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Research Article

THE ANTIMICROBIAL EFFECTS OF SILVER NANOPARTICLES ON THE MULTIDRUG-RESISTANT *KLEBSIELLA* CLINICAL ISOLATES

Alaa El-Dien M.S. Hosny [1], Marwa M. Azab [2], Rasha M. EL-Morsi [2]*

[1]Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, Egypt

[2]Department of Microbiology and Immunology, Faculty of Pharmacy, Suez Canal University, Egypt

*Corresponding Author Email: rashamedhat07@gmail.com

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ABSTRACT

The aim of the present work is to study the properties of Silver nanoparticles prepared by chemical reduction method and their antimicrobial effects on the multidrug-resistant (MDR) *Klebsiella* clinical isolates. Silver nitrate was taken as the metal precursor and sodium citrate as a reducing agent. The Ultraviolet–visible (UV-Vis) absorption spectroscopy used for monitored the formation of the silver nanoparticles. The average size and morphology of silver nanoparticles were determined by transmission electron microscopy (TEM) and the diameter of particle was in the range of 5 to 45 nm and spherical in shape. The combined effect of these nanoparticles with Tetracycline, Doxycycline and Ampicillin was investigated and showed enhanced activity against MDR Klebsiella isolates. The maximum synergistic effect was observed for Ampicillin and Tetracycline with AgNPs.

Keywords: Silver nanoparticles, (MDR) *Klebsiella* clinical isolates, chemical reduction, inhibition zone, transmission electron microscopy.

INTRODUCTION

The most important results of nanoscience are the nanotechnology. Recently, nanotechnology is predicted to be one of the key technologies of the 21st century[1]. The prefix 'nano' indicates one billionth or 10^{-9} units. It is widely agreed that nanoparticles are clusters of atoms in the size range of 1–100 nm[2].

Frequently, nanometer-size metallic particles show unique and considerably changed chemical, physical and biological properties compared to their macro scaled counterparts, due to their small size and high surface-to-volume ratio. Thus, these nanoparticles have been the subject of substantial research in recent years[3-6].

Silver nanoparticles (AgNPs) have bactericidal activity against a wide variety of drug-resistant bacteria and they have particular characteristics provided by the silver itself. This metal tends to induce low bacterial resistance. As anti-bacterial agents, AgNPs were applied in a wide range of applications from home appliances and disinfecting medical devices to water treatment[7, 8].

Silver nanoparticles (SNPs) are non-toxic to humans and most effective against bacteria, virus and other eukaryotic micro-organism at low concentrations without any side effects[9]. In small concentrations, silver is safe for human cells, but lethal for microorganisms[4].

Chemical reduction is the most frequently applied method for the preparation of AgNPs as stable, colloidal dispersions in water or organic solvents were prepared. Commonly used reductants are borohydride, citrate, ascorbate and elemental hydrogen. The reduction of silver ions (Ag^+) in aqueous solution generally yields colloidal silver with particle diameters of several nanometers. Initially, the reduction of various complexes with (Ag^+) ions leads to the formation of silver atoms (Ag0), which is followed by agglomeration into oligomeric clusters. These clusters eventually lead to the formation of colloidal Ag particles[10-14].

Characterization of silver nanoparticles is performed using a variety of different techniques such as transmission and scanning electron microscopy (TEM, SEM), atomic force microscopy (AFM), dynamic light scattering (DLS), X-ray photoelectron spectroscopy (XPS), powder X-ray diffractometric (XRD), Fourier transform infrared spectroscopy (FTIR), and UV–Vis spectroscopy. These techniques are used for determination of different parameters such as particle size, shape, crystallinity, fractal dimensions, pore size and surface area. Moreover, orientation, intercalation and dispersion of nanoparticles in nanocomposite materials could be determined by these techniques for instance, the morphology and particle size could be determined by TEM, SEM and AFM. UV–Vis spectroscopy is used to confirm sample formation by showing the Plasmon resonance[15-21].

MATERIALS AND METHODS

Isolation and identification of clinical isolates

A total of 15 *Klebsiella* clinical isolates were obtained from Mansoura University Hospital during period extending from August 2012 to August 2015. Clinical specimens were examined and identified by standard microbiological procedures and biochemical reactions[22,23].

Reference Strains

Escherichia coli strain ATCC® 25922™ (American Type culture collection, USA) was given by NAMRU -3 in Cairo to the Microbiology Diagnostic and Infection Control Unit (MDICU) in the department of Medical Microbiology and Immunology, Faculty of Medicine, Mansoura University.

Antimicrobial susceptibility testing

Susceptibilities of the tested isolates were tested against 16 antimicrobials of different classes using the Kirby–Bauer method as recommended on Mueller–Hinton agar with commercially available antimicrobial disks (Oxoid, UK), according to Clinical Laboratory Standards Institute[24,25].

Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations of all isolates were determined by broth micro dilution method against 6 antimicrobial agents. Pure forms of 6 antibiotics were used (Manufacturers: E.I.P.I.CO, CID and Pharco B International, Egypt). The scheme for preparing dilutions of antimicrobial agents and the methodology was according to CLSI guidelines[25].

Preparation of silver nanoparticles

The silver colloid was prepared by using chemical reduction method. All solutions of reacting materials were prepared in distilled water. In this experiment 50 ml of 0.001 M Silver nitrate ($AgNO_3$) was heated to boiling temperature then, 5 ml of 1 % trisodium citrate was added drop by drop and the solution was mixed vigorously. Solution was heated until the appearance of pale yellow color. After that, the heating element was turned off and the resulting solution stirred until cooled to room temperature[12, 26.] All chemicals and reagents used were of analytical grade purity.

Characterization techniques of silver Nanoparticles (AgNPs)

UV/VIS spectroscopy was used to prove the existence of nanoparticles. The confirmation of nanoparticle formation was done by UV/Visible spectrophotometer double – beam spectrophotometer (Labomed spectro UV-VIS 2700, USA) operated at a resolution of 2 nm. The UV/VIS absorption spectra of the Silver nanoparticles were screened in a range of 300 to 600 nm.

Morphological analysis of silver nanoparticles was done using TEM (Transmission Electron Microscopy)

TEM is used to characterize the morphology and size of the silver nanoparticles. TEM analysis of the AgNPs was performed using a JEM 2100 instrument operated at an accelerating voltage at 200 kV (JEOL, Japan). The preparation of samples was done by drop coating on formavar- coated copper TEM grids. The diameter of nanoparticles was measured from the TEM images and the size distribution of the AgNPs was calculated.

Antibacterial assay of SNPs against MDR *Klebsiella* isolates

The antimicrobial susceptibility of silver nanoparticles was evaluated using the disc diffusion. The sterile discs were dipped in the prepared silver nanoparticles solution and placed in the nutrient agar plate and kept for incubation at 37°C for 24 hours. Zones of inhibition for control, SNPs were measured. The experiments were repeated thrice and mean values of zone diameter were presented[27].

Disc diffusion assay to evaluate combined effects

The tests were performed on Müller-Hinton agar by using the disc diffusion (DD) method. In this experiment we used the following discs:

First, Ampicillin (AMP, 10ug), Tetracycline (TE, 30ug) and Doxycycline discs (D, 30ug). Second paper disc (5mm) saturated with 10 µL of AgNPs. And third, Ampicillin (AMP, 10ug), Tetracycline (TE, 30ug) and Doxycycline (D, 30ug) discs saturated with 10 µL of AgNPs.

Saline solution was used to suspend MDR *Klebsiella* isolates. After that they plated in the culture medium, and then the discs were placed. The plates were incubated at 35 °C for 24 hours, then the inhibition zones were measured. The synergism was evaluated by the formula $\{(B2-A2)/A2\} \times 100$, where, A = the inhibition zone diameter of the antibiotic alone (Ampicillin, Tetracycline or Doxycycline alone) and B = the inhibition zone diameter of the antibiotics + AgNPs. This formula allowed us to evaluate the increment of the inhibition zone around the bacteria caused by the antibiotic in association with AgNPs[28- 30].

RESULTS

In this study, the susceptibility of the 15 tested *Klebsiella* isolates to 16 different antimicrobial agents was performed. Out of 15 tested isolates, 13 isolates were extensive drug resistance (XDR) and 2 isolates were multidrug resistance MDR. Table 1 and 2 represents the incidence of resistance and MIC of *Klebsiella isolates* to different antimicrobial agents.

Silver nanoparticles were synthesized by chemical reduction method[12,26], the colorless solution turned pale yellow indicating the formation of silver nanoparticles. Figure 1 shows aqueous extract of silver nitrate solution 1.10^{-3} (colorless) and a solution of silver nanoparticles (pale yellow).

The color change of the $AgNO_3$ solution from colorless to pale yellow was observed by naked eye and UV/Visible spectroscopy was used to prove the existence of nanoparticles and it is one of the most widely used techniques for structural characterization of silver nanoparticles. The absorption spectrum in Figure 2 of the pale yellow silver colloids showed a surface Plasmon absorption band with a maximum of 433 nm, indicating the presence of spherical or roughly spherical Ag nanoparticles.

Morphological analysis of silver nanoparticles was done using TEM (Transmission Electron Microscopy)

TEM has been utilized to characterize the size and morphology of the formed silver nanoparticles. The particle size was in the range of 5 to 45 nm and spherical in shape as shown in Figure 3.

Evaluation of the antimicrobial activity

The combined effect of the formed nanoparticles with different antimicrobial agents was investigated against the tested Klebsiella isolates using the disc diffusion method (Table 3). The diameter of inhibition zones (in millimeters) around the tested antibiotic discs in the presence and in the absence of Ag-NPs are shown in Table 3. The antibacterial activities of Doxycycline, Ampicillin, and Tetracycline were increased after addition of Ag-NPs. The highest fold increases in area were observed for Ampicillin and Tetracycline and so, the maximum synergistic effect was observed for Ampicillin and Tetracycline with AgNPs.

DISCUSSION

Nanotechnology has a very important role in the life sciences especially Biotechnology. Nanoparticles have new or improved properties due to their specific characteristics such as distribution, size and morphology[31]. Chemical reduction method is widely used to synthesize Ag-NPs as it can generate Ag-NPs on a large scale under gentle conditions[32].

In this study, the synthesis of AgNPs was initially indicated by a color change from colorless to pale yellow color and the confirmation of nanoparticle formation was done by UV-VIS spectrophotometer (Figure 2).

UV-visible spectroscopy is one of the most widely used techniques for structural characterization of silver nanoparticles. In our study, the absorption spectrum (Figure 2) of the silver colloids synthesized by chemical reduction method according to the description of Lee and Meisel, 1982, showed a surface Plasmon absorption band with a maximum of 433 nm indicating the presence of spherical or roughly spherical Ag nanoparticles, and TEM imaging confirmed this result. As illustrated in UV/Visible spectra (Figure 2), a strong surface Plasmon resonance was centered at approximately 433 nm. Observation of this strong but broad surface Plasmon peak has been well documented for various Me-NPs, with sizes ranging all the way from 2 to 100 nm[33,34].

Figure 3 represents the transmission electron microscopy (TEM) image of the formed silver nanoparticles and indicates well dispersed spherical particles. The particle size distributions of the synthesized silver nanoparticles show that the particles range in size from 5 to 45 nm (Figure 4).

The infections with drug-resistant microorganisms result in spending more time in the hospital and require a form of treatment that uses two or three different antibiotics and is less effective, more toxic, and more expensive[35]. Nanotechnology offers opportunities to re-explore the biological properties of already known antimicrobial compounds by manipulating their size to alter the effect[36].

In this study, the combined effect of the silver nanoparticles with different antibiotics was investigated against MDR Klebsiella isolates by using the disc diffusion method (Figure 5). Inhibition Zones (mm) of the tested Klebsiella isolates against different antibiotics in presence and absence of AgNPs were illustrated in Table 3. The antibacterial activities of Doxycycline, Ampicillin and Tetracycline increased in the presence of Ag-NPs against MDR *Klebsiella pneumoniae* isolates. These findings are in agreement with the previously reported results of Naqvi *et al.*, Kumar et al., and Birla *et al.*, The highest fold increases in area were observed for Ampicillin and Tetracycline[37-39].

Recently, silver nanoparticles exhibiting antimicrobial activity have been synthesized[40]. Antibacterial activity of the silver-containing materials has numerous applications, such as reducing infections and preventing bacterial colonization on catheters, prostheses, vascular grafts, stainless steel materials, dental materials and human skin[41].

Table 1. Incidence of resistance of *Klebsiella* isolates to different anti-microbial agents

Anti-microbial drugs	No (%) of resistant isolates
Ampicillin	15 (93.8%)
Amoxicillin-Clavulante	15 (93.8%)
Cephradine	15 (93.8%)
Cefotaxime	15 (93.8%)
Ceftazidime	15 (93.8%)
Imipenem	15 (93.8%)
Gentamycin	15 (93.8%)
Amikacin	13 (81.3%)
Ciprofloxacin	15 (93.8%)
Norfloxacin	14 (87.5%)
Nitrofurantoin	13 (81.3%)
Colistin sulphate	0 (0%)
Co-trimoxazole	15 (93.8%)
Tetracyclin	14 (87.5%)
Erythromycin	15 (93.8%)
Chloramphenicol	13 (81.3%)

Table 2. The MIC (ug/ml) of the antibiotics against the selected MDR *Klebsiella* isolates

Isolate	IMP	CIP	CTX	C	CE	AK
K106	64	64	128	128	512	128
K T10	32	32	64	<8	512	256
K 29	32	256	256	128	512	128
K T5	>128	>128	256	16	512	<8
K 7B	16	16	128	32	512	64
K 24B	>128	>128	512	128	512	16
K 32B	32	32	512	256	512	512
K 44	>128	32	512	128	512	128
K 45B	32	32	128	64	512	128
K 4	4	4	512	256	512	512
K41	>128	>128	256	256	512	256
K R17	>128	>128	256	128	512	256
K42	>128	>128	256	64	512	256
K 100	32	32	256	512	512	128
K 41B	32	32	512	256	512	128

Table 3. Zone of inhibition (mm) of the tested *Klebsiella* isolates against different antibiotics in presence and absence of AgNPs

Isolate code	Zone of inhibition (mm)								
	AM	Ag NPs + AM	Increase in fold area	TE	Ag NPs + TE	Increase in fold area	D	Ag NPs + D	Increase in fold area
KT10	0	13	3.69	22	25	0.29	0	0	0
K44	0	15	5.25	0	18	8	8	11	0.9
K24B	0	16	6.1	10	16	1.56	0	0	0
K 4	0	16	6.1	0	16	6.1	0	11	2.4
K T5	0	18	8	0	17	7	0	0	0
K41	0	12	3	0	16	6.1	0	11	2.4
K 29	0	14	4.4	0	14	4.4	9	11	0.49
K 7B	0	12	3	0	10	1.8	13	14	0.16
K 32B	0	11	2.4	0	12	3	0	11	2.4
K42	0	14	4.4	0	15	5.25	10	11	0.21
K 41B	0	11	2.4	0	14	4.4	8	12	1.25
K 106	0	19	9	0	17	7	8	11	0.89
K 45B	0	14	4.4	0	14	4.4	19	22	0.34
K 100	0	10	1.7	0	11	2.4	11	13	0.4
K R17	0	11	2.4	0	14	4.4	11	14	0.62

AM: Ampicillin, **TE:** Tetracycline, **D:** Doxycycline and **Ag NPs:** Silver nanoparticles.

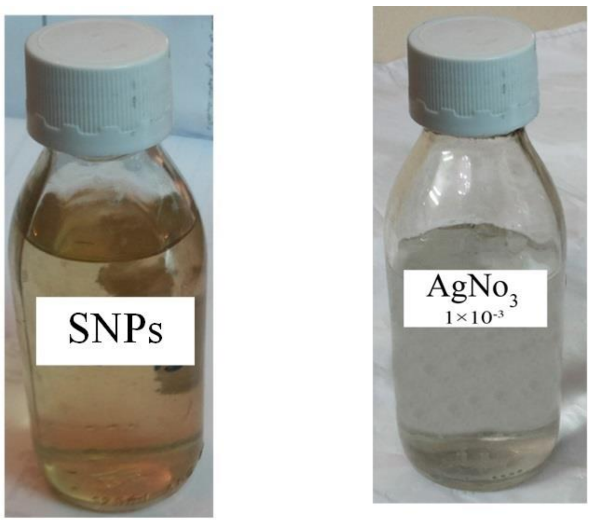

Figure 1. A) Solution of silver nanoparticles (SNPs) and B) Aqueous extract of silver nitrate solution (AgNo3) 1.10^{-3}.

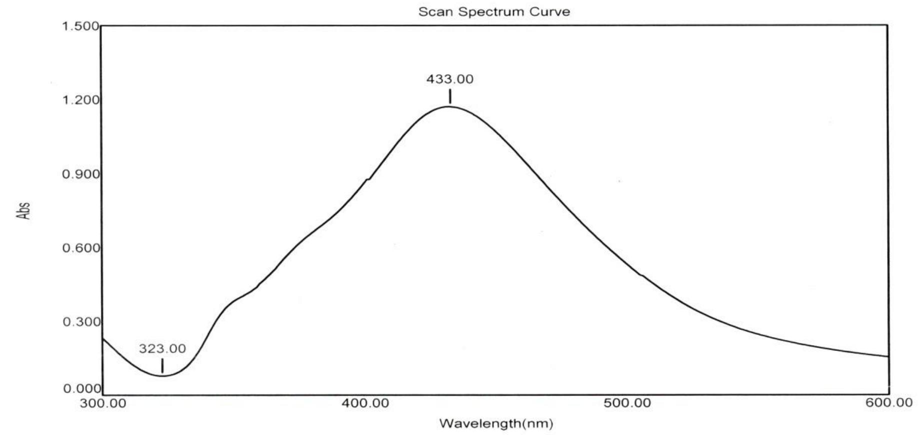

Figure 2. UV- visible absorption spectrum of silver nanoparticles synthesized using chemical reduction method

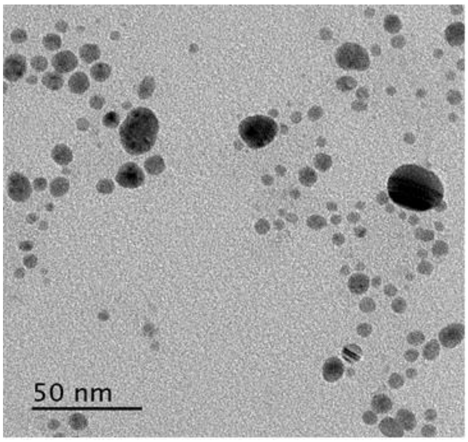

Figure 3. Transmission Electron Microscopy (TEM) image of silver nanoparticles prepared by chemical reduction method.

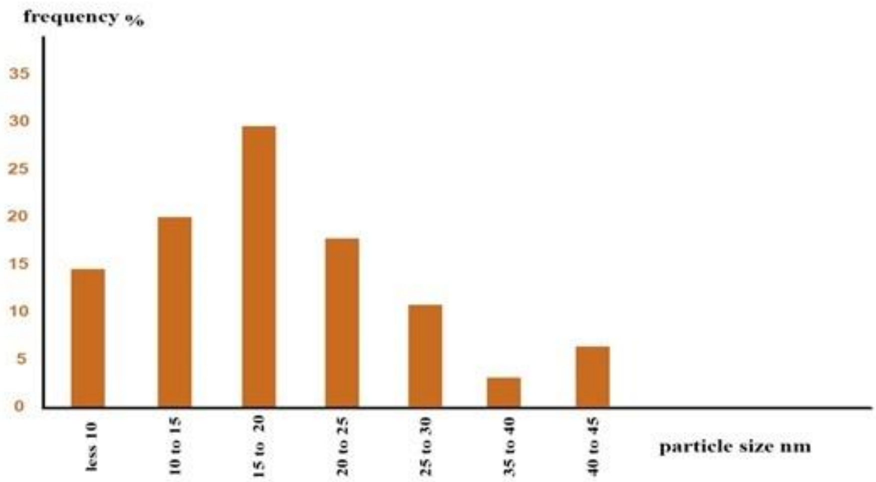

Figure 4. Particle size distribution of the synthesized silver nanoparticles

Figure 5. A) Antimicrobial activity assay of **S0**, **D** and a combination of **S0**+ **D** by disc diffusion method.
B) Antimicrobial activity assay of SNP, TE and a combination of SNP + TE.
Note: **S0 and SNP refer to :** Silver nanoparticle, **TE**, Tetracycline **and D,** Doxycycline**.**

CONCLUSION

Chemical reduction method is a simple and cheap method for preparation of silver nanoparticles. The combined effect of these nanoparticles with Tetracycline, Doxycycline and Ampicillin was investigated and showed enhanced activity against MDR *Klebsiella* isolates. The highest synergistic effect was observed between Ag-NPs with Ampicillin and Tetracycline.

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