Nanomed Res J 6(2):179-187, Spring 2021

RESEARCH ARTICLE

Overcoming the antibiotic resistance of Acinetobacter *baumannii* by using nanofluid containing functionalized carbon nanotubes

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ARTICLE INFO

Article History:
Received 17 Jan 2021
Accepted 01 Apr 2021
Published 01 May 2021

Keywords:
antimicrobial effect
Acinetobacter baumannii
carbon nanotubes
nanofluid
antibiotic resistance

ABSTRACT

Objective(s): Acinetobacter baumannii is crucial for healthcare-associated diseases by significant local variations in the resistance scale, but its risk factors and infection courses have not been adequately studied. Carbon nanotubes are substantially circular molecules made entirely from carbon atoms and can use as nanocarriers. Through their unique characteristics, multi-wall carbon nanotubes hold great promise in the fight against multidrug-resistant bacterial infections. In this research, antimicrobial effects study and the ability to overcome antibiotic resistance evaluation of nanofluid containing functionalized carbon nanotubes on A. baumannii were done.

Methods: Multi-wall carbon nanotubes provided from the United States Research and the nanofluid prepared after carbon nanotube functionalization. Microplate, Alamar Blue Cell viability assay, carried out after incubation of A. baumannii affected with the nanofluid (100µg/ml) for 24h.

Results: Antimicrobial effect of functionalized carbon nanotubes nanofluid was found on the A. baumannii in a dose-specific concentration manner

Conclusions: This study showed that functionalized carbon nanotubes nanofluid could have antimicrobial effects on A. baumannii by overcoming bacterial antibiotic resistance. Although to get more accurate results, to prevent nosocomial infections, more specific cellular and molecular studies are necessary.

How to cite this article
Yazdani M.R., Sheikhpour M., Siadat S.D., Safarian P. Overcoming the antibiotic resistance of Acinetobacter baumannii by using nanofluid containing functionalized carbon nanotubes. Nanomed Res J, 2021; 6(2): 179-187. DOI: 10.22034/nmrj.2021.02.009

INTRODUCTION

Acinetobacter is a compulsory gram-negative, aerobic coccobacillus, essential soil bacteria, and prefers life in wet environments [1, 2]. The bacterium was previously classified as a Neisseria family, but later it was determined by the RNA analysis, as part of the Moraxella family. The most important species of this genus are *Acinetobacter Baumannii(A. baumannii*), which generates different infections like pneumonia, urinary tract diseases, skin infections, ulcers, and meningitis. The rate of expansion of *A. baumannii* into hospitalized cases, particularly in those with prolonged hospitalization or extensive antibiotic or anticancer treatment, is increasing [3]. One of the problems with *A. baumannii* is the emergence of drug-resistant strains to antibiotic classes such as beta-lactams, aminoglycosides, and fluoroquinolones. These resistances are further negotiated with genes placed on moving genetic elements such as transposons and introns and are quickly spread between bacteria [4]. *A. baumannii* is one of the most critical factors in the development of hospital infections around the world. *A.baumannii* could be isolated from all samples collected from the soil and water surface, so it can say that they are present everywhere. Most of the Acinetobacter species isolated from human clinical samples have at least several characteristics being human

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pathogens. *A. baumannii* has also lately caused a variety of infectious symptoms in army personnel wounded into the struggles [5]. Meningitis is one of the diseases most likely to occur by gram-negative pathogens, which is caused by *A. baumannii*, which produces 70% mortality [6].

A. baumannii causes 1.6% of diseases in the ICU, but in the non-ICU, 0.9% causes infections, the mortality rate caused by *A. baumannii* in the ICU environment 34% - 43.4%, and in the outside of the ICU was 32.3%. *A. baumannii* is one of the common reasons for urinary tract diseases, which is accountable for 1.6% of urinary tract infections in ICU. Usually, the organism is linked to diseases caused by the use of catheters [3].

The cases may possess multiple risk factors for pneumonia, including mechanical ventilation, continued consumption of the underlying cardiac also pulmonary conditions, reduced gastric acidity, and immune deficiencies. Also, the microorganisms faced by a person in the hospital are often different from those facing the community [3, 7]. In studies conducted on ICU patients, it has been found that 5 to 10% of these people are affected by the presence of *A. baumannii* in pneumonia, and there is no suspicion that ventilator-dependent pneumonia (VAP) occurs due to opportunistic pathogens [6]. Nosocomial infections are associated with a new disease that affects a person in nosocomial, which is a problem in the ICU's intensive care unit than in other areas [8].

One of the most critical problems with nosocomial infections is the final release of drug resistance. Resistance to antimicrobial agents has spread to a wide range of pathogens, especially pathogens involved in nosocomial infections [9].

In recent studies, the treatment of Acinetobacter infections was possible with several antibiotics like beta-lactams, aminoglycosides, and tetracycline, but now resistance to all known antibiotics is visible in *A. baumannii* [10]. A new therapeutic strategy presented in the world and one of the most important and least costly procedures is the use of nanoscience in the treatment of diseases or the addition of nanomaterials in hospital disinfectants. Nowadays, nanoparticles, especially carbon nanotubes, are used to treat infections as well as cancers [11].

The widespread arrangement of antimicrobial resistance mechanisms was reported for Acinetobacter spp. There is similar to that of other non-fermented gram-negative pathogens. Fast resistance of the *A. baumannii* strains through every beta-lactam, such as carbapenems, indicates the organism's potential in responding rapidly to differences into careful pressure. [12]. The efflux systems are widely found in microorganisms, and in the same order, by pumping antibiotics from the bacteria into where the outside by ATP, cause increased antibiotic resistance. These multiple transmitters can divide into four different groups: The primary facilitator (MF), some small multidrug resistance (SMR), the multidrug also toxic mixture extrusion (MATE), and This resistance-nodule-cell division (RND) group. *AbeM* is part of the MATE pump family, and aminoglycosides are substrates for the *AbeM* pump. The *TetA* gene is a family of MF pumps that is responsible for code-packing ampicillin and tetracycline secretions pumps [13, 14]. Nanoparticles are produced from a wide range of materials and exhibit different shapes [15]. By increasing the level of metal particles, the effectiveness of these materials in special bio-applications, such as the use of silver nanoparticles in antimicrobial applications, also increases [16]. Also, the nanoparticles have unique optical, electrical, chemical, mechanical properties, and so on [15]. Carbon nanotubes nowadays have many medical applications, such as biosensors and drug delivery systems [17-19].

Recently, nanofluids that contain suspensions of metallic or non-metallic materials have been reported as potential anticancer and antimicrobial agents and these properties make them essential in industry and medical sciences [20, 21]. Also, the use of nanoparticles in a suspension form of nanofluids increases their stability and decreases their agglomeration in drug delivery conditions. In this investigation, the study of the antimicrobial effects of functionalized carbon nanotubes nanofluids about *A. baumannii* was done. Then the bacteria were treated with different doses of nanofluid and antibiotic to overcoming the bacterial antibiotic resistance. Then, the gene expression pattern of two genes *TetA* and *AbeM* which belong to the efflux pump system of this bacteria was evaluated in treated pathogen bacteria with different doses of antibiotics, MWCNTs nanofluid, and functionalized MWCNTs nanofluid. Finally, Transmission Electron Microscopy (TEM) observation was done on successfully treated individuals.

MATERIALS AND METHODS

Sample Collection

In this study, a standard strain from the *A. baumannii* with the ATCC code (BAA-7U7) and the pathogen strain were prepared from the microbial bank of mycobacteriology and Pulmonary Research Department, Pasteur Institute of Iran. Samples were re-cultivated and isolated in an agar medium and the Analytical Profile Index (API) test was done to determine the definitive identity and diagnosis [22].

Functionalization of carbon nanotubes

Carbon Nanotubes prepared from United States Research. For modification of Multi-Walled Carbon Nano Tubes (MWCNTs) with a COOH group, 0.5 g of MWCNTs was involved within an 80 ml mix of **H2SO4/HNO3** (3:1, v/v) at 70°C to several times following consecutive sonication. Then the black solid recovered after filtration, washed many times by distilled water also dried at 80°C into a vacuity for eight hours [23, 24]. The characterization of obtained carboxyl Multiwalled carbon Nanotube, MWCNT (COOH) was done by TEM and X-ray diffraction.

Preparation of nanofluid

0.2 g of functionalized powder was added to 100 ml of deionized water slowly, on the Magnetic Stirrer. 6 ml of ethanol and add 0.06 g of Arabic gum were added to the solution and the total solution was stirred for 20 minutes. Then the suspension was transferred in a frozen container to the ultrasonic device (Ultrasonic Homogenizer 400w, 200 kHz), with the power of 200W for 20 minutes.

Effect study of MWCNT and MWCNT(COOH) on standard and pathogen strains of A. baumannii

Standard and pathogen strain suspensions were prepared based on 0.5 McFarland in TSB fluid medium. Then they were incubated with different concentrations of 0.5, 1, 2, and 4 mg/ml from nanofluids containing MWCNT and also MWCNT (COOH) for 24 hours at 37 ° C in a shaker incubator.

Antibiogram

Bacteria were grown in an agar supplemented with a turbidity equivalent to 0.5 McFarland. Then, sterilized suspensions were inoculated into three different directions on the Muller Hinton Agar medium. The antibiotic sensitivity of the standard and pathogen strains was done with the disk diffusion method; the used antibiotic discs for these bacteria were ampicillin, colistin, and meropenem. After 24 hours of contamination, the observed zone of inhibition results was confirmed conformably to the Clinical and Laboratory Standards Institute (CLSI) instruction, and ampicillin was selected for antibiotic resistance and nanofluid effect evaluation.

Minimum Inhibitory Concentration (MIC)

Bacterial suspensions were been ready based on 0.5MacFarlend's turbidity and then the serial dilution of antibiotic in dilutions of 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 µg/ml was prepared. In the following 100 µl of bacterial suspensions were added to each microplate well, and were treated with different dilutions of antibiotics and the microplate was end incubated at 37°C for 24 hours. Each sample was repeated triplet and after that, the turbidity test was done for each well [25].

Minimum Bactericidal Concentration (MBC)

For all treated pathogen and standard bacteria, samples consist of; all dilutions of antibiotics alone, non-functionalized MWCNT nanofluid alone, Functionalized MWCNT nanofluid alone, and combination of antibiotics with each of them, culture on the Muller Hinton agar medium was done. After incubation of samples at 37°C for 24 hours, the results of bacterial growth were observed.

RNA extraction, cDNA synthesis, and Real-Time PCR

The total RNA of all samples extracted utilizing that DNA-Technology PREP-NA DNA / RNA Extraction Kit kit (REF: P-002 / 1INT) extraction kit according to its instructions. cDNA synthesis was done by using the cDNA Synthesis kit (Cat No: YT4500) and gene expression pattern studies were performed for *TetA* and *AbeM* genes by use of related primers with the Real-time PCR method (Table 1).

Table1. The primer sequences for the *16srRNA* (control), *TetA*, and *AbeM* genes.

Name	Sequences5′-3′
16srRNA F	CGGACGGGTGAGTAACGCGTGA
16srRNA R	GCTAGGACTACWGGGGTAT
TetA F	GCTACATCCTGCTTGCCTTC
TetA R	CTGCCTGGACAACATTGCTT
AbeM F	TTAACGGTTGGTGAGGTTGC
AbeM R	CACCACTAGAGTAAAACGGCG

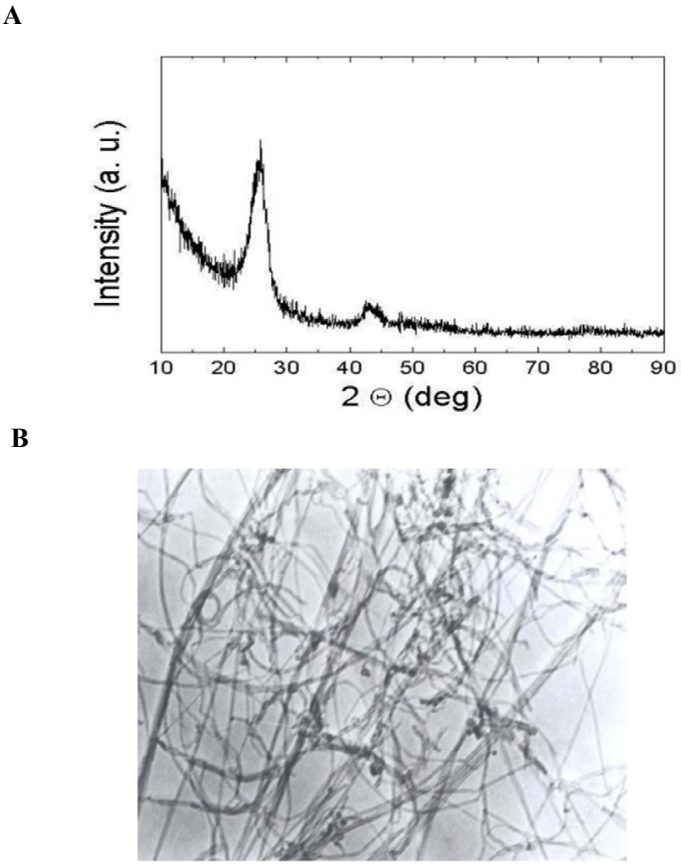

Fig. 1. A) x-ray diffraction pattern and B) Transmission Electron Microscopy (TEM) of functionalized MWCNTs.

RESULTS

Characterization of functionalized carbon nanotubes

As shown in Fig. 1. A, and B, The functionalization of CNTs was approved by X-ray diffraction pattern and Transmission Electron Microscopy (TEM).

Effect study of MWCNT and MWCNT(COOH)

As shown in Fig. 2 in both standard and pathogen strains, functionalized CNTs nanofluid had a substantial influence against the reduction of bacterial growth to a combination of 4mg/ml. For this reason, this concentration of nanofluid was been used for antibiotic-resistant studies.

The results of the antibiogram

The results of the disk diffusion agar method according to the CLSI table showed that the standard strain is resistant to ampicillin and sensitive to meropenem and colistin. The pathogenic strain is also resistant to ampicillin and meropenem and is sensitive to colistin (Fig. 3).

MIC & MBC tests

The bacterial resistant study of standard and pathogen strains was done by the use of ampicillin which was observed that both strains have resistance to it based on CLSI. The strains were treated with different doses of ampicillin at concentrations of 8 µg/ml up to 1024 µg/ml alone, in combination with MWCNTs nanofluid and functionalized MWCNTs nanofluid separately. After the MIC tests, all of the treated wells were confirmed by MBC tests. So, the treated bacteria were cultured on Müller Hinton agar medium also incubated for 24h at 37° C. Then the results of the bacterial growth rate in these treatment conditions were compared to each other. Lack of standard strain bacterial growth was observed because of the antibiotic effect from the concentration of 128µg/ml to 1024 µg/ml. whereas the pathogen strain has resistant to this concentration range. For standard bacteria treated with antibiotics

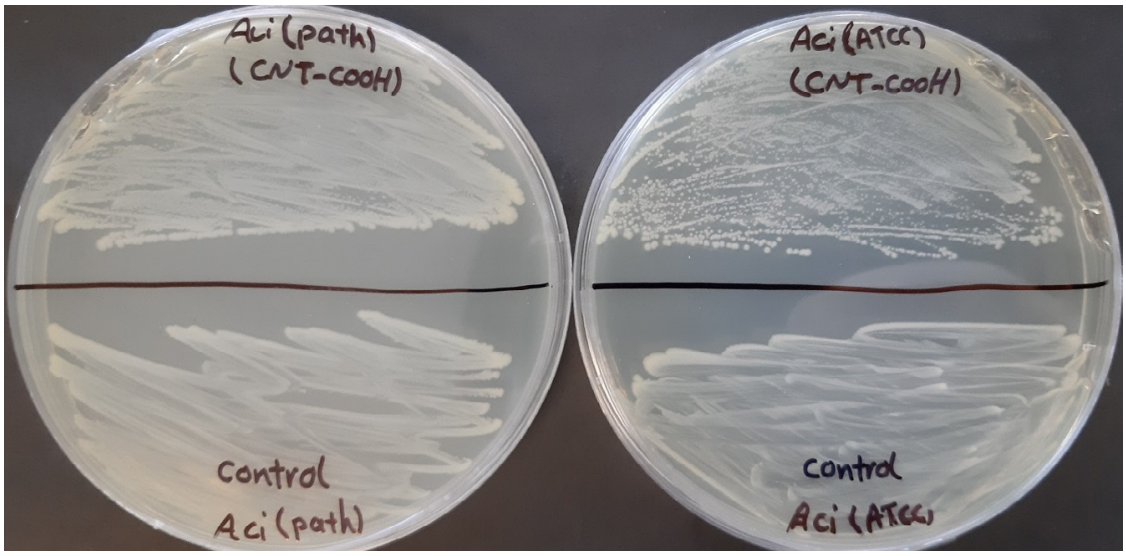

Fig. 2. Nanofluid containing functionalized carbon nanotubes reduces bacterial growth at a concentration of 4mg/ml. The left Fig. is a pathogen and the right Fig. is a standard strain. Aci: *A. baumannii*, CNT-COOH: Functionalized carbon nanotubes, ATCC: Standard strain, code (BAA-7U7), Path: Pathogen strain, Control: Non-treated group.

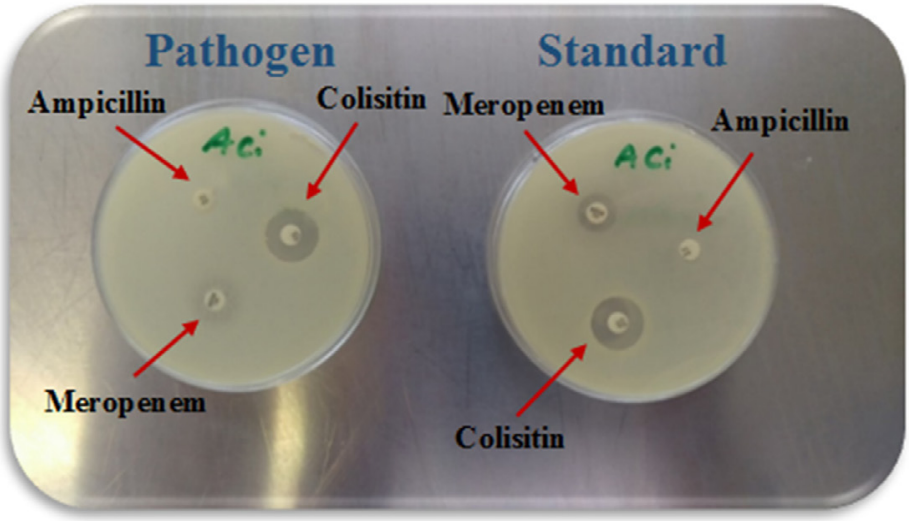

Fig. 3. Antibiogram testing by disk diffusion agar method for pathogen and standard strains of *A. baumannii.*

and MWCNTs nanofluid, it was found that the concentrations of 64 µg/ml to 1024 µg/ml were effective. Also, in the presence of functionalized MWCNTs nanofluid beside the antibiotic the standard strain has no growth from 8 µg/ml to 1024 µg/ml dosage of ampicillin. About the pathogen strain, it was observed that the bacterium has resistant to all of the dosage of antibiotics even with MWCNTs nanofluid at concentrations of 2mg/ml. While, it was found that in antibiotic in addition to functionalized MWCNTs nanofluid at concentrations of 2mg/ml, are no any growths of bacteria from 8 µg/ml to 1024 µg/ml concentration of antibiotic (Table 2).

Gene expression studies

Mathematical interpretation utilizing SPSS software explained a meaningful differentiation (P-value<0.05) in *TetA* and *AbeM* genes in different treated conditions with comparison to untreated bacteria. The expression level of *TetA* and *AbeM*

genes in the treated groups was significantly lower than in the control. Also in the group treated by functionalized MWCNTs nanofluid and antibiotic was lower than the antibiotic alone condition (Fig. 4 and Fig. 5).

TEM microscopy

Fig.s. 6 (A and B) show the bacterial membrane destruction mechanism of functionalized nanofluids. In this way, it can help increase the penetration of the antibiotic inside the bacteria.

Table 2. MBC test results of ampicillin-resistant for standard and pathogen strains. The + symbol indicates bacterial growth and the - symbol indicates a lack of bacterial growth.

A: Standard Strain

Treatments/AB µg/ml	8	16	32	64	128	256	512	1024
A.b	+	+	+	+	-	-	-	-
A.b+AB+NF(-)	+	+	+	-	-	-	-	-
A.b+AB+NF(+)	+	-	-	-	-	-	-	-

B : Pathogen Strain

Treatments/AB µg/ml	8	16	32	64	128	256	512	1024
A.b	+	+	+	+	+	+	+	+
A.b+AB+NF(-)	+	+	+	+	+	+	+	+
A.b+AB+NF(+)	-	-	-	-	-	-	-	-

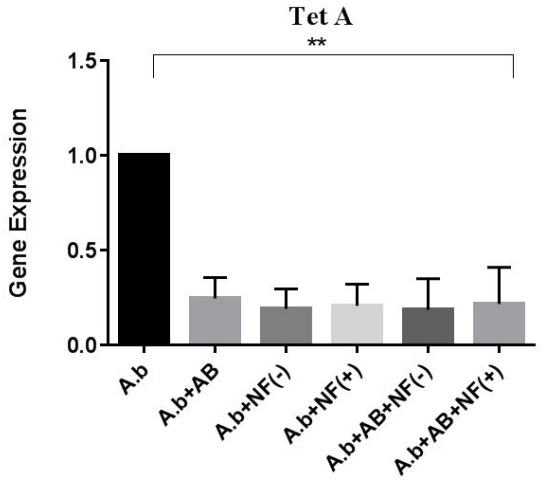

Fig. 4. *TetA* gene expression analysis of the treated pathogen *strain of A. baumannii*. (The y-axis represents the fold change, and the x-axis represents the treated groups.) A.b: *A. baumannii* , AB: Antibiotic, NF(-): Non functionalized carbon nanotubes nanofluid, NF(+): Functionalized carbon nanotubes nanofluid

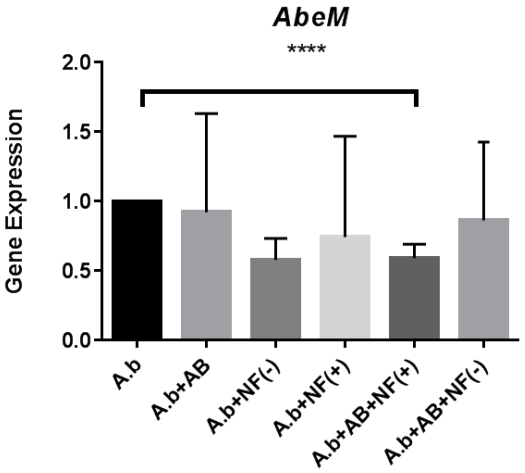

Fig. 5. *AbeM* gene expression analysis of the treated pathogen *strain of A. baumannii*. (The y-axis represents the fold change, and the x-axis represents the treated groups). A.b: *A. baumannii* , AB: Antibiotic, NF(-): Non functionalized carbon nanotubes nanofluid, NF(+): Functionalized carbon nanotubes nanofluid

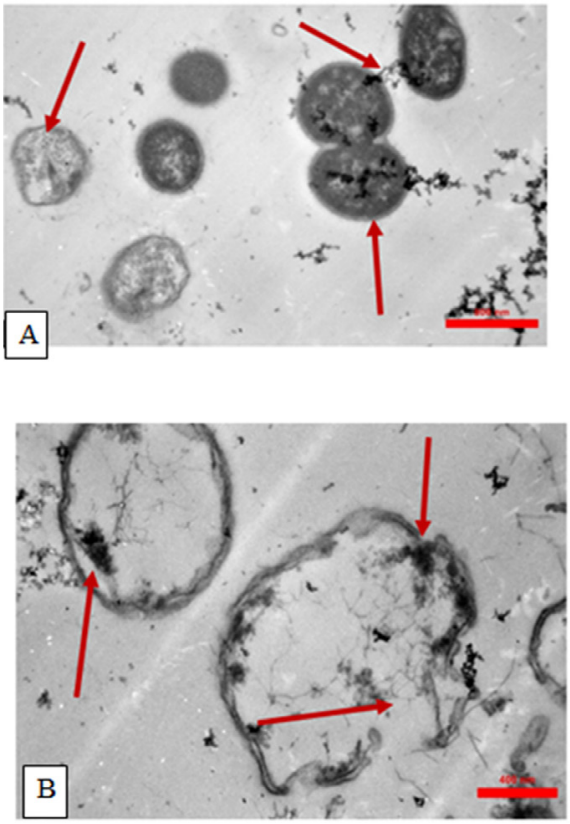

Fig. 6. A and B in different magnifications show the effect mechanism of functionalized MWCNTs nanofluid on cell membrane destruction and antibiotic delivery to the bacteria.

CONCLUSION

Despite recent scientific advances, nosocomial infections are the leading cause of mortality after heart disease and cancer. According to a study by the World Health Organization in 55 hospitals in 14 countries, an average of 8.6% of hospitalized patients are infected with nosocomial infections, which are generally resistant to a wide range of antibiotics. Therefore, the design of new drug delivery systems with the help of nanotechnology methods can help the early and successful treatment of the patients.

In a study conducted by Kang et al. in 2011-11, silver-functionalized carbon nanotubes had higher antibacterial properties than other samples [26].

Proudhana et al. (2011) compared the antibacterial performance of silver-functionalized and non-functionalized carbon nanotubes. They reported that silver-functionalized carbon nanotubes have antibacterial effects on Escherichia coli [27].

Wei Feng et al. In 2013 examined the effect of carbon nanotubes on human intestinal microbes (pathogenic and non-pathogenic, gram-positive and harmful, spherical, also rod). In that research, they used carboxylated multiwall carbon nanotubes. According to the results of the study, carbon nanotubes could reduce the potential risk for probiotic bacteria [28]. In a recent investigation, a unique chemical mixture was created to combat *A. baumannii* diseases. The new carbon nanotube was covered, including an antibacterial mixture, and its effect was evaluated at Broadly Drug-Resistant (XDR), Multidrug-Resistant (MDR); also Pan-Drug-Resistance (PDR) strains of *A. baumannii* Was reviewed. Related results of this study showed that this carbon nanotube mixed with mercury had an antibacterial result against another *A. baumannii* species and that it also was ready to improve the expression from an epidermal growth factor [29]. Similar to our study, this research showed successful treatment with combined carbon nanotubes on *A. baumannii* species.

There has been no study of the effect of functionalized carbon nanotubes on *A. baumannii* to overcoming antibiotic-resistant. Also, *TetA* and *tetM* gene expression studies in this research showed that these two efflux pump genes have the lowest expression level in the combined usage of functionalized MWCNTs nanofluids and antibiotics. That way, the expression of the *TetA* gene, which belongs to the MF family of secretory pumps, was significantly down-regulated, and bacterial resistance was decreased.

Microbial resistance is not only one of the most critical health problems in developing countries, but also, the increasing trend of nosocomial infections and microbial resistance has become a significant concern and challenge in the health system of our country. So, in this novel study, by functionalization of MWCNTs and its administration wit antibiotic in a fluid condition, both of the antibacterial and delivery potential of this formulation was tested and confirmed. Although, more in-depth studies should be conducted in this area.

ACKNOWLEDGMENTS

The authors thank Dr. Alibakhsh Kasaeian from the University of Tehran and Dr. Zahra Taherian from the University of Semnan, who assisted the research team in the process of nanofluid preparation. They also appreciate all the partners of the Mycobacterial and Pulmonary Research Group, Pasteur Institute of Iran, for their assistance in the study of antibacterial effects.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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