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Investigation of the Efficacy of Synthesized Silver and Zinc Oxide Nanoparticles against Multi-Drug Resistant Gram Negative Bacterial Clinical Isolates

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Abstract

Background/aim: The increasing number of multidrug-resistant (MDR) bacteria and the need to synthesize new antimicrobials, nanoparticles have attracted interest in the scientific community. This study aimed to evaluate the synergistic effect of nanoparticles with some antibiotics against MDR pathogenic bacteria.

Material and methods: Seventeen bacterial isolates including; *Escherichia coli* (n=5) and *Klebsiella pneumoniae* (n=12) were collected from different clinical samples of patients suffering from burn wound and fistulae wounds infections. The susceptibility of the collected isolates to different antibiotics was investigated. Six MDR isolates were selected as representatives to different patterns were used for the determination of the antibacterial activities of two metals nanoparticles; silver (Ag-NPs) and zinc oxide (ZnO-NPs). The antibacterial activities of the synthesized nanoparticles alone and their combination with the selected antibiotics were determined against the tested isolates using disc-diffusion method according to EUCAST Clinical Breakpoint.

Results: Well dispersed spherical Ag-NPs with average particle size 12.65 ± 0.55 nm and ZnO-NPs of 7.6 ± 0.5 nm were chemically synthesized by the chemical reduction method and chemical precipitation method, respectively. The obtained results suggested that the Ag-NPs alone had higher antibacterial activity than ZnO-NPs. Furthermore, Ag-NPs show an observable synergistic effect with some antibiotics; more than ZnO-NPs.

Conclusions: It was concluded that Ag-NPs have potential as a combination therapeutic agent for the treatment of infectious diseases by bacteria.

Keywords: MDR bacteria; Ag-NPs; ZnO-NPs; Antibiotics-nanoparticles combination; Antibacterial activity

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Introduction

Microbiologists and pharmacists are in continuous challenge to deal with, control and treat diseases caused by microbes we face daily. Recently the global public health is in a serious threat with the increasingly introduced antimicrobial resistance of the infecting pathogens to the ordinary known treatments. The antimicrobial resistance is defined as the resistance of a microorganism to an antimicrobial drug that was originally effective for the treatment of infections caused by it. Resistant microorganisms such as bacteria, fungi, viruses and parasites are able to withstand the attack by antimicrobials such as; antibacterial (antibiotics), antifungal, antiviral and antimalarial drugs, respectively [1]. As a result, those standard treatments become ineffective and infections persist with prolonged illness, higher health care expenditures and greater chance for death with the risk of spreading to the others [2]. It is important to mention that the antimicrobial resistance's development is present in either developing or advanced countries with impressive increase in the numbers of victims who died by infections caused

by resistant microorganisms every year. Melake et al. [3] studied the prevalence of MDR bacterial isolates causing burn infections from 105 patients admitted the Burn Unit of Menoufia University Hospital. They reported that about 36% of patients had burn wound infections and about 63% of the bacterial growth was MDR isolates.

Although, resistance of bacteria to antibiotics could occur normally as a natural phenomenon, certain human actions accelerate its emergence and spread where; the inappropriate use of antibiotics either by overuse or misuse can lead to the evolution of new MDR bacteria. If these trends persist and resistance continues to rise, some reports estimated that by 2050 there will be ten million antimicrobial resistance related deaths worldwide, costing the world up to 100 trillion $ (2). Unfortunately, the discovering of new antibiotics is considered a real challenge to the researchers and pharmaceutical companies due to the difficulty of identifying novel bacterial targets that could be used for the innovation of new classes of safe and effective antimicrobial agents by a rate covering the incredible increase in bacterial resistance among the world [4].

Nowadays, there is a great interest in integrating nanotechnology with biology in order to develop new antimicrobial drugs based on nanotechnology with higher affectivity. It was reported that some noble metals like gold and silver exhibit interesting antibacterial activity [5-7]. However, only few studies have compared the efficacy of noble metals nanoparticles such as silver nanoparticles (Ag-NPs) with that of other metals oxides like Zinc-oxide (ZnO-NPs). Also the effect of those nanoparticles on enhancing the activity of different antibiotics belonging to different antimicrobial classes is still under investigation. It is important to note that although there are some reports on the antibacterial activity of Ag-NPs and ZnO-NPs against bacterial pathogens, no available study has been done to evaluate the synergistic potential of those nanoparticles with antibiotics with respect to the known CLSI standards. The present study aims to tested whether the combination of synthesized nanoparticles with the selected antibiotics can pose distinguishing antibacterial efficacy against tested clinical isolates, when compared to antibiotic alone, which might have implications on the control and treatment of infections. The susceptibility of the selected isolates to antibiotic-nano combination according to EUCAST Clinical Breakpoint was performed. Consequently detecting however the isolates became more susceptible to antibiotic after nano-combination or the combination resulted in only slight increase in the inhibition zone whiles the organism still resistant. Furthermore, the nanoparticles were characterized using different chemical techniques including; UV-visible Spectrophotometer, Transmission Electron Microscopy (TEM) and Energy dispersive X-ray analysis (EDX).

Materials and Methods

Isolation and identification of clinical bacterial isolates

A total of twenty five clinical specimens were randomly collected in sterile screw capped containers from patients with burn wound infection and fistulae wounds. The clinical samples used for this study were collected from patients attending different hospitals including; Burn unit of Tanta Emergency Hospital, Mubarak Hospital and El-Salam Hospital in Tanta, Egypt. Each specimen was cultured on blood agar and nutrient agar plates. The growing colonies on these media were subcultured on MacConkey agar medium and Eosin methylene blue agar (EMB-agar). The recovered isolates were subjected to different morphological and biochemical tests for identification to the species level as described by Bergey's Manual of Determinative Bacteriology 9th edn. [8].

The isolates were identified as *Escherichia coli* (n=5) and *Klebsiella pneumoniae* (n=12). Identification of tested isolates was confirmed with an API 20E (bioMérieux) identification kit. Stock cultures were stored in 0.05 M K-Na-phosphate buffer, pH 7.0, containing 15% glycerol at - 20°C.

Antibiotics

For studying the combination effect of metal-nanoparticles with antibiotics; the antibiotics used in our study were selected to cover almost all the different antibiotic classes suitable for Enterobacteriaceae isolates. The antibiotics used with their antimicrobial subclasses along with the discs' potencies are presented in **Table 1.**

Chemical synthesis of metal nanoparticles

Synthesis of Ag-NPs by the chemical reduction method: The Ag-NPs used in this study were synthesized using the chemical reduction method [9]. This method involves the reduction of silver nitrate aqueous solution ($AgNO_3$) by using trisodium citrate ($C_6H_5O_7Na_3$) which acts as reducing agent and stabilizing agent [10]. $AgNO_3$ and $C_6H_5O_7Na_3$ (Sigma Aldrich, UK) of analytical grade purity were used as starting materials. In that method, 50 ml of 1 × 10^{-3} M $AgNO_3$ was heated to boiling. Later, 5 ml of 1% $C_6H_5O_7Na_3$ was added drop by drop to this solution with vigorous mixing. The mixture was heated until color change was evident (pale yellow) indicating the formation of Ag-NPs. The suspension was removed from the heating element and stirred until cooled to room temperature.

Table 1 Antibiotic susceptibility disks (Bioanalyse) along with their codes and potencies.

Antimicrobial class	Antimicrobial agent	Disc content
Penicillins	Ampicillin (Am)	10 µg
β-lactam/β-lactamase inhibitor combinations	Amoxycillin/Clavulanic acid (20/10 µg) (AMC)	30 µg
Cephems	Cefotaxime (CTX)	30 µg
Monobactams	Aztreonam (ATM)	30 µg
Carbapenems	Imipenem (IPM)	10 µg
Aminoglycosides	Gentamycin (GEN)	10 µg
Quinolones	Levofloxacin (LEV)	5 µg
Foliate pathway inhibitor	Trimethoprim-sulfamethoxazole (SXT)	25 µg
Phenicols	Chloramphenicol (C)	30 µg
Tetracyclines	Tetracycline (TE)	30 µg

The mechanism of reaction could be expressed according to Šileikaitė et al. [11] as follows: $2\ Ag^{+} + C_6H_5O_7Na_3 + 2\ H_2O \rightarrow 4\ Ag^{0} + C_6H_5O_7H_3 + 3\ Na^{+} + H^{+} + O_2 \uparrow$

Synthesis of ZnO-NPs by precipitation method: ZnO-NPs were synthesized by the precipitation method described by Salahuddin et al. [12] in the Chemistry Department, Faculty of Science, Tanta University. Zinc nitrate hexahydrate ($Zn(NO_3)_2.6\ H_2O$) (0.1 M, solution **A**) and sodium carbonate (Na_2CO_3) (0.12 M, solution B) were prepared then solution A was added to solution B drop wise under vigorous stirring. The white precipitate formed was collected by filtration and rinsed with distilled water three times. The solid was then washed with ethanol and dried at 100°C for 6 h.

Characterization of the chemically synthesized nanoparticles

Visual inspection: In case of Ag-NPs, the conversion of the colorless solution of $AgNO_3$ to a pale yellow color after the reduction with sodium citrate clearly indicated the formation of Ag-NPs [9]. While, in case of ZnO-NPs, the formation of a white precipitate from the precipitation of zinc nitrate indicated the formation of ZnO-NPs [12].

UV-Vis spectroscopy: The formation of the nanoparticles and their optical properties were analyzed via UV-visible Spectrophotometer according to the method of Mie [13] at wavelength 300-800 nm with Ag-NPs and 360-500 nm with ZnO-NPs using Shimadzu dual beam spectrophotometer (Model UV 1650 PC) operated at a resolution of 1nm in the Chemistry Department, Faculty of Science, Tanta University, Egypt.

Transmission electron microscopy (TEM): The morphology and the mean size of the synthesized Ag-NPs and ZnO-NPs were measured by JEOL, JEM-100 SX electron microscope (Japan) in Faculty of Medicine, Tanta University. The transmission electron microscopy was operated at 200 kv and the samples were prepared by dropping the nanoparticles onto carbon coated Cu grid underlying tissue paper, leaving behind a film [10].

Energy dispersive x-ray analysis (EDX): In order to detect the elemental composition of the synthesized nanoparticles, their nano-powders were analyzed by EDX [14]. Samples were collected in the powder form and analyzed by EDX (model FEI) in the Chemical War Labs, Armed Forces, Cairo, Egypt.

Assessment of antibiotic susceptibility, resistance pattern and calculating the multiple antibiotics resistance (MAR) index of the tested bacteria

All the isolates were tested for their susceptibility to the selected antibiotics using Disc Diffusion Method [15]. Bacterial inoculum (100 µl) of 1×10^8 CFU/ml for each isolate was used to inoculate Mueller-Hinton agar (MHA). The plates were allowed to set and the selected antibiotic discs were placed using sterile forceps onto the agar plates. The diameters of the inhibition zones were determined and the results were interpreted as susceptible, intermediately resistant or resistant by referring to the Performance Standards for Antimicrobial Susceptibility Testing [16].

The resistance patterns of the isolates were recorded and the MAR index was calculated for each isolate [17,18] as follow:

$$\text{MAR index} = \frac{\text{Number of antibiotics isolates are resistant to}}{\text{Total number of antibiotics tested}}$$

Determination of the antibacterial efficacy of the synthesized nanoparticles

The antibacterial activity of synthesized Ag-NPs and ZnO-NPs was investigated by well diffusion method using six isolates; (two isolates of *E. coli* and four isolates of *K. pneumoniae*) selected randomly as representatives to the different resistant patterns obtained. The tested inoculums were adjusted to concentration 1×10^8 CFU/ml then 100 µl of each inoculum was applied onto the surface of MHA plates free of nanoparticles using sterile cotton swab. The plates were allowed to dry and a sterile well - cutter of diameter 6.0 mm was used to bore two wells in each plate. Subsequently, two volumes (50 µl and 100 µl) of the Ag-NPs or ZnO-NPs suspensions were introduced, respectively into the wells. The plates were allowed to stand for 1 h for the diffusion to take place and then incubated at 37°C for 18 h. After incubation, the diameters of the inhibitory zones (mm) were measured [19]. All the steps were made in triplicates.

Determination of the efficacy of nanoparticles on enhancing the activity of different antibiotics

Only antibiotics to which the selected isolates were found to be resistant were selected for investigation. The antibacterial activities of these antibiotics alone were compared with those with their combinations with Ag-NPs and ZnO-NPs, respectively by disc diffusion method against the selected isolates [5,20]. Bacterial inocula were prepared and the plates were inoculated as mentioned before. The plates were allowed to set and then the standard antibiotics' discs with and without nanoparticles were placed onto the surface of the inoculated agar plates using sterile forceps. For determining the synergistic effects between antibiotics and nanoparticles, antibiotic discs impregnated with 50 µl of Ag-NPs or ZnO-NPs, respectively were used. Plates were labeled carefully and incubated for 18 h at 37°C. After incubation, the diameters of the inhibitory zones (mm) around the discs were measured. All experiments were conducted in triplicates to confirm the results.

The fold increase in the diameter of inhibition zone of each antibiotic after the combination with Ag-NPs or ZnO-NPs was calculated according to the equation;

$$\text{The fold increase} = (b^2-a^2)/a^2,$$

Where; (a) is the inhibition zone of antibiotic alone and (b) is the inhibition zone of antibiotic plus nanoparticles [20].

The number of isolates changed from resistant to an antibiotic to intermediately resistant or sensitive to the same antibiotic after the combination with nanoparticles were recorded.

Results and Discussion

Chemical synthesis and characterization of nanoparticles

In this study, two different metals nanoparticles including silver nanoparticles (Ag-NPs) and zinc oxide nanoparticles (ZnO-NPs) were chemically synthesized and analyzed by multiple techniques in order to insure the completion of the nano formation and detection of their characteristics.

Visual inspection

The synthesis of Ag-NPs was detected by visual observation for the conversion of the colorless solution of $AgNO_3$ to a pale yellow color by the reduction with sodium citrate [9]. While ZnO-NPs, was indicated by the formation of a white precipitate **(Figure 1)** [20].

UV-Vis spectroscopy: The UV–Vis absorption spectrum of the synthesized Ag-NPs is shown in **Figure 2** where, a well defined surface plasmon band centered at around 420 nm, characteristic to Ag-NPs was obtained. For ZnO-NPs, Chieng and Loo [21] reported that the broad absorption peak observed in spectrum at 360-380 nm is a characteristic band for the pure ZnO nanoparticles. No other peak was observed in the spectrum which confirms that the synthesized products are ZnO only **Figure 3.**

Energy dispersive x-ray analysis (EDX): The EDX profiles obtained in the present study confirmed the presence of Ag-NPs and ZnO-NPs with strong signal energy peaks for Ag and Zn atoms in the standard range as shown in **Figures 4 and 5.**

Transmission electron microscopy (TEM): The morphology and the average size of the synthesized nanoparticles were analyzed by Transmission Electron Microscopy (TEM). The particles of Ag-NPs synthesized by the chemical reduction method and ZnO-NPs synthesized by precipitation method are well separated single spherical particles without aggregation. The presence of the particles without aggregation clearly insured the efficiency of the techniques used in the synthesizing processes. The average size for the particles was calculated and found to be 12.65 ± 0.55 and 7.6 ± 0.5 nm for Ag-NPs and ZnO-NPs, respectively (**Figure 6).**

Antibiotic resistance pattern and MAR index of the isolated bacteria.

In the present study a total of seventeen isolate; *E. coli* (n=5) and *K. pneumoniae* (n=12) isolates were recovered from clinical specimens. The susceptibility of the tested isolates was performed against 10 different antibiotics using disc diffusion method. The incidence of resistance to the selected antibiotics was presented in **Table 2.** It was found that all the isolates were resistant to Ampicillin, Amoxicillin/ Clavulanic acid and Cefotaxime**.** Moreover, 15 isolates were found to be resistant

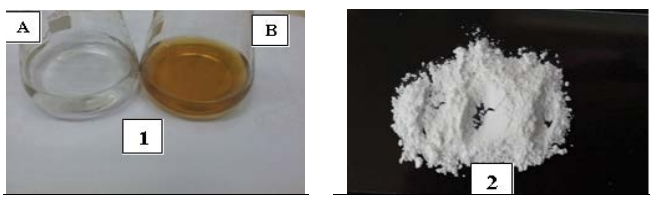

Figure 1 Ag-NPs synthesized by the chemical reduction of silver nitrate salt **(1)** where; (A) silver nitrate solution, (B) colloidal Ag-NPs formed. ZnO-NPs synthesized by the precipitation method as solid dry precipitate **(2)**.

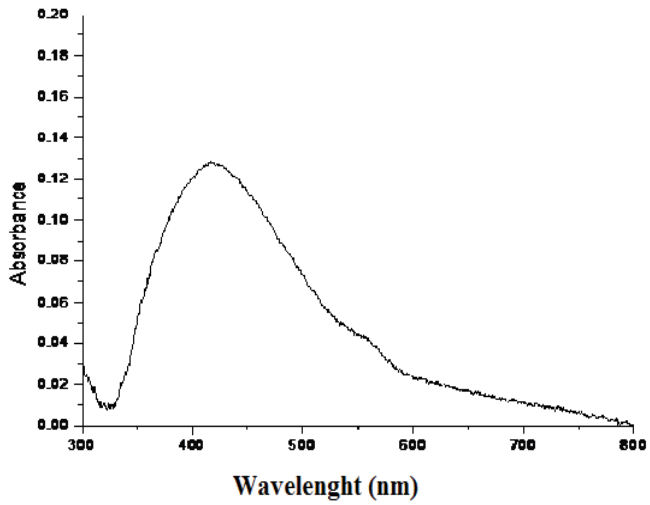

Figure 2 Ultraviolet visible (UV-Vis) spectrum of the synthesized Ag-NPs.

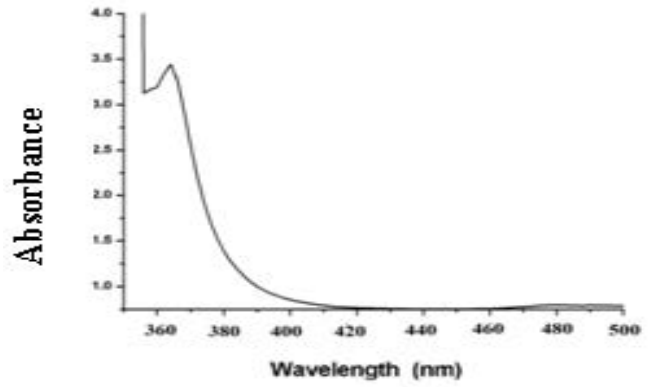

Figure 3 Ultraviolet visible (UV-Vis) spectrums of the synthesized ZnO-NPs.

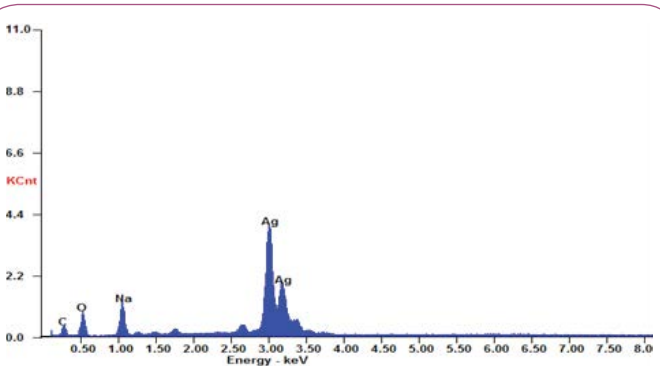

Figure 4 Energy dispersive X-ray (EDX) analysis of the synthesized Ag-NPs.

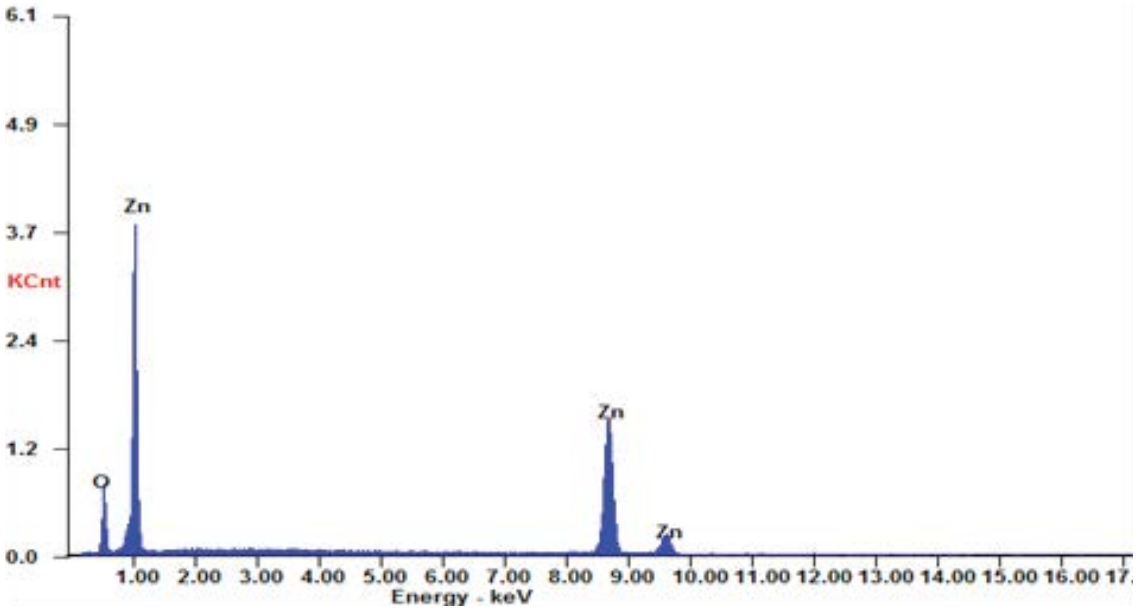

Figure 5 Energy dispersive X-ray (EDX) analysis of the synthesized ZnO-NPs.

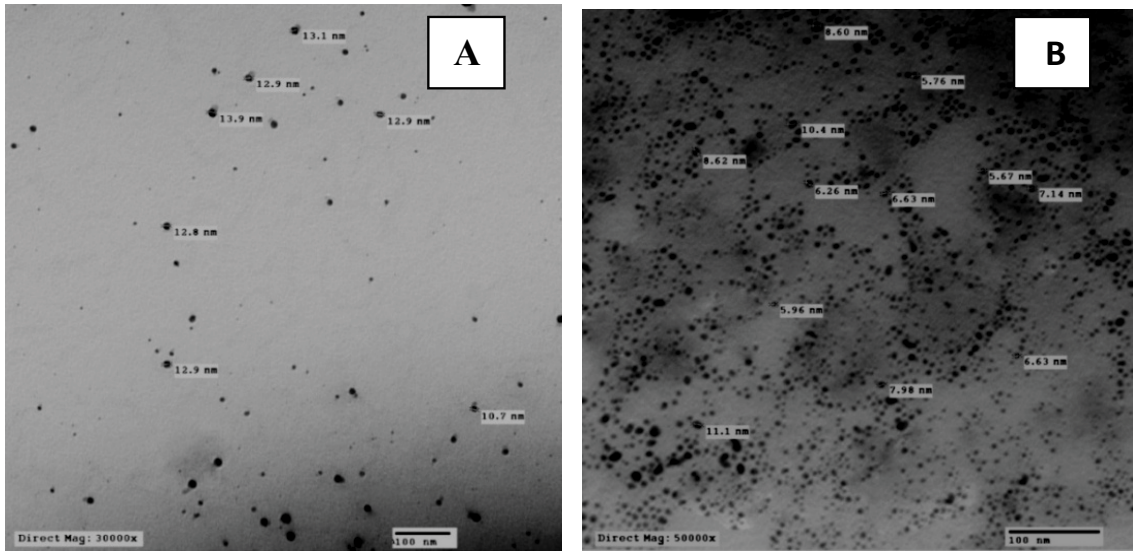

Figure 6 Transmission electron micrographs showing spherical well separated and dispersed synthesized Ag-NP **(A)** with average size 12.65 ± 0.55 and ZnO-NPs **(B)** with average size 7.6 ± 0.5 nm.

Table 2 The incidence of resistance of different antibiotics among different isolates.

Antibiotic	No. of resistant isolates (%)
Ampicillin	17 (100)
Amoxicillin/Clavulanic acid	17 (100)
Cefotaxime	17 (100)
Aztreonam	11 (64.7)
Imipenem	0 (0)
Gentamicin	0 (0)
Levofloxacin	4 (23.5)
Trimethoprim/sulfamethoxazole	15 (88.2)
Chloramphenicol	10 (58.8)
Tetracycline	9 (52.9)

to Trimethoprim/Sulfamethoxazole followed by Aztreonam (11 isolates) and Chloramphenicol (10 isolates) the lowest resistance among the isolates was obtained against Levofloxacin antibiotic where only 4 isolates were found to be resistant. None of the isolates showed any resistance to Imipenem or Gentamycin. From the obtained data, it was concluded that all the isolates were highly MDR isolates. Abbas et al. [22] investigated the bacterial infections in patients with burn wounds in a burn unit in Hehia General Hospital in Egypt. Total of 160 isolates were recovered; where; *S. aureus* was the most common pathogen besides *P. aeruginosa*, *K. pneumoniae* and *S. epidermidis E. coli*, *Enterobacter cloacae* and *Citrobacter freundii* were also present. Most of the isolates were highly MDR as 100% of the *S. aureus*

isolates were found to be resistant to Ampicillin and Cefazolin while, *E. coli* and *Klebsiella* sp. were found to be highly resistant to Tobramycin, Cefoperazone, Amoxicillin-Clavulanic acid, Gentamycin, Cefepime and Amoxicillin (100% of the isolates). Moreover, all the *Pseudomonas* isolates were found to be resistant to Amoxicillin - Clavulanic acid, Amoxicillin, Tetracycline and Cefepime.

According to the data in **Table 3** it was found that, the tested isolates were found to be heterogeneous in their antibiotic resistance patterns where; the twelve tested isolates had resulted in ten different patterns. The most represented patterns were (I B and II B), each represented by three isolates, while patterns (IA, II A and II E) were represented by two isolates and the other patterns were represented by one isolate. The resistant patterns and the MAR index of the isolates were conducted and the data were recorded in **Table 3.**

The antibacterial efficacy of Ag-NPs and ZnO-NPs against *E. coli* and *K. pneumoniae* isolates.

The antibacterial efficacy of both tested nanoaprticles was evaluated against two *E. coli* and four *K. pneumoniae* isolates. According to the obtained results, it was found that all the isolates were sensitive to Ag-NPs with zone of inhibition with diameter ranging between 14 and 15 mm except two *K. pneumoniae* isolates (K2 and K4) that were resistant. On the other hand, the sensitivity of the isolates to ZnO-NPs were found to be less than that to silver one; where only two isolates (E2 and K1) were sensitive to ZnO-NPs while all the other isolates did not show any inhibition zone with ZnO-NPs treatment as shown in **Table 4.** Generally, there was no difference between the diameter of inhibition zones obtained by 50 and 100 µl of either Ag-NPs or ZnO-NPs **Figure 7.**

The efficacy of tested nanoparticles on enhancing the activity of different antibiotics

In this experiment, the antibiotics to which the isolates were found to be resistant were selected from the resistant pattern

Table 3 Resistance patterns and MAR index of tested isolates.

Pattern Code		Antimicrobial resistance pattern	MAR index	No. of isolates exhibit this pattern
I	A	Am, AMC, CTX, C, TE	0.5	2
	B	Am, AMC, CTX, ATM, SXT		3
II	A	Am, AMC, CTX, SXT, C, TE	0.6	2
	B	Am, AMC, CTX, ATM, SXT, C		3
	C	Am, AMC, CTX, LEV, SXT, C		1
	D	Am, AMC, CTX, ATM, LEV, SXT		1
	E	Am, AMC, CTX, ATM, SXT, TE		2
III	A	Am, AMC, CTX, LEV, SXT, C, TE	0.7	1
	B	Am, AMC, CTX, ATM, LEV, SXT, TE		1
	C	Am, AMC, CTX, ATM, SXT, C, TE		1

*Am: Ampicillin; AMC: Amoxicillin/clavulanic acid; CTX: Cefotaxime; ATM: Aztreonam; IPM: Imipenem; GEN: Gentamicin; LEV: Levofloxacin; SXT: Trimethoprim/sulfamethoxazole; C: Chloramphinicol; TE: Tetracycline.

Table 4 Antibacterial activity of Ag-NPs and ZnO-NPs against the selected *E. coli* and *K. pneumoniae* isolates.

Isolates Code	Mean of inhibition zone diameter (mm) ± SD			
	Ag-NPs		ZnO-NPs	
	50 µl	100 µl	50 µl	100 µl
E1	15 ± 0.12	15 ± 0.21	0 ± 0.00	0 ± 0.00
E2	14 ± 0.12	14 ± 0.26	12 ± 0.17	12 ± 0.35
K1	15 ± 0.20	15 ± 0.25	13 ± 0.15	13 ± 0.29
K2	0 ± 0.00	0 ±0.00	0 ± 0.00	0 ± 0.00
K3	15 ± 0.15	15 ± 0.20	0 ± 0.00	0 ± 0.00
K4	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00

*E: *E. coli*; K: *K. pneumonia*; SD: Standard deviation. Each result were in triplicate.

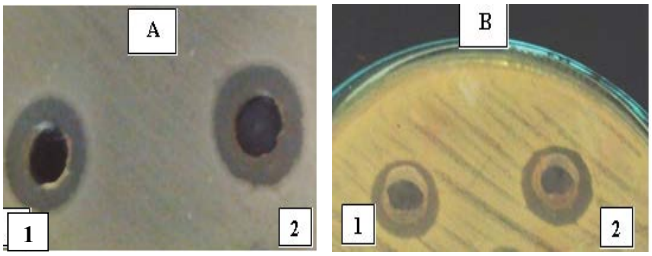

Figure 7 The antibacterial activity of Ag-NPs **(A)** and ZnO-NPs **(B)** against certain *E. coli* isolate; where, **(1)** and **(2)** are representatives to the inhibition zones obtained at 50 and 100 µl of corresponding nanoparticles, respectively.

of each isolate and the antibacterial activities of these antibiotics alone were compared with those with their combinations with Ag-NPs or ZnO-NPs using disc diffusion technique. The effect of these nanoparticles was determined as fold increasing in the inhibition zone diameter and the data was represented in **Table 5.**

According to the obtained results, it was clearly observed that Ag-NPs not only have high antibacterial activity but also they show ability to enhance the activity of other antibiotics illustrated as synergistic effect by increasing the inhibition zone diameter around the antibiotic discs supplemented with Ag-NPs. Moreover, the antibacterial activities of the tested antibiotics discs combined with Ag-NPs were higher than that of the same antibiotics with ZnO-NPs as most of antibiotics remained non-effective even after ZnO-NPs combination (**Figures 8 and 9**).

The highest fold increase in the inhibition zone by Ag-NPs combination (8.878) was observed with Levofloxacin against isolate E2 followed by that of Chloramphenicol (8.000) against isolate K1. In addition, the highest fold increase with ZnO-NPs combination was (1.939) with Amoxicillin/Clavulanic acid and Trimethoprim/Sulfamethoxazole against isolate E2.

The obtained inhibition zone diameters were interpreted to sensitive, intermediately resistant or resistant according to EUCAST Clinical Breakpoint [16]. The change in the susceptibility of each isolate to any of the tested antibiotics was determined in the presence of either Ag-NPs or ZnO-NPs as shown in **Table 5**.

For the ease of comparison, **Table 6** was constructed to summarize the efficacy of tested combinations against the tested isolates.

Figure 8 The enhanced efficacy of Ag-NPs on the antibacterial activity of Aztreonam (A) and Amoxicillin/clavulanic acid (B) on certain *E. coli* isolate; where; the effect of antibiotic disc alone is on the left and the effect of antibiotic disc supplemented with Ag-NPs is on the right.

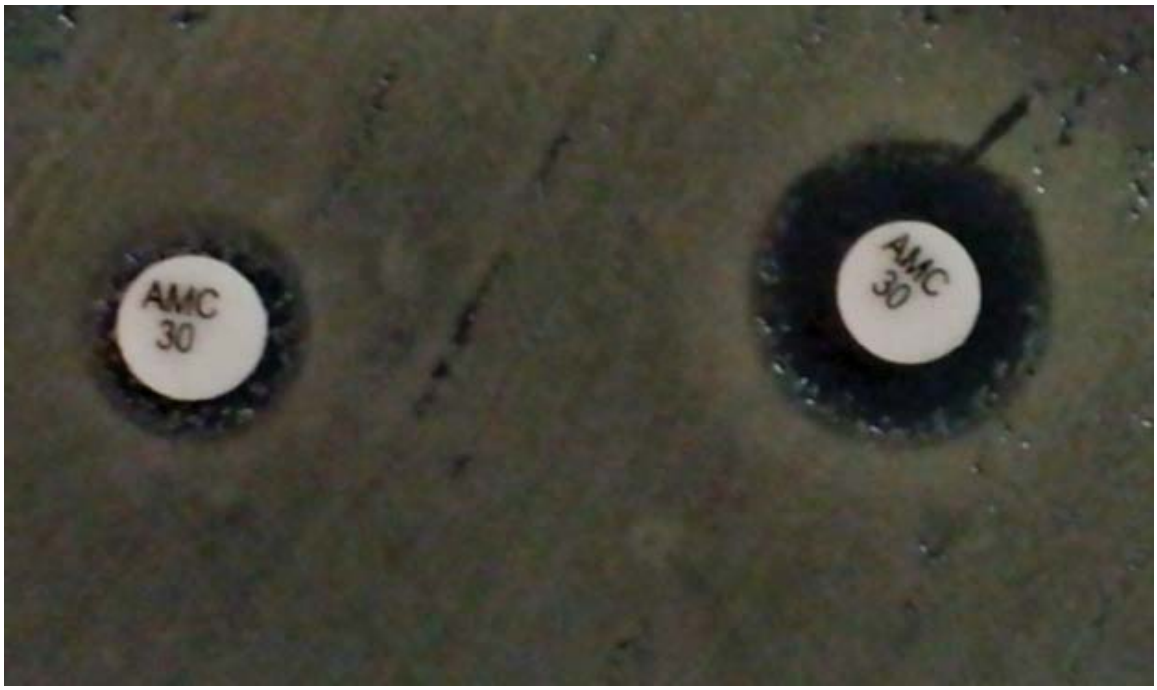

Figure 9 The enhanced efficacy of ZnO-NPs on the antibacterial activity of Amoxicillin/clavulanic on one of *K. pneumoniae* isolates where; the effect of antibiotic disc alone on the left and the effect of the antibiotic disc supplemented with ZnO-NPs on the right.

According to the obtained it was clearly demonstrated that Ag-NPs are able to interestingly enhance the efficiency of some antibiotics. Among six isolates tested with Ampicillin, four isolates had been changed to intermediately resistant, one isolate had been changed to sensitive while only one isolate still resistant. In addition, for Amoxicillin/Clavulanic acid; two isolates had been changed to sensitive, two isolates two intermediately resistant and two isolates still resistant. Moreover, the Tetracycline and Trimethoprim/Sulfamethoxazole combination with Ag-NPs had resulted in increasing the susceptibility of all tested isolates to either sensitive or intermediately resistant. However, the combination between Ag-NPs and other antibiotics such as Cefotaxime resulted in increasing in inhibition zone diameter but it still in the resistant range indicating that the effect of Ag-NPs could be varied according to antibiotic class and mode of action.

These finding agreed with that obtained by Deng et al. [23] who studied the mechanism of synergistic activity between Ag-NPs and antibiotics (Ampicillin, Penicillin, Enoxacin, Kanamycin and Tetracycline) on MDR *Salmonella typhimurium* DT 104. They reported that Enoxacin, kanamycin, Neomycin and Tetracycline showed synergistic growth inhibition and antibiotic - nano complexes formation was also detected. While, Ampicillin and Penicillin neither form complexes nor showed synergism with

Table 5 Antimicrobial activity of antibiotics alone and in combination with Ag-NPs or ZnO-NPs against selected resistant *E. coli* and *K. pneumoniae* isolates.

Isolate code	Tested antibiotic	Treatment Type	Mean of inhibition zone diameter (mm) ± SD	Fold increase in inhibition zone diameter	Combination's susceptibility
K1	Am	Am only	0 ± 0.00	-	-
		Am + Ag	15 ± 0.58	3.592	**I**
		Am+ ZnO	0 ± 0.00	0.000	R
	AMC	AMC only	0 ± 0.00	-	-
		AMC + Ag	15 ± 0.50	3.592	**I**
		AMC + ZnO	13 ± 0.50	2.449	R
	CTX	CTX only	9 ± 0.29	-	-
		CTX + Ag	13 ± 0.50	1.086	R
		CTX+ ZnO	12 ± 0.00	0.778	R
	C	C only	0 ± 0.00	-	-
		C + Ag	21 ± 1.00	8.000	**S**
		C + ZnO	0 ± 0.00	0.000	R
	TE	TE only	0 ± 0.00	-	-
		TE + Ag	20 ± 0.58	7.163	**S**
		TE + ZnO	0 ± 0.00	0.000	R
K2	Am	Am only	0 ± 0.00	-	-
		Am + Ag	18 ± 0.58	5.612	**S**
		Am+ ZnO	0 ± 0.00	0.000	R
	AMC	AMC only	0 ± 0.00	-	-
		AMC + Ag	17 ± 1.00	4.898	**I**
		AMC + ZnO	0 ± 0.00	0.000	R
	CTX	CTX only	0 ± 0.00	-	-
		CTX + Ag	20 ± 0.89	7.163	R
		CTX+ ZnO	0 ± 0.00	0.000	R
	ATM	ATM only	0 ± 0.00	-	-
		ATM + Ag	17 ± 0.50	4.898	R
		ATM + ZnO	0 ± 0.00	0.000	R
	SXT	SXT only	0 ± 0.00	-	-
		SXT + Ag	17 ± 0.00	4.898	**S**
		SXT + ZnO	0 ± 0.00	0.000	R

Isolate code	Tested antibiotic	Treatment Type	Mean of inhibition zone diameter (mm) ± SD	Fold increase in inhibition zone diameter	Combination's susceptibility
E1	Am	Am only	0 ± 0.00	-	-
		Am + Ag	15 ± 0.50	3.592	**I**
		Am+ ZnO	0 ± 0.00	0.000	R
	AMC	AMC only	0 ± 0.00	-	-
		AMC + Ag	18 ± 0.58	5.612	**S**
		AMC + ZnO	11 ± 0.50	1.469	R
	CTX	CTX only	0 ± 0.00	-	-
		CTX + Ag	0 ± 0.00	0.000	R
		CTX+ ZnO	0 ± 0.00	0.000	R
	ATM	ATM only	0 ± 0.00	-	-
		ATM + Ag	15 ± 0.00	3.592	R
		ATM + ZnO	10 ± 0.58	1.041	R
	SXT	SXT only	0 ± 0.00	-	-
		SXT + Ag	17 ± 0.58	4.898	**S**
		SXT + ZnO	0 ± 0.00	0.000	R
	TE	TE only	11 ± 0.00	-	-
		TE + Ag	22 ± 1.15	3.000	**S**
		TE + ZnO	11 ± 0.00	0.000	R
K3	Am	Am only	0 ± 0.00	-	-
		Am + Ag	14 ± 0.00	3.000	**I**
		Am+ ZnO	0 ± 0.00	0.000	R
	AMC	AMC only	0 ± 0.00	-	-
		AMC + Ag	12 ± 0.76	1.939	R
		AMC + ZnO	0 ± 0.00	0.000	R
	CTX	CTX only	0 ± 0.00	-	-
		CTX + Ag	0 ± 0.00	0.000	R
		CTX+ ZnO	0 ± 0.00	0.000	R
	ATM	ATM only	9 ± 0.00	-	-
		ATM + Ag	11 ± 0.50	0.494	R
		ATM + ZnO	10 ± 0.00	0.235	R
	SXT	SXT only	0 ± 0.00	-	-
		SXT + Ag	15 ± 0.50	3.592	**I**
		SXT + ZnO	0 ± 0.00	0.000	R
	C	C only	0 ± 0.00	-	-
		C + Ag	14 ± 0.29	3.000	**I**
		C + ZnO	0 ± 0.00	0.000	R

Isolate code	Tested antibiotic	Treatment Type	Mean of inhibition zone diameter (mm) ± SD	Fold increase in inhibition zone diameter	Combination's susceptibility
K4	Am	Am only	0 ± 0.00	-	-
		Am + Ag	10 ± 0.58	1.041	R
		Am+ ZnO	0 ± 0.00	0.000	R
	AMC	AMC only	0 ± 0.00	-	-
		AMC + Ag	0 ± 0.00	0.000	R
		AMC + ZnO	0 ± 0.00	0.000	R
	CTX	CTX only	0 ± 0.00	-	-
		CTX + Ag	14 ± 0.58	3.000	R
		CTX+ ZnO	0 ± 0.00	0.000	R
	LEV	LEV only	10 ± 0.00	-	-
		LEV + Ag	19 ± 0.50	2.610	**S**
		LEV + ZnO	11 ± 0.29	0.210	R
	SXT	SXT only	0 ± 0.00	-	-
		SXT+ Ag	14 ± 0.00	3.000	**I**
		SXT+ ZnO	0 ± 0.00	0.000	R
	C	C only	0 ± 0.00	-	-
		C + Ag	12 ± 0.58	1.939	R
		C + ZnO	0 ± 0.00	0.000	R
	TE	TE only	0 ± 0.00	-	-
		TE + Ag	13 ± 0.29	2.449	**I**
		TE+ Zn	0 ± 0.00	0.000	R
E2	Am	Am only	0 ± 0.00	-	-
		Am + Ag	14 ± 0.29	3.000	**I**
		Am+ ZnO	0 ± 0.00	0.000	R
	AMC	AMC only	10 ± 0.00	-	-
		AMC + Ag	19 ± 1.15	1.984	**S**
		AMC + ZnO	16 ± 1.00	1.116	**I**
	CTX	CTX only	0 ± 0.00	-	-
		CTX + Ag	13 ± 0.00	2.448	R
		CTX+ ZnO	0 ± 0.00	0.000	R
	ATM	ATM only	9 ± 0.00	-	-
		ATM + Ag	23 ± 0.58	5.530	**S**
		ATM + ZnO	9 ± 0.00	0.000	R
	LEV	LEV only	0 ± 0.00	-	-
		LEV + Ag	22 ± 0.29	8.878	**S**
		LEV + ZnO	10 ± 0.29	1.041	R
	SXT	SXT only	0 ± 0.00	-	-
		SXT+ Ag	16 ± 1.00	4.224	**S**
		SXT+ ZnO	12 ± 0.00	1.939	**I**
	TE	TE only	0 ± 0.00	-	-
		TE + Ag	12 ± 0.29	1.939	**I**
		TE + ZnO	0 ± 0.00	0.000	R

*E: *E. coli*; K: *K. pneumonia*; *Am: Ampicillin; AMC: Amoxicillin/clavulanic acid; CTX: Cefotaxime; ATM: Aztreonam; LEV: levofloxacin; SXT: Trimethoprim/sulfamethoxazole; C: Chloramphenicol; TE: tetracycline; *R: resistant; I: intermediately resistant and S: sensitive; *SD: Standard deviation.

Table 6 Summary of the efficacy of different antibiotics-nanoparticles combinations on the susceptibility of tested isolates.

Antibiotic	No. of tested isolates	Antibiotic-Nano combination	No. (%) of isolates changed to intermediately sensitive	No. (%) of isolates changed to sensitive
Am	6	Amp – Ag-NPs	4 (66.7)	1 (16.6)
		Amp – ZnO-NPs	0 (0)	0 (0)
AMC	6	AMC – Ag-NPs	2 (33.3)	2 (33.3)
		AMC – ZnO-NPs	0 (0)	0 (0)
CTX	6	CTX – Ag-NPs	0 (0)	0 (0)
		CTX – ZnO-NPs	0 (0)	0 (0)
C	3	C – Ag-NPs	1 (33.3)	1 (33.3)
		C – ZnO-NPs	0 (0)	0 (0)
TE	4	TE – Ag-NPs	2 (50)	2 (50)
		TE – ZnO-NPs	0 (0)	0 (0)
ATM	4	ATM – Ag-NPs	0 (0)	1 (25)
		ATM – ZnO-NPs	0 (0)	0 (0)
SXT	5	SXT – Ag-NPs	2 (40)	3 (60)
		SXT – ZnO-NPs	0 (0)	0 (0)
LEV	2	LEV – Ag-NPs	0 (0)	2 (100)
		LEV – ZnO-NPs	0 (0)	0 (0)

*Am: Ampicillin; AMC: Amoxicillin/clavulanic acid; CTX: Cefotaxime; C: Chloramphenicol; TE: Tetracycline; ATM: Aztreonam; SXT: Trimethoprim/sulfamethoxazole; LEV: levofloxacin.

Ag-NPs. They explained that the suggested mechanism of this synergism with some antibiotics is that firstly, antibiotic–AgNPs complex are formed then they interacts more strongly with the bacterial cells and causes more Ag^+ release, thus creating a temporal high concentration of Ag^+ near the bacteria cell wall that leads to growth inhibition of the bacteria.

Our findings about the enhancing activity of silver agreed with that of Sindhu et al. [20] who compared the activity of Gentamycin and Chloramphenicol antibiotics alone with that with Ag-NPs. They reported that the antibacterial activity of both antibiotics increased in the presence of Ag-NPs against the tested strains. Also, Kora and Rastogi [24] studied the effect of chemically synthesized Ag-NPs capped with three different capping agents; citrate, sodium dodecyl sulfate (SDS) and poly vinyl pyrrolidone (PVP) on the antibacterial activity of Streptomycin, Ampicillin, and Tetracycline against *E. coli* and *S. aureus*. They reported that the highest percentage of enhancement in activity against *E. coli* was obtained in combination of Ampicillin with PVP and SDS capped nanoparticles; while, Streptomycin combination with PVP capped nanoparticles showed the highest activity against *S. aureus.*

Furthermore, the observed lower activities of ZnO-NPs in our study agree with the findings of Meruvu et al. [25] who investigated the antibacterial activity of ZnO-NPs combinations with Gentamycin, Nitrofurantoin and Ciprofloxacin against *E. coli* where, the results showed that the activity of nanoparticles alone was lower than that of antibiotics-alone than that of antibiotics-ZnO-NPs combination.

Conclusion

The increasing antimicrobial resistance among bacterial strains is a great threat that needs rapid and serious actions to be taken. Due to their unique antibacterial activity; silver nanoparticles could be evaluated as alternatives to the traditional antibiotics or used to enhance the efficacy of certain antibiotics.

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