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ORIGINAL RESEARCH

The Efficacy of AgNO3 Nanoparticles Alone and Conjugated with Imipenem for Combating Extensively Drug-Resistant *Pseudomonas aeruginosa*

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Introduction: The extensive drug-resistant (XDR) *Pseudomonas aeruginosa* (*P. aeruginosa*) causes a range of infections with high mortality rate, which inflicts additional costs on treatment. The use of nano-biotechnology-based methods in medicine has opened a new perspective against drug-resistant bacteria. The aim of this study was to evaluate the effectiveness of the AgNO3 nanoparticles alone and conjugated with imipenem (IMI) to combat extensively drug-resistant *P. aeruginosa*.

Methods: Antibiotic susceptibility was carried out using disc diffusion method. Detection of different resistant genes was performed using standard polymerase chain reaction (PCR). The chemically synthesized $AgNO_3$ particles were characterized using scanning electron microscope (SEM), dynamic light scattering (DLS) and X-ray diffraction (XRD) methods. Fourier transform infrared spectroscopy (FTIR) was accomplished to confirm the binding of $AgNO_3$ with IMI. The microdilution broth method was used to obtain minimum inhibitory concentration (MIC) of $AgNO_3$ and IMI-conjugated $AgNO_3$. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out on L929 cell line to study the cytotoxicity of nanoparticles. The data were analyzed by Eta correlation ratio and chi-square (X^2) test.

Results: Analysis of the antibiotic resistance pattern showed that 12 (24%) isolates were XDR, and MIC values of IMI were between 64 and 128 µg/mL. Frequency of SHV, TEM, CTX M, IMP, VIM, OPR, SIM, SPM, GIM, NDM, VEB, PER, KPC, OXA, intI, intII, and intIII genes were 29 (58%), 26 (52%), 26 (52%), 32 (64%), 23 (46%), 43 (86%), 3 (6%), 6 (12%), 3 (6%), 4 (8%), 7 (14%), 6 (12%), 18 (36%), 4 (8%), 19 (38%), 16 (32%), and 2 (4%), respectively. The XRD, SEM, DLS, and FTIR analysis confirmed the synthesis of $AgNO_3$ nanoparticles and their conjugation with IMI. The $AgNO_3$ nanoparticles had antimicrobial activity, and their conjugation with IMI showed enhanced effectiveness against XDR isolates. The synthesized $AgNO_3$ showed no cytotoxic effects.

Conclusion: The results suggest that IMI-conjugated $AgNO_3$ has a strong potency as a powerful antibacterial agent against XDR *P. aeruginosa*.

Keywords: *P. aeruginosa*, $AgNO_3$ nanoparticle, XDR, imipenem

Introduction

As shown in the earlier studies, *P. aeruginosa* is a non-fermenting gram-negative bacterium that correlates clinically and epidemiologically with nosocomial infections. Today, the incidence of antibiotic-resistant *P. aeruginosa* infections has been

increased all over the world.[1,2] Although various antibiotic-resistant mechanisms have been identified in *P. aeruginosa*, one of the most notable mechanisms is extended-spectrum beta-lactamase (ESBL) such as TEM, CTXM, and SHV. Carbapenems are commonly used antibiotics against ESBL-producing *P. aeruginosa* infection, but today, production of carbapenemases and metallobeta-lactamases including IMP, VIM, KPC, SIM, GIM, PER, VEB, SPM, OXA143, and NMD have led to the resistance to these antibiotics. By carrying these resistance genes and other mechanisms, *P. aeruginosa* can become resistant to a wide range of antibiotics and produce multidrug-resistant (MDR) and extensively drug-resistant (XDR) isolates.[3–5] Therefore, the treatment of such isolates in hospitals has resulted in several problems for specialists. The MDR isolates are bacteria that are resistant to at least one agent in three or more classes of antimicrobial agents, but XDR isolates are sensitive to only one or two groups of antimicrobials.[4] Numerous reports have shown that Carbapenem antibiotics are one of the most effective agents in the treatment of infections caused by gram-negative bacilli. Several characteristics including high affinity to Protein Binding Penicillin 2, excellent permeability across bacterial membranes, and stability against most β-lactamases makes carbapenems as potent antibiotics against gram-negative bacteria.[6,7] Although carbapenems are one of the most commonly used antibiotics against drug-resistant *P. aeruginosa* infection, these antibiotics have lost their effectiveness with advent of XDR *P. aeruginosa* isolates. The XDR isolates of the *P. aeruginosa* have been found in different countries, and their clinical impact has been reviewed, however, combat with this challenge is a major concern.[8] Other important resistance mechanisms are integrons (int1, int2, and int3) and specialized porin or efflux pumps genes (Opr) which cause resistance to a wide range of antibiotics.[9,10] Nosocomial infections by antibiotic-resistant bacteria resulted in 25,000 death cases, an increase of 2.5 days in the hospitalization, and an estimated 1.5 billion € extra costs in Europe per year.[11,12] Meanwhile, the United States reports showed that *P. aeruginosa* infections have put a heavy burden on hospitals, including mortality, morbidity, and health-care costs. High-mortality *P. aeruginosa* infections (18% to 60%) increase the cost of treatment between $ 20,000 and $ 80,000 per infection.[13] Therefore, researchers are looking for new methods for treating antibiotic-resistant bacteria in that nanoparticles are one of the most important solutions.[1,3,14,15] The use

of nano-biotechnology-based methods in medicine has opened a new perspective against pathogenic bacteria. In order to achieve this purpose, first, the antimicrobial activity of the nanoparticles must be measured, and then, if possible, used for pharmaceutical applications. The chemical and physical properties of nanoparticles offer researchers the opportunity to use them in various medical fields.[16] In recent years, many studies have been conducted on the use of silver nanoparticles against antibiotic-resistant bacteria that cause nosocomial infections. In a study by Bardania et al, the antimicrobial effect of silver nanoparticles was reported against antibiotic-resistant *P. aeruginosa* and *S. aureus* isolated from the clinical sample.[17] Also, Shariati et al showed that silver nanoparticles inhibited MDR isolates of *P. aeruginosa* and reduced the expression of virulence genes in the bacterium.[1] The purpose of this study was to use $AgNO_3$ nanoparticles alone and conjugated with imipenem for combating extensively drug-resistant *P. aeruginosa*.

Materials and Methods
Chemicals and Apparatus
The reagents including Tryptic Soy Broth (TSB), Stuart transport medium, Mueller–Hinton agar (MHA), and Mueller–Hinton broth (MHB), blood, and MacConkey agar and Dimethyl Sulfoxide (DMSO) were purchased from Merck (Germany). Antibacterial disks and antibiotic powders were provided by Mast (Diagnostics, UK) and Sigma-Aldrich (St. Louis, MO, USA), respectively. The synthetic $AgNO_3$ was also purchased from Sigma-Aldrich. All chemicals were of analytical reagent grade and used as received with no further purification.

Bacterial Isolates
The current study was conducted in Microbiology Section of the Central Laboratory of Iran University of Medical Sciences. A total of 50 clinical isolates of *P. aeruginosa* from blood, urine, trachea, sputum, and burn wounds were collected from a part of the routine laboratory procedure of hospitals in Tehran. It should be noted that the sputum specimens obtained from the patients were microscopically examined for the presence of salivary contamination. Briefly, sputum was incubated with an equal volume of sputolysin for 15 min at room temperature and plated onto chocolate agar. The deep oropharyngeal swab samples were collected and cultured on blood agar and MacConkey agar media. Wound swabs were added to

Stuart transport medium, then immediately transported to the Department of Microbiology, Iran University of Medical Sciences, and inoculated onto TSB, blood, and MacConkey agar for 24 hr. All positive cultures were recognized by the related biochemical tests, including gram-negative bacilli, the ability to grow at 42°C, colony characteristics, motility and oxidase test, citrate utilization, OF-glucose, pigment production on Mueller–Hinton agar and non-fermenting in TSI agar.[18] The reference strain used as positive control was *P. aeruginosa* ATCC 27,853. The isolates identified as *P. aeruginosa* were preserved at –80°C in TSB supplemented with 30% glycerol until further processing.

Antimicrobial Susceptibility Test and β-Lactamase Determination

Antibiotic susceptibility test was carried out using disc diffusion method according to the Clinical and Laboratory Standard Institute guideline (CLSI). Antibiotic disks used in the current study contained ceftazidime, chloramphenicol, cefotaxime, amikacin, co-trimoxazole, ceftriaxone, ciprofloxacin, meropenem, gentamicin, and tetracycline. *P. aeruginosa* isolates that were found resistant to IMI by disk diffusion test were re-checked in broth microdilution assay. The MIC for IMI was obtained in the range of 0.5 μg/mL to 256 μg/mL. *P. aeruginosa* ATCC 27,853 was used as control strain. Double disc synergy test was used for phenotypic ESBL-producing *P. aeruginosa* detection. In this test, antibiotics, including ceftazidime and cefotaxime, were used alone and adjacent to all two antibiotics combination with clavulanic acid.[19]

Antibiotic-Resistant Genes Genotyping

Detection of different genes involved in antibiotic resistance including VIM, IMP, OPR, SPM, SIM, GIM, NDM, SHV, TEM, VEB, PER, CTXM, OXA, intI, intII, intII and KPC were performed using standard PCR amplification. DNA was extracted using DNA extraction kit (GeNet Bio Company, Daejeon, Korea) according to the manufacturer's instruction. PCR reaction was performed in a final volume of 25 μL using 3 μL DNA solution, 12.5 μL Qiagen HotStar Taq Polymerase Master Mix, 10 pmol primer and water. PCR cycling conditions were as follows: denaturation at 94°C for 5 min, followed by 30 amplification cycles at 94°C for 1 min and extension at 72°C for 40 s and a final extension at 72°C for 5 min.[20] All primers used in the present study are listed in Table 1. Finally, PCR

products were detected by electrophoresis on 1%-1.5% agarose gel stained with safe stain.

Synthesis of AgNO3

The synthetic $AgNO_3$ had an average purity of 99.7% metals base. Then, 0.05 mg $AgNO_3$ was added to 5 mL sterile distilled water containing 5% polyethylene glycol (PEG). The reason for the use of PEG was to disperse the colloidal nanoparticles, which had no inhibitory effect on the concentration used above. Subsequently, $AgNO_3$ nanoparticles were incubated at 37°C for 30 minutes and homogenized by sonication at 100 w, at 40 kHz to disperse the nanoparticles. Then, the dispersed solutions were autoclaved at 121°C for 15 min and stored at 4°C. The morphology of Ag nanoparticles was evaluated by SEM.

Analysis of Synthesized Nanoparticles

The SEM analysis was conducted using Hitachi S-4500 SEM machine. To perform this evaluation, thin films of the sample were placed on a carbon-coated copper grid by dropping a very small amount of the sample on the grid. The extra solution was removed using a blotting paper, and the films were allowed to dry by putting them under a mercury lamp for 5 min. Also, using DLS equipment on a polystyrene cuvette, the size of the nanoparticles was examined in 3 mL of the reaction mixture. The XRD method was used to identify the phase formation of nanoparticles under different conditions. The samples were prepared as powder and recorded on an X-ray diffractometer (model PW1730, PHILIPS company, Netherlands) with CuKα source radiation (λ=1.5418 Å), accelerator voltage kV40, and current mA30. The sample was analyzed in the range of 5 to 70 degrees.

Conjugation of AgNO$_3$ with Imipenem

The FTIR was performed to confirm the binding of $AgNO_3$ with IMI. The amount of 20 mg of nanoparticles were added to 50 mL distilled water at room temperature under magnetic stirring. Furthermore, 25 mg IMI was poured in 5 mL distilled water and dissolved gently by magnetic stirring. After complete dissolution, the prepared IMI aqueous solution was mixed with $AgNO_3$ suspension under gentle stirring at 25°C. It was kept at room temperature for 24 hr. Then, dissolved suspension was centrifuged at 4000 rpm for about 15–20 min, and the supernatant was separated. Eventually, the resulting solid product was collected by centrifugation and dried at room temperature overnight. The dried structure of the sample was analyzed

Table 1 Primers Used for Detection of Resistance Genes in *P. aeruginosa* Isolates

References	Product Size(bp)	Primer Sequence (5'-3')	Genes
[18]	105 bp	5'-TGCGATCACCACCTTCTACTTC-3' 5'-CGCTGACCGCTGCCTTTC-3'	OprD
[10]	428 bp	5'-CATGGTTTGGTGGTTCTTGT −3' 5'-ATAATTTGGCGGACTTTGGCC −3'	IMP
[10]	512 bp	5'-ATTGGTCTATTTGACCGCGTC −3' 5'-AATGCGCAGCACCAGGATAGC −3'	VIM
[38]	621 bp	5'-GGTTTGGCGATCTGGTTTTC −3' 5'-CGGAATGGCTCATCACGATC −3'	NDM
[39]	893 bp	5'-ATGTCACTGTATCGCCGTCT −3' 5'-TTTTCAGAGCCTTACTGCCC −3'	KPC
[40]	796 bp	5'-TTAACTCCCTGTTAGCCA −3' 5'-GATTGCTGATTTCGCCC −3'	SHV
[10]	375 bp	5'-GGAACAACTTTGACGATTGA −3' 5'-CCCTGTTTTATGAGCAACAA −3'	VEB
[30]	927 bp	5'-ATGAATGTCATTATAAAAGC −3' 5'-TTAATTTGGGCTTAGGGCAGAA −3'	PER
[38]	519 bp	5'-TTTGCGAGTGCAGTACCAGTAA −3' 5'-CTCCCCTGCCGGTTTTATC −3'	CTXM
[6]	867 bp	5'-ATGAGATTCAACATTTCCG −3' 5'-CTGACAGTTACCAATGCTTA −3'	TEM
[10]	570 bp	5'-TAC AAG GGA TTC GGC ATC G −3' 5'-TAA TGG CCT GTT CCC ATG TG-3'	SIM
[10]	271 bp	5'-AAA ATC TGG GTA CGC AAA CG −3' 5'-ACA TTA TCC GCT GGA ACA GG −3'	SPM
[10]	477 bp	5'-TCG ACA CAC CTT GGT CTG AA −3' 5'-AAC TTC CAA CTT TGC CAT GC −3'	GIM
[10]	908 bp	5'-AGTTAACTTTCAATAATTG −3' 5'-TTGGAAAATTATATAATCCC −3'	OXA$_{143}$
[24]	893 bp	5'-ATCATCGTCGTAGAGACGTCGG −3' 5'-GTCAAGGTTCTGGACCAGTTGC −3'	INT1
[24]	467 bp	5'-GCAAATGAAGTGCAACGC −3' 5'-ACACGCTTGCTAACGATG −3'	INT2
[41]	761 bp	5'-GCAGGGTGTGGACGAATACG −3' 5'-ACAGACCGAGAAGGCTTATG −3'	INT3

using FTIR spectrometer in the 400–4000 cm^{-1} wavenumber region.

Antibacterial Activity Test

Microdilution broth method was used according to CLSI to obtain MIC of nanoparticles and IMI-conjugated AgNO$_3$. To prepare nanoparticles and IMI-conjugated AgNO$_3$ stock solution, 1.024 mg of each substance was completely dissolved in 1 mL sterile water. Then, the stock solution was serially diluted in the range of 0.5–1024 μg/ mL, and the nanoparticles and IMI-conjugated AgNO$_3$ dilutions were added to the wells of 96-well plates starting at 256 μg/mL. In the next step, each well was inoculated with 10 μL (5×10^6 CFU/mL) of the XDR isolates of the

P. aeruginosa in Mueller-Hinton broth to a final concentration (5×10^5 CFU/mL). The positive and negative controls were *P. aeruginosa* PAO1 and media alone, respectively. The lowest concentration of nanoparticles and IMI-conjugated $AgNO_3$ that prevented the growth of XDR isolates of the *P. aeruginosa* and did not cause opacity in the well after 24 hr incubation at 37°C was determined as MIC.

Cytotoxicity Assay of Nanoparticles

Cytotoxicity assay was carried out on human epithelial cell lines (L929 ATCC CCL-6364TM purchased from Pasteur Institute of Iran) to study the cytotoxicity level of nanoparticles. To perform the cytotoxicity assay, L929 cell lines were cultured in RPMI 1640 medium (Biosera, USA) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamic acid, 1% non-essential amino acid, and 1% penicillin-streptomycin and incubated at 37°C in CO_2 humidified incubator. For cell viability studies, the cells were seeded in 96-well plates (50,000 cells/well). Following 24 hr incubation, the cells reached 80% confluency. Subsequently, different concentrations of nanoparticles were added to the microtiter wells to evaluate the mitochondrial functions of the cells by MTT assay. After 24 hr of incubation, the cell morphology was evaluated with an Olympus IX70 Inverted Microscope. Then, 20 μL of 10X MTT purchased from Roche (Mannheim, Germany) was directly added to the wells. After 4 hr incubation in dark and dissolving formazan crystals in DMSO, the absorbance was obtained at 570 nm with a standard microplate reader.[21] The viability of the cells exposed to different concentrations of nanoparticles was measured.

Statistical Analysis

All data were analyzed using SPSS 22. The interpretation of the results was based on the frequencies. Eta correlation ratio was exerted to investigate the relationship between the age of patients and different antibiotic resistance rates. The P-value less than 0.05 was considered statistically significant for association between all antibiotics, resistance genes, type of samples, and clinical isolates by the chi-square (X^2) test.

Results
Characteristics of Infectious Samples and Patients

A total of 50 isolates from February 2018 to June 2018 were collected from Tehran hospitals. The specimens were collected from different wards of the hospitals including Burn, Infectious, Intensive Care Unit (ICU), Orthopedic, Gynecological and Outpatient Wards. The number of *P. aeruginosa* isolates from blood, urine, trachea, sputum, burn wounds and bedsore were 8 (16%), 13 (26%), 4 (8%), 9 (18%), 9 (18%) and 7 (14%), respectively. Eight (16%) isolates were obtained from outpatients and 42 (84%) isolates were from hospitalized patients. The age range of the patients infected with *P. aeruginosa* was 21 to 87 years (58.73±19.96). The prevalence rate of *P. aeruginosa* differed among men and women; 20 samples were isolated from females and 30 samples were isolated from males.

Prevalence of ESBL-Producing *P. aeruginosa* and Antimicrobial Susceptibility

The results of antibiogram showed that the most effective antibiotic against *P. aeruginosa* isolates was ciprofloxacin, and the highest resistance was observed to cefotaxime. The resistance of *P. aeruginosa* isolates to all antibiotics was found higher than 50%. The details of the antibiogram results can be seen in Table 2. By disk diffusion test, carbapenem antibiotics susceptibility was evaluated for all the isolates, and the results showed among 50 *P. aeruginosa*, 25 (50%), and 31 (62%) isolates were resistant to meropenem and imipenem, respectively. The results from MIC demonstrated that all the 31 isolates were resistant to IMI with MIC between 32 and 128 μg/mL. The statistical analysis of data indicated that there was a significant relationship between resistance to all antibiotics and age of the patients. On the other hand, some antibiotics such as meropenem, ceftriaxone, co-trimoxazole, chloramphenicol, and tetracycline, had a significant correlation with the type of samples, especially urine, bed ulcer, and sputum. Different wards of the hospitals, especially the ICU ward, also had a direct relationship with the resistance to a number of antibiotics such as ciprofloxacin, gentamicin, amikacin, meropenem, ceftriaxone, chloramphenicol, and tetracycline. Combination disk test results showed that 45 (90%) isolates produced ESBL.

Prevalence and Characterization of Resistance Genes

Among 45 isolates, only 8 (16%) isolates had all three genes (SHV, TEM, and CTXM) together, and 29 (58%) had SHV gene, 26 (52%) had TEM gene, and 26 (52%) carried CTXM gene. The frequency of other resistance

Table 2 Antibiotics Resistance Pattern of *P. aeruginosa* Clinical Isolates

Antibiotics	Total Samples			Type of Samples	Hospital Wards	Age of Patients
	Resistance	Intermediate	Sensitive	(P-value)	(P-value)	(P-value)
Ceftazidime	25 (50%)	6 (12%)	19 (44%)	0.05	0.06	< 0.001
Chloramphenicol	23 (56%)	2 (4%)	20 (40%)	0.05	0.00	< 0.001
Cefotaxime	37 (74%)	4 (8%)	9 (18%)	0.06	0.08	< 0.001
Amikacin	30 (60%)	2 (4%)	18 (36%)	0.03	0.03	< 0.001
Co-trimoxazole	35 (70%)	1 (2%)	14 (28%)	0.00	0.07	< 0.001
Ceftriaxone	28 (56%)	0	22 (44%)	0.01	0.04	< 0.001
Tetracycline	34 (68%)	0	16 (32%)	0.05	0.02	< 0.001
Gentamicin	27 (54%)	1 (2%)	22 (44%)	0.06	0.02	< 0.001
Meropenem	25 (50%)	4 (8%)	22 (42%)	0.02	0.01	< 0.001
Imipenem	31 (62%)	2 (4%)	17 (34%)	0.06	0.06	< 0.001
Ciprofloxacin	25 (50%)	1 (2%)	24 (48%)	0.04	0.06	< 0.001

genes in *P. aeruginosa* isolates was different. Among 50 *P. aeruginosa* isolates, 32 (64%), 23 (46%), 43 (86%), 3 (6%), 6 (12%), 3 (6%), 4 (8%), 7 (14%), 6 (12%), 18 (36%), 4 (8%), 19 (38%), 16 (32%), and 2 (4%) isolates contained IMP, VIM, OPR, SIM, SPM, GIM, NDM, VEB, PER, KPC, OXA, intI, intII, and intIII genes, respectively. Figure 1A shows the band size and location of the resistant genes on agarose gel. The data analysis showed a significant relationship between the presence of all resistance genes and the age of the patients (*P*<0.05). It was also found that resistance genes were more present in the samples isolated from ICU, and a direct relationship was observed between the hospital ward and the presence of these genes (*P*<0.05). The specimens isolated from wound, urine, and sputum had higher resistance genes, which showed a significant correlation between the presence of these genes and the type of samples (*P*<0.05).

Using antibiotics resistance pattern analysis, 12 (24%) isolates were identified as XDR, which were resistant to all antibiotics used in this study. Also, there was a significant relationship between XDR isolates and IMP gene (*P*<0.05), and the highest number of XDR isolates was isolated from ICU. A significant relationship was observed between these antibiotic-resistant isolates and different parts of the hospital.
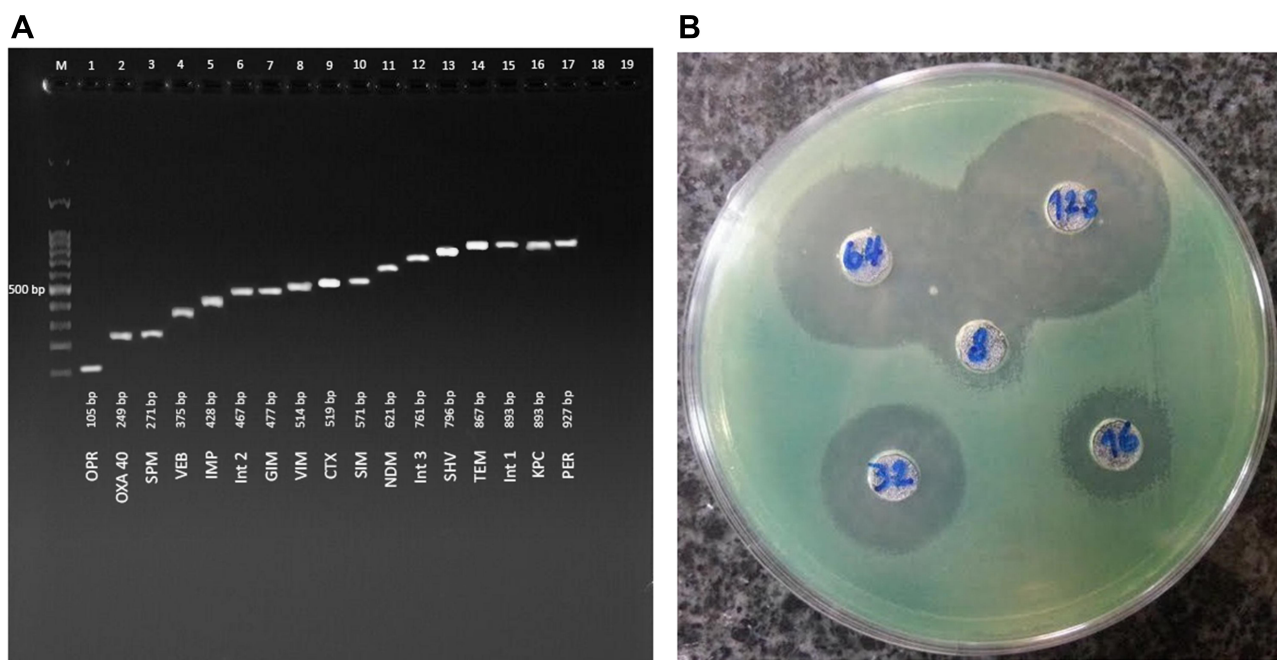
Figure 1 (**A**) The location of resistance genes on electrophoresis gel (**B**) Antimicrobial activity of AgNO₃ nanoparticles against *P. aeruginosa*.
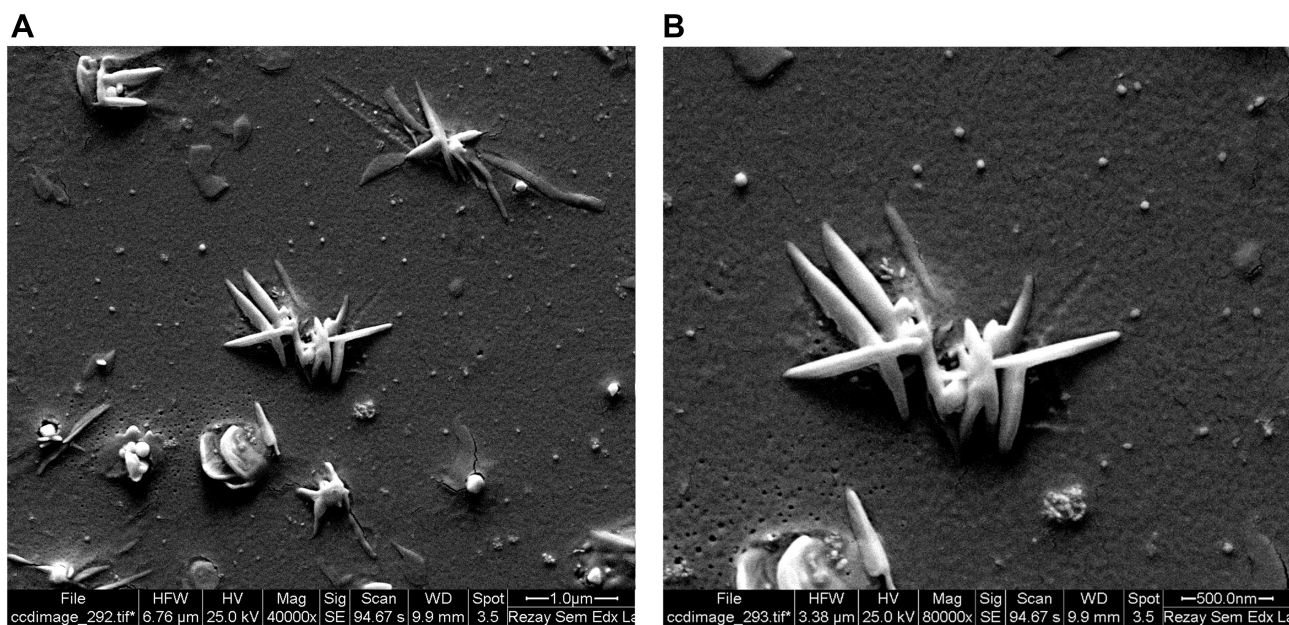
A

B

Figure 2 AgNO3 Nanoparticles with magnify (**A**) 40000× and (**B**) 80000×.

Characterization and Antibacterial Effect of AgNO₃ on Resistant *P. aeruginosa*

All XDR isolates were tested to investigate the antimicrobial effects of nanoparticles and IMI-conjugated AgNO₃. The SEM analysis confirmed different sizes and shapes of the nanoparticles, as can be seen in Figure 2. According to the SEM image in Figure 2, AgNO₃ nanoparticles with a cylindrical shape and diameters of 15–50 nm were fabricated. Also, DLS was employed to obtain the size distribution of AgNO₃ nanoparticles. The results demonstrated that the AgNO₃ nanoparticles had a very narrow size distribution with an average particle size of about 25 nm. The XRD for the AgNO3 nanoparticles showed that the scattering peaks at
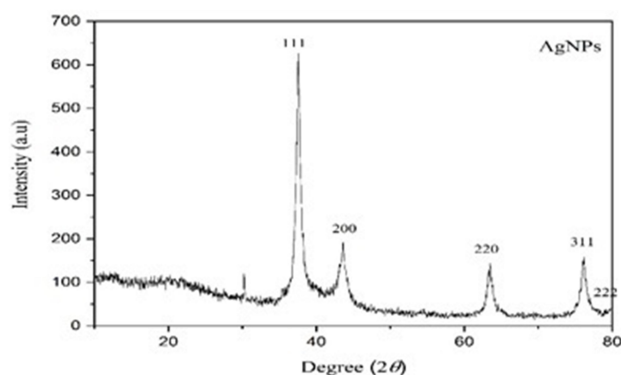

Figure 3 XRD pattern of produced silver nanoparticles. Three intense peaks at 37.34°, 43.61°, and 63.51° revealed the presence of produced silver nanoparticles.

angles 37.34, 43.61, and 63.51 were related to the scattering plates (111), (200), and (220), respectively, and were matched with the structure of AgNO₃ nanoparticles with the card number JCPDS (04–0783). This confirms the correct synthesis of silver nanoparticles (Figure 3).[22,23]

The XDR isolates were resistant to high concentrations of IMI. The antimicrobial activity of the nanoparticles showed that the nanoparticles had antimicrobial activity at low concentrations (Table 3 and Figure 1B). The conjugation of AgNO₃ with IMI showed greater antimicrobial effect on XDR isolates compared to AgNO₃ alone. These XDR isolates carried different resistance genes; Table 3 shows the details of these genes and the MIC. Data analysis revealed no significant relationship between the presence of resistance genes and XDR isolates.

FTIR spectroscopy was used to investigate the interaction between IMI and AgNO₃. Figure 4 shows the presence of O-H stretching bond in the range 3400 cm^{-1}, which was related to water absorption in all samples. The presence of absorption bonds in the range of 3422 cm^{-1}, 2921 cm^{-1}, 2856 cm^{-1}, 1743 cm^{-1}, 1631 cm^{-1}, 1450 cm^{-1}, 1377 cm^{-1}, 1240 cm^{-1}, 1043 cm^{-1} and 596 cm^{-1} confirms the presence of AgNO₃ nanoparticles (Figure 4B). The bonds in the range of 1368 cm^{-1} and 1092 cm^{-1} are characteristic of the absorption bonds of IMI (Figure 4C). These peaks are present in the IMI-conjugated AgNO₃ and confirm the interaction between the two substances, which is related

Table 3 Effect of Nanoparticles Alone and Conjugated with Imipenem Against XDR Isolates of *P. aeruginosa*

Number of Isolates	Harbor Genes	Imipenem MIC (µg/mL)	AgNO₃ Nanoparticle MIC (µg/mL)	Imipenem-Conjugated AgNO₃ MIC (µg/mL)
3	IMP, VIM, OPR, CTXM	128	8	4
5	TEM, PER	128	16	4
6	SHV	64	16	8
8	SHV, TEM, VEB	64	16	8
12	OPR, TEM, VEB, KPC, INT1	64	16	4
13	IMP, VIM, OPR, SHV, TEM, KPC, CTXM	128	16	4
14	IMP, VIM, OPR,SPM, PER, KPC, CTXM, INT1	64	16	8
23	OPR, TEM, KPC, CTXM, INT2, INT3	64	8	8
24	OPR, SHV, CTXM, INT1	64	8	4
40	IMP, VIM, OPR, SHV, VEB, CTXM, INT1	128	8	4
41	OPR, SPM, NDM, TEM, CTXM, INT1, INT2	128	8	8
50	IMP, VIM, OPR, SHV,CTXM, INT1, INT2	64	8	4

to the strong S=O stretching bond (Figure 4A). On the other hand, a minor shift in the peaks in the IMI-conjugated AgNO₃ sample indicates chemical bonding or electrostatic adsorption between IMI and AgNO₃ nanoparticles. Importantly, the presence of a sulfide group on the surface of IMI has made it predispose to form a bond with AgNO₃ nanoparticles. As a result, both components are present in the IMI-conjugated AgNO₃, and this observation confirms the correct synthesis of this compound.
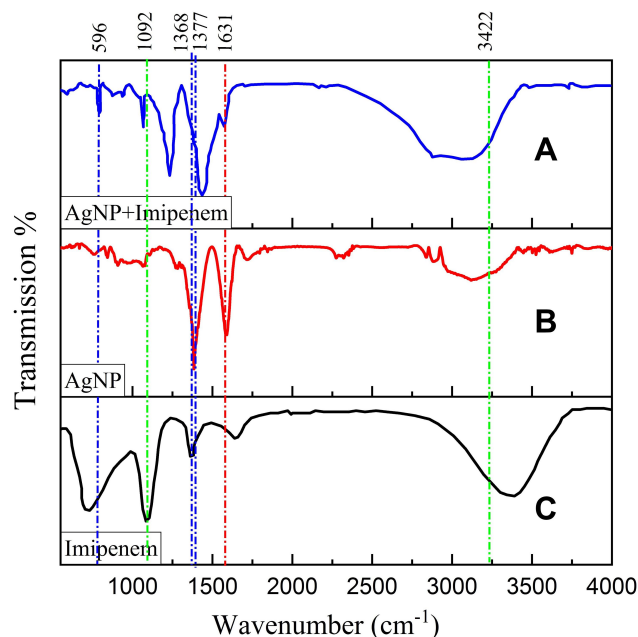
Cytotoxicity Effect of AgNO₃ Nanoparticles

The results of cytotoxic activity of AgNO3 nanoparticles, IMI, IMI-conjugated AgNO₃ (AgNO₃+IMI), and polystyrene tissue culture (TCPS) in L929 ATCC CCL-6364TM cell line demonstrated that cells exposed to nanoparticles were well proliferated compared to the TCPS. Since IMI alone was more toxic than TCPS, when combined with nanoparticles, its toxic property reduced significantly (Figure 5). The nanoparticles alone did not exhibit any toxicity and changes in cell morphology until 5 days; however, the AgNO₃+IMI on day 5 caused minor changes in the cell morphology compared to the TCPS.

Discussion

Antibiotic resistance in the pathogens that cause nosocomial infection is an undeniable challenge that results in mortality and increased hospitalization.[24–26] The results of this study showed that the most effective antibiotic against *P. aeruginosa* isolates was ciprofloxacin, and the highest resistance was observed to cefotaxime. It was also found
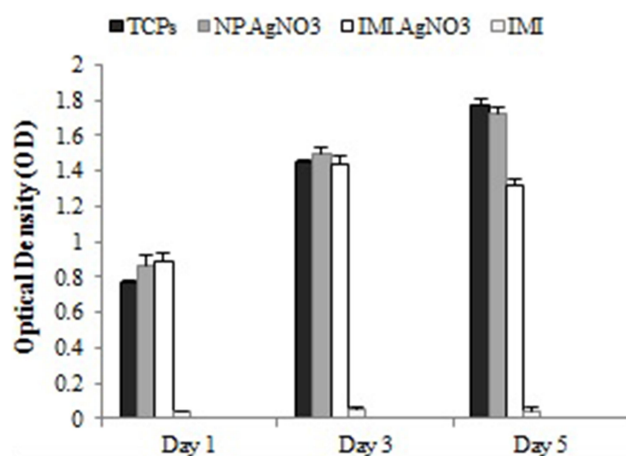

Figure 4 FTIR spectra of imipenem and imipenem conjugated AgNO₃. As shown in this figure, AgNO₃ nanoparticles bond to sulfide groups of imipenem, (**A**) AgNP +Imipenem, (**B**) AgNP, (**C**) Imipenem.

Figure 5 MTT assay results confirmed the in vitro cytotoxicity of silver nanoparticles against L929 ATCC CCL-6364TM cell lines after 1, 3, and 5 days exposures.

that the resistance of *P. aeruginosa* isolates to all antibiotics was above 50%. Similar to our results, a study by Shariati et al confirmed high resistance to most of the antibiotics (70%) in *P. aeruginosa* isolates.[1] Another study found that *P. aeruginosa* isolates were highly resistant to antibiotics (approximately 50%), and the results showed that the highest resistance was to cefotaxime.[27] In this study, the highest number of XDR isolates was isolated from ICU patients, and the antibiotics resistance was higher in the ICU ward than other parts of the hospital. Another study showed a relationship between antibiotics resistant *P. aeruginosa*, especially MDR, and different wards of the hospital, such as ICU.[28] Nseir et al also reported hospitalization in ICU as one of the risk factors for the ones infected with MDR *P. aeruginosa*, which confirms the results of our study.[29] The reason that *P. aeruginosa* strains isolated from the ICU were more resistant than other wards maybe for the patients admitted to this ward who had been hospitalized for a long time and were on long-term antibiotic therapy. Also, admitting to the ICU room where patients were previously hospitalized with MDR *P. aeruginosa* infection is a risk factor for the transmission of this resistant bacterium to the new hospitalized patients.[29] Among 50 *P. aeruginosa* isolates, 32 (64%), 23 (46%), 43 (86%), 3 (6%), 6 (12%), 3 (6%), 4 (8%), 7 (14%), 6 (12%), 18 (36%), 4 (8%), 19 (38%), 16 (32%), and 2 (4%) isolates contained IMP, VIM, OPR, SIM, SPM, GIM, NDM, VEB, PER, KPC, OXA, intI, intII, and intIII genes, respectively. Many studies around the world have investigated the genes associated with antibiotic resistance in clinical isolates of *P. aeruginosa*. In 2014, a study examined common resistance genes in

P. aeruginosa including OXA, VEB, VIM, IMP, SIM, GIM, SPM, and NDM and the results showed that the prevalence of VIM, OXA, VEB, NDM, and IMP were 58.1%, 41.7%, 10.4%, 4.2% and 2.1%, respectively. But, contrary to our results, the genes of GIM, SPM and SIM were not found in *P. aeruginosa* isolates in another study.[10] In the other study, researchers also examined the genes affecting the resistance of *P. aeruginosa* isolates with 3%, 65%, and 52% harbored ESBL, carbapenemase, and AmpC, respectively. The ESBL, carbapenemase and AmpC contained OXA (97%), TEM (61%), VIM (55%), PER (13%), IMP (3%) and AIM (1%). A study investigated the presence of the oprD gene in carbapenem-resistant *P. aeruginosa* isolates. The rate of this gene was 79.1%, which is very close to the outcome of our study.[30] Also, our results demonstrated that 45 (90%) isolates produced ESBL and among these 45 isolates, only 8 (16%) isolates had all three genes (SHV, TEM, and CTXM) together. Chen et al found that 87.5% of *P. aeruginosa* isolates were ESBL-producers and only 5 isolates (2.7%) carried all of the three TEM, SHV and CTXM genes, simultaneously.[31] In 2107, study on integrons in clinical isolates of *P. aeruginosa* showed that the results of integron 1, integron 2, and integron 3 were 27.5%, 25.5% and 15%, respectively. The results were in agreement with our results that showed class 3 integrons were less abundant than other integrons.[27]

High or low prevalence of some resistance genes in *P. aeruginosa* isolates can be due to differences in the geographical areas which indicate that prevalence of resistance genes differs across the world and even in different parts of a country. Other factors that may explain the differences in the prevalence of resistance genes include antibiotic abuse patterns, antimicrobial stewardship program, degree of virulence in strains, and infection control methods. In this study, it was also found that resistance genes were more present in the samples isolated from ICU ward and a direct relationship was observed between hospital ward and the presence of these genes. Other studies have also pointed to the association between the high levels of different antibiotic resistance genes and *P. aeruginosa* isolated from the ICU ward, which confirms the results of our study.[32,33]

In the current study, AgNO$_3$ nanoparticles with a cylindrical shape and diameters between 15 and 50 nm were fabricated (average particle size of about 25 nm). Interestingly, AgNO$_3$ nanoparticles had favorable solubility and bioavailability, which could be attributed to the

large surface area. Our results also showed that the presence of a sulfide group on the surface of IMI has predisposed it to form a bond with AgNO$_3$ nanoparticles. Previous research has proven that small size and high surface area of the nanoparticles increases the loading of antibiotics, and enhances the concentration of antibiotic at the site of contact with the bacteria and thereby further inhibiting the pathogens.[22] Some studies have shown that reducing the size of nanoparticles increases their solubility, which is in agreement with the results of our study.[1,34]

The antimicrobial activity of the AgNO$_3$ nanoparticles was shown at low concentrations. The conjugation of AgNO$_3$ with IMI showed a greater antimicrobial effect on XDR isolates compared to AgNO$_3$ alone. These results indicated that the AgNO$_3$ nanoparticles increased the antimicrobial effect of IMI. In recent years, the use of nanoparticles as one of the new approaches against lethal antibiotic-resistant bacteria, such as *P. aeruginosa* has attracted the attention of researchers. Mapara et al investigated the effects of silver nanoparticles against XDR *P. aeruginosa* and found that the nanoparticles have antimicrobial activity against these resistant isolates.[4] It has also been shown that the use of silver nanoparticles with IMI resulted in greater antibiotic efficacy and reduced the effective dose of IMI which is consistent with our study.[22] In another study, nanoparticles were used against MDR and XDR isolates of *P. aeruginosa* and were found to kill these resistant bacteria, which demonstrates the antimicrobial effects of the nanoparticles.[35]

One of the main concerns of using nanoparticles as a drug is their toxicity. According to our results, human epithelial cell lines exposed to synthesized AgNO$_3$ nanoparticles were well proliferated and no morphological change was observed. Our finding was in accordance with Zendegani et al and Shariati et al studies who demonstrated no toxicity of nanoparticles to the human cells.[1,22] In addition, our results showed that IMI alone was more toxic than TCPS. After conjugation with nanoparticles, its toxic property reduced significantly. Various studies confirm that the use of nanoparticles as coating or in combination with organic or inorganic compounds can reduce their toxicity.[36,37]

Conclusion

In summary, AgNO$_3$ nanoparticles had ideal antibacterial effects on XDR *P. aeruginosa*, and its conjugation with IMI showed greater antimicrobial effects. Despite widespread antibiotic resistance and the presence of ESBL, metallo-β-lactamase, carbapenemase and other resistance genes in *P. aeruginosa*, the AgNO$_3$ alone and in combination with

IMI indicated strong antibacterial activity. Due to the antimicrobial properties of conjugated AgNO$_3$ nanoparticles with IMI, they have the capacity to be introduced as a suitable candidate for targeting antibiotic-resistant bacteria. However, it is essential to develop new nano-antibiotic formulations that can improve their efficacy and reduce dose and toxicity of the antibiotics. Finally, it is recommended to measure the toxic effects of AgNO$_3$ alone and in combination with IMI in animal models.

Ethics Approval

Ethics Research Committee of Microbial Science Research Center approved this study with ethics code IR.BMSU.REC.1396. 880.

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Disclosure

The authors report no conflicts of interest in this work.

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