RESEARCH PAPER

Improvement the Antibacterial Effect of Zinc Oxide Nanoparticles by Conjugation with Tetracycline

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ABSTRACT

Nanoparticles when used along with antibiotics offer a novel strategy to combat bacterial infections. In the present, the synergistic antibacterial effect of Zinc Oxide (ZnO) nanoparticles with Tetracycline antibiotic in two classes of bacteria, including Gram-negative and Gram-positive bacteria such as Klebsiella pneumoniae and Staphylococcus aureus, were assayed. Experimental studies have shown that ZnO nanoparticles and Tetracycline bound ZnO nanoparticles are both antibacterial. Nevertheless, Tetracycline-conjugated ZnO NPs demonstrated strong antibacterial efficacy in comparison with ZnO NPs or Tet alone against both bacteria species tested. This increased potency indicates a possible additive effect of the nanoparticles and antibiotic. This research provides an important introduction to the ability of nanoparticles to potentiate the activity of classical antibiotics. It also highlights the possibility of synergistic effects of these combinations for surmounting resistance by bacteria. Altogether, this study opens up new ways of exploring nanoparticle-antibiotic conjugates as promising and innovative antibacterial products for clinical and industrial purposes.

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INTRODUCTION

Nanotechnology is at the top of the list of scientific and research interests in all countries of the world due to its expected impact on many areas such as agricultures, medicine and industrial... etc [1, 2]. Various studies have been conducted in recent years on the use of nanomaterials, including metal nanoparticles and their oxides such as TiO_2, silver, silver oxide, zinc oxide, gold, calcium and copper oxides, silica and magnesium oxide as antibacterial agents [3-6].

Many studies have proven the antibacterial effectiveness of nano zinc oxide, which is characterized by its ability to inhibit bacteria even at very low concentrations [7,8] and [9]. Many studies have also investigated the antibacterial activity of nanoparticles in combination with antibiotic. combination of NPs with antibiotics may produce or may not produce a synergetic activity [10]. The antibacterial effect of zinc oxide nanoparticle in combination with Tetracycline needs more scientific interest.

Therefore, in this study, we describe the antibacterial activity of ZnO nanoparticles and Tetracycline conjugated ZnO against *Klebsiella pneumoniae* (gram negative bacteria)

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Staphylococcus aureus (and gram positive bacteria).

MATERIALS AND METHODS

Zinc Oxide nanoparticles were obtained from XFNano company, an average size of it 50 nm (characterized by TEM (Transmission Electron Microscopy), SEM (Scanning Electron Microscopy), UV-Vis (Ultra Violet- Visible) Spectroscopy and XRD (X-Ray Diffraction).

Two bacterial isolates *Klebsiella pneumoniae* (gram negative bacteria) *Staphylococcus aureus* (and gram positive bacteria) were obtained from advanced microorganisms laboratory in biology department/ university of Babylon, Bacteria were characterized by conventional methods. Standard laboratory powder of Tetracycline was utilized in the present study.

For the production of Tetracyclin-ZnONPs, solutions of ZnONPs and Tetracycline were prepared (0.01g was suspended in 1ml deionized water). Then Conjugation was made by mixing 1 mL of the antibiotic and ZnO NPs followed by sonication for 30 seconds. The mixture was left for 24h to allow the transfer of Tetracycline to the surface of nanoparticles. Unbounded Tetracycline were washed several times and removed by centrifugation at 10000 rpm for 15minutes. After that, the supernatant was removed, the pellet was washed 3 times by centrifuge with suspending into sterile distilled water, the solution was kept for overnight at dark to dry [11].

The antibacterial susceptibility test for ZnO nanoparticle and Tetracyclin conjugated ZnO nanoparticle against *Klebsiella pneumoniae* (gram negative bacteria) *Staphylococcus aureus* (and gram positive bacteria) was made by agar well diffusion method and broth macro-dilution method, the MIC and MBC were determined from the broth macro-dilution assay.

The concentration 2000 µg/ml for ZnO nanoparticle and Tetracyclin conjugated ZnO nanoparticle was prepared with distilled water, 20 ml of Muller Hinton agar was put in petri plates. After agar solidification, 0.1 ml of each isolate was spread, the petri plates were left for an 5 minutes then 6 mm diameter wells were made in each plate with sterile cork borer. Fifty microliters for ZnO nanoparticle and Tetracycline conjugated ZnO nanoparticle and Tetracycline was

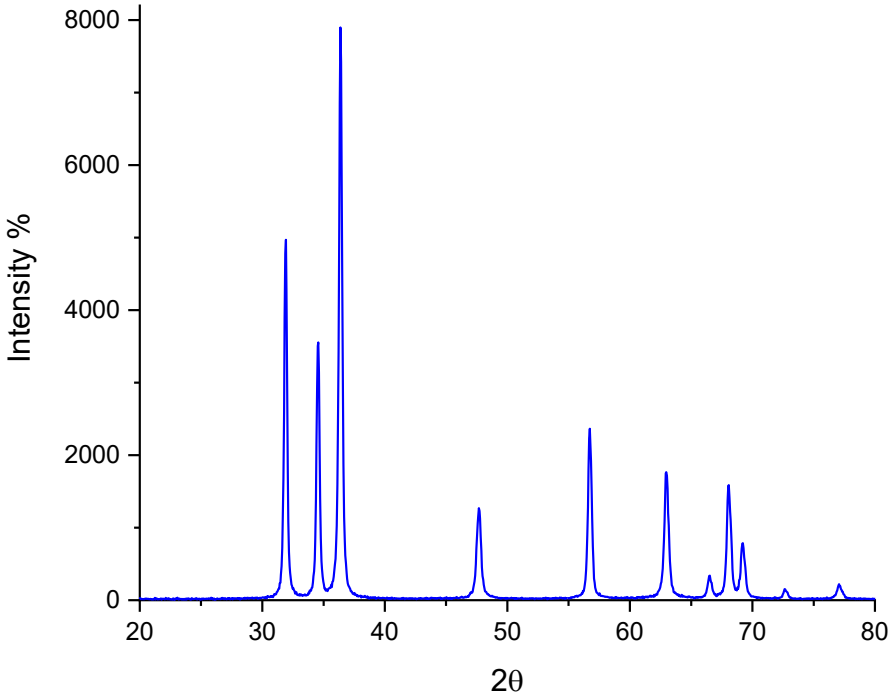

Fig. 1. XRD pattern of ZnO nanoparticles showing distinct peaks corresponding to the hexagonal wurtzite structure.

added to each well and diffuses for 10 minutes. wells containing distilled water were considered as negative control, the plates then incubated at 37oC for 24hrs. Inhibition zones was measured as millimeter in diameter, the experiment was performed in triplicate. The MICs for ZnO nanoparticle and Tetracycline conjugated ZnO nanoparticle against bacteria was determined using broth Macro-dilution method as reported by [12]. The turbidity of the suspension of bacteria in Muller Hinton broth was adjusted to 0.5 McFarland standard to yield 1 × 106 CFU/mL. The MIC was performed by two fold dilution series for ZnO nanoparticle and Tetracyclin conjugated ZnO nanoparticle. Initially, serial dilutions of for ZnO nanoparticle and Tetracycline conjugated ZnO nanoparticle (2000,1000,800,400,200,100) µg/ml were prepared in Muller Hinton broth media. then each tube was inoculated with 100 µL suspension of the bacteria. The positive control tube containing broth medium and bacterial isolate, the negative control was un-inoculated broth. Experiments were assayed in triplicate, the tubes then incubated at 37 °C for 24 h. The turbidity of the tubes was noted to confirm the MIC value. the lowest concentration where no visible growth is seen in the tubes was considered the MIC.

The MBC was determined by sub-culturing all the tubes which showed no bacterial growth in nutrient agar plates using 0.01 ml and incubated for overnight at 37oC, MBC was regarded as the lowest concentration that did not show growth of bacterial colony on the nutrient agar plates [13].

RESULTS AND DISCUSSION

X-ray Diffraction (XRD) Analysis

The crystalline structure of the synthesized ZnO nanoparticles was examined using X-ray diffraction (XRD). The diffraction pattern (Fig. 1) exhibits distinct and sharp peaks, indicating a high degree of crystallinity. The major diffraction peaks appear at 2θ values of approximately 31.8°,

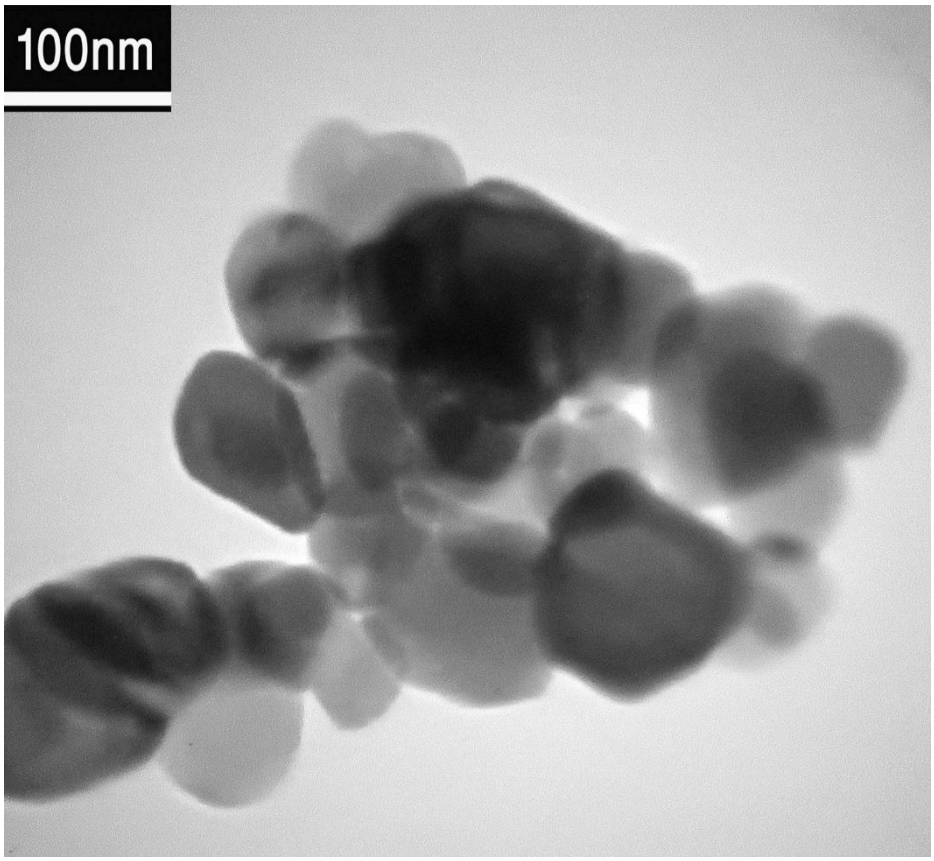

Fig. 2. TEM image of ZnO nanoparticles indicating quasi-spherical morphology and particle size in the range of 20–50 nm.

34.4°, 36.3°, 47.5°, 56.6°, 62.8°, 66.3°, 68.0°, and 69.1°, which correspond well to the (100), (002), (101), (102), (110), (103), (200), (112), and (201) planes, respectively, of the hexagonal wurtzite ZnO crystal structure (JCPDS Card No. 36-1451). No secondary phases or impurity peaks were observed, confirming the purity of the synthesized ZnO nanoparticles [14, 15]. The sharpness and intensity of the peaks further suggest well-defined crystalline domains. Using the Scherrer equation, the average crystallite size was estimated to be in the nanometer range (typically between 20–40 nm), indicating successful synthesis of ZnO in nanocrystalline form [16].

Transmission Electron Microscopy (TEM) Analysis

The morphology and size of the ZnO nanoparticles were investigated using Transmission

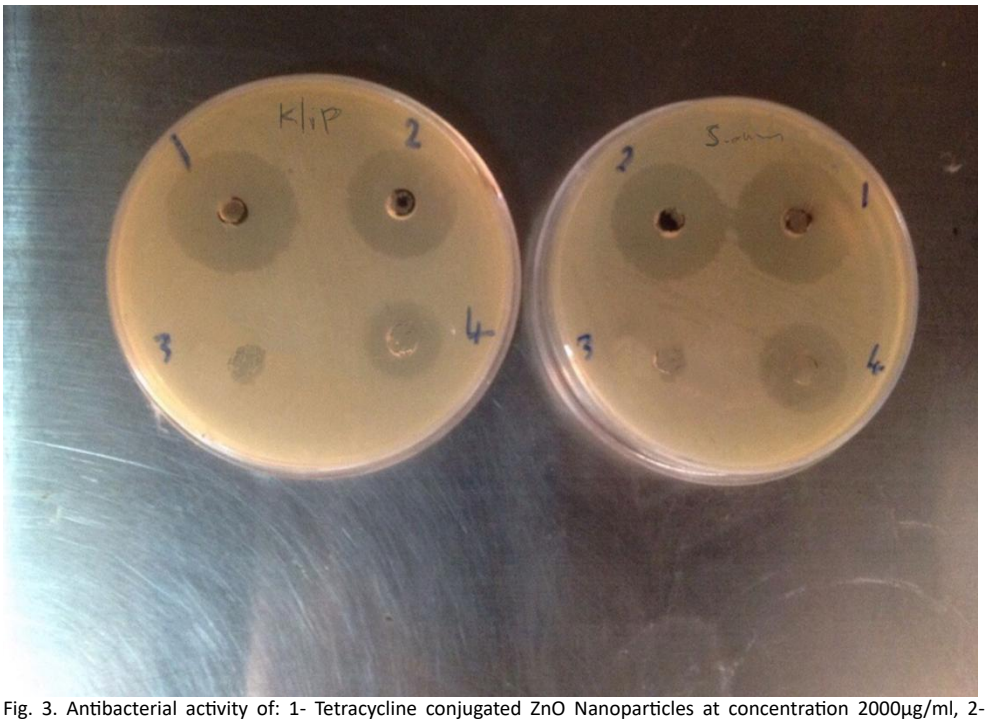

Fig. 3. Antibacterial activity of: 1- Tetracycline conjugated ZnO Nanoparticles at concentration 2000µg/ml, 2- ZnO Nanoparticles at concentration 2000µg/ml , 3- Tetracycline , 4- deionized water (control) against *Klebsiella pneumoniae* and *Staphylococcus aureus.*

Table 1. Antibacterial activity of ZnO nanoparticles and Tetracycline conjugated ZnO nanoparticles and Tetracycline against *Klebsiella pneumoniae* and *Staphylococcus aureus*, Data represent the mean ± SD.

Concentration 2000µg/ml	Zones of Inhibition in Millimeter		
	Tetracycline conjugated ZnO Nanoparticles	ZnO Nanoparticles	Tetracycline
Klebsiella pneumoniae	24.6±0.577	20.0±1.000	14.0±2.000
Staphylococcus aureus	26.6±0.6	23±0.7	13±1.000

Electron Microscopy (TEM), as shown in Fig. 2. The TEM micrograph reveals agglomerated nanoparticles with quasi-spherical shapes. The individual particle size appears to range between 20–50 nm, which is consistent with the crystallite size calculated from XRD data. The particles show moderate agglomeration, which is typical for ZnO nanoparticles due to their high surface energy and tendency to form clusters. Despite agglomeration, the particle boundaries are distinguishable, and the nanoscale nature of the material is evident. Overall, the combined XRD and TEM analyses confirm the successful synthesis of pure, crystalline ZnO nanoparticles with nanometer-scale dimensions and wurtzite phase structure.

Antibacterial Effect

The antibacterial effect of synthesized ZnO nanoparticle and Tetracycline conjugated ZnO nanoparticle at concentration 2000µg/ml against *Klebsiella pneumoniae* (gram negative bacteria) *Staphylococcus aureus* (and gram positive bacteria) were investigated by agar well diffusion assay and the antibacterial sensitivity was measured by measuring the diameter of zones of inhibition in millimeter. The results showed that ZnO nanoparticle and Tetracycline conjugated ZnO nanoparticle have antibacterial effect against *Klebsiella pneumonia* and *Staphylococcus aureus*. The result showed that tetracycline conjugated ZnO Nanoparticles has more antibacterial effect than ZnO nanoparticle or tetracycline alone for both bacteria utilized in the study as shown in Fig. 3.

The MIC and MBC for synthesized ZnO nanoparticles and tetracycline conjugated ZnO Nanoparticles was determined from broth macro dilution method, two-fold dilution series of synthesized ZnO nanoparticles and tetracycline conjugated ZnO Nanoparticles (2000,1000,800,400,200,100) µg/ml were tested, the results are shown in Table 2.

The result showed that the nanoparticles have the same effect for both Gram positive and negative bacteria, this result disagree with [7], he found that gram positive bacteria is more sensitive than gram negative bacteria. he explained that the antibacterial effect of silver nanoparticles could be due to several mechanisms, the main mechanism suggested is the oxidative stress generated by Reactive oxygen species.

ROS, including ^{-}OH (hydroxyl radicals), superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and singlet oxygen ($1O_2$), can cause damage to DNA and proteins in bacteria [17, 18], In this case, ZnO nanoparticles could generated ROS that inhibit bacteria.

Researchers found that ZnO nanoparticles inhibited the growth of bacteria even its multidrug-resistant as well, he found that ZnO nanoparticles damages the outer membrane of *E. coli*, by destroying the lipopolysaccharide layer, followed by damaging the inner membrane, then inter within the cell and create ROS-mediated damage [19].

For all bacteria tested, the results showed that tetracyclene conjugated ZnO nanoparticles more activity than ZnO nanoparticles and Tetracycline alone used in the study, The synergistic effect of ZnONPs with ciprofloxacin and Ampicillin was reported against gram positive negative bacteria including *Pseudomonas aeruginosa*, *Escherischia coli*, *Klebsiella pneumoniae*, *Bacillus subtilis, Salmonella typhi* and *Staphylococcus aureus* [20] who showed the increasing effect of ciprofloxacin and Ampicillin in combination with ZnONPs against bacteria. the improvement in antibacterial effect might be due to the difference in the mode of action of nanoparticles. Conjugation of nanoparticles with antibiotic might increased the binding affinity to their targets and improved penetration through the cell wall, thus, increased the drug effect against bacteria. In addition, the using of two antibacterial agents with different mechanisms of effect, is also important, because if bacteria give rise resistance to one of them, the other antibacterial agent would inhibit the bacteria [21]. In addition, high surface area and

Table 2. The MIC and MBC for synthesized ZnO nanoparticles

Bacteria	Tetracycline conjugated ZnO Nanoparticles		ZnO Nanoparticles	
	MIC	MBC	MIC	MBC
K.pneumoniae	100 µg/ml	200 µg/ml	1000 µg/ml	2000 µg/ml
S. aureus	100 µg/ml	200 µg/ml	1000 µg/ml	2000 µg/ml

small size of NPs make them more drug loading which enhance concentration of antibiotic at the site of antibiotic-bacteria contact and this may cause increasing in the inhabitation of bacteria [22]. Moreover, the conjugants have lower dose of both antimicrobial agents, which reduces the harmful effects [21].

CONCLUSION

Tetracycline conjugated ZnO nanoparticles and ZnO nanoparticles possess antibacterial activity against *Klebsiella pneumoniae* (gram negative bacteria) *Staphylococcus aureus* (and gram positive bacteria). Conjugation of Tetracycline with ZnO nanoparticles increases the antibacterial activity of ZnO nanoparticles.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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