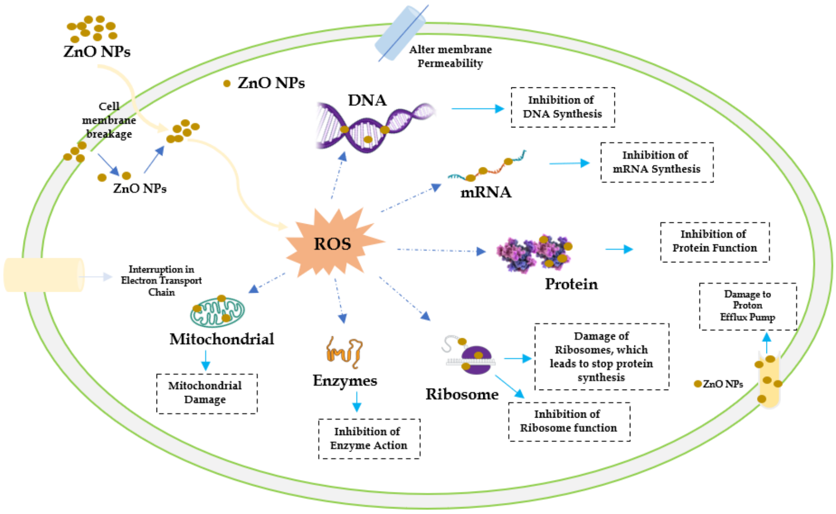

Figure 7. Possible mechanisms of antibacterial activity of ZnO NPs against pathogenic bacteria [9, 17, 48]

Conclusion

The present study showed a concentration-dependent growth inhibitory effect of ZnO NPs against *Salmonella typhimurium*. The result depicted the highest percentage of growth inhibition of 53.79% at 1280 µg/mL. FTIR results showed the possible involvement of alcohol, alkene, carboxylic, and phosphate groups which corresponded to biomolecules such as polysaccharides, fatty acids, and carbohydrates from the cell wall of *S. typhimurium* in the interaction with ZnO NPs after 24 hours of treatment. Besides, treatment with ZnO NPs also induced morphological changes on the surface of *S. typhimurium* which include curvature, bending of cells, and pore formation. The present study proposed the potential usage of zinc oxide nanoparticles as an alternative treatment for the infection caused by *S. typhimurium*. Further study is recommended to increase the concentration of ZnO NPs in future research to determine the MIC for *S. typhimurium*. The major mechanism of toxicity induced by ZnO NPs was proposed to be due to the release of Zn^{2+} ions. The present study could not investigate the amount of Zn^{2+} ions released during the treatment with ZnO NPs. Hence, it is recommended to assess the amount of Zn^{2+} ions released along with a growth inhibition test.

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Author contributions

Investigation, C.K.Y., Supervision, study design, review and editing, S.D., study design, review and editing L.S.W., writing, review, R.R., review, editing, P.K.G and S.K. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

Authors declare no conflict of interest.

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