Nano-strategies in pursuit of efflux pump activeness in Acinetobacter baumannii and Pseudomonas aeruginosa

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Abstract

Multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are the two bacteria notorious for nosocomial infections and threats in healthcare settings. Efflux pump is an important mechanism of the antibiotic resistant phenomenon. The emergence of antibiotic-resistant bacteria demands the development of either new antibacterial agents to overcome the distressing situation. The aim of this study was to evaluate the efficacy of chitosan and silver (Ag) nanoparticles (NPs) and their combination with antibiotics to alter the expression level of efflux pumps and any increase in the inhibitory capacity of antibiotics against clinical *A.baumannii* and *P.aeruginosa* isolates. To conduct the experiments, initially antibiotic resistant *A. baumannii* and *P. aeruginosa* (*PAO1*) strains were exposed to chitosan and AgNPs with and without ciprofloxacin and gentamicin in their sub inhibitory levels. RNA was then extracted to study the antibacterial effects of the nanoparticles in relation to the expression of the efflux pump using real-time PCR. The present investigation found expression levels of *abeM* efflux pumps in *A.baumannii* and *mexY* efflux pumps in *P.aeruginosa* decreased after exposing the bacteria to sub-inhibitory concentrations of chitosan, chitosan NPs, and their combination with ciprofloxacin and gentamicin. Conversely, the minimum inhibitory concentration (MIC) levels of

ciprofloxacin and gentamicin were found to have increased after exposure to the synthetic substances. Though nanoparticles have found their place in the modern scientific therapeutic world however, before they step into treatment strategies it is necessary to determine their effects either alone or with antibiotics in lowering antibiotic resistance.

Keywords: Efflux pumps, Nanoparticles; Chitosan; Silver; Antibacterial resistance, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*

Abbreviations:

Silver nanoparticles (AgNPs), AgNPs + ciprofloxacin (ANC), AgNPs + gentamicin (ANG), N, O-carboxymethyl chitosan (NOCC), N, O-carboxymethyl chitosan nanoparticles (NOCCNPs), NOCCNPs + ciprofloxacin (CNC) and NOCCNPs + gentamicin (CNG).

1.1 Introduction

Acinetobacter baumannii and *Pseudomonas aeruginosa* are of the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species) organisms and threaten global public health because of their resistance to several antibiotics(1). Antibiotic resistance is a universal mechanism evolved by the bacteria through various mechanisms, including efflux pumps.

Efflux pumps, which are membrane-bound transporter proteins with a wide spectrum of substrate specificity and immense drug exclusion capacity, are one of the attributing factors for the evolution of multidrug-resistance (MDR) and even extensive drug-resistance (XDR) in many bacteria (2). MexXY (-OprA) is RND type of efflux pump in *Pseudomonas aeruginosa* that causes resistance to antibiotics such as aminoglycosides, erythromycin, specific β-lactams (cefepime and cefpirome, but not ceftazidime), tetracycline, and fluoroquinolones (3, 4). The AbeM is the multidrug and toxic-compound extrusion (MATE) type efflux pump in

Acinetobacter baumannii that causes bacteria to develop resistance to norfloxacin, ofloxacin, ciprofloxacin, and gentamicin (5, 6). Their exceptional contribution in turning *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, the opportunistic pathogens into nosocomial ones (7) has attracted researchers to discover new antibiotics or other modalities which can neutralize the effects of the emergence of antibiotic-resistant pathogens. The RND family is the main efflux pump mediated in antimicrobial resistance in *A. baumannii* and *P. aeruginosa* that utilizes the proton motive force to discharge the antibiotics (8). In *Pseudomonas aeruginosa*, MexAB-OprM and MexXY-OprM RND efflux pumps play crucial roles, (9) while the presence of AbeM efflux pumps in *A. baumannii* leads to the exertion of their effects. AbeM has its place in MATE family of efflux pumps, and their expression leads to the enhancement of inhibitory concentrations of norfloxacin, ofloxacin, ciprofloxacin, gentamicin, triclosan, acriflavine, ethidium bromide, kanamycin, erythromycin, chloramphenicol, and trimethoprim (6).

Recent advances in nanotechnology have opened some horizons and offered new predictions for developing novel formulations based on distinct types of nanoparticles (NPs) with different sizes and shapes and flexible antimicrobial properties. These nanoparticles have found their role in enhancing antimicrobial effects, particularly when they are coupled with antibiotics (2). In fact, coupling nanoparticles and natural-based antimicrobials is just one of the strategies for rejuvenating several bacterial modalities utilized by them to emerge as MDR or XDR (10). Among many, chitosan has been proven to facilitate both paracellular and transcellular transport of drugs through various routes of administration. The compound is a biodegradable, biocompatible polymer regarded as safe for human dietary use and approved for wound dressing applications (11). Cationic chitosan-based nanoparticles interact with the anionic surfaces of the microbial cell membrane promoting antimicrobial activity however, selection of an adaptable,

suitable, and cost effective synthesis method is much important (2, 12). Among inorganic NPs, silver nanoparticles (Ag-NPs or nanosilver), due to their novel chemical, physical, and biological properties, have attracted the attention of researchers to be used for medical purposes (13, 14). The advantage of using nanosilver is that it is comparatively less reactive than silver ions, and therefore, is well suited for use in clinical and therapeutic applications. It has been tested against both MDR and non-MDR strains of many gram-positive and several gram-negative bacteria (15-17) .

Ciprofloxacin and gentamicin are the conventional antibiotics used to treat infections caused by gram-positive and gram-negative bacteria including, *A.baumannii* and *P.aeruginosa*. These antibiotics are referred for routine antibacterial susceptibility test of *A.baumannii* and *P.aeruginosa* according to standard Clinical Laboratory Standard Institute (CLSI; M100-S24) (18). In case of non-susceptibility to the above-mentioned antibiotics, monobactams and carbapenems have shown promising outcome for MDR *A.baumannii* and *P.aeruginosa* however, these drugs are reserved for patients who have a special need for them. Moreover, hypersensitivity, other adverse effects associated with their use and emergence of resistance mechanisms have constraint their use (19, 20). Thus, in the absence of new compounds to treat these antibiotic resistant organisms, other therapeutic alternatives must be sought. Moreover, ciprofloxacin (the usual quinolone chosen for the treatment of *P. aeruginosa* and other gram-negative infections) (21) and gentamicin non-susceptibility have raised synergistic protocol (22) to be applied against these bacteria. *A.baumannii* and *P.aeruginosa* are manifested as MDR or EDR in recent years in our hospital setting and are seemingly of concern. Conventional antibiotics are unable to combat the emergence of these antibiotic resistant strains and it is difficult to develop new antibiotics to treat infections caused by multidrug resistant

microorganisms. Thus, the current study aimed to evaluate the efficacy of chitosan and silver NPs for their antibacterial activity with and without ciprofloxacin and gentamicin, their efficacy to alter the expression level of the efflux pumps and to assess their effects by MIC levels.

1.2 Materials and methods

1.2.1 Preparation and characterization of nanoparticles

1.2.1.1 Silver nanoparticles (AgNPs) were prepared by adding 5ml of 10^{-2} M L-cysteine as a stabilizer to the 10^{-2}M silver nitrate ($AgNO_3$) followed by blending for 30 minutes. 10^{-2} M Potassium iodide (KI) was then added drop wise to the above solution in the stable mode without mixing to produce silver iodide (AgI) colloid. Sodium tetrahydridoborate ($NaBH_4$) was used as a revitalizer (23). Nanoparticles were obtained after centrifugation for 45 minutes at 12,000 g at 4°C (24). Characterization of NPs was performed by Transmission electron microscopy (TEM) and Electrokinetic measurements. The TEM images were made at an accelerating voltage of 200 kV (TEM, Leo 906, Zeiss, 100KV, Germany) and Zeta potential of the NPs were measured with high-throughput dynamic light scattering DLS (Dynamic Light Scattering) instrument, Malvern, Zetasizer Nanosize ZN3500 England (25) .

1.2.1.2 N, O-carboxymethyl chitosan (N, O-CMC) was prepared from chitosan as described previously (26). Briefly, chitosan nanoparticles (NOCCNPs) were produced by adding 1ml of 0.25% Tripolyphosphate (TPP) as the ionic cross-linking to 0.1% N, O-CMC solution and mixing for 30 min. Nanoparticles were obtained after centrifugation for 45 minutes at 12,000 g at 4°C (24). Characterization of NPs was performed by Transmission electron microscopy (TEM) and Electrokinetic measurements. The TEM images were made at an accelerating voltage of 200 kV (TEM, Leo 906, Zeiss, 100KV, Germany) and Zeta potential of the NPs were measured with high-throughput dynamic light scattering DLS (Dynamic Light Scattering) instrument, Malvern,

Zetasizer Nanosize ZN3500 England (25) .

1.2.2 Interaction of NPs with antibiotics

Chemical interactions of AgNPs and NOCCNPs with Ciprofloxacin (ANC and CNC, respectively) and Gentamicin (ANG and CNG respectively) were prepared by using N-Hydroxysuccinimide (NHS) and N′-ethylcarbodiimide hydrochloride (EDC) as cross linkers for the interaction of nanoparticles and antibiotics as described previously (27).

In addition, combination of nanoparticles (AgNPs, NOCCNPs) with antibiotics (CNG, CNC, ANG, and ANC) was accomplished by Fourier transform Infrared Spectroscopy (FTIR). Analysis on an FT-IR spectrometer (Bruker Tensor 27 FT-IR spectrophotometer, USA). Scanning was done from 400 to 4000 cm^{-1}.

Loading efficiency of antibiotics in interaction with nanoparticles was calculated at the wavelength of 270 nm for ciprofloxacin. For measuring the concentration of gentamicin, fluorometric method was used (24). Standard curves of several dilutions of gentamicin and ciprofloxacin was obtained to determine the amount of drug to be used in combination with nanoparticles.

1.2.3 Cell viability assay

MTT assay as a colorimetric method was used to determine the cell viability percentage after exposure to any experimental ingredients. Cell metabolic and viability activity was assessed by detecting purple colored formazan that is produced after reducing tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide by NAD(P)H oxidoreductase enzymes. This enzyme is produced in metabolically active cells. So by using MTT assay we can assay the viable cells.

For cell viability experiments, stem cells (bone marrow type) were added in Dulbecco modified

Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells with a density of 3000 were seeded to wells of 96-well test plates and incubated for 24hr. Different concentrations of the synthetic materials (0.01-2.5mg/ml) were prepared by dilution in DMEM supplemented with 10% FBS. They were added to the wells (each well 100 µL) with adherent stem cells and incubated for a period of 24, 48, and 72 hours at 37˚C and 5% CO2. Well without any nanoparticle was considered as cultural control.

MTT stock solution was added to the wells and incubate the plate for 4 h at 37°C. After that medium of the wells was removed and replaced to 100 µL of DMSO and mix by a pipette. After incubation at 37°C for 10 min in shaking Incubator, the absorbance was read at 570 nm in Epoch Micro plate Spectrophotometer (Biotek, Germany). Each experiment was analyzed in triplicate(28, 29).

1.2.4 Addition of Bacteria to Nanoparticles and/or Antibiotics on their sub-MIC concentration

Antibiotic resistant and efflux pump positive strains of *A.baumannii* were obtained from our earlier clinical study performed on the expression analysis of *abeM* in different phenotypic groups of *A.baumannii* (30). *Pseudomonas aeruginosa* strain PAO1, positive for the presence of efflux pumps was also included in this study. These strains were stocked at -80˚C in Tryptic soy broth with glycerol. The MIC was defined as the lowest concentration of nanoparticles that prevented visible growth of the *A.baumannii* and *P.aeruginosa* in susceptibility test by micro dilution method (31). MIC was discerned by micro dilution method using above-mentioned *A.baumannii* and *P.aeruginosa* strains. Then *A. baumannii* and *P.aeruginosa* were exposed to chitosan, CNC, CNG, AgNPs, ANC, ANG, gentamicin and ciprofloxacin at their sub inhibitory concentration (0.5xMIC). MICs of ciprofloxacin and gentamicin was calculated before and after

exposure with nanoparticles by using micro dilution method.

1.2.5 RNA extraction and cDNA synthesis

Bacterial culture (1.5x 10^5CFU/ml) in 100µl Mueller Hinton Broth and with nanoparticles at their sub inhibitory concentration (0.5xMIC) was incubated for 24h at 37°C followed by RNA extraction, which was done directly using RNA extraction kit (Sinaclon Co, Tehran, Iran). A.*baumannii* and *PAO1* without specified nanoparticles was used as control. Concentration of RNA was confirmed by Nano drop spectrophotometer and further stored at -80°C.

Complementary DNA (cDNA) was synthesized using cDNA Reverse Transcriptase Kit (Takara) and stored at 4°C for further use.

1.2.6 Real time efflux study

Quantitative real -Time PCR (Step One version 2.3) was performed using SYBR premix (Takara) and specific primers (Table 1) for *abeM*, *MexY* and *16srRNA* as the internal control gene. Bacteria were combined with NPs adjunct with or without antibiotics. Gene expression of target genes (*abeM* and *MexY*) and the reference gene (16srRNA) in antibiotic resistant *A.baumannii* and *PAO1* strains was compared before and after exposure to materials according to a relative quantification method (32).

The protocol used comprised of the following amplification program: Reverse transcription for the amplification of *abeM* was at 95˚C for 2min and 40 cycles each for 10s at 95˚C and 1min at 60˚C. The amplification of *mexY* was comprised at 95˚C for 5min followed by 40 cycles, each for 15s at 95˚C, 10s at 57˚C and 15s at 72˚C (3, 33). The cycling of PCR method for the amplification of *16srRNA* was at 95˚C for 2min and 40 cycles of 10s at 95˚C, 25s at 55˚C and 25s at 72˚C. Melting point data and their curve were collected after PCR cycling and checked for each well. A control reaction without cDNA was included each run as no template control. The

primers was shown in table 1.

The relative expression of the efflux genes was done by using comparative quantification cycle method (34). The relative expression of each target gene was specified by comparing the relative quantity of the mRNA in the presence and absence (control) of the antibiotic and nanoparticles. Each strain was assayed in duplicate.

The cycle number of the amplification plot, CT values, which passed a fixed threshold during the exponential phase of amplification was gathered for the analysis of the quantitative RT-PCR. A real-time PCR is a relative quantification experiment for comparing the expression of a target gene in one sample to the expression of the same gene in control sample. The expression of the target genes between two groups (treated and non-treated bacteria) was expressed as a fold change. In this study *16srRNA* gene was used as a housekeeping gene. *A.baumannii* ATCC 19606 and PAO1 wild-type strain of *P. aeruginosa* were taken as control strains. The amplification efficiencies of the genes in real time PCR was determined by using standard curve method. Five dilution series of cDNA of each sample was prepared and then amplified in real time PCR for obtaining the CT values of reference gene and target genes to construct standard curves. The relative quantification of each target sample was calculated by using Pfaffl formula as follows: (35)

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target}}(control-sample)}}{(E_{\text{ref}})^{\Delta \text{CP}_{\text{ref}}(control-sample)}}$$

Table 1: The primer sequences, amplicon sizes and references.

Genes	Primers	Amplicon size (bp)	Reference
abeM	F: 5′-GGTAGGTGTAGGCTTATGGA-3′ R: 5′-CTTCGGCAACTAATGGTGT-3	80	(30)
MexY	F: 5′-TCGCCCTATTCCTGCTG-3′ R: 5′-AGTTCGCTGGTGATGCC-3′	118	(33)
16srRNA	F: 5′-CAGCTCGTGTCGTGAGATGT-3′	150	(30)

	R: 5′-CGTAAGGGCCATGATGACTT-3′		

1.2.7 Statistical analysis

Viability of the cells was applied by using Graph pad prism 8 software and efflux pump expression (using delta delta cycle threshold method) was calculated by REST 2009 version 2.0.13. *P-value* < 0.05 was considered as statistical significant.

1.3 Results

1.3.1 Characterization of AgNPs and NOCCNPs

The presence and the amount of zeta potential of NPs was characterized by the TEM images and DLS (25, 36, 37) TEM analysis showed colloidal morphology of NOCCNPs in size range of 50-100 nm and the average diameter of the spherical AgNPs was less than 20nm. Zeta potential of AgNPs and NOCCNPs were -21.2 MV and +35.9 MV respectively. Figure 1and 2 shows the TEM images of silver and chitosan nanoparticles.

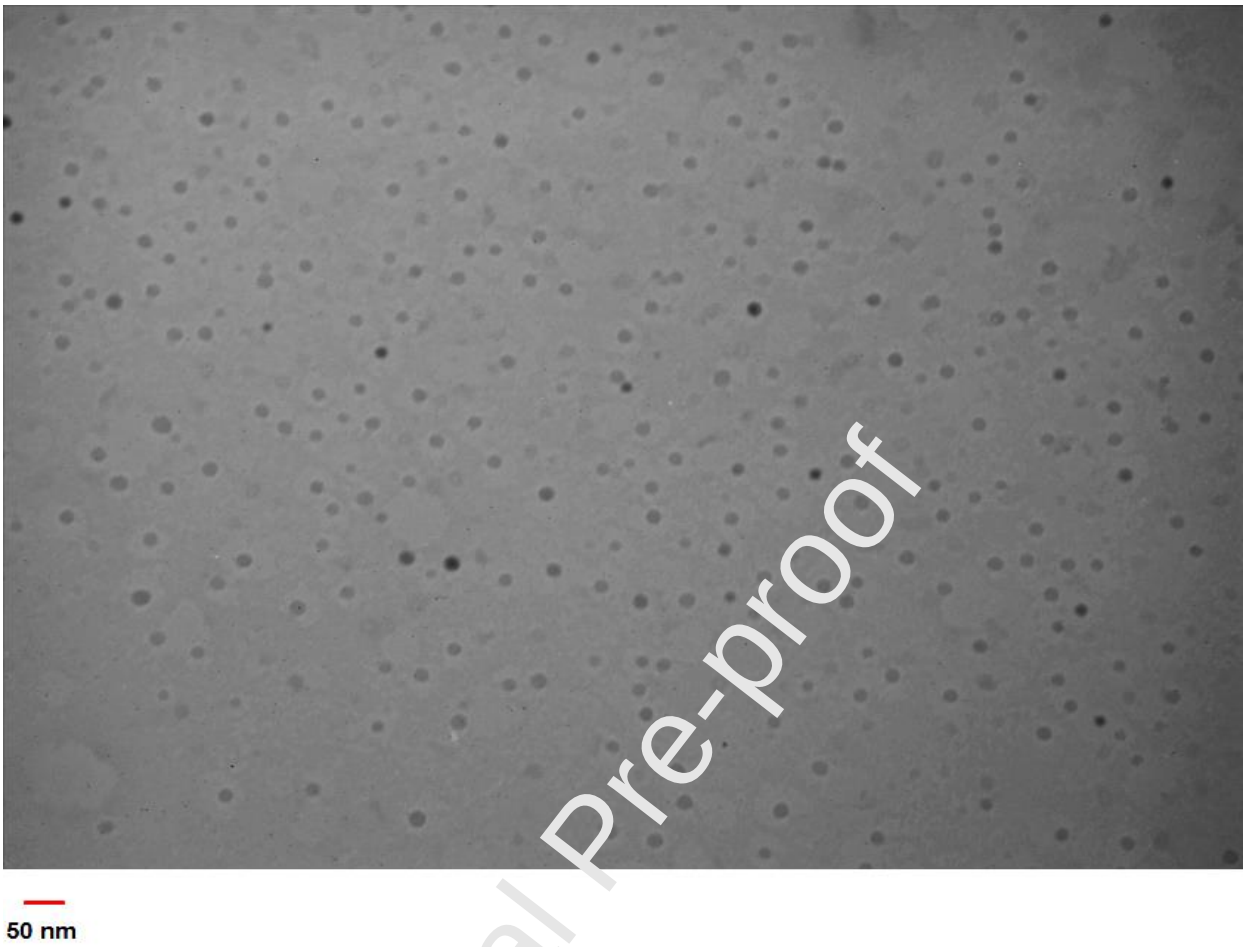

Figure1: TEM image of AgNPs

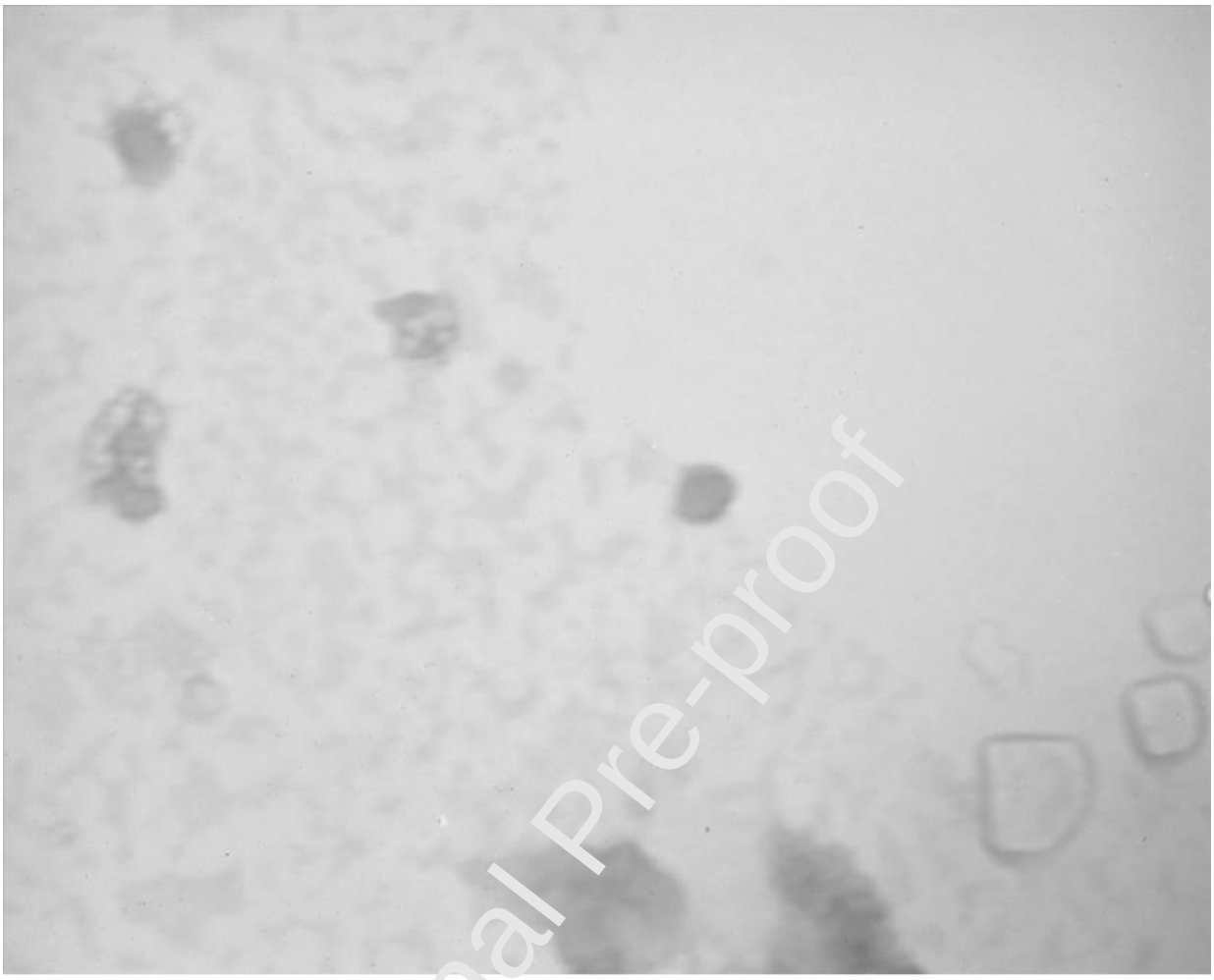

Figure2: TEM image of NOCCNPs

1.3.2 Cytotoxicity assay results

The cell viability was determined by using MTT assay. The percentage of viable cells was calculated as follows:

$$viability\ \% = \frac{(\text{OD sample} - \text{OD blank})}{(\text{OD control} - \text{OD blank})} \times 100$$

To identify the cell viability of stem cells after exposed to [Chitosan, NOCC, NOCCNPs (ChNPs), CNC, CNG, AgNPs, ANC and ANG], stem cells were treated to the materials (0/01 –

2.5 mg/ml) at 24, 48 and 72 hours.

Concentrations of the materials was chosen based MIC rates of them against *Acinetobacter baumannii* and *Pseudomonas aeruginosa*.

As shown in Figure3, Chitosan, ChNPs and CNC in their effective MIC doses had no toxic and reduction effects on cell viability of stem cells at 24, 48 and 72 hours.

Although ANG had no toxic effect on stem cells in concentration of 0/01 – 2.5 mg/ml at 24 hour, it showed toxic properties at 48 and 72hours.

In contrast above mentioned results, we saw reduction in cell viability of stem cells after exposure to 0/01 – 2.5 mg/ml of [NOCC, AgNPs, ANC and ANG] at 24, 48 and 72 hours (Figure 4).

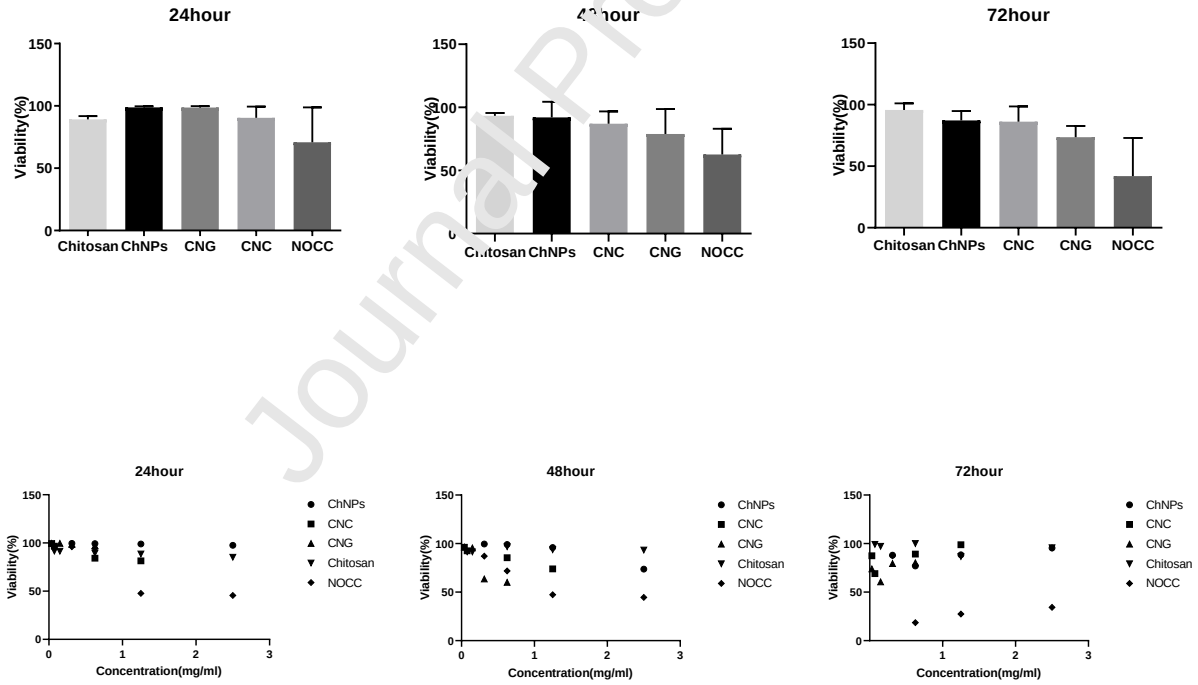

Figure 3: Cell viability of stem cells on (chitosan, ChNPs (NOCCNPs), CNG, CNC and NOCC) after 24, 48 and 72 hour.

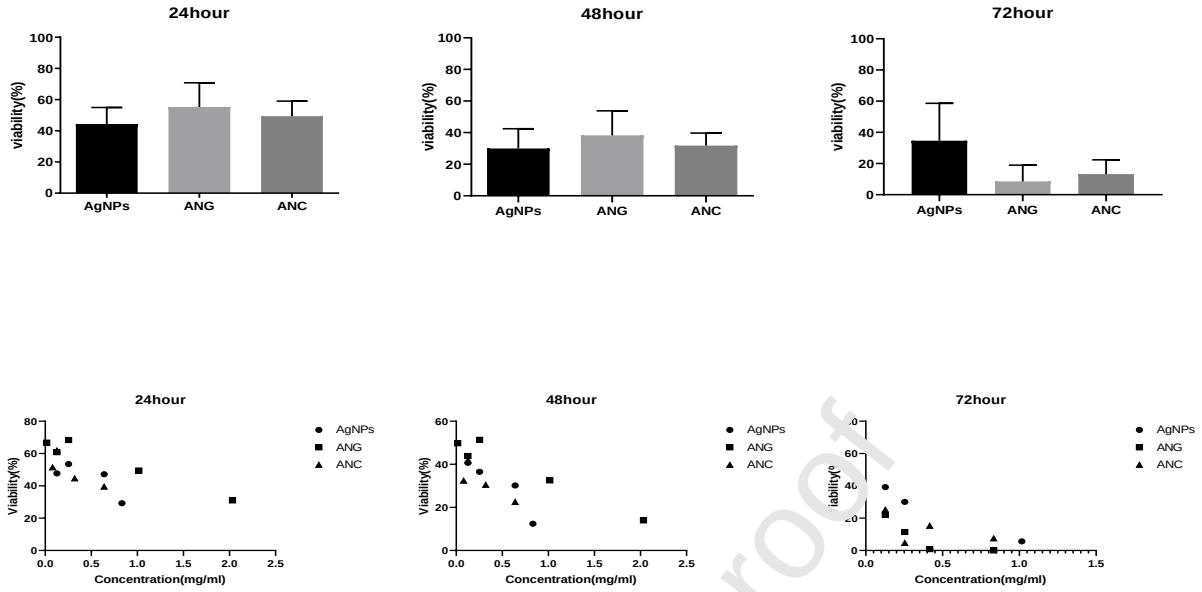

Figure 4: Cell viability of stem cells on (AgNPs, ANG and ANC) after 24, 48 and 72 hour.

1.3.3 MIC level of ciprofloxacin and gentamicin after exposing to nanoparticles in *Acinetobacter baumannii* and *Pseudomonas aeruginosa* at Sub-MIC concentration of them

MIC range of chitosan, CNC, CNG, AgNPs, ANC and ANG, ciprofloxacin and gentamicin against *Acinetobacter baumannii* and *Pseudomonas aeruginosa PAO1* were measured separately. Each MIC range was assayed in triplicate.

A.baumannii and *PAO1* in the final concentration of (1.5x 10^5CFU/ml) were treated with nanoparticles at their sub-MIC concentration **(**0.5×MIC) of chitosan, CNC, CNG, AgNPs, ANC and ANG, and ciprofloxacin and gentamicin for 24h at 37°C.

After bacterial exposure to the mentioned materials, MIC rates of ciprofloxacin and gentamicin against *A.baumannii* and *PAO1* were determined.

Before exposure to the mentioned materials, MIC rates of gentamicin and ciprofloxacin against *A.baumannii* was 16 and 128 (µg/ml) respectively.

However, the MIC ranges of gentamicin against *Acinetobacter baumannii* was increased 16 fold

after exposure to gentamicin and AgNPs and increased 8 fold after exposure to chitosan, ANC and ANG and increased 4 fold after exposure to CNC and CNG.

We did not see any changes in MIC rate of ciprofloxacin after exposing to antibacterial materials in clinical *A.baumannii* after exposure to mentioned substances.

MIC rates of gentamicin and ciprofloxacin against *PAO1* before exposure to the mentioned materials was 0.5 and 0.25(µg/ml) respectively.

However the MIC ranges of gentamicin against *PAO1* was increased 4 fold after exposure to gentamicin, AgNPs, and ANC, and increased two fold after exposing to CNC, CNG and ANG. Also, MIC rate of ciprofloxacin against *Pseudomonas aeruginosa PAO1* was increased 4 fold after exposure to ANC, chitosan, ANG, CNC, CNG and AgNPs.

The amount of MIC in bacteria before and after exposing to antibacterial materials has been shown in Table 2 and 3.

Table 2: MIC of ciprofloxacin and gentamicin against *A.baumannii* before and after exposure to nanoparticles and antibacterial agents

***A.baumannii* exposed to**	**MIC of Gentamicin (µg/ml)**	**MIC of ciprofloxacin (µg/ml)**
No exposure	16	128
Ciprofloxacin	-	128
Gentamicin	256	-
Chitosan	128	128
CNC	64	128
CNG	64	128
AgNPs	256	128
ANC	128	≥128
ANG	128	≥128

*AgNPs (silver nanoparticles), ANC (silver NPs + ciprofloxacin), ANG (silver NPs + gentamicin), CNC (N, O-carboxymethyl chitosan nanoparticles + ciprofloxacin) and CNG (N, O-carboxymethyl chitosan nanoparticles +

gentamicin).

Table 3: MIC of ciprofloxacin and gentamicin against *Pseudomonas aeruginosa* PAO1 before and after exposure to nanoparticles and antibacterial agents

***P.aeruginosa* exposed to**	**MIC of Gentamicin (µg/ml)**	**MIC of Ciprofloxacin (µg/ml)**
No exposure	0.5	0.25
Ciprofloxacin	-	1
Gentamicin	2	-
Chitosan	0.5	1
CNC	1	1
CNG	1	1
AgNPs	2	1
ANC	2	1
ANG	1	1

*AgNPs (silver nanoparticles), ANC (silver NPs + ciprofloxacin), ANG (silver NPs + gentamicin), CNC (N, O-carboxymethyl chitosan nanoparticles + ciprofloxacin) and CNG (N, O-carboxymethyl chitosan nanoparticles + gentamicin).

1.3.4 Expression rates of *abeM* before and after exposure to synthetic materials in *A.baumannii*

Expression of *abeM* efflux pump genes was decreased after exposure to chitosan (0.2-fold), chitosan NPs (0.05-fold), CNC (0.003-fold), CNG (0.009-fold), and ANG (0.5-fold), while an increased expression was noticed after exposure to ciprofloxacin (12-fold), AgNPs (2-fold), and ANC (4-fold). The results are shown in Figure 5.

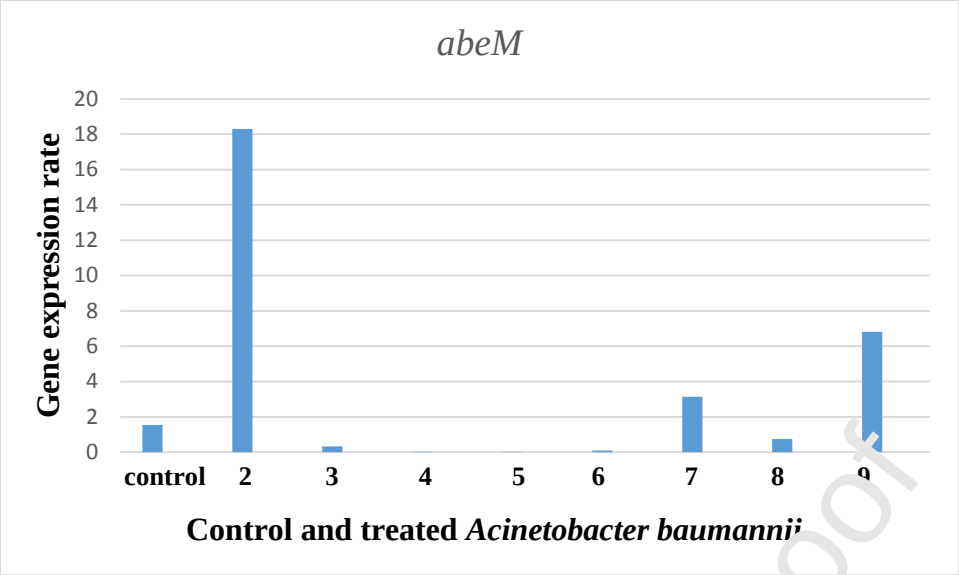

Fig5: Expression rates of *abeM* in *Acinetobacter baumannii* before and after exposure to antibiotics and nanoparticles (NPs).

Control: Expression rate of efflux pumps in clinical *Acinetobacter baumannii* (Ab).
2- Expression rate of efflux pumps in Ab exposed to ciprofloxacin.
3- Expression rate of efflux pumps in Ab exposed to chitosan. 4- Expression rate of efflux pumps in Ab exposed to chitosan nanoparticles with ciprofloxacin (CNC). 5- Expression rate of efflux pumps in Ab exposed to chitosan nanoparticles with gentamicin (CNG). 6- Expression rate of efflux pumps in Ab exposed to chitosan NPs. 7- Expression rate of efflux pumps in Ab exposed to silver nanoparticles (AgNPs). 8- Expression rate of efflux pumps in Ab exposed to silver nanoparticles with gentamicin (ANG). 9- Expression rate of efflux pumps in Ab exposed to silver nanoparticles with ciprofloxacin (ANC).

1.3.5 Expression rates of *mexY* before and after exposure to antibiotics in PA01 *P.aeruginosa*

A decreased level of expression was observed for the *mexY* gene after exposure to chitosan (0.06-fold), CNC (0.02-fold), CNG (0.05-fold), and ANC (0.05-fold), but an increased expression was noted after exposure to ANG (3.8-fold), AgNPs (1-fold), and ciprofloxacin (3.8-fold). The results are shown in Figure 6.

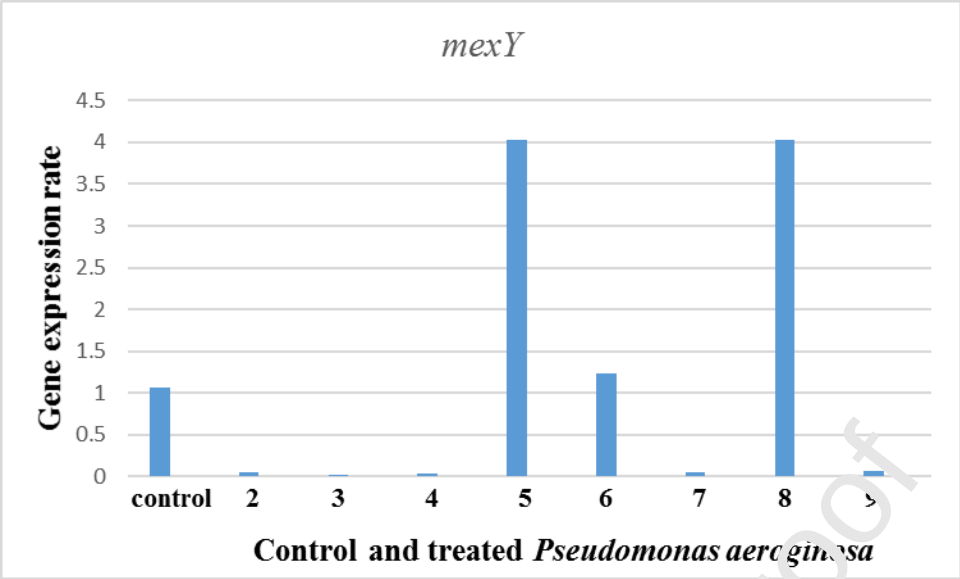

Fig6: Expression rates of *mexY* in *Pseudomonas aeruginosa* PAO1 before and after exposure to antibiotics and nanoparticles (NPs)

Control: Expression rate of efflux pumps in *Pseudomonas aeruginosa* (PAO1).

2- Expression rate of efflux pumps in PAO1 exposed to chitosan nanoparticles with gentamicin (CNG). 3- Expression rate of efflux pumps in PAO1 exposed to chitosan nanoparticles with ciprofloxacin (CNC). 4- Expression rate of efflux pumps in PAO1 exposed to chitosan NPs.

5- Expression rate of efflux pumps in PAO1 exposed to silver nanoparticles with gentamicin (ANG). 6- Expression rate of efflux pumps in PAO1 exposed to silver nanoparticles (AgNPs).

7- Expression rate of efflux pumps in PAO1 exposed to silver nanoparticles with ciprofloxacin (ANC). 8- Expression rate of efflux pumps in PAO1 exposed to ciprofloxacin. 9- Expression rate of efflux pumps in PAO1 exposed to chitosan.

1.4 Discussion

Nanotechnology has found many applications in the medical fields such as drug delivery, biosensors, and medical imaging. Their small size and large surface area enriches their potentiality to deliver drug in the intracellular uptake and extensive structural stability of them helps in their delivery to the targets for an extended period without degradation (38-40). However, inflammation and toxicity are some of the constraints encountered due to reactive oxygen species and chemical reducing agents (41). *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are the two opportunistic pathogens of great medical concern because of their association with nosocomial infections and increasing resistance to many antibiotic classes (3, 42, 43). The up-regulation of RND-type efflux pump in *P.aeruginosa* and *A.baumannii* is one of

the important factors accountable for the multidrug-resistance in these bacteria (44, 45). One of the options suggested is to use efflux pump inhibitors (EPI) to block efflux pumps activity in MDR bacteria (46). Silver has been used as a broad-spectrum antimicrobial and anti-biofilm agent in eye drops to prevent trachoma as well as topical creams to heal burn wounds (47-50). Researchers have shown that AgNPs cause more destruction permeability affecting respiratory activities of cell membranes and collapse of the proton motive force that can affect efflux pump activity (51, 52). Chitosan, is a derivative of chitin with non-toxic bacteriostatic effect has antibacterial effectives against a broad spectrum of bacteria (53, 54)Ch. R. Randall et al. also demonstrated increasing resistance to silver after 6 days of exposure because of loss of OmpC/F porins and activation of CusCFBA efflux pump in *Escherichia Coli* (55). High levels of MIC of silver nanoparticles is due to continues use of Ag NPs in purification of air/water, textile products, wound dressing, food packaging and poultry in the world(56). . Other studies indicated that metal nanoparticles can disrupt proton motive force (PMF) of many bacteria that is essential for efflux pump activity (57-59) Another study has demonstrated that the cell membrane morphology (using TEM and SEM) of *P. aeruginosa* and *S. aureus* was changed after exposure to silver-coated carbon nanotubes (AgCNTs) and AgNPs compared with the cell membrane of non-treated bacteria (60, 61). Any change in cell membrane of the bacteria may be cause of the down regulation of efflux pumps. Oliver Gordon et al. analyzed genes expression related to respiratory chain, glycolysis, TCA cycle, iron hemostasis, oxidative stress response, cell wall and biofilm by microarray method. They demonstrate after treatment of S*taphylococcus epidermidis* with silver, respiratory chain was blocked and genes of TCA cycle enzymes coding were downregulated(62). As we know the proton motive force is very important as energy source for efflux pumps(8). They also showed the *dltABCD* operon which is important in biofilm formation upon silver

treatment was upregulated. Also *lytS* , *lytR, sitABC* and *feoAB* were downregulated after treatment with silver(62). As in the current study, down regulation of efflux pump was shown after exposed the bacteria to silver nanoparticles and upregulation of *abeM* was seen after exposed to silver nanoparticles loaded to ciprofloxacin (ANC) and upregulated of *mexY* was seen after exposed to silver nanoparticles loaded to gentamicin (ANG). In the other study hydroxyl propyl trimethyl ammonium chloride chitosan loaded with poly methyl methacrylate PMMA bone cement showed downregulation of the expression of *icaAD* and *mecA* in *Staphylococcus* spp by using Real-time PCR (63). In the other study electron microscopy showed that the positive charge of chitosan can destroy and alter the negatively-charged bacterial cell wall and can help lose the barrier function of the bacteria (64). Although there are a few research about the effect of nanoparticles against the gene expression of bacteria, in the current study gene expression of *abeM* and *mexY* was downregulated after exposed to chitosan and its combination to gentamicin and ciprofloxacin respectively, but upregulated of *abeM* was seen after exposed to silver nanoparticles loaded to ciprofloxacin (ANC) and upregulated of *mexY* was seen after exposed to silver nanoparticles loaded to gentamicin (ANG). That is showed chitosan has better efflux pump inhibitory effect than AgNPs. In addition, chitosan can inhibit transcription and translation in bacteria by binding to DNA (65). That is cause of down regulation of efflux pumps in our study. Dosunmu E et al. exposed silver-coated carbon nanotubes (AgCNTs) against *Pseudomonas aeruginosa* reported that the expression levels of virulence genes such as *lasA, prtR, mexR, RpoS,* creD, mexT, and rpoS were down-regulated, but gentamicin-treated strains showed an upregulation of gene expression, except for *oprD* gene expression (60). Wen-Ru Li et al. also demonstrated that after exposing *Staphylococcus aureus* to AgNPs, the expression level of acetyltransferase was increased and the expression levels of a glycerol-3-

phosphate dehydrogenase and ABC transporter ATP-binding protein, and recombinase A protein were decreased (66). Hiroaki Saito et al. conjugated chitosan with lysozyme then studied the antibacterial activity of conjugated chitosan against *A.baumannii* and *P.aeruginosa*. (67), MIC values of conjugated chitosan 200 μg/mL in *P.aeruginosa PAO1*. While in our study MIC values of chitosan after conjugated with gentamicin (CNG) was 10 μg/mL in PAO1. That is showed chitosan in combination to gentamicin, have very good antibacterial effect against PAO1. In another study silver nanoparticles was conjugated with ceftriaxone then antimicrobial effects was calculated by disc diffusion method against *Bacillus subtilis*, *Klebsiella pneumonia*, *Staphylococcus aureus* and *Salmonella typhi*. They showed conjugated AgNPs with ceftriaxone had better antimicrobial effect than AgNPs(68). In the current study cysteine was used as stabilizer for AgNPs then the chemical combination of antibiotics and nanoparticles was done by creating peptide bonds between carboxyl grope of cysteine and amine grope of antibiotics. Unexpectedly we did not show significant reduction of MIC combination of AgNPs with antibiotics (ANC and ANG) in comparing to AgNPs. Although down regulation of *abeM* and *mexY* was seen after treatment with AgNPs, MIC rates of AgNPs against *Pseudomonas aeruginosa and A. baumannii* was high compared to other studies. That is maybe because of the activation of resistance mechanism because of variation in outer membrane (62) and maybe any variation in efflux pumps.

In the present investigation, the MIC rate of ciprofloxacin and gentamicin against *PAO1* was found to increase after exposure to respective nanomaterial. Compatible result was observed for the MIC of gentamicin against *Acinetobacter baumannii* after exposure to the antibiotic while, the MIC rate of ciprofloxacin against *Acinetobacter baumannii* remained same before and after exposure to antibacterial agent. Similar to our study, Kaweeteerawat C et al. demonstrated

increased antibiotic resistance of penicillin, chloramphenicol, kanamycin, ampicillin in *Escherichia coli* and *Staphylococcus aureus* by 3–13-fold after exposing the bacteria to sub MIC dose of nanoparticles. They also demonstrated that AgNPs by inducing intracellular ROS can increase bacterial resistance to antibiotics.(69). Other study by Christena LR(57) showed CuNPs at 1× MIC level and 0.5 × MIC level had an efflux inhibitory effect in wild type, MRSA and MDR strains of *Staphylococcus aureus* and wild type *Pseudomonas aeruginosa*. They also showed that CuNPs could decrease the MIC level of ciprofloxacin in the mutant *Staphylococci aureus* by 4 fold (from 64 µg/ml to 16 µg/ml). In another study by Lowrence Rene Christena LR et al. CuNPs treatment could not completely desensitized MDR strain of *E.coli* against ciprofloxacin that is because of the presence of chromosomally encoded Quinolone Resistance genes (57). In the other study hydroxyl propyl trimethyl ammonium chloride chitosan loaded with PMMA bone cement showed downregulation of the expression of *icaAD* and *mecA* in *Staphylococcus* spp by using Real-time PCR (63).

In another study, the MIC value of sulfamethoxazole decreased five-fold in the highly expressive MexEF-OprN efflux pump after exposure to chitosan along with sulfamethoxazole (70). Ma Z et al. showed increasing in MIC levels of kanamycin, ampicillin and tetracycline up to two fold after treating *E. coli* O157:H7 with ampicillin at 0.25X MIC. Whereas they did not show any increase in the MIC levels of that antibiotics after exposing of *E. coli* O157:H7 to 0.25X MIC of chitosan micro particles. (25). In contrast with them, our study showed that the MIC level of gentamicin in *Acinetobacter baumannii* was increased 16-fold (after exposure to antibiotics and AgNPs at 0.5×MIC), 8-fold (after exposure to chitosan, ANC, and ANG at 0.5×MIC), and 4-fold (after exposure to CNC and CNG at 0.5×MIC). MIC level of gentamicin in *PAO1* was increased 4-fold (after exposure to gentamicin, AgNPs and ANC at 0.5×MIC)

and 2-fold (after exposure to ANG, CNC and CNG at 0.5×MIC), and the MIC level of ciprofloxacin was increased 4-fold after exposure to the mentioned materials in *PAO1*.

In our previous study, MDR bacteria were correlated with PAβN as an efflux pump inhibitor, and then the expression of the efflux pump was investigated (33). Some of the bacteria that showed one- to two-fold reduction in their MIC against ciprofloxacin and levofloxacin exhibited increased levels of *mexY* expression (33), while in the current pilot study, bacteria were exposed to nanoparticles and their efflux pump expression and MIC levels were calculated. In contrast with our previous study, even though a decrease in the expression level of the efflux pump was seen, the MIC levels of ciprofloxacin and gentamicin were increased. This result may have been because of the efflux pump inhibitor effects of nanoparticles (used in this study) against bacteria. On the other hand, even though efflux pump expression was decreased in correlation with nanoparticles, other antibiotic resistant genes may become active because of exposure to nanoparticles.

1.5 Conclusion

In this study the effect of chitosan and sliver nanoparticles and their combination with ciprofloxacin and gentamicin was studied on the expression level of efflux pumps in *Acinetobacter baumannii* and *Pseudomonas aeruginosa.* In addition the MIC level of ciprofloxacin and gentamicin was checked after exposing the bacteria to the above mentioned substances. Clinical antibiotic resistant *A. baumannii* and *P.aeruginosa* (PAO1) were exposed to the nanoparticles in their sub MIC concentrations. The efflux pump expression of *abeM* and *mexY* was observed to decrease after exposing to chitosan and its combination to antibiotics. While the efflux pump expression of *mexY* was increased after exposing to ANG, it was decreased after exposing to ANC and also the efflux pump expression of *abeM* was increased

after exposing to ANC and decreased after expose to ANG.

The above experiment showed chitosan nanoparticles, CNC and CNG have anti efflux pumps impact in *A. baumannii* and *P.aeruginosa.* Overall, the expression levels of all 2 efflux pumps in this study were decreased after exposure to AgNPs, chitosan and its combination with antibiotics. Thus, they may be a good candidate for efflux pump inhibitor to be used in research and clinical laboratories. While decreasing expression of efflux pumps was observed, the MIC rate of them had been higher than before exposing, which may be because of activation of other resistant genes. Thus, it is necessary to study other resistance mechanisms and effect of nanoparticles to inhibit them before nanoparticles step in the treatment strategies.

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Highlights

- Chitosan and its nanoparticle form in combination with antibiotics (ciprofloxacin and/or gentamicin) have ability to decrease the efflux pump expression of *abeM* in *A. baumannii* and *mexY* in *P. aeruginosa.*
- AgNPs in combination with gentamicin have the potentiality to decrease the efflux pump expression of *abeM* in *A. baumannii* and in combination with ciprofloxacin can decrease the efflux pump expression of *mexB* in *P. aeruginosa.*
- Overall chitosan and silver nanoparticles may be trico for reducing the efflux pump activity however, in accurate concentration to find their effect either alone or with antibiotics in lowering the resistance.