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RESEARCH ARTICLE

Antimicrobial activity and drug delivery ability of Functionalized Multi-Walled Carbon Nanotubes Nanofluid on *staphylococcus* aureus

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ABSTRACT

Background: Staphylococcus aureus (S. aureus) is a gram-positive bacterium, which has been considered an important nosocomial pathogen worldwide, owing to its increasing antibiotic resistance. Carbon Nanotubes (CNTs) made entirely of carbon atoms, through their unique properties, hold great promise in the fight against multidrug-resistant bacterial infections. Aim: In this study, antimicrobial activity and drug delivery ability of Functionalized Multi-Walled Carbon Nanotubes Nanofluid (F-MWCNTsN) on S. aureus were studied. Methods: MWCNTs were provided from the United States Research and were functionalized with the COOH group, and the nanofluid was prepared. After bacterial treatment with F-MWCNTsN at the concentration range of %0.1 to %1, the bacterial growth was investigated by the Microplate Alamar Blue Assay (MABA) method. Then, the TetM and TetO gene expression studies for evaluating the drug delivery ability of the Nanofluid containing F-MWCNTs were done. Results: The results showed that functionalized multi-wall carbon nanotubes could have antimicrobial effects on S. aureus. Also, by using nanofluid-containing functionalized carbon nanotubes, the researchers could overcome the antibiotic resistance of the photogenic strain of S. aureus. Conclusion: This study is successful in vitro research and a new approach to Nano drug therapy and delivery to antibiotic-resistant strains of bacteria, S. aureus, which causes a wide range of nosocomial infections.
Keywords: Staphylococcus aureus; antimicrobial activity; Multi-Wall Carbon Nanotubes; nanofluid; antibiotic resistance; nosocomial infections

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INTRODUCTION

Bacteria can be resistant to antimicrobial agents through various mechanisms such as changes in metabolic pathways, the production of inactivating antimicrobial enzyme agents, and changes in the antibiotic effect pathways [1]. Several antibiotics can act via different pathways include inhibition of cell wall synthesis, ribosomal inhibition, polymerase RNA or gyrase inhibition, and others can act as anti-metabolites [2, 3]. *Staphylococcus aureus* (*S. aureus*) is gram-positive and anxiolytic cocci that are the most important species in the Staphylococcus genus [4]. It estimated that 20% of the people have long been carriers of bacteria. *S. aureus* is one of the strongest pathogen bacteria, which reduces yellow colonies because of the production of a golden-colored carotene called Staphyloxanthin. This pigment acts as a part of bacterial virulence factors, and also it works as a bacterial antioxidant factor that makes bacteria gain from reactive oxygen species [5]. *S. aureus* is one of the five common causes of infectious diseases, especially post-surgical ulcers [6]. The

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mature range of *S. aureus* pathogens is responsible for the production of proteins and virulence factors of various strains. Some of these proteins stick to the cell wall and deliver themselves at the cellular level. Thus, they can bind proteins in the blood such as immunoglobulin G and fibrinogen or other proteins such as fibronectin and collagen. Two essential properties that these proteins create in a bacterium include escaping from the host immune system, depending on the coverage of the host proteins. Furthermore, the other is to adhere to the host tissues and begin to invade the host cells [7].

The expression of pathogenic staphylococcal factors regulated by various systems that are sensitive to environmental stimuli; these systems consist of two proteins, kinase sensors, and response regulators. The sensor connects to the external ligand or receptor itself, activates the phosphorylation cascade, and the regulator binding to a specific region of the DNA and ultimately activates the transcription [8]. At least four subunit regulation systems that are involved in the expression of *Staphylococcus aureus* genes were shown. These systems include Sae (*S. aureus* exoprotein expression), srrAB (staphylococcal respiratory response), arlS (autologous dependent sensors), and LYtRS. The Sae system regulates the expression of genes at the transcriptional level and acts independently of the air system. This system plays a significant role in the production of alpha-toxin, hemolysin beta, and coagulase. The srrAB system plays a vital role in regulating the expression of pathogenic factors and is affected by oxygen. The early system plays a crucial role in control from the autolysis as well as degradation from the air system. The LytRS system is also involved in the autolysis process [9-12]. Ribosome protection proteins (RPPs) confer tetracycline resistance on connecting to the ribosome and hunting the drug of its binding site. *Staphylococcus* spp. Have two mechanisms of resistance to tetracyclines, which are active efflux resulting from the acquisition of the plasmid-located genes, and ribosomal protection mediated by transposon-located or chromosomal *tetM* or *tetO* determinants. The *tetM* gene confers resistance to all available drugs of tetracycline and minocycline groups[13, 14].

Diseases that are cased with *S. aureus* are associated with toxic shock syndrome[15], staphylococcal scalded skin syndrome[16], bacteremia[17], and respiratory infections. Mortality is high in these diseases, especially when it leads to respiratory distress or septic shock. Clinical manifestations of *S. aureus* pneumonia are indistinguishable from other pathogens of pneumonia [18].

Through developing the new methods for the treatment of nosocomial infections, silver nanoparticles have successful antimicrobial applications[19]. Also in the field of diagnosis, using nanotechnology take attention in recent years. In a similar study, researchers developed an electrochemical immunosensor based on an electrospun carbon nanofiber mat decorated with gold nanoparticles and carbon nanotubes for the detection of breast cancer [20]. Nanoparticles possess unique optical, electrical, chemical, and mechanical characteristics [21]. Among these, carbon nanotubes (CNTs) now possess various medical applications, such as in antimicrobial agents and drug delivery systems [22-24]. Moreover, nanoparticles in suspensions which been called nanofluids have been presented as potential anticancer and antimicrobial agents, and these characteristics make them necessary in the industry and medical sciences[25, 26]. the usage of nanoparticles within a suspension improves their stability and reduces their agglomeration into drug delivery requirements[27]. During this study, antimicrobial effects study of functionalized carbon nanotubes nanofluids (FCNTsNF) on *S. aureus* has been done. The bacteria were treated by several doses from nanofluid and antibiotic to defeating bacterial antibiotic resistance. Then, the gene expression studies of two genes *TetM* and *TetO*, which relates to the efflux pump, were performed before and after treatments. Transmission Electron Microscopy (TEM) observation was done to confirm of drug delivery ability of this nanofluid.

MATERIALS AND METHODS

Sample collection and confirmation tests

In this study, a sample of the *S. aureus*, (ATCC, USA-300) and the pathogen strain were obtained from the microbial bank of Pasteur Institute of Iran. Samples were re-cultivated and isolated in an agar medium to ensure that they were pure. Subsequently, the catalase test and salt agar mannitol test used to determine the definitive identity and diagnosis of *S. aureus*

Functionalization of carbon nanotubes

CNTs were prepared from the United States Research. During adjustment of Multi-Walled

Carbon Nano Tubes (MWCNTs) by a COOH group, 0.5 g of MWCNTs was covered in an 80 ml mix of H_2SO_4/HNO_3 (3:1, v/v) at 70°C to several times below continuous sonication. Next, the black solid obtained after filtration washed some times by distilled water and dried at 80°C in vacuity for eight hours [28, 29]. The characterization of acquired carboxyl MWCNTs or MWCNT (COOH) performed by TEM and X-ray diffraction.

Preparation of nanofluid

For the preparation of each nanofluid (MWCNTs & MWCNT (COOH)), 0.2 g of each nanopowder was added into 100 ml of deionized water gently, on the magnetic stirrer. 6 ml of ethanol add 0.06 g of Arabic gum added into the suspension, and the total suspension was stirred for 20 minutes. Next, the suspension was conveyed in a frozen receptacle into the ultrasonic device (Ultrasonic Homogenizer 400w, 200 kHz), with the power of 200W for 20 minutes.

Nanofluids Effect studies on standard and pathogen strains of **S. *aureus***

Standard and pathogen strains suspensions were provided based on 0.5 McFarland in TSB liquid medium. Next, all of them incubated by different densities of 0.5, 1, 2, and 4 mg/ml of nanofluids containing MWCNT and MWCNT (COOH) for 24 hours at 37 ° C into a shaker incubator.

Antibiogram

Bacteria raised into an agar completed by a turbidity equivalent to 0.5 McFarland. Next, purified suspensions inoculated in three several sides on the Muller Hinton Agar medium. The antibiotic sensitivity from the standard and pathogen strains done by the disk diffusion method; the related antibiotic discs to these bacteria were cefalexin, vancomycin, penicillin, and tetracycline. After 24 hours, the observed district from inhibition outcomes confirmed according to the Clinical and Laboratory Standards Institute (CLSI) guidance.

Minimum Inhibitory Concentration (MIC)

Bacterial suspension of 0.5 MacFarland and serial dilution of antibiotic (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 µg/ml) were prepared. 100µl of bacterial suspension was added to each microplate well and were treated with different dilutions of antibiotics. The microplate was incubated at 37°C for 24 hours. Each sample was repeated triplet, for both standard and pathogen strains and after that, the turbidity test does for each well[30].

Minimum Bactericidal Concentration (MBC)

For all samples of pathogen and standard bacteria with all dilutions of antibiotics alone, non-functionalized MWCNT nanofluid alone, Functionalized MWCNT nanofluid alone, and combination of antibiotics with each of them cultivated a concentration of 4 mg/µl to 512 mg/µl on the Muller Hinton agar medium. After incubation of samples at 37°C for 24 hours, the results of bacterial growth observed.

RNA extraction, cDNA synthesis, and real-time PCR

The total RNA from all samples extracted utilizing the DNA-Technology PREP-NA DNA / RNA Extraction Kit kit (REF: P-002 / 1INT). cDNA synthesis does with utilizing the cDNA Synthesis kit (Cat No: YT4500); also, *TetM* and *TetO* genes expression examinations were performed by use by the Real-time PCR method with the related primers (Table 1).

RESULTS

Characterization of functionalized carbon nanotubes

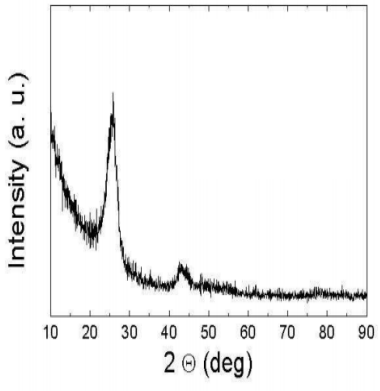
As shown in Fig. 1. A, and B, The functionalization of CNTs confirmed by X-ray diffraction analysis and TEM observation.

Effect study of MWCNT and MWCNT(COOH) nanofluids

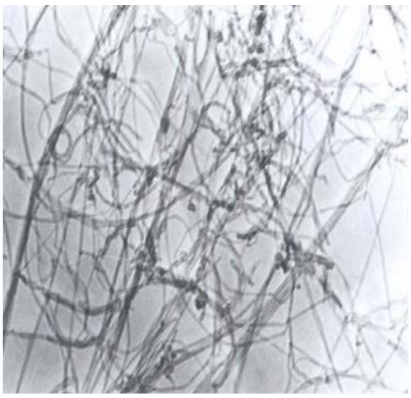
As displayed in Fig. 2 in both standard and pathogen strains, functionalized CNTs nanofluid

Table 1. The primer sequences for the *16srRNA* (control), *TetM,* and *TetO* genes.

Name	Sequences5′-3′
16srRNA F	CGGACGGGTGAGTAACGCGTGA
16srRNA R	GCTAGGACTACWGGGGTAT
TetM F	AGGAAGCGTGGACAAAGGTA
TetM R	TCCTGGCGTGTCTATGATGT
TetO F	AGGAAGCGTGGACAAAGG
TetO R	CCTGGCGTGTCTATGATGT



A



B

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

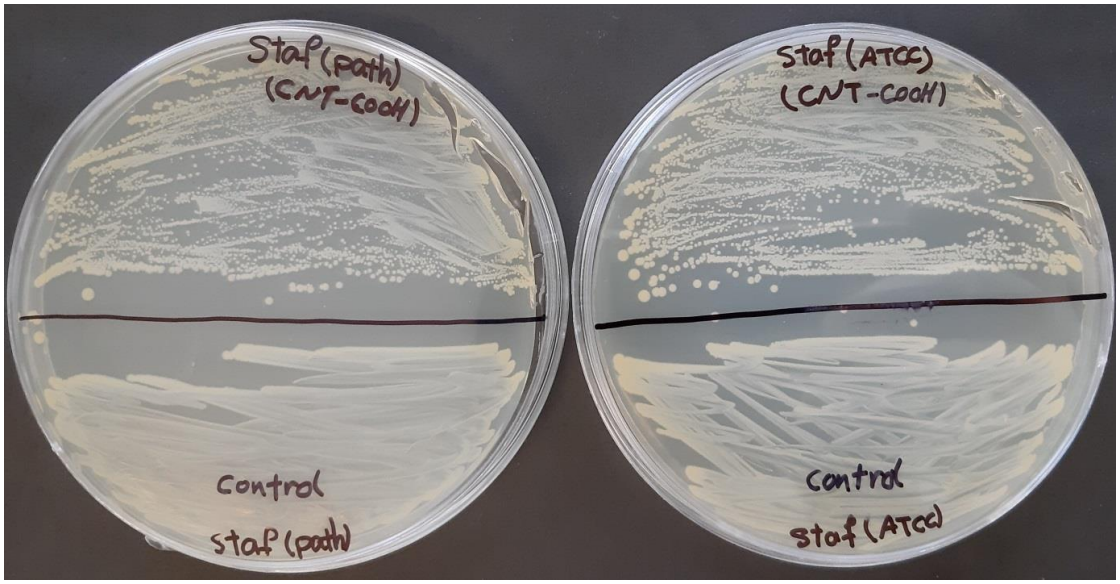


Fig. 2. The left Fig. is a pathogen, and the right Figure is a standard strain. Nanofluid containing functionalized carbon nanotubes reduces bacterial growth at a concentration of 4mg/ml. The left Figure is a pathogen and the right Figure is a standard strain. Staf: S. aureus, CNT-COOH: Functionalized cacbon nanotubes, ATCC: Standard strain, code (USA-300), Path: Pathogen strain, Control: Non-treated group.

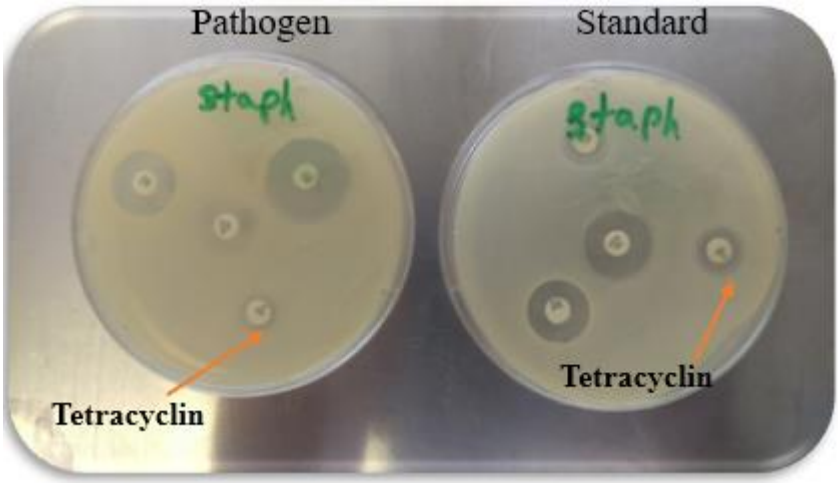


Fig. 3. Antibiogram results by agar disk diffusion method. The arrows each indicate the type of antibiotic.

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. Results of MIC and MBC tests of standard and pathogen strains. The + symbol indicates bacterial growth, and the - symbol indicates a lack of bacterial growth.

had a meaningful effect on the decrease of bacterial growth at a density of 4mg/ml. For this reason, this concentration of nanofluid used for antibiotic-resistant studies.

The results of antibiogram

The results of antibiogram testing by disk diffusion agar method according to the CLSI Table, showed that the standard strain is resistant to tetracycline and sensitive to penicillin. The pathogenic strain is also resistant to tetracycline and penicillin (Fig. 3).

MIC & MBC tests

The bacterial resistant study of standard and pathogen strains was done with the use of tetracycline, which was observed that both strains have resistant to it. The strains were treated by different doses of antibiotics at concentrations of 8 µg/ml up to 512 µg/ml alone, in joining by MWCNTs nanofluid and also FMWCNTs NF. After the MIC tests, all of the treated wells were confirmed with MBC tests. Then, the treated bacteria were cultured on Müller Hinton agar medium and incubated for 24h at 37° C. Next, the results of the bacterial growth were compared to each other.

In contrast, the pathogen strain has resistant to the concentration range. While standard bacteria treated by antibiotics and MWCNTs nanofluid, it has resistant to all of the dosage. Also, in the appearance of FMWCNTs NF by the antibiotic, the standard strain has no growth from 128 µg/ml to 512 µg/ml dosage of tetracycline. About the pathogen

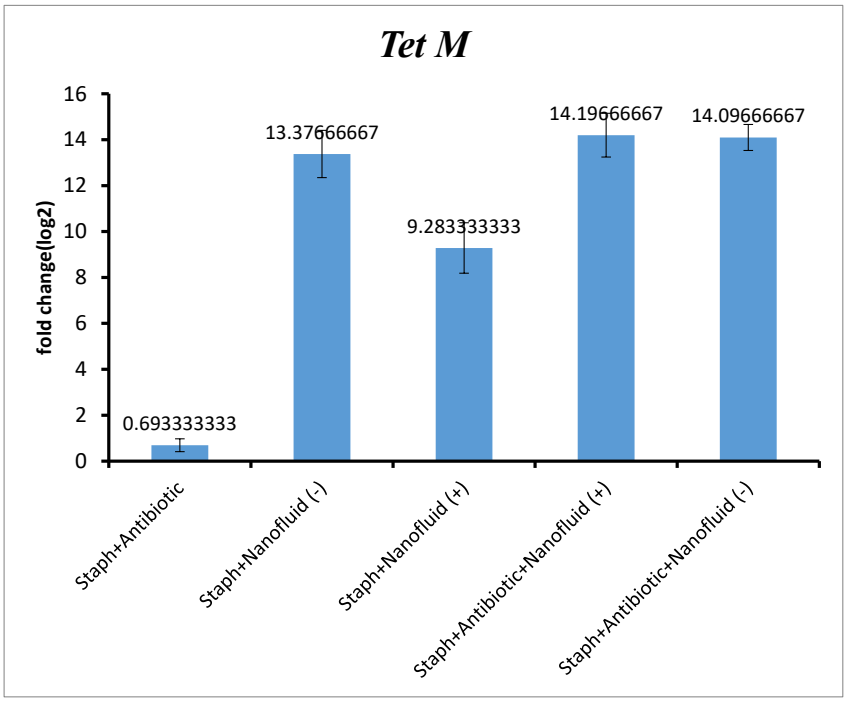


Fig. 5. *TetM* gene expression analysis of the treated pathogen strain of *S. aureus*. (The y-axis represents the fold change, and the x-axis represents the treated groups.) Nanofluid(-) : MWCNTs NF, Nanofluid(+) : FMWCNTs NF.

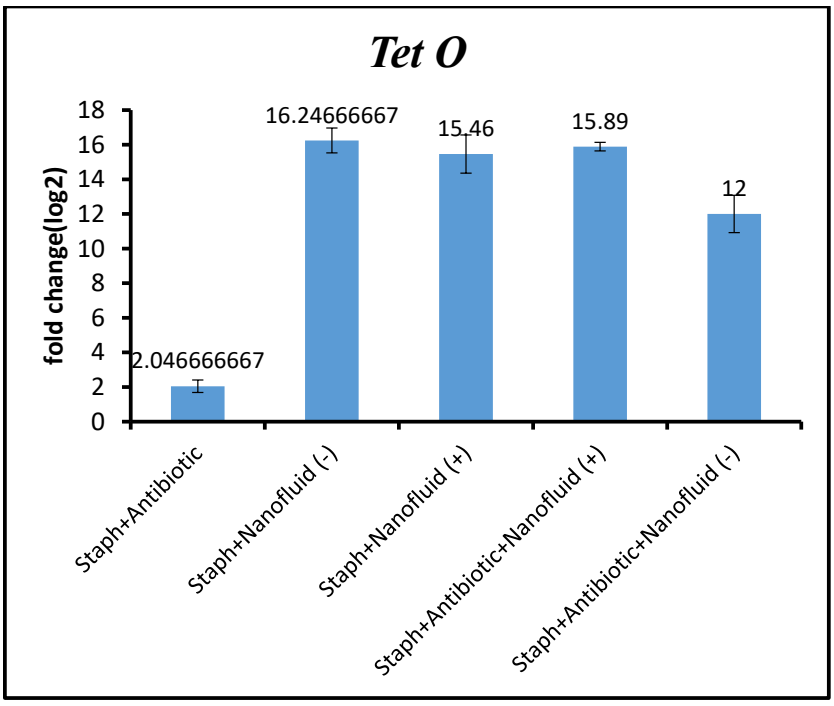


Fig. 6. *TetO* gene expression analysis of the treated pathogen strain of *S. aureus*.
(The y-axis represents the fold change, and the x-axis represents the treated groups.) Nanofluid(-) : MWCNTs NF, Nanofluid(+) : FMWCNTs NF.

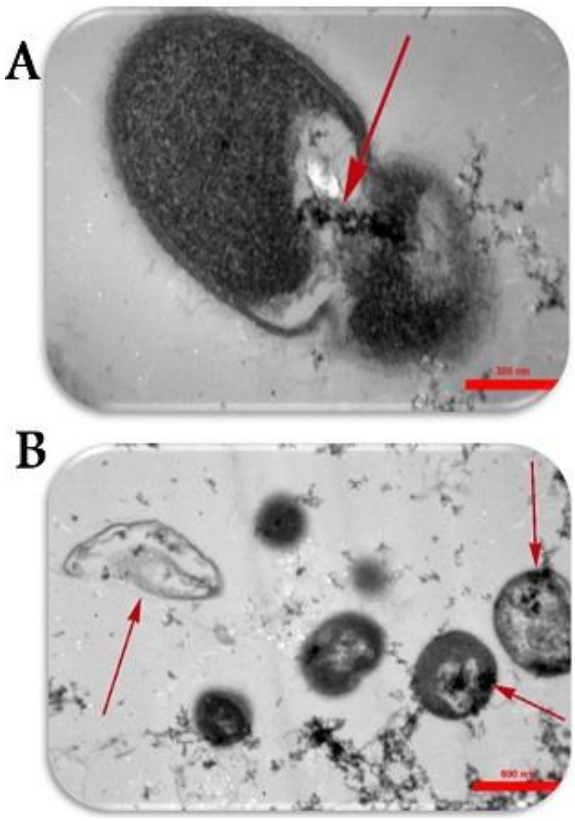


Fig. 7. A and B in different magnifications exhibit the impression mechanism from functionalized MWCNTs nanofluid on cell membrane demolition and antibiotic delivery to the bacteria.

strain, it was observed that the bacterium has resistant to all of the dosages from antibiotics even by MWCNTs nanofluid at concentrations of 2mg/ml. It was noticed that into antibiotics in addition to FMWCNTs NF at concentrations of 2mg/ml, there are no growths of bacteria from 256 µg/ml to 512 µg/ml concentration of antibiotic (Fig. 4).

Gene expression studies

Analytical reports by using SPSS software presented a meaningful difference (P-value<0.05) in *TetM* and *TetO* genes in various treated situations by measuring over untreated bacteria. The expression of the *TetM* gene in the antibiotic-treated group was deficient, while the highest expression of this gene was in the presence of multicellular carbon nanotubes with and without antibiotics. The expression of the *TetO* gene relative to control was high in all but the affected group with antibiotics. However, these genes, which belong to the efflux pumps family, have the highest expression of the gene in the bacterium, which has been treated with functionalized MWCNTs nanofluid and antibiotic. (Fig. 5 and Fig. 6).

TEM microscopy

Figs. 7 (A and B) displays the bacterial membrane disintegration mechanism of functionalized nanofluids. This method can assist improve the insertion of the antibiotic inside 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 infects with nosocomial infections, which are generally resistant to nosocomial infections resistant to antibiotics. Since it is always the best and most effective way to prevent the disease, unfortunately, it has now increased its resistance against large numbers of antibiotics. Therefore, the production of new antibiotics with the help of new nanotechnologies can help the early treatment

of the patient. In a study conducted by Kang et al. in 2011, silver-functionalized carbon nanotubes had higher antibacterial properties than other samples[31]. Prodana et al. (2011) compared the antibacterial performance of silver and non-silver-coated carbon nanotubes. They reported that silver-stained carbon nanotubes could have antibacterial effects on *Escherichia coli* [32]. Wei Feng et al. (2013) also investigated the effect of carbon nanotubes on the intestinal microbes of pathogenic and non-pathogenic, gram-positive and negative, spherical, and rod cells [33]. In this study, they used carburized multi-walled carbon nanotubes. Carbon nanotubes can reduce the potential risk for probiotic bacteria. Not all studies of carbon nanotubes in the fluid have used to treat antibiotic-resistant nosocomial infections against *Staphylococcus aureus*.

In general, studies of the expression of *TetM* and *TetO* genes in these experiments showed that the expression of these two genes, which are from the efflux pump genes, have the most expression level into the mixed usage of FMWCNTsNFs and antibiotics. Consequently, an increase in gene expression indicates that the effectiveness of functional nanoparticles with antibiotics has been dramatically increased.

Bacterial treatment and diagnosis of all types of cancer have attracted, and due to the increasing global population and the crisis of the dangers of the spread of nosocomial infections and the severe threat to human health, more in-depth studies are needed.

Overall, the present study showed that the efficacy of nanofluids containing multi-walled carbon nanotubes functionalized to carboxylic acid was different compared to nanofluids containing multi-walled carbon nanotubes without an agent. This agent appears to have changed after bacterial exposure to the nanofluid and is likely due to a decrease in bacterial growth due to the binding of nanofluids containing functionalized carbon nanotubes to the bacterial membrane, eliminating membrane integrity and enhancing antibiotic efficacy.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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